THE ROLE OF 5-HYDROXYTRYPTAMINE IN PRE-ECLAMPSIA

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DECLARATION

This work has not been accepted for any other degree and is not concurrently being submitted for any other degrees.

We certify that the work submitted was carried out by the author. Due acknowledgement has been made of any assistance received.

Signed.....(Candidate)

Signed.....(Director of Studies)

Sevgili anne ve babama.....

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ABSTRACT

Pre-eclampsia is a disease specific to human pregnancy. It is characterised by increased blood pressure and proteinuria. The main aim of this work was to investigate comparatively the vasoactive actions of 5-HT on vascular tone in normotensive and pre-eclamptic placental vessels and also to compare 5-HT_{2A} receptor protein levels in these vessels. Two representative vessels from each placenta were used in these investigations; arteries and veins close to the insertion of the umbilical cord (primary artery / vein) and arteries and veins close to the periphery of the placenta (secondary artery / vein).

The main finding of the present study was a reduced contractile response of pre-eclamptic placental (primary and secondary) veins and (secondary) arteries to 5-HT. This reduction was more pronounced in the secondary vessels. In addition this study revealed that in pre-eclamptic patients maximal contraction of secondary arteries to 5-HT was significantly reduced in comparison to primary arteries; this was not the case for normotensives. This finding demonstrates the importance of studying different branches of the chorionic vessels comparatively and also suggests that chorionic plate vessels may indeed be differently modified in pre-eclamptic conditions.

Blood / urine 5-HT and its metabolite 5-HIAA levels (in urine) were also investigated in these patients. Circulating 5-HT levels were generally higher in pre-eclamptic samples. However, there was no significant correlation between a patient's response to 5-HT and blood (or urine) 5-HT levels. Although the maximum difference in the response of normotensive and pre-eclamptic vessels to 5-HT was observed at μM levels, circulating 5-HT levels were found to be around 50 nM in pre-eclamptic patients. However, circulating 5-HT levels could reach μM levels locally due to platelet activation especially in pre-eclamptic patients. Therefore a reduced contractile response to 5-HT may have implications for the maintenance and control of blood flow to the foetus and may also provide a protective mechanism.

Western blotting analysis of the 5-HT_{2A} receptor demonstrated similar total receptor levels in primary and secondary vessels and the levels were not affected by pre-eclampsia. This suggests receptor desensitisation may have a role in the reduced response to 5-HT in pre-eclamptic vessels. Preliminary studies using a cultured placental cell line also indicated no effects of oxidative stress and hypoxia on total 5-HT_{2A} receptor levels.

A reduced 5-HT turnover was found in pre-eclamptic samples as determined by a reduced urine 5-HIAA: 5-HT ratio. Since previous studies in our laboratory showed that the catalytic turnover of MAO-A was reduced in pre-eclampsia, km values of MAO-A for its amine substrate was also investigated. Km values were found to be similar in normotensive and pre-eclamptic placental samples suggesting that the affinity of the enzyme for its substrate is not reduced in pre-eclampsia. Possible oxidative / nitrative damage to the enzyme was further investigated using recombinant MAO-A protein. Preliminary studies indicate that MAO-A was highly resistant to short term exposure to hydrogen peroxide and more sensitive to peroxynitrite.

In conclusion these findings suggest that 5-HT may have a role in the control of blood flow to the foetus and the reduced responsiveness in pre-eclamptic vessels may be a protective mechanism which prevents excessive vasoconstriction to compensate for the reduced utero-placental blood flow in pre-eclampsia.

LIST OF ABBREVIATIONS

5-HIAA: 5-Hydroxyindoleacetic acid
5-HT: 5-Hydroxytryptamine (serotonin)
5-HTP: 5-Hydroxytryptophan
AADC: Aromatic-$_L$-amino acid decarboxylase
AP: Alkaline phosphatase
APS: Ammonium persulphate
BCA: Bicinchoninic acid
BCIP: 5-bromo-4-chloro-3indolyl phosphate
BMI: Body Mass Index
BSA: Bovine serum albumin
CAMP: 3'-5' cyclic adenosine monophosphate
CNS: Central nervous system
CSF: Cerebrospinal fluid
CT: Computed tomography
DAB: 3,3'-Diaminobenzidine
DAG: Diacylglycerol
DCDHF: 2'-7'-dichlorodihydrofluorescein
DCF: 2'-7'-dichlorofluorescein
DMEM: Dulbecco's modified eagle's medium
DMF: Dimethyl formamide
DMSO: Dimethyl sulphoxide
DNA: Deoxyribonucleic acid
DTT: Dithiothreitol
ECL: Enhanced chemiluminescence
EDTA: Ethylenediamine tetraacetic acid
ELISA: Enzyme linked immunosorbent assay
FAD: Flavin adenine dinucleotide
FBS: Foetal bovine serum
FDA: Food and drug administration
G_i: G protein inhibitory
GLUT1: Glucose transporters 1
GLUT3: Glucose transporters 3
GPCRs: G-protein-coupled receptors
$G_{q/11}$: G protein q/11
G_s: G protein stimulatory
HCG: Human chorionic gonadotropin
HELLP: Haemolysis elevated liver enzymes and low platelet count
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP: Horse radish peroxidase
IP_3: Inositol 1,4,5-triphosphate
IUGR: Intrauterine growth restriction
iv: Intravenous
KIR: Killer immunoglobulin receptor
Km: Michaelis-Menten constant
KS: Kolmogorov-Smirnov
LDH: L-Lactic dehydrogenase
MAO: Monoamine Oxidase
MAPK: Mitogen activated protein kinase

MDA: Malondialdehyde
MHC: Major histocompatibility complex
MLCK: Myosin light chain kinase
MMps: Matrix metalloproteinases
mN: Milli Newton
mRNA: Messenger Ribonucleic acid
NAD: Nicotinamide adenine dinucleotide
NBT: Nitro blue tetrazolium
NHBPEP: National high blood pressure education program
NK cells: Natural killer cells
NO: Nitric oxide
NOS: Nitric oxide synthase
NSS: Normal swine serum
NT: Normotensive
PA: Primary artery
PBS: Phosphate buffered saline
PE: Pre-eclamptic
PET: Positron emission tomography
PIGF: Placental growth factor
PIP_2: Phoshatidylinositol 4,5-bisphosphate
PLC: Phospholipase C
PMSF: Phenylmethylsulfonyl fluride
PN: Peroxynitrite
PP-13: Placental protein 13
PPP: Platelet poor plasma
PRP: Platelet rich plasma
PV: Primary vein
RNA: Ribonucleic acid
RNS: Reactive nitrogen species
ROS: Reactive oxygen species
SA: Secondary artery
SDS: Sodium dodecyl sulphate
SDS-PAGE: Sodium dodecylsulphate polyacrylamide gel electrophoresis
SEM: Standard error of the mean
sEng: Soluble endoglin
SERTs: Serotonin transporters
sFlt-1: Soluble fms-like tyrosine kinase-1
SOD: Superoxide dismutase
SR: Sarcoplasmic reticulum
SV: Secondary vein
TBS: Tris buffered saline
TEMED: N,N,N',N'-tetramethyl-ethylenediamine
TPH: Tryptophan hydroxylase
TRP: Tryptohan
tsA: Temperature sensitive A
VSM: Vascular smooth muscle
X: Xanthine
XDH: Xanthine dehydrogenase
XO: Xanthine oxidase

LIST OF CONTENTS

PAGE

LIST OF FIGURES

PAGE

LIST OF TABLES

CHAPTER I

GENERAL INTRODUCTION

1 CHAPTER I General Introduction

1.1 The placenta

The placenta is an organ that develops from both maternal and foetal components and serves as the interface between the mother and the developing foetus. It also serves as an endocrine organ and produces the hormones which are necessary for maintaining a healthy normal pregnancy.

1.1.1 Development of the human placenta

Following fertilization the zygote undergoes rapid cell division and forms a morula inside the fallopian tube. During its journey through the fallopian tube into the uterine cavity the morula develops into a blastocyst, which is composed of an outer cell mass, the trophectoderm, and an inner cell mass. The trophectoderm gives rise to the trophoblast, which develops into the placenta and external membranes while the foetus develops mainly from the inner cell mass. Stages in development from ovulation to implantation of the blastocyst are summarised in Figure 1.1.

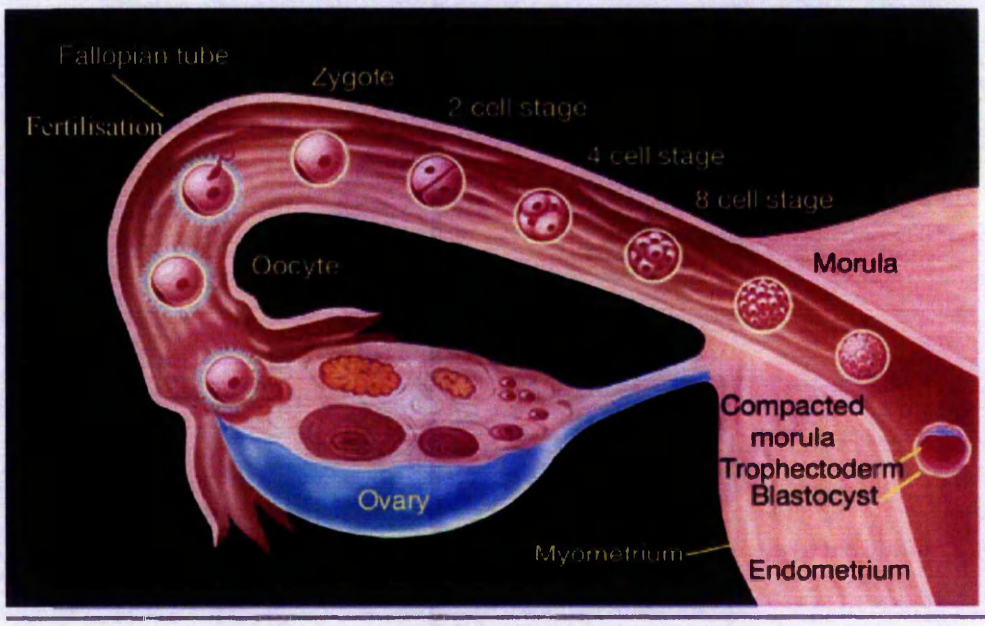


Figure 1.1 Pre-implantation events of the fertilised ovum

Reproduced from Red-Horse *et al.* (2004)

Implantation of the blastocyst into the uterine wall is the first stage in the development of the placenta. The effect of implantation is to obtain very close apposition between embryonic and maternal tissues. There are, however, considerable differences among species in the process of implantation, especially with regard to invasiveness (Carter *et al.*, 2006). In humans, implantation involves penetration of trophoblasts deeply into the endometrium of the uterus. Hormones such as human chorionic gonadotropin (hCG) play a very important role in implantation (reviewed in Srisuparp *et al.*, 2001). Once the embryo is attached to the uterine wall, the next stage is the formation of the extraembryonic lineages in order to build a shell lining the uterine cavity (maternal-foetal interface). During placental development, the embryonic cells are always separated from maternal tissues and blood by a layer of cytotrophoblasts (mononuclear trophoblasts) and syncytiotrophoblast (multi-nucleated trophoblasts). This is very important for nutrient exchange and also to protect the developing foetus. During human placentation the uterine spiral arteries must undergo physiological modification in order to provide an increased blood supply to the growing foetus. This is achieved with the interaction of invasive cytotrophoblast cells with the spiral artery wall. Interstitial cytotrophoblast cells invade the decidual stroma and by the eighth week of gestation reach the endometrial / myometrial border (Pijnenborg *et al.*, 1981). Endovascular cytotrophoblast cells invade into the lumen of the spiral arteries (Figure 1.2). This process is thought to start at 4-6 weeks of gestation and reaches the myometrial arteries by 10 weeks (Pijnenborg *et al.*, 1983). The cytotrophoblast becomes embedded in the vessel wall and replaces the endothelial cell lining of the spiral arteries. Muscular and elastic components of the vessel are replaced with fibrinoid material. This cytotrophoblast invasion results in the transformation of the normally small muscular arteries into low resistance and high capacitance vessels in order to meet increasing demands for sufficient blood flow as the pregnancy progress. Blood flow to the intervillous space begins by 10-12 weeks of gestation. Maternal blood is delivered to the forming placenta via endometrial spiral arteries while being drained away via uterine veins (Figure 1.2). Oxygen tension plays an important role during these stages. Studies have shown that trophoblasts limit access to uterine blood by plugging the lumina of the vessels, thus early stages of placental development take places in a relatively low oxygen environment (Jauniaux *et al.*, 2003a).

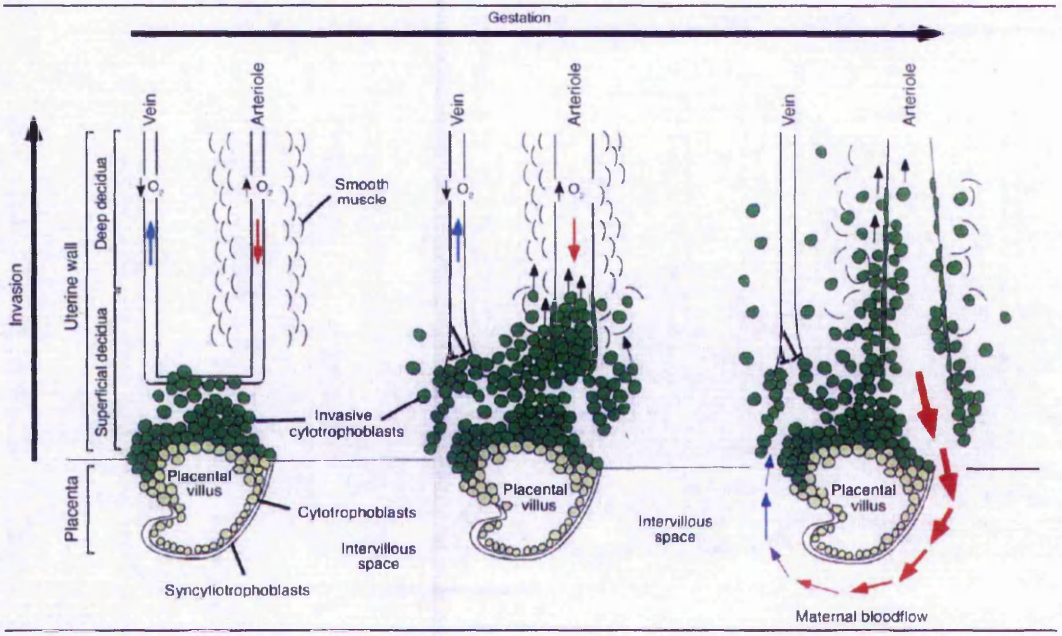


Figure 1.2 Cytotrophoblast invasion of spiral arteries

Cytotrophoblast cells rapidly increase in numbers and invade decidual and myometrial segments of spiral arteries. Plugs are formed in the artery lumen in order to keep low oxygen tensions. Invasion continues and by 10-12 weeks of gestation blood flow to the intervillous space is established. Blood is delivered to the developing placenta via the endometrial spiral arteries and is drained away via uterine veins.

Reproduced from Red-Horse *et al.* (2004)

The chorionic villi closest to the maternal blood supply continue to develop and expand into a mass of chorionic tissue while the chorionic villi away from the maternal blood supply are gradually pushed into the uterine cavity by the expanding amniotic sac.

At term the human placenta has a diameter of 15 - 20 cm and a thickness of 2 - 3 cm and weighs about 500 grams. However, there is a direct relationship with the fetal weight. A mature placenta is demonstrated in Figure 1.3

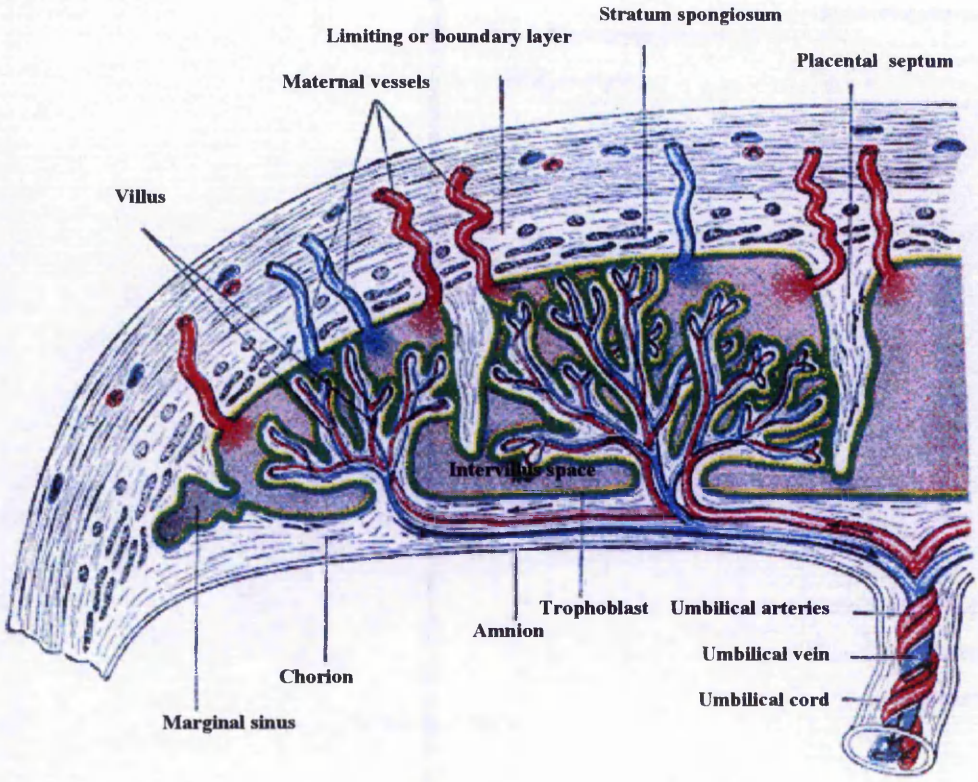


Figure 1.3 Cross section of a placenta showing different components and the placental circulation

Reproduced from Gray's Anatomy of The Human Body, 20th edition –by Lea & Febier Bartleby.com

1.1.2 Functions of the placenta

The placenta has many functions. It serves as an efficient gas exchange layer, allowing oxygen and carbon dioxide to pass between the maternal and foetal circulations without allowing them to mix. Involvement of the placenta in gas exchange has been suggested since the discovery of oxygen (1774-1775). Erasmus Darwin, grandfather of Charles Darwin devoted a chapter of his famous "*Zoonomia; or the laws of organic life*" (1794-1796) to the respiratory function of the placenta. He stated that the placenta "*consists of arteries carrying the blood to its extremities, and a vein bringing it back, resembling exactly in structure the lungs and gills……and that the blood changes its colour from a dark to a light red in passing through these vessels*" (quoted in Pijnenborg and Vercruysse, 2007), pointing out the similarities between the function of the placenta and that of lungs. However, as transmembrane transport was poorly understood at the time, the role of the placenta in the transport of nutrients was disregarded.

The placenta accomplishes the selective transport of substrates for foetal metabolism and disposes of waste products (Cross, 2006). This is facilitated by the close approximation of maternal and foetal vascular systems within the placenta. The transport of water as well as gases is carried out by simple diffusion. Gases like oxygen and carbon dioxide diffuse through and across tissues due to differences in partial pressure. In addition the placental membrane is permeable to nutritional substances such as vitamins, glucose, free fatty acids and electrolytes. Glucose being the major energy substrate provided to the placenta and foetus is transported across the placenta via glucose transporters 1 and 3 (GLUT1 and GLUT3) (Jansson *et al.*, 1993). Although the foetus receives large amounts of intact glucose, a substantial amount is also oxidized within the placenta to lactate. Amino acids are transported to the foetus by active transport, as amino acid concentrations in foetal blood are higher than in maternal blood. Sodium-dependent and sodium-independent amino acid transporters have been identified in placenta, which serve this function (reviewed in Kudo and Boyd, 2002). It is also known that some amino acids are metabolised as they cross the placenta before delivery to the foetus (Battaglia and Regnault, 2001).

In addition to its role in transporting molecules between mother and foetus, the placenta acts as an endocrine organ (Evain-Brion and Malassine, 2003). It synthesises steroid hormones that have major influences on ovarian, uterine, mammary and foetal systems. Progesterone and estrogens are produced by the placenta to maintain pregnancy by inhibiting endometrial shedding. Several protein and peptide hormones are also synthesised in placentae. Human chorionic gonadotropin (hCG) is produced by foetal trophoblast cells, which serves as the signal for maternal recognition of the pregnancy (Evain-Brion and Malassine, 2003). In summary during pregnancy the placenta plays a critical role in providing communication between mother and foetus. This interaction and communication between mother, placenta and foetus is summarised in Figure 1.4.

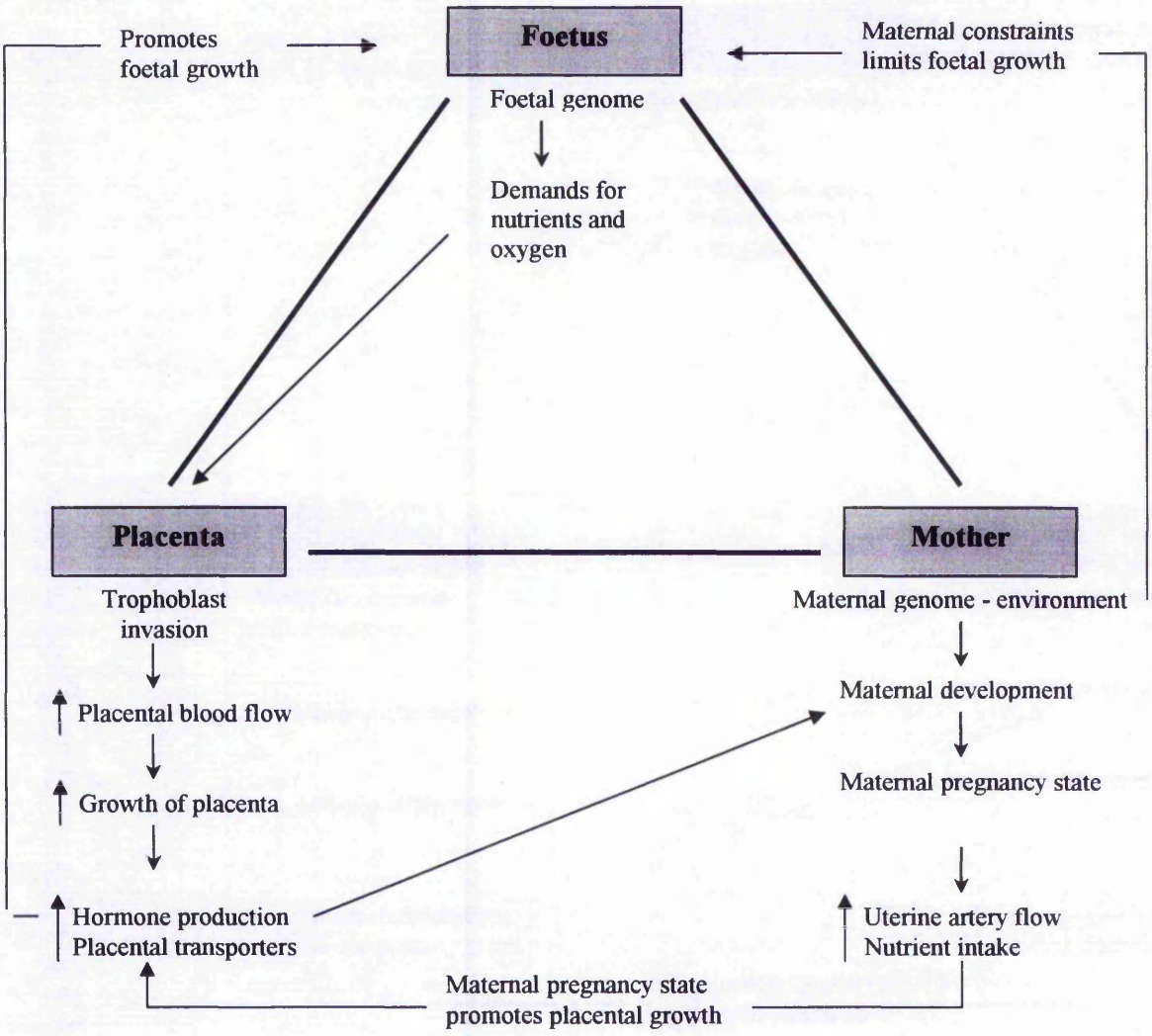


Figure 1.4 Interactions and communication between the foetus, placenta, and mother during pregnancy

Maternal / foetal genome and environment influence maternal development during pregnancy. Factors such as maternal health, smoking, and nutritional status in turn influence the maternal pregnancy state. Placental trophoblast invasion and blood flow promote the growth of the placenta, which produces hormones. Hormones facilitate the signalling between the mother and foetus and transporters in order to transfer nutrients and waste products. Placental function promotes foetal growth, which is also under the influence of the foetal genome.

Adapted from Murphy *et al.* (2006)

1.2 Pre-eclampsia

Pre-eclampsia is a disease specific to human pregnancy. It is characterised by increased blood pressure and proteinuria. It affects about 3–5 % of pregnancies and is a leading cause of maternal and perinatal mortality (de Swiet, 2000; Chang *et al.*, 2003). In the past pre-eclampsia was defined as an elevation of >30 mmHg systolic or >15 mmHg diastolic pressure compared to patients' baseline blood pressure. This has been shown to be a poor indicator of the condition, and criteria for pre-eclampsia were reviewed in 2000 by the National High Blood Pressure Education Program (NHBPEP) working group.

According to NHBPEP (2000) four classes of hypertensive disorders of pregnancy were identified, these are:

1) Pre-eclampsia – eclampsia: Increase in blood pressure equal or greater than 140 mm Hg systolic or 90 mm Hg diastolic in association with proteinuria equal to or greater than 300 mg in 24 hour urine after 20^{th} week of pregnancy.
2) Chronic hypertension: Blood pressure equal to or greater than 140 mm Hg systolic or 90 mm Hg diastolic before pregnancy or diagnosed before 20^{th} week of pregnancy.
3) Pre-eclampsia superimposed upon hypertension: new establishment of 300 mg or greater proteinuria in a 24 hour urine specimen in a pregnant woman who had blood pressure equal to or greater than 140 mm Hg systolic or 90 mm Hg diastolic before pregnancy or 20^{th} week of pregnancy.
4) Gestational hypertension: gestational hypertension term is used during pregnancy until a specific diagnosis can be made postpartum.
    a) Transient hypertension: if pre-eclampsia is not present at the time of delivery and blood pressure goes back to the normal levels within 12 weeks from postpartum.
    b) Chronic hypertension: if increase in blood pressure persists and does not return to normal postpartum.

Pre-eclampsia is regarded as severe if one or more of the following criteria are met. Blood pressure ≥ 160 mmHg systolic or ≥ 110 diastolic on two occasions at least 6 hours apart, proteinuria ≥ 5 gram in 24 hour urine or ≥3+ on two random urine samples four hours apart, oliguria of < 500 ml in 24 hours, cerebral or visual disturbance, pulmonary edema or cyanosis, epigastric or right upper-quadrant pain, impaired liver function, thrombocytopenia (platelet count <100000/µl), foetal growth restriction.

Pre-eclampsia if untreated can rapidly progress to eclampsia, which is defined as the occurrence of seizures in woman with pre-eclampsia that cannot be attributed to any other cause. As oedema is common even in many normal pregnancies it was not included as a marker in recent classifications (Mushambi *et al.*, 1996; Green, 2001).

1.2.1 Risk Factors for Pre-eclampsia

Prevalence of pre-eclampsia is thought to be under the influence of several factors such as maternal age and multiple pregnancy. Many studies have investigated the effect of maternal age on the pregnancy outcome. Mothers aged 40 and over have nearly twice the risk of developing pre-eclampsia (Bianco *et al.*, 1996). Young maternal age on the other hand does not seem to affect the risk of developing pre-eclampsia (Berenson *et al.*, 1997). Multiple pregnancy is another risk factor for developing pre-eclampsia. The risk of developing pre-eclampsia in women pregnant with twins is more than double compared to singleton pregnancies (Conde-Agudelo and Belizan, 2000; Sibai *et al.*, 2000; Catov *et al.*, 2007). Additional factors include parity, body mass index, previous incidence of pre-eclampsia, family history of pre-eclampsia, pre-existing medical conditions, paternity and time between pregnancies (Duckitt and Harrington, 2005). Morover, the prevalence of hypertensive disorders of pregnancy varies with ethnicity, countries and different geographical areas (Svensson *et al.*, 1985; Samadi *et al.*, 1996; Knuist *et al.*, 1998; Palmer *et al.*, 1999; Conde-Agudelo and Belizan, 2000; Lee *et al.*, 2000; Walker, 2000; Gaio *et al.*, 2001; Barton *et al.*, 2002; Zareian, 2004). Interestingly, smoking during pregnancy is suggested to reduce the risk of pre-eclampsia (Conde-Agudelo and Belizan, 2000; England *et al.*, 2002). However, data concerning women who smoked but quit before they became pregnant is conflicting.

Some studies suggest a reduced risk (Zhang *et al.*, 1999) while others have not found a significant association between smoking and pre-eclampsia. Although carbon monoxide is thought to have vasodilating properties, the exact mechanisms of protective effects of smoking on pre-eclampsia is not clear (Bainbridge *et al.*, 2005).

The effect of having pre-eclampsia on long-term health of the mother has also been investigated. Pre-eclampsia is associated with the risk of developing some medical conditions later in life such as cardiovascular disease (Irgens *et al.*, 2001; Ramsay *et al.*, 2003; Wilson *et al.*, 2003; Haukkamaa *et al.*, 2004; Arnadottir *et al.*, 2005; Ness and Hubel, 2005) and diabetes (Libby *et al.*, 2007). In addition low birth weight is now accepted as a major risk factor in the baby for coronary heart disease, stroke, hypertension and adult-onset diabetes (Barker, 2004; Hack, 2006). Realisation of these possible long-term health effects on mothers and their infants indeed is an important achievement and it is hoped that this will promote further research in pre-eclampsia.

1.2.1.1 Parity, paternity and time between pregnancies

Pre-eclampsia is generally accepted as a disease of first pregnancy (Coonrod *et al.*, 1995; Odegard *et al.*, 2000b; Stamilio *et al.*, 2000). On the other hand, some studies with multiparous women with previous normotensive pregnancies demonstrated that a change of partner increased the risk of pre-eclampsia compared to women with the same partner (Gunnlaugsson *et al.*, 1989; Trupin *et al.*, 1996; Misra and Kiely, 1997; Dekker *et al.*, 1998). Moreover changing paternity decreased the risk of reoccurrence in women with a history of pre-eclampsia (Li and Wi, 2000). As a result, primipaternity rather than primiparity has been suggested to be a risk factor. However, partner change is often associated with a long birth interval. Therefore this has recently been challenged by the studies demonstrating increased risk with increasing time interval between deliveries for women without pre-eclampsia in the first pregnancy. (Trogstad *et al.*, 2001; Mostello *et al.*, 2002; Zhang and Patel, 2007). The birth interval hypothesis however has received critical comments (Dekker and Robillard, 2003) and the effect of partner cahenge on the risk of developing pre-eclampsia is inconclusive.

1.2.1.2 Body mass index (BMI)

Several studies have investigated the relationship between body mass index (BMI) before pregnancy and the risk of pre-eclampsia. Despite the fact that they all used different BMI ranges, these studies suggest an overall increase in the risk of developing pre-eclampsia with higher BMI (Bianco *et al.*, 1998; Thadhani *et al.*, 1999; Conde-Agudelo and Belizan, 2000; Lee *et al.*, 2000; Catov *et al.*, 2007). Recently Bhattacharya *et al.* (2007) reported a linear relationship between increasing BMI and the risk of developing pre-eclampsia, the risk was 3 times higher for obese (BMI 30 to 39.9 Kg/m^2) and 7 times higher for morbidly obese (BMI >40 Kg/m^2) pregnant women. In addition some studies also noted that underweight women had a reduced risk of developing pre-eclampsia (Eskenazi *et al.*, 1991; Lee *et al.*, 2000; Sebire *et al.*, 2001; Bhattacharya *et al.*, 2007).

1.2.1.3 History of medical conditions

Several pre-existing medical conditions are known to increase the risk of pre-eclampsia. Hypertension (Conde-Agudelo and Belizan, 2000; Odegard *et al.*, 2000b; Catov *et al.*, 2007), diabetes (Garner *et al.*, 1990; Hanson and Persson, 1993; Ros *et al.*, 1998; Conde-Agudelo and Belizan, 2000; Catov *et al.*, 2007) and renal disease (Sacks *et al.*, 1987; el-Khatib *et al.*, 1994) prior to pregnancy are all known to increase the risk for developing pre-eclampsia. In addition some believe a history of pre-eclampsia also increases the risk of pre-eclampsia in a subsequent pregnancy (Sibai *et al.*, 1986; Lee *et al.*, 2000; Odegard *et al.*, 2000b; Trogstad *et al.*, 2004; van Rijn *et al.*, 2006; Ananth *et al.*, 2007). The highest risks for the re-occurence of pre-eclampsia were when the initial case was preterm, severe, or complicated by eclampsia and the HELLP (hemolysis, elevated liver enzymes, and low platelet count) syndrome (Dildy *et al.*, 2007). Moreover, a family history of pre-eclampsia is thought to increase the risk of developing pre-eclampsia (Arngrimsson *et al.*, 1990; Skjaerven *et al.*, 2005).

1.2.2 Pathophysiology of pre-eclampsia

Pre-eclampsia is a pregnancy specific disease, which affects virtually all maternal organ systems. The pathophysiology of pre-eclampsia remains unknown. As most studies use specimens collected after pre-eclampsia is established, there have been difficulties in defining the relationship between specific mechanisms and clinical manifestations. Pre-eclampsia can develop to eclampsia, characterized by the occurrence of convulsions, or the HELLP syndrome. Both of these conditions are linked with severe complications such as, liver hemorrhage and rupture, cerebral complications (such as convulsions, cortical blindness, cerebral haemorrhage) and lung edema. Vascular endothelial damage and dysfunction are common pathological changes observed in the placenta, kidneys and brain. In addition pulmonary edema is common in pre-eclampsia /eclampsia which occurs as a result of increased intravascular hydrostatic pressure and increased capillary permeability (Benedetti *et al.*, 1985).

1.2.2.1 Cardiovascular system

In order to accommodate the demands of pregnancy substantial changes occur in the cardiovascular system even in normal pregnancy. There is a rapid expansion of total blood volume, increase in heart rate and stroke volume and therefore increase in cardiac output. In early pregnancy blood pressure declines progressively until about 20–24 weeks gestation and returns to the baseline levels at term. Subsequently, peripheral vascular resistance is reduced throughout pregnancy. In pre-eclampsia the hemodynamic and vascular adaptations to pregnancy are altered resulting in increased blood pressure, reduced plasma volume, increased peripheral vascular resistance and vasoconstriction (Visser and Wallenburg, 1991). Low platelet count (thrombocytopenia) is also common in pre-eclampsia. Increased levels of platelet specific protein β-thromboglobulin suggest platelet activation (Ballegeer *et al.*, 1992).

1.2.2.2 Central nervous system (CNS)

Neurological complications of pre-eclampsia may vary. Headache, hyperreflexia, confusion and clonus are considered as warning signs of increased cerebral irritation. As well as headache, visual disturbances such as light sensitivity (photophobia), double vision (diplopia) or blurred vision are common in pre-eclampsia. These visual disturbances are thought to be the result of ischemia of the posterior cerebral arteries or oedema in the occipital region (Cunningham *et al.*, 1995). Although rare, more severe cerebral manifestations such as paralysis and visual loss have also been documented (Royburt *et al.*, 1991). Suggested pathogenesis in the central nervous system includes vasospasm, ischemia, haemorrhage, intravascular coagulation and oedema. Cerebral oedema, haemorrhages and infarcts have been demonstrated by Computed tomography (CT) scanning in patients with pre-eclampsia (Kirby and Jaindl, 1984; Milliez *et al.*, 1990). Although the exact mechanism for convulsions in eclampsia is still not clear it is thought to be due to cerebral vasospasm, microinfarcts and oedema.

1.2.2.3 Renal and hepatic changes

Glomerular swelling, narrowing of the glomerular capillary lumen and fibrin deposition in the endothelial cells (glomerular capillary endotheliosis) result in a decrease in glomerular filtration. Proteinuria in pre-eclampsia is different to that of renal disease. Increased permeability allows larger molecular weight proteins to appear in urine, unlike in renal disease that has selective proteinuria. Oliguria (urine output < 20-30 ml/h over 2 hour) is common in severe cases although progression to renal failure is rare. Acute tubular necrosis is thought to be the cause of reversible renal failure while cortical necrosis may result in permanent renal failure (Sibai *et al.*, 1990).

Pathological changes in the livers of women with pre-eclampsia / eclampsia have also been described (Anday and Cohen, 1990). Attenuated liver function can affect clearance of drugs metabolised by the liver. The cause of hepatic dysfunction is not clear.

1.2.2.4 Foetus

Pre-eclampsia can affect the child in addition to the mother. Women with pre-eclampsia have increased occurrence of intrauterine growth restriction (IUGR) (Xiong *et al.*, 1999; Odegard *et al.*, 2000a) and of small-for-gestational-age infants (Kolben *et al.*, 1996). However weight reductions differ between clinical subgroups (early on-set versus late on-set or mild versus severe pre-eclampsia) and mainly affects the early-onset and severe pre-eclampsia (Odegard *et al.*, 2000a; Rasmussen and Irgens, 2003). Hence the risks for the foetus can be listed as perinatal death, intrauterine growth restriction and preterm delivery, the latter being as a consequence of concerns about maternal safety. Generally maternal and perinatal outcomes are worse in severe cases, which develop before 33 weeks of gestation (Hauth *et al.*, 2000; Buchbinder *et al.*, 2002).

1.2.3 Aetiology of pre-eclampsia

The cause of pre-eclampsia remains unclear despite many attempts to understand the mechanisms responsible for its pathogenesis. Studies over the past decades underline several possible contributory factors to the origin of pre-eclampsia. Abnormal placentation, inflammatory response to pregnancy and oxidative stress are now widely accepted to contribute to the aetiology of pre-eclampsia. Contributory factors include endothelial dysfunction (Roberts *et al.*, 1989), circulating angiogenic growth factors (Lash *et al.*, 1999), the activation of angiotension receptor (AbdAlla *et al.*, 2001), placental ischemia (Wang and Walsh, 2001; Vanderlelie *et al.*, 2005; Wang *et al.*, 2005), increased levels of circulating 5-Hydroxytryptamine (Middelkoop *et al.*, 1993; Hutter *et al.*, 1996; Carrasco *et al.*, 1998; Bertrand and St-Louis, 1999) and reduced placental monoamine oxidase (MAO) activity (Carrasco *et al.*, 2000; Sivasubramaniam *et al.*, 2002).

1.2.3.1 Placentation

The cause of pre-eclampsia is thought to be placental in origin. The presence of a foetus or the uterus is probably not required as pre-eclampsia can occur in the absence of a foetus (hydatidiform mole) (Lee, 1965) and may develop in abdominal pregnancy. Pre-eclampsia is also associated with hyperplacentation (increased mass of placenta) as in diabetic and multiple pregnancies. It is now generally accepted that in pre-eclampsia trophoblastic invasion of the spiral arteries is abnormal and this could result in reduction in uteroplacental perfusion. As described earlier (section 1.1.1 and Figure 1.2) in normal pregnancy decidual spiral arteries undergo remodelling. These vessels change to flaccid tubes with a diameter of at least four times greater than that of vessels from non-pregnant individuals. In pre-eclampsia these adaptations of normal pregnancy, which result in an increase in placental perfusion, are found to be incomplete (Pijnenborg *et al.*, 1991; Khong and Mott, 1993; Pijnenborg *et al.*, 1996). Endovascular invasion is thought to be shallow. Morover, uterine spiral arteries retain their endothelial lining and muscular wall and, as a result, remain as narrower and high resistance vessels (Pijnenborg *et al.*, 1991). Subsequently blood flow into the intervillous space is reduced. This has been confirmed by Doppler ultrasound studies of maternal uterine blood flow (Papageorghiou *et al.*, 2004). Both the invasive depth and numerical density of interstitial trophoblasts are reduced in pre-eclampsia (Kadyrov *et al.*, 2003; Kadyrov *et al.*, 2006). Primary mechanisms of normal and failed trophoblast invasion are very complex and not fully elucidated. Studies suggest that many factors will influence trophoblast invasion. Some of these can be listed as growth factors and their receptors (Bass *et al.*, 1994; Librach *et al.*, 1994; Lyall *et al.*, 2001), matrix metalloproteinases (MMPs) and their inhibitors (Huppertz *et al.*, 1998; Xu *et al.*, 2000), adhesion molecules (Damsky *et al.*, 1994), cytokines (Librach *et al.*, 1994), oxygen tension (Genbacev *et al.*, 1997; Caniggia *et al.*, 2000), and presence of natural killer cells (NK cells) (Loke and King, 1997). Other mediators of implantation include nitric oxide (NO) (Martin and Conrad, 2000) and carbon monoxide (CO) (Lyall *et al.*, 2000). Cytotrophoblasts may produce these vasoactive mediaters in order to change spiral arteries prior to endovascular invasion.

It has been suggested that altered expression of these factor are involved in trophoblast invasion in pre-eclampsia (Zhou *et al.*, 1993).

Studies have also demonstrated the importance of oxygen tension during early placentation. Genbacev *et al.*, (1997) demonstrated that cytotrophoblasts maintained *in vitro* at lower oxygen tension (2%) preferentially proliferated and poorly differentiated. On the other hand when cultured at 20 % oxygen (imitating the environment close to uterine arteries) they differentiated normally. This clearly showed the importance of oxygen tension in the regulation of placental growth and trophoblast invasion (Genbacev *et al.*, 1997).

Early placental development occurs in a relatively low oxygen environment (Rodesch *et al.*, 1992) and the utero-placental circulation establishes progressively, first starting at the periphery and gradually extending towards the centre (Jauniaux *et al.*, 2003b). In addition, in order to keep efficient oxygen tension regulation of the placental bed, the efficient plugging of the spiral arteries and, consequently, the timely establishment of the blood flow to the intervillous space are very important. This is shown by the association of early pregnancy loss with early and excessive entry of the maternal blood into the intervillous space (Jauniaux *et al.*, 2003b). The overall effect of inadequate invasion of the spiral arteries is incomplete remodelling and decreased placental perfusion. This is illustrated in Figure 1.5.

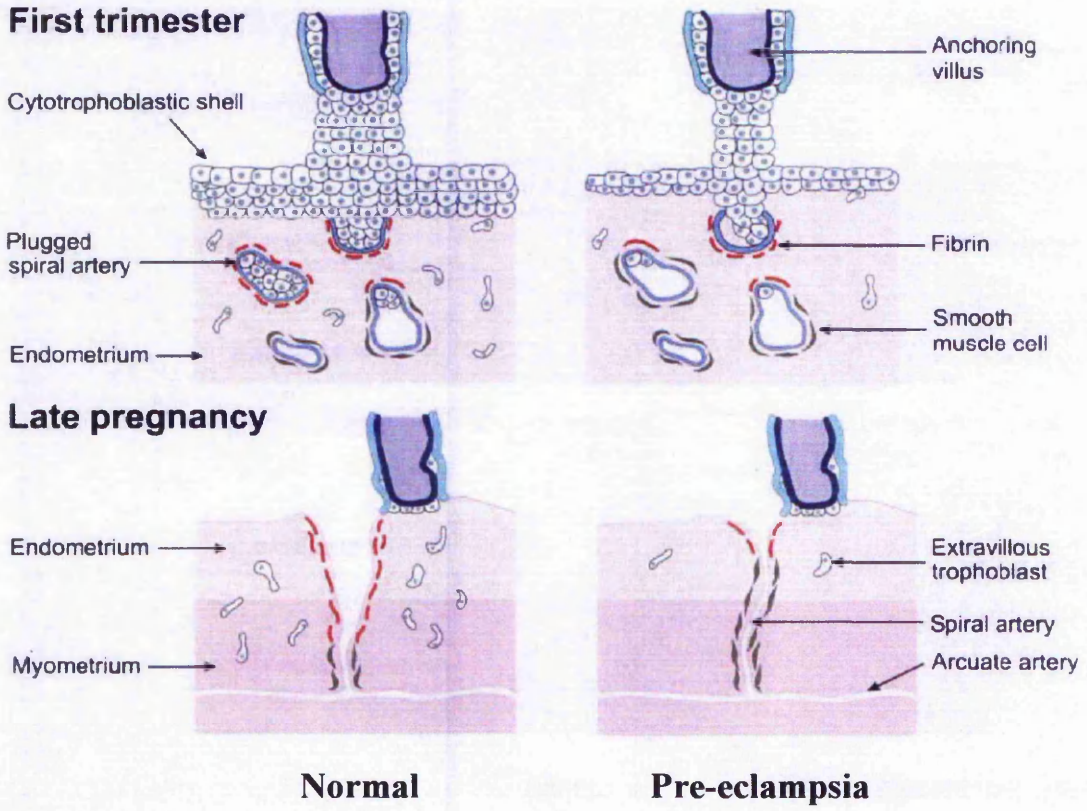


Figure 1.5 Incomplete transformation of the spiral arteries in pre-eclampsia

In pre-eclampsia cytotrophoblastic plugging is reduced and transformation of the spiral arteries are incomplete. Reproduced from Jauniaux *et al.* (2006)

1.2.3.2 Oxidative stress

Oxidative stress is defined as an inbalance between the generation of free radicals (substances with one or more unpaired electrons) and the capacity of antioxidant defence. Biologically the most common free radical species are the superoxide anion ($O_2^{\cdot-}$) and nitric oxide ($NO^{\cdot}$). As well as free radicals there are also nonradical but reactive intermediates such as hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$). Hence, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are collective terms that includes not only the oxygen / nitrogen radicals but also nonradical derivatives. ROS and RNS play a dual role, they can be either harmful or beneficial to living systems (Lowenstein *et al.*, 1994; Palmer and Paulson, 1997). Enzymes such as NAD(P)H oxidase, xanthine oxidase (XO) and nitric oxide synthase (NOS) can all contribute to the generation of ROS and RNS. Mitochondria are known to be a significant source of ROS and RNS (Jezek and Hlavata, 2005). The superoxide anion is produced by electron leakage from the mitochondrial electron transport chain and can be converted to hydrogen peroxide by superoxide dismutase. Whilst the superoxide ion is membrane impermeable, hydrogen peroxide can freely diffuse through the mitochondrial membranes into the cytoplasm. In the presence of iron, hydrogen peroxide can form the highly reactive hydroxyl radical ($OH^{\cdot}$) through the Fenton reaction. Superoxide ion can also react with nitric oxide and form higly reactive peroxynitrite ($ONOO^-$). The formation of reactive oxygen and nitrogen species is illustrated in Figure 1.6.

ROS and RNS can cause indiscriminate damage to all types of biomolecules such as lipids, proteins and DNA (Halliwell, 2006). The state of oxidative stress in a given tissue can be assessed by measuring antioxidant capacity (by measuring individual antioxidant levels), the activity of antioxidant ezymes or by measuring products of oxidative modification such as malondialdehyde (MDA), isoprostanes (lipid oxidation products), carbonyls (oxidative products of proteins) and nitrotyrosine residues (Mates *et al.*, 1999; Adams *et al.*, 2001; Yeum *et al.*, 2004).

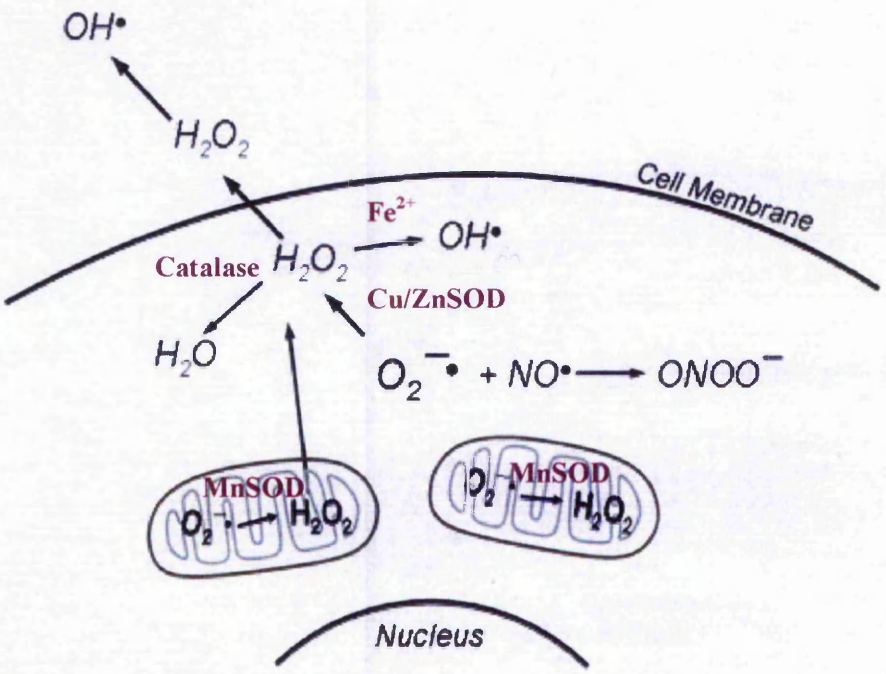


Figure 1.6 Formation of reactive oxygen and nitrogen species

Adapted from Szeto (2006) with modifications. SOD= superoxide dismutase.

Reduced placental perfusion and hypoxia – reoxidation may result in the generation of ROS (Hung *et al.*, 2001). Within the placenta this is thought to be caused by contractions of the spiral arteries, external compression to the arteries (mainly due to uterine contractions) and redistribution of maternal blood flow (such as maternal exercise, change of posture) (reviewed in Burton and Jauniaux, 2004). Although a certain degree of hypoxia – reoxygenation type injury (hence oxidative stress) is observed in normal pregnancy, this stuation seems to be worse in pre-eclamptic pregnancies. There are reports of increased oxidative and nitrative damage and reduced activity of antioxidant enzymes as well as antioxidant molecules in pre-eclampsia (Myatt *et al.*, 1996; Wang and Walsh, 2001; Vanderlelie *et al.*, 2005; Wang *et al.*, 2005; Sharma *et al.*, 2006). This oxidative stress and damage is thought result in endothelial cell dysfunction observed in pre-eclampsia.

1.2.3.3 Inflammatory response

As explained in previous sections, placental implantation involves admixture of genetically dissimilar cells of foetus and those of the mother. Hence the relationship between placenta and the uterus has been compared to a transplanted graft and a recipient. In the early 1950's Medawar put forward the idea of 'foetus as an allograft'. As the feto-placental unit contains paternal antigens which are foreign to the mother, it has been assumed that implantation of the placenta would be under the control of the maternal immune response mediated by T cells recognising paternal driven allo-antigens expressed by the placenta. Consequently, studies trying to explain the contribution of the maternal inflammatory response to the development of pre-eclampsia was based on a T cell mediated immune response. However, recent studies suggest NK cell involvement rather than a T cell mediated immune response (Moffett-King, 2002; Hiby *et al.*, 2004). In the human placenta the syncytiotrophoblast surface (that forms the villous surface and interacts with the circulating maternal immune cells) is major histocompatibility complex (MHC)-negative and consequently unable to induce the antigenic stimulation of maternal T cells. In addition extravillous trophoblasts express a unique combination of HLA-C, HLA-E and HLA-G rather than HLA-A, HLA-B and HLA-D antigens that are known to stimulate a T cell dependent graft-rejection response (Hiby *et al.*, 1999; King *et al.*, 2000a; King *et al.*, 2000b). Uterine NK cells, shown to express a variety of receptors including members of the killer immunoglobulin receptor (KIR) family, recognise HLA-C molecules (Vilches and Parham, 2002). It is suggested that in the placental implantation site a novel form of allorecognition is present and this is based on uterine NK cells interacting with paternal HLA. This immune interaction is unique, couple-specific and does not involve T cells (Moffett and Hiby, 2007). Some KIR/HLA-C combinations are suggested to be unfavourable for trophoblast invasion and this is thought to play a role in the aetiology of pre-eclampsia (Hiby *et al.*, 2004). Nevertheless, this is not applicable to syncytiotrophoblast surfaces that lacks MHC antigens. It is hence suggested that syncytiotrophoblast debris relased to the maternal circulation cause a systemic inflammatory response that develops into pre-eclampsia (Sargent *et al.*, 2006). It should be noted that syncytiotrophoblast debris (or subcellular microparticles) shedding is part of a normal turnover and renewal of the placental surface and present during normal pregnancy, although increased levels are

reported in pre-eclampsia (Knight *et al.*, 1998). Oxidative stress is suggested to be one of the factors that leads to an overload of debris by stimulating apoptosis in pre-eclamptic placentae. The continual clearance of this debris from the maternal circulation could cause a systemic inflammatory response that develops into pre-eclampsia (Redman and Sargent, 2003; Borzychowski *et al.*, 2006).

1.2.4 Prediction and prevention of pre-eclampsia

Despite extensive research in the last decade there has not been a substantial advance in the methods of prediction and prevention of this disorder. It is very difficult to carry out preventive treatment without complete understanding of the aetiology of pre-eclampsia. Several biomarkers have been proposed for the prediction of pre-eclampsia (Chaiworapongsa *et al.*, 2004; Levine *et al.*, 2004; Tjoa *et al.*, 2004; Madazli *et al.*, 2005; Levine *et al.*, 2006; Papageorghiou and Campbell, 2006; Smets *et al.*, 2006). Concentrations of these biomarkers have been reported to be increased / decreased before the onset of the condition. The most promising biomarkers to date are placental protein 13 (PP-13), soluble fms-like tyrosine kinase-1 (sFlt-1), placental growth factor (PIGF) and soluble endoglin (sEng). However, data available so far for these markers are not consistent and do not justify their routine clinical use (Conde-Agudelo *et al.*, 2004; Baumann *et al.*, 2007). Pregnancies complicated by abnormal uterine artery Doppler findings in the second trimester are usually associated with an increase in the risk of pre-eclampsia. Therefore, Doppler ultrasonography is a useful tool to asses any abnormal velocity wave form of uterine artery. However, studies investigating its use for predicting pre-eclampsia concluded that it has a limited value as a screening test for pre-eclampsia (Chien *et al.*, 2000).

Several randomised trials studied the use of various methods to reduce the severity or risk of pre-eclampsia. Antiplatelet drugs (mainly low-dose aspirin) have been suggested to reduce the risk of pre-eclampsia. Recent systematic reviews suggested low-dose aspirin has small to moderate benefits when used to prevent pre-eclampsia with no apparent adverse effects on mother or foetus (Askie *et al.*, 2007; Duley *et al.*, 2007). Calcium supplementation is suggested to reduce the incidence of pre-eclampsia especially in high-risk women with low baseline calcium intake (Herrera *et al.*, 2005) but this is not conclusive (Hofmeyr *et al.*, 2006; Villar *et al.*, 2006).

As pre-eclampsia has been associated with increased oxidative stress, several studies have investigated the use of antioxidants, but recent studies with vitamins C and E found no protection against the development of pre-eclampsia (Poston *et al.*, 2006; Rumbold *et al.*, 2006; Polyzos *et al.*, 2007). Other potential preventive therapies investigated, including magnesium sulphate, fish oil, progesterone, diuretics and garlic, have shown no proven beneficial effects (Sibai *et al.*, 1989; Makrides *et al.*, 2006; Meher and Duley, 2006a,, 2006b; Churchill *et al.*, 2007). Thus at present there is no accepted preventive treatment for pre-eclapmsia in either the general population or in high-risk pregnancies. Clearly frequent screening of high-risk pregnancies for the emergence of hypertension is very important however; probably the most important aspect remains to be the effective management of the condition. Timing of the delivery is crucial in order to prevent pre-term delivery and maternal complications.

1.2.5 Treatment of pre-eclampsia

As the aetiology and pathogenesis of pre-eclampsia remain to be elucidated, delivering the infant and the placenta is the only effective cure. The main goal of treatment of pre-eclampsia is to safeguard the mother against complications and ensuring the delivery of a healthy infant while considering possible effects of intrauterine drug exposure on foetal development (Bolte *et al*, 2001). Treatment of hypertension in pregnancy is different to treatment in nonpregnant individuals in the sense that the duration of therapy is shorter and the exposure to drugs will include both mother and the foetus. As each case differs greatly, duration of foetal exposure to antihypertensive drugs will be highly variable. Drugs administered during the second and third trimester of pregnancy may affect foetal growth and development. In addition, drugs given close to term may have adverse effects on the labour process and the neonate. Parenteral hydralazine, labetalol and oral nifedipine are the most commonly used drugs to control hypertension in pre-eclamptic women. Table 1.1 lists the drugs used in the management of hypertension in pregnancy.

	Drug	Mode of action	Onset of action	Duration of action	Comments / Concerns	FDA Category
Centrally acting agonists	Methyldopa	Dopa decarboxylase inhibitor CNS α_2-receptor agonist	4-6h	2h	Slow onset of action Not recommended for hypertensive crises	B
	Clonidine	CNS α_2-receptor agonist				C
α / β Blocker	Labetalol	α_1 non-selective β-blocker	5-10 min	3-6h	Scalp tingling, Bradycardia, Hypoglycaemia	C
β Blockers	Atenolol	β1 antagonist	2 to 4 h (Oral); 5 min (iv)	24h (Oral)	Hypotension; bradycardia Associated with fetal growth retardation	D
	Metoprolol	β antagonist	1 to 2 h (Oral); 20 min (iv)		Hypotension; edema; flushing; bradycardia	C
	Pindolol	β antagonist			Bradycardia; hypotension	B
	Acebutolol	β1 antagonist			hypotension, bradycardia, heart failure	B
Calcium channel antagonists	Nifedipine	Calcium antagonist	10-15 min	4-5 h	Synergistic interaction with $MgSO_4$, headache, tachycardia, Tocolytic activity	C
	Nicardipine	Calcium antagonist	5-10 min	1-4 h	headache	C
	Isradipine	Calcium antagonist	15-20 min	8-10h	Headache, tachycardia, Effective for nonproteinuric hypertension	C
5-HT_2 receptor antagonist	Ketanserin	5-HT_{2A} receptor antagonist	Minutes	13-16h	QT_c prolongation, dizziness, tiredness	N/A

Vasodilators	Hydralazine	Arterial vasodilator		2-6 hours	Possible hypotension, long experience with its use, numerous side effects	C
	Diazoxide	peripheral arteriolar vasodilator	5-10 min	28±8 h	Rapid hypotension, Hyperglycaemia, Tocolytic activity, use is waning	C
	Nitroglycerin	Relaxation of venous (and arterial) vascular smooth muscle	2-5 min	3-5 min	Requires arterial line for continuous BP monitoring, Potential methemoglobinemia	C
	Sodium nitroprusside	Vasodilator	immediate	1-2 min	Requires arterial line for continuous BP monitoring, Potential cyanide toxicity	C
diuretics	Thiazide	inhibits the reabsorption of sodium and chloride in the proximal and distal tubules also in the loop of Henle			There are no adequate and well-controlled studies in pregnant women	C
Rennin angiotensin aldosterone system	Captopril	Angiotensin-converting enzyme inhibitor	60-90 min (Oral)	2-3h	Contraindicated during pregnancy, foetal exposure second and third trimester associated with oligohydramnios, neonatal anuria or renal failure, skull hypoplasia, foetal death	C (1st trimester) D (2nd and 3rd trimester)
	Enalapril	Angiotensin-converting enzyme inhibitor	60 min (Oral) iv minutes	11h		

Table 1.1 Drugs used for treatment of severe hypertension in pregnancy

N/A: not available, iv: intravenous, FDA: Food and Drug Administration, FDA classification key is given in appendix I.

Adapted from Bolte *et al.* (2001b) with modifications.

Hydralazine shows its effect on blood pressure control by acting directly on vascular smooth muscle. The mechanism of action for hydralazine is not yet clear (Vidrio *et al.*, 2003) but it is considered to be a drug of first choice for the treatment of acute severe hypertension in pregnancy.

Labetalol acts as a competitive antagonist at the α_1-adrenoceptor, β_1-adrenoceptor and the β_2-adrenoceptor. It also has some partial agonist activity at β_2-adrenoceptor. Hypotensive effect of labetalol is a result of vasodilatation induced by blockage of α_1-adrenoceptor and activation of β_2-adrenoceptor on the vascular smooth muscle. Labetalol have been associated with foetal growth restriction in some studies (Sibai *et al.*, 1987) but not in others (Pickles *et al.*, 1989) and generally regarded as safe to use during pregnancy (Sibai, 2002; Podymow and August, 2007; von Dadelszen *et al.*, 2007).

Nifedipine is known to show its anti-hypertensive effects by blocking the voltage-gated Ca^{2+} channels in the vascular cells. Nifedipine does not appear to represent a major teratogenic risk (Magee *et al.*, 1996) although use of short-acting nifedipine for severe hypertension in pregnancy has been associated with maternal hypotension and foetal distress (Waisman *et al.*, 1988; Impey, 1993).

Methyldopa is a prodrug, which requires metabolic conversion to α-methylnoradrenaline in order to produce its effects. α-methylnoradrenaline is handled by the noradrenergic neuron in exactly the same way as noradrenaline, thus it functions as a false transmitter. It has no known major adverse events on foetal development (Elhassan *et al.*, 2002) however, due to the slow onset of action it is not suitable for hypertensive crises (Magee, 2001).

Angiotensin converting enzyme inhibitors (such as captopril and enalapril) are associated with potential adverse foetal effects especially when used in the second and third trimester of pregnancy (Rosa *et al.*, 1989; Barr and Cohen, 1991; Hanssens *et al.*, 1991; Pryde *et al.*, 1993; Murki *et al.*, 2005). Diuretics inhibit sodium-potassium chloride co-transport hence they block the re-uptake of these ions to the plasma. Diuretics are known to impair the plasma volume expansion of normal pregnancy. This remains as a concern for their use during pregnancy (reviewed in Churchill *et al.*, 2007).

1.2.5.1 Ketanserin: a serotonin 2 receptor blocker

In 1981 Leysen *et al.* discovered ketanserin (3-{2-[4-(4-Fluorobenzoyl) piperidino] ethyl} quinazoline-2,4 (1H,3H)-dione also named R-41468) which selectively binds to 5-HT_2 receptors and has no significant effect on the $5HT_1$, $5HT_3$ or $5HT_4$ receptor families. Within the 5-HT_2 receptor family ketanserin has the highest affinity for 5-HT_{2A} receptor and usually considered to be selective for 5-HT_{2A} receptor. Ketanserin also has a low affinity for α_1-adrenergic receptors although in humans the antihypertensive properties of ketanserin do not appear to involve inhibition of these receptors (Vanhoutte *et al.*, 1983). The chemical structure of ketanserin is shown in Figure 1.7

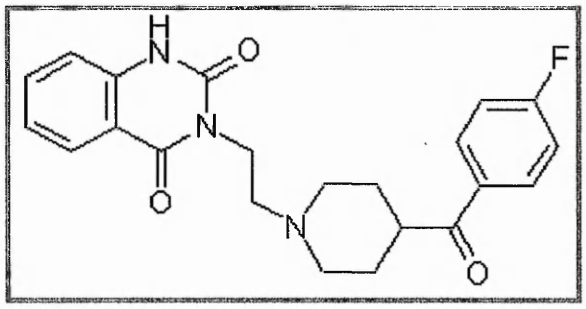


Figure 1.7 Chemical structure of ketanserin

The drug acts as an antagonist of the $5HT_{2A}$ receptor in blood vessels, counteracting the vasoconstrictive response to 5-HT (Bolte *et al.*, 2001b). Ketanserin has been suggested to have therapeutic potential in hypertension as well as in peripheral vascular disease. It causes few side-effects such as dizziness, tiredness oedema, dry mouth and weight gain but its positive effect on platelet aggregation and thrombus formation is considered as an advantage (Brogden and Sorkin, 1990). Ketanserin has been shown to be metabolised by keton reduction and oxidative N-dealkylation to ketanserinol. There seems to be an oxidation-reduction equilibrium between ketanserin and ketanserinol. The metabolite ketanserinol has a 1000 fold lower affinity for the 5-HT_{2A} receptor than ketanserin and it is excreted mainly in urine. (Van Peer *et al.*, 1986).

Similar to other drugs, when used during pregnancy both ketanserin and ketanserinol cross the placenta and are detectable in neonatal plasma after birth (Hanff *et al.*, 2004).

Ketanserin is classified as an anti-hypertensive by the World Heath Organization; however, its use is limited to the Netherlands and South Africa. First studies using ketanserin in human pregnancy were conducted in the immediate postpartum period (Montenegro *et al.*, 1985). This was shortly followed by a report on the use of ketanserin with viable foetuses (Hulme and Odendaal, 1986). Subsequently ketanserin was reported to effectively lower blood pressure in the treatment of hypertension during pregnancy both orally (Voto *et al.*, 1987; Steyn and Odendaal, 2001) and intravenously (Steyn and Odendaal, 2000). A few studies have compared the maternal and foetal outcomes and antihypertensive efficacy of ketanserin to other drugs. Rossouw *et al.* (1995) compared intravenous ketanserin with hydralazine in late pregnancy. They reported a more gradual blood pressure control in the ketanserin group (no hypotension) while both groups had similar umbilical blood flow, hence they suggested ketanserin was safer to use. Moreover, in another study with early onset, severe pre-eclamptic women the anti-hypertensive efficacies of ketanserin and dihydralazine were found comparable while fewer maternal complications occurred in the ketanserin group (Bolte *et al.*, 1999). However, Steyn and Odendaal (1997) reported that ketanserin was less effective in controlling blood pressure compared to dihydralazine but concluded this might be due to sub-optimal dosage. In addition, a recent study reported lack of efficacy of intravenous ketanserin when used in a subgroup of severe early onset pre-eclamptic patients (Hanff *et al.*, 2006). Studies on pharmacokinetics of ketanserin in pre-eclamptic patients suggested that the differences in anti-hypertensive responses might not be due to pharmacokinetic differences, but related to severity of the disease and / or differences in 5-HT_{2A} receptor activity within a pre-eclamptic population (Hanff *et al.*, 2005).

1.3 5-Hydroxytryptamine (5-HT)

5-Hydroxytryptamine (serotonin or 5-HT) was isolated for the first time by Erspamer and Vially (1937) from enterochromaffine cells of the gut. Colour tests suggested that the substance behaved like an indole so they have named it enteramine. Later Rapport, Green, and Page (1948) isolated a substance from bovine serum and due to its vasoconstrictor properties they named it serotonin which depicted the source (serum) and the activity (constriction) (Whitaker-Azmitia, 1999). It was not until 1952 that enteramine and serotonin, discovered by the two independent groups, were revealed to be the same substance, 5-hydroxytryptamine (Erspamer and Asero, 1952). Following studies on tissue distribution, 5-HT was found in extracts of mammalian brain (Twarog and Page, 1953) and this brought it into the field of neuroscience (Woolley and Shaw, 1954). 5-HT is involved in several physiological processes such as smooth muscle contraction, blood pressure regulation, peripheral and central nervous system neurotransmission. In addition studies have reported a mitogenic effect of 5-HT in a wide variety of cell types including placental cells (Fecteau and Eiler, 2001; Sonier *et al.*, 2005). Although the involvement of 5-HT in the CNS has been long established, its role in the periphery is far less defined.

1.3.1 Synthesis, metabolism, transport and storage of 5-HT

The majority of 5-HT is synthesised in the enterochromaffin cells of the intestine, while the remaining is synthesised in raphe nuclei of the brain, the pineal gland and neuroendothelial cells lining the lung (Kuhn, 1999). A two-step enzymatic pathway is employed in the synthesis of 5-HT from the amino acid tryptophan. The first step is the hydroxylation of tryptophan to 5-hydroxytryptophan (5-HTP) by the rate limiting enzyme tryptophan hydroxylase (TPH). Further decarboxylation of 5-HTP by aromatic-$_L$- amino acid decarboxylase (AADC) forms 5-HT. Approximately 2 % of dietary tryptophan is converted into 5-HT. As well as playing a role in the synthesis of 5-HT, enterochromaffin cells are also storage sites for it. About 80 % of total body 5-HT is synthesised and stored in enterochromaffin cells (Erspamer and Testini, 1959).

Another major storage site are dense granules of platelets. As 5-HT is a vasoactive amine, elevated plasma concentrations may be hazardous; therefore in the circulation it is mostly confined to platelets. Three processes limit any increase of free 5-HT in blood:

1) 5-HT is taken up rapidly by platelets and stored in dense granules
2) Plasma 5-HT binds to transport proteins
3) Free 5-HT is removed rapidly from plasma by endothelial cells, or it is enzymatically degraded

The function of 5-HT in the blood is to promote platelet aggregation and blood clotting. Platelets also contain 5-HT_{2A} receptors, these receptors when activated promote further platelet activation and aggregation (Vanhoutte, 1991). During aggregation usually on the site of vascular injury platelets release 5-HT thus providing a unique delivery mechanism (Kema *et al.*, 2000).

Under physiological conditions 5-HT is not capable of crossing the membrane lipid bilayer, hence serotonin transporters (SERTs) facilitate uptake and release of 5-HT from cells. The SERT protein has been located in several tissues, including the placenta, heart, lung, blood vessels, brain, platelets, adrenal gland, liver and kidney (Cortes *et al.*, 1988; Lesch *et al.*, 1993; Mortensen *et al.*, 1999; Ni *et al.*, 2004). The functions of SERT in these tissues include regulation of cardiac and smooth muscle contractility, platelet aggregation, neuronal activity and urinary excretion. SERT has been a target in the treatment of CNS disorders such as depression where selective serotonin reuptake inhibitors are used (Dalack *et al.*, 1995).

The majority of 5-HT is metabolised by Monoamine Oxidase (MAO; EC 1.4.3.4) to form 5-hydroxyindole acetaldehyde, which in turn is oxidised by aldehyde dehydrogenase to produce 5-hydroxyindole acetic acid (5-HIAA). 5-HIAA is water soluble therefore it is excreted almost entirely in the urine. Synthesis and metabolism of 5-HT is demonstrated in Figure 1.8 and Table 1.2 summarises tryptophan, 5-HT and 5-HIAA concentrations in several biological matrices.

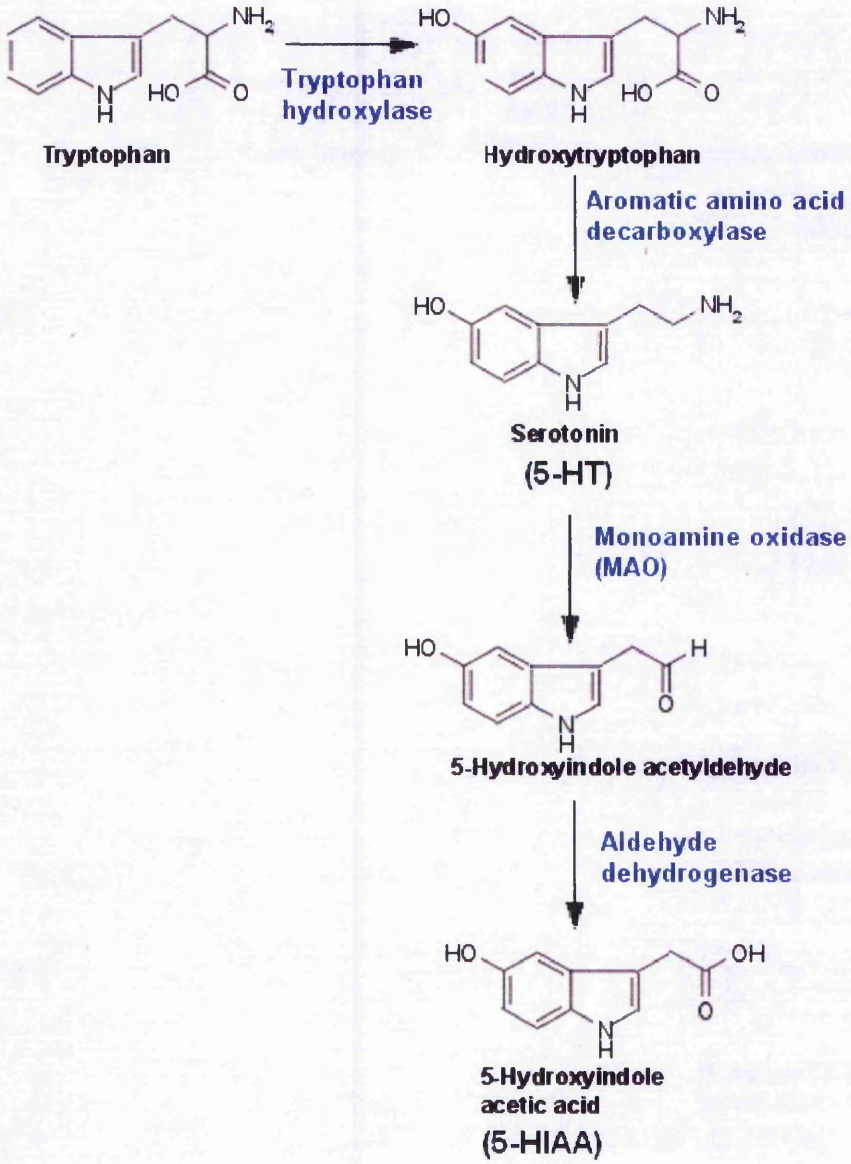


Figure 1.8 Synthesis and metabolism of 5-HT

	Tryptophan	5-HT	5-HIAA
Urine	73-330 µmol / 24h	340-950 nmol / 24h	2-50µmol / 24h
Platelet Rich Plasma (PRP)		3.81±0.87 nmol / 10^9 platelets	
Platelet Poor Plasma (PPP)	61±5 µmol/l (total) 6.1±0.7µmol/l (free)	2.8±1.4 nmol / l	11.0±0.5 nmol / l
CSF	1257-2757 nmol / l	5.7-12.0 nmol / l	80.8-143.2 nmol / l

Table 1.2 Concentrations of tryptophan, 5-HT and 5-HIAA in several biological matrices

CSF: cerebrospinal fluid, 5-HT: 5-Hydroxytryptamine, 5-HIAA: 5-hydroxyindoleacetic acid (Reproduced from Kema *et al.*, 2000)

1.3.2 Factors affecting 5-HT

In the circulation 5-HT is mostly confined to platelets but a small amount is present in plasma as free 5-HT (Ortiz *et al.*, 1988). For measuring blood concentrations of 5-HT researchers have used mainly three pools of 5-HT separated from whole blood; platelets, platelet rich plasma (PRP) and platelet poor plasma (PPP). PRP has been used as a measure of whole blood concentrations and PPP as a measure of circulating or free 5-HT. However, in order to use 5-HT concentrations as a diagnostic measure or experimentally one should first define the normal physiology of 5-HT. As a result there has been a great deal of research into factors affecting 5-HT levels, such as age, gender, diet, menstruation and time of day.

Studies investigating involvement of circadian rhythm on platelet 5-HT levels failed to show any differences in both healthy male (Eynard *et al.*, 1993) and female subjects (Montero *et al.*, 1989). In addition they have not found significant seasonal variances (Eynard *et al.*, 1993). Moreover, 5-HT levels do not seem to vary between male and females. For example, in a large-scale study Jernej *et al.* (2000) compared platelet 5-HT levels from male and female subjects and they found no significant differences between the sexes. However, they noticed a progressive decrease of platelet 5-HT levels with age, reaching statistical significance between the extreme age groups (under 25s versus over 55s). PPP 5-HT levels on the other hand do not seem to be affected by age except in very old subjects (85-92 years old) where higher levels were repoted (Hindberg and Naesh, 1992). Variations in 5-HT levels have been reported during the menstrual cycle. PPP 5-HT levels were higher at the premenstrual and ovulation period when compared to the first day of menstruation (Hindberg and Naesh, 1992).

The effects of diet and consumption of certain foods containing biogenic amines such as nuts, banana, kiwi fruit, plums, pineapple, tomato, grapes and chocolate on plasma and urinary 5-HT / metabolite 5-HIAA levels have also been studied. While urinary 5-HIAA levels was found to be influenced by ingestion of these foods (Feldman and Lee, 1985; Mashige *et al.*, 1996) platelet 5-HT levels on the other hand does not seem to be affected (Kema *et al.*, 1992).

1.3.3 5-HT receptors

The physiological effects of 5-HT are produced through a variety of membrane-bound receptors. The first classification of 5-HT receptors was suggested by Gaddum and Picarelli (1957) following their observation that functional responses of the guinea pig ileum could be partially inhibited by morphine (M) while the rest of the response was blocked by dibenzyline (D). Hence they proposed subdivision of 5-HT receptors and naming them M and D receptors, respectively. This was followed by the studies utilising radioligands (Bennett and Snyder, 1976; Peroutka and Snyder, 1979) and a new classification system was proposed consisting of three main groups of 5-HT receptors, namely 5-HT_1-like, 5-HT_2 (formerly named D receptor) and 5-HT_3 (formerly named M receptor) (Bradley *et al.*, 1986). The development of molecular biology and cloning techniques allowed the confirmation of the existence of multiple 5-HT receptors previously identified using pharmacological and biochemical techniques (Fargin *et al.*, 1988). In addition several gene products for putative 5-HT receptors have been identified (Matthes *et al.*, 1993). Currently 5-HT receptors are divided into seven families (or classes), $5HT_1$ to $5HT_7$, according to their pharmacological, transductional and structural profiles (Table 1.3) (Hoyer *et al.*, 2002). According to this classification system the use of lower case in the nomenclature of a receptor designates that the receptor has not been definitely demonstrated to function in native systems. All 5-HT receptor families belong to the G-protein-coupled receptors (GPCRs) super family, with the exception of the $5HT_3$ receptor, which is a ligand gated ion channel (Hoyer and Martin, 1996; Hoyer *et al.*, 2002). G-protein-coupled 5-HT receptors namely $5HT_1$, 5-HT_2, $5HT_4$, $5ht_5$, $5HT_6$ and $5HT_7$ display the basic architecture of the GPCRs. These are integral membrane proteins with seven transmembrane helical domains connected by three intercellular (i_1, i_2 and i_3) and three extra cellular (e_1, e_2 and e_3) loops. The amino terminus is oriented toward the extra cellular space, in contrast the carboxyl terminus is oriented toward the cytoplasm (Baldwin, 1993). The 5-HT_2 receptors (5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}) are preferentially coupled to Gq/11 to increase the hydrolysis of inositol phosphates and elevate cytosolic calcium. 5-HT_4, 5-HT_6 and 5-HT_7 receptors activate adenylate cyclase while 5-HT_1 and $5ht_5$ inhibit (Hoyer *et al.*, 2002).

Receptor (Previous nomenclature)	**Subtypes** (Previous nomenclature)	**Functional response**	**Primary transduction mechanisms**	
			Effector	**Transducer**
5-HT$_1$	5-HT$_{1A}$	Anxiety (Gardner, 1986) Depression (Cervo *et al.*, 1988) Hypotension (Dreteler *et al.*, 1990)	cAMP ↓	(G_i / G_o)
	5-HT$_{1B}$ (5-HT$_{1D\beta}$)	Behavioural effects (Lucki, 1992) Vasoconstriction (rat caudal artery) (Craig and Martin, 1993)		
	5-HT$_{1D}$ (5-HT$_{1D\alpha}$)	Vasoconstriction (cerebral arteries) (Nilsson *et al.*, 1999a)		
	5-ht$_{1E}$	Not known		
	5-HT$_{1F}$ (5-HT$_{1E\beta}$, 5-HT6)	Not known		
5-HT$_2$	5-HT$_{2A}$ (D, 5-HT$_2$)	Vasoconstriction (MacLean *et al.*, 1996) Platelet aggregation and thrombus formation (De Clerck *et al.*, 1984b) Behavioural effects (Bhatnagar *et al.*, 2001)	PLC ↑	($G_{q/11}$)
	5-HT$_{2B}$ (5-HT$_{2F}$)	Rat stomach fundus muscle contraction (Cohen and Fludzinski, 1987) Vascular smooth muscle relaxation (Glusa and Pertz, 2000)		
	5-HT$_{2C}$ (5-HT$_{1C}$)	Feeding (Heisler *et al.*, 1998)		

5-HT$_3$ (M)	5-HT$_{3A}$ 5-HT$_{3B}$ 5-ht$_{3C}$ 5-ht$_{3D}$ 5-ht$_{3E}$	Vasodilation (human forearm) (Blauw *et al.*, 1988) Anxiety (Smith *et al.*, 1999) Unknown Unknown Unknown		Ion Channel
5-HT$_4$		Contraction (colon) (Ono *et al.*, 2005) Relaxation (sheep pulmonary vein) (Cocks and Arnold, 1992)	cAMP ↑	(G_s)
5-ht$_5$	5-ht$_{5A}$ (5-HT$_{5a}$) 5-ht$_{5B}$	unknown	cAMP ↓	(G_i / G_0)
5-HT$_6$		May be related to behavioral disorders (Bentley *et al.*, 1999; Lindner *et al.*, 2003)	cAMP ↑	(G_s)
5-HT$_7$ (5-HT$_x$, 5-HT$_1$-like)		May be related to depression (Guscott *et al.*, 2005)	cAMP ↑	(G_s)

Table 1.3 Classification for 5-HT receptors

Receptor subtypes represented with lower case designates that these receptors have not been demonstrated to be functional in native systems. PLC = Phospholipase C, cAMP = 3'-5' cyclic adenosine monophosphate, G_s = G protein stimulatory, G_i = G protein inhibitory.

↑ Arrow represents increase; ↓ arrow represents decrease.

(Hoyer and Martin, 1997; Hoyer *et al.*, 2002)

1.3.3.1 5HT$_1$ receptors

The 5-HT$_1$ receptor class contain five different receptors. In humans these receptors share 41-63% overall sequence identity and all are found in the brain (Waeber *et al.*, 1988; Beer *et al.*, 1992; Varnas *et al.*, 2001). The 5-HT$_{1A}$ receptor subtype is widely distributed in the central nervous system (CNS) particularly in the dorsal raphe, hippocampus and cortex (Burnet *et al.*, 1995). In the periphery the 5-HT$_{1A}$ receptor is expressed in kidneys (Raymond *et al.*, 1993). Unlike 5-HT$_{1A}$ receptors 5-HT$_{1B}$ receptors are also found to be expressed in coronary arteries and thought to be involved in vasoconstriction of these arteries (Ishida *et al.*, 1999; Nilsson *et al.*, 1999b).

The human 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors are very similar in sequence although they are encoded by two distinct genes (Hartig *et al.*, 1996). Both receptors are believed to have potential roles in the pathogenesis of migraine headaches. Due to lack of selective ligands the precise function of these receptors remains to be elucidated. However there is a great interest in developing selective agonists and antagonist for these receptors mainly due to antimigraine properties of sumatriptan (a non selective 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor agonist with low selectivity against other receptors). This compound is thought to act either via direct vasoconstriction of cerebral arteries (via 5HT$_{1B}$ receptors) or by inhibiting release of vasoactive neuropeptides (Goadsby, 1998).

5-ht$_{1E}$ and 5-HT$_{1F}$ receptor subtypes are very closely related, they share 70 % sequence homology across the seven transmembrane domains (Miller and Teitler, 1992; Adham *et al.*, 1993). However the physiological function of these receptors remains unknown.

1.3.3.2 5-HT$_2$ receptors

The 5-HT$_2$ receptor class consists of 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors which demonstrate 46-50 % overall sequence identity (Kursar *et al.*, 1992). 5-HT$_2$ receptors have been a clinical interest as they are thought to be involved in many physiological function of 5-HT both in the CNS and the periphery.

1.3.3.2.1 5-HT$_{2A}$ receptors

The 5-HT$_{2A}$ receptor is coded for on human chromosome 13q14-q21 (Sparkes *et al.*, 1991) and consists of three exons separated by two introns (Chen *et al.*, 1992a). These introns must be removed by ribonucleic acid (RNA) splicing prior to RNA translation. As alternative RNA splice sites can be utilised in the removal of introns, multiple protein isoforms may occur. The 5-HT$_{2A}$ receptor messenger ribonucleic acid (mRNA) indeed undergoes alternative splicing that produces a 118-base insert in the fourth transmembrane domain and codes for a truncated protein of the receptor. Studies with transfected cell lines however indicated this variant of the receptor is not functionally coupled to calcium mobilisation (Guest *et al.*, 2000).

The 5-HT$_{2A}$ receptor consists of 471 amino acids in mouse, rat and human. The receptor is widely distributed in peripheral and central tissues (Burnet *et al.*, 1995; Laporte *et al.*, 1996; Nilsson *et al.*, 1999b). In the periphery, 5-HT$_{2A}$ receptors are located in platelets (De Clerck *et al.*, 1984a; de Chaffoy de Courcelles *et al.*, 1985), vascular smooth muscle (Cohen *et al.*, 1981), uterine smooth muscle (Wilcox *et al.*, 1992). In blood vessels 5-HT$_{2A}$ receptors are known to be expressed in smooth muscle cells (Ullmer *et al.*, 1995). These receptors are involved in contractile responses of vascular smooth muscle (de Chaffoy de Courcelles *et al.*, 1985; MacLean *et al.*, 1996; Banes *et al.*, 1999) as well as non-vascular smooth muscle (uterine contraction) (Ichida *et al.*, 1983). 5-HT$_{2A}$ receptors have also been shown to be involved in platelet aggregation (De Clerck *et al.*, 1984b).

1.3.3.2.2 5-HT$_{2B}$ receptors

The 5-HT$_{2B}$ receptor gene is localised to chromosome 2q36.3-2q37.1 and contain two introns hence the gene structure is similar to 5-HT$_{2A}$ receptor. 5-HT$_{2B}$ receptor mRNA is expressed in liver, kidney, pancreas and spleen (Bonhaus *et al.*, 1995). However the presence of the receptor in the brain appears to be controversial (Kursar *et al.*, 1994; Pompeiano *et al.*, 1994). 5-HT$_{2B}$ receptors are involved in rat stomach fundus muscle contraction (Cohen and Fludzinski, 1987) and vascular smooth muscle relaxation (Glusa and Pertz, 2000).

1.3.3.2.3 5-HT$_{2C}$ receptors

The 5-HT$_{2C}$ receptor gene is localised to chromosome Xq24 and contains three introns. 5-HT$_{2C}$ receptors are expressed nearly in every part of the brain (Mengod *et al.*, 1990; Pompeiano *et al.*, 1994). This receptor plays an important role in food intake and body weight (Sargent *et al.*, 1997; Vickers *et al.*, 2000; Somerville *et al.*, 2007). Activation of 5-HT$_{2C}$ receptors decreases body weight in obese subjects (Sargent *et al.*, 1997) and there has been a great interest in developing agonists for the receptor as potential anti-obesity agents in order to regulate appetite and control body weight (Halford *et al.*, 2005).

1.3.3.3 5-HT$_3$ receptors

This ligand-gated ion channel receptor (Maricq *et al.*, 1991) consists of 5 subunits encoded by HTR3A, HTR3B, HTR3C, HTR3D and HTR3E genes. As in other ligand gated ion channels subunits are arranged around a central ion conducting pore. Binding of 5-HT to the receptor opens the channel which is permeable to sodium, potassium, and calcium ions and results in an excitatory response. Functional channels can consist of five identical 5-HT$_{3A}$ subunits (homopentameric) or a mixture of 5-HT$_{3A}$ and one of the other four subunits (heteropentameric). However only 5-HT$_{3A}$ homopentameric channels are functional (Niesler *et al.*, 2007).

1.3.3.4 Other 5-HT receptors

5-HT_4, 5-HT_6 and 5-HT_7 receptors are all coupled preferentially to Gs however they are classified as distinct receptor classes as they show < 40% overall sequence identity with other 5-HT receptors. 5-HT_4 receptors are found in the brain as well as in peripheral organs such as the gastrointestinal tract, bladder and heart. The main physiological role of the receptor seems to be in the gastrointestinal tract (Rivkin, 2003). Genes for putative 5-ht_5 receptors (5-ht_{5A} and 5-ht_{5B}) have been cloned (Matthes *et al.*, 1993). In humans only the 5-ht_{5A} receptor gene encodes a functional protein.The human 5-ht_{5B} gene on the other hand gives rise to a non-functional protein due to stop codons in the gene (Grailhe *et al.*, 2001). Both 5-HT_6 and 5HT_7 receptors have been identified in the brain and both have high binding affinity to antidepressant and antipsychotic drugs. In addition 5-HT_7 receptor is thought to mediate vasodilation (Villalon *et al.*, 2001) and smooth muscle relaxation (Terron and Falcon-Neri, 1999; Janssen *et al.*, 2002).

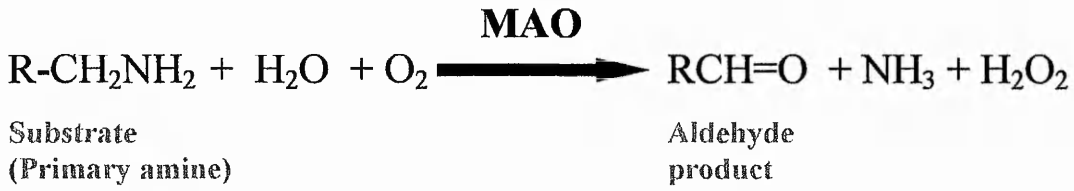
1.3.4 5-HT and disease relationship

5-HT is known to be involved in a wide variety of physiological process and is thought to be involved in several pathological conditions both in the CNS and in the periphery. It is known to be involved in several important brain functions, ranging from the regulation of circadian changes in the sleep-wake cycle to processing of complex information relating to emotion and cognition. Hence aberrations in its central nervous system function are believed to be involved in anorexia (Brewerton and Jimerson, 1996), anxiety (Hedges *et al.*, 2007), depression (Cowen, 1993) and schizophrenia (Iqbal and van Praag, 1995). In addition in Alzheimer's (Meltzer *et al.*, 1998) and Parkinson's disease (Halliday *et al.*, 1990) degeneration of serotonergic neurons have been documented. In the periphery abnormalities in 5-HT related processes have been reported in drug-induced emesis (Endo *et al.*, 2000), migraine (Hamel, 2007), hypertension (Myers *et al.*, 1985), some symptoms of carcinoid syndrome (Feldman, 1978) and pre-eclampsia (Hutter *et al.*, 1996; Bolte *et al.*, 2001a). Since the major part of this thesis focuses on the role of 5-HT in pre-eclampsia a more detailed description of changes concerning 5-HT is given in chapter V.

1.4 Monoamine oxidase

Monoamine Oxidase (MAO; EC 1.4.3.4) is a flavin-containing enzyme mainly located in the mitochondrial outer membranes. It is involved in the oxidative deamination of biogenic amines such as the neurotransmitters norepinephrine, dopamine and 5-HT. MAO was first identified as tyramine oxidase by Mary Hare in liver (Hare, 1928). It was later revealed that tyramine oxidase, noradrenaline oxidase and aliphatic amine oxidase were the same enzyme (Blaschko *et al.*, 1937). MAO is capable of metabolizing primary, secondary and tertiary amines but does not metabolize diamines (such as histamine).

MAO contains a covalently bound cofactor flavin adenine dinucleotide (FAD) and catalyses oxidative deamination of monoamines to their corresponding aldehydes, ammonia and hydrogen peroxide (Weyler *et al.*, 1990b). The oxidation of amine substrates is achieved through the reduction of the FAD. The imine product is non-enzmatically hydrolysed to an aldehyde and ammonia is released. Oxidised flavin is then regenerated by conversion of oxygen to hydrogen peroxide (Houslay and Tipton, 1973). The aldehyde intermediate is rapidly metabolized, usually by oxidation (by the enzyme aldehyde dehydrogenase) to the corresponding acid, or in some cases to the alcohol (by the enzyme aldehyde reductase). The general reaction mechanism is presented in Figure 1.9.



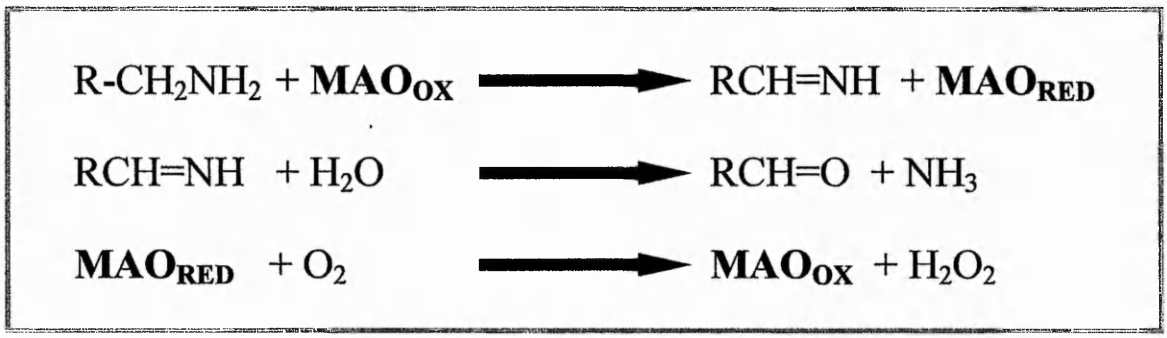


Figure 1.9 Reaction mechanism of MAO

The primary product of MAO acting on a monoamine is the corresponding aldehyde, ammonia and hydrogen peroxide. Steps involved in the reaction mechanism of MAO are presented in the box. OX= oxidised, RED= reduced

1.4.1 Isoforms

In 1963 for the first time Gorkin suggested that MAO could exist in at least two forms; MAO-A and MAO-B (Gorkin, 1963). Studies showed that these isoforms differed in their substrate and inhibitor specificity. MAO-A was found to be sensitive to the inhibitor clorgyline and preferentially metabolized noradrenaline and 5-HT (Johnston, 1968), whereas MAO-B was sensitive to the inhibitor deprenyl and preferred benzylamine and phenylethylamine as substrates (Magyar and Knoll, 1977). The presence of the two isoforms has been confirmed with immunological studies (Kochersperger *et al.*, 1985; Westlund *et al.*, 1985; Church *et al.*, 1994; Finch *et al.*, 1995), by molecular genetics (Bach *et al.*, 1988) and most recently differences in protein structure (Binda *et al.*, 2002; Ma *et al.*, 2004). The A and B isoforms are encoded by distinct genes located in a 240 kb interval of the chromosome region Xp11.23–11.4 and a comparison of the deduced amino acid sequences shows that they share approximately 70% sequence identity (Bach *et al.*, 1988; Chen *et al.*, 1992b). Both proteins are attached to the outer mitochondrial membrane (Schnaitman *et al.*, 1967) by a transmembrane helix that extends out from the main body of the enzyme (Schnaitman *et al.*, 1967; Ma *et al.*, 2004). The insertion of MAO into the outer mitochondrial membrane is dependent on the polypeptide ubiquitin (Zhuang *et al.*, 1992). Both isoforms are divided into the three functional domains: membrane binding, flavin binding and substrate binding regions. The structure of the two isoforms is similar; both enzymes contain two identical subunits each with a cofactor FAD. However, comparison of the inhibitor binding sites of the two isoform has revealed major differences in the side-chains within the active centres; explaining the differences in their substrate and inhibitor preference (Ma *et al.*, 2004). The active site of MAO-B consists of an entrance cavity and a substrate binding cavity. Passage of the ligands from the entrance cavity to the flavin containing region depends on the confirmation of Ile-199. The equivalent residue in MAO-A is Phe-208, which has a strong impact on the binding of ligands in the active site (Hubalek *et al.*, 2005). The two most important residues in the active site are tyrosines that are located perpendicular to the plane of the flavin: tyrosines 407, 444 in MAO-A and 398 and 435 in MAO-B. It has been demonstrated that mutating these residues results in inactive enzyme (Geha *et al.*, 2002).

1.4.2 Factors affecting monoamine oxidase

MAO-A and MAO-B activities are regulated independently by endogenous and exogenous factors including genetic determinants, hormones, diet and aging. Both *in vivo* and *in vitro* studies have shown that hormonal influences are involved in MAO expression (Carlo *et al.*, 1996; Holschneider *et al.*, 1998; Slotkin *et al.*, 1998; Bompart *et al.*, 2001). Changes in MAO activity levels have also been observed in relation to aging (reviewed in Nicotra *et al.*, 2004). Several studies have reported high brain MAO-B activity in neurodegenerative diseases such as Alzheimer's diseases (Jossan *et al.*, 1991; Saura *et al.*, 1994a; Saura *et al.*, 1994b). The strong relationship between changes in MAO levels and age-related neuropsychiatric and neurological diseases has stimulated research on brain MAO activity during aging. Studies using human brain from post-mortem samples have shown an age-related increase in MAO-B activity and little or no variation in MAO-A activity (Fowler *et al.*, 1980; Kornhuber *et al.*, 1989; Sparks *et al.*, 1991). These findings were also confirmed with studies employing other techniques such as high-resolution radioautography and positron emission tomography (PET) (Fowler *et al.*, 1997; Saura *et al.*, 1997). Differences in activity levels and expression of MAO-A and MAO-B are also apparent during early development. For example, studies carried out in both human and rat brain demonstrate that during development MAO-A appears before MAO-B (Mantle *et al.*, 1976; Lewinsohn *et al.*, 1980). Following birth MAO-B levels rise rapidly, which is thought to be due to proliferation of astrocytes in the brain. Similarly in (human) foetal lung, aorta and digestive tract, MAO-A emerges before MAO-B (Lewinsohn *et al.*, 1980).

β-carbolines and tetrahydro-β–carbolines, which have a similar structure to tryptamine, are known to have MOA competitive reversible inhibitor properties (Kim *et al.*, 1997; Herraiz and Chaparro, 2006). These are naturally occurring compounds, found in many plant and mammalian tissues, and can also occur during food production, processing and storage. Roasted, fermented and smoked foodstuffs are known to have high amounts of tetrahydro-β–carbolines (reviewed in Herraiz, 2000). Tobacco smoke also contains β-carbolines. Studies have demonstrated that tobacco smokers have up to a 28% lower brain MAO-A and a 40% lower brain MAO-B activity compared to non-smokers (Fowler *et al.*, 1996a; Fowler *et al.*, 1996b).

In addition smokers exhibit lower MAO activity in their platelets (Oreland *et al.*, 1981; Norman *et al.*, 1987). Other compounds found in tobacco smoke with MAO inhibitor properties are 2,3,6-trimethyl-benzoquinone (Khalil *et al.*, 2000) and 2-naphthylamine (Hauptmann and Shih, 2001).

1.4.3 Tissue distribution of monoamine oxidase

MAO probably is one of the most widely distributed enzyme in humans. Moreover its presence is not limited to mammalian species; it also appears that MAO is present in avians (Fowler and Callingham, 1977; Hall *et al.*, 1985), piscines (Hall *et al.*, 1982; Hall and Uruena, 1982) as well as invertebrates (Blaschko and Hawkins, 1952).

The level of MAO expression shows tissue specificificty and the rates of synthesis and degradation of the enzyme, hence the half-life is thought to vary in different tissues (Weyler *et al.*, 1990a). Morover, the two isoforms seem to be under different control. In tissues MAO-A and MAO-B may either co-localise in the same cell type, or one of the two isoform may be predominant. For example, in brain MAO-A localises in adrenergic, noradrenergic and dopaminergic neurons, while MAO B is expressed in serotoninergic and histaminergic neurons and astrocytes (Luque *et al.*, 1995). Thus the enzyme localisation does not always correspond with the substrate preference of the isotype. Outside the nervous system, MAO-A and MAO-B may be co-expressed, such as in human liver, or one of the isoforms might be prevalent, such as MAO-A in placenta or MAO B in platelets (Sivasubramaniam *et al.*, 2003; reviewed in Billett, 2004).

1.4.3.1 Monoamine oxidase in peripheral tissues

In mammals MAO has been identified in all cell types except erythrocytes (reviewed in Billett, 2004). In the majority of peripheral tissues both isoforms co-localise (Rodriguez *et al.*, 2000a; Rodriguez *et al.*, 2000b; Rodriguez *et al.*, 2001). In adults the highest activities of MAO-A are detected in placenta and liver. In the blood vessels (areteries and veins), muscular layers are reported to contain moderate levels of both isoforms whilst only low levels of MAO proteins are found in the endothelial layer (Rodriguez *et al.*, 2001; Sivasubramaniam *et al.*, 2003).

1.4.3.2 Monoamine oxidase in placenta

Although most human tissues contain both isoforms placenta has been shown to contain only MAO-A protein (Sivasubramaniam *et al.*, 2002; Sivasubramaniam *et al.*, 2003) while both MAO-A and MAO-B mRNAs were present (Auda *et al.*, 1998; Sivasubramaniam *et al.*, 2002). Moreover, the use of MAO-A specific monoclonal antibodies allowed the study of cellular localisation of the protein in placental tissue. These studies demonstrated that MAO-A is localised in the cytoplasm of syncytiotrophoblast and intermediate trophoblasts (Church *et al.*, 1994).

The functional role of MAO in placenta is not fully understood. However it is generally accepted that it protects the foetus from the harmful effects of catecholamine (and 5-HT) and by catabolising vasoactive substances it limits their transfer through the placenta (Gujrati *et al.*, 1985). Studies carried out on rat reported increased levels of MOA activity in uterus, ovary and placenta near term, which partly explains the importance of MAO for the maintenance of healthy pregnancy (Kono *et al.*, 1994). There have been reports of decreased placental MAO-A activity and catalytic turnover in pre-eclampsia (Carrasco *et al.*, 2000; Sivasubramaniam *et al.*, 2002). However, the causative factor for this reduction in enzyme activity is not known. Among placental cells syncytiotrophoblasts being the outermost are exposed to highest levels of oxygen coming from the maternal circulation and surprisingly express low levels of antioxidant enzymes, especially in early pregnancy (Hung *et al.*, 2001). As MAO-A is localised in the syncytiotrophoblasts, oxidative damage to the enzyme could be a contributory factor to the reduction in its activity in pre-eclampsia. Oxidative stress and its potential effects on MAO-A activity will be further discussed in chapter VI.

5-HT has been reported to induce vascular contraction of umbilicoplacental vessels at physiologic concentrations (Altura *et al.*, 1972; Gonzalez *et al.*, 1990). The tone of placental vessels is important for the delivery of nutrients and oxygen to the foetus. Since the umbilicoplacental circulation is devoid of autonomic innervation (Reilly and Russell, 1977; Fox and Khong, 1990), humoral factors such as 5-HT are thought to play an important role in the regulation of the umbilicoplacental vascular tone (Gonzalez *et al.*, 1990).

Conflicting results however, have been obtained in a limited number of studies that compared the vasoactive effects of 5-HT on normotensive and pre-eclamptic umbilical / placental vessels (Johnstone *et al.*, 1987; Bertrand and St-Louis, 1999; Gupta *et al.*, 2006). Since increased levels of 5-HT (Middelkoop *et al.*, 1993; Gujrati *et al.*, 1996; Carrasco *et al.*, 1998; Laskowska *et al.*, 2001) and decreased placental MAO-A catalytic catalytic turnover have been reported in pre-eclampsia, 5-HT and MAO may be involved in the pathogenesis of pre-eclampsia.

1.5 Aims

This study aims to investigate the role of 5-HT in pre-eclampsia with specific emphasis on vascular tone and reactivity. Thus the main aims of this study are:

- To investigate the vasoactive actions of 5-HT on vascular tone in normotensive and pre-eclamptic placental vessels
- To investigate the effect of 5-HT_{2A} receptor blocking on 5-HT mediated changes in vascular tone in both normotensive and pre-eclamptic placental vessels
- To investigate 5-HT_{2A} receptor protein levels in the vessels from normotensive and pre-eclamptic placentae
- To investigate 5-HT and 5-HIAA levels in blood / urine from normotensive and pre-eclamptic patients
- To investigate changes in $5HT_{2A}$ receptor protein levels and MAO-A activity in an *in vitro* model following hypoxia and oxidative stress

CHAPTER II

MATERIALS AND METHODS

2 CHAPTER II Materials and Methods

2.1 Materials

2.1.1 Specialised laboratory reagents

- 3,3'-Diaminobenzidine tablet sets (DAB Sigma Fast™; D-4168), Sigma-Aldrich Company, Poole, UK.
- 5-Bromo-4-chloro-3-indolyl-phosphate (di-sodium salt; BCIP; MB1018), Melford Laboratories Ltd., Ipswich, UK.
- 5-HT ELISA kit (BA10-0900), LDN Labor Diagnostika Nord GmbH & Co. KG, Germany.
- 5-HTIAA ELISA kit (BA10-1900), LDN Labor Diagnostika Nord GmbH & Co. KG, Germany).
- Acrylogel 3 solution Electran (containing 2.5 % NN′-methylenebisacrylamide, final ratio 29:1:0.9; 443735T), VWR International Ltd., Lutterworth, UK.
- Calcium Chloride (1 mol l^{-1}) volumetric solution (19046 4k), VWR International Ltd., Lutterworth, UK.
- Dimethlsulfoxide (DMSO; D/4120/PB08), Fisher Scientific UK Ltd., Loughborough, UK.
- Folin-Ciocalteu´s phenol Reagent (J/4100/08), Fisher Scientific UK, Leicester, UK.
- Human Chorionic Ganodotropin (HCG) ELISA kit (0400), Autogen Bioclear UK Ltd., Wiltshire, UK.
- Hydrogen peroxide 30 % solution (1.07209), VWR International Ltd., Lutterworth, UK.
- Ketanserin (R 41468; 3-[2-(4-[4-Fuorobenzoyl]-1-piperidinyl0ethyl]-2,4[1H.3H]-quinazolinedione) tartrate salt (S006), Sigma-Aldrich Company, Poole, UK.
- Krebs-Henseleit Buffer (K3753), Sigma-Aldrich Company, Poole, UK.
- L-Lactic dehydrogenase (EC 1.1.1.27) solution, type II: from rabbit muscle (L2500), Sigma-Aldrich Company, Poole, UK.
- Monoamine Oxidase A (EC 1.4.3.4.) human, recombinant, expressed in baculovirus infected BTI insect cells (MAO-A; M7316), Sigma-Aldrich Company, Poole, UK.

- Monoamine Oxidase insect cell control, recombinant, expressed in wild type baculovirus transfected BTI cells (M7566), Sigma-Aldrich Company, Poole, UK.
- Myo-Inositol (^{3}H) sterile aqueous solution MP Biomedicals, London, UK.
- Nitro Blue Tetrazolium (NBT; MB1019), Melford Laboratories Ltd., Ipswich, UK.
- Nitrocellulose 0.22 µM pore size (WP2HY00010), Genetic Research Instrumentation, Essex, UK.
- Pierce ECL Western Blotting Substrate (32209), Perbio Science UK Ltd., Northumberland, UK.
- Potassium Chloride (10198 3k), VWR International Ltd., Lutterworth, UK.
- Precision Plus protein all blue standards (161-0376), Bio-Rad Laboratories Ltd., Hemel Hempstead, UK.
- Prestained Protein marker, Broad range, (P7708S), New England Biolabs (UK) Ltd., Herts., UK.
- Protease Inhibitor cocktail (P8340), Sigma-Aldrich Company, Poole, UK.
- Pyruvic acid sodium salt (P-2256), Sigma-Aldrich Company, Poole, UK.
- Serotonin Hydrochloride (5-HT; H9523), Sigma-Aldrich Company, Poole, UK.
- Sodium Nitrate (431605), Sigma-Aldrich Company, Poole, UK.
- ß-Nicotinamide adenine dinucleotide, reduced disodium salt (N8129), Sigma-Aldrich Company, Poole, UK.
- Tyramine 7- ^{14}C Hydrochloride (T0661), Sigma-Aldrich Company, Poole, UK.
- Xanthine Oxidase from bovine milk Grade I (X1875), Sigma-Aldrich Company, Poole, UK.
- Xanthine sodium salt (X3627), Sigma-Aldrich Company, Poole, UK.

2.1.2 General laboratory reagents

All general laboratory reagents were of the highest grade available and purchased from Sigma-Aldrich Chemical Company, Poole, UK, unless otherwise specified in the text.

2.1.3 Cell culture

2.1.3.1 Reagents

The following were purchased from Lonza Wokingham, Ltd., Berkshire, UK:

- Dulbecco's Modified Eagle's medium (DMEM; BE12-614F),
- Foetal bovine serum (DE14-801F),
- HAM's F12 medium (BE12-615F),
- Leibovitz (L-15) medium,
- L-glutamine (BE17-605E),
- Penicillin/ streptomycin (DE17-603E),
- trypsin/EDTA solution (BE02-007E).

The following were obtained from Sigma-Aldrich Company, Poole, UK:

- MEM non-essential amino acids solution (M7145),
- Trypan blue solution 0.4 % (v/v; T8154).

2.1.3.2 Plastic ware

- Cryotube vials (Nunc brand products), from Merk Ltd., Leicester, UK.
- Poly-L-lysine coated (3" x 1") slides from Sigma-Aldrich Company, Poole, UK
- Nunc Lab-Tech CC chamber slides (glass, 177402) from Scientific Laboratory Supplies, Ltd., Nottingham, UK.
- All sterile plastic ware used in cell culture studies was purchased from Sarstedt, Leicester, UK.

2.1.4 Antibodies

Primary antibodies

- Mouse monoclonal Anti-Monoamine Oxidase A (clone 6G11/E1) antibody,
- Mouse monoclonal Anti-Monoamine Oxidase B (clone 3F12/G10/2E3) antibody

were produced in house and are available in our laboratory.

- Anti-Serotonin receptor 5-HT_{2A}R mouse monoclonal (clone G186-1117) antibody (556326), BD Biosciences, Oxford, UK.
- Mouse monoclonal anti-Nitrotyrosine (HM11) antibody (sc-32731), Autogen Bioclear UK Ltd., Wiltshire, UK

Secondary antibodies

The following were purchased from DAKO Ltd., Cambridgeshire, UK:

- Goat anti-mouse immunoglobulins, alkaline phosphatase conjugated (D0486).
- Goat anti-mouse immunoglobulins, horseradish peroxidase conjugated (P0447).

2.1.5 Specialised equipment

- ATTO HorizBlot AE6675, Atto Corporation, Japan.
- Beckman Coulter DU 530 Life Science UV / VIS Spectrophotometer, Buckinghamshire, UK.
- Bio-Rad mini protean III Gel system, Bio-Rad Laboratories Ltd., Hemel Hempstead, UK.
- Bio-Rad mode 680 microplate reader, Bio-Rad Laboratories Ltd., Hemel Hempstead, UK.
- Bio-Rad power Pac 300, Bio-Rad Laboratories Ltd., Hemel Hempstead, UK.
- CBS Isothermal liquid nitrogen storage system 2300 series, Sanyo Biomedical division, Leicestershire, UK.
- Cell scraper (C2808), Sigma-Aldrich Company, Poole, UK.
- Coulter Counter (model Z_B), Coulter Electronics Ltd., Beds., UK.
- Dot Blotter, Schleicher & Schull SRC 96D S & S manifold I Dot blotter, Dassel, Germany.

- Electronic caliper Rolson Quality Tools, Twyford, UK.
- Force displacement transducers, Harvard Instruments, UK.
- Fujifilm FLA-5100 gel scanner, Fujifilm Life Sciences Products, Sheffield, UK.
- Fujifilm intelligent dark box, Fujifilm Life Sciences Products, Sheffield, UK.
- Leica CLMS confocal laser microscope, Leica, Germany.
- Mettler AE 260 Delta Balance, Mettler, UK.
- MIKRO 22R microfuge, Hettich, Germany.
- MSE Centaur 2 centrifuge, MSE Scientific Instruments, UK.
- MSE Falcon 6/300R Centrifuge, MSE (UK) Limited, London, UK.
- Neubauer double cell clear sight haemocytometer (AC1000), Weber Scientific International (Division of Hawksley Technology), West Sussex, UK.
- Nikon Eclipse TS 100 inverted microscope, Nikon, Japan.
- Philips pH Meter – PW9409, Pye-Unicam, UK.
- Power 1401 data acquisition system Cambridge Electronic Design Limited, Cambridge, UK.
- Radnoti 4 chamber tissue bath system ADInstruments, Chalgrove, UK.
- Sanyo CO_2 incubator MCO-17AIC, Sanyo Gallenkamp PCL, Leicestershire, UK.
- Sanyo Harrier 18/80 refrigerated centrifuge, Sanyo Gallenkamp PCL, Leicestershire, UK.
- Sanyo O_2 / CO_2 incubator MCO-175M, Sanyo Gallenkamp PCL, Leicestershire, UK.
- SM1 Magnetic stirrer, Stuart Scientific, UK.
- Soniprep 150, MSE scientific instruments, UK.
- Two-blade carbon steel knife (2 mm gap) manufactured in house.
- Walker class II microbiological safety cabinet, Walker safety cabinets Ltd., Derbyshire, UK.
- Water bath 20-90° C, Grant, UK.
- Water purification system, Millipore, USA.

2.2 Methods

2.2.1 Sample collection

Placenta, blood and urine samples were obtained using procedures in accordance with the ethical approval by the Local Research Ethics Committee, Trent Health Authority (NHS), City Hospital, Nottingham. Pre-eclampsia was defined as a blood pressure of 140 / 90 on two or more occasions after 20th week of pregnancy together with the presence of significant proteinuria (more then 0.3 g in 24 hour urine), in a previously normotensive women. Patients with previous hypertension and medical complications such as diabetes and renal disease as well as multiple births were excluded from this study. Normal pregnant control subjects had uncomplicated singleton pregnancies with no proteinuria and normal blood pressure throughout their pregnancy. All samples were obtained within 30 minutes of routine elective Caesarean sections or vaginal delivery.

2.2.1.1 Placenta

Umbilical cords were clamped immediately after delivery in order to maintain pressure inside the blood vessels. The outer diameter of vessels was measured with an electronic caliper, only arteries and veins with approximately 2 mm outer diameter were marked for dissection.

2.2.1.2 Blood

Maternal blood samples were obtained before caesarean section, or before induction in the case of vaginal delivery. 10 ml of blood was slowly transferred in to a tube containing 100 µl of anti coagulant solution (10 % [w/v] Ethylenediamine tetraacetic acid [EDTA] pH 7.4).

Umbilical cord blood (5-10 ml) was collected at the time of delivery. After clamping the cord was cut and blood (arterial and venous) collected into a tube containing 100 µl of anti coagulant solution.

Placental blood was collected after marking blood vessels for dissection. Several incisions were made to arteries and veins, approximately 10 ml of the draining blood was immediately collected into a tube containing100 µl of anti coagulant solution.

A small number of non-pregnant female volunteers were also involved in the study to conduct preliminary experiments with blood samples. Volunteers were healthy, normotensive and taking no medication or oral contraceptives. Blood samples were collected approximately midway through their menstrual cycle.

Since some foods (tomato, avocado, walnuts etc.) are known to interfere with 5-HT measurements, all pregnant and non-pregnant subjects completed a short questionnaire to inform the study whether they had eaten (24 hour prior to blood collection) any of these foods.

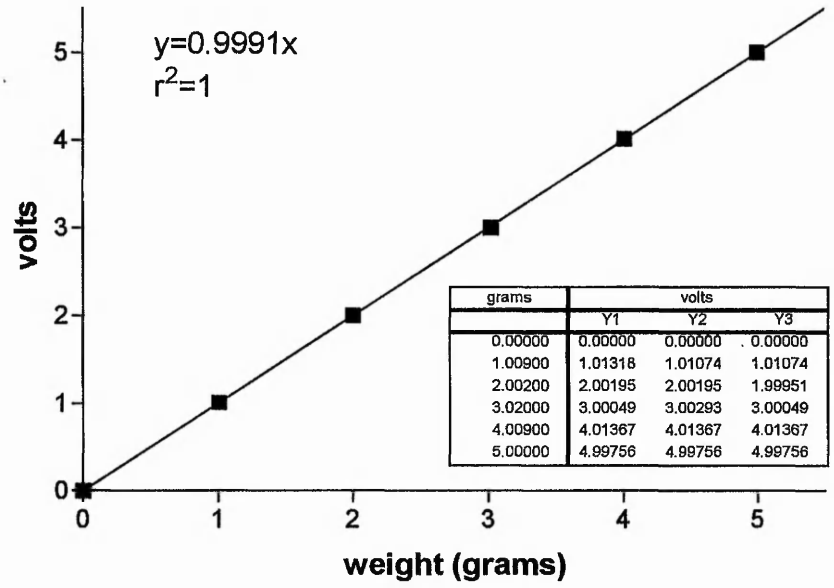
2.2.1.3 Urine

A 24-hour urine sample was collected the day before delivery, acidified with the addition of 10 ml 6 M HCl and the total volume was recorded. Several aliquots were stored at -80 °C until needed.

2.2.2 In vitro tissue bath analysis

2.2.2.1 Tissue bath calibration

Force displacement transducers were calibrated following the installation of the tissue bath system. A stainless steel hook was attached to each transducer with a stainless steel wire. The Power 1401 data acquisition system was turned on and a baseline, which was set to zero, was recorded while no weight was hanging from the stainless steel hook. Increasing amounts of known weights were loaded on to the hook and changes in the voltage recorded at each weight increment. A standard line was then produced showing voltage against weight. This procedure was repeated three times and the system was calibrated by equating voltage difference to weight. As a result, a calibration factor for each transducer was determined, converted to milli Newton (mN) and stored on the Power 1401 data acquisition system for recording. A sample calibration line is given in Figure 2.1.



grams	volts		
	Y1	Y2	Y3
0.00000	0.00000	0.00000	0.00000
1.00900	1.01318	1.01074	1.01074
2.00200	2.00195	2.00195	1.99951
3.02000	3.00049	3.00293	3.00049
4.00900	4.01367	4.01367	4.01367
5.00000	4.99756	4.99756	4.99756

Figure 2.1Calibration of force displacement transducers used in tissue bath experiments

Changes in voltage were recorded at each weight increment and these values were plotted to produce a standard calibration.

The calibration factor was calculated as follows:

$$\text{Response (mN)} = \text{Signal (volts)} \times \underbrace{\left[\frac{9.797}{\text{Slope}}\right]}_{\textbf{Calibration factor (CF)}}$$

(1 gram-force =9.797 mN, mN= milli Newton)

Thus, the calibration factor is calculated as 9.8058 for the sample transducer. These values were then loaded in to the Spike 2 program so that responses could be directly recorded in mN.

2.2.2.2 Preparation of human placental vessels for tissue bath analysis

Krebs-Henseleit buffer (containing 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.17 mM $MgSO_4$, 1.18 mM KH_2PO_4, 19.8 mM $NaHCO_3$, and 11.1 mM glucose; pH 7.4) was prepared fresh for each experiment and cooled to 4° C. Chorionic arteries and veins of 2 cm length with an outer diameter of approximately 2 mm were carefully dissected, immediately immersed in pre-cooled Krebs-Henseleit buffer and transported to the laboratory on ice.

In order to maintain consistency, only the following arteries and veins were selected for this study;

(a) arteries and veins close to the insertion of umbilical cord (primary artery / vein)

(b) arteries and veins distal to the umbilical cord (secondary artery / vein)

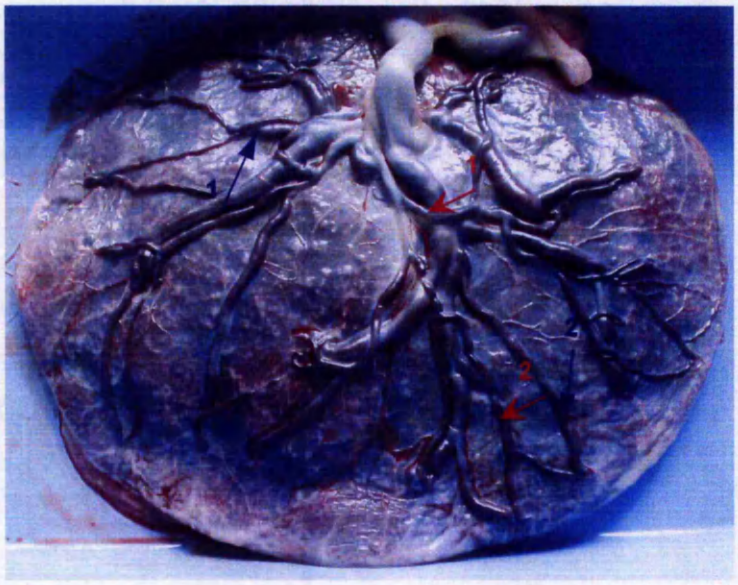


Figure 2.2 Normotensive placenta showing the chorionic vessels

Image of a normotensive placenta taken before dissection. Red and blue arrows indicate the respective chorionic arteries and veins.

1 = primary artery / vein; 2 = secondary artery / vein.

2.2.2.3 Tissue bath set-up

A two-blade carbon steel knife (with a 2 mm gap between them) was manufactured in house to produce consistent lengths. Blood traces and adherent tissues were removed, and a 2 mm long vessel ring was cut and mounted between two stainless steel hooks in a 15 ml tissue bath. The system was continuously gassed with 95 % oxygen and 5 % carbon dioxide. Baths and Krebs-Henseleit buffer were maintained at 37°C. Isometric tension changes were measured through a force displacement transducer, which was connected to the Power 1401 data acquisition system. Tissues were allowed to equilibrate for 90 minutes and the Krebs-Henseleit buffer was changed at 20-minute intervals.

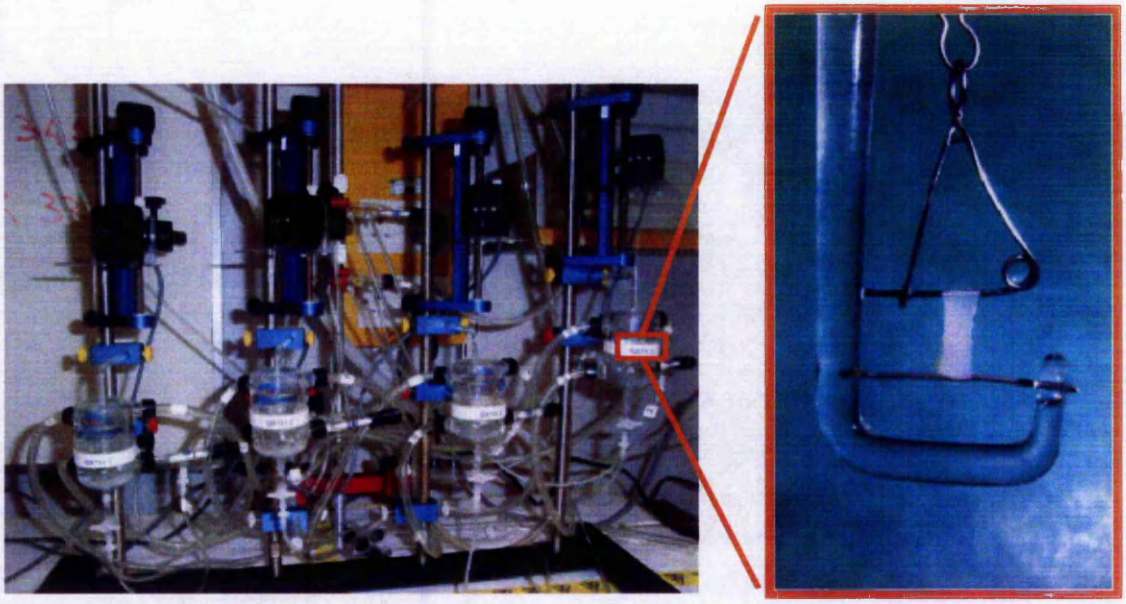


Figure 2.3 Radnoti 4 chamber tissue bath system

Image of Radnoti 4 chamber tissue bath system used in experiments and a close up image showing a vessel ring mounted between two stainless steel hooks.

2.2.2.3.1 Passive tension and dose determination studies

In order to investigate the influence of passive tension on the response to vasoactive agents, arteries and veins were adjusted and equilibrated at different initial passive tensions. Vessels were equilibrated at 0.5 g passive tension and the contractile response to 120 mM of KCl was evaluated. After several washes with Krebs-Henseleit solution, vessels were adjusted to 1 g passive tension and their response to 120 mM KCl was evaluated. This procedure was repeated at 1.5, 2 and 3 g passive tension for all vessel types under investigation.

In order to determine a concentration-response curve to KCl chorionic arterial and venous rings were adjusted and equilibrated at passive tension (1.5 and 2 g) and subjected to increasing concentrations of KCl (5, 10, 20, 40, 60, 80 and 120 mM).

2.2.2.3.2 Concentration-response studies

Tissues were set up as explained in section 2.2.2.3 and, following equilibration and adjusting passive tension, vessel response to 60 mM KCl was recorded. This was repeated after washing tissue with Krebs-Henseleit solution. This was done to ensure vessel contractility prior to addition of 5-HT and was used to normalise vessel response.

In order to examine the effects of 5-HT on isolated chorionic arterial and venous rings, concentration-response curves (1 nM - 10 μM) were obtained cumulatively in these vessels at a passive tension of 1.5 g (found to be optimal, see chapter III for details).

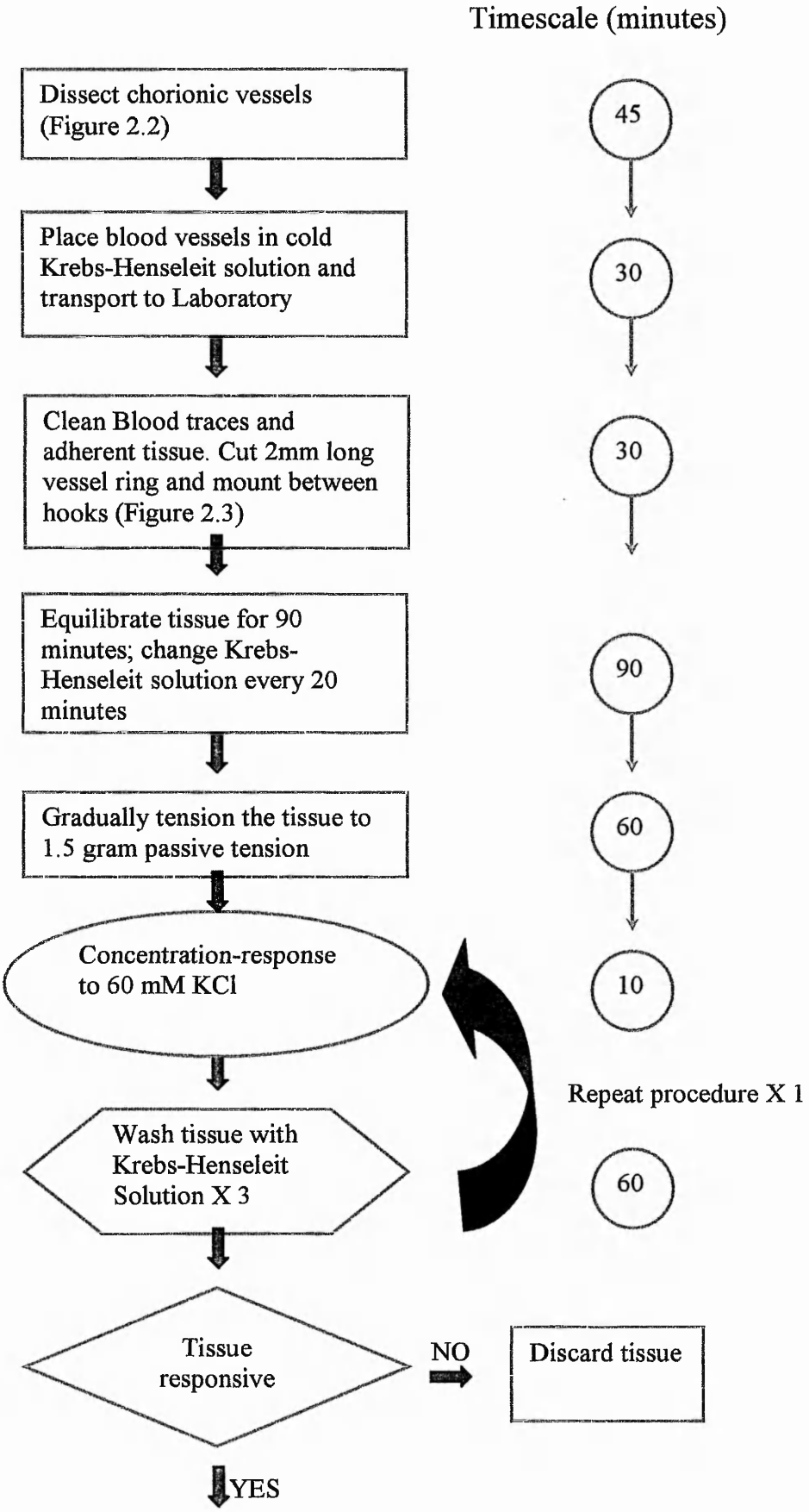
The effect of 5-HT on vessel contraction in the presence of the 5-HT_{2A} receptor antagonist ketanserin was also investigated. Ketanserin was solubilised to a final concentration of 100 mM in Dimethlsulfoxide (DMSO), further diluted to a final concentration of 1 mM in distilled water and aliquots were kept at –20 °C. In order to asses ketanserin antagonism the vessels were left to equilibrate (with 20 minute washes) for 2 hours after the first concentration-response curve to 5-HT. Ketanserin (1μM, final concentration) was added to each bath and equilibrated for a further 30

minutes, then the concentration-response curve to 5-HT was repeated in the presence of ketanserin. Contractions produced by 5-HT were measured as changes in tension from baseline and expressed as a percentage of the mean contraction induced by two consecutive 60 mM KCl response at the start of the experiment. Concentration response curves were analysed by non-linear regression analysis using GraphPad Prism Software (GraphPad Prism version 3.00 for Windows, Graph Pad Software, San Diego, California, USA). Agonist potency (EC_{50}; concentration of the agonist to produce half the maximal response) expressed as pEC_{50} (-log EC_{50}) and efficacy of the agonist was expressed as E_{max} (maximal response).

The following diagram (Figure 2.4) demonstrates an overview of the experimental procedure used in the tissue bath experiments with placental vessels. Approximate timescale (in minutes) are given for each procedure. From receipt of tissue, the average time spent conducting the tissue bath experiments was around 12-15 hours.

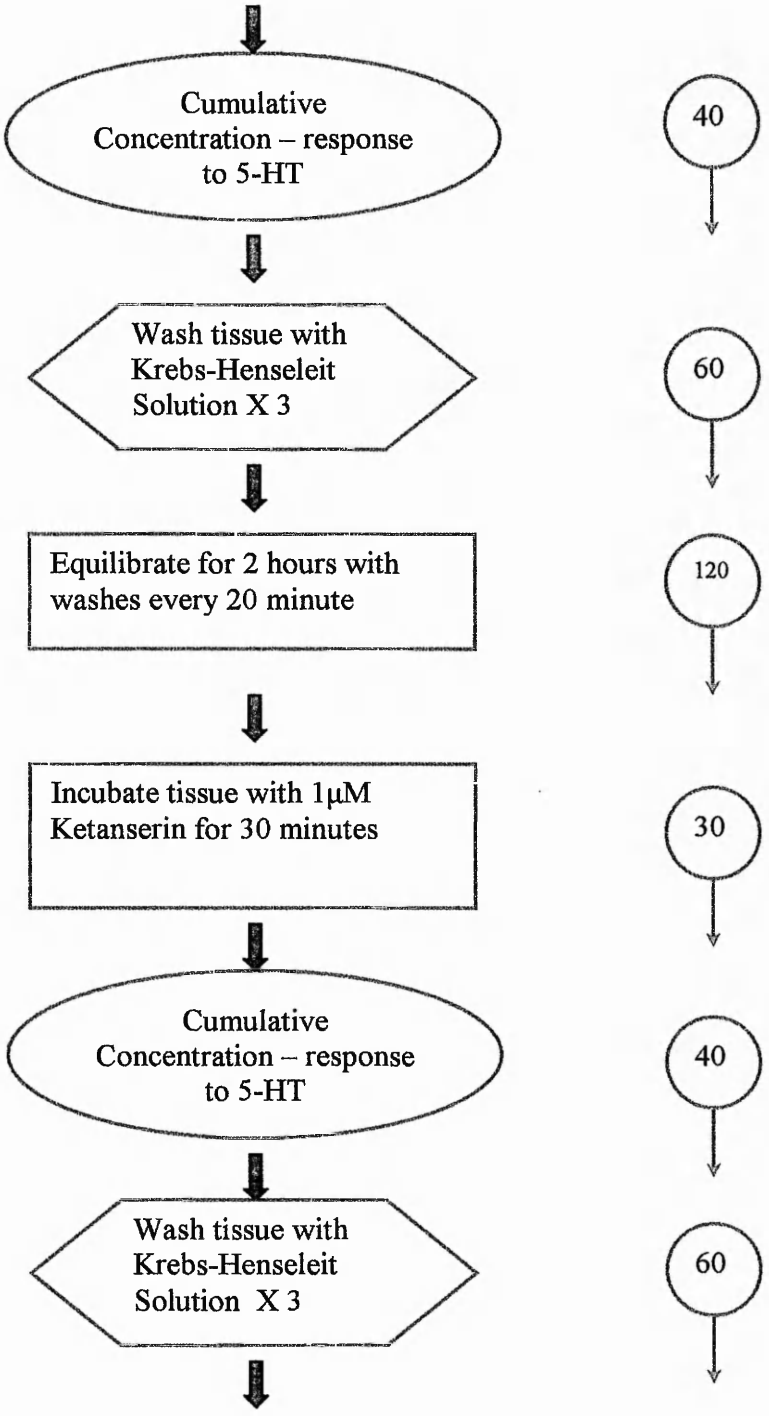
A)

TISSUE PREPARATION AND RESPONSE TO KCl



B)

5-HT CONCENTRATION RESPONSE



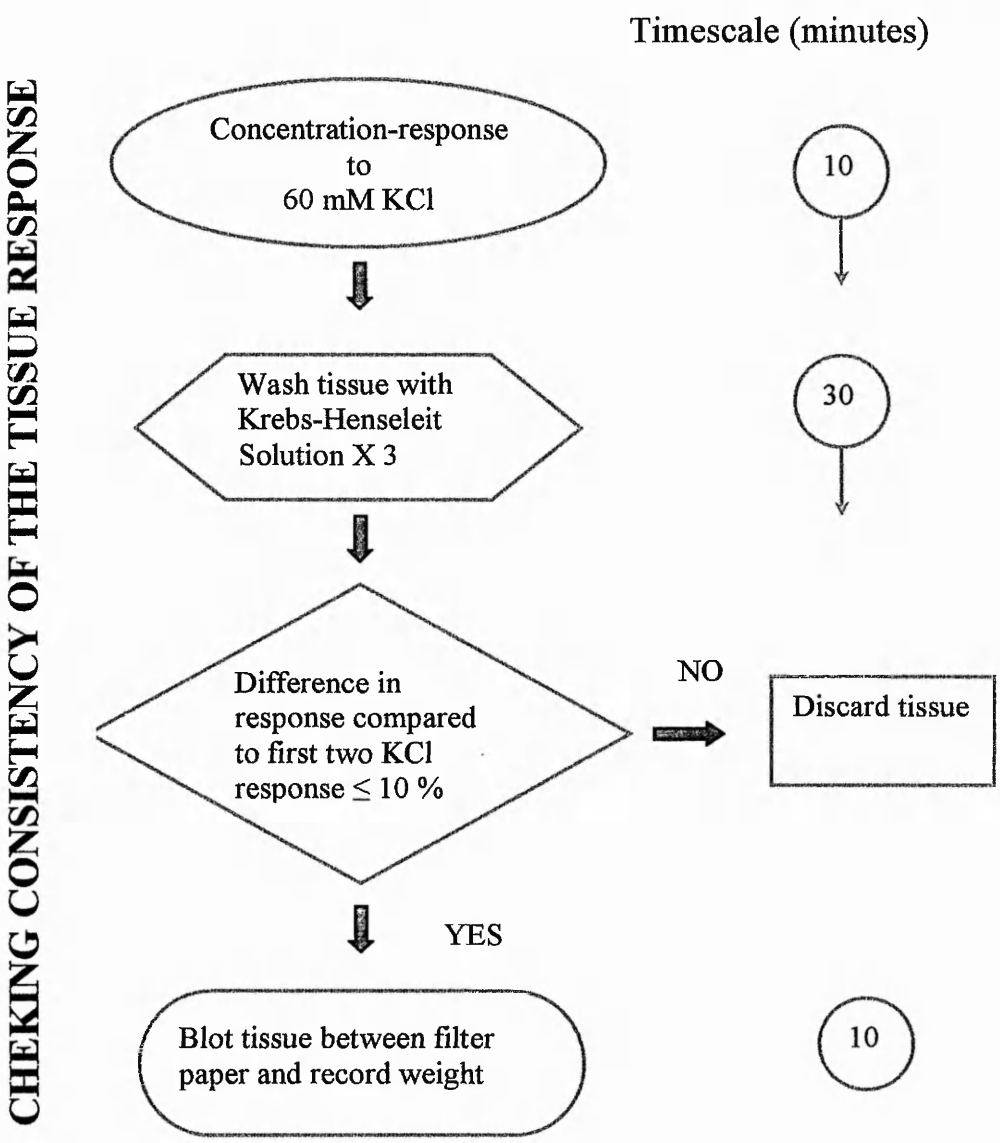


Figure 2.4 Flow diagram of tissue bath experiment with placental vessels

Flow diagram showing the stages involved in tissue bath experiments with placental vessels. Approximate timescale (in minutes) is shown next to each step.

A) Tissue preparation and response to KCl in order to ensure vessel contractility prior to experiment with agonist (5-HT).

B) Concentration-response to 5-HT alone and in presence of antagonist (ketanserin).

C) Final check with KCl contraction at the end of the experiment in order to ensure consistency of the tissue response and viability.

2.2.3 Preparation of tissue samples for analysis

Following dissection of blood vessels for tissue bath analysis, remaining blood vessels were cleaned of blood traces, and snap frozen in liquid nitrogen. Approximate 10-gram portions of placenta were also taken avoiding the area close to cord insertion, for MAO activity assays. Rat placentas were obtained immediately after birth. Rat brain was obtained from a male Wistar Rat. All samples were stored at -80 °C until proteins extracted from them as detailed in sections 2.2.3.1 and 2.2.3.2.

2.2.3.1 Homogenisation of human and rat tissues

Tissue samples were removed from -80 °C and thawed on ice, washed in excess volumes of ice-cold Phosphate Buffered Saline (PBS; 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4 and1.47 mM KH_2PO_4, pH 7.4) to remove blood traces. Samples were then sliced finely (by scalpel) and homogenised in 5 volumes of ice-cold extraction buffer (50 mM Trizma base pH 6.8, 150 mM NaCl, 5 mM EDTA, 1 mM Sodium Orthovanadate, 0.5 % [v/v] Triton X-100, 2 mM phenylmethylsulfonyl fluride [PMSF] and 0.2 % protease inhibitor cocktail) using a glass-teflon homogeniser (Potter-Elvehjem, 0.1 to 0.15 mm clearance). Extracts were mixed at 4 °C for 90 minutes in order to solubilise proteins, and then centrifuged at 14 000 x g for 10 minutes at 4 °C. Supernatants were collected and stored at –80 °C in working aliquots.

2.2.3.2 Homogenisation of placenta for MAO activity assay

Tissue samples (approximately 10 g) were thawed on ice and washed in excess volumes of ice-cold buffered sucrose solution (0.25 M sucrose pH 7.4, 3 mM Imidazole, 2 mM PMSF) in order to remove all traces of blood. Tissue samples were then cut finely with scissors and homogenised in 5 volumes in buffered sucrose solution containing 0.5 % (v/v) Triton X-100 using a mill attachment of a blender (Kenwood type BL446). Following two hours of tumbler mixing at 4 °C homogenates were centrifuged at 27,000 x g for 30 minutes. Resulting supernatants were aliquoted, their protein content estimated and stored at –80 °C.

2.2.4 Estimation of MAO activity

The activity of MAO-A in placental homogenates was measured using a radioactivity assay (Russell and Mayer, 1983). This assay is based on the ability of MAO to convert water-soluble substrates to their corresponding aldehyde products. Samples were removed from –80 °C and thawed on ice. After vortex mixing, an aliquot was warmed to 37 °C and incubated with Deprenyl (1μM, final concentration) for 15 minutes in a water bath, in order to inhibit residual MAO-B activity. Samples (typically, 5μl) were then diluted to a final volume of 180 μl using 20 mM potassium phosphate buffer (20 mM K_2HPO_4, 20 mM KH_2PO_4, pH 7.4), mixed and returned to water bath. Following addition of 20 μl (0.02 μCi) of 1 mM Tyramine 7- ^{14}C Hydrochloride (1μCi/μmole) each sample was swirl mixed and incubated for a further 30 minutes at 37 °C. The reaction was terminated by the addition of 200 μl of 0.5 M HCl. A blank of each sample was also made by adding the acid before the substrate. Radiolabelled product was then extracted into 3 ml of scintillation counting solution (1 % [w/v] diphenyloxazole in toluene: ethylacetate [mixed in a 1:1 ratio]) and counted using a liquid scintillation counter (Canberra Packard, UK).

For the recombinant MAO-A activity determination, potassium phosphate buffer was replaced with 50 mM HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) buffer, pH 7.4, in order to achieve a higher buffering capacity. Method optimisation is detailed in section 6.2.2.1.

For the MAO km determination studies the activity assay was modified as follows. A separate stock solution of 0.1 mM Tyramine 7- ^{14}C Hydrochloride (1 μCi / 0.2 μmole) was prepared to give a final concentration of 10μM in the assay. Unlabelled tyramine was added to give a range of substrate concentration (10μM-100μM).

2.2.5 Preparation and analysis of human blood and urine samples

2.2.5.1 Separation of different fractions of blood

Maternal, cord and placental blood samples were centrifuged at 200g for 10 minutes at room temperature in order to separate platelet rich plasma (PRP). Collected PRP was transferred in to another clean tube using a plastic Pasteur pipette and 100µl samples were taken for platelet count. In order to separate platelets, PRP was aliquoted (200 µl and 500 µl) in eppendorf tubes and centrifuged at 1500 g for 20 minutes at room temperature. The supernatant, containing platelet poor plasma (PPP) was carefully removed without disturbing the platelet pellet and transferred into another eppendorf, a 100 µl aliquot of PPP was then taken for platelet count. This was a quality check for the procedure. Aliqouted PRP, PPP and platelet pellets were stored at –80 °C.

2.2.5.2 Counting platelets

The Coulter Counter® was used to count platelets. 20 µl of PRP or PPP was diluted in Isoton (Bechman Coulter) to a final volume of 50 ml in a volumetric flask and particles sizing 1.6 µm to 3.2 µm counted according to Wilkinson *et al.* (1986). Briefly, six blank measurements (Isoton only) were performed prior to the counting of each sample. The average of six to eight consecutive particle counts were used to calculate platelet numbers for each sample. Platelet counts in PPP samples were also taken as a quality control procedure to ensure effective separation of platelet pellets from PRP samples.

2.2.5.3 5-HT analysis by ELISA

5-HT in platelets, platelet poor plasma and urine samples was assayed using a commercially available kit (BA10-0900, LDN Labor Diagnostika Nord GmbH & Co. KG, Germany) according to the manufacturer's instructions. The assay is a competitive enzyme linked immunosorbent assay (ELISA), which can quantitatively determine derivatised 5-HT in a wide range of sample formats. Hence sample preparation includes a quantitative derivatisation of 5-HT to N-acylserotonin. 5-HT is bound to the solid phase of the microtiter plate, acylated 5-HT (in solution) and

bound 5-HT compete for a limited number of antibody binding sites. Therefore the amount of antibody bound to solid phase 5-HT is inversely proportional to the amount of 5-HT present in the sample assayed.

All reagents supplied in the kit were allowed to reach room temperature and all incubations were carried out at room temperature on an orbital plate shaker (Titertek, Flow Laboratories) at 4000 rpm unless otherwise specified. Assay sensitivity was 0.3 ng/ml for PPP and 5ng/ml for PRP and urine.

2.2.5.3.1 5-HT measurements in platelets

Maternal, cord and placental blood platelet pellets, obtained from 200µl of PRP as explained in section 2.2.5.1, were removed from –80 °C, resuspended in 200 µl of distilled water and centrifuged at 3000 g for 2 minutes at room temperature. 10 µl of supplied standards, controls and samples were pipetted into the respective wells of the acylation plate supplied. 250 µl of acylation buffer and 25 µl of acylation reagent were added to the samples, standards and controls. The plate was sealed and incubated for 15 minutes. 10 µl of acylated samples, standards, and controls were then transferred to a 96 well ELISA plate (pre-coated with 5-HT), and 50 µl of primary antibody (rabbit anti-serotonin antibody) was added to each well. The plate was sealed and incubated in dark for 1 hour. The plate was washed three times with wash buffer (1:10 [v/v] diluted) and excess liquid was blot dried on absorbent paper. 100 µl of secondary antibody (HRP conjugated anti-rabbit antibody) were added to the each well, sealed and incubated for 30 minutes. The ELISA plate was washed as before with wash buffer. Following the addition of 100 µl of substrate, the plate was incubated for a further 30 minutes, the reaction stopped by addition of 100 µl stop solution, and absorbance was read at 450 nm with a reference reading at 655 nm.

A standard calibration was constructed by plotting log 5-HT concentration of the standards against absorbance (450 nm-655 nm). The amount of 5-HT in each sample was calculated using the equation of the standard line. An example of a standard calibration and absorbance values for each standard are given in Figure 2.5.

A)

Standards	A	B	C	D	E	F
5HT (ng / ml)	0	15	50	150	500	2500
Log (5HT)		1.176	1.699	2.176	2.699	3.398
Absorbance (450-655 nm)	1.975	1.857	1.439	1.1040	0.734	0.374

B)

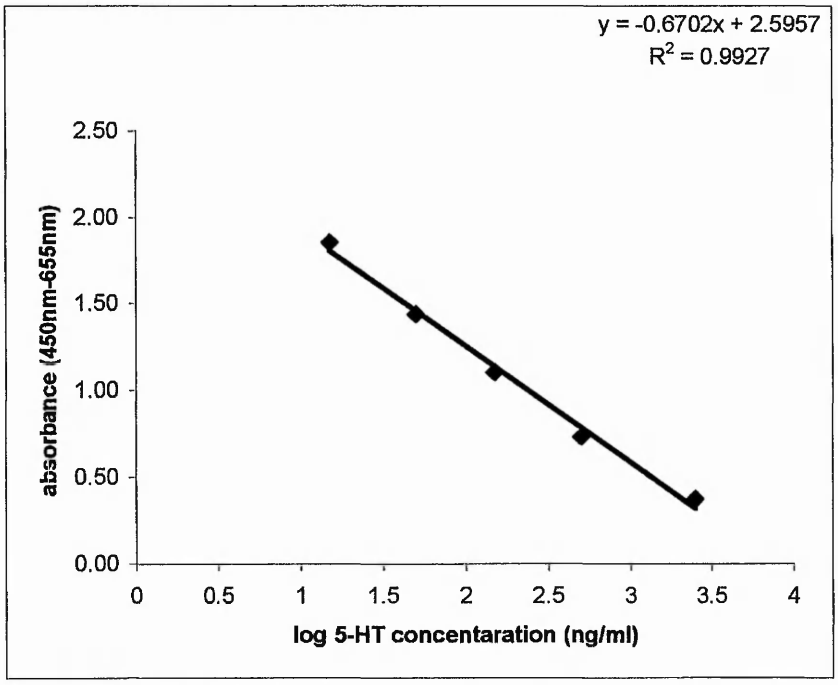


Figure 2.5 Standard calibration for 5-HT ELISA assay for PRP

A) Table listing the amount of 5-HT in each standard supplied with the kit and their absorbance values measured at 450 nm with a reference reading at 655 nm.

B) Standard calibration obtained by plotting the absorbance values measured for the standards on the y-axis (linear) against the corresponding concentrations on the x-axis (logaritmic). The concentrations of 5-HT in PRP samples were calculated using the equation obtained from the line of best fit.

2.2.5.3.2 5-HT measurements in platelet poor plasma

The same assay was also used to measure 5-HT in platelet poor plasma samples. Maternal, cord and placental blood were assayed in order to establish circulating 'free' 5-HT levels. Platelet poor plasma samples were allowed to thaw at room temperature. In this case supplied 5-HT standards and controls were diluted 1:25 (v/v) in distilled water and 100µl of PPP, standards and controls were added to the respective wells of the acylation plate supplied. 100 µl of acylation buffer and 10 µl of acylation reagent were added to the samples, standards and controls. Following 15 minutes incubation, 30 µl of acylated samples, standards, and controls were transferred to a 96 well ELISA plate, probed with primary and secondary antibodies and developed as explained previously.

2.2.5.3.3 5-HT measurements in urine

5-HT in urine was also determined following the same protocol as previously described for platelets. An aliquot (ml) of urine was removed from –80 °C and thawed, 10 µl of urine sample was used for each assay as explained in section 2.2.5.3.1.

2.2.5.4 5-HTIAA analysis by ELISA

5-HTIAA in urine samples was assayed using a commercially available kit (BA10-1900, LDN Labor Diagnostika Nord GmbH & Co. KG, Germany) according to manufacturers instructions. The assay is a competitive ELISA, which can quantitatively determine the levels of derivatised 5-HTIAA in urine. As in the 5-HT ELISA, sample preparation includes a quantitative derivatisation of 5-HTIAA. Acylated 5-HTIAA and bound 5-HTIAA compete for a limited number of antibody binding sites. Therefore the amount of antibody bound to the solid phase 5-HTIAA is inversely proportional to the amount of 5-HTIAA present in the sample assayed. Assay sensitivity was 0.17 mg/l. A standard line is plotted in order to determine the concentration of 5-HTIAA in urine samples. The absorbance values measured for the supplied 5-HT standards are plotted on the y-axis (linear) against the corresponding concentrations on the x-axis (logaritmic). The concentration of 5-HTIAA in each

sample was determined using the equation from the line of best fit. This procedure is the same as explained previously for determination of 5-HT levels (Figure 2.5).

2.2.6 Cell culture

Two human placental cell lines, BeWo and 3A Sub E, were used for this study. Both cell lines were obtained from European Collection of Cell Culture (ECACC, UK).

2.2.6.1 Maintenance of placental cell lines

Cell culture was carried out in a class II safety cabinet using aseptic techniques. Cells were incubated at 37 °C (or 40 °C depending on application) in a humidified atmosphere of 95 % (v/v) air / 5 % (v/v) carbon dioxide. Cells were cultured in 25 cm^2 (T25), 75 cm^2 (T75) and 175 cm^2 (T175) flasks until 70-90 % confluent in growth medium containing at final concentration:

- BeWo: Ham's F12 containing 10 % (v/v) heat inactivated foetal bovine serum (heat inactivated at 60 °C for 30 min), 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin.
- 3A Sub E: Dulbecco's Modified Eagles Medium (DMEM) containing: 10 % (v/v) heat inactivated foetal bovine serum (heat inactivated at 60 °C for 30 min), 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 1 % (v/v) non essential aminoacids.

2.2.6.2 Sub-culturing cell lines

Cell growth was checked daily and, when cells reached 70-90 % confluency, growth medium was removed and the cell monolayer was washed twice with warmed DMEM (3A Sub E) or Ham's F12 (BeWo). Cells were detached from the flask surface using a solution of trypsin (250 µg/ml) and Ethylenediamine (EDTA; 100 µg/ml) in DMEM (3A Sub E) or Ham's F12 (BeWo) at 37 °C. Cells were observed via light microscopy; when cells became detached growth medium (ten times the volume of trypsin solution) was added in order to quench trypsin activity. The cell suspension was transferred to a sterile centrifuge tube and centrifuged at 150 x g for 5 min. The supernatant was discarded, the pellet re-suspended in growth medium and

an aliquot was taken for cell count. The required volume of cells was then transferred to a flask and warmed growth medium was added. Cells were maintained as described in section 2.2.6.1.

2.2.6.3 Viable cell count and seeding cells

To estimate viable cell numbers equal volumes (typically 20 µl) of cell suspension and Trypan blue solution (0.4 % [v/v] were mixed. Using light microscopy a viable cell count was performed in five fields of view in a 0.1 mm depth Neubauer haemocytometer. Cell density was estimated using the following calculation:

Cell Numbers / ml = (average cell number / 0.1 mm^3) x 10^4 x 2 (dilution factor)

The required volume of cell suspension to achieve a specific cell density in a known volume of growth medium was then calculated. The required volume of cell suspension and growth medium were placed in an appropriate flask and incubated as described in section, 2.2.6.1, 2.2.6.4 or 2.2.6.5 depending on application.

2.2.6.4 Growing placental cell lines in hypoxic (2 % O_2) conditions

Cells were counted and seeded (500,000 cells in a T25 flask) as detailed in section 2.2.6.3 and allowed to recover overnight. Approximately 16-24 hours after seeding growth medium was removed and replaced with warmed fresh medium and flasks were transferred to the hypoxia incubator, where cells were incubated in 2 % (v/v) oxygen at 37 °C for the required time period. Duplicate flasks were grown under normoxic conditions (95 % O_2 [v/v], 5 % CO_2 [v/v]) as controls.

2.2.6.5 Growing placental cell lines under oxidative stress

Cells were counted and seeded (500,000 cells in a T25 flask) as detailed in section 2.2.6.3 and allowed to recover overnight. Oxidative stress was achieved from the reaction of xanthine oxidase with its sustrate xanthine to generate hydrogen peroxide. Growth medium was removed approximately 16-24 hours after seeding the cells, and replaced with warmed fresh medium containing Xanthine (100 µM final concentration) and increasing concentrations of Xanthine oxidase (0.25-5 mM final concentration). Flasks were then transferred to a 37 °C CO_2 incubator for the

required time period. Duplicate flasks were grown containing growth medium only and substrate only as controls.

2.2.6.6 Cryo-preservation of cells

Cells stocks were cryopreserved in order to ensure a constant supply. Viable cell count was performed as detailed in section 2.2.6.3 and cells resuspended in ice-cold freezing medium (95 % [v/v] foetal bovine serum [FBS] and 5 % [v/v] sterile dimethyl sulphoxide [DMSO]) at a densiy of 2 x 10^6 cells / ml. Aliquots (1 ml) of the cell mixture were dispensed into cryovials on ice and immediately transferred to –80 °C for gradual cooling before transferring to liquid nitrogen for long term storage.

2.2.6.7 Resuscitation of cryo-preserved cells

Cells were removed from liquid nitrogen storage and quickly thawed in a 37 °C water bath. Thawed cell suspensions were immediately transferred with a Pasteur pipette into a sterile tube containing 10 ml of fresh growth medium at 37 °C, centrifuged 150 x g for 5 minute and the supernatant removed. Cells were re-suspended in 1 ml of fresh growth medium using a Pasteur pipette before being transferred to a T25 flask containing an appropriate volume of growth medium. Cells were maintained as described in section 2.2.6.1.

2.2.6.8 Preparation of cell extracts for western and dot blotting

Following removal of growth medium cell monolayers were washed twice with PBS before addition of 100µl of ice-cold extraction buffer (50mM Trizma base, pH 6.8, 150 mM NaCl, 5 mM EDTA, 1 mM Sodium Orthovanadate, 0.5 % [v/v] Triton X-100, 2 mM PMSF and 0.2 % [v/v] protease inhibitor cocktail) directly on to the monolayer and cells extraction with a cell scraper. Cell suspensions were transferred to a 1.5 ml Eppendorf tube and incubated for 20 minutes on ice. Cell extracts were centrifuged at 14,000 x g for 10 minutes at 4 °C and supernatants were stored at –20 °C prior to estimation of protein concentration.

2.2.7 Protein estimation of extracts

2.2.7.1 Bicinchoninic acid (BCA) protein assay

The BCA protein assay was used in accordance with the manufacturer's guidelines to estimate protein content of cell extracts. A standard graph was constructed using different dilutions of bovine serum albumin (BSA), prepared in extraction buffer. 25 µl of both samples and standards were dispensed into the wells of an ELISA plate. 200 µl of BCA working solution (containing 50 parts reagent A and 1 part reagent B) were added to each well, mixed on a plate shaker and after an incubation period of 30 minutes at 37 °C, cooled to room temperature for 5 minutes. Absorbance was read at 570 nm.

2.2.7.2 Micro-Lowry method

Protein content of tissue samples was estimated using the Lowry method (Lowry *et al.*, 1951) with modifications. A standard graph was constructed using bovine serum albumin (BSA), prepared in extraction buffer. Samples and standards were made up to a final volume of 100 µl in distilled water. The working Lowry reagent (2 % [w/v] $NaCO_3$, 0.01 % [w/v] $CuSO_4$, 0.027 % [w/v] NaK tartrate in 0.1 M NaOH) was prepared immediately before use. 1 ml of working Lowry reagent was then added to each standard and sample, vortex mixed and incubated at room temperature for 15 min. Following the addition of 100 µl of Folin Ciocalteu's phenol reagent (diluted 1:1 in distilled water) the reaction was developed over 30 minutes at room temperature. Samples and standards were vortex mixed, 200 µl of samples and standards were then dispensed into an ELISA plate and absorbance measured at 750 nm.

2.2.8 Human chorionic gonadotropin (hCG) analysis by ELISA

A commercially available ELISA kit was used to measure Human Chorionic Gonadotrophin (hCG) secreted into the cell culture medium by placental cell lines. The assay is based on the sequential binding of hCG from a sample to two antibodies, one is immobilised on the ELISA plate and the other conjugated to enzyme horseradish peroxidise (HRP). The assay was performed according to the manufacturers instructions. Briefly, 25 µl aliquots of standards, controls and samples (cell culture medium collected after growing cells for the required time) were added to the wells of the plate. A set of blanks were also prepared using fresh growth medium.100 µl of HRP conjugated antibody was then added to each well and covered. After 30 minutes incubation at room temperature wells were washed four times with distilled water, 100 µl of substrate solution was added to each well and incubated for a further 10 minutes. Following the addition of 50 µl of stop solution, absorbance was measured at 450 nm. The amount of hCG in the samples were determined using the standards (detection limit for the assay is 1.5 mIU/ml).

2.2.9 Measurement of inositol phosphate accumulation in BeWo cells

Cells were cultured as detailed in section 2.2.6.1 until reaching 70-80 % confluency, cells were trypsinised and pelleted by centrifugation at 300 x g for 5 minutes. The cell pellet was re-suspended in 1 ml of growth medium; 50 µl of which was then used for a viable cell count. Each well in a 24 well plate was seeded with ~150,000 cells and left to settle for 24 hours. Cells were then pre-labelled by replacing growth medium with 600 µl of fresh serum free medium containing 0.42 mM [^{3}H] myo-inositol (5µCi/ml final concentration). After 24 hours, free [^{3}H] myo-inositol was removed by washing with Krebs-Henseleit buffer, and cells were equilibrated with phosphatase inhibitor Lithium Chloride (LiCl-20 mM) dissolved in L-15 Leibovitz medium. After 30 minutes incubation at 37°C, different concentrations of 5HT (0.01nM-1µM) were added to wells and incubated for a further 30 minutes at 37°C. The reaction was stopped by aspirating the medium from the wells. One millilitre of termination mix (Methanol / 0.1 M HCl mixed in a 1:1 ratio) was added to each well

and the plate was stored at -20°C overnight. Subsequently, 800 µl of sample were removed from each well and added to vials containing 4.2 ml of neutralizing solution (3.75 ml of 0.5 M NaOH, 27.5 ml Trizma base pH 7, and 85 ml of distilled water). Inositol phosphate was isolated from samples by ion exchange chromatography (Dowex anion exchange columns) and elution was performed using 1M HCl. Scintillation fluid (Ultima Gold, Perkin Elmer, UK) was added to eluted samples and radioactivity detected using a liquid scintillation counter (Packard TriCarb 2250CA).

2.2.10 Studies with recombinant MAO-A

2.2.10.1 Peroxynitrite synthesis and concentration determination

Peroxynitrite was synthesised from sodium nitrate and acidic hydrogen peroxide as described by Koppenol *et al.* (1996). Briefly, equal volumes (typically, 5 ml) of ice-cold 0.6 M $NaNO_2$ and 0.6 M HCl / 1.2 M H_2O_2 were mixed together. The same volume of 0.9 M NaOH was added instantly to preserve peroxynitrite, which is stable at an alkaline pH. In order to remove residual hydrogen peroxide, the solution was mixed with 1 gram of MnO_2 and stirred inside a fume cupboard until the bubbles disappear. Following filtration, peroxynitrite solution was incubated overnight at –20 °C in 1 ml aliquots and the resulting liquid fraction that formed the upper layer was collected, pooled together and stored at –80 °C. Several dilutions (ranging from 1:10 to 1:10,000) of the solution were prepared in 0.45 M NaOH and concentrations were determined spectrophotometrically at 302 nm according to the Beer-Lambert Law using an extinction coefficient of 1670/Mcm. Typically 80-100 mM peroxynitrite was obtained following freeze fractionation.

2.2.10.2 Nitration

Nitration of human recombinant MAO-A (EC1.4.3.4.) expressed in baculovirus infected insect cells (BTI-TN-5B1-4) was performed by incubation of the protein with peroxynitrite. Typically, 15 µg of MAO-A were re-suspended in 50 mM HEPES buffer pH 7.4. The nitrating reaction was initiated by the addition of peroxynitrite (0.1-1 mM) to the protein solution while mixing on a shaker. For control experiments peroxynitrite was added to the buffer 15 minutes before the addition of the enzyme in order to initiate degradation of peroxynitrite.

All samples were incubated at 37 °C for 30 minutes before assessing their activity (see section 2.2.4) or where applicable sample buffer was added for SDS-PAGE analysis (section 2.2.12.3).

2.2.10.3 Treatment with hydrogen peroxide

Similar to nitration assay detailed above, 15 µg of MAO-A were re-suspended in 50 mM HEPES buffer pH 7.4. In this case hydrogen peroxide was added to samples to give a range of final concentrations (1µM-10mM) while mixing on a shaker. A control sample without hydrogen peroxide was also prepared in HEPES buffer. All samples were incubated at 37 °C for 30 minutes before assessing their activity (see section 2.2.4).

2.2.11 Estimation of Lactate dehydrogenase (LDH) activity

LDH activity was determined using a spectrophotometric method that quantifies the conversion of pyruvate to lactate. LDH (0.075 unit) was treated with hydrogen peroxide in the same manner as MAO-A enzyme (detailed in section 2.2.10.3). Following incubation with hydrogen peroxide or buffer only (controls) LDH activity was measured. Briefly, 800 µl of 50 mM HEPES buffer, pH 7.4 (pre-warmed to 37 °C), 50µl of 27 mM pyruvate and 50 µl of 4 mM reduced ß-Nicotinamide adenine dinucleotide (NADH) and 100 µl of sample (containing 0.075 unit enzyme) were successively added in to a quartz cuvette. The decrease of absorbance due to the oxidation of NADH was recorded each 5 s for 5 minutes in a Beckman Coulter DU 530 Life Science UV / VIS Spectrophotometer at 340 nm. LDH activity was calculated from the slope of the absorbance curve.

2.2.12 Denaturing sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)

2.2.12.1 Preparation of acrylamide resolving gels

The Bio-Rad mini protean III apparatus was assembled according to the manufacturer's guidelines. For each gel 10 ml of gel mix was prepared as detailed in Table 2.1. Immediately before the gel was poured, 50 µl of 10 % (w/v) ammonium persulphate (APS) and 5 µl N, N, N', N'-tetramethyl-ethylenediamine (TEMED) were added to the gel mix as polymerisation agents. Gels were poured between the glass plates leaving enough space (approximately 2 cm) at the top for stacking gel and covered with 600 µl of distilled water in order to create a smooth surface. The gel mix was allowed to polymerise at room temperature for 30-45 min.

Separation of proteins within a sample can be varied depending on the percentage of acrylamide within the resolving gel. The table below details the reagents used in the preparation of polyacrylamide resolving gels.

Reagent	7.5% (w/v) gel	10 % (w/v) gel
40 % Acrylamide stock	1.9 ml	2.5 ml
1.5 M Tris buffer pH 8.8	2.5 ml	2.5 ml
10 % (w/v) SDS	100 µl	100 µl
Distilled water	5.5 ml	4.9 ml

Table 2.1 Preparation of acrylamide resolving gels for SDS-PAGE

2.2.12.2 Preparation of stacking gel

Once the resolving gel had polymerised, water was removed by blotting the top of the gel with filter paper. 2.5 ml of 4 % (w/v) acrylamide stacking gel mixture were used per gel. The reagent volumes required to prepare 50 ml of stacking gel mix are shown in Table 2.2. To polymerise 2.5 ml of the stacking gel stock, 12.5 µl APS and 5 µl of TEMED were added immediately before pouring on top of the resolving gel. Combs were positioned within the gel to form individual wells. The gel was allowed to polymerise for at least 30 min at room temperature before removal of the combs. Ready gels were then transfer to an electrophoresis chamber and submerged in SDS-PAGE buffer (Tris 25 mM pH 8.3, 192 mM glycine, 0.1 % [w/v] SDS).

Reagent	Volume
40 % Acrylamide stock	5 ml
0.5 M Tris buffer pH 6.8	12.5 ml
10 % (w/v) SDS	0.5 ml
Distilled water	32 ml

Table 2.2 Preparation of 50 ml stock 4 % polyacrylamide stacking gel

2.2.12.3 Preparation of samples for SDS-PAGE

Samples (typically 20-60 µg protein) were diluted 3:1 in 4 x concentrated reducing electrophoresis sample buffer (8 % [w/v] SDS; 40 % glycerol; 0.2 M Tris- HCl pH 6.8; 200 mM Dithiothreitol [DTT]; 0.02 % [w/v] Bromophenol blue). Samples were vortex mixed and heated to 100 °C for 5 minutes. Samples were then microfuged for 10 seconds to recover all the condensation prior to loading on to the gel. Proteins were separated at a constant current of 200 Volts. The process was stopped when the dye front approached the bottom of the gel.

2.2.13 Western Blotting and Immunoprobing of proteins

2.2.13.1 Western Blotting

Proteins separated by SDS-PAGE were transferred onto a nitrocellulose membrane by Western blotting using an Atto semi-dry blotting apparatus. Twelve pieces of filter paper and one piece of nitrocellulose were cut to the same size as the gel and were soaked in electroblotting buffer (39 mM glycine, 48 mM Trizma base, 0.0375 % [w/v] SDS, 20 % [v/v] methanol) (Towbin *et al.*, 1979). The gel was laid on the top of the nitrocellulose and this was sandwiched between six pieces of filter paper and plate electrodes on each side. The running conditions in the semi-dry blotter were 2 mA / cm^2 of gel for 100 minutes, which is optimal for the transfer of most proteins. Following electrophoretic transfer, the blots were stained using 0.05 % Copper phythocyanine in 12 mM HCl in order to assess the protein transfer efficiency. Following imaging using a Fujifilm intelligent dark box, the blots were fully destained in 12 mM NaOH solution.

2.2.13.2 Immunoprobing of Western Blots

Following destaining blots were blocked with 3 % (w/v) non-fat milk powder (Marvel) in Tris buffered saline (TBS; 50 mM Trizma base, 200 mM NaCl, pH 7.4) for 1 hour with gentle shaking followed by overnight incubation with primary antibody (diluted in blocking agent) at 4 °C with gentle shaking. Blots were then washed for six 10 minute periods with TBS containing 0.1 % (v/v) Tween-20 (TBS-Tween) with vigorous shaking in order to remove unbound primary antibody. Blots were then incubated for 2 hours at room temperature with gentle shaking with a horseradish peroxidase (HRP) or alkaline phosphatise (AP) conjugated secondary antibody diluted in 3 % Marvel/TBS, to allow development via enhanced chemiluminescence or colorimetric methods respectively (see sections 2.2.13.3 and 2.2.13.4). Blots were washed again with TBS-Tween for six 10-minute periods in order to remove unbound antibodies.

2.2.13.3 Developing Western Blots by Enhanced Chemiluminescence (ECL)

Blots were rinsed in distilled water and ECL was performed using a kit from Pierce (Perbio Science UK Ltd.) according to manufacturer's instructions. In brief, equal volumes of reagent 1 and reagent 2 were mixed to a final volume of 1 ml / 9 x 6 cm^2 nitrocellulose then immediately incubated for 1 minute while agitating. Blots were removed from the ECL reagent, excess was removed by blotting with a filter paper and positioned protein-side up in the Fuji Film Intelligent Dark Box. Blots were exposed to the camera for the required time, depending on the primary antibody used. The image was revealed digitally following the manufacturer's instructions.

2.2.13.4 Developing Western Blots with Alkaline Phosphatase

Blots were washed for 5 minutes in distilled water and immersed in substrate buffer (0.75 M Tris pH 9.5) for a further 5 minutes to equilibrate. Antibody binding was visualised by developing in the dark by addition of alkaline phosphatase substrate solution (20 ml substrate buffer; 33 µl 5-bromo-4-cloro-3indolyl phosphate [BCIP, 50 mg/ ml]; 44 µl nitro blue tetrazolium [NBT, 75 mg/ ml in 70 % (v/v) dimethyl formamide [DMF]) prepared immediately prior to use. The reaction was allowed to proceed at room temperature until bands appeared and stopped with extensive washing with distilled water. Developed blots were dried between two pieces of filter paper for further analyses or storage.

2.2.13.5 Stripping and re-probing membranes

In some cases it was necessary to re-probe membranes with a different primary antibody. In order to remove bound antibodies, blots were submerged in stripping buffer (6.25 mM Tris-HCl pH 6.7, SDS 2 % [w/v], 100 mM, 2-mercaptoethanol) and incubated at 50 °C for 30 minutes with occasional agitation. The membrane was washed six times for 10 minutes in TBS-Tween, then blocked and re-probed as detailed in section 2.2.13.2.

2.2.13.6 Quantification of Western blots and Dot blots

Quantitative comparison of protein band / dot intensity following Western / Dot blotting and immunoprobing was performed using the Aida Image Analyser v.4.03, according to the manufacturer's guidelines. In brief, pixel intensity of equal sized areas around bands /dots were obtained and quantified based on the number of pixels in each area, multiplied by the grey shade value of each pixel. The same analysis was also performed for the copper stain images. For western blots whole lane for each well was scanned while for dot blots each dot was used. Changes in protein loading were corrected against positive control or for the first well. Bands / dots corrected for loading using copper stains were then expressed as % positive control or % of the sample in the first well where applicable.

2.2.14 Dot Blotting

Dot blotting apparatus was assembled and carried out according to manufacturer's instructions. Briefly, one sheet of filter paper and nitrocelluse membrane was cut to the size of the apparatus and equilibrated in TBS. Moistened filter paper was placed on the unit and nitrocellulose was placed on top of the filter paper. The apparatus was closed and connected to a vacuum pump. Samples were diluted to a required final protein concentrations (typically, 4 – 80 µg / ml protein) in TBS depending on the application and 250 µl aliquot of sample were loaded per well. Under a vacuum wells were then washed with 250 µl of TBS. When the entire wash buffer had filtered through the membrane, the dot blotting apparatus was dismantled and the nitrocellulose membrane removed. Nitrocellulose membrane was blocked, probed with primary and secondary antibodies and developed as described in section 2.2.13.3 and 2.2.13.4.

2.2.15 Immunocytochemistry

Immunocytochemistry was used for the detection of MAO proteins in placental cell lines BeWo and 3A Sub E. Cells were grown as explained in sections 2.2.15.1. Liver sections, which were already available in our laboratory, were used as positive controls.

2.2.15.1 Seeding, growing and fixing cells on microscopic slides

BeWo and 3A Sub E cells were seeded and grown on (3" x 1") poly-L-lysine coated slides for immunocytochemical analysis. Cells were harvested as described in section 2.2.6.1 and seeded (at a cell density of 200,000 cells / slide) on to the slide, which was placed into a sterile Petri dish containing growth media. Slides were observed daily and when the cells reached ~ 60 % confluency, slides were removed from Petri dishes and washed in PBS twice to remove serum. Slides were then fixed by immersion in ice-cold acetone for 20 minutes then washed twice in PBS for 5 minutes.

2.2.15.2 Immunoprobing and developing of slides

Cells were permeabilised by incubating in a bath containing 0.5 % (v/v) Triton X-100 in PBS for 5 minutes at room temperature, then washed twice for 5 minutes in PBS. Prior to immunoprobing non-specific antibody binding was prevented by blocking with 20 % (v/v) normal swine serum (NSS) in PBS for 30 minutes at room temperature in a humidified chamber. Blocking solution was removed by tipping onto a tissue and slides were incubated with primary antibody (or RPMI medium for negative control) overnight at room temperature in a humidity chamber. Cells were washed with PBS three times for 5 minutes then incubated with HRP labelled secondary antibody (1:100 diluted in 5 % [v/v] NSS) for 1 hour at room temperature. Excess secondary antibody was removed by three 5-minute washes in PBS. Antibody binding was revealed by the addition of Sigma Fast DAB® (diaminobenzidine) solution prepared immediately before addion on to the slides. Slides were developed in the dark and observed for colour development. Excess DAB® was washed away in a bath of running water for 15 minutes. Slides were then counter stained with Gill's Haematoxylin. For this, slides were immersed into a bath of copper sulphate solution

(0.5 % [v/v] $CuSO_4.5H_2O$, 0.9 % [w/v] NaCl) for 15 minutes and washed with water for 2 minutes. Slides were then immersed into Gill's haematoxylin for 20 seconds before washing with running water. Following this, slides were incubated in Scott solution (0.2 % [w/v] $KHCO_3$, 2 % [w/v] $MgSO_4$) for 20 seconds before washing under running water for 1 minute. Slides were then transferred into an acid alcohol bath (75 % [v/v] ethanol, 10 mM HCl) briefly until excess haematoxylin was removed. Finally the cells were dehydrated by 1-minute washes in increasing ethanol baths (70 %, 90 % and 100 % [v/v]) and finally rinsed in xylene. DPX mountant (BDH, UK) was applied to the slide before a cover slip was placed over the cells and sealed into place. Slides were air-dried in a fume cupboard prior to viewing and imaging by phase contrast microscopy.

Liver sections were also treated as explained above except prior to incubation with primary antibodies, endogenous peroxidise activity of liver sections was inhibited by a 10 minute incubation in 3 % (v/v) H_2O_2 in PBS.

2.2.16 Statistical analysis

Data were presented ± the standard error of the mean (SEM) at a 95 % confidence limit. Statistical analysis was performed using a two-tailed Student's t-test and statistical significance was accepted at $p < 0.05$ (*) or 0.01 (**).

All values from tissue bath data were also presented as ± the standard error of the mean (SEM), *n* represents the number vessel rings, each vessel was obtained from a different patient unless specified in the text. Statistical analysis was performed using a two-tailed Student's t-test (unless otherwise specified in the text) after confirming normal distribution using the Kolmogorov-Smirnov (KS) test employing GraphPad Software. For the comparison of normotensive vessels to pre-eclamptic vessels unpaired Student's t-test was employed. For the comparison of primary vessel to secondary vessels, Student's t-test for the paired data was employed. In both cases statistical significance was accepted at $p < 0.05$ (*) or 0.01 (**).

CHAPTER III

IN VITRO TISSUE BATH OPTIMISATION AND 5-HT_{2A} RECEPTOR ANTIBODY CHARACTERISATION

3 Chapter III *In vitro* tissue bath optimisation and 5-HT_{2A} receptor antibody characterisation

3.1 Introduction

Blood vessels play crucial roles in the maintenance of blood pressure and regulation of tissue blood flow and distribution by means of changing their tone. Vascular tone of a blood vessel can be defined as the degree of constriction to its maximally dilated state. Contraction of the smooth muscle cells reduces the diameter of the vascular lumen decreasing blood flow, whilst relaxation of the smooth muscle cells increases blood flow. The degree of constriction of the smooth muscle cells in the vascular wall determines the vessels diameter and hence tone of the vessel. This tone is thought to depend on innervation (Bevan, 1978), circulating and locally synthesized vasoactive substances (Luscher, 1990) as well as physical forces such as pressure and shear force interacting with endothelial and vascular smooth muscle cells (Price, 1991).

3.1.1 Vascular smooth muscle contraction

A number of chemical stimuli such as 5-HT, angiotensin II, endothelin-1 and thromboxane A_2 can cause vascular smooth muscle (VSM) contraction. Each of these substances binds to a specific receptor on the VSM cells or to receptors on the endothelium adjacent to the VSM and initiates contraction. The mechanism of contraction involves several signal transduction pathways, however intracellular calcium plays a major role as a second messenger (Horowitz *et al.*, 1996). A simplified VSM contraction mechanism is illustrated in Figure 3.1. Activation of receptors on the cell surface with the binding of an agonist results in an increase in free intracellular calcium via release from internal stores (such as sarcoplasmic reticulum). Similarly an increase in free intracellular calcium can result from increased influx into the cell through calcium channels on the cell membrane. Free calcium binds to calmodulin, which in turn results in the activation of calcium-calmodulin dependent myosin light chain kinase (MLCK). MLCK is an enzyme that is capable of phosphorylating 20-kDa myosin light chains in the presence of ATP, facilitating cross-bridge formation between the myosin heads and actin filaments, hence resulting in increased vascular tone (or contraction) (Horowitz *et al.*, 1996).

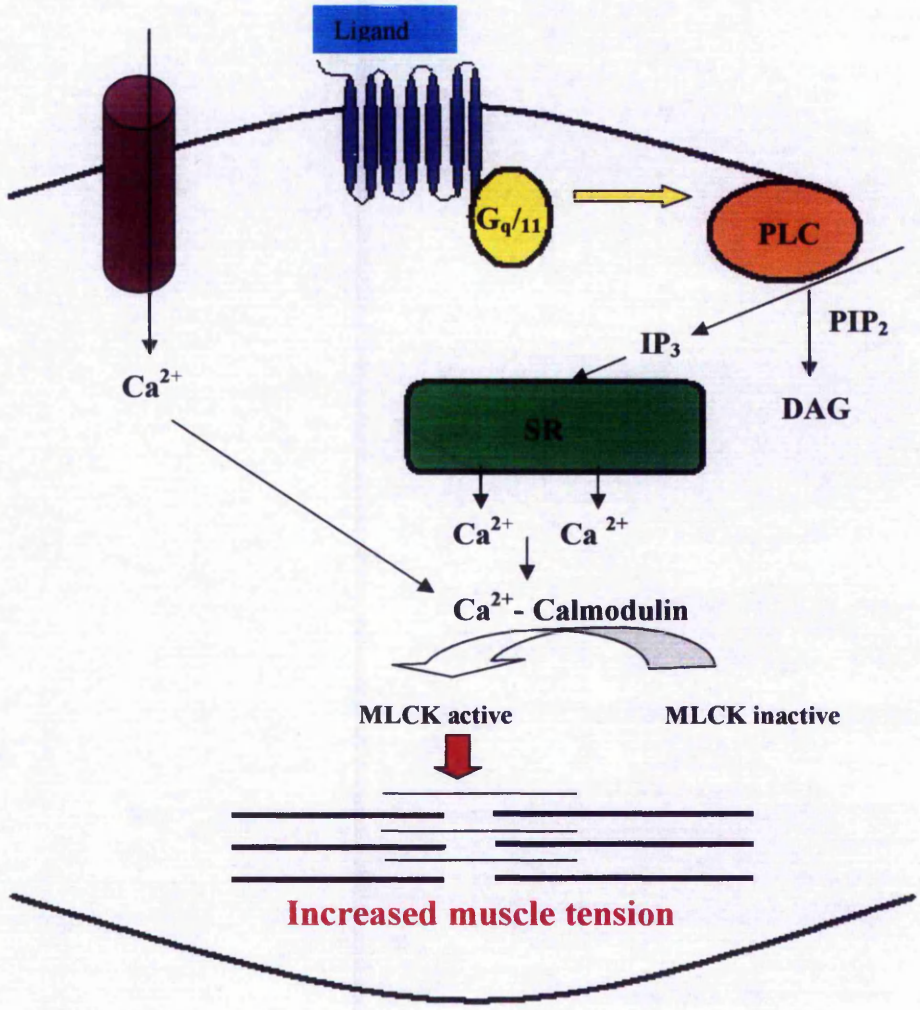


Figure 3.1 Signal transduction mechanism in vascular smooth muscle contraction

Vascular smooth muscle (VSM) contraction requires the binding of Ca^{2+} to calmodulin. This complex then activates myosin light chain kinase (MLCK). MLCK phosphorylates the light chain of myosin, which facilitates cross-bridge formation between the myosin heads and actin filaments resulting in contraction. The Ca^{2+} for activation of this pathway can enter through Ca^{2+} channels or alternatively, can be released from intracellular stores (such as sarcoplasmic reticulum, SR) after activation of membrane receptors coupled to Inositol 1,4,5-triphosphate (IP_3) production.

$G_{q/11}$=heterotrimeric GTP-binding protein, PLC= phospholipase C, PIP_2= phoshatidylinositol 4,5-bisphosphate, DAG= diacylglycerol.

3.1.2 Vascular tone of placental vessels

Placental blood vessels facilitate nutrient exchange between the maternal and foetal circulations, thus the function of these vessels is vital for the maintenance of a healthy pregnancy and foetal growth. The placental circulation consists of umbilical, chorionic plate and stem villi arteries and veins and the tone of these vessels is important for the delivery of the nutrients and oxygen to the foetus. The mechanisms responsible for the regulation of placental blood flow are poorly understood, however it is well established that the umbilicoplacental circulation is devoid of autonomic innervation (Reilly and Russell, 1977; Fox and Khong, 1990). Therefore humoral factors are suggested to be responsible for the regulation of the umbilicoplacental vascular tone. These include for example, amines such as 5-HT (Gonzalez *et al.*, 1990), histamine (Cruz *et al.*, 1991), peptides such as angiotensin II (Rosenfeld and Naden, 1989) and eicosanoids such as prostaglandins (Bjoro and Stray-Pedersen, 1986). 5-HT has been reported to induce vascular contraction of umbilicoplacental vessels at physiologic concentrations (Altura *et al.*, 1972; Gonzalez *et al.*, 1990).

3.1.3 Aims of chapter

Tissue bath techniques allow isometric tension measurement of isolated vessels under different experimental conditions. However, several factors might affect the subsequent evaluation of the contractions of these vessels. Therefore the main aim of this chapter was to investigate optimal experimental conditions for *in vitro* tissue bath studies. Initial studies concentrated on establishing the optimal passive tension and KCl concentration for chorionic vessels. Although KCl has been used as pre-contracting agent for human chorionic vessels by most of the previous studies (Cruz *et al.*, 1995; Estan *et al.*, 1998; Bertrand and St-Louis, 1999), the concentration of KCl used varies in different studies. Therefore preliminary experiments investigated the minimum KCl concentration that elicited the maximal response in human placental vessels.

Since this study aims to compare normotensive and pre-eclamptic chorionic vessel response to 5-HT and the contribution of 5-HT_{2A} receptor in this contractile response (see chapter IV), it was also important to ensure that the contractile response to 5-HT was reproducible within the experimental timeline.

At the time the experiments were conducted blood concentrations of ketanserin (5-HT_{2A} receptor antagonist) in patients treated with this drug for pre-eclampsia were not available therefore preliminary experiments were conducted in order to assess ketanserin antagonism at varying concentrations.

Finally, the final aim of this chapter was to characterise a commercially available anti-5HT_{2A} receptor mouse monoclonal antibody (clone G186-1117), using western blotting. This peptide antibody was directed against human 5-HT_{2A} receptors and originally characterised using rat and human brain samples (Wu *et al.*, 1998). The immunogen was the entire first extracellular domain of the N-terminus of the 5-HT_{2A} receptor, where 5-HT_2 receptor subtypes share less than 30% homology. Therefore the antibody specifically binds to the 5-HT_{2A} receptor subtype while showing no immunoreactivity against 5-HT_{2B} and 5-HT_{2C} receptors (Wu *et al.*, 1998). In this study, reactivity of G186-1117 with a range of rat and human samples was investigated.

3.2 Results

3.2.1 Optimal conditions for studying contractile responses of placental arteries and veins

3.2.1.1 Optimal passive tension for placental arteries and veins

Optimal passive tension for placental arteries was determined using a standard supra-maximal concentration of KCl (120 mM). Arteries were adjusted and equilibrated to different initial tensions (0.5, 1, 1.5, 2 and 3 g) as detailed in section 2.2.2.3.1. The contractile response increased with increasing tension reaching a plateau with around 1.5-2 g (Figure 3.2). However differences between contractions achieved with 1.5-2 g and other passive tensions studied were not statistically significant.

Optimal passive tension for placental veins was also determined. The maximal response to KCl was achieved at 1.5 g for both primary and secondary chorionic veins (Figure 3.3). However, the only significant difference was between passive tensions of 0.5 g and 1.5 g.

3.2.1.2 Concentration – response to KCl

In order to determine an optimal passive tension together with an optimal concentration of KCl as a pre-contracting agent, vessel response to increasing amounts of KCl was recorded at passive tensions of 1.5 and 2 g. Chorionic arterial and venous rings from a single placenta were used. Vessels were pre-adjusted and equilibrated to passive tensions of either 1.5 g or 2 g before KCl was added cumulatively (5 mM-120 mM) to the tissue bath. Contraction increased with increasing KCl, and plateaued with 60 mM KCl for both arteries and veins. In addition the response to KCl was similar at both passive tensions. In subsequent experiments a passive tension of 1.5 g and pre-contraction with 60 mM KCl were used in order to avoid overstretching the tissue. Concentration response curves to KCl are presented in Figures 3.4 and 3.5 for arteries and veins, respectively.

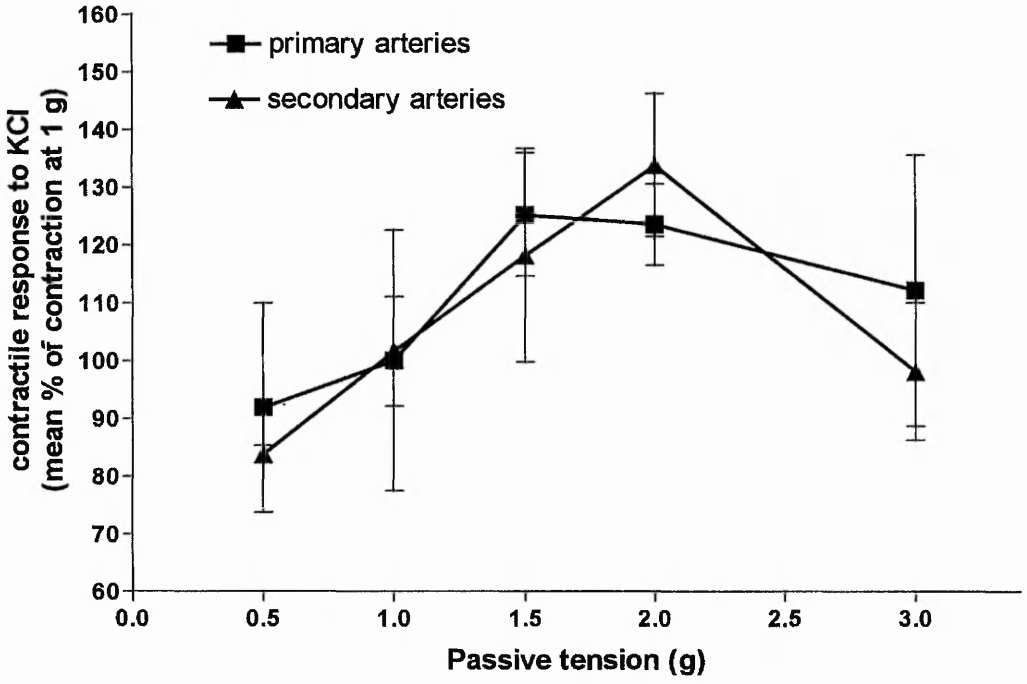


Figure 3.2 Response of the placental arteries to KCl at different passive tensions

Optimal passive tension for primary arteries (squares) and secondary arteries (triangles) was determined by studying the response to a supra-maximal concentration of KCl (120 mM). Following adjustment and equilibration to 0.5, 1, 1.5, 2 and 3 g passive tensions contractile response to KCl was recorded at each passive tension and expressed as percentage of contraction achieved at 1 g passive tension (*n=3-7*). Experiments were conducted using normotensive placentae, results expressed as mean ± SEM and *n* represents number of vessels used for the study (each vessel obtained from a different placenta). Maximum contractile response (1.5 gram and 2 g for primary and secondary arteries respectively) was compared to other passive tensions studied. Statistical significance was accepted at $p< 0.05$ (*) and $p<0.01$ (**).

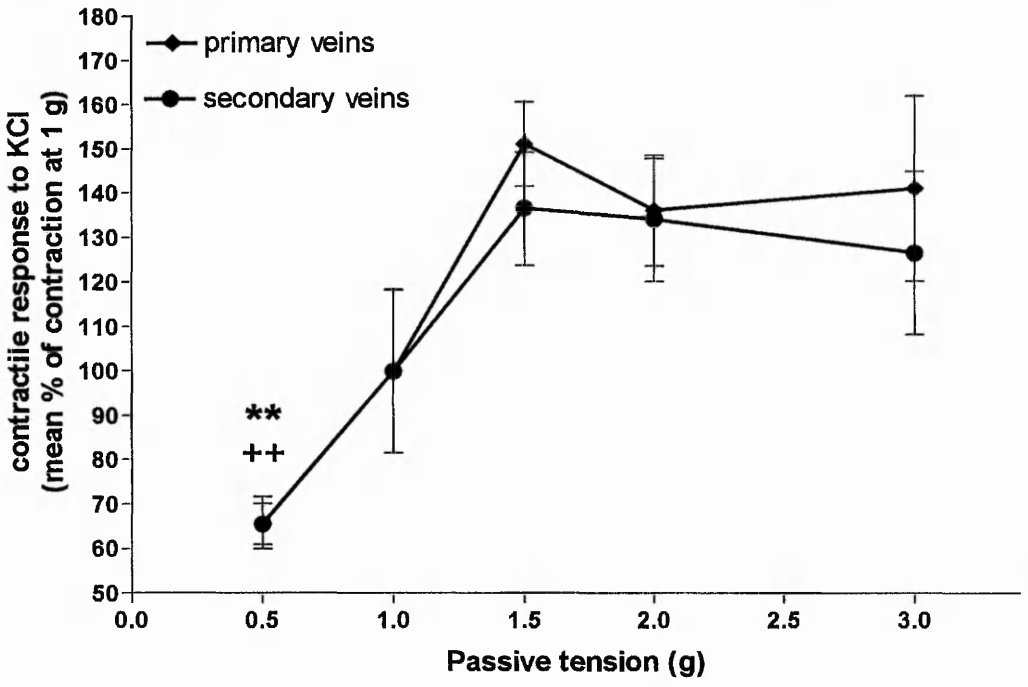


Figure 3.3 Response of the placental veins to KCl at different passive tensions

Optimal passive tension for primary veins (diamonds) and secondary veins (circles) was determined by studying the response to a supra-maximal concentration of KCl (120 mM). Following adjustment and equilibration to 0.5, 1, 1.5, 2 and 3 g passive tensions contractile response to KCl was recorded at each passive tension and expressed as percentage of contraction achieved at 1 g (*n=3-7*). Experiments were conducted using normotensive placentas, results expressed as mean ± SEM and *n* represents number of vessels used for the study (each vessel obtained from a different placenta). Maximal contractile response (1.5 g) was compared to 0.5, 1, 2 and 3 g. Statistical significance was accepted at $p < 0.05$ (*), $p<0.01$ (**) for primary veins and $p < 0.05$ (+), $p<0.01$ (++) for secondary veins.

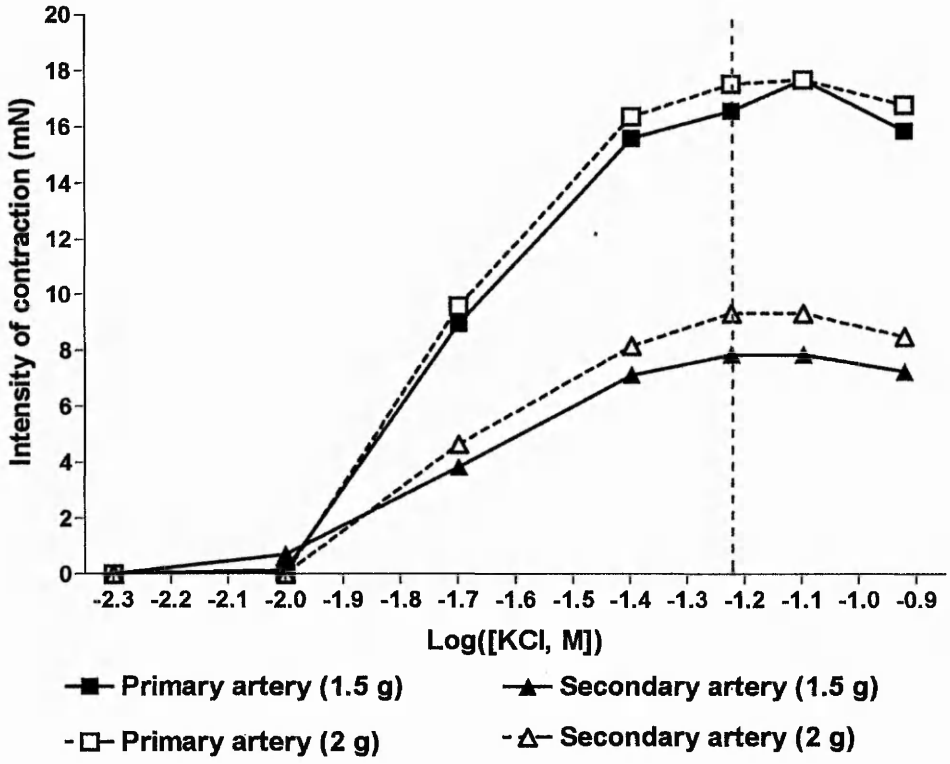


Figure 3.4 Response of human chorionic arterial rings to KCl at passive tensions of 1.5 g and 2 g

Primary arteries (squares) and secondary arteries (triangles) were adjusted and equilibrated at a passive tension of 1.5 g (filled squares and triangles) and 2 g (open squares and triangles). Contraction was recorded after adding KCl cumulatively (5 mM-120 mM) to the tissue bath. KCl induced a concentration dependent contraction of the chorionic vessels. Maximal contraction was achieved with 60 mM KCl (labelled with dashed line on graph) for both primary and secondary arterial rings (*n=1*).

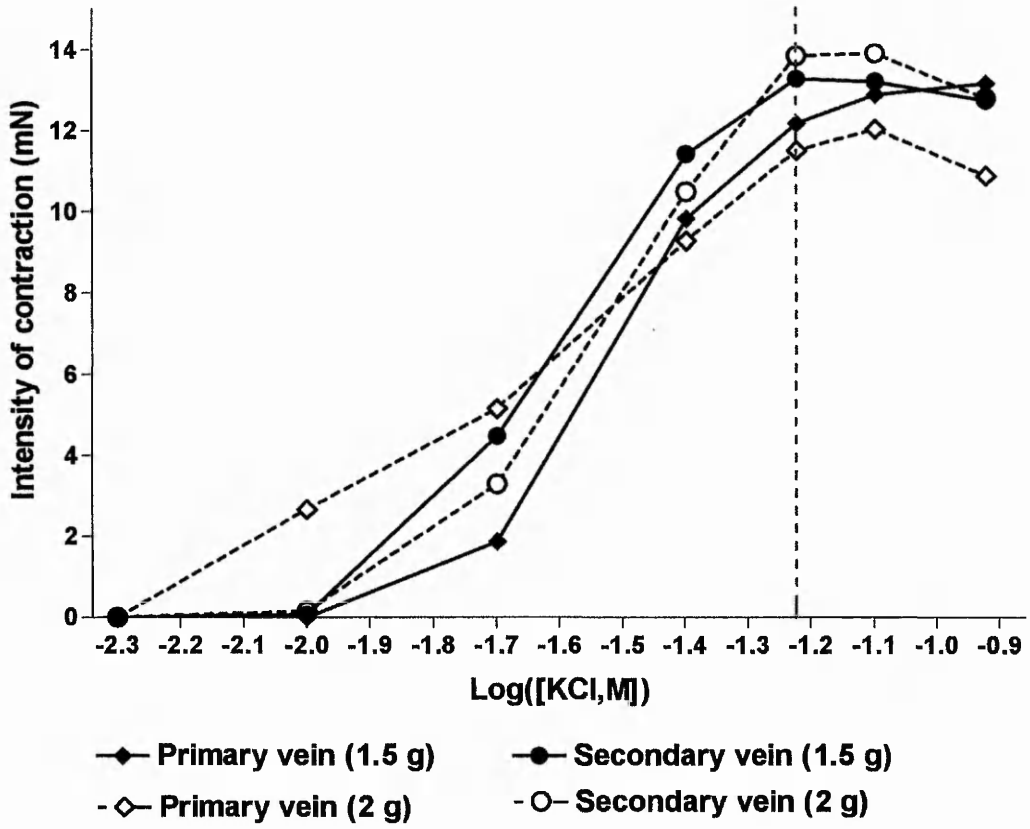
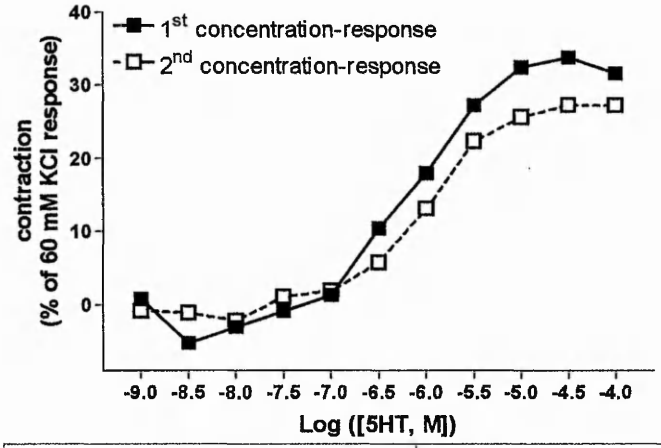


Figure 3.5 Response of human chorionic venous rings to KCl at passive tensions of 1.5 g and 2 g

Primary veins (diamonds) and secondary veins (circles) were adjusted and equilibrated at a passive tension of 1.5 g (filled diamonds and circles) and 2 g (open diamonds and circles). Contraction was recorded after adding KCl cumulatively (5 mM-120 mM) to the tissue bath. KCl induced a concentration dependent contraction of the chorionic vessels. Maximal contraction was achieved with 60 mM KCl (labelled with dashed line on graph) on both primary and secondary venous rings ($n=1$).

3.2.1.3 Profiles of consecutive concentration-response to 5-HT

The purpose of this study was to ensure that the response to ketanserin was not affected by a previous concentration-response to 5-HT and also to investigate whether the contractile response to 5-HT was reproducible within the experimental time frame. 5-HT response was investigated using primary arteries from a single normotensive placenta. Vessels were treated according to standard protocol outlined in section 2.2.2.3.2, except that for the second concentration-response to 5-HT vessels were incubated without the addition of ketanserin. The time difference between the two concentration-responses was approximately 3.5 hours (section 2.2.2.3.2). Figure 3.6 shows that the two consecutive concentration–responses are very similar and the differences are deemed insignificant in comparison with the variation involved with these experiments.



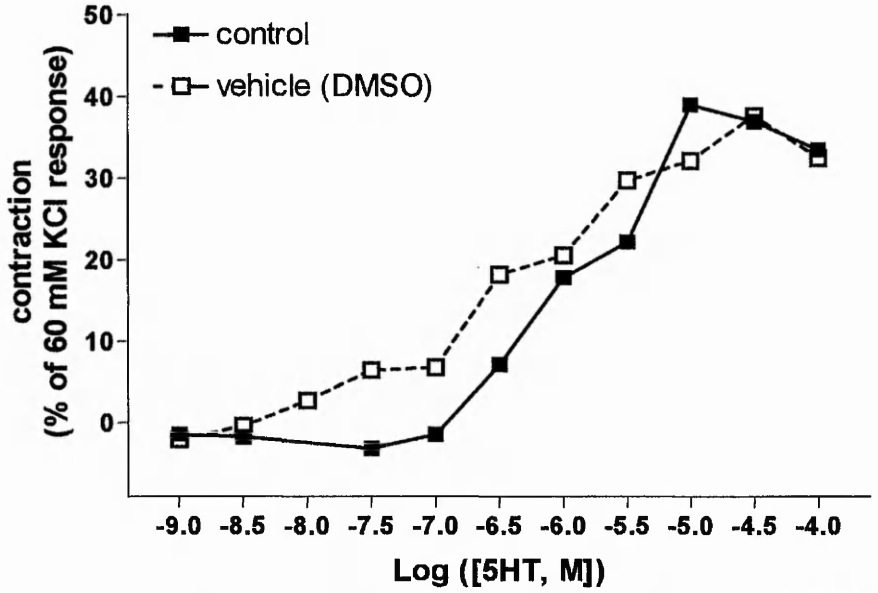
	Emax (% KCl)	pEC50
1st Concentration-response	33.53	6.179
2nd Concentration-response	28.08	6.018

Figure 3.6 Profiles of consecutive concentration response to 5-HT

Profiles of two consecutive concentration-responses to 5-HT were investigated using primary arteries from a single normotensive placenta (*n=1*). Vessel response to cumulative addition of 5-HT was recorded (first concentration-response, represented by filled squares). After washing and a 30 minute incubation concentration-response was repeated (second concentration-response, represented by open squares). Contraction is expressed as % 60 mM KCl response. Agonist potency (EC_{50}) expressed as pEC_{50} (-log EC_{50}) and efficacy of the agonist expressed as Emax (maximal response).

3.2.1.4 Effect of vehicle on vascular reactivity

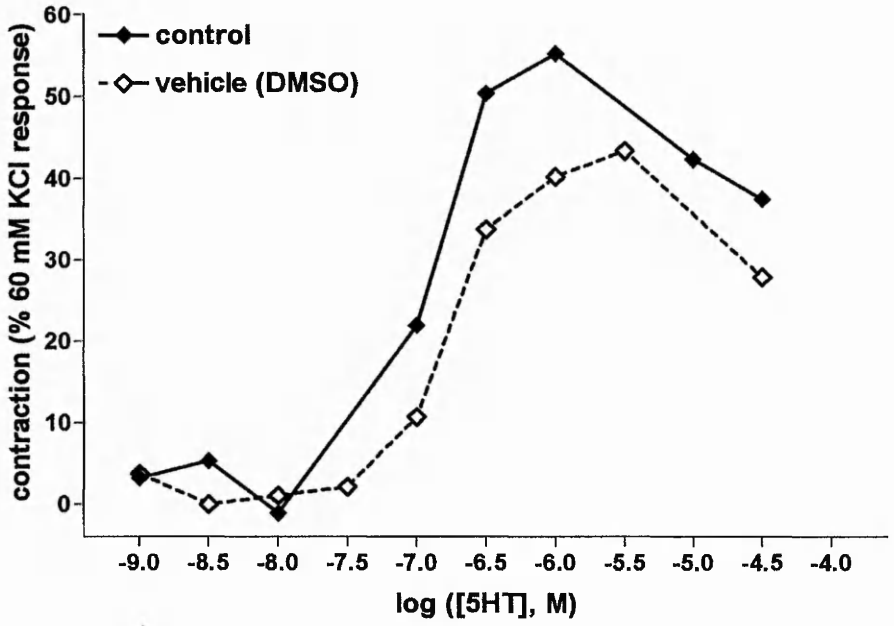
The effect of the vehicle (DMSO) used for solubilising ketanserin on the response to 5-HT was also investigated. Primary arteries and veins were obtained from a single normotensive placenta. Vessels were treated according to the standard protocol (section 2.2.2.3.2) except they were incubated for 30 minutes in DMSO (not ketanserin) before the second concentration-response to 5-HT. Figures 3.7 and 3.8 demonstrate little effect of DMSO for primary arteries and veins respectively.



Concentration-response	Emax (% KCl)	pEC_{50}
Control	39.04	5.952
Vehicle (DMSO)	37.67	6.392

Figure 3.7 Effect of vehicle on vascular reactivity of arteries

The effect of vehicle (DMSO) was investigated using primary arteries obtained from a single normotensive placenta (*n=1*). Vessels were incubated in DMSO instead of ketanserin before conducting a second concentration-response to 5-HT. First concentration-response (control), represented by filled squares and second concentration-response in presence of DMSO is represented by open squares. Contraction is expressed as % 60 mM KCl response. Agonist potency (EC_{50}) expressed as pEC_{50} (-log EC_{50}) and efficacy of the agonist expressed as Emax (maximal response).



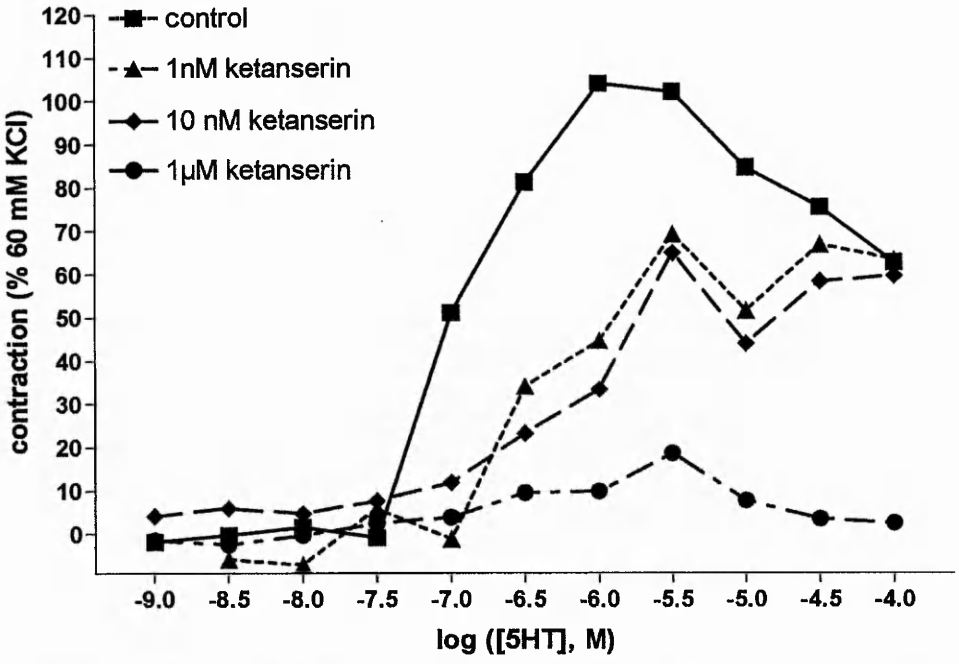
Concentration-response	Emax (% KCl)	pEC_{50}
control	55.23	7.059
Vehicle (DMSO)	43.43	6.755

Figure.3.8 Effect of vehicle on vascular reactivity of veins

The effect of vehicle (DMSO) was investigated using primary veins obtained from a single normotensive placenta (*n=1*). Vessels were incubated in DMSO instead of ketanserin before conducting a second concentration-response to 5-HT. First concentration-response (control) represented by filled diamonds and second concentration-response (in presence of DMSO) represented by open diamonds. Contraction is expressed as % 60 mM KCl response. Agonist potency (EC_{50}) expressed as pEC_{50} (-log EC_{50}) and efficacy of the agonist expressed as Emax (maximal response).

3.2.1.5 Effect of different concentrations of ketanserin on antagonism to 5-HT

Due to limited tissue availability primary veins from a single normotensive placenta were used. Figure 3.9 demonstrates 5-HT-responses with three concentrations of ketanserin. Increasing concentrations of ketanserin reduced Emax while having little effect on EC_{50}. Maximum antagonism of the 5-HT_{2A} receptor was required in subsequent experiments; thus 1μM was chosen for future studies.



	Ketanserin concentration			
	control	**1nM**	**10 nM**	**1μM**
Emax	103.67	69.41	65.07	18.73
pEC_{50}	7.063	6.477	6.189	6.467

Figure 3.9 Ketanserin at different concentration

Adjacent vein rings, cut out from the same vessel, were used to assess ketanserin antagonism to 5-HT at different concentrations (*n=1*). Vessel response to 5-HT was separately assessed in the presence of different concentrations of ketanserin (1nM, 10 nM, and 1μM). Contraction is expressed as % 60 mM KCl response. Agonist potency (EC_{50}) expressed as pEC_{50} (-log EC_{50}) and efficacy of the agonist expressed as Emax (maximal response).

3.2.2 5-HT_{2A} receptor antibody characterisation

Commercially available mouse monoclonal anti-5-HT_{2A} receptor antibody (5HT2AR, clone G186-1117) was characterised using western blotting. This peptide antibody was directed against human 5-HT_{2A} receptors. Initial studies concentrated on establishing the reactivity of the antibody with platelets since they are a rich source of the receptor (McBride *et al.*, 1983). G186-1117 reacted strongly with a 55 kDa protein and displayed small reactivity with a 63 kDa protein in a platelet sample (Figure 3.10 lane 1). Incubating blots only with the secondary antibody revealed no reactivity (Figure 3.10 lane 2).

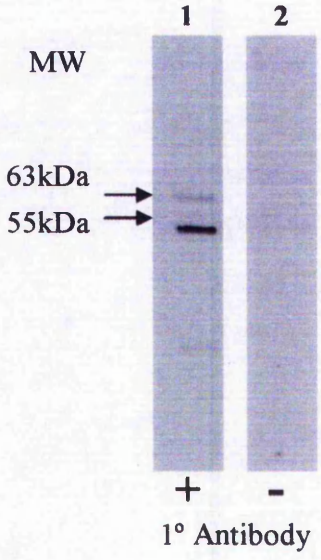


Figure 3.10 Western blot analysis of a 5-HT_{2A} receptor antibody

Platelet extracts (40 µg) were separated by SDS-PAGE and transferred electrophoretically on to nitrocellulose membrane. Shown are blot of platelet extracts probed with anti-5HT_{2A} mouse monoclonal antibody (clone G186-1117) followed by goat anti-mouse horseradish peroxidase conjugated secondary antibody (lane 1) or goat anti-mouse horseradish peroxidase conjugated secondary antibody (lane 2). Antibody binding was detected by ECL as described in section 2.2.13.3.

MW= molecular weight

Reactivity of G186-1117 with a range of rat and human samples was then investigated. Since G186-1117 was originally characterised using rat and human brain samples (Wu *et al.*, 1998), a rat brain sample was used as a positive control. Figure 3.11 shows that the antibody binding profile varied with different tissue types. As with platelets (lane 4) with rat brain samples (lane 1) the antibody reacted with a 55 kDa protein and had a weak reaction with a 63 kDa band. With whole rat placenta (lane 2), however, the dominant reaction was with a 40 kDa protein. But other proteins ranging in size from 40 to 55 kDa as well as 63 kDa also reacted. Human placental arteries (lane 6) and veins (lane 5) displayed a very similar profile to the rat placental sample (lane 2). This was not surprising as rat placenta included blood vessels. Human placental tissue (devoid of blood vessels) (lane 3) and BeWo cell extracts (lane 7) contained a major immunoreactive protein band of approximately 63 kDa and faint bands of 55 kDa. Since the antibody is raised against a peptide immunogen (Wu *et al.*, 1998) which shows predictable binding to human platelets and rat brain it is believed that all reactive bands are at least derived from the 5-HT_{2A} receptor (further considered in section 3.3.4)

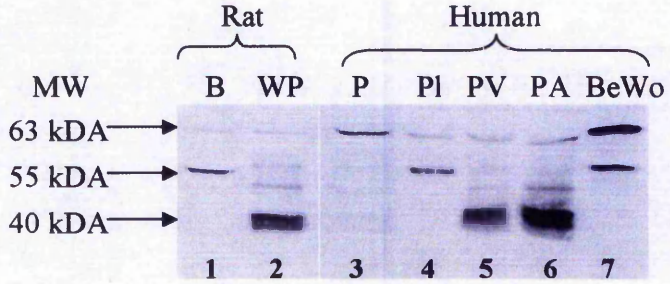


Figure 3.11 Comparison of 5-HT_{2A} receptor protein profile in rat and human tissues

Rat, human tissue samples and BeWo cells extracted as detailed in sections 2.2.3.1 and 2.2.6.8. 40 µg protein was loaded / lane, separated by SDS-PAGE and transferred electrophoretically on to nitrocellulose membrane. Shown are blot of rat, human tissue and BeWo cells extracts probed with anti-5HT_{2A} mouse monoclonal antibody (clone G186-1117) followed by goat anti-mouse horseradish peroxidase conjugated secondary antibody. Antibody binding was detected by ECL as described in section 2.2.13.3.

MW= molecular weight, B=Brain, WP=Whole placenta (including blood vessels), P= Placental tissue (without blood vessels) Pl=Platelets, PV=Placental veins, PA=Placental arteries, BeWo cell extract.

3.3 Discussion

3.3.1 Passive tension and KCl concentration

In order to maintain consistency, chorionic arteries and veins of approximately 2 mm of outer diameter and 2 mm long were used in all experiments. The optimal tissue bath conditions were all established using these constant sized vessel rings (both arteries and veins). In order to avoid overstretching of the tissue, an optimum passive tension of 1.5 g was selected to use in the tissue bath experiments.

KCl has been used as pre-contracting agent for human chorionic vessels by most previous studies (Cruz *et al.,* 1995; Estan *et al.,* 1998; Bertrand and St-Louis, 1999). However, the concentration of KCl used varies in different studies. It was therefore important to determine the minimum KCl concentration that elicited the maximal response in order to avoid unnecessary stressing of the tissue. It should be noted that the dose response to KCl was obtained using vessel rings (2 arteries and 2 veins) from the same placenta, and that 60mM KCl has given the maximal contraction in all of them. The KCl concentration found to give maximum contraction in this study is only slightly higher than that of Estan *et al.* (1998), who reported maximal response in chorionic vessels with 40 mM KCl.

3.3.2 Effect of time and vehicle on vascular response

It is well-known that prolonged storage of vascular specimens at low temperature adversely affects endothelial and smooth muscle cell viability and function (Stanke-Labesque *et al.*, 1999; Abad *et al.*, 2003). For example Abad *et al.* (2003) noted a significant decrease in chorionic vascular contractility when specimens were stored for 24-hour in cold, compared to fresh preparations. In this study therefore, in order to avoid any variations caused by tissue viability and function, blood vessels were dissected and experiments were performed immediately after delivery. It was also important to ensure that the contractile response to 5-HT was reproducible within the experimental timeline and response to ketanserin was not affected by previous concentration-response to 5-HT. Therefore preliminary experiments were conducted using primary vessels (arteries and veins) from a single normotensive placenta.

In these experiments, vessels were treated according to the standard protocol, except that for the second concentration-response to 5-HT, vessels were incubated without the addition of ketanserin. A typical time interval between the two concentration-responses to 5-HT was approximately 3.5 hours (section 2.2.2.3.2). Due to limited tissue availability it was not possible to repeat the experiments; however, preliminary experiments showed that within the experimental timescale the difference between the two consecutive concentration–response curves was very small (Figure 3.6). The possible effects of the vehicle (DMSO) used for solubilising ketanserin on response to 5-HT was also investigated (Figure 3.7 and Figure.3.8). The difference between the two consecutive concentration-response curve in the absence and presence of vehicle was very small. Thus, these differences were deemed insignificant in comparison with the variation involved with these experiments. As a result, it could be suggested that any changes in response to 5-HT in the presence of ketanserin would be due only to receptor blockage by ketanserin and unlikely to be due to changes in the receptor levels caused by either time or previous exposure to vehicle or 5-HT.

3.3.3 Ketanserin concentration

At the time the experiments were conducted, blood concentrations of ketanserin in patients treated with this drug for pre-eclampsia were not available. It was only known that ketanserin could cross the placenta as shown in pregnant ewes (Schneider *et al.*, 1996). Therefore approximate ketanserin concentration in blood was estimated from therapeutic doses (Bolte *et al.*, 2001) to be in the range of 0.1-1μM. In addition preliminary experiments were conducted with primary veins from a single normotensive placenta in order to assess ketanserin antagonism at varying concentrations. For this adjacent vein rings response to 5-HT were separately recorded in the presence of different concentrations of ketanserin (Figure 3.9). In order to obtain maximal antagonism of the 5-HT_{2A} receptor, a concentration close to the therapeutic dose and also in line with other studies was chosen (Cruz *et al.*, 1998), namely 1μM for use in all experiments. This was also found to be in agreement with subsequent published data for transplacental transmission of ketanserin in pre-eclamptic patients (Hanff *et al.*, 2004).

In the present study, increasing concentrations of ketanserin reduced Emax while having little effect on pEC50 (Figure 3.9), typical characteristics of non-competitive inhibition. This is in good agreement with the findings of Cruz *et al.* (1998) who also reported non-competitive inhibition by ketanserin (0.1 and 1μM) using human placental veins. However, ketanserin seems to act as a competitive inhibitor at low concentrations (VanNueten *et al.*, 1986; O'Donohue *et al.*, 2004) and converts to a non-competitive inhibitor at higher concentrations (Monopoli *et al.*, 1990; Cruz *et al.*, 1998). For example, this was observed with isolated human mammary arteries. Monopoli *et al.* (1990) reported that ketanserin reversed the contractions elicited by 5-HT in a competitive manner at low concentrations (10 nM), while non-competitive inhibition was observed at higher concentrations (0.05, 0.1 and 0.5 μM).

3.3.4 5-HT_{2A} receptor protein profile in rat and human tissues

Of particular interest in the present study was a comparison of 5-HT_{2A} receptor protein levels in various chorionic plate vessels (normotensive versus pre-eclamptic or primary versus secondary) to allow an assessment of whether receptor levels contributed to possible changes in vascular response to 5-HT (see chapter IV). Initial experiments characterised a commercial anti-5HT_{2A} mouse monoclonal antibody (clone G186-1117), using western blotting. Wu *et al.* (1998) noted that G186-1117 detected an immunoreactive band of about 55 kDa in rat brain extracts, which is the predicted size for the 5-HT_{2A} receptor protein. On the other hand a smear of immunoreactive proteins of sizes ranging from 40-55 kDa was observed in human brain extracts. These authors explained the differences in rat and human brain extracts to be the result of protein degradation since human tissues would be obtained several hours post mortem.

In the present study, reactivity of G186-1117 with a range of rat and human samples was investigated. 5-HT_{2A} receptor protein profile varied within different tissue types (Figure 3.11). The antibody reacted with a 55 kDa protein and had a weak reaction with a 63 kDa band in rat brain and human platelets. Whole rat placenta and human placental vessels, however, displayed immunoreactive proteins of sizes ranging from 40 to 55 kDa as well as 63 kDa. Since rat placental samples included blood vessels this was not surprising.

Profiles were in good agreement with Wu *et al.* (1998) for human and rat brain extracts. Moreover, human placental tissue extracts and BeWo cell extracts revealed a major protein band of approximately at 63 kDa and faint bands of 55 kDa. Interestingly a doublet bands of 63 kDa and 51 kDa 5-HT_{2A} receptor protein was reported in mouse cumulus cells using a polyclonal rabbit antibody (Amireault and Dube, 2005). Indeed as noted in chapter I, a variant of the 5-HT_{2A} receptor that appears to be generated by insertion of a 118 base pair cassette into the fourth transmembrane domain splice site has been identified (Guest *et al.*, 2000). Although the origin of this cassette is not known, it is thought to be derived from the intron between exon II and III which is normally spliced out (Guest *et al.*, 2000). It is possible that the 63 kDa protein observed in the present study and others could be this variant of the 5-HT_{2A} receptor. Therefore in the present study, 5-HT_{2A} receptor protein quantification in placental vessels included both 63 kDa and 40-55 kDa proteins. Nevertheless, differences in the relative expression of 55 and 63 kDa proteins in different tissue samples could not be readily explained by this study. More research will be necessary to confirm the identity of the 63 kDa protein and to show possible functional role of this variant. However it has been demonstrated that variant (truncated) receptors might form heterodimers with corresponding full length receptors and regulate their function (Schoneberg *et al.*, 1995). In addition variant receptors might play a role in disease states (Schmauss *et al.*, 1993).

CHAPTER IV

VASCULAR TONE STUDIES USING *IN VITRO* TISSUE BATH

4 CHAPTER IV Vascular tone studies using *in vitro* tissue bath

4.1 Introduction

4.1.1 Vascular tone of placental vessels in pre-eclampsia

5-HT-induced modifications of vascular tone in umbilical / chorionic arteries and veins from normotensive placentae have been studied by several investigators (Reviriego and Marin, 1989; Gonzalez *et al.*, 1990; MacLean *et al.*, 1992; Cruz *et al.*, 1995; Cruz *et al.*, 1998). 5-HT is known to exert its vasoconstrictive effect through mainly activation of 5-HT_2 receptors in peripheral vascular beds (Cohen *et al.*, 1981; Connor *et al.*, 1989; Nilsson *et al.*, 1999). In placental vessels 5-HT is thought to mediate vasoconstriction mainly through activation of 5-HT_{2A} and 5-HT_{1B} receptors, as identified by application of various 5-HT receptor agonists and antagonists (Cruz *et al.*, 1998; Lovren *et al.*, 1999). In their study using human placental veins from normotensive placentae Cruz *et al.* (1998) showed that 5-HT_{2A} receptors mediated contraction of these vessels, whilst 5-HT_7 or 5-HT_3 receptors were not involved. In addition Lovren *et al.* (1999) demonstrated the presence of 5-HT_{1B} receptors in human umbilical arteries.

Conflicting results have been obtained in a limited number of studies that compared the vasoactive effects of 5-HT on normotensive and pre-eclamptic umbilical / placental vessels. Umbilical arteries are the most studied vessels. Johnstone *et al.* (1987) reported increased contraction in response to 5-HT by umbilical arteries of severe pre-eclamptic patients, whilst others have either found no difference or a reduction in contractile response to 5-HT in pre-eclamptic vessels. For example, Gupta *et al.* (2006) found no significant difference between normotensive and pre-eclamptic umbilical arteries. In contrast, Bertrand and St-Louis, (1999) reported a reduced response to 5-HT by both arteries and veins from pre-eclamptic umbilical cords. In addition Parsons *et al.* (1988) who studied stretch-response of umbilical arteries to a single dose of (0.1 μM) 5-HT, reported a significant reduction in severe pre-eclamptics (with IUGR). Moreover, the stretch-response of these vessels was in

decreasing order when normal, mild, severe pre-eclamptic and severe pre-eclamptic (with IUGR) vessels were compared.

Although chorionic vessels are more representative of the villous circulation than the umbilical vessels (Tulenko, 1979), placental vasculature, especially the vasoactive effects of 5-HT on these vessels in pre-eclampsia, has been poorly studied. Bertrand and St-Louis, (1999) reported decreased vasoactive effects of 5-HT on pre-eclamptic veins while the effects of 5-HT on arteries were not significantly different between normotensive and pre-eclamptic samples. In addition, Inayatulla *et al.* (1993) reported that there was no significant difference in the responsiveness of chorionic plate arteries (but this studied only 4 vessels from each group). However, each study used different sized specimens and also expressed the response levels in different ways, making comparisons difficult. Details of each study investigating vessel (umbilical / placental) response to 5-HT in pre-eclampsia are summarised in Table 4.1.

Vessel type	Location	Vessel type and dimension Diameter (D) length (L)	Number of specimen	Response to 5-HT in PE	Source
Umbilical arteries	10 cm from placental insertion	Rings L: 4mm	NT: 23 PE: 10	Increased (Expressed as mg tension)	Johnstone *et al.* (1987)
	Not specified	Helical strips L: 4 X 10 mm	NT: 12 Mild PE: 11 Severe PE: 7 Severe PE (IUGR): 5	Severe PE (IUGR)< Severe PE< Mild PE< NT (Expressed as stretch tension)	Parsons *et al.* (1988)
	Not specified	Rings D: 1.5 –2 mm; L: 3-4 mm	NT: 18-39 PE: 10-12	No difference (Expressed as % KCl response)	Gupta *et al.* (2006)
	5 cm from placental insertion	Rings L: 4-5 mm	NT: 9 PE: 9	Reduced (Expressed as mN/mm)	Bertrand and St-Louis, (1999)
Umbilical veins	5 cm from placental insertion	Rings L: 4-5 mm	NT: 9 PE: 9	Reduced (Expressed as mN/mm)	Bertrand and St-Louis, (1999)
Placental arteries	Chorionic plate (Not specified)	Helical strips D: 2-3 mm; L: 3mm X 20mm	NT: 4 PE: 4	No difference (Expressed as mg tension)	Inayatulla *et al.* (1993)
	Second branch after insertion of umbilical vessels	Rings L: 4-5 mm	NT: 8 PE: 9	No difference (Expressed as mN/mm)	Bertrand and St-Louis, (1999)
Placental veins	Second branch after insertion of umbilical vessels	Rings L: 4-5 mm	NT: 8 PE: 8	Reduced (Expressed as mN/mm)	Bertrand and St-Louis, (1999)

Table 4.1 Summary of umbilical and placental vessel response to 5-HT in pre-eclampsia as detailed in literature
NT: Normotensive, PE: Pre-eclamptic

Studies investigating the chorionic plate vessel response to 5-HT used one representative vessel from each placenta (Inayatulla *et al.*, 1993; Bertrand and St-Louis, 1999). It is possible that differences between even the different branches of the same vessel may exist. However, at present the comparative responsiveness of primary and secondary chorionic plate arteries / veins to 5-HT within the same normotensive (or pre-eclamptic) placentae are not known. Recent work by Gupta *et al.* (2006) has shown that the contribution of 5-HT_{2A} receptors to the vascular tone of umbilical arteries is similar in normotensive and pre-eclamptic patients. However, there is no comparison between normotensive and pre-eclamptic chorionic plate vessels in terms of 5-HT_{2A} receptor contributions to 5-HT mediated contractile response. In addition the effects of pre-eclampsia on receptor protein levels in either vessel (umbilical or placental) has not been studied. Therefore, it is not clear whether 5-HT has a different effect on pre-eclamptic versus normotensive placental vessels.

4.1.2 Aims of chapter

The aims of this chapter were to answer the following questions

1) Is there a difference in the response of chorionic primary and secondary vessels (arteries / veins) to 5-HT and are the levels of the 5-HT_{2A} receptor the same in these vessels?
2) Does pre-eclampsia modify the response of chorionic vessels to 5-HT (primary and secondary arteries / veins)?
3) Is the 5-HT_{2A} antagonist ketanserin effective in reducing the vasoconstriction produced by 5-HT in these vessels?
4) If so, are any of these changes due to altered 5-HT_{2A} receptor expression?

4.2 Results

4.2.1 Clinical details of patients

Table below summarises the clinical details of the patients who donated the placental samples. Five of the pre-eclamptic patients were medicated as detailed in Table 4.3 (section 4.2.5) while three were bed rested and had no medication.

Parameter	Normotensive (*n=9*)	Pre-eclamptic (*n=8*)	P value *
Maternal age (years)	34.2 ± 1.4	28.5 ± 2.2	0.0745
Gestational age (weeks)	38.9 ± 0.1	37.5 ± 0.7	0.0360
Systolic blood pressure (mmHg)	122.2 ± 3.5	153.8 ± 3.4	<0.0001
Diastolic blood pressure (mmHg)	70.6 ± 3.4	98.5 ± 1.9	<0.0001
Protein in urine (g / l)	N/D	1.149 ± 0.328	
(g / 24 hour)	N/D	2.687 ± 0.852	
Placental weight (g)	657.4 ± 37.3	506.3 ± 35.8	0.015
Mode of delivery			
Caesarean section	9	2	
Vaginal delivery	0	6	
Gestational Weight (kg)	3.4 ± 0.1	2.6 ± 0.2	0.015
Gestational sex (male : female)	7:2	7:1	
Ethnicity (White European: Black African : Asian)	7:1:1	7:1:0	

Table 4.2 Clinical details of pre-eclamptic and normotensive patients

The details are expressed as mean ± SEM, N/D= none detected.

* Mann – Whitney U test

4.2.2 Vessel weight analysis

The effective and accurate comparison of vessels from different placentae relies on stringent selection. For this study, vessels, which were approximately 2 mm in diameter, were excised and dissected into 2 mm lengths. Hence any changes in tissue weight would be mainly due to changes in vessel wall thickness. For this reason blotted tissue weights were recorded after each experiment. The scatter plot in Figure 4.1 demonstrates that there were no significant differences in the weights of primary and secondary vessels from both normotensive and pre-eclamptic women. Moreover, tissue weights of normotensive and corresponding pre-eclamptic vessels were similar.

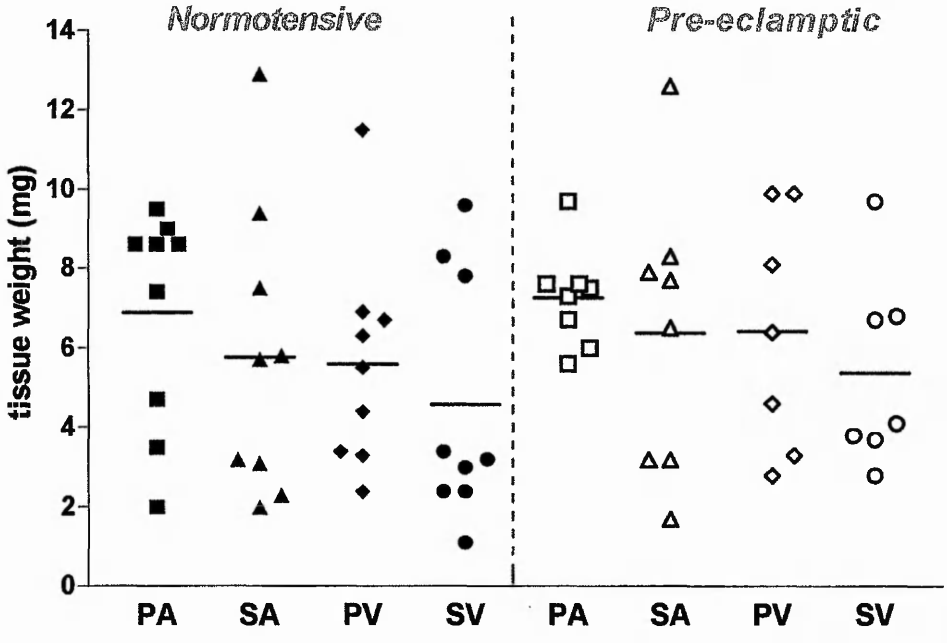


Figure 4.1 Vessel weights

Scatter plot of blotted tissue weights (mg) recorded after each experiment. *n* represents number of vessels used for the study (each vessel obtained from a different patient). *n= 9* normotensive vessels, *n= 8* pre-eclamptic arteries, *n=7* pre-eclamptic veins. Blotted weights of primary and secondary vessels were compared. In addition weights of normotensive and corresponding pre-eclamptic vessels were compared. Statistical significance was accepted at $p< 0.05$ (*) and $p<0.01$ (**).

PA= primary artery, SA= secondary artery, PV= primary vein, SV= secondary vein.

4.2.3 Responses of normotensive and pre-eclamptic vessels to KCl

The contractility of each vessel was evaluated using the mean of two consecutive KCl responses at the start of each experiment as detailed in section 2.2.2.3.2 and the results are presented in Figure 4.2. The response of primary vessels (arteries and veins) to KCl was similar to that of secondary vessels in normotensive samples. Similarly there were no significant difference between pre-eclamptic primary and secondary veins. However pre-eclamptic secondary arteries were less responsive to KCl than primary arteries. KCl response was similar when normotensive and corresponding pre-eclamptic vessels were compared.

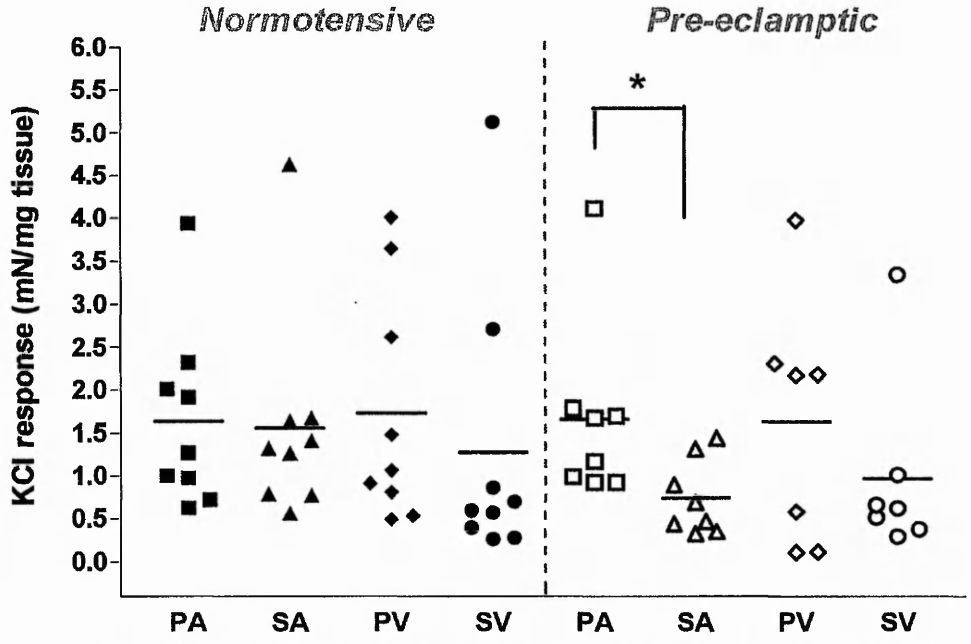


Figure 4.2 Contraction of normotensive and pre-eclamptic vessels in response to KCl

Scatter plot of KCl response of each vessel in mN / mg tissue. Contractile response to KCl was obtained at the start of each experiment for each vessel as detailed in section 2.2.2.3.2. The mean value from two consecutive responses to KCl was corrected for tissue weight (mg). *n* represents number of vessels used for the study (each vessel obtained from a different patient). *n= 9* normotensive vessels, *n= 8* pre-eclamptic arteries, *n=7* pre-eclamptic veins. KCl response of primary and secondary vessels was compared. In addition KCl response of normotensive and corresponding pre-eclamptic vessels were compared. Statistical significance was accepted at $p< 0.05$ (*) and $p<0.01$ (**).

PA= primary artery, SA= secondary artery, PV= primary vein, SV= secondary vein.

4.2.4 Comparison of 5-HT response and 5-HT_{2A} receptor levels in normotensive and pre-eclamptic vessels

In this part of the study the contractile responses to 5-HT of chorionic vessels (arteries and veins) from placentas delivered after normal and pre-eclamptic pregnancies were compared. Using four-representative normotensive and pre-eclamptic placentae, 5-HT_{2A} receptor protein levels in these vessels were also compared employing western blotting and antibodies specific to the 5-HT_{2A} receptor protein. Clinical details of the latter patients are summarised in appendix II. The possible differences between primary and secondary vessels are considered in section 4.2.5.

4.2.4.1 Arteries

4.2.4.1.1 Response of arteries to 5-HT

Figure 4.3 represents a separate concentration-response for primary and secondary arteries from normotensive and pre-eclamptic samples. Emax values of pre-eclamptic primary and secondary arteries were reduced by 28.24 % and 59.80 % respectively compared to the corresponding normotensive vessels; however differences were significant for only the secondary arteries. In contrast EC_{50} was unaffected by pre-eclampsia.

4.2.4.1.2 5-HT_{2A} receptor levels in primary and secondary arteries

Figure 4.4 and 4.5 show that pre-eclampsia appear to reduce 5-HT_{2A} receptor protein levels in primary arteries (Figure 4.4 A) and secondary arteries (Figure 4.5 A), but this was statistically not significant. Similarly there was no significant change in the 63 kDa to 40-55 kDa band ratios in the pre-eclamptic arteries (Figures 4.4 B and 4.5 B).

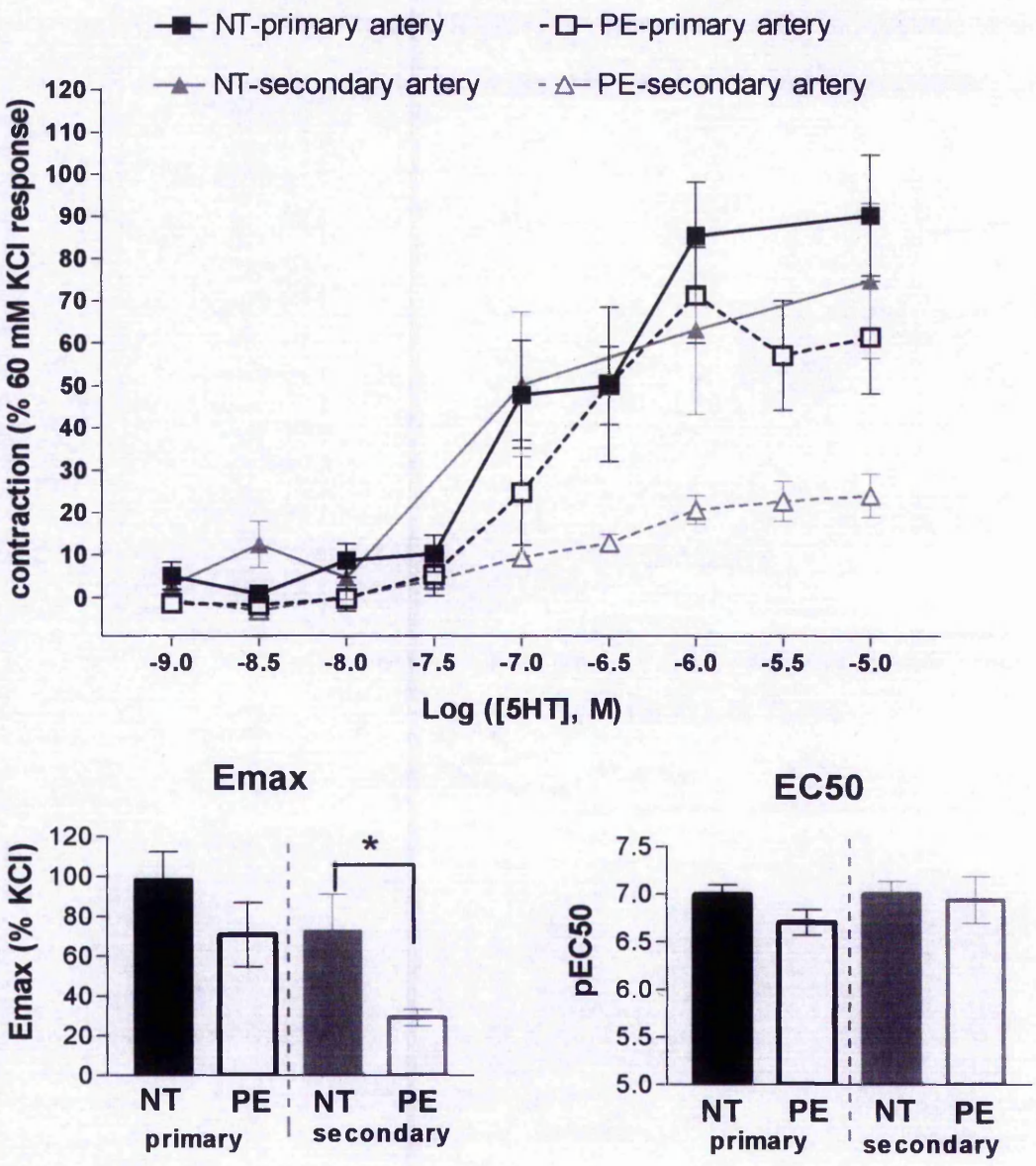


Figure 4.3 Comparison of normotensive and pre-eclamptic chorionic arteries contractile response to 5-HT

Concentration-response to 5-HT for chorionic plate primary (squares) and secondary (triangles) arteries from placentae delivered after normal and pre-eclamptic pregnancies. Contraction produced by 5-HT is expressed as % 60 mM KCl response. *n* represents number of vessels used for the study (each vessel obtained from a different patient) *n= 9* normotensive primary arteries, *n= 8* normotensive secondary arteries, *n= 8* pre-eclamptic primary arteries, *n= 8* pre-eclamptic secondary arteries. Normotensive and pre-eclamptic vessels were compared. Statistical significance was accepted at $p< 0.05$ (*) and $p<0.01$ (**). NT= normotensive, PE= pre-eclamptic

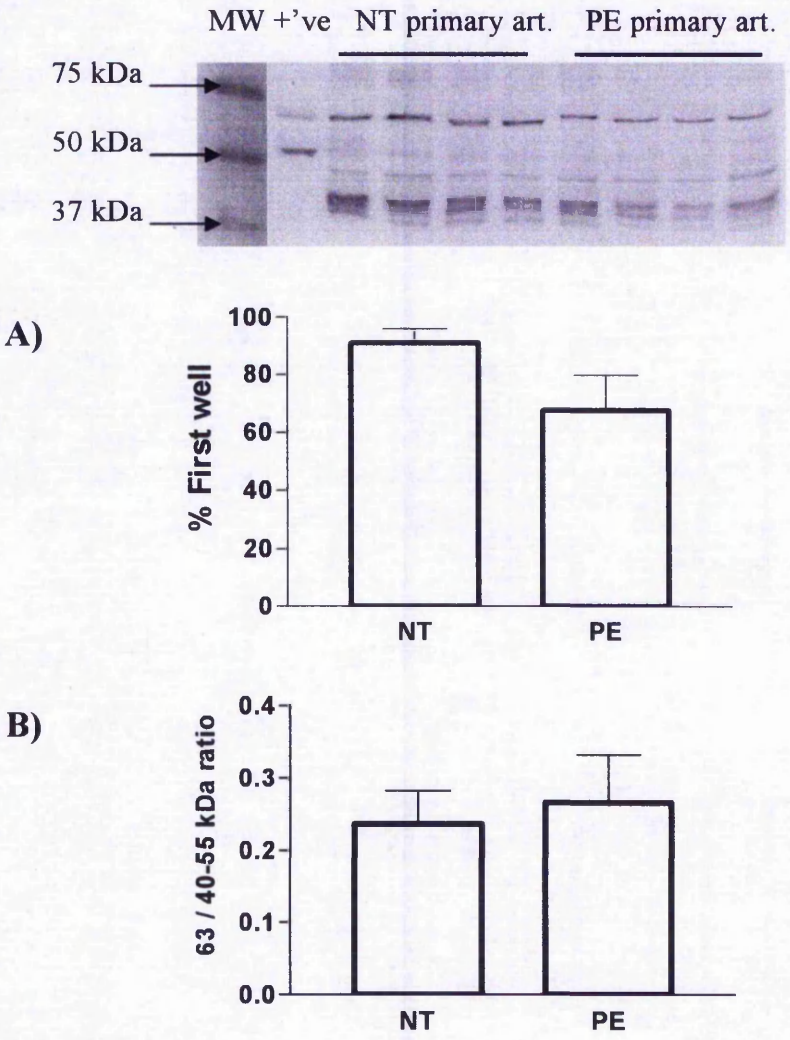


Figure 4.4 Expression of 5-HT_{2A} receptor protein in chorionic plate primary arteries from normotensive and pre-eclamptic patients

Primary arteries were extracted as detailed in section 2.2.3.1. Proteins separated by SDS-PAGE, transferred to nitrocellulose and probed with anti-5-HT_{2A} receptor antibody (G186-1117, 1:250). Bands were corrected using copper staining for differences in protein loading. A representative blot of arteries from four individuals is shown (*n=4*). Blots are repeated three times.

A) Results are presented as mean % of first experimental sample (first NT primary artery sample, assigned as 100 %) ± SEM

B) Results are presented as mean 63 kDa to 40-55 kDa band ratio ± SEM.

MW= molecular weight standards, +'ve= positive control (platelet extract), NT=normotensive, PE=pre-eclamptic, art.=artery.

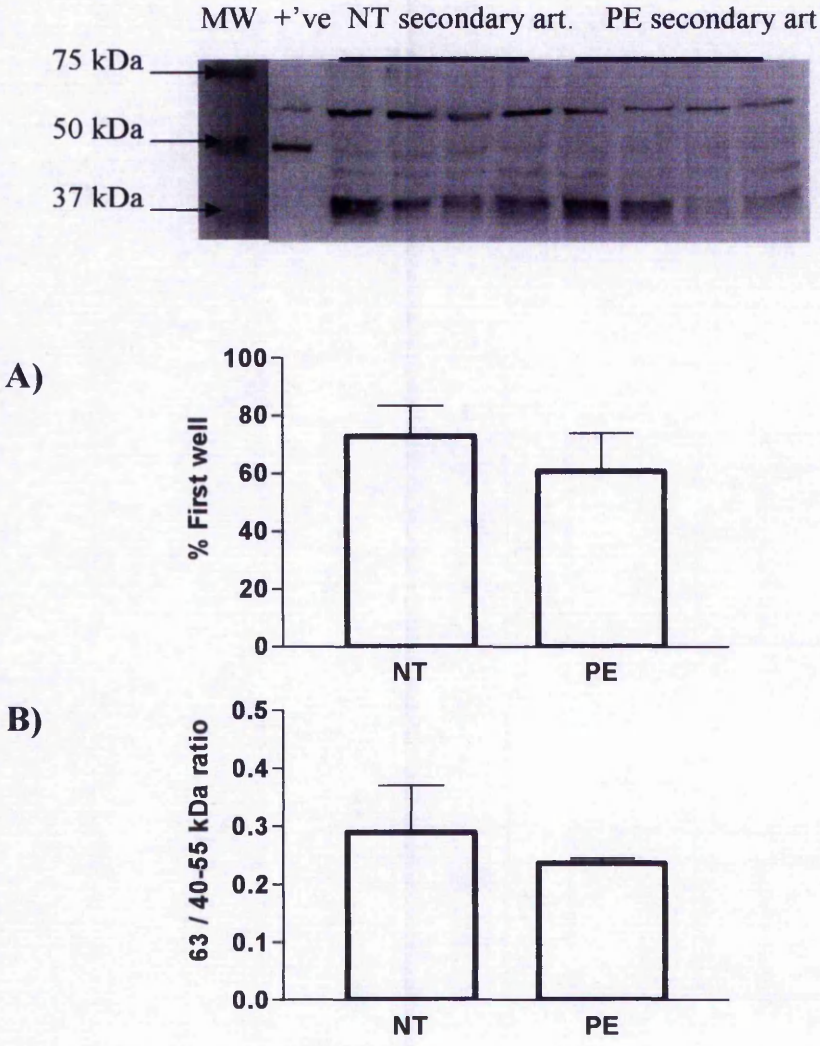


Figure 4.5 Expression of 5-HT_{2A} receptor protein in chorionic plate secondary arteries from normotensive and pre-eclamptic patients

Secondary arteries were extracted as detailed in section 2.2.3.1. Proteins separated by SDS-PAGE, transferred to nitrocellulose and probed with anti-5-HT_{2A} receptor antibody (G186-1117, 1:250). Bands were corrected using copper staining for differences in protein loading. A representative blot of arteries from four individuals is shown ($n=4$). Blots are repeated three times.

A) Results are presented as mean % of first experimental sample (first NT secondary artery sample, assigned as 100 %) ± SEM

B) Results are presented as mean 63 kDa to 40-55 kDa band ratio ± SEM.

MW= molecular weight standards, +'ve= positive control (platelet extract), NT=normotensive, PE=pre-eclamptic, art.=artery.

4.2.4.2 Veins

4.2.4.2.1 Response of veins to 5-HT

Normotensive primary and secondary veins were also compared to pre-eclamptic primary and secondary veins (Figure 4.6). Emax for both pre-eclamptic primary and secondary veins was significantly lower than the corresponding normotensive veins. Emax values of pre-eclamptic primary and secondary veins were reduced by 42.87 and 51.57 % respectively compared to normotensives. EC_{50} values for primary and secondary veins on the other hand did not change significantly when normotensive and pre-eclamptic vessels were compared.

4.2.4.2.2 5-HT_{2A} receptor levels in primary and secondary veins

Figure 4.7 A shows that there were no significant differences in 5-HT_{2A} receptor protein levels in primary veins. In secondary veins receptor protein levels appear to increase in pre-eclampsia (Figure 4.8 A) however, this was not significant. Similarly there was no significant change in the 63 kDa to 40-55 kDa band ratios in the pre-eclamptic veins (Figures 4.7 B and 4.8 B).

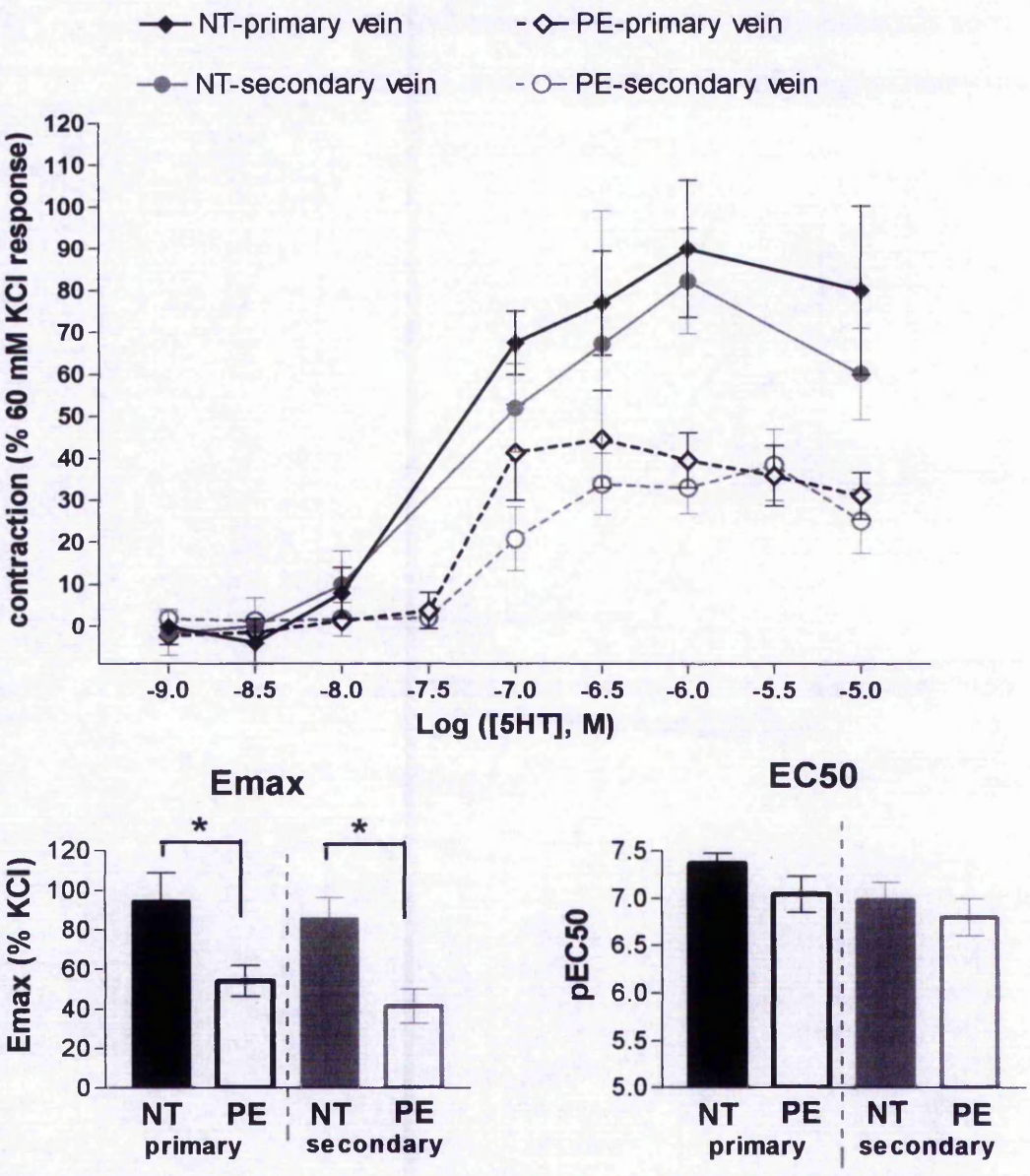


Figure 4.6 Comparison of normotensive and pre-eclamptic chorionic veins contractile response to 5-HT

Concentration-response to 5-HT for chorionic plate primary (diamonds) and secondary (circles) veins from placentae delivered after normal and pre-eclamtic pregnancies. Contraction produced by 5-HT expressed as % 60 mM KCl response. *n* represents number of vessels used for the study (each vessel obtained from a different patient) *n= 8* normotensive primary veins, *n= 9* normotensive secondary veins, *n= 6* pre-eclamptic primary veins, *n= 6* pre-eclamptic secondary veins. Normotensive and pre-eclamptic vessels were compared. Statistical significance was accepted at $p< 0.05$ (*) and $p<0.01$ (**).

NT= normotensive, PE= pre-eclamptic

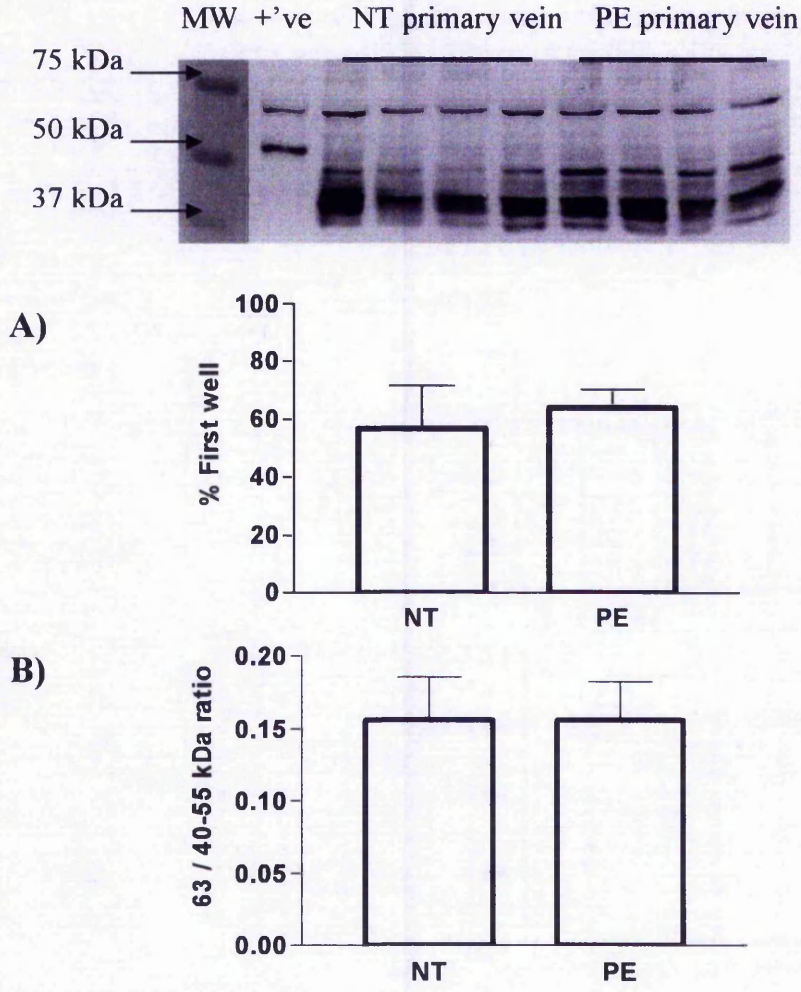


Figure 4.7 Expression of 5-HT_{2A} receptor protein in chorionic plate primary veins from normotensive and pre-eclamptic patients

Primary veins were extracted as detailed in section 2.2.3.1. Proteins separated by SDS-PAGE, transferred to nitrocellulose and probed with anti-5-HT_{2A} receptor antibody (G186-1117, 1:250). Bands were corrected using copper staining for differences in protein loading. A representative blot of arteries from four individuals is shown (*n=4*). Blots are repeated three times.

A) Results are presented as mean % of first experimental sample (first NT primary vein, assigned as 100 %) ± SEM.

B) Results are presented as mean 63 kDa to 40-55 kDa band ratio ± SEM.

MW= molecular weight standards, +'ve= positive control (platelet extract), NT=normotensive, PE=pre-eclamptic.

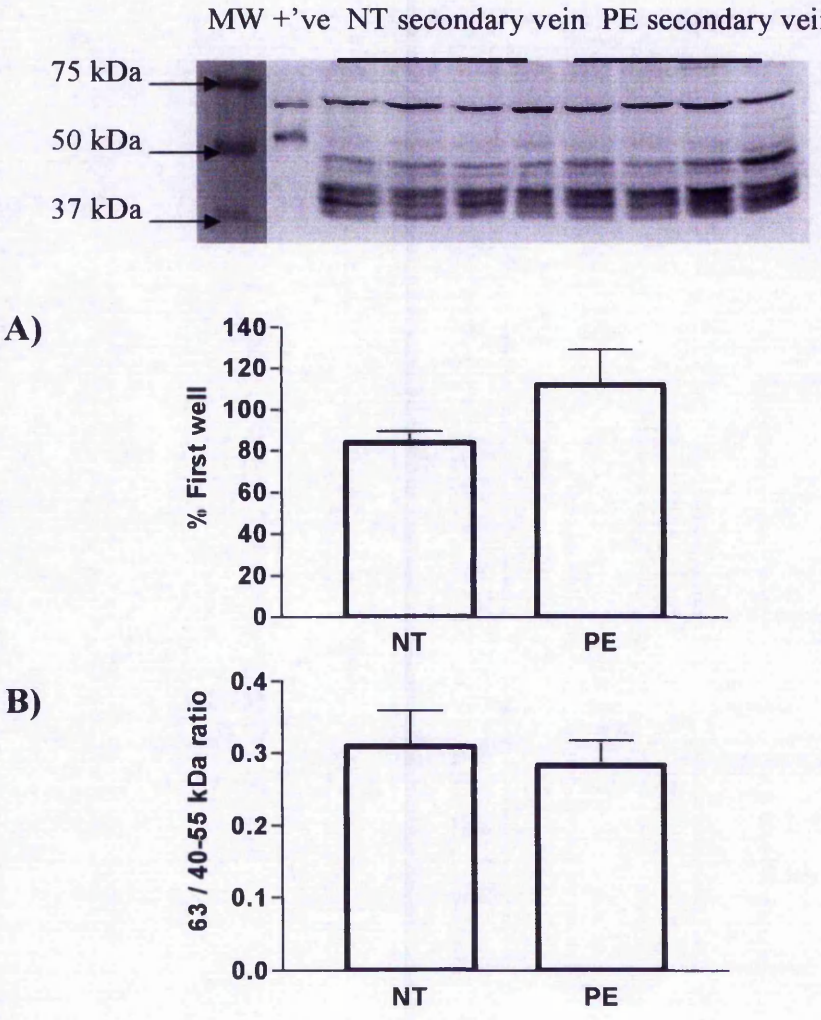


Figure 4.8 Expression of 5-HT_{2A} receptor protein in chorionic plate secondary veins from normotensive and pre-eclamptic patients

Secondary veins were extracted as detailed in section 2.2.3.1. Proteins separated by SDS-PAGE, transferred to nitrocellulose and probed with anti-5-HT_{2A} receptor antibody (G186-1117, 1:250). Bands were corrected using copper staining for differences in protein loading. A representative blot of arteries from four individuals is shown (*n=4*). Blots are repeated three times.

A) Results are presented as mean % of first experimental sample (first NT secondary vein, assigned as 100 %) ± SEM

B) Results are presented as mean 63 kDa to 40-55 kDa band ratio ± SEM.

MW= molecular weight standards, +'ve= positive control (platelet extract), NT=normotensive, PE=pre-eclamptic.

4.2.5 Contribution of the 5-HT_{2A} receptor to 5-HT mediated responses in primary and secondary vessels

In this part of the study the contractile responses of primary and secondary vessel to 5-HT were compared, and the contribution of the 5-HT_{2A} receptor to these contractile responses was estimated using the antagonist ketanserin (1μM).

4.2.5.1 Arteries

The response of normotensive primary and secondary arteries to 5-HT (alone) was similar with average Emax 98.59 ± 13.97 and 72.62 ± 18.67 respectively for primary and secondary vessels. Pre-eclamptic secondary arteries on the other hand, were significantly less responsive to 5-HT (alone) compared to primary arteries (Emax 29.20 ± 4.09 and 70.75 ± 16.03 respectively). Data for each individual patient are presented in Table 4.3.

4.2.5.1.1 Ketanserin antagonism to 5-HT response

Table 4.3 also shows % reduction in Emax for normotensive and pre-eclamptic samples in the presence of ketanserin and appendix II shows representative concentration-response to 5-HT in the presence and absence of ketanserin. Ketanserin reduced the average contractile response to 5-HT in a similar manner in both primary and secondary vessels while average pEC_{50} was unaffected by ketanserin. % Reduction in Emax for pre-eclamptic arteries appears to be greater than normotensive arteries however, these differences were not statistically significant. Table 4.3 also indicates how pre-eclamptic patients were managed during pregnancy (medication).

	NORMOTENSIVE						PRE-ECLAMPTIC						
	Primary arteries			Secondary arteries			Primary arteries			Secondary arteries			
Sample	5-HT	5-HT + ket	% red	5-HT	5-HT + ket	% red	5-HT	5-HT + ket	% red	5-HT	5-HT +ket	% red	medication
1	163.56	143.43	12.31	103.70	57.36	44.69	78.72	23.59	70.03	48.55	4.50	90.73	Nifedipine
2	145.56	88.22	39.40	128.15	90.99	29.00	75.14	10.61	85.88	19.61	8.60	56.14	Labetalol Hydralazine
3	107.61	78.56	27.00	42.35	21.77	48.61	64.44	26.03	59.61	24.76	42.85	0.00	Not used
4	112.97	118.11	0.00	100.17	86.52	13.62	15.75	2.69	82.92	24.22	0.00	100.00	Labetalol
5	53.64	N / A	N / A	13.92	17.09	0.00	49.79	7.23	85.48	31.99	0.00	100.00	Methyl dopa
6	124.53	10.38	91.66	12.61	6.31	49.99	19.13	8.45	55.83	25.95	18.37	29.21	Not used
7	68.33	27.91	59.15	145.50	54.51	62.53	150.99	30.36	79.89	14.95	2.58	82.74	Labetalol Hydralazine
8	59.17	24.38	58.81	34.59	8.65	75.00	112.00	71.89	35.81	43.60	33.91	22.22	Not used
9	51.93	20.82	59.91	N / A	N / A	N / A							
Mean Emax	**98.59**	**63.98 ***	**43.53**	**72.62**	**42.90 ***	**40.43**	**70.75**	**22.61 ****	**69.43**	**29.20 †**	**13.85**	**60.13**	
± SEM	± 13.97	± 17.75	± 10.54	± 18.67	± 12.09	± 8.81	± 16.03	± 7.88	± 6.31	± 4.09	± 5.81	± 13.79	
Mean pEC_{50}	**6.994**	**6.951**		**6.998**	**6.772**		**6.703**	**6.569**		**6.941**	**6.915**		
± SEM	± 0.100	± 0.142		± 0.136	± 0.224		± 0.134	± 0.165		± 0.248	± 0.505		

Table 4.3 Ketanserin antagonism to 5-HT response and changes in Emax and EC_{50} values in normotensive and pre-eclamptic chorionic arteries

Emax and EC_{50} values for 5-HT for chorionic plate primary and secondary arteries in the absence and presence of ketanserin (ket) (1 μM). Contractions produced by 5-HT are expressed as % 60 mM KCl response. Agonist potency (EC_{50}) expressed as pEC_{50} (-log EC_{50}) and efficacy of the agonist expressed as Emax (maximal response). % Reduction (% red) represents the reduction in Emax values in the presence of ketanserin. Emax in the absence and presence of ketanserin was compared. Statistical significance was accepted at $p< 0.05$ (*) and $p< 0.01$ (**). In addition the response of primary and secondary arteries to 5-HT only was compared. Statistical significance was accepted at $p< 0.05$ (†) and $p< 0.01$ (‡). N/A= not available

4.2.5.1.2 5-HT_{2A} receptor levels in primary and secondary arteries

The 5-HT_{2A} receptor protein levels in primary and secondary arteries were compared. Figure 4.9 shows that receptor levels were similar in four normotensive patients. Similarly receptor protein levels in primary and secondary arteries from pre-eclamptic placentae (PE1, PE2, PE4 and PE6; see Table 4.3 for drug treatments) were similar although 63 kDa to 40-55 kDa band ratio was increased in secondary arteries compared to primary arteries (Figure 4.10). Clinical details of patients from whom the samples were collected are summarised in appendix II.

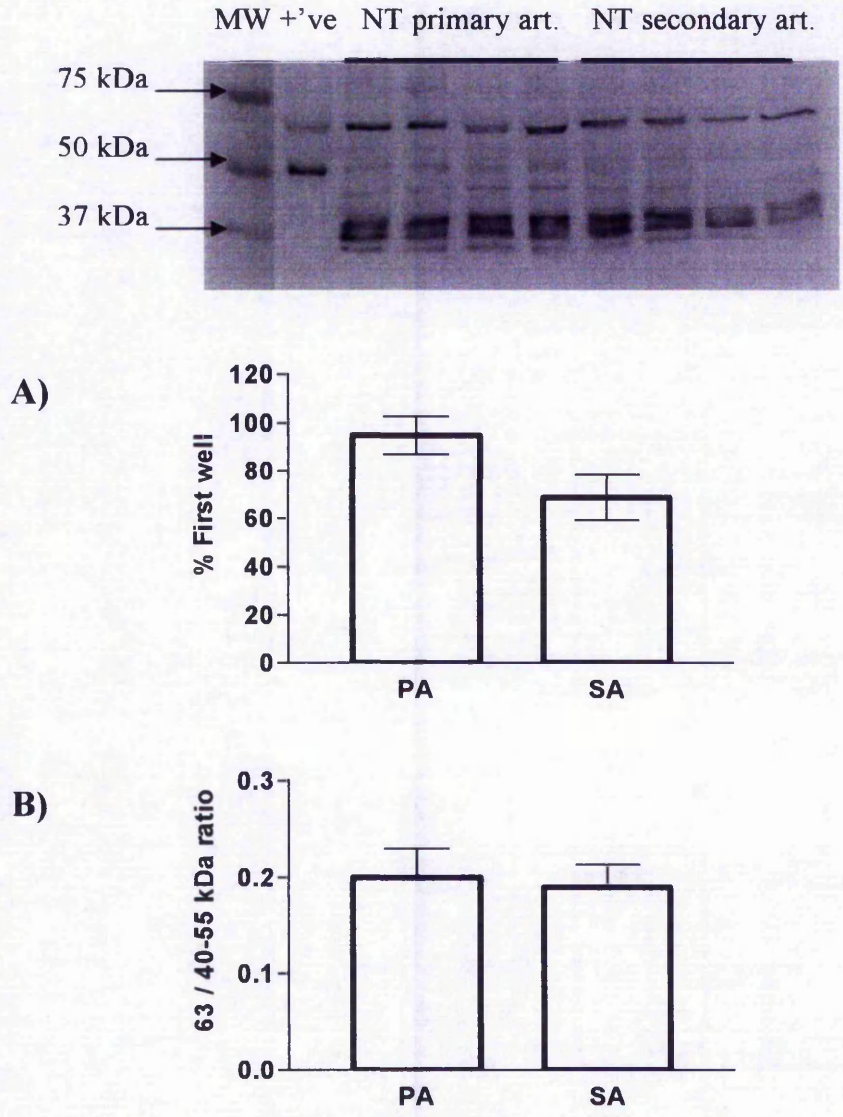


Figure 4.9 Expression of 5-HT_{2A} receptor protein in chorionic plate primary and secondary arteries from normotensive patients

Arteries were extracted as detailed in section 2.2.3.1. Proteins separated by SDS-PAGE, transferred to nitrocellulose and probed with anti-5-HT_{2A} receptor antibody (G186-1117, 1:250). Bands were corrected using copper staining for differences in protein loading. A representative blot of arteries from four individuals is shown (*n=4*). Blots are repeated three times.

A) Results are presented as mean % of first experimental sample (first NT primary artery sample, assigned as 100 %) ± SEM

B) Results are presented as mean 63 kDa to 40-55 kDa band ratio ± SEM

MW= molecular weight standards, +'ve= positive control (platelet extracts), NT=normotensive, art=artery.

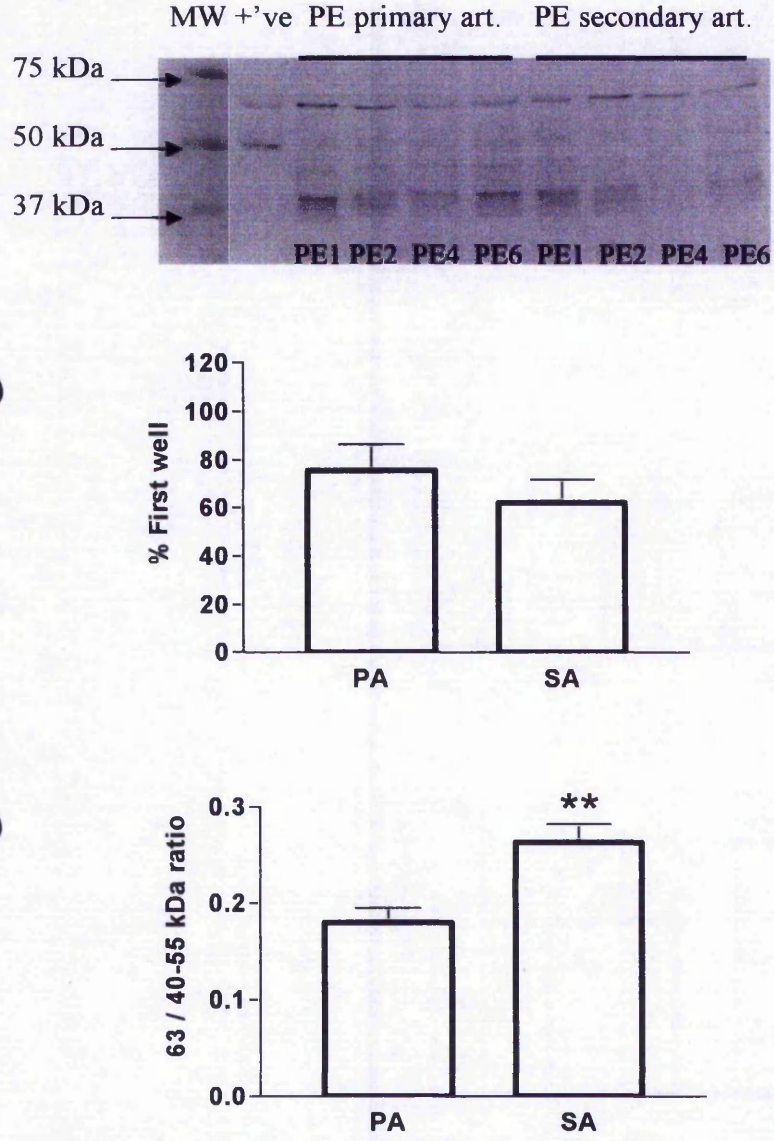


Figure 4.10 Expression of 5-HT_{2A} receptor protein in chorionic plate primary and secondary arteries from pre-eclamptic patients

Arteries were extracted as detailed in section 2.2.3.1. Proteins separated by SDS-PAGE, transferred to nitrocellulose and probed with anti-5-HT_{2A} receptor antibody (G186-1117, 1:250). Bands were corrected using copper staining for differences in protein loading. A representative blot of arteries from four individuals is shown (*n=4*). Blots are repeated three times.

A) Results are presented as mean % of first experimental sample (first PE primary artery sample, assigned as 100 %) ± SEM

B) Results are presented as mean 63 kDa to 40-55 kDa band ratio ± SEM

MW= molecular weight standards, +'ve= positive control (platelet extracts), PE= pre-eclamptic, art.=artery, PA=primary artery, SA=secondary artery.

4.2.5.2 Veins

The response of normotensive primary and secondary veins to 5-HT (alone) was similar with average Emax 94.53 ± 14.22 and 85.61 ± 10.86 respectively for primary and secondary vessels. Similarly pre-eclamptic primary and secondary veins response to 5-HT (alone) did not change significantly (Emax = 54.11 ± 7.68 and 41.46 ± 8.45 for primary and secondary veins respectively). Data for each individual patient are presented in Table 4.4.

4.2.5.2.1 Ketanserin antagonism to 5-HT response

Table 4.4 also shows % reduction in Emax for normotensive and pre-eclamptic samples in the presence of ketanserin and appendix II shows representative concentration-response to 5-HT in the presence and absence of ketanserin. Ketanserin reduced contractile response to 5-HT in all vessels. In both normotensive and pre-eclamptic vessels % reduction in Emax was greater in secondary veins than in primary vessels; however, the difference was not statistically significant. Table 4.4 also indicates how pre-eclamptic patients were managed during pregnancy (medication).

4.2.5.2.2 5-HT_{2A} receptor levels in primary and secondary veins

The 5-HT_{2A} receptor protein levels in primary and secondary veins were compared. Figure 4.11 shows there were no significant differences in 5-HT_{2A} receptor protein levels in normotensive primary veins compared to those in secondary veins. Similarly receptor protein levels in primary and secondary veins from pre-eclamptic placenta (PE1, PE2, PE4 and PE6; see Table 4.4 for drug treatments) were similar. 63 kDa to 40-55 kDa band ratio appeared to be increased in secondary vessels although these differences were not statistically significant. Clinical details of patients whom the samples were collected from are summarised in appendix II.

	NORMOTENSIVE						PRE-ECLAMPTIC						
	Primary veins			Secondary veins			Primary veins			Secondary veins			
Sample	5-HT	5-HT + ket	% red	5-HT	5-HT + ket	% red	5-HT	5-HT + ket	% red	5-HT	5-HT +ket	% red	medication
1	84.46	14.44	82.90	125.73	99.20	21.10	44.16	0.00	100.00	15.96	0.00	100.00	Nifedipine
2	124.65	132.17	0.00	113.80	59.74	47.50	42.15	26.01	38.29	70.37	0.00	100.00	Labetalol Hydralazine
3	N / A	N / A	N / A	86.27	101.96	0.00	N / A	N / A	N / A	19.99	12.00	39.97	Not used
4	58.89	59.29	0.00	112.06	12.72	88.65	77.04	68.87	10.60	N / A	N / A	N / A	Labetalol
5	175.06	94.51	46.01	69.23	12.09	82.54	78.51	78.69	0.00	40.76	43.31	0.00	Methyl dopa
6	95.38	70.24	26.36	90.91	31.17	65.71	N / A	N / A	N / A	47.87	27.54	42.47	Not used
7	66.94	53.23	20.48	29.03	9.68	66.67	35.05	4.20	88.02	53.80	5.84	89.14	Labetalol Hydralazine
8	52.30	18.73	64.18	44.34	43.89	1.01	47.74	18.21	61.86	N / A	N / A	N / A	Not used
9	98.60	60.84	38.30	99.08	40.37	59.26							
Mean Emax	**94.53**	**62.93 ***	**35.48**	**85.61**	**45.65 ****	**48.05**	**54.11**	**32.66 ***	**49.80**	**41.46**	**14.78**	**61.93**	
± SEM	± 14.22	± 13.54	± 9.17	± 10.86	± 11.77	± 11.10	±7.68	±13.61	±16.61	±8.45	±7.07	±16.67	
Mean pEC_{50}	**7.365**	**6.876**		**6.980**	**6.876**		**7.042**	**6.627**		**6.800**	**6.844**		
± SEM	± 0.111	± 0.155		± 0.189	± 0.212		± 0.190	± 0.507		± 0.197	± 0.617		

Table 4.4 Ketanserin antagonism to 5-HT response and changes in Emax and EC_{50} values in normotensive and pre-eclamptic chorionic veins

Emax and pEC50 values for 5-HT on chorionic plate primary and secondary veins in the absence and presence of ketanserin (ket) (1 μM). Contractions produced by 5-HT are expressed as % 60 mM KCl response. Agonist potency (EC_{50}) expressed as pEC_{50} (-log EC_{50}) and efficacy of the agonist expressed as Emax (maximal response). % Reduction (% red) represents the reduction in Emax values in the presence of ketanserin. Emax in the absence and presence of ketanserin was compared. Statistical significance was accepted at $p< 0.05$ (*) and $p<0.01$ (**). In addition primary and secondary veins response to 5-HT only was compared. Statistical significance was accepted at $p< 0.05$ (†) and $p<0.01$ (‡). N/A= not available.

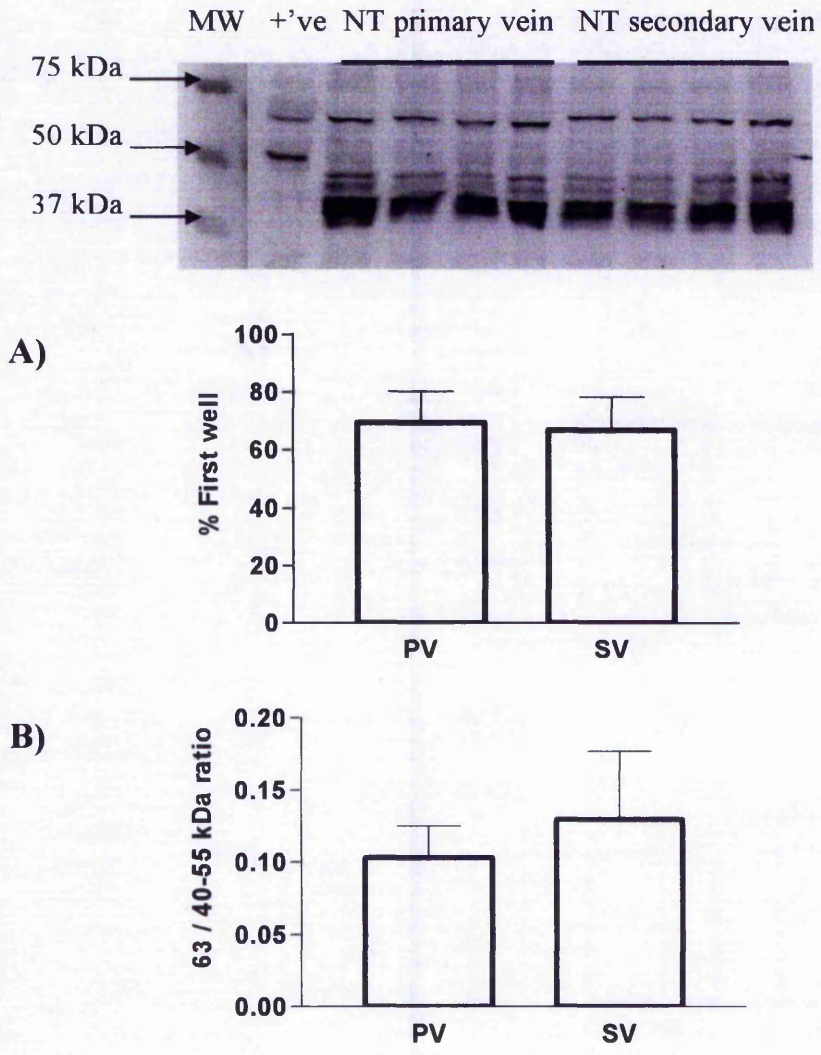


Figure 4.11 Expression of 5-HT_{2A} receptor protein in chorionic plate primary and secondary veins from normotensive patients

Veins were extracted as detailed in section 2.2.3.1. Proteins separated by SDS-PAGE, transferred to nitrocellulose and probed with anti-5-HT_{2A} receptor antibody (G186-1117, 1:250). Bands were corrected using copper staining for differences in protein loading. A representative blot of arteries from four individuals is shown ($n=4$). Blots are repeated three times.

A) Results are presented as mean % of first experimental sample (first NT primary vein sample, assigned as 100 %) ± SEM

B) Results are presented as mean 63 kDa to 40-55 kDa band ratio ± SEM

MW= molecular weight standards, +'ve= positive control (platelet extracts), NT=normotensive, PV=primary vein, SV=secondary vein.

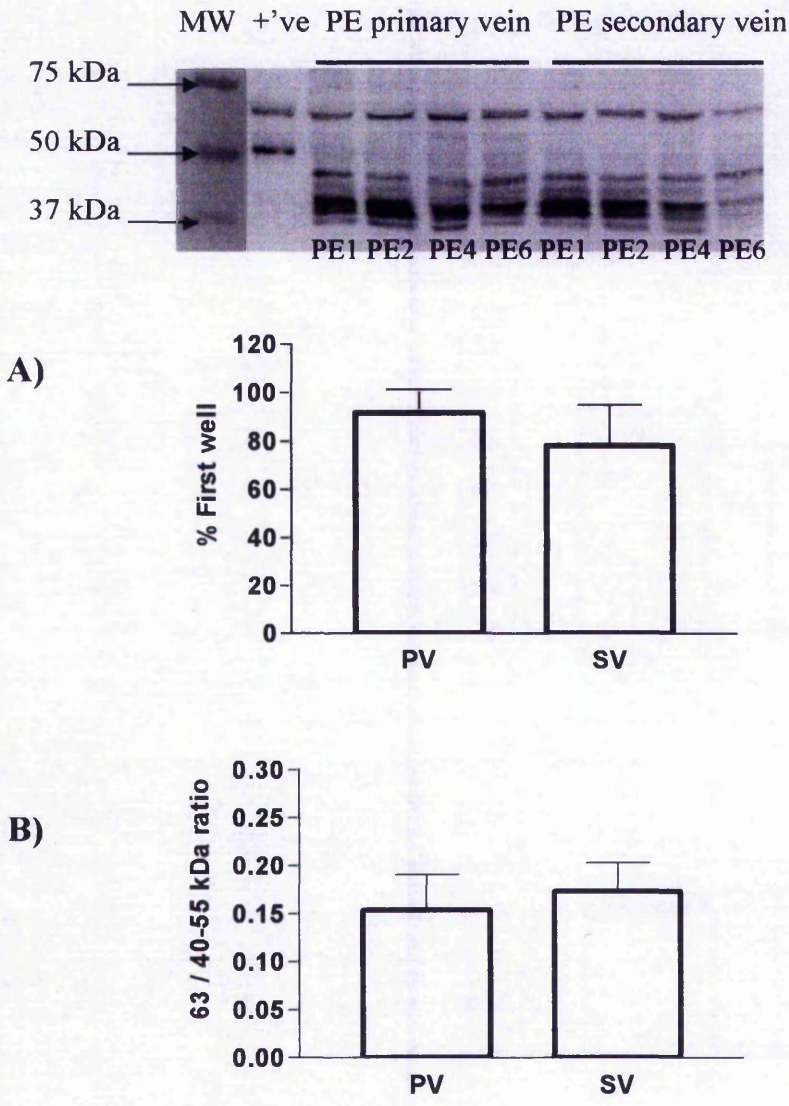


Figure 4.12 Expression of 5-HT_{2A} receptor protein in chorionic plate primary and secondary veins from pre-eclamptic patients

Veins were extracted as detailed in section 2.2.3.1. Proteins separated by SDS-PAGE, transferred to nitrocellulose and probed with anti-5-HT_{2A} receptor antibody (G186-1117, 1:250). Bands were corrected using copper staining for differences in protein loading. A representative blot of arteries from four individuals is shown (*n=4*). Blots are repeated three times.

A) Results are presented as mean % of first experimental sample (first PE primary vein sample, assigned as 100 %) ± SEM

B) Results are presented as mean 63 kDa to 40-55 kDa band ratio ± SEM

MW= molecular weight standards, +'ve= positive control (platelet extracts), PE=pre-eclamptic, PV=primary vein, SV=secondary vein.

4.2.5.3 Overview of ketanserin antagonism

Table 4.5 and Figure 4.13 summarise normotensive and pre-eclamptic vessel responses to 5-HT and ketanserin antagonism in these vessels. Ketanserin reduced the contractile response to 5-HT in both normotensive and pre-eclamptic primary / secondary vessels. As can be seen from Figure 4.13 mean % reduction in Emax in the presence of ketanserin was greater in all pre-eclamptic vessels compared to normotensives, however the differences were not statistically significant.

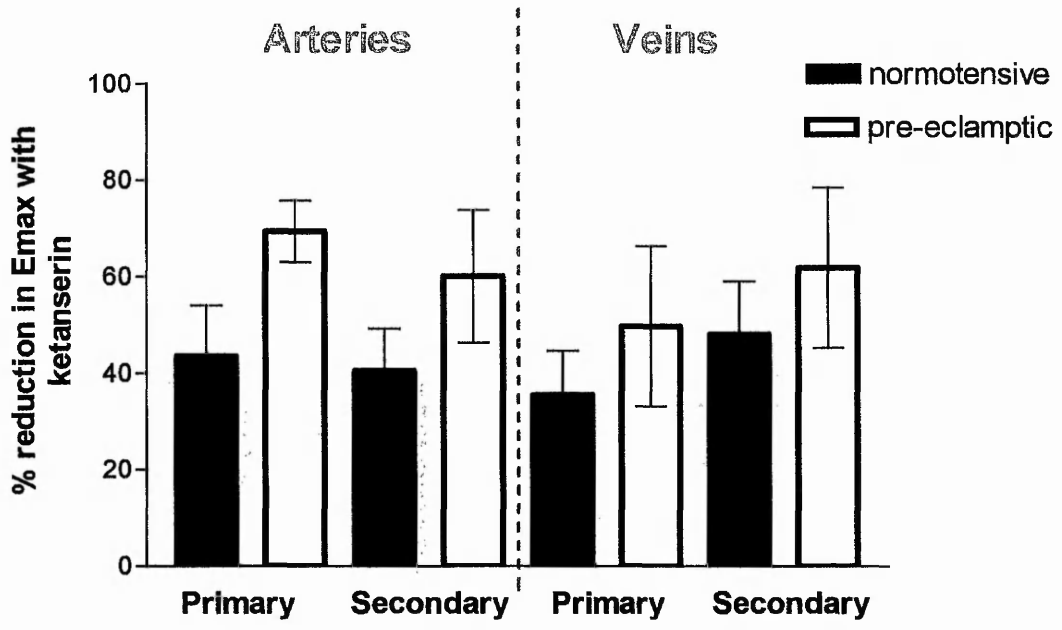


Figure 4.13 Overview of ketanserin antagonism to 5-HT response in normotensive and pre-eclamptic vessels

Contraction produced by 5-HT is expressed as % 60 mM KCl response. % Reduction represents the reduction in Emax values in response to 5-HT in the presence of ketanserin. Statistical significance was accepted at $p< 0.05$ (*) and $p<0.01$ (**).

			Emax (% KCl)	pEC_{50}	*n*
Normotensive Vessels	**Primary Arteries**	**Control**	98.59 ± 13.97	6.994 ± 0.100	9
		Ketanserin	63.98 ± 17.75 §	6.951 ± 0.142	8
	Secondary Arteries	**Control**	72.62 ± 18.67	6.998 ± 0.136	8
		Ketanserin	42.90 ± 12.09 §	6.772 ± 0.224	8
	Primary Veins	**Control**	94.53 ± 14.22	7.365 ± 0.111	8
		Ketanserin	62.93 ± 13.54 §	6.876 ± 0.155	8
	Secondary veins	**Control**	85.61 ± 10.86	6.980 ± 0.189	9
		Ketanserin	45.65 ± 11.77 §§	6.876 ± 0.212	9
Pre-eclamptic Vessels	**Primary Arteries**	**Control**	70.75 ± 16.03	6.703 ± 0.134	8
		Ketanserin	22.61 ± 7.88 §§	6.569 ± 0.165	8
	Secondary Arteries	**Control**	29.20 ± 4.10 *†	6.941 ± 0.248	8
		Ketanserin	13.85 ± 5.81	6.915 ±0.505	8
	Primary Veins	**Control**	54.11 ± 7.68 *	7.042 ± 0.190	6
		Ketanserin	32.66 ± 13.61 §	6.579 ± 0.488	6
	Secondary Veins	**Control**	41.46 ± 8.45 *	6.800 ± 0.197	6
		Ketanserin	14.78 ± 7.07	6.844 ± 0.617	6

Table 4.5 Overview of normotensive and pre-eclamptic vessel Emax and EC_{50} values to 5-HT alone and in the presence of ketanserin

Emax values for 5-HT for chorionic plate primary and secondary arteries / veins in the absence and presence of ketanserin (1 μM) from placentae delivered after normal and pre-eclamptic pregnancies. Maximal contraction (Emax) produced by 5-HT are expressed as % 60 mM KCl response and agonist potency (EC_{50}) expressed as pEC_{50} (-log EC_{50}). Results are expressed as mean ± SEM where *n* represents number of vessels used for the study (each vessel obtained from a different patient).

A) Emax and EC_{50} of normotensive and pre-eclamptic vessels were compared. Statistical significance was accepted at $p< 0.05$ (*) and $p<0.01$ (**)

B) Emax and EC_{50} of primary and secondary vessels were compared. Statistical significance was accepted at $p< 0.05$ (†) and $p<0.01$ (‡)

C) Emax and EC_{50} in the absence and presence of ketanserin were compared. Statistical significance was accepted at $p< 0.05$ (§) and $p<0.01$ (§§)

4.3 Discussion

4.3.1 Clinical details of patients

Placentae for this study were obtained from 9 normotensive women and 8 women with pre-eclampsia. Women with essential hypertension and with medical complications such as diabetes and renal disease were excluded from the study. As is characteristic of the condition, both systolic and diastolic blood pressures were significantly higher in the pre-eclamptic group. In addition, pre-eclamptic women had proteinuria (2.687 ± 0.852 g/24 hour) while none was detected in normotensive women. The mean maternal age in the two groups was similar at 34.2 ± 1.4 and 28.5 ± 2.2 respectively. Gestational age at delivery tended to be lower in the women with pre-eclampsia (38.9 ± 0.1 weeks for normotensives and 37.5 ± weeks for pre-eclamptics). This is due to concerns about maternal and foetal safety and in reality pre-eclamptic patients hardly ever deliver at term. Considering this, the difference in gestational age between normotensive and pre-eclamptic patients was minimal. Placental and gestational weights were slightly lower in the pre-eclamptic group. This was not surprising as placental and gestational weights are known to correlate with gestational age (Williams *et al.*, 1997; Salafia *et al.*, 2005). All normotensive patients were delivered by caesarean section while most of the pre-eclamptic patients (6 out of 8) were induced and delivered vaginally. However, the mode of delivery does not appear to affect the umbilicoplacental vessels response to 5-HT or KCl, as demonstrated by several investigators (Johnstone *et al.*, 1987; Abad *et al.*, 2003). Five of the pre-eclamptic patients were medicated (none of these patients were treated with ketanserin) while the remaining three were bed rested only. None of the normotensive controls were under any type of medication. In order to avoid possible variations introduced by the use of medications it would have been ideal to include only bed rested, pre-eclamptic patients; however this was not possible in this study due to time constraints. Since patient numbers was small it was not possible to asses whether medication affected 5-HT responses and / or 5-HT_{2A} receptor levels.

4.3.2 Comparison of primary and secondary vessels

All studies investigating placental vessel function in response to 5-HT have used one representative vessel from each placenta from a variety of locations (such as close to or distal to umbilical cord insertion) or have only defined the vessel diameter (Inayatulla *et al.*, 1993; Cruz *et al.*, 1998; Bertrand and St-Louis, 1999). This is the first study investigating different branches of chorionic plate vessels in response to 5-HT. Primary and secondary chorionic vessels with a diameter of approximately 2 mm were chosen. In order to achieve consistency these vessels were also cut to 2 mm length. In order to check potential variations in the vessel wall thickness blotted tissue weights were recorded after each experiment. Results indicate that there were no significant differences in vessel weight between primary and secondary vessels. In addition, tissue weights were similar when normotensive and corresponding pre-eclamptic vessels compared.

The contractility of each vessel was evaluated using 60 mM KCl responses at the beginning and end of each experiment. The response of primary vessels to KCl was similar to that of secondary vessels although secondary vessels contractility tended to be generally lower than primary vessels. However, these differences were only significant for pre-eclamptic arteries. More importantly the KCl response was similar when normotensive and corresponding pre-eclamptic vessels were compared.

The maximal contraction of (both normotensive and pre-eclamptic) primary and secondary veins to 5-HT was similar. In addition the response of normotensive primary and secondary arteries to 5-HT was similar. On the other hand the maximal contraction of secondary arteries to 5-HT was significantly reduced in pre-eclamptic samples. This finding suggests that chorionic plate vessels may indeed differentially modified in pre-eclamptic conditions and also emphasises the importance of studying different branches of the chorionic vessels comparatively. Although total 5-HT_{2A} receptor levels were similar, 63 kDa to 40-55 kDa band ratios was increased in pre-eclamptic secondary arteries compared to primary arteries (Figure 4.10). This may suggest that relative expression levels of 63 kDa and 40-55 kDa proteins have a functional role.

4.3.3 Comparison of 5-HT response in normotensive and pre-eclamptic vessels

The aims of this study were to compare the contractile response to 5-HT of chorionic vessels (arteries and veins) from placentae delivered after normal and pre-eclamptic pregnancies. And to investigate whether 5-HT_{2A} receptor protein levels contributed to possible changes in vessel function. 5-HT induced dose-dependent contractile response in the vessels studied (arteries and veins).

4.3.3.1 Arteries

Comparison of separate concentration-response curves for primary and secondary arteries from normotensive and pre-eclamptic samples revealed that for both arteries maximal response (Emax) was lower in the pre-eclamptic vessels (Figure 4.3). Although Emax of pre-eclamptic primary and secondary arteries were reduced by around 28 % and 60 %, respectively, compared to the corresponding normotensive vessels, the differences were significant only for the secondary arteries. As mentioned (section 4.1.2) the general contractility (as determined by KCl response) of pre-eclamptic secondary arteries is lower than that of primary arteries. However, this is unlikely to be the cause of the differences observed in response to 5-HT since maximal response to 5-HT is expressed as % KCl response and therefore contractile response to 5-HT is normalised for these differences (vessels general contractility). Primary arteries used in this study are comparable to those studied by Bertrand and St-Louis, (1999) as determined by their location on chorionic plate. Interestingly in their study they have also demonstrated a non-significant (approximately 20 %) reduction in the maximal response to 5-HT in pre-eclamptic samples. The data reported in this thesis demonstrate that only one representative vessel from the chorionic plate might underestimate the differences between normotensive and pe-eclamptic vessels. In order to demonstrate this, contractive responses of primary and secondary vessels were pooled together and a concentration-response curve was created using the mean contraction values produced by each vessel at each dose (Figure 4.14).

As expected Emax of pre-eclamptic arteries was lower than normotensives but the difference was not statistically significant. This clearly demonstrates that (a) one representative vessel from the chorionic plate may not be sufficient to study functional responses of the vessels and (b) there may be differences between vessels close to cord insertion and vessels at the periphery of the chorionic plate.

4.3.3.2 Veins

To date only one study has investigated the response of chorionic plate veins to 5-HT in pre-eclampsia (Bertrand and St-Louis, 1999). Since chorionic plate veins contain smooth muscle they may have important contractile potential. It should also be noted that, as in the pulmonary circulation, placental veins carry oxygenated blood from the placenta to the foetus. Hence changes in venous contractility to vasoactive substances such as 5-HT in pre-eclampsia may have important consequences for the delivery of oxygen and nutrients to the foetus.

Results from the present study show that maximal contraction of both primary and secondary veins to 5-HT was significantly reduced by pre-eclampsia (43 % and 52 % reduction) (Figure 4.6). Sensitivity to 5-HT (pEC50) in both vessels on the other hand was not significantly different between normotensive and pre-eclamptic vessel. As both vessels were significantly less responsive to 5-HT in pre-eclamptic vessels, pooling the data together did not change the outcome (Figure 4.15).

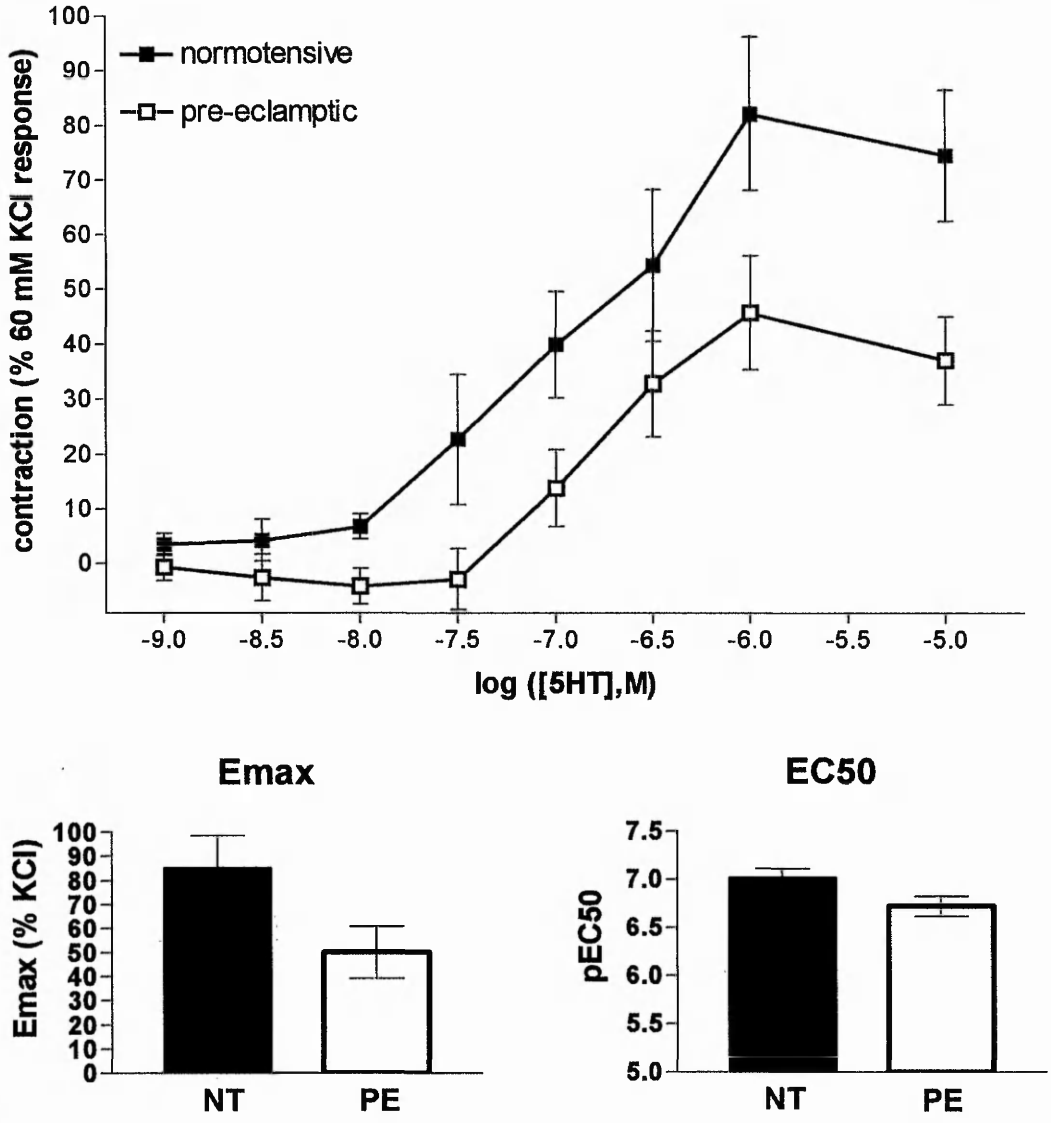


Figure 4.14 Response of normotensive and pre-eclamptic chorionic arteries to 5-HT (pooled data)

Concentration-response to 5-HT for chorionic plate arteries from placentas delivered after normal (filled squares) and pre-eclamptic pregnancies (open squares). Contraction produced by 5-HT expressed as % 60 mM KCl response. The primary and secondary artery contractive responses to 5-HT were pooled. A concentration-response curve was created using the mean values of contraction produced by each vessel at each dose. *n* represents number of vessels used for the study (each vessel obtained from a different patient). *n= 9* normotensive artery, *n= 8* pre-eclamptic artery). Statistical significance was accepted at $p< 0.05$ (*) and $p<0.01$ (**).

NT= normotensive, PE= pre-eclamptic.

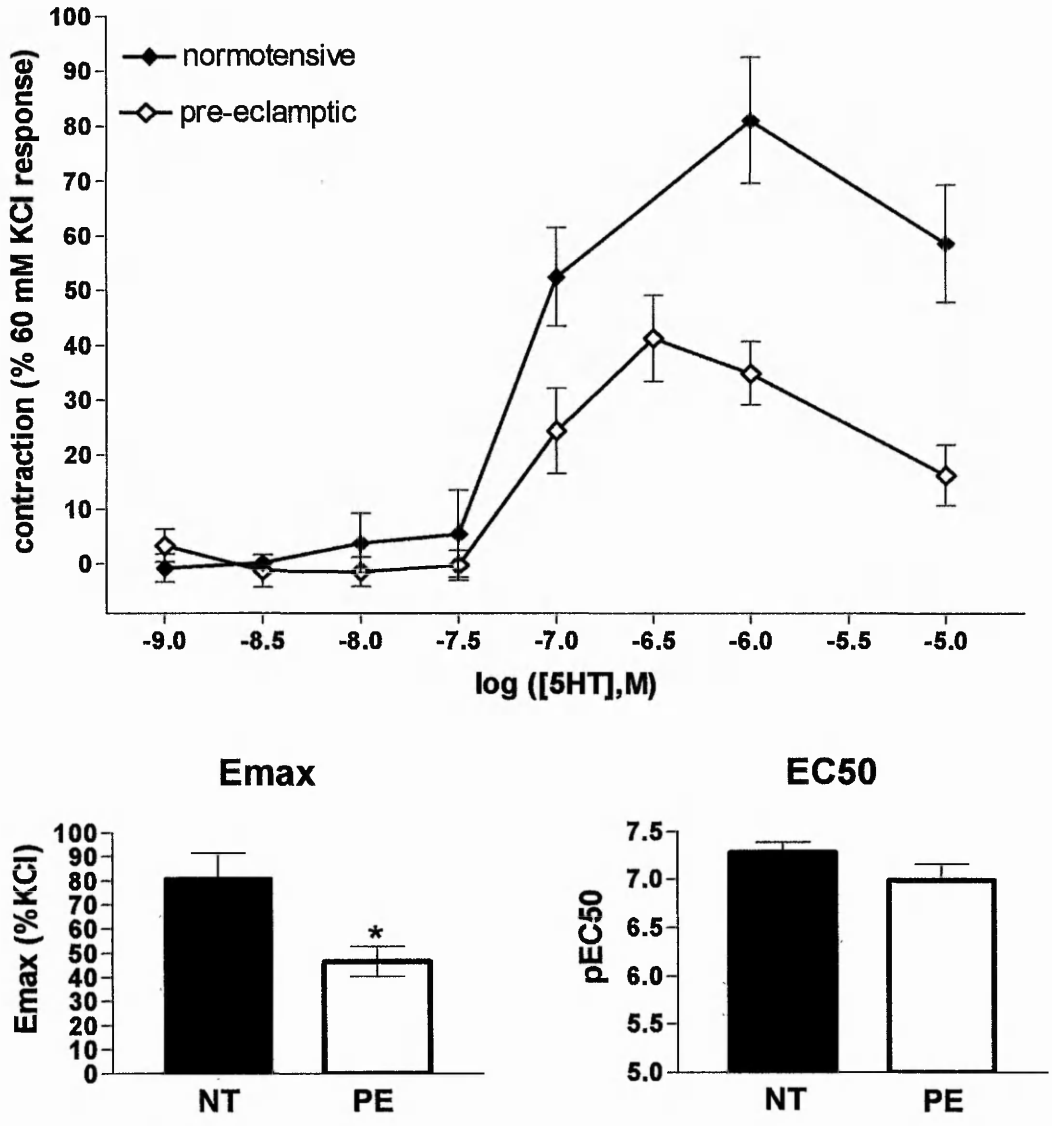


Figure 4.15 Response of normotensive and pre-eclamptic chorionic veins to 5-HT (pooled data)

Concentration-response to 5-HT for chorionic plate veins from placentas delivered after normal (filled diamonds) and pre-eclamptic pregnancies (open diamonds). Contraction produced by 5-HT expressed as % 60 mM KCl response. Primary and secondary artery contractive response to 5-HT was pooled. Concentration-response curve was produced using mean values of contraction at each dose by each vessel. *n* represents number of vessels used for the study (each vessel obtained from a different patient). *n= 9* normotensive veins, *n= 8* pre-eclamptic veins). Statistical significance was accepted at $p< 0.05$ (*) and $p<0.01$ (**).
NT= normotensive, PE= pre-eclamptic.

4.3.4 Contribution of 5-HT_{2A} receptor to 5-HT mediated response

4.3.4.1 5-HT_{2A} receptor expression levels

Pre-eclamptic arteries were less responsive to 5-HT, although 5-HT_{2A} receptor protein levels were not significantly reduced in these vessels (Figure 4.4 and Figure 4.5). In addition 5-HT_{2A} receptor protein expression levels in (primary and secondary) veins were similar in normotensive and pre-eclamptic vessels (Figure 4.7 and Figure 4.8) as were the ratio of the 63 kDa to 40-55 kDa proteins. However, receptor desensitization might contribute to this reduced responsiveness to 5-HT in pre-eclamptic vessels. As with many other GPCRs, 5-HT_{2A} receptors are known to desensitise upon repeated agonist exposure, demonstrated by *in vivo* and *in vitro* cell culture models (Bhatnagar *et al.*, 2001; Hanley and Hensler, 2002; Damjanoska *et al.*, 2004). Receptor desensitisation (or a decrease in response) can occur as a result of increased receptor internalisation (sequestration of the receptor away from the cell surface), receptor uncoupling from G protein, or down regulation of total receptor numbers. The results suggest the latter is unlikely to be responsible for the reduced response to 5-HT. Nonetheless, there may be differences in receptor levels available at the cell surface due to agonist-induced receptor internalisation; alternatively receptor coupling to G protein may be altered in pre-eclampsia. It must be noted that triton soluble total tissue extracts were analysed in the present study and this does not allow receptors expressed at the cell surface to be distinguished from receptors within the cell (internalised receptors). Due to time limitations it was not possible to investigate cellular localisation of the 5-HT_{2A} receptors and therefore whether expression of these receptors at the cell surface level are reduced in pre-eclamptic vessels remains to be elucidated.

4.3.4.2 Ketanserin antagonism to 5-HT response

Ketanserin reduced contractile response to 5-HT in both normotensive and pre-eclamptic primary / secondary vessels. The mean % reduction in Emax in the presence of ketanserin was greater in all pre-eclamptic vessels compared to normotensives (Figure 4.13), albeit the difference was not statistically significant. This would suggest that 5-HT_{2A} receptor contributions to 5-HT mediated contraction are greater in pre-eclampsia. Ketanserin antagonism varied greatly between patients even within the normotensive group. This variation was also evident in 5-HT_{2A} receptor expressions as described earlier. Insufficient efficacy of ketanserin has been reported in the treatment of severe early onset pre-eclamptic patients (Hanff *et al.*, 2006). Nonetheless, pharmacokinetic parameters of ketanserin have been reported to be comparable to that of healthy non-pregnant subjects (Hanff *et al.*, 2005) and the variations in the antihypertensive response within pre-eclamptic patients therefore could be due to variations in 5-HT_{2A} receptor activity (Hanff *et al.*, 2005). Moreover in some pre-eclamptic samples ketanserin completely antagonised the response to 5-HT; this was not observed in the normotensive group (Table 4.3 and Table 4.4). However this effect might not be specific to 5-HT_{2A} receptors as ketanserin antagonism generally tended to be higher in the medicated group (Table 4.3, Table 4.4 and Figure 4.16). Since the patient numbers are small a definitive conclusion cannot be made. As most of the pre-eclamptic patients were medicated and none of the controls were under any medication, it cannot be completely disregarded that these differences might also have an effect on overall vessel response.

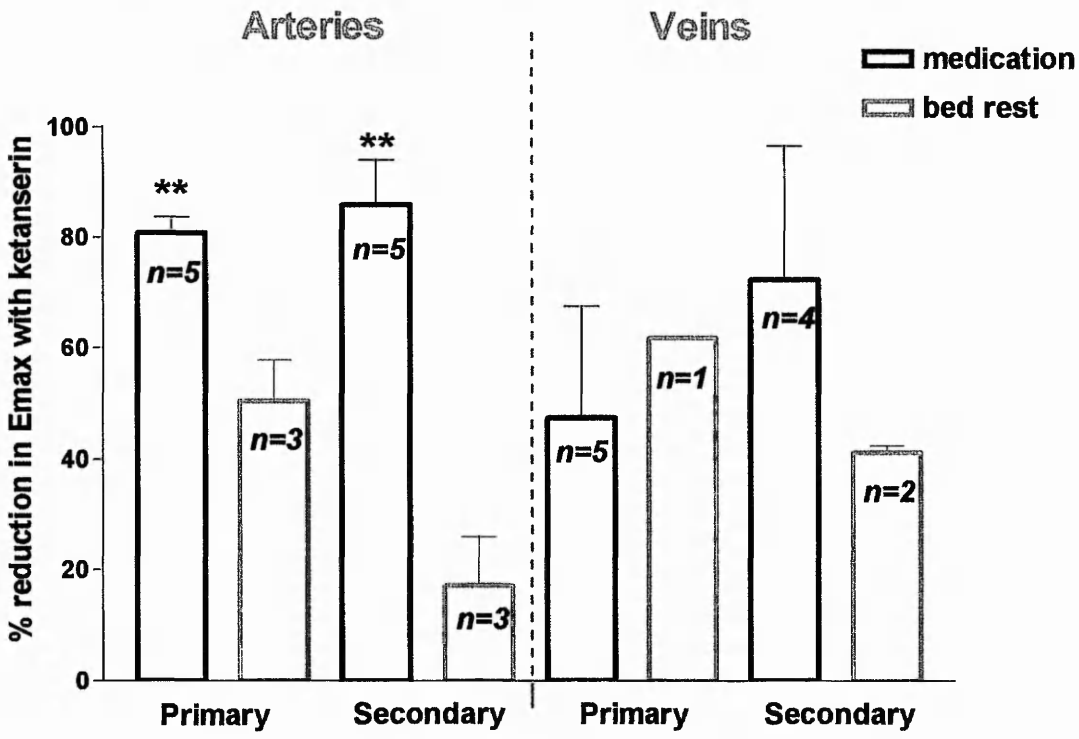


Figure 4.16 Ketanserin antagonism to 5-HT response in pre-eclamptic vessels

Contraction produced by 5-HT are expressed as % 60 mM KCl response. % Reduction represents the reduction in Emax values in response to 5-HT in the presence of ketanserin. Pre-eclamptic patients was divided into two subgroups: medicated and bed rested. % Reduction in Emax was compared between samples collected from medicated and bed rested patients. Statistical significance was accepted at $p< 0.05$ (*) and $p<0.01$ (**). Number of samples used from each group is shown in figure on each column.

CHAPTER V

5-HT TURNOVER

5 CHAPTER V 5-HT turnover

5.1 Introduction

5.1.1 Possible role of 5-HT and MAO in pre-eclampsia

5-HT is suggested to have an important physiological role during pregnancy and labour (Fecteau and Eiler, 2001). It has a mitogenic effect in a wide variety of cell types including placental (Fecteau and Eiler, 2001; Sonier *et al.*, 2005) and foetal cells (Lauder, 1990; Whitaker-Azmitia *et al.*, 1996; Butkevich *et al.*, 2003). Higher levels of 5-HT have been detected in foetal blood compared to maternal blood in pregnant cows which is in line with its mitogenic effects (Fecteau and Eiler, 2001). On the other hand, although 5-HT is important for the development of the foetus, high levels have been demonstrated to have a detrimental effect on pregnant animals and their foetuses (Robson and Sullivan, 1966).

Increased levels of 5-HT have been reported in pre-eclampsia although there are some differences between studies. In pre-eclampsia increased levels of serum 5-HT (Laskowska *et al.*, 2001), platelet poor plasma (PPP) 5-HT (Middelkoop *et al.*, 1993; Carrasco *et al.*, 1998) and placental (tissue extracts) 5-HT levels (Gujrati *et al.*, 1996) have been reported. Platelet serotonin concentrations have found to be unaffected by pre-eclampsia in some studies (Carrasco *et al.*, 1998) whilst increased levels have been reported in others (Gujrati *et al.*, 1994). However, Carrasco *et al.* (1998) studied platelet uptake and content of 5-HT in severe-preclamptic patients whilst in the study of Gujrati *et al.* (1994) disease was less advanced in the pre-eclamptic group. Hence the different results obtained in the two studies are thought to be due to differences in the severity of pre-eclampsia in the patient groups (Carrasco *et al.*, 1998). Methods used for analysis can also contribute to these differences. Indeed platelets are very fragile and could be activated easily resulting in the release of 5-HT from the platelets adding to the error during analysis. In addition some studies have taken an indirect approach and investigated urinary 5-HT and 5-HIAA (5-HT metabolite) levels. Urinary 5-HT levels were reported to be increased in pre-eclampsia (Vural *et al.*, 1999).

5-HIAA levels were also fund to be increased by pre-eclampsia in some studies (Filshie *et al.*, 1992) but have decreased in others (Vural *et al.*, 1999). Again these differences are probably due to differences between the patient groups studied (severity of disease, mild or severe pre-eclampsia)

The majority of 5-HT is synthesised in the enterochromaffin cells of the intestine. These cells appear in the intestine of the developing foetus as early as 2 months gestation (Facer *et al.*, 1989). Therefore one of the main sources of increased 5-HT in maternal blood has been suggested to be the foetus (Gujrati *et al.*, 1985; Weiner, 1987). However, a major factor in the regulation of circulating 5-HT levels in the body is its metabolism by the enzyme MAO-A; hence placental MAO-A activity is important in the control of 5-HT levels. There are two limiting factors in the metabolism of 5-HT; first the transport of the amine by SERT (serotonin transporter) to the syncytiotrophoblast (where MAO-A is located) and secondly the activity of the enzyme. The uptake and transport of 5-HT (as determined by the activity of SERT) into the placenta is not affected in pre-eclampsia (Carrasco *et al.*, 2000a; Carrasco *et al.*, 2000b). On the other hand, the catalytic turnover of placental MAO is reduced in placental tissue from pre-eclamptic pregnancies (Sivasubramaniam *et al.*, 2002). Therefore decreased MAO-A activity could be a major factor in the increased 5-HT levels in maternal blood.

5.1.2 Specific aims of chapter

Since 5-HT levels are reported to vary with the severity of the disease it was necessary to monitor 5-HT and 5-HIAA concentrations in the pre-eclamptic and normotensive patients used for this study. Increased levels of 5-HT have been reported in pre-eclamptic placental tissue but levels in placental blood from pre-eclamptic patients have not yet been investigated. Therefore it was aimed to investigate 5-HT levels in various blood samples from pre-eclamptic and normotensive patients namely cord blood, placental blood and maternal (peripheral) blood. Since 5-HIAA concentrations are very low in blood, 5-HT turnover was investigated by measuring 5-HT and 5-HIAA levels in urine samples from normotensive and pre-eclamptic patients. In addition placental MAO-A activity in both groups of patients was determined in order to investigate a possible relationship between 5-HT turnover and placental MAO-A activity. As reduced MAO-A activity in placental tissue from pre-eclamptic patients have been reported, possible changes in enzyme affinity for its substrate was also investigated. This was achieved by estimating the Michaelis-Menten constant (Km) of MAO-A for tyramine in normotensive and pre-eclamptic placental samples.

5.2 Results

5.2.1 Preliminary studies with non-pregnant subjects

Preliminary experiments concentrated on establishing the effects of sampling time on platelet and PPP 5-HT levels. Since the majority of blood samples would be collected in the morning from pregnant subjects it was important to establish whether 5-HT levels changed significantly with collection time. This was investigated by measuring 5-HT in four non-pregnant subjects three times on the same day (in the morning, midday and in the afternoon). Blood samples were collected from volunteers who were healthy, normotensive and not taking any medication or oral contraceptives. In order to achieve consistency, samples were collected approximately midway through their menstrual cycle. In addition since some foods (tomato, avocado, walnuts etc.) are known to interfere with 5-HT measurements, blood samples were collected before meals and all subjects completed a short questionnaire to inform the study whether they had eaten (24 hour prior to blood collection) any of these foods.

Blood was first fractionated to obtain PRP; this was further fractionated to produce PPP and platelet pellets (section 2.2.5.1), which were analysed for 5-HT levels as detailed in section 2.2.5.3.

5.2.1.1 Changes in platelet 5-HT levels during the day

Figure 5.1 shows there were no significant differences between 5-HT levels at each sampling time. Mean 5-HT levels in platelets were 488.2 ± 57.38, 495.8 ± 62.51 and 519.5 ± 79.20 ng / 10^9 platelets in the morning, midday and afternoon respectively.

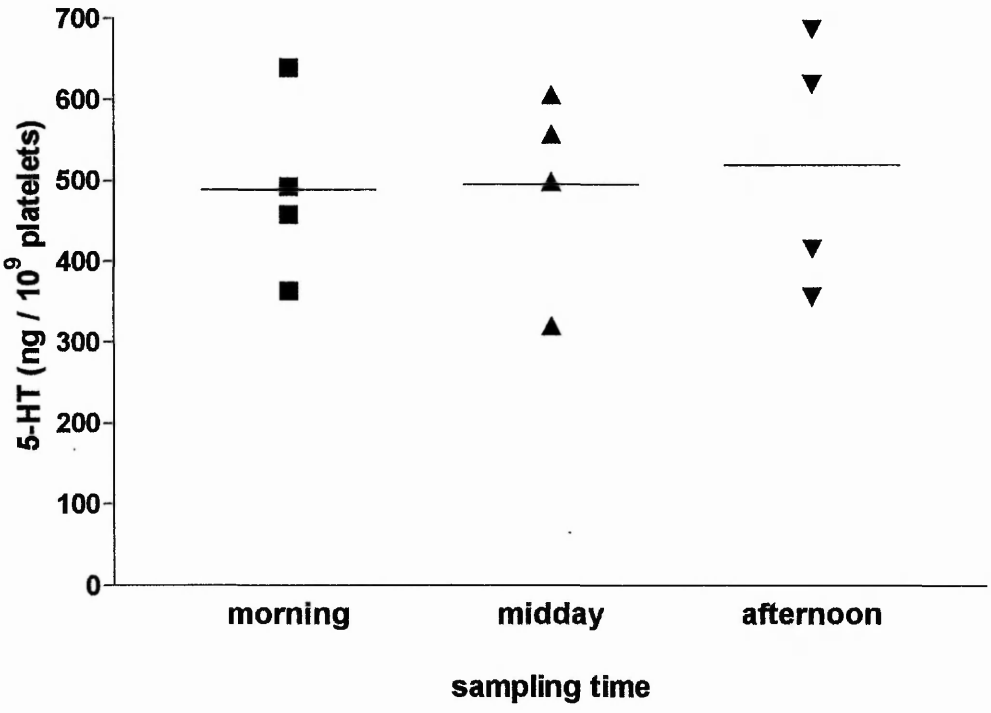


Figure 5.1 Changes in platelets 5-HT levels during the day

Scatter plot of 5-HT levels in platelets determined as detailed in section 2.2.5.3.1. Statistical analysis of comparison of 5-HT levels at each time point was carried out using ANOVA one-way analysis of variance where statistical significance was accepted at $p < 0.05$ (*) and $p<0.01$ (**), ($n=4$).

5.2.1.2 Changes in platelet poor plasma 5-HT levels during the day

Figure 5.2 shows that there were no significant differences between 5-HT levels in PPP at each sampling time. Mean 5-HT levels were 2.582 ± 0.144, 2.311 ± 0.168 and 2.614 ± 0.201 ng / ml in the morning, midday and afternoon respectively.

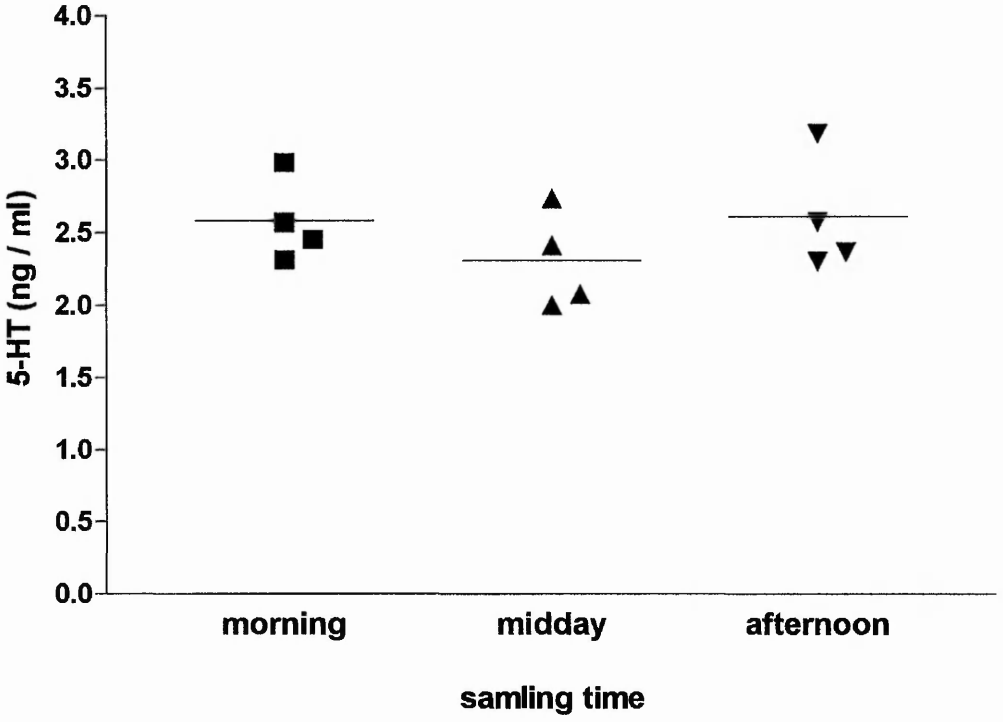


Figure 5.2 Changes in PPP 5-HT levels during the day

Scatter plot of 5-HT levels in PPP fractions determined as detailed in section 2.2.5.3.2. Statistical analysis of comparison of 5-HT levels at each time point was carried out using ANOVA one-way analysis of variance where statistical significance was accepted at $p < 0.05$ (*) and $p<0.01$ (**), ($n=4$).

5.2.2 Platelet and platelet poor plasma 5-HT levels in normotensive and pre-eclamptic subjects

Blood samples were collected from normotensive and pre-eclamptic women by venipuncture. Since the study included both elective caesarean and vaginal delivery patients, blood samples were collected immediately before caesarean section or induction of labour. As a standard procedure at the Nottingham City Hospital, women undergoing caesarean section are advised to starve from midnight and women to be induced are given a light breakfast and do not eat once in labour. Similar to non-pregnant subjects, pregnant subjects also completed a short questionnaire to inform the study whether they had eaten (within 24 hour prior to blood collection) any of the foods known to interfere with 5-HT measurements.

5.2.2.1 Platelet 5-HT levels in maternal peripheral blood

Mean 5-HT levels in platelets was 420.8 ± 74.72 and 573.9 ± 152.4 ng / 10^9 platelets for normotensive and pre-eclamptic women respectively (Figure 5.3). Although mean platelet 5-HT level was slightly higher in the pre-eclamptic group, the difference was not statistically significant. However, further analysis revealed that pre-eclamptic women who were on medication (pre-eclapmtic samples 1, 4 and 5-see table 4.3 for medication used) had significantly higher platelet 5-HT concentrations than normotensive women (Figure 5.3 b - also see Table 5.1 for mean and median values). On the other hand, pre-eclamptic women who were not medicated and were simply bed rested (pre-eclamptic samples 3, 6 and 8 - see table 4.3) had a similar platelet 5-HT level to the normotensive controls (Figure 5.3 b- also see Table 5.1 for mean and median values). Platelet numbers did not vary significantly between groups, mean platelet numbers were 218.4 ± 15.21, 243.0 ± 58.56 and 216.7 ± 16.38 10^9 / L for normotensive, pre-eclamptic women on medication and pre-eclamptic bed rested patients, respectively.

There was no correlation between platelet 5-HT levels and maximal vessel (primary / secondary arteries and veins) response to 5-HT in either normotensive or pre-eclamptic subjects (data not shown).

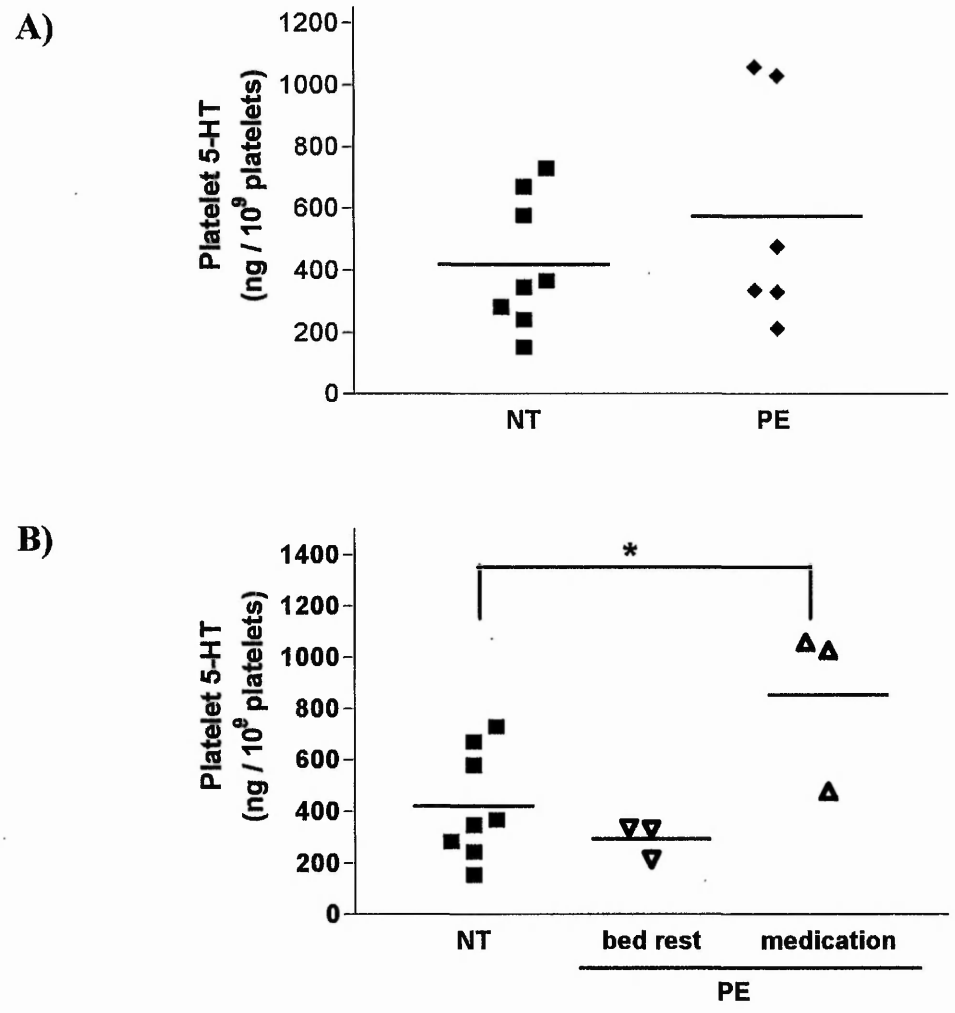


Figure 5.3 Platelet 5-HT concentrations in normotensive and pre-eclamptic women

A) Scatter plot of 5-HT levels in platelets from normotensive and pre-eclamptic women

B) Scatter plot of 5-HT levels in platelets from normotensive and pre-eclamptic women divided into two sub groups, medicated and bed rested

Statistical significance was accepted at $p< 0.05$ (*) and $p<0.01$ (**), (*n=10* normotensive, *n=3* pre-eclamptic on medication and *n=3* pre-eclamptic bed rested).

NT= normotensive, PE= pre-eclamptic

5.2.2.2 Platelet poor plasma 5-HT levels in maternal peripheral blood

5-HT concentrations determined in PPP from normotensive and pre-eclamptic women were not significantly different. Mean 5-HT concentrations were 4.539 ± 0.502 and 6.674 ± 1.386 ng / ml in normotensive and pre-eclamptic women respectively (Figure 5.4 a). However, further analysis revealed that pre-eclamptic women who were not on medication and were bed rested only had significantly higher PPP 5-HT concentrations than normotensive women (Figure 5.4 b). On the other hand, pre-eclamptic women who were on medication had similar PPP 5-HT levels to normotensive controls. Mean and median PPP 5-HT levels are given in Table 5.1.

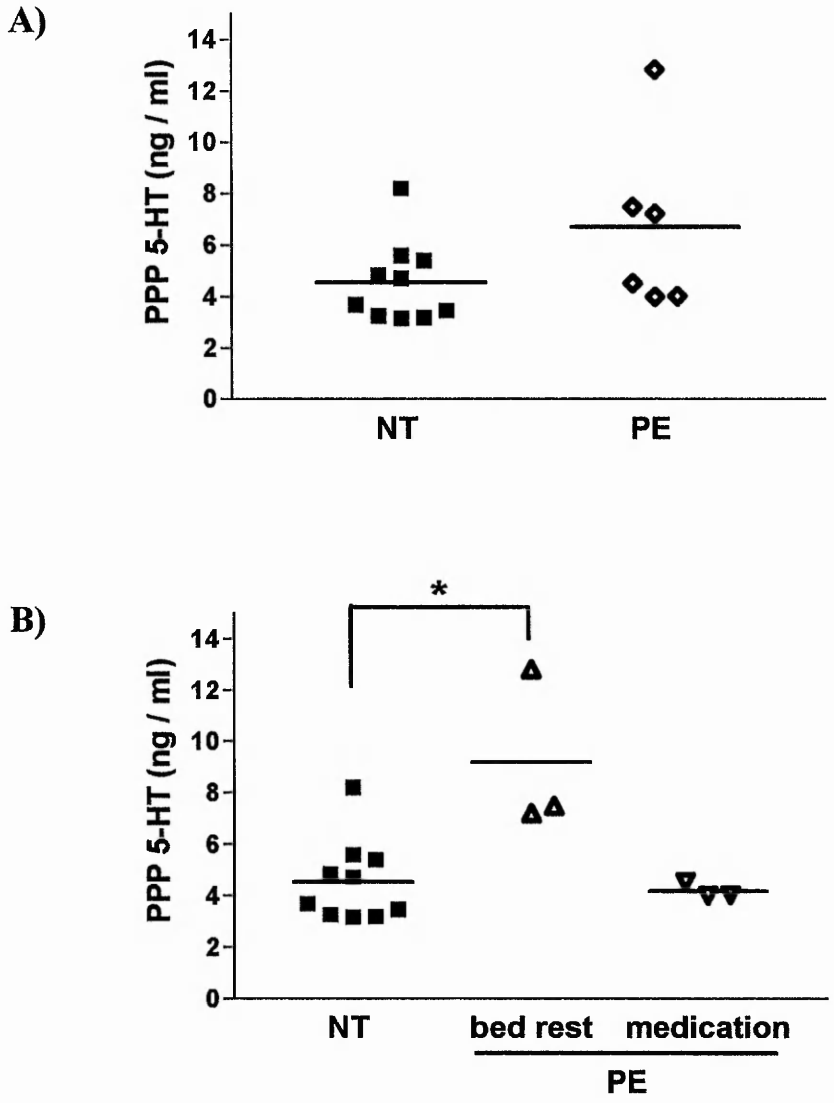


Figure 5.4 PPP 5-HT levels in normotensive and pre-eclamptic subjects

A) Scatter plot of 5-HT levels in PPP from normotensive and pre-eclamptic women

B) Scatter plot of 5-HT levels in PPP from normotensive and pre-eclamptic women divided in to two sub groups who had medication and bed rested

Statistical significance was accepted at $p< 0.05$ (*) and $p<0.01$ (**), (*n=10* normotensive, *n=3* pre-eclamptic on medication and *n=3* pre-eclamptic bed rested).

NT= normotensive, PE= pre-eclamptic

5.2.2.3 Platelet poor plasma 5-HT levels in cord and placental blood

Mean 5-HT concentrations in placenta and cord blood PPP fractions were unaffected by pre-eclampsia. Further analysis revealed that placental PPP 5-HT levels were higher in the non-treated (bed rested pre-eclamptic patients) group compared to normotensive controls although these differences were not statistically significant (Figure 5.5b).

Moreover there was no correlation between PPP 5-HT levels and maximal vessel (primary / secondary arteries and veins) response to 5-HT in either normotensive or pre-eclamptic subjects (data not shown).

Table 5.1 gives an overview of PPP and platelet 5-HT values in maternal, cord and placental blood samples from normotensive and pre-eclamptic patients. Median values in addition to mean values are given.

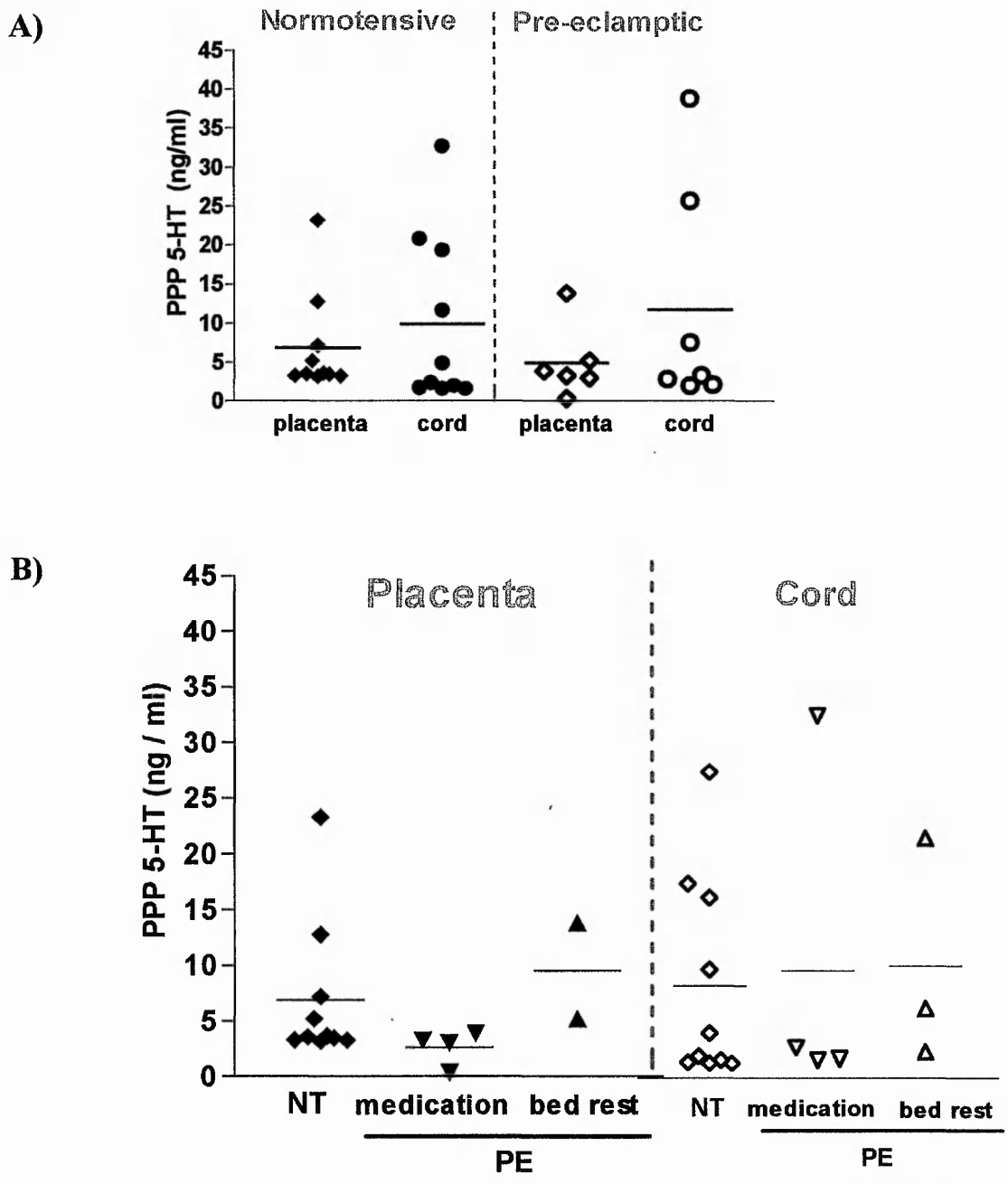


Figure 5.5 PPP 5-HT levels in normotensive and pre-eclamptic samples

A) Scatter plot of 5-HT levels in PPP obtained from cord and placenta blood collected from normotensive and pre-eclamptic patients

B) Scatter plot of 5-HT levels in PPP obtained from cord and placenta blood collected from normotensive and pre-eclamptic patients divided in to two sub groups who had medication and bed rested

Cord versus placental blood samples and normotensive versus pre-eclamptic samples compared. Statistical significance was accepted at $p< 0.05$ (*) and $p<0.01$ (**). (*n=10* normotensive samples, *n= 6-7* pre-eclamptic samples).

			Normotensive	Pre-eclamptic	Pre-eclamptic	
					Medication	Bed rest
PPP 5-HT (ng/ml)	Maternal blood	Mean ± SEM	4.539 ± 0.502	6.674 ± 1.386	4.180 ± 0.1705	9.167 ± 1.831 **
		Median (Range)	4.184 (3.153-8.196)	5.861 (3.996-12.830)	4.024 (3.996-4.521)	7.474 (7.201-12.83)
	Placental blood	Mean ± SEM	6.890 ± 2.046	4.936 ±1.894	2.640 ± 0.7783	9.528 ± 4.306
		Median (Range)	3.618 (3.181-23.220)	3.592 (0.371-13.830)	3.151 (0.371-3.888)	9.528 (5.222-13.83)
	Cord blood	Mean ± SEM	9.912 ± 3.463	11.76 ± 5.517	11.56 ± 9.073	12.04 ± 6.955
		Median (Range)	3.660 (1.668-32.730)	3.315 (2.003-38.760)	2.730 (2.003-38.76)	7.582 (2.865-25.69)
Platelet 5-HT (ng / 10^9 platelets)	Maternal blood	Mean ± SEM	420.8 ± 74.72	573.9 ± 152.4	854.8 ± 188.9 *	293.1 ± 39.90
		Median (Range)	356.4 (152.8-729)	406.5 (213.4-1058)	1029 (477.3-1058)	330.3 (213.4-335.7)

Table 5.1 Summary of PPP and platelet 5-HT levels in blood samples from normotensive and pre-eclamptic patients

Normotensive and pre-eclamptic maternal, cord and placental blood samples were compared using Student's t-test and statistical significance was accepted at $p < 0.05$ (*) and $p < 0.01$ (**).

5.2.3 Levels of 5-HT and its metabolite 5-HIAA in urine

Urine samples were collected prior to induction or caesarean section. Although the intention was to collect 24-hour urine samples this proved not to be possible in all cases and a minimum of 12-hour collection was accepted for analysis. Sample collection duration was 21.88 ± 1.01 hours and 19.33 ± 2.40 hours for normotensive and pre-eclamptic samples respectively. Mean total urine volumes were 1328 ± 205.1 ml for normotensive and 1253 ± 346.0 ml for pre-eclamptic samples.

Figure 5.6 shows 5-HT and 5-HIAA levels in normotensive and pre-eclamptic urine samples. Pre-eclamptic women had significantly higher 5-HT in their urine while 5-HIAA levels were similar in the two groups. Moreover, the 5-HIAA: 5-HT ratio was significantly lower in pre-eclamptic women compared to normotensive controls.

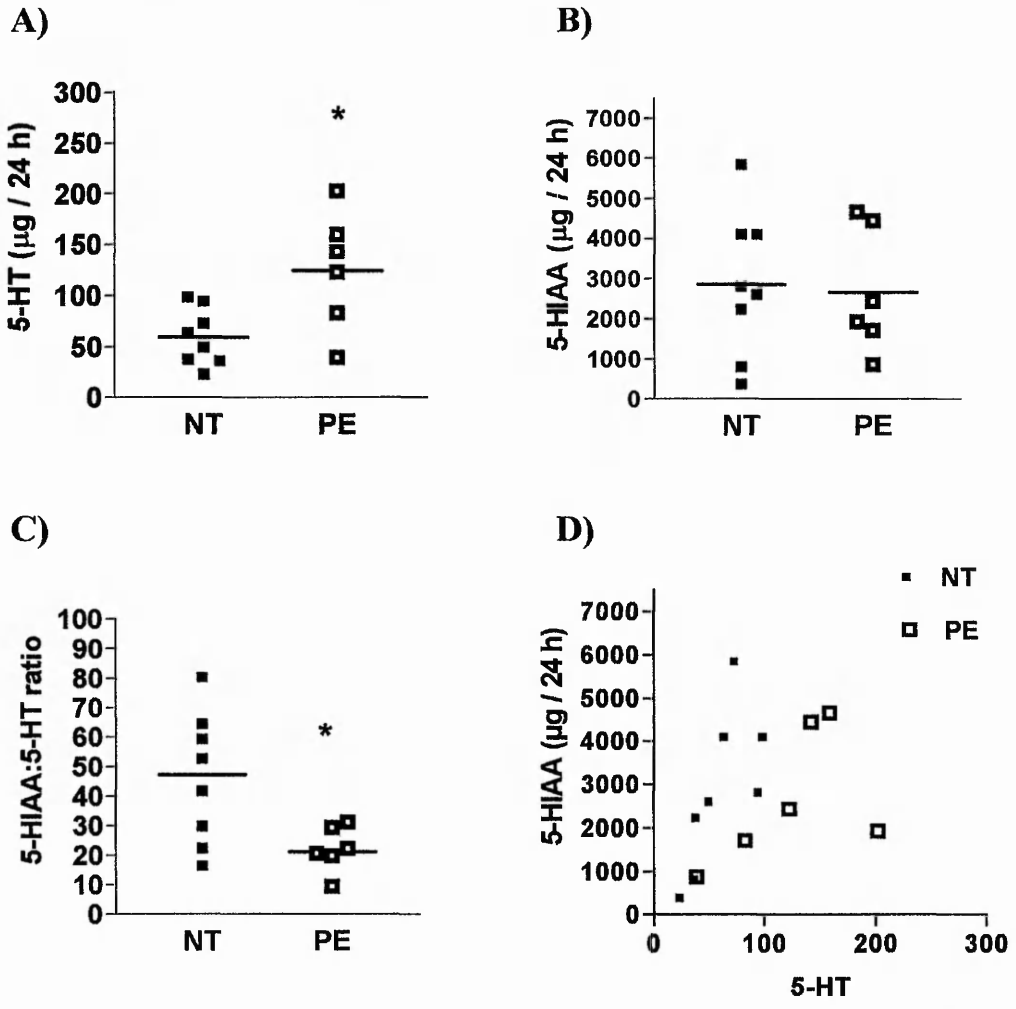


Figure 5.6 Urine 5-HT and 5-HIAA levels in normotensive and pre-eclamptic women

Urine 5-HT and 5-HIAA concentrations were determined as detailed in sections 2.2.5.3.3 and 2.2.5.4.

A) Scattergram showing 5-HT levels

B) Scattergram showing 5-HIAA levels

C) Scattergram showing 5-HIAA: 5-HT ratios

D) Scattergram showing 5-HIAA and 5-HT values for each sample

Statistical significance was accepted at $p < 0.05$ (*) and $p < 0.01$ (**), ($n=8$ normotensive, $n=6$ pre-eclamptic). NT = normotensive, PE = pre-eclamptic.

5.2.4 Monoamine Oxidase-A activity in placental tissue

5.2.4.1 MAO-A activity assay optimisation

Initial studies concentrated on assay optimisation. Ranges of protein concentrations (i.e. sample volume) and incubation times with substrate were investigated. Using representative normotensive and pre-eclamptic placental extract samples, MAO-A activity was measured at three different protein concentrations (2-4 mg/ml) and was shown to be propotional with these protein concentrations (Figure 5.7). In addition using these samples (3 mg/ml) the linearity of the assay was investigated over a 40 minute incubation period. Figure 5.8 shows that the assay was linear for 40 minutes of reaction time.

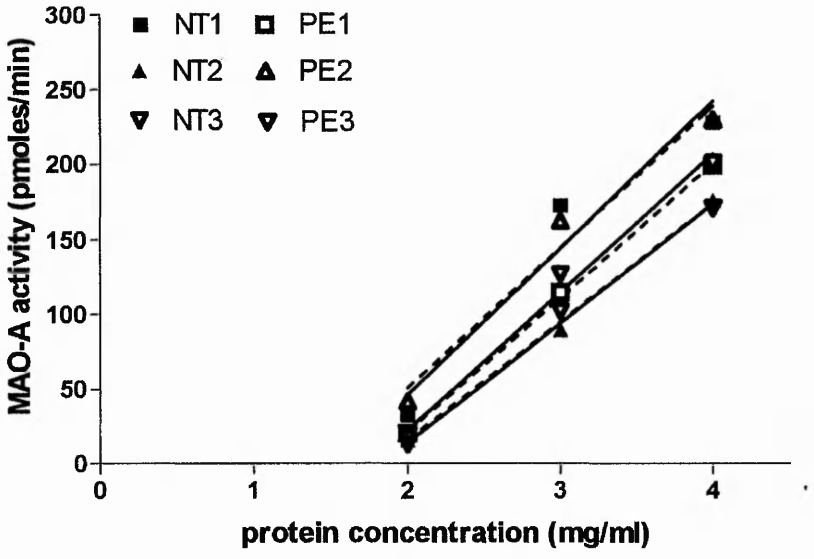


Figure 5.7 MAO-A activity optimisation for sample protein concentration

Three representative normotensive and pre-eclamptic placenta samples were extracted and protein estimated as detailed in sections 2.2.3.2 and 2.2.7.2. Samples were diluted to desired final protein concentrations (2-4 mg/ml) and MAO-A activity was assayed as described in section 2.2.4. Resultant activities are presented as pmoles/min. NT = normotensive, PE = pre-eclamptic

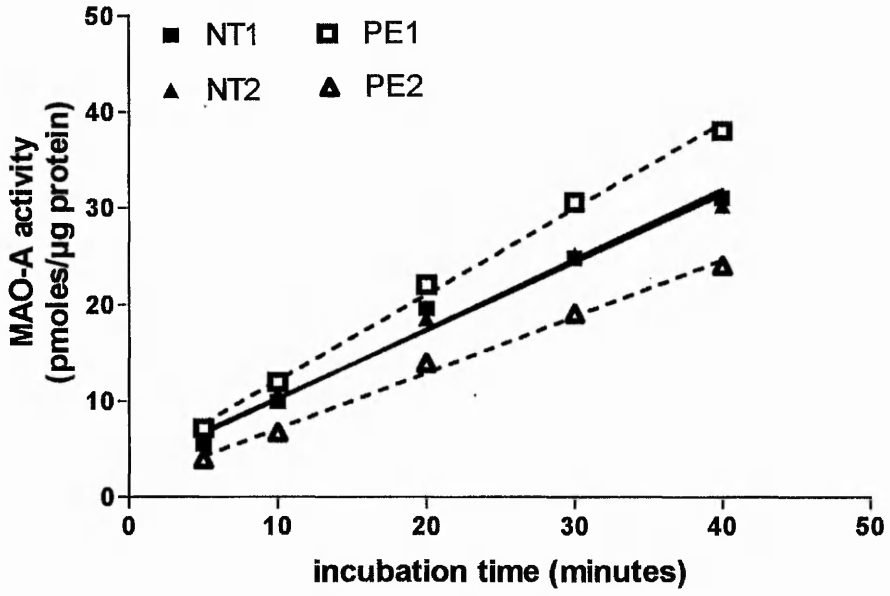


Figure 5.8 MAO-A activity optimisation for substrate incubation time

Two representative normotensive and pre-eclamptic placenta samples were extracted and protein estimated as detailed in sections 2.2.3.2 and 2.2.7.2. Samples were diluted to desired final protein concentrations (3 mg/ml) and MAO-A activity was assayed for 5-40 min. Resultant activities are presented as pmoles/µg protein.

NT = normotensive, PE = pre-eclamptic

5.2.4.2 MOA-A activity in normotensive and pre-eclamptic samples

MAO-A activity was determined in placental extracts from normotensive and pre-eclamptic pregnancies. Mean activity was 1.588 ± 0.071 and 1.245 ± 0.164 pmoles/min/µg protein for normotensive and pre-eclamptic samples respectively. Figure 5.9 presents MAO-A activity in normotensive and pre-eclamptic samples and Table 5.2 summarises urine 5-HT and 5-HIAA levels and placental MAO-A activity. Although not significant, a decrease in placental MAO-A activity resulted in a significant increase in urine 5-HT and a reduced 5-HIAA : 5-HT ratio.

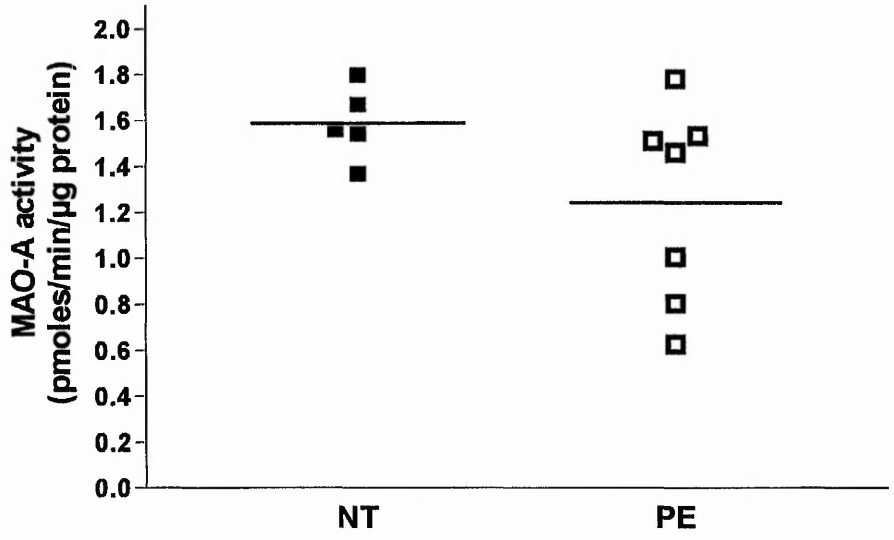


Figure 5.9 MOA Activity in normotensive and pre-eclamptic placenta samples
MAO-A activity was determined in placental extracts from normotensive and pre-eclamptic pregnancies as detailed in section 2.2.4. Enzyme activity is expressed as pmoles/min/µg protein. Statistical significance was accepted at $p < 0.05$ (*) and $p < 0.01$ (**).
NT = normotensive, PE = pre-eclamptic ($n=5$ normotensive, $n=7$ pre-eclamptic).

	Normotensive	Pre-eclamptic	P value
5-HT (μg / 24 hour urine) (mean ± SEM)	59.47 ± 9.76	124.8 ± 23.55*	0.0152
5-HIAA (μg / 24 hour urine) (mean ± SEM)	2863 ± 637.5	2668 ± 628.7	0.8351
5-HIAA: 5-HT ratio	45.91 ± 7.84	22.05 ± 3.16*	0.0278
MAO-A activity (pmoles/min/μg protein) (mean ± SEM)	1.588 ± 0.071	1.245 ± 0.164 (21.6 % reduction)	0.1251

Table 5.2 Summary of 5-HT and 5-HIAA levels in urine samples and MAO-A activity in placental tissue in normotensive and pre-eclamptic patients

Urine 5-HT and 5-HIAA concentrations were determined as detailed sections 2.2.5.3.3 and 2.2.5.4. MAO-A activity was determined in placental extracts from normotensive and pre-eclamptic pregnancies as detailed in section 2.2.4. Results are expressed as mean ± SEM. Statistical significance was accepted at $p < 0.05$ (*) and $p < 0.01$ (**).

5.2.4.3 Determination of MAO-A Km for tyramine

Next whether pre-eclampsia affected the km of MAO-A for its amine substrate was investigated. MAO-A activity was measured employing the usual assay using different final concentrations of tyramine. Lineweawer-Burk and Eadie-Hofstee plots are demonstrated for three representative normotensive and pre-eclamptic samples in Figure 5.10 and Figure 5.11 respectively. Km values for all samples were calculated from Eadie-Hofstee plots. Km values for MAO-A from normotensive and pre-eclamptic placenta were similar (Figure 5.12).

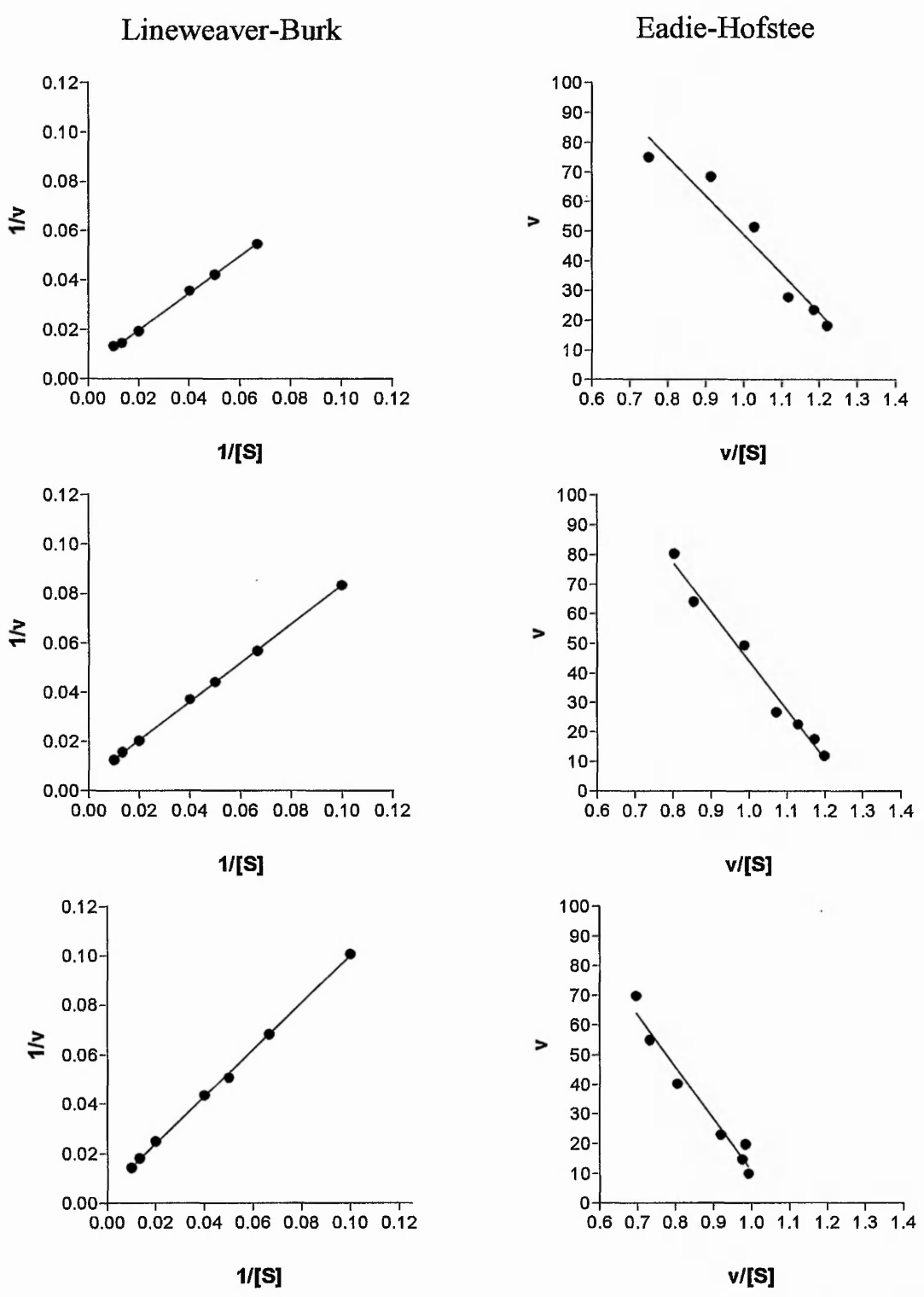


Figure 5.10 MAO-A enzyme kinetics for normotensive samples

Lineweawer-Burk and Eadie-Hofstee plots of MAO-A activity of normotensive placental samples measured with different substrate (tyramine) concentrations.

[S] = Substrate concentration (μM), v = reaction velocity (pmoles / min)

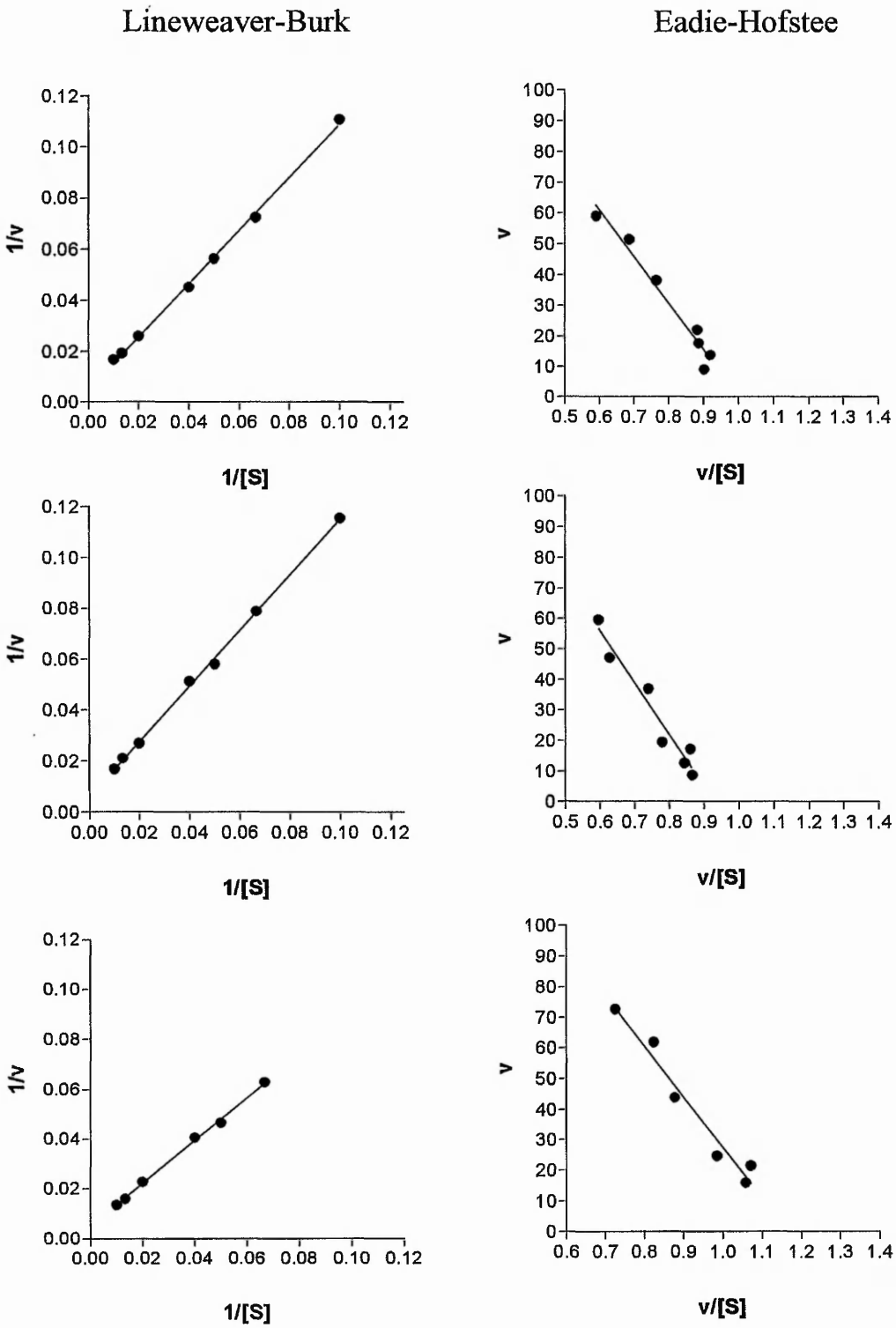


Figure 5.11 MAO kinetics for pre-eclamptic samples

Lineweaver-Burk and Eadie-Hofstee plots of MAO-A activity of pre-eclamptic placental samples measured with different substrate (tyramine) concentrations.

[S] = substrate concentration (μM), v = reaction velocity (pmoles / min)

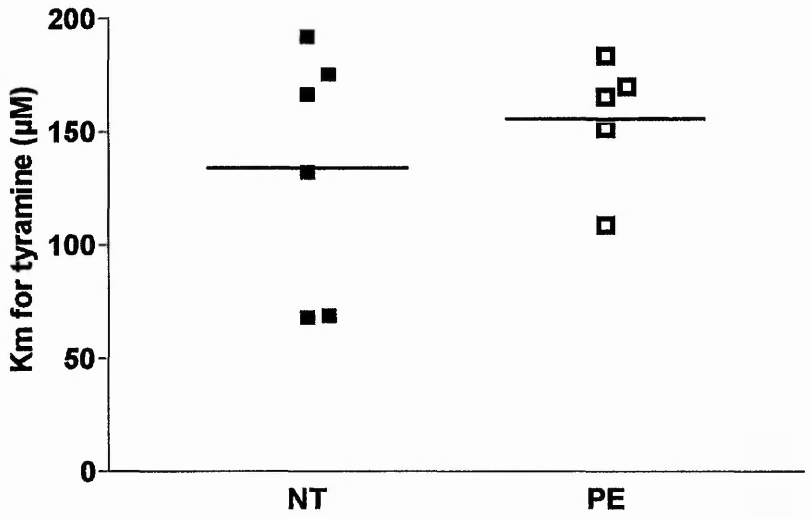


Figure 5.12 Km comparison for normotensive and pre-eclamptic samples

Km values for MAO-A using tyramine as substrate were calculated from Eadie-Hofstee plots. Normotensive and pre-eclamptic placental samples were compared. Statistical significance was accepted at $p < 0.05$ (*) and $p < 0.01$ (**).

NT = normotensive, PE = pre-eclamptic

5.3 *Discussion*

5.3.1 5-HT levels in pre-eclampsia

Since 5-HT and 5-HIAA levels in the amniotic fluid are higher during labour compared to the levels present before the onset of labour (Jones and Pycock, 1978), 5-HT is suggested to have a role during labour. As indicated earlier, in addition to its possible role during parturition, 5-HT has mitogenic effects on placental (Fecteau and Eiler, 2001; Sonier *et al.*, 2005) and foetal tissues (Lauder, 1990; Whitaker-Azmitia *et al.*, 1996; Butkevich *et al.*, 2003). Therefore it is important for foetal development. However, high levels of 5-HT have been demonstrated to have a deleterious effect on pregnant animals and their foetuses. Animal studies suggest that the foetus is very tolerant to 5-HT, which is in line with its mitogenic effects. Indeed injecting the rat foetus directly with 5-HT was well tolerated (Robson and Sullivan, 1966). However injecting mothers with 5-HT resulted in the death of the foetus, usually in less than one hour (Robson and Sullivan, 1966). This indicated that 5-HT did not have a direct toxic affect on the foetus but an action through the mother. This is thought to be a result of deterioration of foetal nutrition and oxygen availability (Robson and Sullivan, 1966). Interestingly, in a recent study carried out on pregnant rats, administration of 5-hydroxytryptophan (5-HTP) a serotonin precursor, lowered plasma volume and increased urinary protein levels; whilst this effect was not observed in non-pregnant animals (Salas *et al.*, 2007) . In addition both placental and foetal weights were reduced (Salas *et al.*, 2007). This finding further supports the importance of balanced 5-HT levels during pregnancy and also the possible role of 5-HT in complications of pregnancy such as pre-eclampsia.

As discussed earlier increased levels of 5-HT have been reported in pre-eclampsia (Middelkoop *et al.*, 1993; Gujrati *et al.*, 1996; Carrasco *et al.*, 1998; Laskowska *et al.*, 2001). However, since 5-HT levels appear to vary between subgroups of pre-eclamptic patients (Backe *et al.*, 1997), it was necessary to define 5-HT levels in the patients used for this study. In addition it was important to study possible differences in 5-HT turnover in pre-eclamptic patients in comparison to normotensive controls.

Preliminary experiments carried out using blood samples of non-pregnant female subjects showed no significant variation in platelet or PPP 5-HT levels during the day. Although this study involved only a small number of subjects this finding was in agreement with earlier studies (Eynard *et al.*, 1993). In addition platelet and PPP 5-HT levels found in this study were in good agreement with reported 5-HT levels for healthy subjects (Table 5.3), confirming the reliability of the blood preparation and 5-HT measurement methods used.

5-HT in platelets (ng / 10^9 platelets)	5-HT in circulation (PPP) (ng / ml)	n	Reference
414 ± 18		36	(Meyerhoff and Dorsch, 1981)
605 ± 17		68	(Jernej *et al.*, 2000)
420.0 ± 31.6	2.97 ± 0.4	8	(Carrasco *et al.*, 1998)
228 - 1783	0.2 – 9.6	28	(Kumar *et al.*, 1990)
488.2 ± 57.4 (am) 495.8 ± 62.5 (midday) 519.5 ± 79.20 (pm)	2.582 ± 0.14 (am) 2.311 ± 0.17 (midday) 2.614 ± 0.20 (pm)	4	This study

Table 5.3 Platelet and Platelet poor plasma (PPP) 5-HT levels

n = number of subjects involved in the study

Platelet and PPP 5-HT levels were compared in normotensive and pre-eclamptic maternal blood (Figure 5.3 and Figure 5.4). Since some pre-eclamptic women were medicated whilst others were only bed rested, the pre-eclamptic patient group was further divided into two subgroups. Platelet numbers did not vary significantly between these groups. Pre-eclamptic patients who had only bed rest had significantly higher circulating (PPP) 5-HT levels compared to normotensive controls whilst the medicated group had similar circulating 5-HT level to normotensives (Figure 5.4 b). The opposite was observed for platelet 5-HT levels. In this case platelet 5-HT levels were significantly higher in the medicated group compared to normotensives (Figure 5.3 b). Interestingly a study by Carrasco *et al.* (1998), which included blood samples collected before any treatment was commenced, reported similar platelet 5-HT levels in normotensive and pre-eclamptic patients and increased circulating 5-HT levels in the pre-eclamptic group. Clearly non-medicated pre-eclamptic patients would be better representative of the disease, however due to time constraints it was not possible to exclude medicated pre-eclamptic patients from this study. Although number of samples was limited, it could be suggested that platelet 5-HT levels were higher in more severe cases of pre-eclampsia (needing medication) and medication appears to prevent release of 5-HT from platelets resulting in lower circulating (PPP) 5-HT levels (compared to bed rested patients). However, different anti-hypertensive drugs with different mechanisms of action were used in the treatment of the patients (calcium channel antagonist nifedipine, α / β blocker labetalol, vasodilator hydralazine with unknown mechanism, centrally acting α_2 agonist methyldopa) therefore it is not possible to conclude their mode of action on platelet and PPP 5-HT levels.

5-HT concentrations in placenta and cord blood PPP fractions from normotensive and pre-eclamptic subjects were also investigated. Compared to maternal blood samples, cord and placental blood samples had greater variance. This is possibly due to activation of the platelets during blood collection. Nevertheless the same trend as for maternal blood was observed. Placental and cord PPP 5-HT levels were all higher in the non-treated (bed rested pre-eclamptic patients) group compared to normotensive controls although these differences were not statistically significant (Table 5.1).

5.3.2 5-HT turnover and MAO-A activity in pre-eclampsia

5-HT and its metabolite 5-HIAA were determined in urine of normotensive and pre-eclamptic women (Figure 5.6 and Table 5.2). Pre-eclamptic women had significantly higher 5-HT in their urine while its metabolite 5-HIAA levels were not changed. Thus the 5-HIAA: 5-HT ratio was significantly lower in pre-eclamptic women compared to normotensive controls, suggesting reduced 5-HT turnover. Vural *et al.* (1999) has also reported increased urinary 5-HT levels in pre-eclamptic women. On the other hand 5-HIAA levels were found to be increased by pre-eclampsia in some studies (Filshie *et al.*, 1992) but decreased in others (Vural *et al.*, 1999). In this study 5-HIAA levels were found to be similar in normotensive and pre-eclamptic samples. Although not statistically significant, placental MAO-A activity was lower in the pre-eclamptic group. It should be noted that in pre-eclampsia the transport and uptake of 5-HT into the placenta is not affected (Carrasco *et al.*, 2000b) but increased 5-HT levels have been reported in placental tissue (Gujrati *et al.*, 1996). Therefore it is likely that the increased levels of 5-HT in PPP and urine are due to a reduction in 5-HT turnover via MAO-A activity. Indeed previous studies in our laboratory showed that MAO-A activity was reduced in pre-eclamptic placenta although the number of MAO-A molecules / mg placental tissue was similar in normotensoive and pre-eclamptic placenta. This suggested that the catalytic turnover of the enzyme is reduced in pre-eclampsia (Sivasubramaniam *et al.*, 2002). Such a reduction could be result of endogenous inhibitors or some damage to the enzyme due to conditions present in pre-eclamptic placenta. The presence of possible endogenous inhibitors of MAO-A activity was investigated by mixing extracts of normotensive and pre-eclamptic placentae but none were revealed (Sivasubramaniam *et al.*, 2002). In the present work MAO-A from normotensive and pre-eclamptic placentae had similar Km values for tyramine (Figure 5.12) indicating that the affinity of the enzyme for its substrate is unaffected by pre-eclampsia. In turn this suggests that in pre-eclamptic placentae some of the MAO-A protein are inactive, whilst those that are active have a similar affinity for substrate to MAO-A in normotensive placenta. Therefore reduced MAO-A activity could be the result of damage a proportion of the MAO-A molecules. Possible oxidative and / or nitrative damage to MAO-A is further discussed in chapter VI.

Finally it was demonstrated that:

- Circulating 5-HT levels in non-medicated pre-eclamptic women were increased compared to normotensives,
- Urinary 5-HT levels were increased in pre-eclamptic women,
- Placental MAO-A activity was reduced in pre-eclamptic placenta (although not significant)

Since all these findings are in good agreement with previous studies, this clearly indicates that the pre-eclamptic group included in present study was representative.

CHAPTER VI

PRELIMINARY ANALYSIS OF THE EFFECTS OF OXIDATIVE STRESS AND HYPOXIA ON MAO-A ACTIVITY AND 5-HT_{2A} RECEPTOR EXPRESSION

6 CHAPTER VI Preliminary analysis of the effects of oxidative stress and hypoxia on MAO-A activity and 5-HT_{2A} receptor expression

6.1 Introduction

6.1.1 Oxidative stress

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are products of metabolism in cells under normal physiological conditions. However, ROS and RNS are also known to be involved in a wide variety of pathological conditions and induce oxidative and nitrative stress (Burdon, 1995). Mitochondria are a significant source of ROS through electron leakage from the electron transport chain (Jezek and Hlavata, 2005). In addition enzymes such as NADPH oxidase and xanthine oxidase can both contribute to superoxide anion generation (Granger, 1988; Griendling *et al.*, 2000). Xanthine oxidase exists in two inter-convertible forms; an oxidase (capable of reacting with oxygen) and a dehydrogenase (utilizing nicotinamide adenine dinucleotide, NAD, as a co-factor [Nishino *et al.*, 2005]). In normal conditions the enzyme exist mainly as xanthine dehydrogenase (XDH) which displays high xanthine / NAD reductase activity and a low xanthine / O_2 reductase activity even in the presence of O_2 (Della Corte and Stirpe, 1968). Both forms convert hypoxanthine to xanthine and xanthine to urate. Hypoxia-reoxygenation is thought to contribute to conversion of the XDH to the XO form (resulting in increased superoxide production) and also contributes to the inactivation of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase (Parks and Granger, 1983; Gonzalez-Flecha *et al.*, 1993).

ROS can modify cell function by damaging all types of biomolecules such as proteins, lipids or DNA. The oxidative modifications of proteins can occur at the polypeptide backbone (peptide bond cleavage) and at the amino acid side chain (reviewed in Berlett and Stadtman, 1997). Oxidative modifications of side chains differ with different amino acids. Although the side chains of arginine, lysine, proline, histidine, tryptophan, and tyrosine are known to be oxidatively modified, the sulphur containing amino acids cysteine and methionine are especially sensitive to

oxidation (reviewed in Berlett and Stadtman, 1997). Hydrogen peroxide is demonstrated to react with cysteine and methionine (Hampton and Orrenius, 1997). Oxidation of methionine residues by the addition of an extra oxygen atom results in methionine sulfoxide. Oxidation of both cysteine and methionine can be reversed since oxidised cysteine and methionine can be converted back to their unmodified forms by the respective actions of disulfide reductases and methionine sulfoxide reductase (Figure 6.1) (Berlett and Stadtman, 1997; Finkel, 2000). Reversible oxidation of cysteine is suggested to work as a cellular redox sensor (Finkel, 2000) and in addition reversible oxidation of methionine may have an important antioxidant defence role (Levine *et al.*, 1999).

Similar to ROS, RNS can also modify cell function through nitration of cellular components, such as proteins, lipids or DNA (Droge, 2002). In proteins, most nitration reactions occur on a tyrosine residue to form chemically stable 3-nitrotyrosine (Figure 6.1). Nitration of tyrosine affects the maintenance of protein conformation and thus can alter a protein's function (Ara *et al.*, 1998; Eiserich *et al.*, 1999). Reactive oxygen and nitrogen species are thought to play an important role in the maintenance of vascular homeostasis as well as endothelial dysfunction (reviewed in Szocs, 2004).

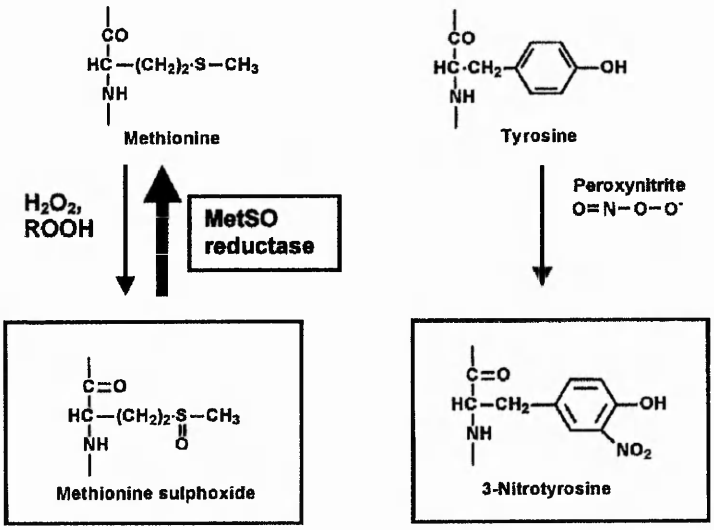


Figure 6.1 Oxidation products of methionine and tyrosine

Adapted from Ahmed and Thornalley (2003).

6.1.2 Oxidative stress and pre-eclampsia

There is now increasing evidence of the involvement of oxidative stress in the pathophsiology of pre-eclampsia even though its precise mechanism of action is not clear. A certain degree of oxidative stress (possibly due to hypoxia-reoxygenation) is observed in normal pregnancies but this appears to be exacerbated in pre-eclampsia (Hung and Burton, 2006). There are many reports of increased oxidative stress markers and decreased antioxidant capacity in pre-eclampsia. For example increased superoxide synthesis rates in placental tissue (Wang and Walsh, 2001) and increased XO activities in maternal, foetal plasma and placental tissues in pre-eclamptics (Many *et al.*, 2000; Yildirim *et al.*, 2004; Karabulut *et al.*, 2005). In addition increased levels of products of oxidative stress such as lipid oxidation products, including isoprostanes (Walsh *et al.*, 2000) and malondialdehyde (Gulmezoglu *et al.*, 1997), oxidative products of proteins, (such as protein carbonyls [Zusterzeel *et al.*, 2000]) and protein nitrative products such as nitrotyrosines (Myatt *et al.*, 1996; Many *et al.*, 2000) have been reported in pre-eclampsia. Moreover decreased antioxidant enzyme capacity and antioxidant molecule levels are documented in pre-eclamptic patients (Yanik *et al.*, 1999; Kharb, 2000; Vanderlelie *et al.*, 2005; Sharma *et al.*, 2006). Markers of oxidative and nitrative stress have been observed in the villous vascular endothelium and syncytiotrophoblasts (Myatt *et al.*, 1996; Many *et al.*, 2000; Hung *et al.*, 2001). Mitochondria of syncytiotrophoblast cells at early gestation are found to be especially susceptible to oxygen mediated damage (Watson *et al.*, 1998). Since MAO-A is located on the mitochondrial outer membrane, MAO-A in syncytiotrophoblast cells may be exposed to oxidative and nitrative stress and its reduced activity in pre-eclampsia may be due to such oxidative or nitrative damage.

In vitro cell culture studies are valuable since they allow the growth and maintainance of cells under desired experimental conditions. In addition to placental villous explants and primary cell cultures, trophoblast cell lines derived from normal and choriocarcinoma cells have been extensively used in placental research (Liu *et al.*, 1997; Bhat and Anderson, 2007; Hu *et al.*, 2007). Thus an *in vitro* model would

be valuable to study the status of both the MAO-A enzyme and 5-HT_{2A} receptor expression under conditions that are thought to exist in pre-eclampsia.

Two human placental cell lines namely, 3A Sub E and BeWo were used. The cell line 3A Sub E was established by the transformation of placental cells by temperature sensitive A (*tsA*) mutants of simian virus 40 (SV 40) (Chou, 1978). At permissible temperature (33-37 °C) this clone can be propagated whilst at the restrictive temperature (40 °C) the cells are reported to regain their normal trophoblastic behaviour secreting hCG and other proteins expressed by term placental cells *in vivo* (Chou, 1978). The second cell line, BeWo, was derived from a malignant gestational choriocarcinoma of the placenta (Pattillo and Gey, 1968). Choriocarcinoma cells were transplanted to the cheek pouch of the hamster, after maintaining through serial transfers a tumor was removed from the cheek pouch and established *in vitro* (Pattillo and Gey, 1968). Since then BeWo cells have been extensively used for placental research (Liu *et al.*, 1997; Bhat and Anderson, 2007; Hu *et al.*, 2007).

This chapter specifically aims to investigate the effects of oxidative and nitrative stress on MAO-A activity and also to investigate any potential effects of hypoxia and oxidative stress on 5-HT_{2A} receptor protein expression in these cell lines.

MAO proteins were analysed using immunocytochemistry and dot blotting. Levels of 5-HT_{2A} receptor proteins were analysed using western blotting. Since the 5-HT_2 receptors (5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}) are coupled to the activation of phospholipase C-β / protein kinase C, indicating a coupling to Gq protein (Hoyer *et al.*, 2002), the presence of functional receptors was investigated by measuring inositol phosphate accumulation in response to 5-HT treatment. Receptor levels were investigated under hypoxia and oxidative stress. In line with other studies, 20 % oxygen was used for controls and 2 % oxygen was used to create a hypoxic environment (Strohmer *et al.*, 1997; Hu *et al.*, 2007). Oxidative stress was generated employing a xanthine / xanthine oxidase system. The possible effects of serum withdrawal under these conditions were also investigated.

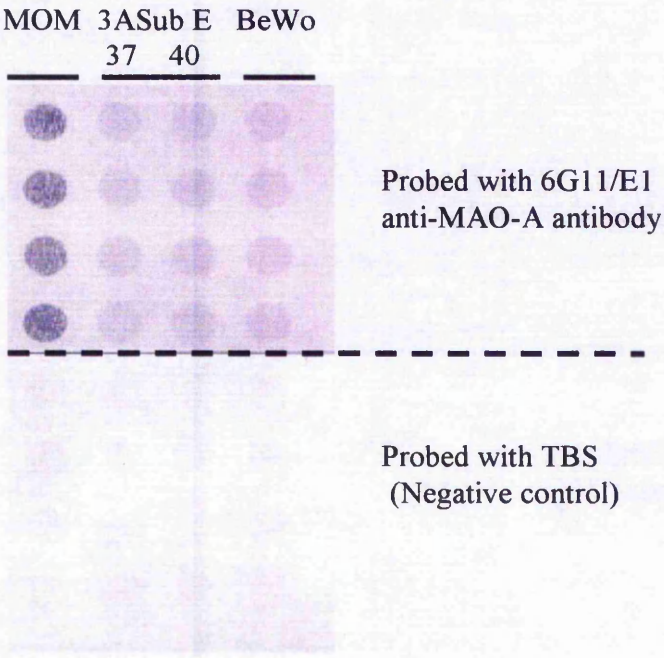
6.2 Results

6.2.1 Partial characterisation of placental cell lines

Initial studies concentrated on partial characterisation of the two placental cell lines, 3A Sub E and BeWo in terms of expression of MAO-A and MAO-B proteins and human chorionic gonadotropin secretion.

6.2.1.1 Analysis of placental cell lines for MAO protein expression

Human placental cell lines 3A Sub E and BeWo were analysed for MAO-A and MAO-B protein expression using dot blotting and immunocytochemistry techniques. 3A Sub E cells were grown at both 37 °C and 40 °C in order to reveal any potential differences at these growing conditions. Cell extracts from both cell lines were assayed for their MAO-A and MAO-B protein expression using the dot blot assay (section 2.2.14). In addition both cell lines were grown and fixed on microscope slides for immunocytochemical analysis (section 2.2.15.1). As a positive control for the dot blot assays human liver outer mitochondrial membranes were used, whilst human liver sections were used as a positive control for the immunocytochemistry. Human liver is known to contain high levels of both MAO-A and MAO-B (reviewed in Billett, 2004). These analyses revealed that both cell lines express low levels of MAO-A protein (Figure 6.2 and Figure 6.3). Immunocytochemistry analysis revealed a punctuate staining within the cytoplasm; MAO-A expression in 3A Sub E cells was similar at 37 °C and 40 °C. Neither cell line however expressed MAO-B protein (Figure 6.4 and Figure 6.5). In Figure 6.5 it is clear that only haematoxylin staining of the nucleus is evident in the cells. However, the liver section is strongly positive for MAO-B protein.



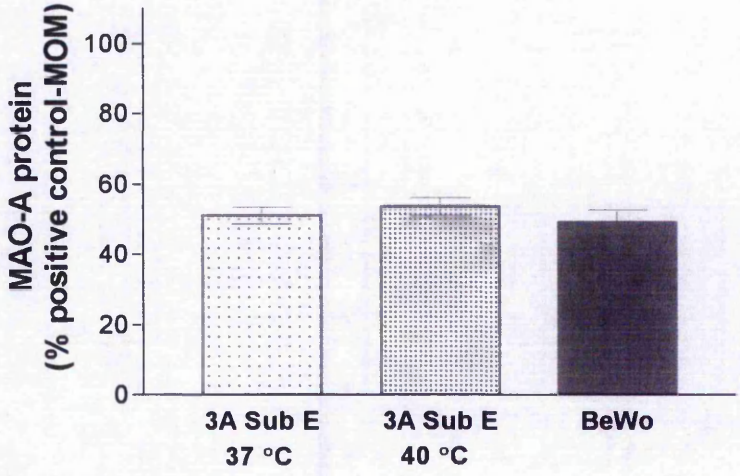


Figure 6.2 Dot blot analysis of 3A Sub E and BeWo cell lines for MAO-A protein expression

3A Sub E and BeWo cells were grown, and extracted as described in sections 2.2.6.1 and 2.2.6.8. Shown are dot blots of extracts containing 2 μg of protein / well. Upper panel is probed with 6G11 / E1 anti-MAO-A antibody (hybridoma tissue culture supernatant, undiluted) and lower panel is incubated with TBS as no primary antibody control. Histogram shows relative MAO-A protein expression in cell lines, represented as mean % of positive control (MOM).

3A Sub E 37 = cells grown at 37 °C, 3A Sub E 40 = cells grown at 40 °C,

MOM = mitochondrial outer membranes from human liver as positive control.

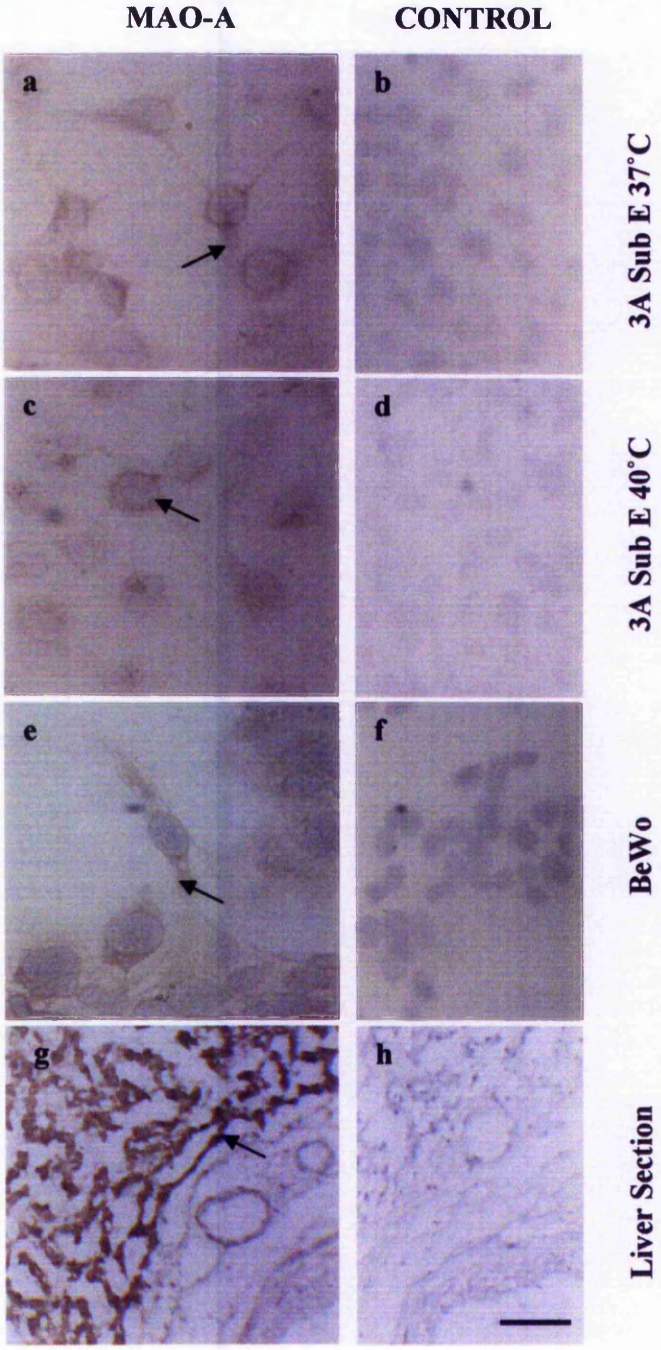


Figure 6.3 Immunocytochemistry with cell lines for MAO-A protein analysis

Cells were seeded at 200,000 cells /slide and fixed when reached ~ 60 % confluency. Cells were permeabilised and immunocytochemistry performed as described in section 2.2.15.2. Human liver section used as a positive control (panels g and h). In panels Plates a, c, e and g cells were probed with 6G11 / E1 anti-MAO-A antibody; panels b, d, f and h (controls) were probed with hybridoma growth medium. Panels a, c and e demonstrate MAO-A protein is expressed by cell lines 3A Sub E grown at 37°C, 3A Sub E grown at 40°C and BeWo respectively. Arrows show the staining for MAO-A. Magnification X 200, bar = 20μm.

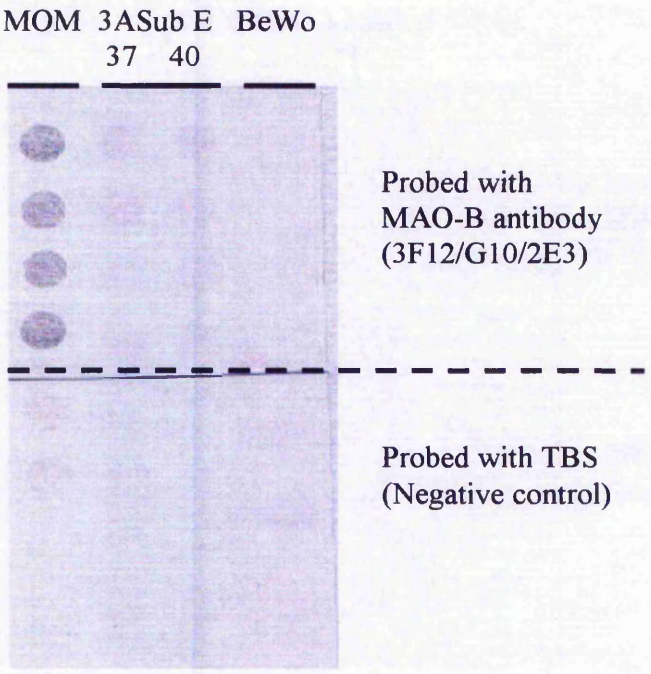


Figure 6.4 Dot blot analysis of 3A Sub E and BeWo cell lines for MAO-B protein expression

3A Sub E and BeWo cells were grown, and extracted as described in section 2.2.6.1 and 2.2.6.8. Shown are the dot blots of extracts containing 2 μg of protein / well. Upper panel is probed with clone 3F12/G10/2E3 anti-MAO-B antibody (hybridoma tissue culture supernatant, undiluted) and lower panel is incubated with TBS as no primary antibody control.

3A Sub E 37 = cells grown at 37 °C, 3A Sub E 40 = cells grown at 40 °C,

MOM = mitochondrial outer membranes from human liver as positive control.

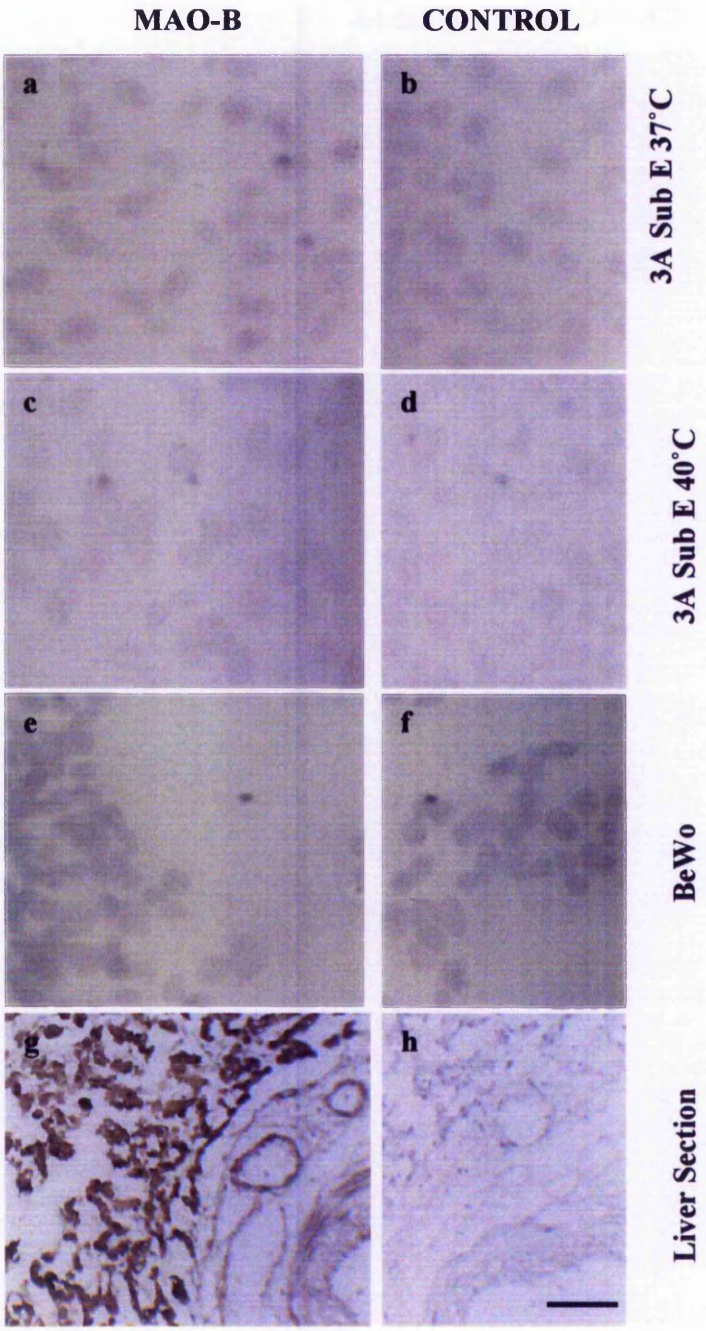


Figure 6.5 Immunocytochemistry with cell lines for MAO-B protein analysis

Cells were seeded at 200,000 cells /slide and fixed when reached ~ 60 % confluency. Cells were permeabilised and immunocytochemistry performed as described in section 2.2.15.2. Human liver section used as a positive control (panels g and h). In panels Plates a, c, e and g cells were probed with 3F12/G10/2E3 anti-MAO-B antibody; panels b, d, f and h (controls) were probed with hybridoma growth medium. Panels a, c and e demonstrate MAO-B protein is not expressed by cell lines 3A Sub E grown at 37°C, 3A Sub E grown at 40°C and BeWo respectively. Magnification X 200, bar = 20μm.

6.2.1.2 MAO-A activity

MAO-A activity in both BeWo and 3A Sub E placental cell lines was measured using tyramine 7- ^{14}C as a substrate. Despite optimising the assay and changing cell numbers, time of incubation and specific activity of the substrate, activity levels were too low to be detected in both cell lines.

6.2.1.3 Human chorionic gonadotropin (hCG) secretion

Both placental cell lines were assessed for their human chorionic gonadotropin secretion into the growth medium during culturing. Figure 6.6 shows that the BeWo cell line secreted hCG during 72 hours of culturing. No detectable levels of hCG were secreted by the 3A Sub E cell line grown at 37 °C and 40 °C (results not shown). Based on these findings the BeWo cell line was used for further analysis.

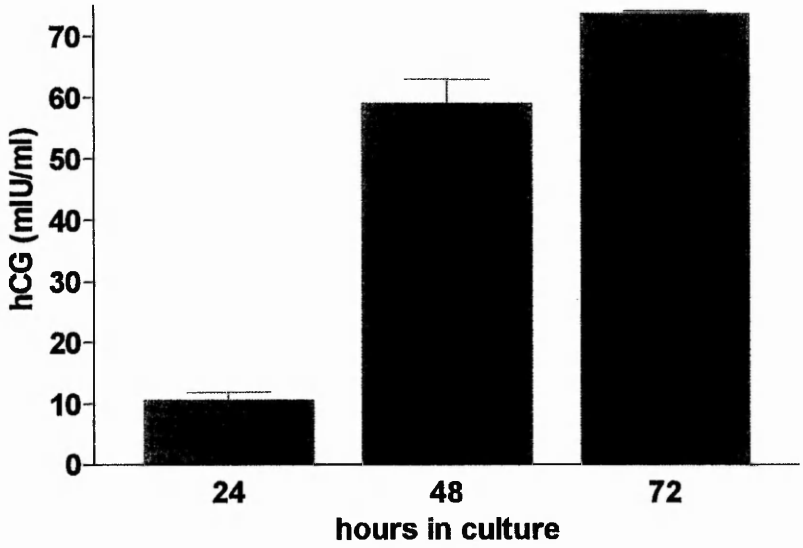


Figure 6.6 BeWo cell line hCG measurements over 72 hour in culture.

Cells were seeded at 500,000 cells in a T25 flask, and media samples collected after 24, 48 and 72 hours and assayed for hCG content; fresh growth medium served as a blank (section 2.2.8). Results are expressed as mean ± SEM of two independent experiments.

6.2.2 Effects of oxidative stress on MAO-A activity

Since MAO-A activity was too low to be estimated in both cell lines human recombinant MAO-A was used in order to investigate the effects of oxidative stress on enzyme activity. This recombinant protein was commercially available and expressed using the baculovirus–insect cell system. The microsomal product was obtained from insect cells (BTI-TN-5B1-4) infected with baculovirus containing cDNA inserts for human MAO-A. This system also allows post-translational processing and modifications of the recombinant protein. Oxidative stress was generated using either hydrogen peroxide or peroxynitrite. Initial experiments concentrated on optimising MAO-A activity assay for the recombinant enzyme.

6.2.2.1 Recombinant MAO-A activity assay optimisation

Activity assays were usually performed in 20 mM potassium phosphate buffer but, in order to assay MAO-A activity after treatment with peroxynitrite, increased buffering capacity was needed. Peroxynitrite is stable only in basic solutions (pH > 9) (Beckman *et al.*, 1990). Thus MAO-A activity was undertaken in 50 mM HEPES buffer pH 7.4. Degradation of peroxynitrite in the assay buffer was confirmed by measuring its concentration spectrophotometrically, peroxynitrite degraded to background levels in about 30 seconds (Figure 6.7).

A range of protein concentrations (0.25-2 μg/assay) and substrate incubation times (5-50 minutes) were investigated. Activity was linear up to 2 μg protein / assay (r^2 = 0.9951) and linear for at least 50 minutes incubation time (r^2 = 0.9928) when 1 μg protein / assay was used (Figure 6.8). Based on these findings 1 μg of protein and 20 minutes of incubation time were used for subsequent analysis. Figure 6.8c demonstrates that 1μM clorgyline (a MAO-A inhibitor at μM concentrations) completely abolished enzyme activity, as expected.

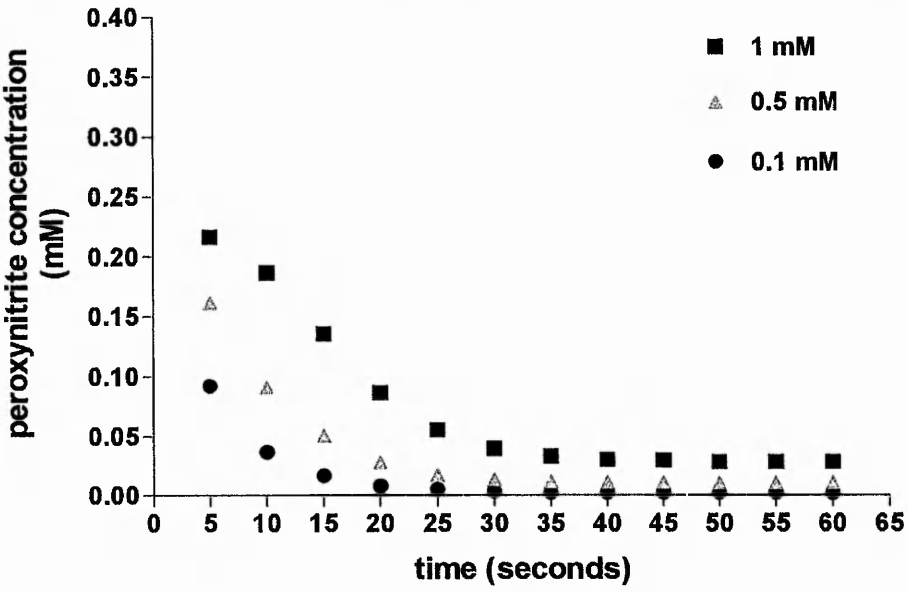


Figure 6.7 Peroxynitrite degradation in MAO-A activity assay buffer

Degradation of peroxynitrite in MAO-A activity assay buffer (50 mM HEPES buffer, pH: 7.4) determined by measuring its concentration spectrophotometrically at 302 nm as described in section 2.2.10.1. Peroxynitrite solution was prepared in 0.45 M NaOH and diluted in assay buffer to final concentration of 0.1, 0.5 and 1 mM. Change in peroxynitrite concentration was determined over 1 minute every 5 seconds.

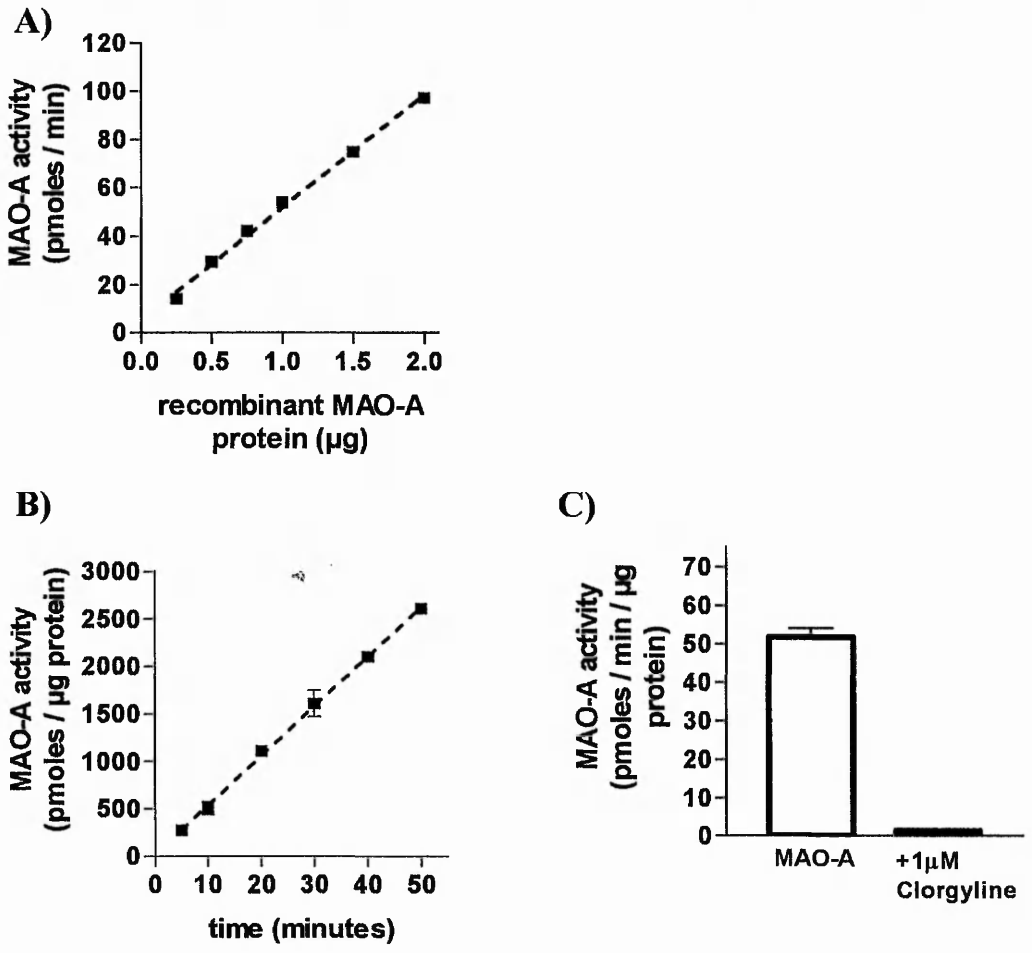


Figure 6.8 Recombinant MAO-A activity assay optimisation

Recombinant MAO-A activity was determined using 50 mM HEPES buffer (pH 7.4) as detailed in section 2.2.4.

A) Recombinant MAO-A activity at increasing protein concentration (0.25-2 µg/assay). Enzyme activity is expressed as pmoles/minutes, (*n=2*).

B) Recombinant MAO-A activity (1 µg/assay) at increasing substrate incubation time (5-50 minutes). Enzyme activity is expressed as pmoles/µg protein, (*n=2*).

C) Recombinant MAO-A activity was determined in the presence of the MAO-A-specific inhibitor clorgyline. Enzyme was re-suspended in assay buffer and incubated with clorgyline (1 µM final concentration in assay) or only assay buffer (as control) for 15 minutes at 37 °C prior to incubation with the substrate. Enzyme activity is expressed as pmoles/minute/µg protein, (*n=3*).

6.2.2.2 Effect of H_2O_2 treatment on MAO-A activity

The effect of H_2O_2 treatment on MAO-A activity was assessed by pre-treating recombinant protein with a range of H_2O_2 concentrations (1 μM - 10 mM) prior to activity assay. Only at higher concentrations of H_2O_2 (10 mM) was enzyme activity significantly reduced compared to control levels.

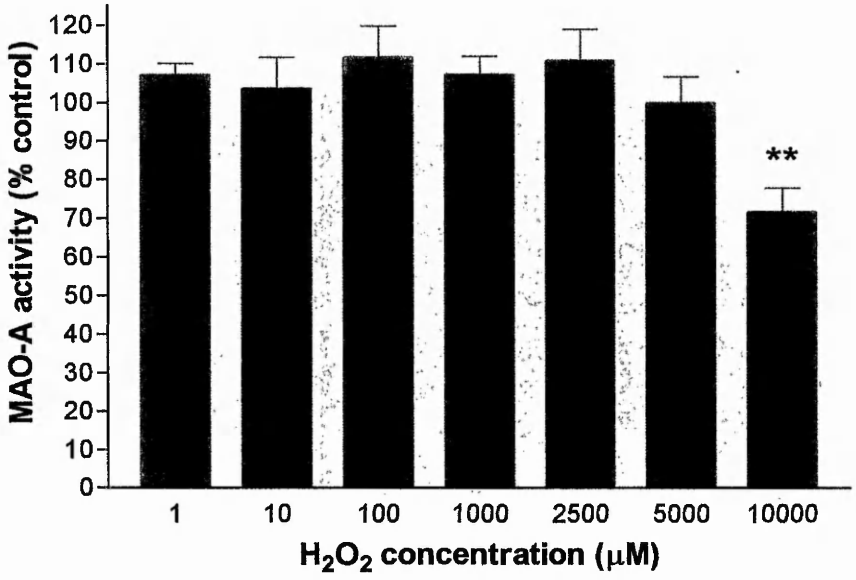


Figure 6.9 Effect of H_2O_2 treatment on MAO-A activity

Recombinant MAO-A activity was determined in the presence of H_2O_2. Enzyme was re-suspended in assay buffer and incubated with a range of H_2O_2 (1 μM-10mM) or in assay buffer (as control) at 37 °C for 30 minutes prior to activity assay as detailed in section 2.2.10.3. Results are shown as mean % control ± SEM for three independent experiments (*n=3*). Statistical significance was accepted at $p<0.05$ (*) and $p<0.01$ (**).

6.2.2.3 Effect of H_2O_2 treatment on Lactate dehydrogenase activity

Since MAO-A activity was only affected at high concentrations of hydrogen peroxide, similar experimental conditions were tested with another enzyme as a positive control. Commercially available Lactate dehydrogenase (LDH) enzyme was used. LDH activity was assessed by pre-treating the enzyme with a range of H_2O_2 concentrations (1 µM - 10 mM) prior to activity assay. LDH activity was reduced in a dose dependent manner and this reduction was significant compared to control levels when 1 mM and higher concentrations of hydrogen peroxide used.

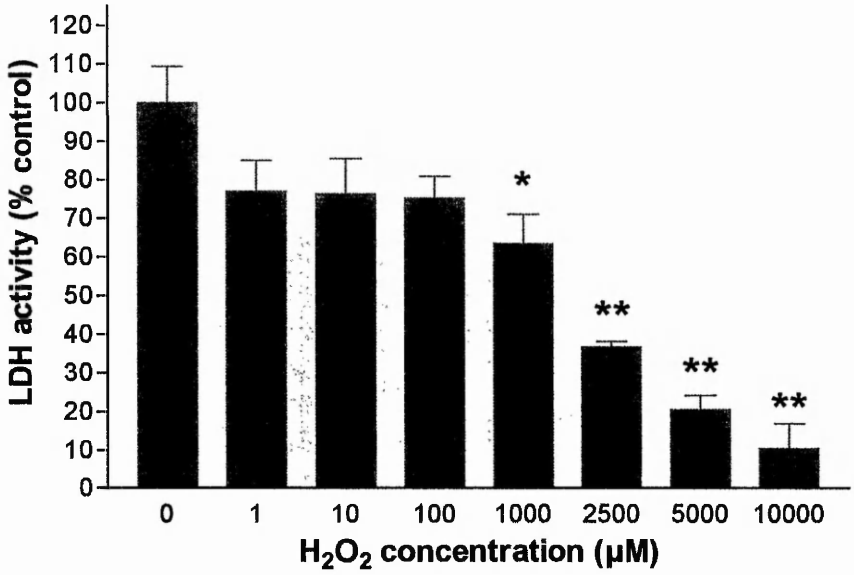


Figure 6.10 Effect of H_2O_2 treatment on LDH activity

LDH activity was determined in the presence of H_2O_2. Enzyme was re-suspended in assay buffer and incubated with a range of H_2O_2 (1 µM-10mM) or in assay buffer only (as control) at 37 °C for 30 minutes prior to activity assay as detailed in section 2.2.10.3. Results are shown as mean % control ± SEM for three independent experiments (*n=3*). Statistical significance was accepted at $p<0.05$ (*) and $p<0.01$ (**).

6.2.2.4 Effect of Peroxynitrite treatment on MAO-A activity

Experiments were performed to establish whether recombinant MAO-A could be nitrated *in vitro* by peroxynitrite. Western blot analysis using an anti-nitrotyrosine antibody revealed no reactivity with either untreated MAO-A (C) or with MAO-A treated with degraded peroxynitrite (D), but evidence of nitration following treatment with peroxynitrite (PN) (Figure 6.11 a). The blot was then stripped and probed for MAO-A protein (Figure 6.11 b) confirming position of MAO-A protein. MAO-A was not the main target for peroxynitrite as other less dominant proteins were more susceptible.

The effect of nitration on MAO-A activity was assessed by pre-treating recombinant protein with a range of peroxynitrite concentrations (0.1-1mM final concentration in assay) prior to activity assay. Incubation with peroxynitrite resulted in a concentration dependent decrease in the enzyme activity, which was significant at 1 mM (Figure 6.12).

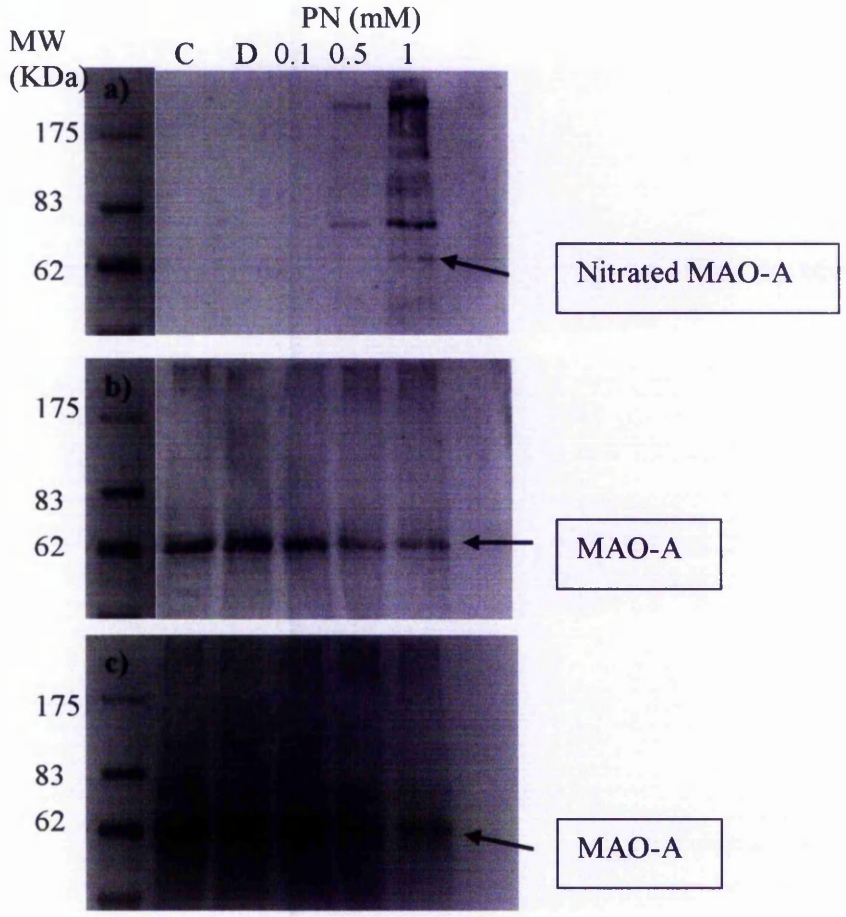


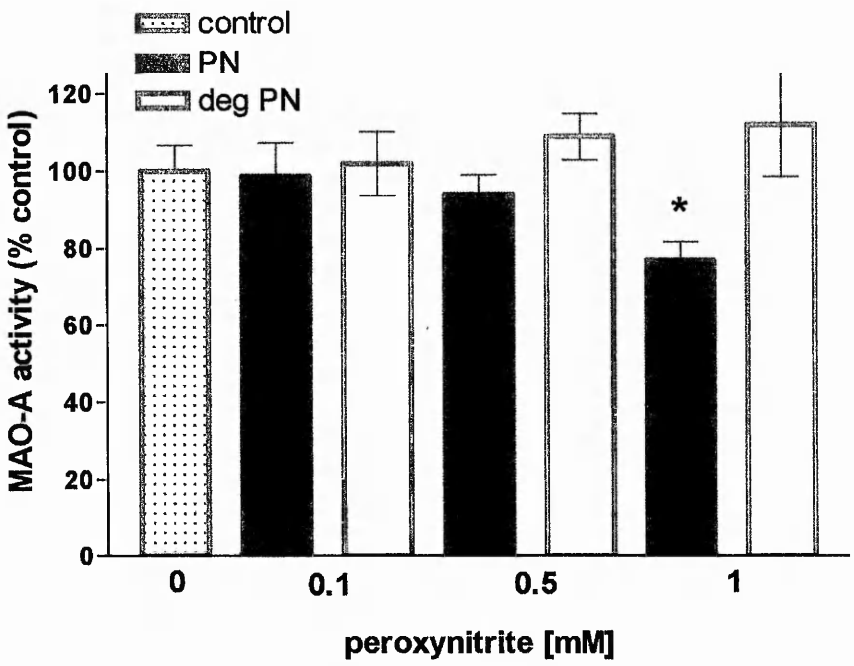
Figure 6.11 Western blot analysis of MAO-A nitration

a) Western blot determination of recombinant MAO-A nitration. Proteins (15 μg) were separated by SDS-PAGE and transferred electrophoretically on to nitrocellulose membrane. Blot was probed with anti-nitrotyrosine antibody HM11.

b) Immunoblot was stripped and reprobed with anti MAO-A antibody 6G11/E1. Primary antibody binding was detected by ECL as described in section 2.2.13.3.

c) Copper stained blot before immunoprobing

C=Control, D= degraded peroxynitrite, PN= peroxynitrite



	MAO-A activity (pmoles / min / µg) (Mean ± SEM)	
Peroxynitrite concentration (mM)	**peroxynitrite**	**Degraded peroxynitrite**
0.1	59.00 ± 6.42	63.83 ± 1.08
0.5	55.70 ± 1.59	68.25 ± 0.65
1	46.13 ± 5.11	69.92 ± 3.88

Figure 6.12 Effect of peroxynitrite treatment on MAO-A activity

Recombinant MAO-A activity was determined in the presence of peroxynitrite. Enzyme was re-suspended in assay buffer and incubated with peroxynitrite (0.1, 0.5 and 1 mM) or in assay buffer only (controls). A set of negative controls were also prepared, for this peroxynitrite (0.1. 0.5 and 1 mM) was added to the buffer 15 minutes before the addition of the enzyme in order to initiate degradation of peroxynitrite (degraded PN). All samples were incubated at 37 °C for 30 minutes and enzyme activity was determined as detailed in section 2.2.4. Results are shown as mean % control ± SEM and pmoles / min / µg protein for three independent experiments (*n=3*). Nitrated MAO-A activity at each concentration was compared to degraded peroxynitrite controls. Statistical significance was accepted at $p<0.05$ (*) and $p<0.01$ (**).

6.2.3 Effects of oxidative stress and hypoxia on $5HT_{2A}$ receptor protein expression in the BeWo cell line

6.2.3.1 5-HT_{2A} receptor protein expression

Western blotting analysis of BeWo cell extracts revealed a major protein band of approximately 63 kDa and a faint band of 55 kDa; in human platelets, on the other hand the 55 kDa band was most reactive whilst the 63 kDa band was very faint.

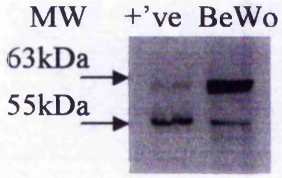


Figure 6.13 $5HT_{2A}$ receptor protein expression in the BeWo cell line

BeWo and platelet extracts (40 µg) were fractionated by SDS-PAGE, transferred electrophoretically on to nitrocellulose membrane and probed with anti-$5HT_{2A}$ mouse monoclonal antibody (clone G186-1117). A goat anti-mouse horseradish peroxidase conjugated secondary antibody was used as a secondary antibody (sections 2.2.12 and 2.2.13).

+'ve= positive control (human platelets).

6.2.3.2 Functionality of 5-HT$_2$ receptors expressed in BeWo cells

In order to assess the functionality of 5-HT$_2$ receptors expressed in BeWo cells, radiolabelled inositol phosphate accumulation in cells was measured. Following stimulation with 5-HT a dose dependent increase in radiolabelled inositol phosphate was observed in these cells, suggesting that the 5-HT$_2$ receptors are functional. EC_{50} value of 5.277 x10^{-11} M and Emax of 127.9 (% control) were found for 5-HT in this cell line.

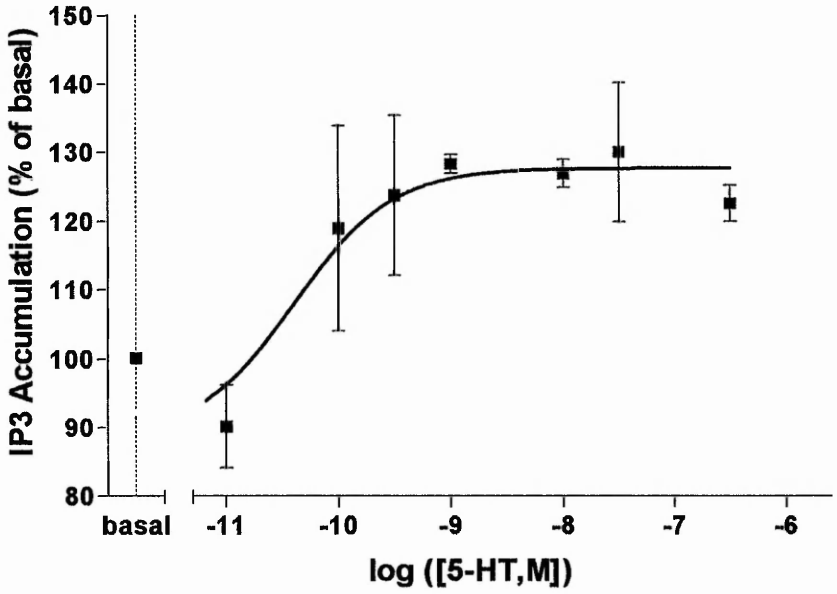


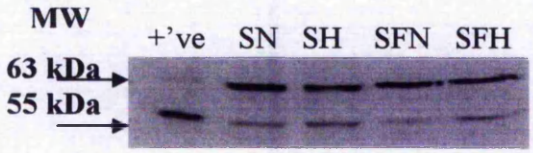
Figure 6.14 Measurement of inositol phosphate accumulation in the BeWo cell line

Each well in a 24 well plate was seeded with ~150,000 cells and inositol phosphate accumulation at increasing concentrations of 5-HT was determined as described in section 2.2.9. A concentration-response curve of three independent experiments is shown. Results are expressed as mean ± SEM of control (no 5-HT).

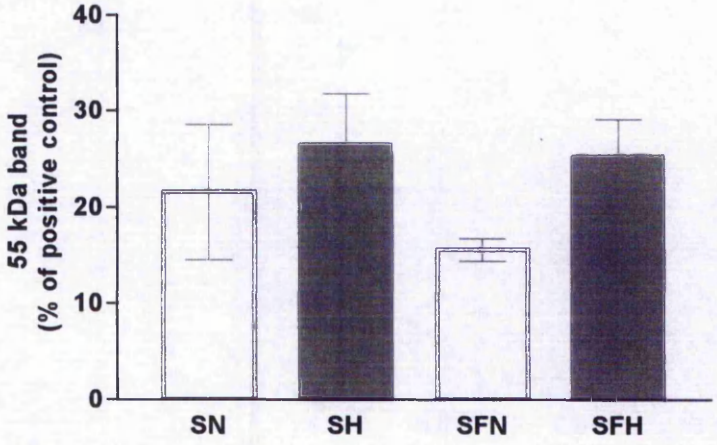
6.2.3.3 Effects of hypoxia on $5HT_{2A}$ receptor protein expression

Receptor levels of cells grown under normoxia (20 % O_2) and hypoxia (2 % O_2) in the presence (10 %) and absence of serum were compared by western blotting (Figure 6.15). Although not statistically significant the overall trend was for hypoxia to result in an increase in the 55 kDa band (panel a) and a decrease in the 63 kDa band (panel b) irrespective of serum; thus the 63 kDa / 55 kDa band ratio was reduced following hypoxia (panel c). Since the 63 kDa was dominant, hypoxia reduced overall receptor levels (panel d) but this was not significant.

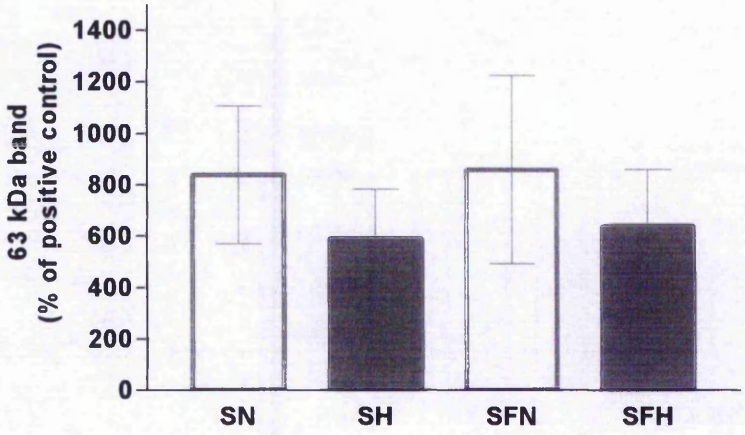
Attempts were made in order to assess possible changes in the receptor function under hypoxia but high cell death especially during radiolabelling and incubation with 5-HT resulted in a very high variability in the data. Since even under normal conditions Emax was low (Figure 6.14) it was not possible to measure receptor functionality under hypoxia reliably (data not shown).


MW
+'ve
SN
SH
SFN
SFH
63 kDa
55 kDa

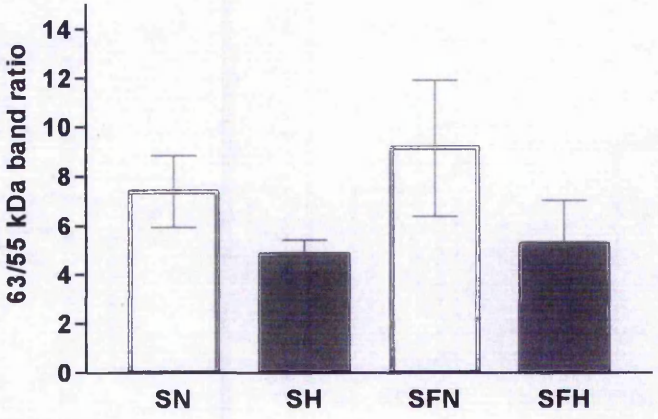
A)


55 kDa band
(% of positive control)
40
30
20
10
0
SN
SH
SFN
SFH

B)


63 kDa band
(% of positive control)
1400
1200
1000
800
600
400
200
0
SN
SH
SFN
SFH

C)


63/55 kDa band ratio
14
12
10
8
6
4
2
0
SN
SH
SFN
SFH

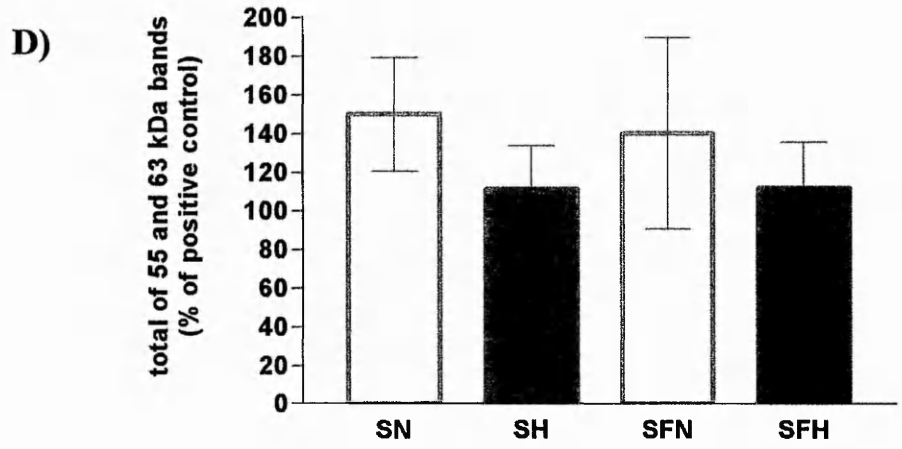


Figure 6.15 Western blot comparison of $5HT_{2A}$ receptor expression in BeWo cells under hypoxic and normoxic conditions.

Cells were seeded at 500,000 cells in T25 flasks, after 24 hours growth medium was removed and replaced with warmed fresh medium. Cells were extracted after a further 24 hours incubation in normoxic or hypoxic conditions +/- serum. Proteins (40 µg) were fractionated by SDS-PAGE and transferred electrophoretically on to nitrocellulose membrane. Cell and platelet extracts (as positive controls) were probed with anti-$5HT_{2A}$ mouse monoclonal antibody (clone G186-1117) followed by detection using goat anti-mouse horseradish peroxidase conjugated secondary antibody as described in section 2.2.13.3. Shown are representative blots from three independent experiments. Results are expressed as mean ± % positive control (platelet extracts) SEM (*n=3*). Statistical significance was accepted at $p<0.05$ (*) and $p<0.01$ (**).

A) 55 kDa band expression

B) 63 kDa band expression

C) 63 / 55 kDa band ratios

D) Total of 63 and 55 kDa bands

+'ve= Positive control (human platelets); SN= Normoxia (20 % oxygen) with serum (10 %) containing growth medium; SH= Hypoxia (2 % oxygen) with serum (10 %) containing growth medium; SFN= Normoxia (20 % oxygen) with serum free growth medium; SFH= Hypoxia (2 % oxygen) with serum free growth medium.

6.2.3.4 Effects of oxidative stress on $5HT_{2A}$ receptor protein expression

The free radical-generating system of xanthine (X) and xanthine oxidase (XO), which converts xanthine to uric acid liberating a combination of superoxide and H_2O_2 (Repine *et al.*, 1979), has been used in this study in order to induce oxidative stress. Confocal microscopy images (Figure 6.16) confirm the generation of ROS in BeWo cells after incubation with xanthine and xanthine oxidase (2.5 mU/ml).

Increasing ROS levels for 24 hours resulted in a slight increase in total $5HT_{2A}$ receptor levels (Figure 6.17 d) due to an increase in the dominant 63 kDa band (Figure 6.17 b); however this increase was not significant. Unlike hypoxia, ROS increased the ratio of the 63 kDa to 55 kDa band, but again this was not significant (Figure 6.17 c).

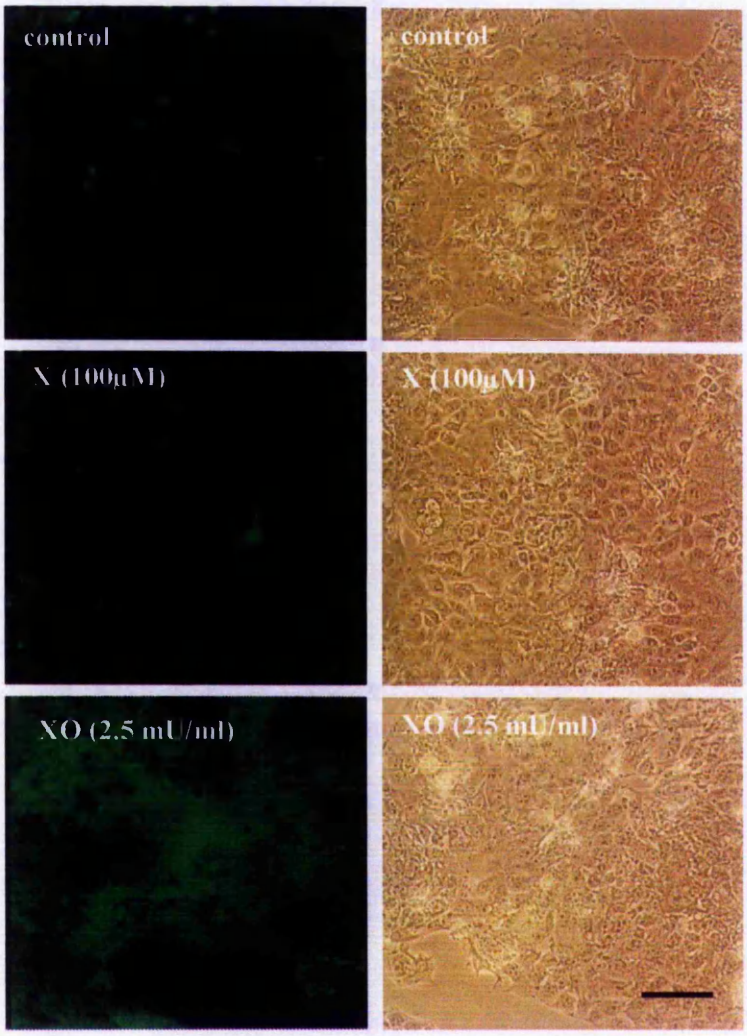
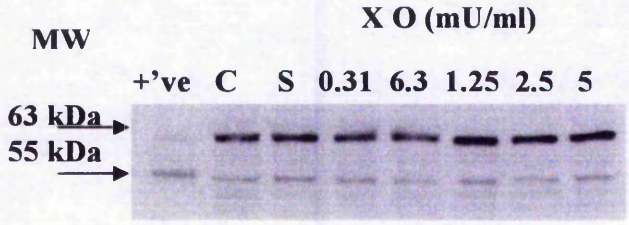


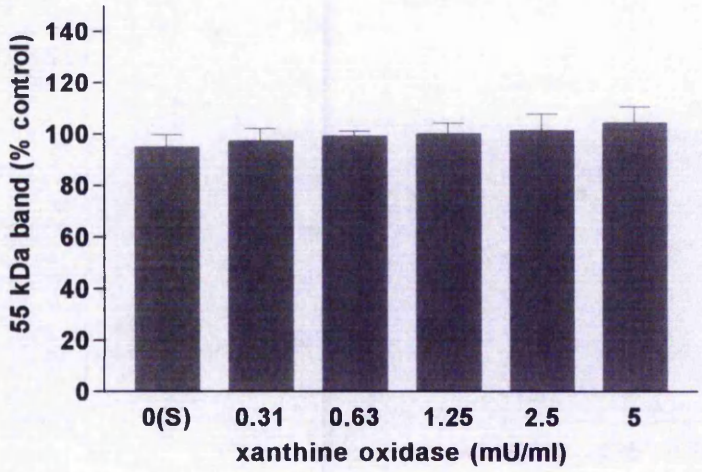
Figure 6.16 Generation of reactive oxygen species with xanthine and xanthine oxidase system

Cells were seeded at 10,000 cells/ chamber, and when they reached~ 60-70 % confluency, growth medium was removed and replaced with warmed fresh medium containing 100 μM xanthine (substrate) alone or xanthine plus xanthine oxidase (2.5 mU/ml) in serum free medium. Following 24 hours incubation, cells were incubated for a further 50 minutes with 2'-7'-dichlorodihydrofluorescein (DCDHF) which is converted to the fluorescent product 2'-7'-dichlorofluorescein (DCF) when oxidised. Confocal microscopy analysis was performed at x 200 magnification, scale bar represents 20 μm. Panels on the right demonstrates the cell morphology.

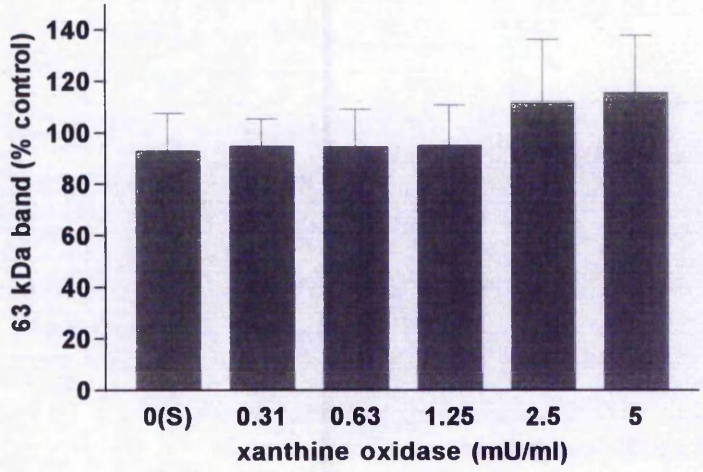
X = Xanthine, XO = Xanthine Oxidase.


X O (mU/ml)
MW
+'ve C S 0.31 6.3 1.25 2.5 5
63 kDa
55 kDa

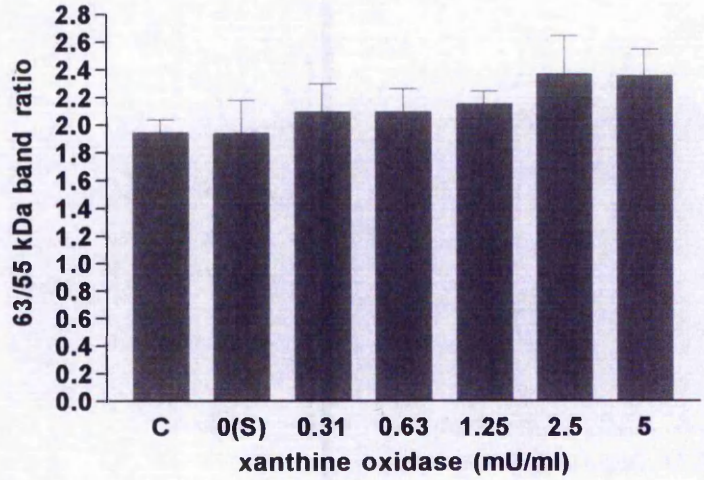
A)


55 kDa band (% control)
140
120
100
80
60
40
20
0
0(S) 0.31 0.63 1.25 2.5 5
xanthine oxidase (mU/ml)

B)


63 kDa band (% control)
140
120
100
80
60
40
20
0
0(S) 0.31 0.63 1.25 2.5 5
xanthine oxidase (mU/ml)

C)


63/55 kDa band ratio
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
C 0(S) 0.31 0.63 1.25 2.5 5
xanthine oxidase (mU/ml)

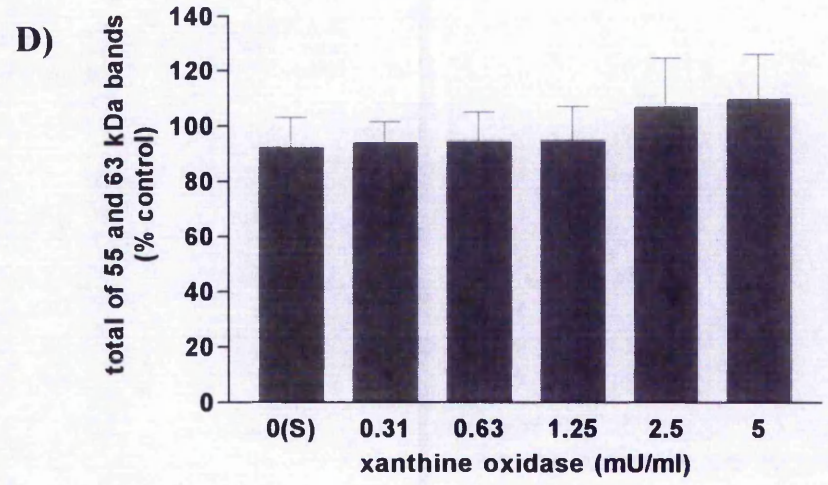


Figure 6.17 Western blot comparison of $5HT_{2A}$ receptor expression by BeWo cells under oxidative stress.

Cells were seeded at 500,000 cells in T25 flasks, after 24 hours growth medium was removed and replaced with warmed fresh medium containing 100 μM xanthine (subtrate) and increasing concentrations of xanthine oxidase in serum free medium. Cells were extracted after a further 24 hours incubation. Proteins (40 μg) were fractionated by SDS-PAGE and transferred electrophoretically on to nitrocellulose membrane. Cell and platelet extracts (as positive controls) probed with anti-$5HT_{2A}$ mouse monoclonal antibody (clone G186-1117) followed by goat anti-mouse horseradish peroxidase conjugated secondary antibody as described in section 2.2.13.3. Shown are representative blots from five independent experiments. Results are expressed as mean % control (no treatment) ±SEM (*n=5*). Statistical significance was accepted at $p<0.05$ (*) and $p<0.01$ (**).

A) 55 kDa band expression

B) 63 kDa band expression

C) 63 / 55 kDa band ratios

D) Total of 55 and 63 kDa bands

+'ve= Positive control (human platelets), C = control, S = Substrate (100μM xanthine as substrate only control)

6.3 Discussion

6.3.1 MAO protein expression in placental cell lines

Both cell lines expressed MAO-A protein but no MAO-B protein as determined by immnoblotting and immunocytochemistry (Figures 6.2, 6.3, 6.4 and 6.5). Immunocytochemistry for MAO-A protein showed a punctuate staining within the cytoplasm. It should be noted that human placenta express only MAO-A protein although both MAO-A and MAO-B mRNAs are present (Auda *et al.*, 1998; Sivasubramaniam *et al.*, 2002). Therefore it could be suggested that both cell lines shows characteristics of placental tissue expressing only the A isoform of MAO enzyme. However, despite many attempts and assay optimisation MAO-A activity was not at detectable levels in either cell line and only BeWo cells secreted hCG to the growth medium but no hCG was detected in 3A Sub E cells even at 40 °C. Therefore BeWo cell line was used for further analysis of 5-HT_{2A} receptor and human recombinant MAO-A enzyme was utilised for studying the effects of oxidative and nitrative stress on enzyme activity.

6.3.2 Effects of oxidative stress on MAO-A activity

In order to test the effects of oxidative stress on enzyme activity, MAO-A was treated with H_2O_2. *In vivo* hydrogen peroxide is generated mainly in the mitochondria by the action of superoxide dismutase enzyme on superoxide radical (Figure 1.6). In addition enzymes such as MAO also produce hydrogen peroxide as a catalytic product. In the presence of transition metal ions, for example iron, hydrogen peroxide can be converted into hydroxyl radical which is far more reactive than hydrogen peroxide itself and causes indiscriminative damage to biomolecules (reviewed in Halliwell *et al.*, 2000). However, as MAO produces hydrogen peroxide as a by-product of its activity, in this study only the direct effects of hydrogen peroxide on enzyme activity were investigated. Hydrogen peroxide levels in human blood are thought to be at micromolar levels, although varying amounts (0.25 - 9.09 μM) have been reported in different studies (Frei *et al.*, 1988; Varma and Devamanoharan, 1991; Deskur *et al.*, 1998; Lacy *et al.*, 1998). These differences are probably due to the health status of the subjects and the sensitivity / specificity of the

methods used for its detection. In this study a wide range of hydrogen peroxide concentrations (1μM – 10 mM) was tested, however only 10 mM reduced MAO-A activity significantly (28.5 %) whilst lower concentrations had no significant effect (Figure 6.9). Thus MAO-A is highly resistant to short term exposure to hydrogen peroxide. In order to test the experimental set-up another enzyme, Lactate dehydrogenase activity was also assessed under same conditions. Hydrogen peroxide reduced LDH activity in a dose dependent manner and significantly at 1mM, confirming MAO-A's high resistance to this oxidant.

There are 9 cysteine residues in human MAO-A (Bach *et al.*, 1988), one of which is linked to the FAD (Cys - 406), leaving 8 cysteine as free thiols (Weyler, 1989). Site-directed mutagenesis studies showed that only mutations on Cys-406 and –374 residues result in activity loss whilst mutations on the other cysteine residues do not alter catalytic activity of the enzyme (Wu *et al.*, 1993). This may partly explain why only very high concentrations of hydrogen peroxide altered the activity of the enzyme in this study. However, it is important to note that the environment of the recombinant enzyme would also affect its fate. Recombinant MAO-A was obtained from the microsomal fractions and microsomes are known to be rich in lipids. Since lipids are highly susceptible to oxidation, this lipid rich environment might also reduce the effects of hydrogen peroxide on MAO-A. It is therefore remains to be investigated whether in situ MAO-A enzyme activity, that is, MAO-A in its normal mitochondrial location, is resistant to exposure to hydrogen peroxide.

The effects of nitrative stress on MAO-A enzyme activity were investigated by treating the enzyme with peroxynitrite. *In vivo* peroxynitrite forms through the reaction of nitric oxide and superoxide in which the rate is limited only by diffusion (Radi *et al.*, 2001). The main degradation products of peroxynitrite include nitrite (NO_2^-) and nitrate (NO_3^-) (Radi *et al.*, 2001). In this study 0.1, 0.5 and 1 mM peroxynitrite was used as 1 mM peroxynitrite was used by many other studies (MacMillan-Crow and Thompson, 1999; Lanone *et al.*, 2002; Webster *et al.*, 2006) and it has been suggested that peroxynitrite concentration can reach up to 1mM / min *in vivo* (Ischiropoulos *et al.*, 1992). Since tyrosine residues are especially susceptible to nitration by peroxynitrite, nitration of the enzyme was confirmed by the western blotting detection of nitrotyrosine residues (Figure 6.11).

It was not a very strong reaction and MAO-A was not the main target since nitration of other less abundant proteins was observed. Nonetheless, nitration of the enzyme was detectable when 1 mM peroxynitrite was used. In addition nitration of the enzyme resulted in a dose dependent reduction in its activity and was significant with 1 mM peroxynitrite. A 35 % decrease in enzyme activity was observed compared to that of incubated with degraded peroxynitrite (as control). Nitration of many other proteins including manganese superoxide dismutase (Yamakura *et al.*, 1998), p38 mitogen activated protein kinase (MAPK), cytochrome P450 (Roberts *et al.*, 1998) sarcoplasmic reticulum Ca-ATPase (Viner *et al.*, 1996) to list a few have been reported. Protein nitration has different effects on different proteins. For example protein nitration resulted in inhibition of the protein function as observed in p38 MAPK (Webster *et al.*, 2006), cytochrome P450 (Roberts *et al.*, 1998) and superoxide dismutase (Yamakura *et al.*, 1998) or resulted in activation of the protein as in case of protein kinase epsilon (Balafanova *et al.*, 2002). In this study nitration of MAO-A protein resulted in reduced enzyme activity. In proteins nitration reactions mainly occur on tyrosine residues (Figure 6.1). There are two tyrosine residues (Tyr-407 and -444) in the active site of MAO-A which are shown to be very important, as mutations on these sites results in inactivation of the enzyme (Geha *et al.*, 2002). Nitration of these tyrosine residues might have caused the reduced activity observed in this study. Although the effects of hydrogen peroxide and peroxynitrite cannot be directly compared, it could be suggested that MAO-A enzyme activity was more susceptible to damage by peroxynitrite than hydrogen peroxide. This is also supported by site-directed mutagenesis studies detailed earlier, which suggests an important role for two tyrosine residues in the active site of the enzyme and limited contribution of cysteine residues to the activity of the enzyme. Since MAO generates hydrogen peroxide as a result of its own activity, oxidation of cysteine residues might serve as a defence mechanism for the enzyme. However it should be noted that hydrogen peroxide can contribute to the formation of highly reactive hydroxyl radical and this might also affect enzyme activity. Indeed it has been reported that exposure to hydroxyl ions results in 15-47 % reduction of MAO-A enzyme activity (Soto-Otero *et al.*, 2001).

In summary *in vitro* nitration of MAO-A resulted in decreased enzyme activity. It should be noted that increased protein nitration has been demonstrated in pre-eclampsia (Myatt *et al.*, 1996). Whether the reduced activity of placental MAO-A in pre-eclampsia is a result of such nitration of the tyrosine residues remains to be investigated.

6.3.3 Effects of oxidative stress and hypoxia on 5-HT_{2A} receptor

In the present study BeWo cells have been demonstrated to express 5-HT_{2A} receptors. Western blotting of BeWo cell extracts revealed a major protein band of approximately 63 kDa and a faint band of 55 kDa (Figure 6.13). The same protein bands were observed in human placental tissue extracts (see section 3.2.2). It has also been shown that the 5-HT_{2A} receptor expressed in this cell line is functional as demonstrated by inositol phosphate release upon stimulation with 5-HT (Figure 6.14). However, it should be noted that the techniques used do not assess whether both the 55 and 63 kDa proteins contribute to inositol phosphate release, i.e. whether only the 55 kDa protein is functional. Although oxidative stress has been generally accepted to play an important role in pre-eclampsia, the effects of oxidative stress and hypoxia on 5-HT_{2A} receptor protein expression is not known. Studies indicate that oxidative stress and hypoxia could alter receptor densities. Acute and chronic hypoxia have been demonstrated to modify β-adrenergic receptor (Voelkel *et al.*, 1981; Marsh and Sweeney, 1989) oxytocin receptor (Mlynarczyk *et al.*, 2003) and α1-adrenergic receptor expression (Ueno *et al.*, 1997). Moreover oxidative stress is thought to increase A_1 adenosine receptor expression (Nie *et al.*, 1998). Hypoxia could also affect 5-HT_{2A} receptors. Indeed exposure of ovine common carotid arteries to hypoxia has been demonstrated to significantly decrease 5-HT_{2A} receptor density (Angeles *et al.*, 2000).

In the present study oxidative stress (generated by the xanthine/ xanthine oxidase system) and hypoxia have been demonstrated to affect 5-HT_{2A} receptor protein expression in different ways. Hypoxia tended to increase 55 kDa protein levels whilst under oxidative stress 55 kDa protein expression did not change. In addition whilst the levels of the 63 kDa band tended to decrease under hypoxia, under oxidative stress they tended to increase. However none of these changes were statistically

significant. It should be noted that pre-eclampsia did not cause a significant change in receptor levels (section 4.2.4). However, it was not possible to study receptor function under hypoxia or oxidative stress conditions. Even under normal conditions Emax was low and there was great variably in the data (possibly due to high cell death during radiolabelling and incubation with agonist). These factors made it difficult to observe any potential change in the receptor function under hypoxia or oxidative stress. Further investigations will be necessary to show whether changing trends in receptor expression have a significant effect on the receptor function. Moreover, future studies will demonstrate whether hypoxia / oxidative stress cause any change in 5-HT_{2A} receptor protein expression in vascular smooth muscle cells.

CHAPTER VII

GENERAL DISCUSSION

7 CHAPTER VII General Discussion

7.1 Tissue bath optimisation and 5-HT_{2A} antibody characterisation

The main aims of this study were to (a) comparatively analyse the vasoactive actions of 5-HT on vascular tone of normotensive and pre-eclamptic placental vessels, (b) to investigate the effects of blocking the 5-HT_{2A} receptor on 5-HT mediated changes in vascular tone of these vessels and (c) to comparatively analyse 5-HT_{2A} receptor levels in placental vessels from normotensive and pre-eclamptic placentae. These were achieved by *in vitro* tissue bath and western blotting assays.

Tissue bath techniques are powerful tools in investigating isolated vessel tone under experimental conditions. However, there are several factors that might affect the subsequent analysis of the contractile responses of the vessels. These include the initial passive tension of the vessels, concentration of the pre-contracting agent and handling of the tissue (collection and cold storage). All these factors were addressed in the present study. The initial passive tension that the vessels are subjected to is crucial since it may influence the evaluation of the induced contraction of these vessels. The optimal passive tension found in this study is in good agreement with previous studies using similar sized vessels (Marin *et al.*, 1990; Reviriego *et al.*, 1990). Although it is well-known that prolonged storage of vessels at low temperature adversely affects tissue viability and function (Abad *et al.*, 2003) there are still recent studies using overnight cold stored vessels for functionality assays (Gupta *et al.*, 2006). In order to avoid variability caused by tissue viability, blood vessels were dissected and experiments were performed immediately after delivery; this meant working 16-hour days!

Ketanserin concentration to be used for 5-HT_{2A} receptor blocking has also been addressed. It would be valuable to comparatively investigate the antagonism of ketanserin using a range of concentrations in normotensive and pre-eclamptic vessels. However, due to experimental restrains it was only possible to use a single concentration of ketanserin, hence 1μM ketanserin (concentration produced the maximal blockage at the receptor) was selected for all analysis.

Although blood concentrations of ketanserin were not available at the time the experiments were conducted, the concentration used (1μM) was found to be in good agreement with subsequent published data for transplacental transmission of ketanserin in patients treated for pre-eclampsia (Hanff *et al.*, 2004).

In order to comparatively analyse 5-HT_{2A} receptor protein levels in normotensive and pre-eclamptic vessels, an anti-5-HT_{2A} receptor antibody was characterised using a range of rat and human samples. Receptor protein profile varied within different tissue types. As well as the expected immunoreactivity with 40-55 kDa proteins, the antibody also reacted with a 63 kDa band in all samples tested. This protein band was also reported with another antibody by a previous study (Amireault and Dube, 2005). As discussed previously (see section 3.3.4) this protein band could be a variant of the 5-HT_{2A} receptor. Interestingly, the relative expression of the 63 kDa protein band was higher in human placental tissue and BeWo cells whilst vascular specimens and platelets contained relatively lower amounts. This may suggest that receptor expression may be regulated differently in different cell types. Nonetheless further studies will be necessary to confirm the identity of the 63 kDa protein and indeed if it is truly a 5-HT_{2A} receptor and whether it is functional. Identification could involve either 1-D or 2-D gel analysis followed by protein digestion, mass spectrometry analysis and identification of the protein band / spot using database searching of peptide fingerprints. Previous studies indicates that this variant may not couple to calcium signalling since it lacks the third intracellular domain (Guest *et al.*, 2000). However, variant receptors may form heterodimers with corresponding full length receptors which regulate their function (Schoneberg *et al.*, 1995). Therefore it would also be interesting to investigate whether the 63 kDa variant may regulate the expression levels or ligand coupling to the 55 kDa protein. Finally, once the identity of the receptor was confirmed, it would also be useful to investigate relative expression in different types of human tissues.

7.2 *Comparison of 5-HT response and 5-HT$_{2A}$ receptor levels in normotensive and pre-eclamptic vessels*

It is generally accepted that the cause of pre-eclampsia to be placental in origin. In pre-eclampsia poor invasion and incomplete remodelling of the spiral arteries would reduce blood flow to the placenta (Pijnenborg *et al.*, 1991). This reduced maternal uterine blood flow has also been suggested by Doppler ultrasound studies (Papageorghiou *et al.*, 2004). In addition studies suggest that endothelium dependent vascular dilation is compromised in pre-eclamptic women. For example reduced endothelial responsiveness has been demonstrated in myometrial small arteries (Ashworth *et al.*, 1997) and arteries from subcutaneous fat (Cockell and Poston, 1997) in pre-eclamptic women.

The main finding of the present study was the generally reduced contractile response of pre-eclamptic placental vessels to 5-HT. Moreover, this reduction was more pronounced in the secondary vessels, which were closer to the periphery of the placenta. There were no significant correlation between a patient's response to 5-HT and blood (or urine) 5-HT levels. However, this is probably due to presence of two smaller groups of medicated and bed rested pre-eclamptic patients. PPP 5-HT levels in non-medicated pre-eclamptic patients were around 50 nM (9.2 ng / ml) whilst normotensives had a PPP 5-HT around 25 nM (4.5 ng / ml). The maximum difference in response of normotensive and pre-eclamptic vessels to 5-HT however was observed around 1μM. Although the circulating 5-HT levels are much lower than 1μM, and response of normotensive and pre-eclamptic vessels did not differ greatly at 50 nM levels, 5-HT levels could reach μM levels locally especially in pre-eclamptic subjects due to higher platelet activation.

Reduced contractile response to 5-HT may have implications for the maintenance and control of blood flow to the foetus and may also provide a protective mechanism. In addition preventing excessive vasoconstriction may be a compensatory action for reduced utero-placental blood flow observed in pre-eclampsia. Several factors such as 5-HT$_{2A}$ receptor levels or maturation of the vessel wall may affect the contractile responsiveness to 5-HT. In the present study 5-HT$_{2A}$ receptor levels were found to be

similar in normotensive and pre-eclamptic vessels. However, receptor desensitisation may contribute to the reduced contractile response (detailed in chapter IV). Indeed several studies demonstrated that 5-HT_{2A} receptors desensitise upon repeated agonist exposure (Hanley and Hensler, 2002; Damjanoska *et al.*, 2004). Considering higher circulating 5-HT levels in maternal and placental blood reported in the present study (chapter V) and by other investigators (Middelkoop *et al.*, 1993; Carrasco *et al.*, 1998), receptor desensitisation and internalisation may explain the reduced responsiveness to 5-HT in pre-eclampsia. The techniques used in the present study however do not allow distinguishing receptors available at the cell surface and internalised receptors. Future studies using immunohistochemisty coupled with confocal microscopy may be useful. Although qualitative, these techniques would allow an investigation of the relative expression of cell surface and internalised receptors in normotensive and pre-eclamptic vessels. Vessel wall maturation could also affect vasocontractility. Since pre-eclamptic and their corresponding normotensive vessel weights were similar, and similar sized specimens were used it is likely that there were no apparent difference in the vessel wall thickness. There was a small non-significant reduction in pre-eclamptic vessel response to KCl (general contractility-independent of receptor levels) compared to corresponding normotensive vessels. However, it should be noted that all 5-HT responses were expressed as a percentage of the KCl contractile response and therefore any potential differences would be normalised. Furthermore, studies indicate that there is no significant relationship between maximal response to 5-HT (by arteries / veins) and neonatal weight (gestation) during third trimester in either group (Bertrand and St-Louis, 1999). Considering the above, the differences in gestational age and hence vessel wall maturation is unlikely to be the reason for reduced responsiveness to 5-HT in pre-eclampsia.

A significant recent study by Meziani *et al.* (2006) suggests that shed membrane microparticles (derived from leukocytes and platelets) from plasma of pre-eclamptic women can cause decreased responsiveness to 5-HT in human resistance vessels. This study also suggests that these microparticles produce nitrative and oxidative stress in the vessel wall partly due to up-regulation of iNOS and subsequent overproduction of NO and partly due to increased superoxide levels. Nevertheless, whether the altered vascular smooth muscle signalling and contraction systems have

a role in decreased vascular tone observed in pre-eclamptic vessels needs further investigation. Vascular smooth muscle signalling and coupling to G protein could be investigated in primary cell cultures from normotensive and pre-eclamptic samples by measuring (radiolabelled) inositol phosphate accumulation. To investigate potential differences in contraction systems western blotting could be used to assess actin to myosin light chain ratios in normotensive and pre-eclamptic vessel extracts (Cavaille *et al.*, 1995).

As any other technique, the tissue bath system has its limitations. First of all vessels are not in their circular confirmation and therefore the pressure applied to the vessel wall is not equally distributed. Secondly in line with other studies tissue baths were aerated with 95 % O_2 – 5 % CO_2 (Inayatulla *et al.*, 1993; Cruz *et al.*, 1998; Bertrand and St-Louis, 1999; Abad *et al.*, 2003; Gupta *et al.*, 2006) and there is some recent evidence that oxygen concentration may have an effect on vasoreactivity of placental vessels (Wareing *et al.*, 2006). Nevertheless, the oxygen tension was the same in all experiments performed and therefore any differences found between normotensive and pre-eclamptic samples are expected to be relevant.

7.3 Comparison of 5-HT response and 5-HT$_{2A}$ receptor levels in primary and secondary vessels

The maximal contractile response of normotensive primary and secondary arteries to 5-HT was similar. Similarly primary and secondary veins (both normotensive and pre-eclamptic) responded similarly to 5-HT. On the other hand the maximal contraction of pre-eclamptic secondary arteries to 5-HT was significantly reduced compared to primary arteries. This finding indicates that chorionic plate vessels may indeed be differentially modified in pre-eclamptic conditions and also demonstrates the importance of studying different branches of the chorionic vessels comparatively. This finding might also explain the discrepancies between the data presented in this thesis and the findings of Gupta *et al.* (2006), who reported that 5-HT response was not altered in pre-eclamptic umbilical arteries. As detailed above, the response of secondary arteries but not primary arteries to 5-HT was altered in pre-eclamptic samples. This suggests that there may be a gradient effect with arteries close to the

periphery of the placenta being affected in pre-eclampsia whilst umbilical and chorionic arteries closer to the cord insertion are not affected. Future studies could investigate contractile responses to 5-HT in several placental (primary secondary and stem villus) and umbilical vessels from same subjects to confirm this finding. This investigation could also include patients from different subgroups of pre-eclampsia (such as early onset versus late onset or mild versus severe pre-eclampsia) patients in order to reveal potential differences with the severity of the disease.

7.4 Ketanserin antagonism to 5-HT response

Ketanserin reduced contractile response to 5-HT in both normotensive and pre-eclamptic primary / secondary vessels. Ketanserin antagonism varied greatly between patients even within the same group. Since pharmacokinetic parameters of ketanserin have been reported to be comparable to that of healthy non-pregnant subjects (Hanff *et al.*, 2005) observed variations are likely to be due to 5-HT_{2A} receptor activity. This is also supported by variable patient response to treatment by ketanserin (Hanff *et al.*, 2006). Nevertheless, the mean % reduction in Emax in the presence of ketanserin was generally greater in all pre-eclamptic vessels compared to normotensives. Moreover in some cases ketanserin completely antagonised the response to 5-HT; interestingly this was only observed in the pre-eclamptic group. These findings suggest a greater 5-HT_{2A} receptor contribution to 5-HT mediated contraction in pre-eclamptic vessels. However, this finding should be interpreted with caution since ketanserin antagonism generally tended to be higher in the medicated group and therefore this effect might not be specific to 5-HT_{2A} receptors. Since the patient numbers are small and most of the pre-eclamptic patients were medicated and none of the controls were under any medication, it cannot be completely disregarded that these differences might also have an effect on overall vessel response. Future studies could investigate the 5-HT_{2A} receptor contributions in a larger pre-eclamptic patient group. It would also be useful to include non-medicated patients only or the patients treated with the same drug in the study so that the potential effects of the drug on the vessel response to 5-HT could also be assessed *in vitro*.

7.5 5-HT turnover

In line with previous studies (Middelkoop *et al.*, 1993; Carrasco *et al.*, 1998; Vural *et al.*, 1999) generally increased 5-HT levels were found in pre-eclamptic PPP samples. Results from present study also suggest a reduced 5-HT turnover in pre-eclampsia as determined by a reduced 5-HIAA: 5-HT ratio. Although not significant, placental MAO-A activity was also reduced in pre-eclampsia. Previous studies in our laboratory showed that the catalytic turnover of MAO-A was reduced whilst the MAO-A molecules / mg tissue was similar in normotensive and pre-eclamptic placenta (Sivasubramaniam *et al.*, 2002). Therefore Km values for MAO-A from normotensive and pre-eclamptic placenta for its amine substrate was investigated. Since the Km values were similar in normotensive and pre-eclamptic placental MAO-A, it could be concluded that the affinity of the active enzyme for its substrate is not affected in pre-eclampsia but that some MAO-A molecules are completely inactive. Therefore possible oxidative / nitrative damage to the enzyme was further investigated using an *in vitro* model (discussed in section 7.6). However, the long term aim would be to study MAO-A isolated from normotensive and pre-eclamptic placentae. This would involve preparation of mitochondrial fractions from placentae and separating proteins on either 1-D or 2-D gels. MAO-A protein then could be selected and digested. Mass spectrometry analysis could be used to monitor possible differences in peptide fragments due to oxidation / nitration. Alternatively using anti-nitrotyrosine or anti-carbonyl antibodies modified proteins could be immunoprecipitated from mitochondrial fractions. These samples could then be separated on 1-D (or 2-D) gels and nitrated / oxidised proteins could be identified using mass spectrometry and database searching.

7.6 In vitro effects of oxidative stress and hypoxia on 5-HT$_{2A}$ receptor expression and MAO-A activity

Since characterisation of a tissue culture system would be valuable in studying the control of expression and activity of MAO enzyme, preliminary experiments were conducted using two placental cell lines (3A Sub E and BeWo). Placental cell lines were selected in order to be able to study the effects of oxidative stress and hypoxia on the MAO-A enzyme and 5-HT$_{2A}$ receptor levels / activities together in the same cell system. Since MAO-A activity was not at detectable levels a recombinant MAO-A protein was used instead. Recombinant MAO-A was highly resistant to short term exposure to hydrogen peroxide whilst peroxynitrite was more affective in reducing MAO-A activity. These differences in sensitivity to hydrogen peroxide and peroxynitrite could be due to the structure of MAO-A and the amino acids involved in the active site of the enzyme. In addition due to its mitochondrial location and since MAO-A produces hydrogen peroxide as a result of its own activity, the enzyme would naturally be exposed to higher concentrations of hydrogen peroxide. However, these findings are preliminary and it is evident that there is a need to confirm using a cell culture system. This would involve using a cell line expressing high amounts of active MAO-A protein. Cells could be treated with xanthine / xanthine oxidase to generate ROS or alternatively ROS production within cells could be increased by using rotenone which is known to inhibit complex I and leads to ROS production. In addition to monitor effects of peroxynitrite cells could be treated with SIN-1, which is a donor of superoxide and nitric oxide to form peroxynitrite. Following exposure to oxidative and nitrative stress, MAO-A activity could be monitored in these cells.

Preliminary *in vitro* cell culture studies suggest no effect of oxidative stress and hypoxia on total 5-HT$_{2A}$ receptor levels. It should be noted that there were also no significant changes in the total receptor levels in normotensive and pre-eclamptic vessels. However, oxidative stress and hypoxia conditions may still have functional consequences on 5-HT$_{2A}$ receptors and also may have different effects on different cell types.

7.7 Summary

The present study investigates the role of 5-HT in pre-eclampsia. This study demonstrates the importance of studying different branches of the chorionic vessels comparatively since they may be differentially modified in pre-eclamptic conditions *In vitro* studies suggest that contractile response of placental vessels to 5-HT are reduced whilst total 5-HT_{2A} receptor levels are not affected in pre-eclampsia. 5-HT turnover was also reduced in pre-eclamptic patients. Altogether these findings suggest that 5-HT may have a role in the control of blood flow to the foetus and reduced responsiveness in pre-eclamptic vessels may also provide a protective mechanism.

CHAPTER VIII

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CHAPTER VIII References

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APPENDICES

9 CHAPTER IX Appendices

9.1 Appendix I

CATEGORY	INTERPRETATION
A	Studies in pregnant women have not shown an increased risk of foetal abnormalities to the foetus in any trimester of pregnancy.
B	Animal studies have not demonstrated evidence of harm to the foetus but there are no well-controlled studies in pregnant women. **OR** Animal studies have shown an adverse effect but this was not confirmed in controlled studies in pregnant in any trimester.
C	Animal studies have shown an adverse effect and there are no controlled studies in pregnant women. **OR** There are no available animal or controlled studies in pregnant women.
D	There is positive evidence of human foetal risk. Controlled or observational studies in pregnant women have demonstrated a risk to the foetus. However, the benefits of therapy may be acceptable despite the potential risks. The drug use may be acceptable in a life-threatening situation, when safer drugs cannot be used or are ineffective.
X	Controlled or observational studies in animals or pregnant women have demonstrated positive evidence of foetal abnormalities or risks. The risk of the use of the drug in pregnant women clearly outweighs any possible benefit. The use of the product is contraindicated in women who are or may become pregnant.

Table 9.1Food and Drug Administration (FDA) category for drug use in pregnancy

9.2 *Appendix II*

9.2.1 Clinical details of pre-eclamptic and normotensive patients for Western Blot analysis

Parameter	Normotensive (*n=4*)	Pre-eclamptic (*n=4*)	P value *
Maternal age (years)	33.25 ±1.315	27.75 ± 4.151	0.3429
Gestational age (weeks)	39.00 ± 0.2401	38.58 ± 0.4376	0.6857
Systolic blood pressure (mmHg)	119.9 ± 6.463	156.4 ± 4.259	0.0286
Diastolic blood pressure (mmHg)	79.75 ± 4.918	99.25 ± 3.711	0.0286
Protein in urine (g / l)	N/D	1.133 ± 0.4782	
(g / 24 hour)	N/D	2.460 ± 1.284	
Placental weight (g)	593.8 ± 62.57	562.5 ± 18.09	0.3429
Mode of delivery			
Caesarean section	4	1	
Vaginal delivery	0	3	
Gestational Weight (kg)	3.185 ± 0.06538	2.993± 0.2542	0.8857
Gestational sex			
Male	3	3	
Female	1	1	

Table 9.2Clinical details of pre-eclamptic and normotensive patients for Western Blot analysis

The details are expressed as mean ± SEM, pre-eclamptic group consisted of patients PE1, PE2, PE4 and PE6, medication details are given in Table 4.3 for each patient. N/D= none detected

* Mann – Whitney test

9.2.2 Ketanserin antagonism to 5-HT response in normotensive chorionic arteries

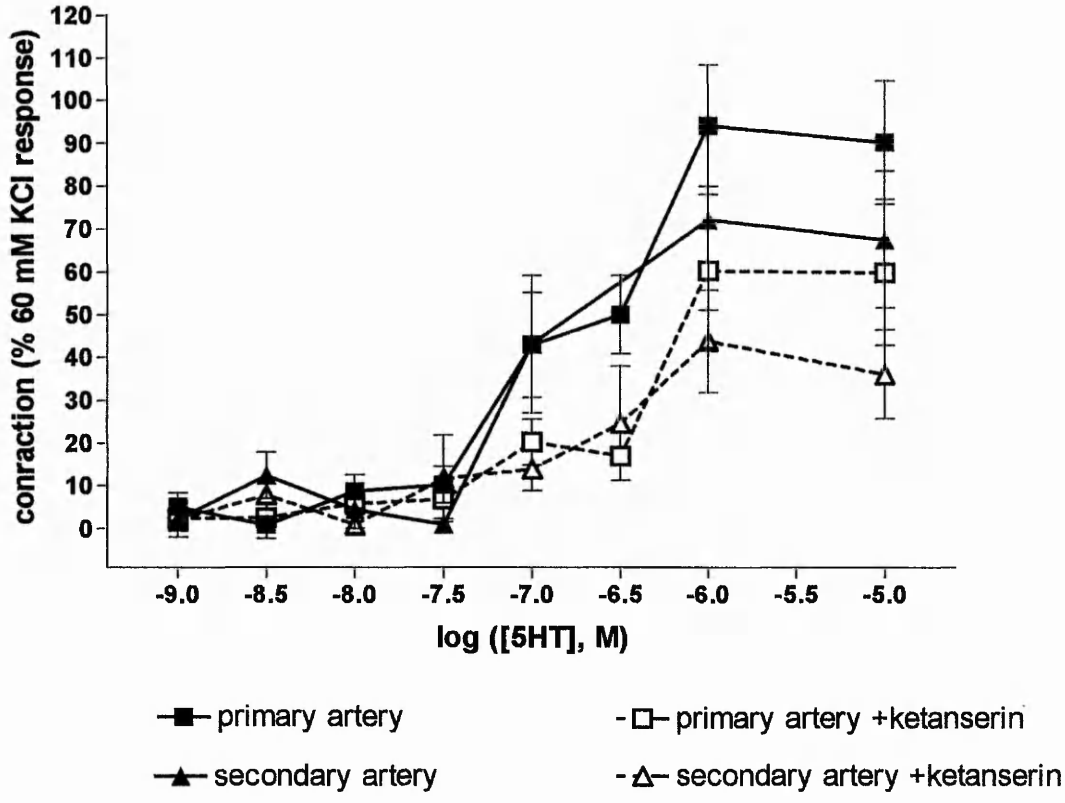

Figure 9.1 Ketanserin antagonism to 5-HT response in normotensive chorionic arteries

Concentration-response to 5-HT for chorionic plate primary (squares) and secondary (triangles) arteries in absence (filled squares and triangles) and in presence (open squares and triangles) of ketanserin (1 μM). Contraction produced by 5-HT are expressed as % 60 mM KCl response. Results expressed as mean ± SEM. *n* represents number of vessels used for the study (each vessel obtained from a different patient); *n= 9* primary arteries, *n= 8* secondary arteries. Statistical significance was accepted at $p< 0.05$ (*) and $p<0.01$ (**).

9.2.3 Ketanserin antagonism to 5-HT response in pre-eclamptic chorionic arteries

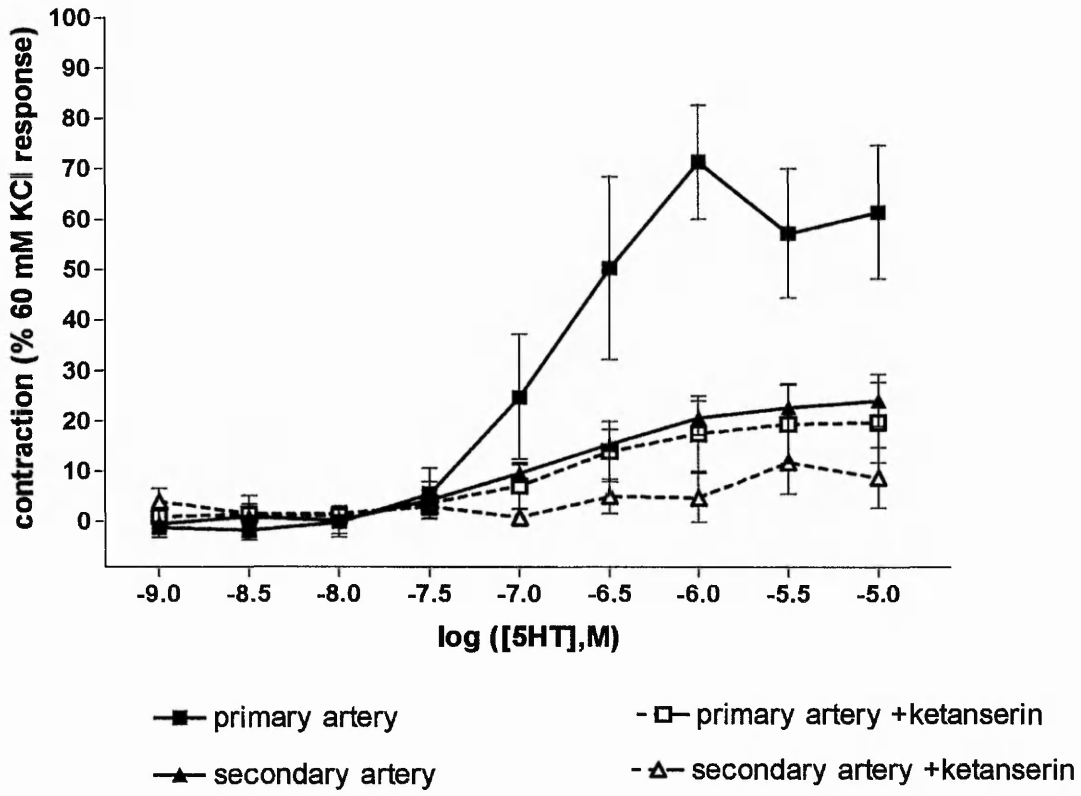

Figure 9.2 Ketanserin antagonism to 5-HT response in pre-eclamptic chorionic arteries

Concentration-response to 5-HT for chorionic plate primary (squares) and secondary (triangles) arteries in absence (filled squares and triangles) and in presence (open squares and triangles) of ketanserin (1 μM). Contraction produced by 5HT expressed as % 60 mM KCl response. Results expressed as mean ± SEM. *n* represents number of vessels used for the study (each vessel obtained from a different patient); *n= 8* primary arteries, *n= 8* secondary arteries. Statistical significance was accepted at $p< 0.05$ (*) and $p<0.01$ (**).

9.2.4 Ketanserin antagonism to 5-HT response in normotensive chorionic veins

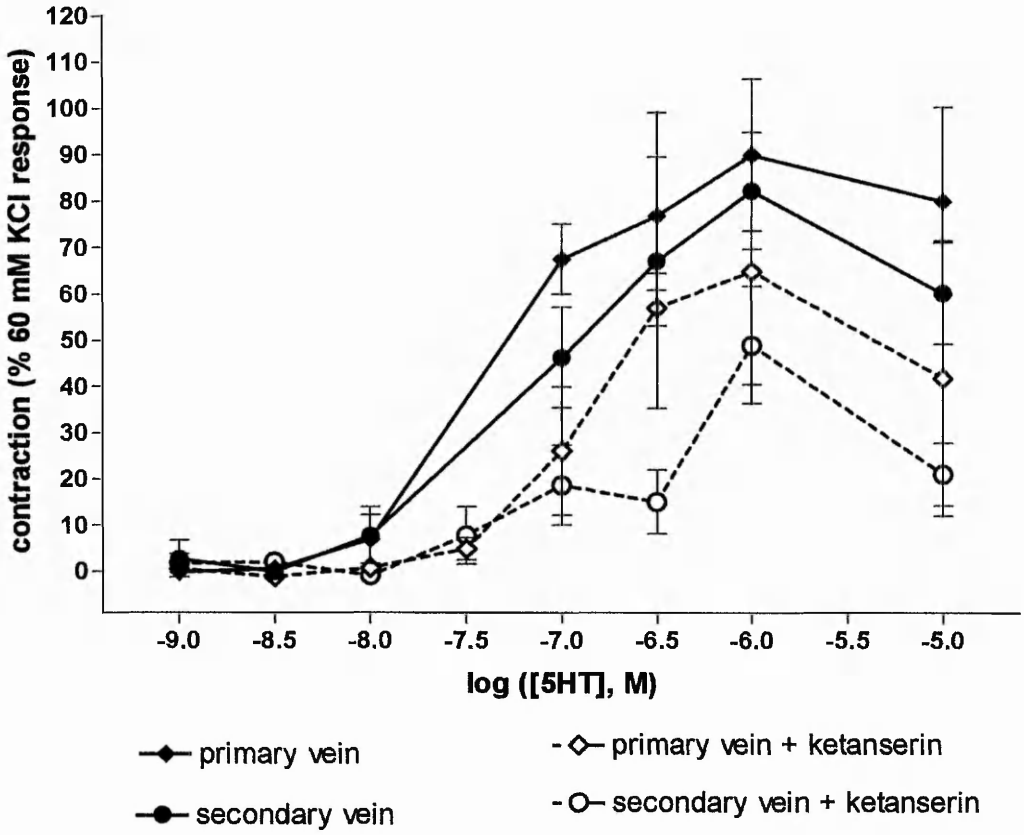

Figure 9.3 Ketanserin antagonism to 5-HT response in normotensive chorionic veins

Concentration-response to 5-HT for chorionic plate primary (diamonds) and secondary (circles) veins in absence (filled diamonds and circles) and in presence (open diamonds and circles) of ketanserin (1 μM). Contraction produced by 5-HT expressed as % 60 mM KCl response. Results expressed as mean ± SEM. *n* represents number of vessels used for the study (each vessel obtained from a different patient); *n= 8* primary veins, *n= 9* secondary veins. Statistical significance was accepted at $p< 0.05$ (*) and $p<0.01$ (**).

9.2.5 Ketanserin antagonism to 5-HT response in pre-eclamptic chorionic veins

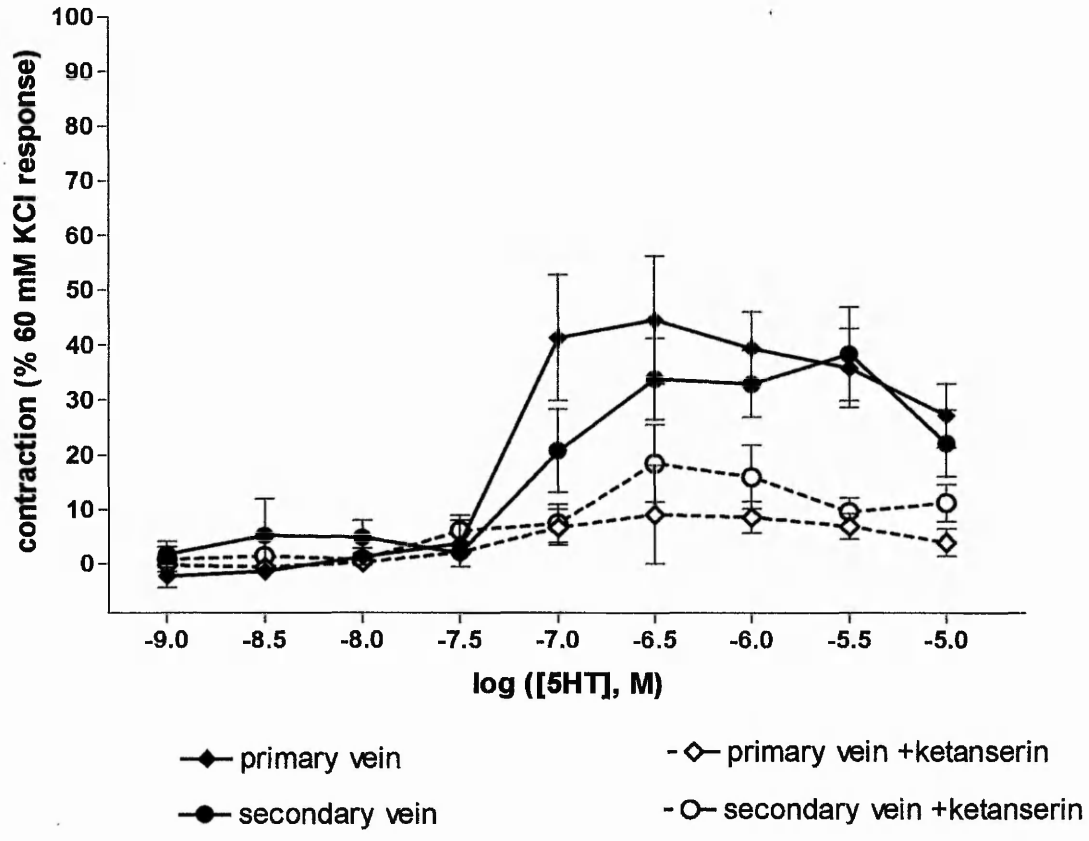

Figure 9.4 Ketanserin antagonism to 5-HT response in pre-eclamptic chorionic veins

Concentration-response to 5-HT for chorionic plate primary (diamonds) and secondary (circles) veins in absence (filled diamonds and circles) and in presence (open diamonds and circles) of ketanserin (1 μM). Contraction produced by 5-HT expressed as % 60 mM KCl response. Results expressed as mean ± SEM. *n* represents number of vessels used for the study (each vessel obtained from a different patient); *n= 6* primary veins, *n= 6* secondary veins. Statistical significance was accepted at $p < 0.05$ (*) and $p < 0.01$ (**).