Pre-eclampsia –

the role of vascular endothelial growth factor and its interaction with the vascular endothelium

By

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Presentations, publication and abstracts

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Serum levels of Vascular Endothelial Growth Factor are elevated in pre-eclampsia.
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2. R.P. Wellings, J.C.Brockelsby and P.N.Baker
Activation of endothelial cells by plasma from women with pre-eclampsia: differential effect on four cell types.
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3. J.C. Brockelsby, R.G. Hayman, A Ahmed, A.Y. Warren, I.R. Johnson and P.N. Baker.
VEGF via VEGF receptor-1 (Flt-1) mimics preeclamptic plasma in inhibiting uterine blood vessel relaxation in pregnancy: implications in the pathogenesis of preeclampsia Lab Invest 1999; 79(9), 1101-1111.

4. J.C. Brockelsby, I.R. Johnson, P.N. Baker
The effect of Vascular Endothelial Growth Factor on endothelial cells- a role in pre-eclampsia
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5. RG. Hayman, A.Y.Warren. J.C.Brockelsby, I.R.Johnson and P.N.Baker
Plasma from women with preeclampsia induces an in vitro alteration in endothelium dependent behaviour of myometrial resistance arteries
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P.N.Baker and J.C.Brockelsby
The key to pre-eclampsia
Contemporary Reviews in Obstetrics and Gynaecology 1999; 12, 251-256.

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Vascular Endothelial Growth Factor causes a loss of endothelium dependent relaxation in myometrial resistance arteries.
The International Society for the Study of Hypertension in Pregnancy, Oxford.1997.

2. J.C.Brockelsby, R.P.Wellings, P.N. Baker.
Vascular Endothelial Growth Factor (VEGF) alters endothelial cell function: a role in pre-eclampsia ?.
3rd International Symposium for the study of resistance arteries, London. 1997.

3. J.C.Brockelsby, R.G.Hayman, F.W. Anthony, F. F. Broughton Pipkin, P.N. Baker
Concentrations of vascular endothelial growth factor (VEGF) correlate with both blood pressure and relaxation to bradykinin in an ex-vivo model of pre-eclampsia
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Abstracts:

1. J.C. Brockelsby, R.P. Wellings, P.N. Baker.
Vascular Endothelial Growth Factor (VEGF) alters endothelial cell function.
Journal of Clinical Science:1997:93 supplement 37:8.

2. T. Wheeler, J.C. Brockelsby, R.P. Wellings, F.W. Anthony, P. Evans, P.N. Baker
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9. J C Brockelsby , F Broughton Pipkin , PN Baker .
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J. Soc. Gynecol. Invest:1999 6 1(Supple) 124A.

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The VEGF flt-1 receptor mediates altered vascular function in pre-eclampsia
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11. Lash G, Brockelsby J, Trew A, Taylor C, Anthony F, WheelerT.
Increased levels of Vascular Endothelial Growth Factor are secreted from isolated trophoblast cells under hypoxic conditions.
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An alteration in resistance vessel endothelial dependent relaxation may be mediated by an upregulation of nitric oxide
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I would like to dedicate this thesis to;

My parents

For their support and encouragement with everything that I have done

Kate Molander

For being there and making everything worthwhile

This writing business

-pencils and what not is overrated.

Silly stuff. Nothing in it.

EOR (Winnie the Pooh)

Hypothesis:

This thesis set out to test the hypothesis, first propsed by Baker et al (1995), that Vascular Endothelial Growth Factor (VEGF) may be involved in the alteration in endothelial function that is observed in the disease of pre-eclampsia.

Aims:

- To investigate concentrations of VEGF in plasma from non-pregnant, and normal pregnant women and women with pre-eclampsia.
- To investigate uterine and placental expression of VEGF in non-pregnant and normal pregnant women and women with pre-eclampsia.
- To investigate some of the vascular adaptations that occur in pregnancy and pre-eclampsia within the uterine and systemic circulations.
- To investigate the effect of plasma from women with PE and VEGF on
    - i) An in vitro endothelial cell culture model.
    - ii) An in vitro isolated vessel model.
- To characterise the mechanism whereby VEGF causes any alteration in vascular function.

Chapter One: Introduction:

1.1: General Introduction:

1.1.1: Pre-eclampsia:

1.1.1.1: Incidence:

Pre-eclampsia complicates approximately 7-10 % of pregnancies in the United Kingdom (HMSO, 1952-1984) and places the mother and fetus at risk, increasing both mortality and morbidity rates. However, despite vast amounts of research its cause remains an enigma of modern medicine. Therefore an increased understanding of the pathophysiology may well enhance our treatment modalities.

1.1.1.2: Definition and Classification:

The unknown aetiology of pre-eclampsia has made its' definition difficult and led to the clinical signs being used not merely for diagnosing but also for defining the condition. Hypertension is the classical clinical sign associated with pre-eclampsia. However, pre-eclampsia is more than just hypertension in pregnancy, it is a syndrome with a combination of abnormal signs. The second clinical sign that has been accepted by most authors as defining pre-eclampsia is proteinuria (Davey and MacGillivary, 1988).

Despite these controversies there is a need for provisional definitions to allow different investigators to compare their experiences. Davey and MacGillivray devised one of the most commonly used systems of classification and it is their definition that is used in this thesis.

" It is that clinical syndrome in which a previously normotensive woman, with no pre-existing cardiovascular or renal disease, after the twentieth week of pregnancy, develops a blood pressure of at least 140/90 mmHg on two or more occasions (separated by greater than four hours) in the presence of proteinuria of at least 500mg (or 300mg/l) in a 24 hour collection of urine, in the absence of urinary tract infection. Blood pressure and proteinuria must have returned to normal levels by the sixth post partum week" (Davey and MacGillivary, 1988).

1.1.2: The pathogenesis of pre-eclampsia:

Numerous hypotheses concerning the pathogenesis of pre-eclampsia have been proposed. These include, an alteration in the circulating prostaglandins, an altered response to angiotensin and an overstimulation of the sympathetic nervous system. These hypothesies have been fully reviewed previously (de Jong et al., 1991) (Broughton Pipkin and Rubin, 1994) (Higgins and Brennecke, 1998) (Dekker and Sibai, 1998) (Dietl, 2000) and therefore these will not be discussed further in this thesis. However, none of these hypotheses fully explain the observed pathophysiology. It has been suggested that an alteration in endothelial cell function accounts for its diverse clinical presentations (Roberts et al., 1989a). This is an attractive hypothesis as all blood vessels are lined by the endothelium and the association of the disease with an alteration in vascular function is well documented. Whether such changes are primary or secondary remain to be determined.

The endothelial hypothesis proposed by Roberts et al (1989) suggests that a genetic predisposition leads to an immunologically-mediated failure of trophoblast invasion into the maternal decidua, resulting in poor placentation and hypoxia. Placental hypoxia then causes an upregulation of a factor(s) which are released into the maternal circulation and these dramatically alter vascular endothelial function (Roberts et al., 1989). This thesis was undertaken to examine the aetiology of this observed alteration in endothelial function and this literature review will assess and critically analyse the evidence for each component of this in turn. However, it does not attempt to address other hypotheses concerning the pathogenesis of pre-eclampsia.

1.1.2.1 Uteroplacental function and pre-eclampsia:

1.1.2.1.1: Placenta:

The pivotal role of the placenta has been established by several clinical observations. The first was the recognition that the fetus is not a disease pre-requisite, as it occurs with hydatiform mole where the fetus is absent (Scott and Beer, 1976). The second was that resolution of the disease occurs on removal of the placenta (Newman and Eddy, 1988). Further grounds for accepting a key role for the placenta come from the observation that the disease incidence increases with pregnancies that are associated with hyperplacentosis: multiple pregnancy, diabetes and hydatiform mole (Chesley, 1978) (Brittain and Bayliss, 1995) (Nugent et al., 1996) (Joffe et al., 1998) (Ros et al., 1998) (Malone et al., 1998).

1.1.2.1.2: Trophoblast:

The placenta consists primarily of trophoblast cells. These can be divided into two distinct morphological cell types- the villous-trophoblast and the semi-allogenic, extra-villous trophoblast (Pijnenborg, 1988) (Bulmer, 1992). The extra-villous trophoblast is invasive in nature, lining both the inter-villous space and spiral arterioles of the uterus, and their semi-allogenic status with respect to their host is an obvious target for the HLA cell mediated immune system.

Studies examining the expression of HLA molecules on trophoblast cells have shown that HLA expression is limited to HLA-C (King, 1996) and the pregnancy-specific HLA class I molecule, HLA-G (Ellis et al., 1990) (Kovats et al., 1990). Kovats et al noted that HLA-G expression was subject to gestational shift, with predominance occurring in the first trimester (Kovats et al., 1990). These observations led both Ellis and Kovats to postulate that HLA-G was important in mediating the lowered immune susceptibility of trophoblastic tissue. McMaster et al (1995) investigated the expression of HLA-G by human trophoblast in both the *in vivo* and *in vitro* situation, utilising immunohistochemical techniques they demonstrated that HLA-G expression was limited to the invasive cytotrophoblast (McMaster et al., 1995). In keeping with this is the observation that isolated first trimester trophoblasts, which develop an invasive pattern in culture, increased their expression of HLA-G antigen during this transformation (McMaster et al., 1995). Two studies have demonstrated that HLA-G confers resistance to natural killer cell (NK) lysis (Chumbley et al., 1994) (Rouas-Freiss N (1997). The evidence cited above both indirectly and directly confirms the hypothesis that HLA-G antigen bestows an immunological shield on the invading trophoblast in normal pregnancy(Rouas-Freiss et al., 1997). However, the role of HLA-G in pre-eclampsia has yet to be elucidated.

1.1.2.1.3: Myometrium:

Spiral arteries run through the myometrium and during pregnancy supply the intervillous space. Normal placentation is characterised by trophoblast invasion into these arteries which causes, through structural changes, their diameter to increase by some 6-fold relative to the non-pregnant state (Robertson et al., 1973) (Robertson and Warner, 1974) (Sheppard and Bonnar, 1974) (Brosens et al., 1978). These vascular changes extend from the intervillous space up to the inner third of the myometrium and lead to a dramatic increase in the blood supply to this junction (Brosens et al., 1972) (Pijnenborg et al., 1991). Trophoblast invasion occurs in two phases, the first at six weeks gestation and the second at sixteen to eighteen weeks. During this process the endothelium and internal elastic lamina are replaced by trophoblast and an amorphous matrix that consists of fibrin deposits (Brosens et al., 1967) (De Wolf et al., 1975) (Pijnenborg et al., 1980).

As an alteration in spiral artery function appears to be crucial for normal placental development, it has been postulated that abnormalities in this process may lead to pre-eclampsia. Several studies have demonstrated that in women who develop pre-eclampsia there is histological evidence of shallow endovascular trophoblast invasion, with the second invading phase failing to reach the myometrial segments of the spiral arteries (Brosens and Renaer, 1972) (Brosens et al., 1977) (Khong et al., 1986) (Pijnenborg et al., 1991). Interestingly Robertson et al (1975) noted that this was not an effect specific to pre-eclampsia. They observed that the spiral arteries of chronic hypertensive women without proteinuria exhibited similar changes (Robertson et al., 1975). Since this observation, several other groups have examined this finding with conflicting results. Brosens et al (1977) demonstrated that in chronic hypertensive subjects the invasion of the spiral arteries was normal, whereas Sheppard et al (1981); Frusca et al (1989) reported the absence of physiological changes (Brosens et al., 1977) (Brosens, 1977) (Sheppard and Bonnar,

1981) (Frusca et al., 1989). One explanation for the discrepancy in these results, is that there was no universal definition of the hypertensive disorders of pregnancy prior to Davey et al (1989) (Davey and MacGillivary, 1988). Both Sheppard et al and Frusca et al included patients who would now not be defined as having pre-eclampsia. Meekins et al (1994) using the definitions of Davey et al (1989) re-examined this question and demonstrated using immunohistochemical techniques that there was both a spectrum of trophoblast invasion, and that spiral arteries displaying no physiological changes had evidence of invasion. Their overall conclusion was that pregnancies complicated by pre-eclampsia had significantly less spiral artery invasion than normal pregnancies (Meekins et al., 1994).

An alteration in spiral artery function may also manifest itself as acute atherosis of the spiral arteries. This is characterised by the appearance of lipid-laden foam cells consisting of damaged intimal smooth muscle cells and infiltrating macrophages and has been considered virtually pathognomonic of pre-eclampsia (Robertson et al., 1975). This view has been refuted by Sheppard et al (1974) who were able to demonstrate similar lesions in the spiral arteries of normotensive women with intra-uterine fetal growth restriction (Sheppard and Bonnar, 1974). This coupled with the demonstration that failure of adequate invasion is also apparent with intra-uterine growth restriction has led to the suggestion that inter-uterine growth restriction and pre-eclampsia lie within a spectrum of the same disease (Gerretsen et al., 1981).

Although a uniform definition of pre-eclampsia was not accepted until the late 1980s, it is apparent from the literature that pre-eclampsia involves shallow endovascular invasion of the spiral arteries by trophoblast.

1.1.3.1: Alteration of endothelial cell function in the pathogenesis of pre-eclampsia:

All blood vessels within the vasculature are lined by a monolayer of flattened, rhomboid-shaped cells termed the endothelium. For many years these cells were considered to be a passive barrier between blood and the underlying vasculature. However, in 1980 Furchgott et al demonstrated that aorta denuded of endothelium was resistant to vasorelaxation initialted by acetlycholine, but was capable of relaxation to other agents (Furchgott and Zawadski, 1980). This observation led to the hypothesis that the endothelium was a prerequsite for some types of vasorelaxation. This relationship has subsequently been confirmed in other smaller blood vessels and other vasodilators (Furchgott RF, 1983).

These observations and the development of techniques for cell culture (Booyse et al., 1975) (Elgjo et al., 1975) have led to much research in the field of endothelial cell biology (Luscher and Barton, 1997) (Kirkpatrick et al., 1997). Endothelial cells offer a unique interface between the intra- and extra-vascular environment and have a diverse function in normal physiology and pathology, including the regulation of angiogenesis, immune responses and inflammation, as well as in the maintenance of vascular tone. Since the vascular endothelium has been shown to have such a diverse function in haemostasis, it has been hypothesised that it is central to the pathogenesis of pre-eclampsia (Roberts et al., 1989). The prominent and defining clinical features of pre-eclampsia; elevated blood pressure, glomerular capillary leakage leading to proteinuria, and oedema, are also consistent with an abnormality of the endothelium (Roberts et al., 1991).

1.1.3.1.1 In vivo evidence:

There is much evidence for an altered endothelial state in the pathogenesis of pre-eclampsia. *In vivo* evidence for a loss of endothelial function comes from the study of Campbell et al who established that Evans blue labelled albumin exhibited an increased dispersion rate from the vascular compartment in women with pre-eclampsia when compared to normotensive controls (Campbell and Campbell, 1983).

Further evidence for endothelial involvement comes from histological assessment of renal biopsies and necropsies, which have revealed that the glomerular endothelial cells exhibit a characteristic engorged appearance with intracellular inclusions, termed glomerular endotheliosis (Sheehan, 1980) (Kincaid-Smith, 1991). However, it should be noted that few other vessels have been histologically examined for endothelial cell injury in women with pre-eclampsia. Umbilical cord arteries have been studied and reports have confirmed endothelial cell disruption (Gilabert et al., 1999). This serves to confirm the hypothesis of endothelial dysfunction and demonstrates that vascular changes in the fetus may parallel the vascular changes observed in the mother.

Many studies have investigated circulating plasma and serum levels of endothelial products in women with pre-eclampsia and compared these to normotensive controls. Freidman et al demonstrated that plasma concentrations of fibronectin, von Willebrand factor, tissue plasminogen activator and plasminogen activator inhibitor-1 were increased in women with pre-eclampsia. Although all of these substances are synthesised by a large number of cells, they concluded that there was evidence of an alteration of endothelial cell function (Friedman et al., 1995).

Endothelial cells produce surface adhesion molecules upon cellular activation; these serve to enhance cell-cell binding to circulating immune cells such as neutrophils and platelets. This has led several groups to hypothesise that these would increase in pre-eclampsia. The cell adhesion molecules are a complex group consisting of vascular cell adhesion molecule (VCAM), intracellular adhesion molecule (ICAM), E-selectin, and P-selectin. Several studies have established circulating levels of ICAM in pregnancies complicated by pre-eclampsia and have shown a significant increase in compromised pregnancies when compared to controls (Austgulen et al., 1997) (Krauss et al., 1997). In contrast, Lyall et al and Djuovic et al were unable to show similar results in their populations (Lyall et al., 1995) (Djurovic et al., 1997). These differences may be explained by the fact that ICAM is not specific for endothelial cells and an increase may represent general cellular activation. E-Selectin and VCAM-1 show greater specificity for endothelial cells, although, measurement of these levels have still produced inconclusive results. All studies that have determined the less specific VCAM-1 levels have noted an increase in pregnancies complicated by pre-eclampsia (Lyall et al., 1994) (Budak et al., 1998) (Higgins et al., 1998). The highly specific E-selectin show differing results, with several groups demonstrating an increase (Heyl et al., 1999) (Austgulen et al., 1997) (Krauss et al., 1997) and another group unable to determine any difference (Lyall et al., 1994). However, the concentration of E-selectin in peripheral blood has been shown to be very dependent on the assay utilised and this may explain the discrepancies within the literature.

1.1.3.1.2 In vitro evidence:

McCarthy et al (1993) provided the first *ex vivo* evidence of altered endothelial function in women with pre-eclampsia. Utilising subcutaneous resistance vessels and the technique of wire myography, they showed that endothelial dependent relaxation to actelycholine was impaired in women with pre-eclampsia when compared to controls (McCarthy et al., 1993a). Knock and Poston, using the same system, have also show a similar diminished relaxation to bradykinin in subcutaneous resistance vessels (Knock and Poston, 1996). Ashworth et al, (1997) have indicated that is may be a more global effect by demonstrating that resistance vessels obtained from the myometrium exhibit an inhibition to relaxation with bradykinin in women with pre-eclampsia.

The above evidence implicates the endothelium as pivotal to the pathogenesis of pre-eclampsia. The observations that the placenta appears to be crucial have led to the hypothesis that it produces a factor(s) that are released into the maternal circulation initiating these alterations in endothelial function. The advent of endothelial cell culture has allowed this hypothesis to be tested further.

1.1.4.1 Circulating factors in the pathogenesis of pre-eclampsia:

1.1.4.1.1 Cell culture studies:

The circulating factor hypothesis has been primarily investigated in a number of *in vitro* studies. These have compared the effects of serum or plasma samples taken from women with pre-eclampsia and normal pregnant controls, on cultured endothelial cell function.

Rodgers et al first presented evidence of a circulating factor in 1988 (Rodgers et al., 1988). They established that 10% sera from women with pre-eclampsia exhibited a greater cytotoxic effect on human umbilical vein endothelial cells (HUVEC) than control plasma, assessed via the release of intracellular chromium 51. This led to the hypothesis that there was a short-lived cytotoxic factor, which was produced by the placenta and released into the maternal circulation. However, other studies have failed to reproduce these findings (Kupferminc et al., 1996) (Endresen et al., 1995). Indeed, Roberts et al re-examined this question and demonstrated that the serum of women with pre-eclampsia did not cause endothelial cell membrane leakage or alteration in morphology. Thus they modified their original description of vascular endothelial cytotoxicity to 'activation'(Roberts et al., 1992). Other groups have demonstrated that endothelial cells cultured in the serum of women with pre-eclampsia exhibited a greater mitogenic effect and that any metabolic changes noted were almost completely reversed within 24 hours by replacing the pathological sera with standard culture medium (Lorentzen et al., 1991).

Many studies of plasma characterisation have utilised human umbilical vein endothelial cells (HUVEC) which are potentially misleading as pre-eclampsia is a disease of microvascular and not macrovascular cells. HUVEC are derived from the fetal macrovasculature and as such their response to plasma from women with pre-eclampsia may not represent what happens at the maternal microvascular level. Other studies have opted for bovine microvascular endothelial cells (B88), and one may question for similar reasons to HUVEC, the use of these in the determination of possible hypotheses regarding the pathopysiology of pre-eclampsia. To date no study has tried to rationalise the use of various endothelial cell lines in the investigation of pre-eclampsia.

1.1.4.1.2 Myometrial vessel model

Recently, Asworth, et al have utilised an *ex vivo* model to negate the problem of cultured endothelial cells (Ashworth et al., 1998). They examined bradykinin-mediated relaxation of myometrial resistance arteries after *in vitro* incubation with either plasma from women with pre-eclampsia or normotensive controls, demonstrating that there was impaired relaxation in the vessels that were exposed to the plasma from women with pre-eclampsia. The group of normal vessels exposed to plasma from women with pre-eclampsia mimicked vessels taken from women with pre-eclampsia (Ashworth et al., 1998). It would be of interest to examine whether plasma factors such as VEGF, which have been hypothesised to be integral to pathogenesis of pre-eclampsia produce the same effect in this vessel model system.

Thus, there is a body of evidence to suggest that a circulating factor(s) exists in the blood of women with pre-eclampsia that causes endothelial cells to alter their behaviour.

1.2 Vascular reactivity:

1.2.1 General:

1.2.1.1 Vascular reactivity and the role of the endothelium:

Blood flow and pressure are regulated in the periphery by a diverse set of vessels termed the 'resistance arteries'. These vessels must achieve a compromise between the often-conflicting demands of the body and individual organs. Adjustments in resistance are achieved by a change in the luminal diameter.

Formerly resistance vessels were considered to be arterioles, defined as those vessels that have no more than one layer of smooth muscle cells. These are arteries that have a diameter of between 30-50μm. It is now clear from functional evidence that this is not the case with approximately one half of resistance vessels lying proximal to arterioles. Bloom and Fawcett give an anatomical definition stating that pre-arteriolar vessels with a diameter of ≤ 500μm are resistance vessels (Bloom and Fawcett, 1968) and this is consistent with the functional definition of resistance vessels (Baumbach and Heistad, 1983) (Chilian et al, 1986) (Faraci and Heistad, 1990).

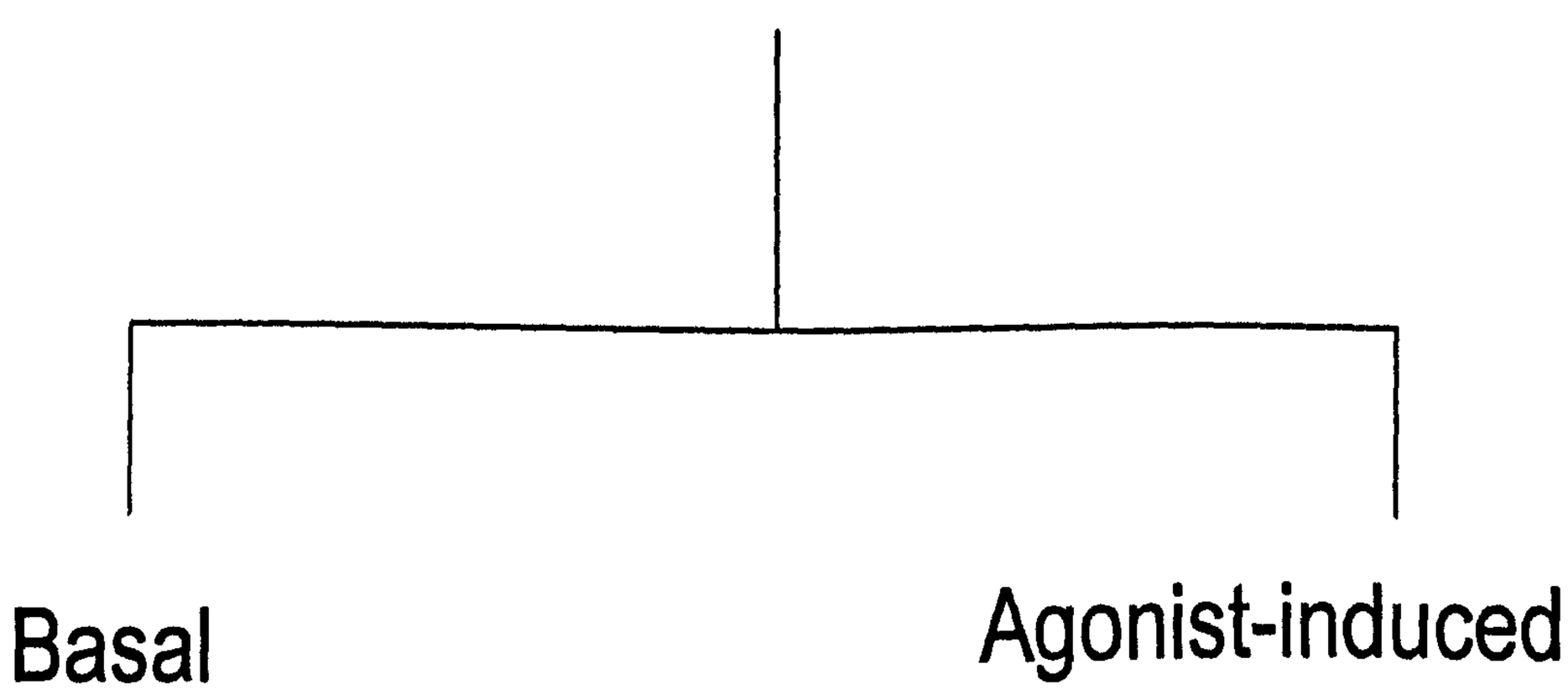
Under physiological conditions, the primary function of the smooth muscle is to generate force. Normal vascular smooth muscle is in a continual state of activation, but the amount of force generated is ultimately regulated by a variety of extracellular factors, both physiological and chemical. The active tension exerted by vascular smooth muscle in a vessel wall is known as

tone. By operating in a state of sustained constriction (or tone) peripheral vascular resistance may be regulated, thereby modulating cardiac output, maintaining systemic blood pressure and regulating organ blood flow.

For the sake of clarity, a simplified model of tone will be considered (Figure 1.1). Overall vascular tone might be considered as a composite phenomenon comprising basal tone and agonist-induced tone, rather than a single entity. Basal tone compromises two elements, the myogenic and intrinsic tone. Myogenic autoregulation is dependent on stretch (pressure or flow on the endothelium), which directly induces compensatory contraction or relaxation of the smooth muscle and will not be considered in this review. Agonist-induced tone is controlled through (i) neurological control, involving the autonomic nervous system, (ii) circulating substances and (iii) locally-produced vasoactive substances.

Sympathetic innervation originates from the cardiac and vasomotor centres of the medulla. The preganglionic neurons synapse in the thoracic sympathetic ganglia. The post-ganglionic fibres travel to supply the arterial and venous circulation. The neurotransmitter at the nerve endings is noradrenaline, synthesised locally from tyrosine, via dopa and dopamine, its action being dependent upon the nature and extent of its receptor stimulation (Smith and Kampine, 1990). At the nerve endings, stimulation of α_1-adrenoceptors causes vasocontriction, particularly in the skin, skeletal muscles, and renal vascular beds. α_2- adrenoceptors on the neuronal membrane provide a negative feedback mechanism limiting the release of noradrenaline from the nerve terminal (Berthelsen and Pettinger, 1977).

Fig 1.1


Overall vascular tone
Basal
- myogenic
- intrinsic
Agonist-induced
- neural
- hormonal
- metabolic
- endothelial

The parasympathetic fibres arise in the motor nuclei of the brainstem and exit by way of the cranial nerves and the sacral division of the spinal cord. The neurotransmitter at the parasympathetic nerve terminal is acetyl choline, which acts on muscarinic receptors on the recipient organ. In the peripheral vasculature, the parasympathetic system causes vasodilatation in the vascular beds of the brain, myocardium, sex organ and the intestine. In other vascular beds, vasodilation occurs by means other than parasympathetic stimulation (Smith and Kampine, 1990).

The vascular endothelium represents another source of vasoactive substances. It produces three major relaxants the eicosanoids, nitric oxide and the yet to be identified endothelial-derived hyperpolarising factor (EDHF), on stimulation by various circulating substances. The vascular endothelium also produces the vasoconstrictor endothelin.

The control of vascular resistance occurs through the dynamic interplay of all these described systems. Therefore, when its control is considered in an experimental sitting, it should ideally investigate all components together. However, this was not possible to achieve in this thesis and investigations were therefore limited to the role that the endothelium had in this system.

1.2.2. Vasorelaxants substances:

1.2.2.1 EDRF – Nitric Oxide:

Discovery of EDRF (Nitric Oxide):

Furchgott et al (1980) elegantly demonstrated that denuded rabbit aorta was unresponsive to acetylcholine and proposed that the stimulated endothelium produced a chemical mediator that induced vascular smooth muscle relaxation. They termed this mediator Endothelium Derived Relaxing Factor (EDRF) (Furchgott and Zawadski, 1980). The chemical nature of EDRF remained unknown until Palmer et al (1987) and Ignarro et al (1987) demonstrated independently that EDRF derived from cultured endothelial cells and aqueous nitric oxide caused similar relaxation profiles (Palmer et al., 1987) (Ignarro et al., 1987).

It is now clear that nitric oxide released from the endothelium contributes to the physiological regulation of blood pressure. In rats, Rees et al (1989) found that the blockade of nitric oxide formation with the inhibitor L-NMMA resulted in an immediate rise in blood pressure and that the subsequent administration of the substrate L-arginine reversed this effect (Rees et al., 1989). This has also been shown to be the case in humans; forearm plethysmography studies demonstrated that infusion of nitric oxide inhibitors caused a reduction in peripheral blood flow, which could be overcome by the use of L-arginine (Vallance et al., 1989).

Formation of Nitric Oxide:

The chemical formation of NO has been demonstrated to involve the hydroxylation of the N terminal group of the guanidino group of L-arginine. This hydroxylation reaction leads to the generation of the free radical nitric oxide and L-citrulline (Sakuma et al., 1988). The L-citrulline has been shown to result in the formation of L-arginine via its combination with ammonia (Anggard, 1994).

Nitric Oxide synthase enzymes:

Synthesis of NO has been demonstrated in various mammalian tissues. These include the vascular endothelium, the brain and activated macrophages (Ignarro et al., 1988) (Marletta et al., 1988) (Garthwaite et al., 1989). As unregulated production has been implicated in many pathological events, tightly controlled biosynthesis of nitric oxide is crucial. To this end nitric oxide synthetase is the most regulated enzyme in the human body. Once these tissues had been demonstrated to produce nitric oxide the search was on for the enzyme.

Initial efforts to purify these enzymes were unsuccessful, as activity was lost during purification. Bredt et al 1990 utilised their observation that these enzymes were calmodulin dependent, to isolate brain nitric oxide synthase (Bredt et al., 1990) (Bredt and Snyder, 1990). Using similar approaches, other isoforms of nitric oxide synthase have been isolated. From early studies of this complex set of enzymes it was apparent that macrophages contained a specific isoenzyme, different to all the others. This enzyme has been demonstrated to be Ca^{2+} independent (Knowles and Moncada, 1994). The calcium independent enzyme has now been demonstrated in several other immune tissues and is not, as was first thought, macrophage specific. Since this

observation, immune cytokines have been shown to increase the expression of calcium independent NOS in various tissue, which has led to the term of inducible NOS (Galea et al., 1995) (Salter et al., 1991) (Nussler et al., 1992) (Nussler et al., 1993) (Geller et al., 1993). The isoenzymes of nitric oxide synthase can be broadly divided into two categories: constitutive and inducible. The accepted nomenclature for the different isoforms of NOS remains under discussion as those parameters by which the different isoforms were initially thought to be differentiated would now appear less mutually exclusive (Sessa, 1994).

1.2.2.2. Prostaglandins: prostacyclin:

Over the last twenty years, much interest has focused on the prostaglandins- a complex group of substances which have been demonstrated to be crucial to the interplay between platelets and the vascular wall. Arachidonic acid has been established as the most common fatty acid present in phospholipids (Moncada and Vane, 1979) and is the precursor of all the bisenoic prostaglandins. It is liberated from membrane phospholipids by the phospolipase group of enzymes (Vankeman and Von Dorp, 1968). There are three distinct stages in the formation of prostaglandins from their arachidonic acid precursor. First, phospholipid cleavage to mobilise arachidonic acid, second the sequential conversion to the prostaglandin endoperoxides, and third the subsequent isomerisation /reduction of these endoperoxides to produce the biologically active compounds (Moncada and Vane, 1979). Prostaglandin synthesis has been shown to be initiated by several stimuli including, shear stress, hormones and proteinases (examples of which include bradykinin and anti diuretic hormone) activating the prostaglandin E_2 synthesis pathway in the renal tubules (Grenier et al., 1981) (Kirschenbaum et al., 1982).

Discovery of prostacyclin:

The importance of prostaglandins in vascular functioning was demonstrated in 1974 with the discovery that the non-prostaglandin metabolite thromboxane, exhibited vasoconstrictor properties (Hamberg and Samuelsson, 1974). Monacada and Vane discovered a second vasoactive metabolite of the prostaglandins; this was demonstrated to have diametrically opposing functions to those of thromboxane. They elegantly demonstrated that endoperoxides of prostaglandin were transformed by microsomal enzymes from rabbit and pig aortas into an unstable substance that inhibited aggregation of platelets and caused the relaxation of various vessels. However, they were unable to demonstrate that arachidonic acid was a precursor, concluding that a pre-requisite conversion to the endoperoxides was required (Moncada et al., 1976a). They postulated that the balance between the pro-aggregatory vasoconstrictor thromboxane and the anti-aggregatory vasodilator PGX may be critical for formation of thrombi. Bunting et al 1976, utilising fresh vessels, confirmed the original finding of Moncada et al (1976) and demonstrated that arachidonic acid was indeed capable of metabolism to prostaglandin X (Bunting et al., 1976). This compound, initially named prostaglandin X (PGX), was later identified as a metabolic intermediate of the known compound, 6-keto-PGF1 α and renamed prostacyclin due to its chemical structure (Johnson et al., 1976).

Formation of prostacyclin:

As stated previously, the final step in the conversion of arachidonic acid to one of its biologically active compounds is dependent on a multitude of product specific enzymes. The predominance of one of these enzymes determines which prostaglandin is the major cellular product. This is best exemplified in the platelet, where the enzyme thromboxane synthetase predominates and leads to

the production of thromboxane (Ullrich and Haurand, 1983). The major prostanoid produced by endothelial cells is prostacyclin (PGI_2):- a potent vasodilator and inhibitor of platelet aggregation (Bunting et al., 1976) (Gryglewski et al., 1976) (Moncada et al., 1976a) (Moncada et al., 1976b). Although many cells within an organ will not produce these agents, all smooth muscle cells can produce prostacyclin and it is probable that all endothelial cells can produce prostacyclin and prostaglandin E_2.

Prostacyclin synthetase (cyclo-oxygenase):

Endothelial cells are known to generate PGI_2 (prostacyclin) upon stimulation by various receptor mediated ligands (Nawroth et al., 1984). Although prostaglandin production has been ascribed to phopholipases for the availability of the substrate archidonic acid, an important control mechanism exists at the cyclo-oxygenase level. Cytokines such as interleukin 1α (IL-1α) or phorbol esters have been demonstrated to induce de novo synthesis of cyclo-oxygenase in endothelial cells (Raz et al., 1988) (Wu et al., 1988) (Lin et al., 1989). However, several studies reported discrepancies in the correlation between enzyme mass and the increase in activity, thus postulating the existence of different isoenzyme of cyclo-oxygenase (Bazan et al., 1994) (Kujubu et al., 1991), which has now been confirmed to be correct (Meade et al., 1993) (Feng et al., 1993).

1.2.2.3 Endothelium-Derived Hyperpolarising Factor (EDHF):

The existence of an endothelium-derived substance which causes vasodilatation by hyperpolarisation of the vascular smooth muscle cell membrane has been postulated since the late 1970s, when acetylcholine was demonstrated to hyperpolarise vascular smooth muscle (Kuriyama and Suzuki, 1978). The discovery of nitric oxide and prostacyclin has allowed the

development of compounds that inhibit their synthesis. These have allowed a classification of the mechanism of vasodilatation to be made and have confirmed the presence of as yet unidentified endothelium-derived hyperpolarising factor (EDHF). The role of EDHF in mediation of endothelium-dependent relaxation, and the precise nature of the substance (or even whether EDHF is a group of substances) are all the subject of much current research.

The nature of EDHF:

The chemical identity of EDHF still remains obscure. Possible candidates included epoxyeicosatrienoic acids (EETs), endogenous cannabinoids, and recently two further candidates have been reported to have EDHF like functions; potassium ions and gap junctions.

Epoxyeicosatrienioc acids (EET):

It has been widely postulated that EDHF(s) derives from arachidonic acid. EETs are labile metabolites of arachidonic acid and are formed through cytochrome P450-dependent mono-oxygenase activity. Bauersachs et al (1994) showed in perfused rat coronary arteries relaxed to bradykinin that the nitric oxide and prostaglandin resistant relaxation was abolished by inhibitors of cytochrome P450 (Bauersachs et al., 1994). Campbell et al (1996) also demonstarted that inhibition of the P450 enzyme attenuated methacholine-induced relaxation of bovine coronary arteries to a similar extent as did tetraethylammonium (an inhibitor of Ca 2+-activated K+ channels) or a high [K+], both of which have been shown to prevent hyperpolaristion of the vascular smooth muscle (Campbell et al., 1996). In vessels incubated with labelled arachidonic acid, methacholine was shown to stimulate release of arachidonic acid metabolites, which induced the EETs. Four different EET isomers were found to induce relaxation in constricted bovine

coronary arteries, all to a similar extent (Campbell et al., 1996), a finding that has been confirmed in other vascular beds (Rosolowsky and Campbell, 1993) (Pfister et al., 1991) (Gebremedhin et al., 1998) (Hecker et al., 1994). ETTs have also been shown to activate potassium channels in vascular smooth muscle cells (Gebremedhin et al., 1998) (Hu and Kim, 1993), further implicating them as a possible EDHF.

Although some cytochrome P450 inhibitors inhibit the EDHF-mediated response, not all have this effect. Moreover some of these inhibitors have been shown to inhibit potassium-channels (Zygmunt et al., 1996) and their use as evidence for ETT's mediating the sole component of EDHF must therefore be questioned.

Gap Junctions:

Although the chemical identity of EDHF remains to be determined there is now increasing evidence to suggest that nitric oxide and prostanoid-independent agonist induced relaxation preferentially diffuses from the endothelium to smooth muscle via gap junctions, rather than through the extracellular space (Javid et al., 1996) (Taylor et al., 1998) (Hutcheson et al., 1999). This hypothesis has been supported by the existence of myoendothelial gap junction plagues in conduit arteries (Christ et al., 1996), and dye transfer experiments have demonstrated direct chemical coupling between the two cell types (Beny and Gribi, 1989) (Beny and Connat, 1992) (Beny and Pacicca, 1994).

Gap junctions are membrane-localised hemichannels, contributed by apposing cells. Each hemichannel is formed by 6 connexin protein subunits arranged around an aqueous central pore

that provides electrical continuity and permits intracellular movement of small molecules < 1 KDa in size. This facilitates intercellular communication and co-ordination of cell activities (Christ et al., 1996). A family of connexins, having at least 13 identified members in mammals, have been described (Christ et al., 1996) (Falk et al., 1997) (Barrio et al., 1999) (Suchyna et al., 1999) (Van Rijen et al., 1997). Three of the characterised connexins (connexin 43, connexin 40,and connexin 37) have been identified in cells of the vascular system, where they may have a role in the control of vasomotor function (Christ et al., 1996). Dantas and colleagues have recently demonstrated that there is an enhanced relaxation in the aorta of pregnant rats when compared with non-pregnant vessels. They also demonstrated that the increased relaxation in pregnant rat aorta is mediated through gap junctions; they blocked gap junctions with heptanol and the demonstration that connexin 43 mRNA was increased in these vessels. However, they demonstrated that these responses, although mediated through gap junctions were predominately nitric oxide mediated, as depolarisation with potassium had little effect on the relaxations obtained and the addition of heptanol to vessels constricted with K+ caused total loss of endothelium dependent relaxation (Dantas et al., 1999).

1.3: Vascular reactivity changes in pregnancy and pre-eclampsia:

1.3 1: General cardiovascular effects:

During pregnancy, dramatic changes occur in maternal physiology to accommodate the growing conceptus. Nowhere are these changes more evident than in the cardiovascular system. Blood volume and cardiac output increase by 40-50% (Duvekot and Peeters, 1994). These increases have occurred by the second trimester and are maintained until after parturition (Bosio et al., 1999). Blood pressure throughout pregnancy remains low until the end of the third trimester, secondary to a reduced peripheral resistance (Duvekot and Peeters, 1994). However, in pre-eclampsia, total peripheral resistance increases, causing the blood pressure to rise (Bosio et al., 1999). It has been postulated that the changes in vascular reactivity observed in both normal pregnancy and pre-eclampsia are caused by alterations in the vascular endothelium.

1.3.2 Effects of pregnancy on vasoreactivity:

Vascular reactivity to the pressor-effects of angiotensin II are decreased in pregnancy (Gant et al., 1973). This allows an increased circulating concentration of angiotensin II without an increase in peripheral resistance. The reduced sensitivity of the vascular endothelium to pressor agents appears to be selective for angiotensin II, as other agents such as noradrenaline show no alteration of their pressor responses with pregnancy (Ramsay et al., 1993). Evidence that this is mediated through changes in vascular reactivity come from the observation that rapid infusions of fluid do not alter angiotensin II sensitivity (Matsuura et al., 1983). The difficulties with *in vivo* studies in human pregnancy have led researchers to examine the vascular changes that occur in

pregnancy with indirect methods. These have included animal models and myography. Myography allows the study of vascular reactivity in *ex vivo* resistance vessels and can be used to determine the contribution of the endothelium to the observed changes.

Considering the increased accessibility of the peripheral vascular system, there has been little direct research into the vascular changes that occur in human pregnancies. However, increases in endothelial derived substances which are known to cause vasodilation have been postulated to be important in this process. The next part of this thesis will consider those directly related to this thesis. Although there are many other alternative explanations for the observed fall in total peripheral resistance, these are outside this thesis and will not be considered.

To date, few studies have addressed the possibility of pregnancy-mediated differences in endothelium-dependent relaxation to various agonists in human resistance vessels. McCarthy et al utilised subcutaneous vessels from pregnant women and compared them to similar vessels from non-pregnant women. They were unable to demonstrate differences between the groups with respect to contraction with epinephrine (noradrenaline) or relaxation to acetylcholine (McCarthy et al., 1993b). Pascoal and Umas have confirmed this observation in human omental vessels exposed to acetylcholine and bradykinin (Pascoal and Umans, 1996).

Uterine resistance vessels are juxtaposed to the developing placental bed and undergo considerable remodelling with pregnancy. Thus it is pertinent to study these vessels when examining the changes that occur during pregnancy. However, there is a paucity of data into the effect of pregnancy on myometrial resistance vessels. This is primarily due to the technical difficulties of sample collection. The studies that have investigated endothelium-dependent

relaxation in myometrial resistance arteries have utilised different vasoconstrictor and vasorelaxant agents making comparison difficult (Ashworth et al., 1996b) (Kublickiene et al., 1997) (Svane et al., 1991).

Svane, et al provided the first report concerning the vascular responses in non-pregnant and pregnant human uteri. They examined the contractile and relaxatory responses of myometrial vessels to angiotensin II, vasopressin, U46619 (a thromboxane memetic), and noradrenaline. Resistance vessels were obtained to allow comparison of the non-pregnant and pregnant state. In addition, within the pregnant group, placental and non-placental bed vessels were compared. Svane et al were unable to produce any evidence that either pregnancy or placental localisation produced any changes in the smooth muscle responses to agonists important in the control of human uteroplacental resistance (Svane et al., 1991). These findings were subsequently confirmed by Steele et al (Steele et al., 1993).

Using different endogenous vasodilators, Ashworth et al, 1996 and Svane et al, 1991 demonstrated that there do not appear to be any changes in the vasculature of the pregnant uterus. However, neither study elucidated whether the mechanism of relaxation altered with pregnancy (Ashworth et al., 1996b) (Svane et al., 1991). An alteration in mechanism of vasodilatation, without a difference in total relaxation, may have important implications for pregnancy outcome; in disease states these alterations may be absent.

1.3.3. The mechanism for the alterations in vascular reactivity in pregnancy:

1.3.3.1 Eicosanoids:

Studies of maternal plasma levels of prostacyclin have yielded conflicting results. Lewis et al, 1980 reported that plasma concentrations of the vasodilator 6-keto-prostaglandin F1α (6-keto-PG F1α), a stable metabolite of prostacyclin were increased in pregnant women when compared to non-pregnant controls (Lewis et al., 1980). Goodman et al, confirmed this finding by measuring the urinary excretion of 6-keto PGF1α (Goodman et al., 1982). However, Ylikorkala were unable to demonstrate any significant increase in the plasma levels of 6-keto-PGF1α in pregnant women (Ylikorkala et al., 1981). These differences in results are probably due to different methodologies. Several longitudinal studies have sought to examine the change of prostacyclin levels during confinement and have shown increased levels of 6-keto-PGF1α during pregnancy until the second trimester (Lewis et al., 1980) (Goodman et al., 1982).

Inhibition of eicosanoid synthesis during pregnancy alters the angiotensin II infusion sensitivity towards that of the non-pregnant state (Everett et al., 1978) (O'Brien and Pipkin, 1979). This implicates an alteration in eicosanoid synthesis as being important in the vascular adaptations of pregnancy. However, *ex vivo* studies of resistance vessels from several vascular beds have been unable to confirm these findings (Ashworth et al., 1997) (McCarthy et al., 1994) (Pascoal and Umans, 1996). Differences in results between the *in vivo* and *ex vivo* situations may have occurred due to the use of non-specific eicosanoid synthesis inhibitors, which have other non-specific *in vivo* effects.

The evidence thus supports the hypothesis that eicosanoids are important in the observed vasodilator responses observed in pregnancy. This has led to the hypothesis that altered eicosanoid production may lead to the observed hypertension that occurs in pre-eclampsia and this will be discussed later in this review.

1.3.3.2 Endothelial Derived Relaxing Factor -Nitric Oxide:

Nitric oxide status in human pregnancy is far from clear. Seligman et al (1994) reported that there was a small but significant increase in plasma nitric oxide production of non-pregnant as compared to pregnant subjects (Seligman et al., 1994) and this was confirmed by Smarasson et al (1997) who compared plasma nitrate concentrations from a cohort of non-pregnant and pregnant women using a vanadium V chloride reduction reaction and chemiluminence (Smarason et al., 1997). Several groups have demonstrated increased urinary and plasma concentrations throughout human pregnancy (Kopp et al., 1977) (Sala et al., 1995). These observations have led to the hypothesis that increased nitric oxide production mediates the vascular changes, which are so indicative of pregnancy. Myatt et al observed an increased urinary nitrate excretion in single voided urine samples (Myatt et al., 1992), however, other groups have failed to show any increase in 24-hour urinary nitric oxide concentrations between non-pregnant and pregnant controls (Brown et al., 1995b). All these studies had insufficient power to draw appropriate conclusions and only one of these studies controlled for nitrogen intake during the course of the investigation. This may have masked any significant results since a high dietary intake of nitrates causes its' increased excretion in the urine.

Steele et al (1993) examined whether pressor responses of myometrial resistance arteries were altered after removal of the endothelium or the inhibition of nitric oxide (Steele et al., 1993). Removal of the endothelium from non-pregnant women produced no significant increase in the pressor response, however, denuded pregnant vessels showed enhanced responses. This effect was independent of nitric oxide. Steele et al (1993) concluded that in the pregnant state, endothelial cells appear to modify the contractile response to noradrenaline of the maternal vascular smooth muscle through a mechanism dependent on substances other than nitric oxide (Steele et al., 1993). This may imply a role for Endothelial Derived Hyperpolarisng Factor (EDHF) in the alteration of vascular responses in pregnancy.

Mechanistic changes in vasodilatation have also been studied with the use of L-nitro L-arginine methylester (L-NAME) / N-mono-methyl-L-arginine (L-NNMA) (Nitric oxide synthetase inhibitors) and indomethacin (eicosanoid synthesis inhibitor). Neither blockade of nitric oxide nor eicosanoid synthesis altered vessel behaviour, again implicating the vasoactivity mediator EDHF as important in the physiology of these vessels (Pascoal et al., 1998) (McCarthy et al., 1993a) (McCarthy et al., 1994).

In summary, the evidence for increased production of nitric oxide accounting for the vascular changes that are observed in human pregnancy is far from convincing. This may be compared with the four-fold rise in the excretion of the major metabolite of prostacyclin (PGI_2), and 6-keto prostaglandin $F_{1\alpha}$, by the end of the first trimester. Although PGI_2 can function as a circulating hormone, nitric oxide seems more likely to be locally synthesised for a local role. Nevertheless, at present there is no clear evidence that nitric oxide increase in human pregnancy.

1.3.3.3 Endothelial Derived Hyperpolarising Factor (EDHF):

Although the chemical identity of EDHF remains to be elucidated the use of both nitric oxide synthase and cyclooxygenase inhibitors has allowed changes in the proportion of the relaxation attributable to EDHF to be investigated. Use of these inhibitors has demonstrated, in various vascular beds, that a reciprocal relationship exists between nitric oxide and EDHF mediated responses (Randall and March, 1998).

Kenny et al have recently examined the mechanism of endothelium-dependent vasodilatation in human myometrial resistance vessels. They demonstrated with the use of both the eicosanoid inhibitor, indomethacin, and the nitric oxide synthase inhibitor, L-NAME, that an increased non-nitric oxide, non-prostanoid mediated component to vascular function occurs in pregnant versus non-pregnant vessels. They postulated that these increases occur through a decrease in nitric oxide and an increase in EDHF mediated responses, in these vessels (Kenny et al., 1999b). Gerber at al have also demonstrated that in pregnant rats EDHF-dependent response predominates when compared to non-pregnant rats (Gerber et al., 1998). These studies suggest an important function for EDHF in normal pregnancy adaptation.

1.3.4. Effect of pre-eclampsia on vascular reactivity:

Pre-eclampsia is associated with an elevation of blood pressure and increasing evidence indicates an involvement of the maternal endothelium.

Although the difficulty of *in vivo* human experimentation has limited the study of vascular reactivity in this condition, several studies exist to indicate that an altered reactivity occurs in pre-eclampsia. Gant et al demonstrated that while normotensive pregnancies exhibited a decreased sensitivity to infused angiotensin II, pregnancies that were destined to develop pre-eclampsia were characterised by increased sensitivity from as early as eighteen weeks (Gant et al., 1973). In a larger study Kyle et al confirmed the enhanced sensitivity to angiotensin II in pre-eclampsia but found that as a diagnostic test, it had a much lower sensitivity and specificity than initially reported by Gant et al (Kyle et al., 1996). Increased sensitivity to infused physiological concentrations of noradenaline has also been noted in pregnancies complicated by pre-eclampsia (Kaaja et al., 1999) (Manyonda et al., 1998). This suggests that an alteration in the vascular reactivity occurs with pregnancy and that this may be lost prior to the onset of disease. As stated previously, *in vivo* studies are difficult to conduct and vascular reactivity has thus been examined by indirect methods.

Several *ex vivo* studies of resistance vessels have demonstrated an alteration in vascular reactivity in pregnancies complicated by pre-eclampsia. These studies have examined endothelium-dependent relaxation and have shown that in pre-eclampsia the agonist concentration required to obtain relaxation is significantly increased (McCarthy et al., 1993a) (Ashworth et al., 1997). This further implicates the vascular endothelium as important in the pathophysiology of this disease. These *ex vivo* studies thus confirm the observation of altered vascular reactivity and implicate the endothelium as pivot in this process.

1.4.5. The mechanism for the alterations in vascular reactivity in pre-eclampsia:

1.4.5.1 Eicosanoids

Numerous studies have attempted to assess eicosanoid status in pre-eclampsia, but there are no clear conclusions. Studies have either considered the maternal vascular compartment or the fetal vascular compartment.

In studying the maternal compartment with regard to the levels of the vasodilator prostacyclin and vasoconstrictor thromboxane, several groups have determined plasma levels. These have produced contradictory results. Several studies have demonstrated decreased prostacyclin and increased thromboxane levels (Downing et al., 1980) (Lewis et al., 1980) (Goodman et al., 1982) (Makila et al., 1984) (Malatyalioglu et al., 2000), whereas others have been unable to elicit any differences (Yamaguchi and Mori, 1985). Varriations in results may stem from the methods of determination. On balance, the evidence from plasma levels is suggestive of a change in the prostacyclin / thromboxane ratio.

The hypothesis of altered eicosanoid synthesis in pre-eclampsia has also been tested via measurement of the urinary excretion of these metabolites. In humans, urinary excretion of prostacyclin has been demonstrated to decrease in women with pre-eclampsia (Goodman et al., 1982) (Barden et al., 1994) (Mills et al., 1999b). Levels of thromboxane showed no difference in pregnancies complicated by pre-eclampsia (Barden et al., 1994) (Mills et al., 1999b). Scafer, et al

investigated the urinary excretion of both prostacyclin and thromboxane in a rat model for pre-eclampsia and demonstrated that both prostacyclin and thromboxane were decreased. They noted that despite an overall decrease in metabolites, the thromboxane / prostacyclin ratio changed in favour of the thromboxane (Scafer et al., 1993).

The observation that prostacyclin production is reduced in pre-eclampsia led investigators to determine if the endothelium was responsible for this altered production. This has been achieved via two methodologies, *ex vivo* resistance arteries and *in vitro* cell culture experiments. *Ex vivo* resistance artery experiments have produced contradictory results. Ashworth et al reported enhanced relaxation of myometrial vessels from women with pre-eclampsia to bradykinin in the presence of indomethacin and from this concluded that vasoconstrictor eciosanoids were partially responsible for the observed reduced relaxation (Ashworth et al., 1997). Other workers, utilising different vascular beds, have been unable to demonstrated alterations in vasodilation upon the addition of eicosanoid synthatase inhibitors (Pascoal et al., 1998) (Knock and Poston, 1996). Ashworth et al can be criticised in their protocol for not using correct time control experiments, and so the observed effect of indomethacin may be due to a return to normal vasodilatation of resistance vessels.

Cell culture experiments have demonstrated that endothelial cells exposed to plasma from women with pre-eclampsia increase their production of the vasodilator prostacyclin (Baker et al., 1996a). This is contrary to the original hypothesis and led Baker et al to investigate this further. Baker et al conducted time course experiments and showed that at twenty-four hours an initial increase in the prostacyclin production was observed. This was followed at forty-eight hours by a reduction in prostacyclin production when compared to controls (Baker et al., 1996a).

1.4.5.2 Endothelial Derived Relaxing Factor -Nitric Oxide:

The role of nitric oxide in the pathogenesis of pre-eclampsia is equally confusing. Several studies have demonstrated that rats infused with L-NAME or various other inhibitors of nitric oxide synthetase exhibit signs that are similar in character to pre-eclampsia (Yallampalli and Garfield, 1993) (Molnar et al., 1994) (Buhimschi et al., 1995) (Zhang and Kaufman, 2000). These include increased systemic blood pressure and proteinuria. This has led to the hypothesis that a decreased nitric oxide production causes the observed vascular changes of pre-eclampsia. One of many problems with the rat model is the fact that it is difficult to extrapolate the findings to humans since rats do not suffer pre-eclampsia. Another problem is with the fact that if male rats are infused with L-NAME similar results are obtained with increased blood pressure (Huang et al., 1995) (Sventek et al., 1997). Pre-eclampsia is specific to pregnant women and therefore any model of this disease that is not pregnancy or even gender specific must be questioned.

The hypothesis that decreased nitric oxide production causes the observed vascular changes that occur in pre-eclampsia has been investigated with varying results. Several studies have examined urinary nitric oxide production as representative of *in vivo* nitric oxide production. Brown et al studied twenty-four hour urinary nitrite/nitrate concentrations in women with varying degrees of pre-eclampsia and compared these to levels of women in the third trimester of normal pregnancies (Brown et al., 1995b). They were unable to show any differences between groups, either when nitrate was expressed alone or as a fraction of creatinine clearance. The expression of results as a function of creatinine clearance negates the problem of reduced renal output in pre-eclampsia. Brown et al concluded that no increase in nitrate/nitrite production occurred in pre-

eclampsia. However, Davidge et al (1996) demonstrated that urinary nitrate / nitrite excretion was decreased in women with pre-eclampsia. Interestingly, they were unable to show significant differences in plasma concentrations between patients with pre-eclampsia and normotensive controls (Davidge et al., 1996b).

The postulated altered production of nitric oxide in pre-eclampsia has also been investigated via the direct measurement of serum nitrites / nitrates and indirectly by cGMP levels. Seligman et al (1994) utilised the Griess reaction and showed that there was a significant reduction in nitrates in the serum of women with pre-eclampsia as compared to normotensive controls (Seligman et al., 1994). However, a more recent study demonstrated that there was an increase in the nitrate levels of women with pre-eclampsia (Smarason et al., 1997). The differences in these studies may again be due to the small numbers investigated. This is of special relevance, given that any differences observed are small. Furthermore, the study of Seligman et al was confounded by the administration of magnesium sulphate to their cohorts prior to blood sampling. Endothelial nitric oxide is calcium dependent and so magnesium may have the effect of reducing endothelial nitric oxide synthase activity. Studies that have measured cGMP provide an indirect measurement of nitric oxide. These have shown that an increase in cGMP occurred in pre-eclampsia (Begum et al., 1996). One further methodological problem not addressed by any of these studies is the lack of control of dietary nitrogen intake, which may bias results with exogenous nitrites. Conrad et al (1999) have recently addressed this and demonstrated that in women with pre-eclampsia there is no alteration in either nitric oxide or cGMP (Conrad et al., 1999).

Evaluations of the mechanistic changes of resistance vessels in pre-eclampsia have provided contradictory results with respect to nitric oxide, probably due to the different vascular beds

studied. Ashworth, et al (1997) reported that myometrial vessels exhibited two possible alterations in mechanism when bradykinin was used as the vasodilator. They showed that eicosanoids may be important as discussed previously. They also showed that the addition of L-NMMA attenuated the endothelium dependent relaxation in vessels from women with pre-eclampsia. From this they concluded that there was a compensatory increase in the nitric oxide component (Ashworth et al., 1997). However, other studies of the mechanisms have failed to demonstrate differences in either eicosanoid or nitric oxide mediated relaxation in systemic vessels (McCarthy et al., 1993a). The resistance to eicosanoids and nitric oxide synthetase inhibitors in normal pregnant and pre-eclamptic blood vessels (Ashworth et al., 1997) must indicate a role for either EDHF or another endothelial-derived product in both pregnancy and pre-eclampsia.

Increased nitric oxide production by the endothelium has been confirmed by cell culture. Baker et al have shown that endothelial cell exposure to the plasma of women with pre-eclampsia generated increased concentrations of nitric oxide when compared with exposure to control plasma. They postulated that this increase in nitric oxide may be detrimental to endothelial cells function through the generation of peroxynitrites (Baker et al., 1995b). Roggensack et al have demonstrated that vessels isolated from women with pre-eclampsia have increased expression of endothelial peroxynitrite, further implicating an abnormal nitric oxide respond in the pathogenesis of this disease (Roggensack et al., 1999).

1.4.5.3 Endothelial Derived Hyperpolarising Factor (EDHF):

To date there are no studies that have directly addressed the role of EDHF in the pathogenesis of pre-eclampsia, although several studies have produced results that have led to the hypothesis that it may be important in the pathophysiology of this disease. McCarthy et al (1993) were the first to suggest a possible role for EDHF in pre-eclampsia. They demonstrated that concentration response curves to acetylcholine from subcutaneous resistance vessels were attenuated in women with pre-eclampsia when compared to normal pregnant controls. They also demonstrated that these observed alterations in endothelial function were independent of eicosanoids and nitric oxide, postulating that they may be due to alterations in EDHF-mediated relaxation. This observation of an alteration in non-eicosanoid, non-nitric oxide dependent relaxation in pre-eclampsia has been confirmed in the omental and myometrial vascular beds (Ashworth et al., 1997) (Pascoal et al., 1998). However, until the chemical nature and specific inhibitors of EDHF are discovered, all studies will remain as indirect conjecture.

In summary the literature suggests that an alteration in vascular reactivity occur in both pregnancy and pre-eclampsia. However, the mechanism of this alteration remains unclear. With pre-eclampsia, as stated previously, there may be a circulating factor that causes the changes in endothelial cell function and vascular reactivity. One such candidate is Vascular Endothelial Growth Factor.

1.5 Vascular Endothelial Growth Factor (VEGF): biological structure:

1.5.1 Vascular Endothelial Growth Factor:

1.5.1.1 Discovery of VEGF:

Ferrara et al first reported Vascular Endothelial Growth Factor (VEGF) in 1989. They demonstrated that medium exposed to bovine pituitary cells was capable of initiating a mitogenic effect that was specific to endothelial cells (Ferrara and Henzel, 1989). However, VEGF had been reported previously in a different context. Senger et al (1983) demonstrated that isolated mammalian tumour cells produced a factor that increased vascular permeability (Senger et al., 1983) (Senger et al., 1986) and termed this Vascular Permeability Factor (VPF).

Connolly et al extended this early work and showed that isolated VPF was capable of producing an endothelial specific mitogenic effect (Connolly et al., 1989a). They postulated that VPF and VEGF might be one and the same as they shared a common NH_2 terminal sequence. Keck et al (1989) confirmed this hypothesis by demonstrating that these factors shared a common gene and molecular weight (Keck et al., 1989).

1.5.1.2 VEGF gene:

The VEGF gene has been isolated from a variety of tissues: mouse, rat and guinea-pig (Leung et al., 1989) (Keck et al., 1989) (Senger et al., 1990) (Conn et al., 1990) (Breier et al., 1992). In

humans it originates from a single gene located on chromosome 6 (Keck et al., 1989) (Mattei et al., 1996).

1.5.1.3 Structure of Vascular Endothelial Growth Factor:

Protein purification yielded a 45-46 kiloDalton (kD) product and subsequent secondary structure analysis revealed a dimeric compound composed of two 23 kd subunits which were cross-linked with disulphide bonds (Ferrara and Henzel, 1989) (Gospodarowicz and Lau, 1989).

Primary (amino acid) sequence analysis of VEGF shows there are eight conserved cysteine residues. These are integral for the inter- and intra- molecular bridging (secondary structure). Platelet derived growth factor (PDGF) has also been shown to contain eight conserved cysteine residues. A comparison of DNA sequence between PDGF and VEGF showed a striking conservation of all eight cysteine residues. Further sequence analysis of VEGF and the two PDGF chains revealed that there was a 21% and 24% homology when the amino and carboxyl terminal regions of VEGF extending beyond the mature form of PDGF were removed (Tischer et al., 1989). This led to the conclusion that VEGF and PDGF are from the same family of cytokines.

1.5.1.4 VEGF splice variant:

Analysis of the human VEGF gene demonstrated that the protein coding regions are arranged in eight exons (Tischer et al., 1991). Alternative splicing of these exons generates at least five different mRNA for VEGF, which encode 121,145,165,189, and 206 amino acids respectively (Leung et al., 1989) (Keck et al., 1989) (Tischer et al., 1991) (Houck et al., 1991). An important

biological property that distinguishes these VEGF isoforms is their ability to bind heparin and heparan-sulphate. The VEGF $_{121}$ transcript, with exon 6 and 7 deleted, encodes a weakly acidic and free soluble polypeptide that fails to bind to heparin. The VEGF $_{145}$ transcript has the exon 7 deleted but contains exon 6 (Charnock-Jones et al., 1993). The amino acids encoded by exon 6 contain à heparin binding domain and also elements that enable the binding of VEGF$_{145}$ to extracellualr matrix (Poltorak et al., 1997). VEGF $_{165}$, with exon 6 deleted but including the cationic polypeptide sequence encoded by exon 7, is a basic and heparin-binding form, and a significant fraction remains bound to the extracellular matrix. All VEGF 121,145 and165 are able to induce proliferation of endothelial cells and *in vivo* angiogenesis (Park et al., 1993) (Zhang et al., 1995) (Poltorak et al., 1997). The full transcript, containing exons 1 to 8, encodes the VEGF $_{189}$ isoform. The additional 17-amino acids insertion in VEGF $_{206}$ is encoded by a 51 base pair nucleotide sequence which begins at its 5' –end with GT, a consequence sequence for the 5'-splice donor site necessary for RNA processing (Houck et al., 1991). Both VEGF 189 and VEGF 206 are secreted but are more basic than the other isoforms and are almost completely bound to the cell surface and the extracellular matrix (Houck et al., 1992) (Park et al., 1993). This may account for their observed lack of mitogenic activity on endothelial cells (Houck et al., 1992). However, the longer isoforms can be released by proteases after cleavage at the C-terminus, which generates an isoform comprised of the first 110 NH2-terminal amino acids of VEGF by plasmin (Keyt et al., 1996) or a 38 kDa fragment by urokinase (Plouet et al., 1997). This may be important in tumours where increased expression of proteases, such as plasminogen activators (PA), has been reported. However, loss of heparin binding, by alternative splicing of RNA or plasmin cleavage, results in a decreased mitogenic activity for vascular endothelial cells (Keyt et al., 1996). All five isoforms of VEGF have been shown to posses vascular permeability activity as

demonstrated by the Miles assay of dye extravasation and mitogenic activity (Houck et al., 1991) (Poltorak et al., 1997).

1.5.2 Regulation of VEGF:

1.5.2.1 Regulation of Vascular Endothelial Growth Factor:

Increased *in vivo* expression of endogenous VEGF has been documented in many pathological tissues (Brown et al., 1992) (Hashimoto et al., 1994) (Plate et al., 1992) (Shweiki et al., 1992) (Banai et al., 1994) (Detmar et al., 1994) (Pe'er et al., 1995). Moreover, increased expression of VEGF receptor mRNA has also been demonstrated to be endothelial cell specific in the same tissues (Plate et al., 1992) (Brown et al., 1995a) (Tuder et al., 1995) (Wizigmann-Voos et al., 1995). This implies that the control of VEGF action is affected by both the expression of VEGF and its receptors.

1.5.2.2 Factors effecting the expression of VEGF:

The female reproductive tract undergoes cyclical changes that are associated with extensive modification of vascular structure. This provides an excellent *in vivo* environment for the study of angiogenesis. Sheweki et al (1993), utilising this phenomenon, reported that sex steroids either directly or indirectly effect VEGF expression in mouse uterus and ovary. Using in situ hybridisation and immunostaining, they showed that during the menstrual cycle mRNA expression for VEGF increased in tandem with oestrogen and progesterone levels (Shweiki et al., 1993). Ovarian steroids and VEGF will be discussed further in this chapter, when the role of VEGF in the reproductive tract is considered.

Although physiologically the sex steroids have been shown to regulate expression of VEGF, in pathological states hypoxia has been implicated in this process (Shweiki et al., 1992) (Brogi et al., 1996). Utilising Western Blot and RNAase protection assays to assess protein and mRNA production, Minhenko et al (1994) exposed cultured endothelial and non-endothelial cells to hypoxic conditions and demonstrated that VEGF was significantly increased in all cell lines, except those of endothelial origin, which showed only weak expression after 24 hours stimulation (Minchenko et al., 1994b). In contrast several other groups have demonstrated that endothelial cells on hypoxic stimulation increase VEGF production. Ladoux et al (1993) have identified transcripts for VEGF in hypoxic rat brain endothelial cells (Ladoux and Frelin, 1993) and Namiki et al demonstrated that cultured micro-and macro vascular endothelial cells require hypoxic exposure of up to 48 hours to reach maximum VEGF protein and mRNA expression (Namiki et al., 1995). Comparison of Namiki et al results after 24 hours showed similar profiles to Minchenko et al (1994) with only a minimal increase in expression. From this they postulated that hypoxic driven angiogenesis is a chronic adaptive process aimed at increasing perfusion to ischaemic tissues (Namiki et al., 1995). Another noteworthy aspect of their study was that microvascular endothelial cells behaved differently to macrovascular cells increasing their production of VEGF within 24 hrs. This may represent the fact that the human microvascular system requires a greater capacity to undergo vascular remodelling in response to hypoxic insults than the macrovascular system. This also emphasises the fact that the correct cell type should be examined when considering endothelial function.

Several studies have extended these *in vitro* observations and examined the effect of hypoxia on VEGF production in an *in vivo* system (Minchenko et al., 1994c) (Tuder et al., 1995). Minchenko

et al (1994) demonstrated, utilising an RNAase protection assay, that hypoxia produced increases in the expression of VEGF in rat organs such as the heart, liver, brain, kidneys, and muscle (Minchenko et al., 1994a) (Minchenko et al., 1994c). Tuder et al exposed rats to low oxygen tensions for varying periods using a barometric chamber. After exposure, lungs were rapidly harvested and using in situ hybridisation, VEGF production was shown to increase in a time dependent manner within alveolar epithelial cells. Northern Blot analysis was used to confirm their original qualitative observation effect (Tuder et al., 1995). Acute hypoxic insults have also been examined in an isolated perfused rat lung system and these produced comparable results to chronic stimulus (Tuder et al., 1995).

The cellular mechanism regulating VEGF expression has been postulated to be similar to that of the erythropoietin gene (Epo) (Minchenko et al., 1994c) (Namiki et al., 1995). The Haem protein acts as the oxygen sensing mechanism for the Epo gene. Haem undergoes structure changes on the binding of Co^{2+} and Ni^{2+} and this stimulates the production of Epo in an analogous manner to hypoxia (Goldberg and Schneider, 1994). Evidence to support this hypothesis is provided from cell culture work, which demonstrates that the presence of cobalt chloride or nickel enhance VEGF expression (Minchenko et al., 1994c) (Namiki et al., 1995).

A further mediator of transcriptional responses to hypoxia is hypoxia-inducible factor-1 (HIF-1), whose binding sites have been identified in the 5'-promoter of the rat and human VEGF gene (Liu et al., 1995) (Forsythe et al., 1996). The accumulation of adenosine under hypoxic conditions has also been shown to induce VEGF expression (Takagi et al., 1996). In addition to increased expression of VEGF by cells, it has recently been demonstrated that hypoxia causes increased mRNA stability (Ikeda et al., 1995) (Stein et al., 1995) (Levy et al., 1996b). Sequences that

mediate increased stability have been identified in the 3'-untranslated region of VEGF mRNA (Levy et al., 1996b). A hypoxia– induced protein, HuR, which binds to this region and stabilises VEGF mRNA, has also been identified (Levy et al., 1996a) (Levy et al., 1998).

A variety of cytokines and growth factors have been shown to modulate VEGF expression in various cultured endothelial cells. Factors that can up-regulate VEGF expression and induce the release of VEGF protein include EGF (Goldman et al., 1993), TGF-α (Detmar et al., 1994), TGF - β (Pertovaara et al., 1994), keratinocyte growth factor (KGF) (Frank et al., 1995), IL-1α (Ben-Av et al., 1995), IL-6 (Cohen et al., 1996), IGF-I (Warren et al., 1996), TNF-α (Ryuto et al., 1996). Additionally, some small molecules such as phorbol esters (Tischer et al., 1991) (Finkenzeller et al., 1992), prostaglandin E_1 and E_2 (Harada et al., 1994) have been shown to modulate VEGF expression. Another small molecule that up-regulates VEGF expression is nitric oxide, which is in turn up-regulated by VEGF (Chin et al., 1997) (Hood et al., 1998).

1.5.3 VEGF receptors:biological function and regulation:

1.5.3.1 VEGF receptors:

Radiolabelling studies have identified high and low affinity binding sites for ^{125}I-labelled VEGF $_{165}$ on various endothelial cells. The dissociation constants for these vary from 10^{-12} to $9x10^{-12}$ M for the high affinity receptor and 10^{-11} to $9x10^{-11}$ M for the low affinity receptor (Vaisman et al., 1990). Receptor densities have been established for both receptor types and range from 3,000-25,000 for the high affinity receptor and 3,000-18,000 for the low affinity receptors (Vaisman et al., 1990).

Cross-linking experiments with VEGF $_{165}$ to bovine vascular endothelial cells show three high molecular weight complexes of 225,195,175 kD. From these weights it was deduced that the receptor sizes were in the order of 180,150,130 kd (Vaisman et al., 1990) (Myoken et al., 1991). As VEGF shared a close structural homology to PDGF it was originally suggested they might share a common receptor system. However, this was proven to be incorrect with the demonstration that when mRNA for the fetal liver tyrosine-kinase (flt) like gene was expressed on the Zenopus oocyte and stimulated with VEGF it caused a five-fold increase in Ca^{2+} efflux (Shibuya, 1990). These *in vitro* experiments support but do not confirm the hypothesis that the flt-1 receptor is a native VEGF receptor. Subsequent receptor purification and cross-linking studies have demonstrated that the flt-1 receptor is VEGF specific on endothelial cells and receptor antagonists have confirmed this finding (Clauss et al., 1990).

Alternative splicing of flt-1 pre-mRNA also generates at least three soluble forms of this receptor that were first identified as a rare cDNA in an endothelial cell cDNA library (Kendall and Thomas, 1993) (Boocock et al., 1995) (Kendall et al., 1996). Soluble flt-1 (sflt-1) contains the six N-terminal immunoglobulin (Ig)-like extracellular loops followed by a unique 31-amino acid residue C-terminal sequence (Kendall and Thomas, 1993). The protein encoded by this cDNA was also identified in the conditioned medium of human umbilical vein endothelial cells (HUVEC) (Kendall et al., 1996). Kendall et al (1996) demonstrated that the sflt-1 binds VEGF with a high affinity (Kd 10-20 pM) and is able to inhibit VEGF-induced *in vitro* endothelial mitogensis at concentrations that are substoichiometric to VEGF (Kendall and Thomas, 1993). In addition to inhibiting VEGF binding to cell surface receptors by sequestering it, sflt-1 also forms dominant-negative heterodimers with full-length VEGF receptors, including dimerization with the mitogenically competent Kinase Domain Receptor (KDR) receptor (Kendall et al., 1996).

The Kinase Domain Receptor (KDR), a novel endothelial cell specific receptor of the tyrosine kinase gene family had previously been demonstrated by PCR techniques (Terman et al., 1992). Cross-linking experiments in an expression vector were used by Terman et al (1992) to establish that the KDR receptor was also specific for VEGF (Terman et al., 1992). The *in vivo* importance of VEGF in vasculogenesis and angiogenesis acting through the KDR receptor has been confirmed in mice (Millauer et al., 1993).

The KDR and flt-1 receptors share structural similarities. Both have been shown to be type III receptor tyrosine kinases with inserted kinase domains of similar length. Each has been demonstrated to contain seven extracellular immunoglobulin-like domains, which exhibit high sequence homology (Terman et al., 1992) (Shibuya, 1990). Heparin also appears to have a differential effect on VEGF $_{165}$ binding with enhancement to KDR expressing cells and inhibition to flt expressing cells (Gitay-Goren et al., 1992) (Terman et al., 1994).

VEGF receptor expression was initially thought to be limited to the endothelium. However, several reports now refute this, with receptors being reported on leucocytes, melanoma cells, trophoblast and oesteoblasts (Clauss et al., 1990) (Gitay-Goren et al., 1993) (Shore et al., 1997a) (Athanassiades et al., 1998) (Bellamy et al., 1999). Although VEGF binding to these cells does not stimulate proliferation it has been demonstrated to confer other functional properties; monocyte chemokinesis and oesteoblast differentiation (Clauss et al., 1990) (Wang et al., 1997).

The first step in ligand-dependent activation of receptor tyrosine kinases is receptor dimerization and 'autophosphorylation' at specific residues which has been shown to involve mainly transphosphorylation of one receptor by its dimerized partner (Heldin, 1995). Presumably VEGF receptors are activated in a similar fashion, but there are as yet no studies of VEGF receptor activation, and the sites of phosphorylation in mammalian cells have not yet been mapped. The bacterially expressed cytosolic domain of KDR undergoes autophosphorylation at tyrosine residues 951, 996, 1054, and 1059 (Dougher-Vermazen et al., 1994).

VEGF-activated endothelial cell receptors phosphorylate several cytoplasmic proteins including some that contain receptor phosphotyrosine–binding SH2 domains and can participate in downstream signal transduction (Guo et al., 1995). These tyrosine-phosphorylated proteins include phosphatidylinositol 3-kinase, which phosphorylates phosphotidylinositols to produce two products; a phosporylated inositol ring and phospolipase C (PLC) (Guo et al., 1995) (Kroll and Waltenberger, 1997). VEGF, probably acting predominantly through KDR, activates the MAP kinase cascade and is able to stimulate the PLC-Y pathway leading to the generation of inositol 1,4,5-trisphosphate, mobilization of Ca^{2+} from intracellular stores, and activation of protein kinase C (Xia et al., 1996). The differences in KDR and flt-1 signal transduction that account for disparity in mitogenic signal generation are not yet known.

A third class of VEGF receptor has recently been characterised. This new class is a low affinity receptor that appears to be specific for $VEGF_{165}$ and has been isolated on endothelial cell and tumour cells (Soker et al., 1996). This has been demonstrated to be Neuropilin – 1(Soker et al., 1998), which has previously been identified to be a neuronal cell guidance receptor for the

collapsin / semaphorin family (He and Tessier-Lavigne, 1997) (Kolodkin et al., 1997). Soker et al, 1998 demonstrated that that neuropilin was an isoform specific for VEGF $_{165}$, but not VEGF $_{121}$ (Soker et al., 1998). It binds to VEGF $_{165}$ via the VEGF exon 7-encoded domain, with a Kd of about 2-3x 10^{-10} M (Soker et al., 1996). Neuropilin-1 has also been shown to bind to the heparin binding form of PlGF, PlGF-2 but not for PlGF-1 (Migdal et al., 1998). Neuropilin is expressed by endothelial and tumour cells, such as breast carcinoma, prostate carcinoma, and melanoma.

Soker et al (1998) proposed that neuropilin-1 may not function as an independent receptor as no responses were obtained when cells expressing only this receptor were stimulated with $VEGF_{165}$ (Soker et al., 1998). However, gene disruption studies have shown that in mouse embryos lacking functional neuropilin –1 genes the cardiovascular system fails to develop properly (Kawasaki et al., 1999). These observations led Soker et al 1998 to postulate that neuropilin was a $VEGF_{165}$ co-receptor. Soker et al (1998) provided evidence to support this hypothesis by demonstrating that neuropilin –1 enhanced the binding of VEGF $_{165}$ to the KDR receptor, and that this binding enhanced chemotactic and mitogenic activity (Soker et al., 1998).

1.5.3.2 Factors regulating expression of VEGF receptors:

Biological responses to VEGF may depend on either the overall cellular expression of each receptor or the relative expression to each other, in a similar manner to VEGF itself (Chapter 1.5.2). Receptor expression appears to be controlled by tissue oxygen tension. In 1996, Brodgi et al were the first to report a paracrine *in vitro* regulatory effect of hypoxia on the production of VEGF receptors (Brogi et al., 1996). They demonstrated that hypoxic conditioned media from smooth muscle cells caused a 13-fold increase of the KDR, but not the flt-1 receptor expression,

in cultured macro and microvascular endothelial cells. This response was evident within 4 hours and peaked at 24 hours. However, culturing endothelial cells in hypoxic conditions had no effect on KDR or flt-1 receptor expression. From this they concluded that the smooth muscle cells produced a soluble factor that evokes this response and hypothesised that this was cytokine mediated. However, direct stimulation with VEGF, TNF-alpha, TGF beta 1, BFGF or with antibodies to the cytokines, was unable to effect the KDR receptor expression (Brogi et al., 1996).

Waltenberger et al, 1996 also examined the effect of hypoxia on cultured endothelial cells. However, they obtained contrasting results to Brodgi et al (1996). They demonstrated that endothelial cells cultured in the presence of low oxygen tensions exhibited up-regulation of KDR, but not flt-1 receptors (Waltenberger et al., 1996). Methodological differences may explain these observations, with Waltenderger et al using lower oxygen tensions than Brogi et al (1996). When the biology of VEGF is considered these differing results may produce a hypothesis. VEGF functions to enhance blood supply to oxygen deficient areas; as such a paracrine factor would be of benefit to enhance distal blood supply. However, if the oxygen tension in the distal tissues was to drop further, then a subsequent enhancement is required, and this may occur with the direct up-regulation of KDR by hypoxia. It has become apparent that individual vascular beds function differently and therefore results may be dependent on the endothelial cell which is studied. Cultured bovine retinal endothelial cells, which express only KDR receptors, exhibited down-regulation of these receptors with acute exposure to hypoxia assessed via Northern blotting (Takagi et al., 1996). This has also been demonstrated in an *in vivo* system, were transient ischemia in rats, produced by raised intraocular pressure, causes a down-regulation of both VEGF and KDR mRNA (Ogata et al., 1998). However, Takagi et al (1996) in their culture system, demonstrated that VEGF binding increased with chronic hypoxia, without an increase in the

mRNA expression. This indicates that post-translation events may also occur to increase membrane expression of the KDR receptor (Takagi et al., 1996).

In vitro cell culture studies have demonstrated that KDR receptor membrane expression is oxygen sensitive. Tuder et al, used an animal model to examine the *in vivo* situation. Utilising Northern blotting they showed that exposure to both acute and chronic hypoxia increased in vivo concentration of the KDR receptor rat lungs (Tuder et al., 1995). Moreover, recently the role of hyperoxia has been demonstrated to control the expression of both the VEGF receptor types in rat lungs. Klekamp et al, 1999 showed that the expression of VEGF, flt-1 and KDR were significantly decreased with chronic hyperoxia and postulated that this may explain the detrimental effects of oxygen therapy (Klekamp et al., 1999).

Although hypoxia appears to be an important regulator of both VEGF and its KDR receptor, other substances have also been show to effect expression. Tumour Necrosis Factor α (TNF α) is one such substance. TNF α is an inflammation and neoplasia-associated cytokine that alters expression of many genes (Vilcek and Lee, 1991) and can promote or inhibit endothelial cell growth and angiogenesis, depending on the system under study (Klagsbrun and D'amore, 1991). Patterson et al were the first to report TNF α effect on VEGF receptor expression. They initially demonstrated that VEGF-stimulated DNA synthesis was inhibited in a dose dependent manner by the addition of TNF α (Patterson et al., 1996). Further investigation of this process, utilising Northern Blot analysis, showed it to be secondary to the down-regulation of both the KDR and flt receptor transcription (Patterson et al., 1996). However, higher doses of TNF α have been observed to upregulate angiogenesis through increased expression of KDR receptor (Yoshida et

al., 1997) (Giraudo et al., 1998). Although the effect of TNF α on endothelial cell angiogenesis remains to be completely understood, it is evident that it may have a biphasic response, initially inhibiting proliferation and then enhancing it.

Despite our incomplete understanding of the regulatory mechanisms of both VEGF and its associated receptors, it is evident that this process it complex and multifactorial in nature

1.5.4 Other members of the VEGF family:

Four VEGF-related proteins have been identified, these are PlGF, VEGF-b/VRF, VEGF-C/VRP and VEGF-D/ FIGF.

1.5.4.1 VEGF–B:

VEGF-B, also named VRF (VEGF-related factor), is a secreted protein but lacks consensus sequence for N-linked glycosylation. VEGF-B is thus unlike other members of this family (Olofsson et al., 1996b). Two alternatively spliced forms have been identified, with 167 and 188 amino acid residues (Olofsson et al., 1996b). VEGF-B forms disulphide-linked homodimers, and is able to form heterodimers with VEGF when co-expressed (Olofsson et al., 1996b) (Olofsson et al., 1996a). However, it has not been established if naturally occurring VEGF/VEGF-B heterodimers exist. VEGF-B binds to flt-1, but not to KDR and flt-4 receptors (Olofsson et al., 1996a). Recombinant VEGF-B_{167} stimulates thymidine incorporation into DNA in human umbilical vein endothelial cells and bovine capillary endothelial cells, suggesting that it is an endothelial mitogen (Olofsson et al., 1996a). However, VEGF-B_{186} homodimers have no detectable

mitogenic activity on endothelial cells (Olofsson et al., 1998). VEGF-B is primarily expressed in the skeletal muscle and myocardium as a membrane-bound protein which can be released by heparin, and is co-expressed with VEGF in many tissues (Olofsson et al., 1996a). VEGF-B is also expressed in human placenta (Vuorela et al., 1997), human granulosa-luteal cells (Laitinen et al., 1997), and most tumours analysed (Salven et al., 1998). Expression of VEGF-B is not responsive to hypoxia, and its mRNA was found to be unusually stable (half-life more than 8 hours), suggesting chronic rather than acute regulation (Enholm et al., 1997).

1.5.4.2 VEGF-C:

VEGF-C, also named VRP (VEGF related protein), is a secreted protein with 339 amino acids residues and has a 32% identity to VEGF (Joukov et al., 1996) (Lee et al., 1996). It is a ligand for flt-4 (Pajusola et al., 1994) (Mustonen and Alitalo, 1995), and KDR (Joukov et al., 1996). VEGF-C stimulates the growth of human lung endothelial cells but with less potency than $VEGF_{165}$, and the migration of bovine capillary endothelial cells in collagen gel (Joukov et al., 1996). VEGF-C, like VEGF, is also a vascular permeability factor (Joukov et al., 1996).

The expression of VEGF-C is associated with the development of lymphatic vessels. Its mRNA was detected in mesenchymal cells of post-implantation mouse embryos, particularly in the regions where the lymphatic vessels undergo sprouting from embryonic veins, and in the adult mouse lung, heart and kidney, where flt-4 was also predominant (Kukk et al., 1996). Expression of VEGF-C has also been demonstrated in human placenta (Vuorela et al., 1997), human granulosa-luteal cells (Laitinen et al., 1997), CD 34 + cells, platelets and T-cells (Wartiovaara et al., 1998). Cytokines, including interleukin (IL)-1α, and tumour necrosis factor-α, can induce

VEGF-C expression (Ristimaki et al., 1998). Hypoxia, which is an important inducer of VEGF expression, has no effect on VEGF-C expression (Enholm et al., 1997).

1.5.4.3 VEGF-D:

VEGF –D, also called FIGF (c-fos-induced growth factor), a new member of the VEGF family and closely related to VEGF-C, is a mitogen for fibroblasts and endothelial cells (Orlandini et al., 1996) (Yamada et al., 1997). It can activate KDR and flt -4, but does not bind to flt-1 (Achen et al., 1998). In adult human tissues, VEGF-D mRNA is mainly expressed in heart, lung, skeletal muscle, colon and small intestine (Yamada et al., 1997) (Achen et al., 1998).

1.5.4.4 Placental Growth Factor:

Placenta growth factor was first described in 1991 (Maglione et al., 1991). At that time it was thought to be specific to placenta and as such was termed Placenta Growth Factor (PlGF). This is now known not to be the case as several tumour cells, including renal cell carcinoma and thyroid tumours, express it (Viglietto et al., 1997).

Structure of Placenta Growth Factor:

Placenta Growth Factor (PlGF) is a 149-amino acid sequence protein which when analysed demonstrates a sequence homology of 53% to VEGF. This increases to 71% when the conserved amino acids are considered (Maglione et al., 1991). These observations led to the inclusion of PlGF in the VEGF family of proteins. PlGF's have been shown to be dimeric glycoproteins that contain N-terminal hydrophobic secretory leader sequences, which promote

active secretion. PlGF gene contains 7 exons that can generate three alternatively spliced forms, PlGF 149, 170 and 221 (also called PlGF –1, -2 and –3) (Maglione et al., 1993) (Cao et al., 1997). PlGF has high affinity binding to the flt-1 receptor with the Kd in the range of 100-200 pM, but not to the KDR receptor (Park et al., 1994).

Observations of molecular mass indicate differences in the secondary structure of PlGF and VEGF. PlGF contains two extra carbohydrate moieties as compared to VEGF, which contains only one. This difference in secondary structure may account for the proposed variations in the function of PlGF as compared to VEGF. PlGF has been established as a dimeric structure similar to VEGF and has recently been demonstrated to form dimers with VEGF *in vitro* and *in vivo* (Maglione et al., 1991) (DiSalvo et al., 1995). This only serves to highlight the emerging similarities between VEGF:PlGF and may account for differing biological functions.

PlGF Receptors:

The close sequence homology between VEGF and PlGF led to the hypothesis that these two growth factors may initiate their biological function through the same receptor system. Kendel et al (1994) demonstrated that like VEGF, PlGF was capable of binding to the flt-1 receptor but was unable to bind the KDR receptor system (Kendall et al., 1994). Park et al (1994) showed that PlGF, although capable of binding to the flt-1 receptor was unable to initiate a stimulus via tyrosine phosphorylation. However, they demonstrated that below threshold concentrations of VEGF in the presence of equimolar PlGF-1 initiated both permeability and mitogenicity and concluded that PlGF-1 had a regulatory effect. This effect of PlGF on VEGF is not synergistic, as it is smaller than the maximal effect of VEGF alone. The authors postulated that the binding of

VEGF to the flt-1 receptor may act to reduce its the biological activity, in an analogous manner to the interleukin system, with flt-1 receptor acting as a decoy and possessing no biological activity. However, they fail to appreciate that the binding kinetics of VEGF and PlGF-1 are similar and that for this system to operate effectively, the PlGF-1 constant would have to be large in comparison to VEGF. This then allows for adequate displacement of VEGF from the flt-1 receptor. Moreover, PlGF-1 produced by eukaryotic cells has recently been demonstrated to induce angiogenesis in the rabbit cornea and chick chorioallantoic membrane assays and also has a high mitogenic effect on cultured endothelial cells (Ziche et al., 1997). PlGF in these assays was demonstrated to be as potent as VEGF (Ziche et al., 1997). The authors suggested the observed differences were due to human recombinant PlGF-1 lacking the two carbohydrate moieties. This is supported by the finding that PlGF-1 stimulates MAP kinase and mitogenicity of porcine aortic endothelial cells expressing flt-1 *in vitro* (Landgren et al., 1998).

PlGF transcripts are expressed abundantly by villous trophoblasts of human term placenta (Khaliq et al., 1996) (Vuorela et al., 1997) (Shore et al., 1997b) (Clark et al., 1998b). Expression of PlGF was also detected in human vascular endothelial cells (Barleon et al., 1994), and in lower levels in adult heart, lung and skeletal muscle (Maglione et al., 1991) (Maglione et al., 1993). The exact roles of PlGF *in vivo* remain to be determined. However, in contrast to VEGF, PlGF expression was found to be down-regulated by hypoxia *in vitro* (Shore et al., 1997b).

1.5.5 General Biological functions;

1.5.5.1 Stimulation of proliferation of endothelial cells:

VEGF stimulates cell growth in a variety of endothelial cells, examples of which include human umbilical vein endothelial cells, bovine aortic, adrenal tissues (Connolly et al., 1989a) (Ferrara and Henzel, 1989) (Plouet et al., 1997). It also stimulates tubular formation of endothelial cells in Matrigel and Collagen gel assays (Connolly et al., 1989a) (Goto et al., 1993). Although the process of angiogenesis in vivo is known to be a complex one, there is accumulating evidence to suggest that VEGF is a direct angiogenic factor *in vivo* as well as *in vitro*.

1.5.5.2 Increase in vascular permeability:

Senger et al (1993) demonstrated that VEGF was a permeability factor (Senger et al., 1983). They showed that ascitic fluid from cancer patients stimulates an increase in blood vessel permeability when assessed using the Miles assay.

VEGF acts to induce increased permeability at concentrations of 1 nM; this is significantly lower than other known vascular permeability factors, such as bradykinin or substance P (Connolly et al., 1989b). VEGF has been shown to be unique among angiogenic and permeability factors, by possessing the ability to induce both responses (Connolly, 1991). VEGF induces a transient increase in vessel permeability which lasts for a maximum of thirty minutes (Wu et al., 1996) (Bates and Curry, 1996). Recently, VEGF has been shown to have both a transient and chronic effect on vascular permeability.

Using frog mesenteric vessels, Bates et al (1996) examined the time effect of a single VEGF exposure on vascular permeability. They noted a transient increase in permeability that subsided after thirty minutes to a base line. Examination of permeability after twenty-four hours demonstrated a subsequent increase in permeability that lasted a further twenty-four hours (Bates et al, 1996). The authors explained this by considering two possible mechanisms. Firstly, VEGF being a mitogenic trigger might enhance vascular permeability via induction of mitosis. It has been previously shown that endothelial cells increase their vascular permeability on entering mitosis (Lin et al., 1988). Secondly, the exposure of cells to VEGF causes fenestrae formation (Roberts and Palade, 1995) and it was this that caused the increased permeability. Both these explanations are possible as they occur over a similar course of time. However, the authors fail to appreciate that fenestrae formation may be a secondary event to the process of mitosis.

The subcellular mechanism for this effect has been postulated to be nitric oxide mediated as permeability effects are lost with prior exposure to nitric oxide synthase inhibitors (Wu et al., 1996). The role of nitric oxide in the function of VEGF remains to be elucidated.

1.5.6 VEGF and its function in reproductive biology:

1.5.6.1 VEGF and placental expression:

Poor placental development and the subsequent failure of trophoblast invasion into the maternal decidua have been associated with intrauterine growth retardation and pre-eclampsia (Brosens et al., 1970) (Fox, 1986) (Meekins et al., 1994). This association implicates abnormal placental angiogenesis as pivotal in the pathology of these diseases. This led to the investigation of cytokines in placental development.

There are several reports that have tried to address this important question. Studies have investigated the expression of VEGF using the polymerase chain reaction (PCR) with similar results. Sharkey et al, (1993) demonstrated that four isoforms of VEGF were expressed by the human placenta, $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{198}$ (Sharkey et al., 1993). Anthony et al, used a two round PCR protocol to confirm this finding, along with the addition of a further VEGF $_{206}$ isoforms (Anthony et al., 1994). This discrepancy may have arisen from the different PCR protocols employed (Anthony et al., 1994). However, examination of the most abundant form has yielded contradictory results. Initially, using Western Blot analysis, $VEGF_{121}$ was reported to be the primary expression product (Houck et al., 1991). Anthony et al (1994) obtained results from cultured placental tissues that suggested that $VEGF_{165}$ predominated. However, there are several methodological differences that may explain these differences. Removal of placental tissue from its regulatory hormone milieu may have profound effects on $VEGF_{121}$ expression and produce inconclusive results. From data in the literature, it is apparent that $VEGF_{121}$ is the most abundant form.

It is evident that the human placenta produces various amounts of all the VEGF isoforms, with the $VEGF_{121}$ predominating. However, the functions of the different isoforms within the uteroplacental environment remain to be determined.

In situ hybridisation for VEGF mRNA has shown that in first trimester human placentae, expression was limited to the cytotrophoblast and syncytiotrophoblast surrounding the villi (Sharkey et al., 1993). However, in term placentae mRNA changed and was confined to the syncytiotrophoblast, extravillous trophoblast and maternal macrophages (Sharkey et al., 1993).

The differing spatio-temporal observations of VEGF in early and late gestation placenta indicate that VEGF may play a role in both placental development and endothelial maintenance. Using an immuniohistochemistry technique, these original observations have been confirmed, with the VEGF protein localised in the first trimester to cyto and syncytiotrophoblast and as gestation progressed to syncytiotrophoblast only (Barleon et al., 1994).

1.5.6.2 VEGF and Pre-eclampsia:

The role of angiogenic growth factors in the pathogenesis of pre-eclampsia has been advocated since Taylor et al (1990) demonstrated that the plasma of women with pre-eclampsia produced a mitogenic response on incubation with cultured fibroblast, but not endothelial cells (Taylor et al., 1990a). Since this report several others have observed that the plasma from women with pre-eclampsia is indeed mitogenic for cultured endothelial cells, assessed both with increased incorporation thymidine and cell number (Endresen et al., 1995). This difference in results may stem from the fact that Rodgers et al used human umbilical vein endothelial cells that were not primary cultures. It has been demonstrated that various cytokines have no mitogenic effect on endothelial cell lines (Cines et al., 1998). Taylor et al (1990) partially characterised their novel growth factor via acid treatment and protein filtrates and determined it to be in the order of 160 kd. However, to date no further characterisation of this factor has been produced in the literature.

The observation that plasma from women with pre-eclampsia has a mitogenic action led Baker et al (1995) to investigated the possible role of VEGF in the pathophysiology of pre-eclampsia (Baker et al., 1995b). They demonstrated that serum from women with pre-eclampsia had significantly increased concentrations of VEGF compared to normotensive controls. However, this study is not without limitation. VEGF levels were only detected in 10 out of 78 women

sampled, with the rest below the limit of detection of the assay. Therefore, whether VEGF is increased in pregnancies complicated by pre-eclampsia and its role in the pathogenesis of this disease remains to be elucidated.

Chapter Two: Materials and Methods:

The following chapter will describe the methods utilised in this thesis and the validation experiments that were performed to ensure that results were consistent. In Chapter 3 the pilot data and final protocol designs will be discussed. Individual chapters will contain experimental design. Demographic details of patients will be displayed as Tables to the relevant chapters.

2.1 Materials

All chemicals were supplied by Sigma, Poole, Dorset, UK unless otherwise stated.

2.2: Blood sample collection:

2.2.1 Ethical approval:

All of the blood and tissue samples used during the course of this project were collected from Nottingham City Hospital. The local ethics committee approved this study prior to its commencement and all subjects gave written, informed consent.

2.2.2.Blood samples:

2.2.2.2 Preparation of plasma:

Venous blood samples were taken within 24hrs of admission and were collected from nulliparous women diagnosed with pre-eclampsia (see Chapter 1.1.1). Patients that received antihypertensive therapy were excluded, preventing any bias occurring within this group. To act as controls, blood was collected from gestation-matched normotensive pregnant women without complications of pregnancy or underlying illness. Blood samples were collected from the median cubital vein through an 18 gauge needle into pre-cooled glass tubes containing EDTA (Beckington, UK) using a Vacutainer®. Blood was then immediately transferred to the laboratory on ice and centrifuged at 2,500 revolutions per minute for 15 minutes at a constant temperature of 4^{0}C. Once centrifuged, 500µl aliquots of plasma were placed into Eppendorff tubes (Anachem-Scotlab;Luton, UK) and then stored at –80^{0}C until use.

The use of plasma in experimental protocols avoids the confounding variable of cellular products, which are released into serum during blood coagulation. These would only complicate an already diverse metabolic process and may produce *in vitro* alterations that are not relevant to the *in vivo* situation (Fox and DiCorleto, 1984).

2.2.2.2 Generation of serum for cell culture:

Blood samples were collected from normotensive women of varying gestations and parity who attended Nottingham City Hospital antenatal clinic for routine antenatal care. Blood samples were collected through an 18 gauge needle into pre-cooled glass tubes (Beckington, UK) using a Vacutainer®. Blood was then immediately transferred to the laboratory on ice and left to clot at 4^0C for 3 hours. After this time samples were centrifuged as described in Chapter 2.2.2.1. Once centrifuged, serum samples taken on the same day were pooled and placed into universal containers (Sterilin, UK) in 5 ml aliquots and then stored at -80^0C until use.

2.2.3 Removal of VEGF from plasma using Dynabeads:

Dynabeads are uniform, monodisperse polymer particles, which are coated with a thin polymer shell to encase a magnetic material and provide a defined surface area for the adsorption or coupling of various molecules. The polymer contained within the beads is superparamagnetic, in that it exhibits magnetic properties only when placed within a magnetic field. This property allows their use in the isolation of specific proteins or cells.

The beads are coated with a specific ligand for the cell or protein under consideration. They are then added to the heterogeneous suspension to bind to the target molecule. The resultant complex can be removed from the suspension by placing within a magnetic field (the Dynal MPC). In this field the beads become magnetised; the suspension can then be removed leaving the target molecule.

Dynabeads were coated with VEGF antibody according to manufacturers instructions (see appendix I). The coated Dynabeads were placed in the Dynal MPC and washed four times: twice in PBS/BSA (pH 7.4 for 5 minutes at 4°C), once in Tris/BSA (pH 8.0 for 4 hours at 37°C), and once in PBS/BSA (pH 7.4 for 5 minutes at 4°C).

The tube containing the pellet of washed Dynabeads was removed from the Dynal MPC, 1 ml of pooled plasma (see Chapter 8.3) from women with pre-eclampsia was added, and the Dynabeads were gently re-suspended. The bead-sample mixture was then rotated for 120 minutes at 4°C, after which time it was placed in the Dynal MPC, the supernatant being removed and stored until required at -80°C.

2.3.1 Vascular Endothelial Growth Factor Assay:

The assay used in this thesis was developed by Anthony et al (1997). The author spent several periods of time at the University of Southampton with Dr F.W.Anthony performing the assay and validating it for this thesis (Anthony et al., 1997).

2.3.1.1 Radioimmunoassay:

Recombinant human VEGF $_{165}$ (Genetech; Code M3-RD280, U.S.A) was used as the standard and the tracer. VEGF was labelled to a specific activity of 35 mCi/mg with ^{125} I using a modified chloaramine t method of iodination (Dr FW Anthony). 100 µl aliquots of standards (0.2 to 50 µg/L) were produced in a solution of 0.05 M phosphate buffer (pH=7.4) containing 5% bovine serum albumin (BSA) by serial dilution from a stock solution of 100 µg/L.

All standards and samples were measured in triplicate. 200 µl of the standards (0.2 to 50µg/L) and plasma samples were aliquoted into LP3 tubes (LIP Ltd, UK) to which 200µL of radioactive tracer (30 000 c.p.m in 0.2% BSA in phosphate buffer containing 200 µg/mL heparin) and 200 µL of rabbit polyclonal antiserum to VEGF (Genetech: Lot No 11094-70B, diluted 1in 20 000 in 0.2% BSA in phosphate buffer) was added. Following overnight incubation, 100µL aliquots of Sal-cel donkey anti-rabbit coated cellulose suspension (Code AA-SACI, IDS, Tyne and Wear. UK) were added to all tubes, which were then incubated at room temperature for thirty minutes. After incubation 1 ml of distilled water was added and tubes centrifuged at 1500g for 15 minutes. After decantation the radioactivity of the pellets was counted and analysed by computer using the RIA-CALC, LKB-Wallac package (see Fig 2.1).

2.3.2 Validation of the assay:

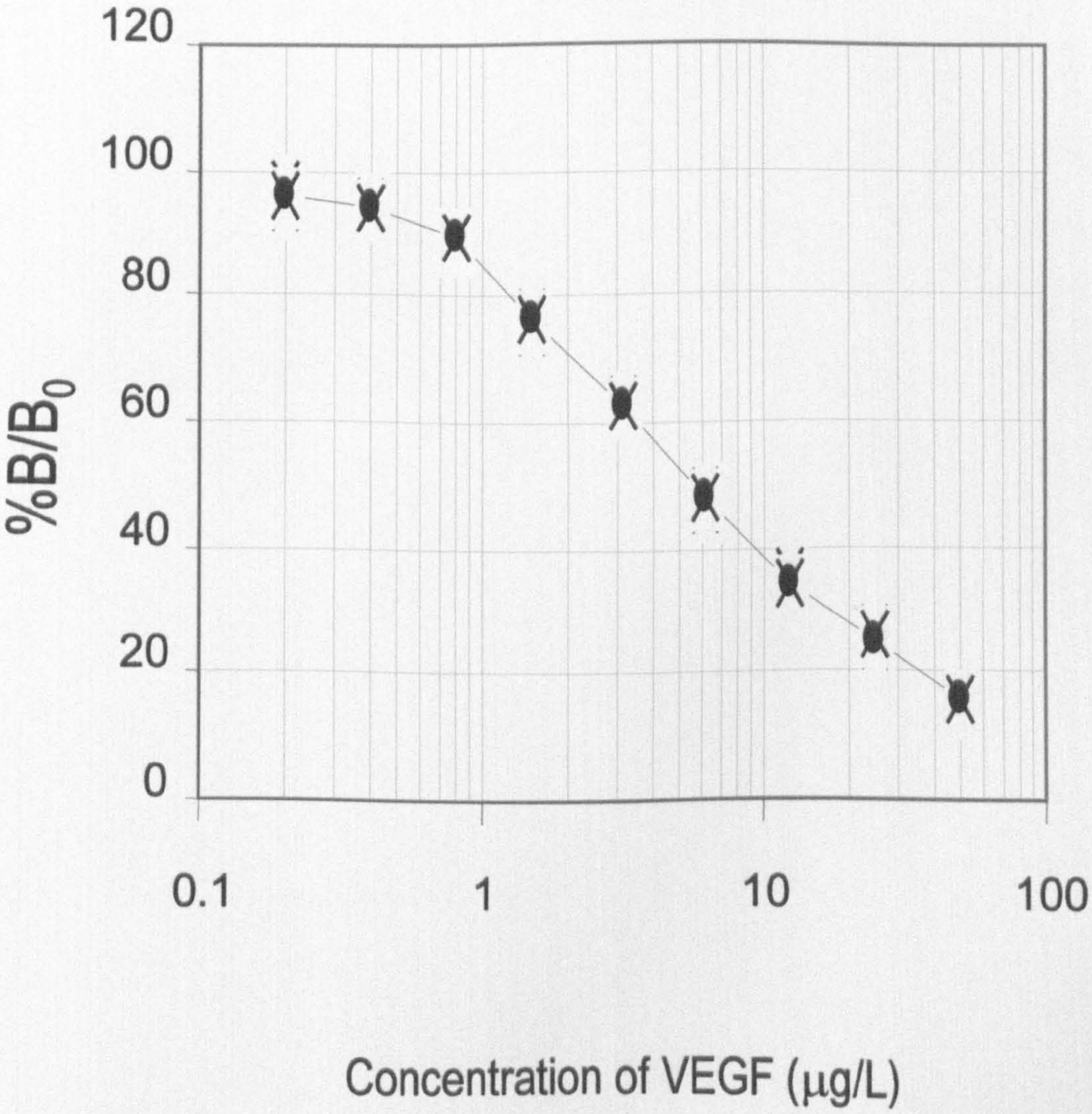
Although Anthony et al (1997) have previously validated this assay, several experiments were performed to confirm these characteristics in the hands of the author of this thesis (Anthony et al., 1997).

2.3.2.1 Reproducibility:

The reproducibility of the assay method was calculated from a plasma standard (pooled plasma from normal pregnant women) aliquoted and stored at -80^0 C until required. The inter-assay variation was evaluated from measuring the plasma standard in thirteen consecutive experiments. The intra-assay variation was calculated from eight measurements in the same experiment.

Fig 2.1

The standard curve for the calculation of the production of Vascular Endothelial Growth Factor (VEGF)



	Mean (µg/l) +/- S.D	Coefficient of variation
Intra-assay	4.02 +/- 0.19	4.81%
Inter-assay	3.22 +/- 0.19	5.97%

2.3.2.2 Sensitivity:

The sensitivity is defined in terms of the slope of the dose-response curve, and is the detection limit of the assay. A significant difference was shown between the percentage binding of the lowest standard (0.2µg/ml) and the zero binding levels (t-test, p=0.015, n=3).

2.3.2.3 Specificity:

Anthony et al, demonstrated that their assay has no cross-reactivity with the closely related placenta growth factor (PlGF), or basic fibroblast growth factor (bFGF) and is not influenced the addition of α2-macroglobulin at a concentration of 100mg/L; this pilot work was therefore not repeated (Anthony et al., 1997).

VEGF	100%
PlGF	0%
BFGF	0%

2.4.Reverse Transcription Polymerase Chain Reaction (RT-PCR):

2.4.1 Sample collection:

It was also wished to study the expression of VEGF and its associated receptors in the feto-maternal unit of control pregnancies and those complicated by pre-eclampsia. The feto-maternal unit comprises two major components, the placenta and the myometrium.

Therefore, biopsies of placentae from women with pre-eclampsia were taken along with normotensive controls. Once the attending midwife had examined the placenta, a 1cm-cube biopsy of the placenta was dissected using scissors from the centre of the placental bed. Samples were then immediately frozen in liquid nitrogen and stored a -80^0C until use.

Uterine biopsy samples were obtained from women undergoing elective Caesarean section. For inclusion, patients had no hypertensive or growth complications in their present or previous pregnancies and had no other medical complications. At Caesarean section, following delivery of the placenta, a full thickness biopsy of myometrium was obtained from the upper margin of the incision. Samples were then immediately frozen and stored in liquid nitrogen until use. In a similar manner, samples were also collected from women diagnosed with pre-eclampsia.

Biopsies were also collected from non-pregnant women of reproductive age undergoing abdominal hysterectomies for non-malignant causes. Once the specimen had been removed, the anatomical area above the cervix was incised and a piece of full thickness myometrium removed

and frozen in liquid nitrogen. The area removed is analogous to the lower segment in late pregnancy

2.4.2 Sample preparation:

Powdering samples:

Myometrial and placental biopsies were ground into a powder using a powdering gun and metal pestle. All samples were powdered under liquid nitrogen so as to reduce RNA degradation. Once samples had been powdered they were stored in liquid nitrogen until RNA extraction.

2.4.3 RNA extraction:

RNA extraction was achieved using a commercially available kit (Purescript RNA Isolation Kit; Gentra Systems, Inc. USA). RNA extraction was carried out as detailed in the manufacturers instructions. The manufacturers RNA handling recommendations were adhered to, working in as far as possible an RNAase free environment. This was achieved by performing RNA extractions in a laminar flow hood, and using machine packaged sterile plasticware.

2.4.3.1 Protein-DNA precipitation:

50-100 mgs of powdered sample was weighed out into a sterile 1.5 ml Eppendorff tube (Anachem-Scotlab;Luton,UK). 1ml of protein-DNA precipitation solution (Gentra Systems, Inc. USA) was then added to the powdered sample. The tube was then gently inverted ten times and

placed on ice. The solution was centrifuged at 15,000xg for 5 minutes, during which time the DNA and protein precipitated out and formed a pellet.

2.4.3.2. RNA precipitation:

The supernatant, which contains the RNA, was aliquoted equally into three 1.5 ml Eppendorff tubes (Anachem-Scotlab;Luton,UK). To these 1 ml of 100% isopropanol was added and mixed thoroughly by gentle inversion of the tube 50 times and placed in a freezer at –20^{0}C for 2 hours. The solution was then centrifuged at 15,000x g for 5 minutes. The RNA was then visible as a small, translucent pellet. The supernatant was poured off, and the RNA washed with 1ml of 70% ethanol. This solution was then centrifuged at 15,000x g for 2 minutes and the ethanol poured off. The tube was then inverted onto clean absorbent paper and allowed to air dry for 15 minutes.

2.4.3.3 RNA hydration:

The air-dried tubes were then placed on ice and 500µl RNA hydration solution (Gentra Systems, Inc. USA) was added and allowed to rehydrate for 30 minutes. Once rehydrated, RNA was stored at –80^{0}C.

2.4.4 Reverse transcription

cDNA was then generated from the total RNA using a commercially available reverse transcription kit (Reverse-iT, 1st strand synthesis kit; Advanced Biotechnologies: UK).

2μl of total RNA template was pipetted into a thin walled PCR tube (Anachem-Scotlab; Luton, UK). 1μl of anchored oligo dT (Advanced Biotechnologies; UK) was added to the RNA template; this was then made up to a final volume of 12 μl with molecular biology grade water (BDH, Poole, UK). The PCR tube was then placed in a heating block (MJ Research PTC-200 DNA Engine). To remove any secondary structure, the reaction mixture was heated to a temperature of 70^{0}C for 5 minutes and then cooled to 4^{0}C. Reverse transcription was achieved using 1μl of murine-monoclonal leukaemia virus (M-MLV) reverse transcriptase (Advanced Biotechnologies). This was performed in the presence of 4μl of 5x 1st strand synthesis buffer, 2μl deoxynucleotide triphosphate (dNTP) mix (5mM), and 1μl of RNAase inhibitor. The mixture was incubated at 42^{0}C for 1 hour to allow cDNA synthesis. Incubating the solution at 90^{0}C for 10 minutes terminated the reverse transcription. This cDNA produced was then stored at –20^{0}C for subsequent use.

2.4.5 Polymerase chain reaction (PCR):

The original description of the polymerase chain reaction (PCR) was by Saiki et al (1985) (Saiki et al., 1985). It relied on the observation that the enzyme DNA polymerase extends DNA in Escherischia coli (E.Coli) (Bambara et al., 1978). Utilising two oligonucleotide sequences as primers and catalysing the reaction with DNA polymerases they were able to generate synthetic DNA. This technique was rapidly confirmed and extended (Mullis et al., 1986) (Mullis and Faloona, 1987). However, early PCR was inhibited by the use of E.Coli DNA polymerase I to catalyse the extension of the annealed primers. This is heat intolerant, and as protocols require heating to high temperatures, each round of synthesis required the addition of fresh enzyme. This was overcome with the incorporation of the heat stable DNA polymerase from the thermophilic bacterium Thermus aquaticus (Taq polymerase) into PCR protocols. This enzyme has been

demonstrated to be stable up to temperatures of 95^{0}C and replacement on every round of annealing is not required. The use of increased annealing temperatures has decreased mispriming and increased the purity of long fragment products (Saiki et al., 1988) (Holland et al., 1991). PCR amplification of DNA was achieved using oligonucleotide primers. These are short stranded DNA molecules that are complementary to a defined sequence of the DNA template. The annealing of primers to the specific region of a single strand of DNA to be amplified is crucial for the initiation of a new DNA molecule (Watson et al., 1992). The primers are extended on single strand DNA template by the enzyme Taq DNA polymerase in the presence of dNTPs under suitable reaction conditions (Watson et al., 1992). The synthesis of new strands can be repeated by heat denaturation of double strand DNA, annealing of primers by cooling the mixture, and extension by setting a suitable temperature for the enzyme reaction.

The expression of β- actin was used as a positive control to check that the PCR technique was working and the reverse transcription was successful. In addition the β-actin was used in the data analysis which will be discussed in Chapter 5.

The same PCR protocol was employed for the different molecules studied. 2μl of cDNA was amplified using a ready load master mix (22.5μl), which contained Mg Cl_2 (2mM), a mixture of dNTPs and Taq DNA polymerase (Advanced Biotechnology) and 0.25μl of each primer (sense and antisense). The PCR was carried out using a thermal cycler (PCR system PTC-200 Peltier).

The conditions and the primers used for each molecule are as follows;

2.4.6 Primers:

β-actin: β-actin mRNA was amplified using the primers; Sense (5'-AAG GAT TCC TAT GTG GGC-3'); and the Antisense (5'-CAT CTC TTG CTC GAA GTC-3') under the following conditions: 95^0 C for 1 minute and 30 seconds, then 95^0 C for 30 seconds, 54^0 C for 1 30 seconds for a total of 9 cycles then 72^0 C for 10 minutes. The reaction was terminated by cooling to 4^0 C.

VEGF: A nested PCR (two round PCR) was used for the amplification of the VEGF mRNA using the previously described primer pairs I and J, and C and H (Boocock et al., 1995). The first round of amplification was performed using the primers I: (Sense 5'-GGC TCT AGA TCG GGC CTC CGA AAC CAT-3'); and J (Antisense 5'-GGC TCT AGA GCG CAG AGT CTC TTC-3') under the following conditions: 95^0C for 1 minute and 30 seconds, 95^0C for 30 seconds, 60^0C for 30 seconds, 72^0C for 1 minute for a total of 34 cycles then 72^0C for 10 minutes. In the second round, the cDNA was amplified with the pair of primers C (Sense 5' –GAC TGT GTG CCC ACT GAG GA-3') and H (Antisense 5'-GGC TCT AGA TCA CCG CCT CGG CTT GTC AC-3') under the conditions: 95^0C for I minute and 30 seconds, then 95^0C for 30 seconds, 64^0C for 30 seconds, 72^0C for 1 minute and repeated for a total of 30 cycles then 72^0C for 10 minutes. Cooling to 4^0C terminated the reaction.

PIGF: PIGF mRNA was amplified using the primers (Sense 5'-AGA TGC CGG TCA TGA GGC TGT-3') and (Antisense 3'-GTT ACC TCC GGC GAA CAG CA-3'). The reaction was held at 95^0C for 1 minute and 30 seconds the PCR was performed for 9 cycles of denaturation at 95^0C for 30 seconds, annealing at 62^0C for 30 seconds and extension at 72^0C for 1 minute. This was followed by a final extension at 72^0C for 10 minutes. Cooling to 4^0C terminated the reaction.

Flt-1: Flt-1 mRNA was amplified using the primers (5'-GTC ACA GAA GAG GAT GAA GGT GTC TA-3') and the antisense primers (5'-AGC CAC AGT CCG GCA CGT AGG TGA TT-3') (Boocock et al., 1995). The reaction was held at 95^{0}C for 1 minute and 30 seconds, denatured at 95^{0}C for 30 seconds, annealing at 62^{0}C for 30 seconds and extension at 72^{0}C for 1 minute. This was followed by a final extension at 72^{0}C for 10 minutes. Cooling to 4^{0}C terminated the reaction.

KDR: A nested PCR was used for the amplification of the KDR cDNA. A two round PCR was performed using the primers C (Sense 5'-CAT CAC ATC CAC TGG TAT TGG-3') and D (Antisense 5'-GCC AAG CTT GTA CCA TGT GAG-3') (Boocock et al., 1995) under the following conditions: 95^{0}C for 1 minute and 30 seconds, the 95^{0}C for 30 seconds, 59^{0}C for 30 seconds, 72^{0}C for 1 minute for a total of 29 cycles then 72^{0}C for 10 minutes. The reaction was terminated by cooling to 4^{0}C.

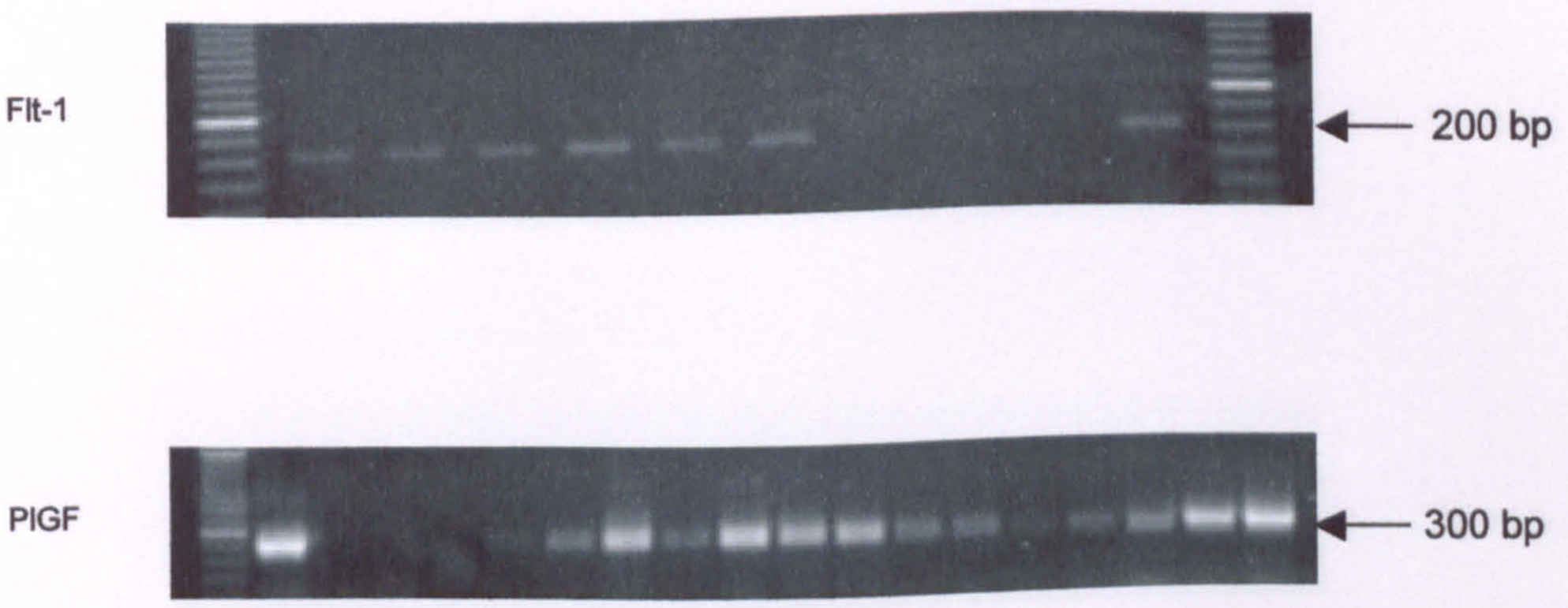
Products were visualised on 1% agarose gel stained with ethidium bromide and viewed under ultraviolet light (Fig 2.2, 2.3,2.4).

2.4.7: Analysis of the data:

Optical densitometry of the bands following PCR were measured using Aida software in a luminescent image analyser PC (Science Systems, Fuji Photo film, UK). The expression of the housekeeping genes is assumed to be similar in the same tissue type between different individuals. Based on this assumption, β-actin was used as an index for the comparison of the expression of VEGF, PlGF, KDR and flt-1 in the placental and myometrial samples. Since PCR

Fig 2.2

RT-PCR of non-pregnant myometrial samples for
VEGF, PlGF, Flt-1, KDR and ß-actin



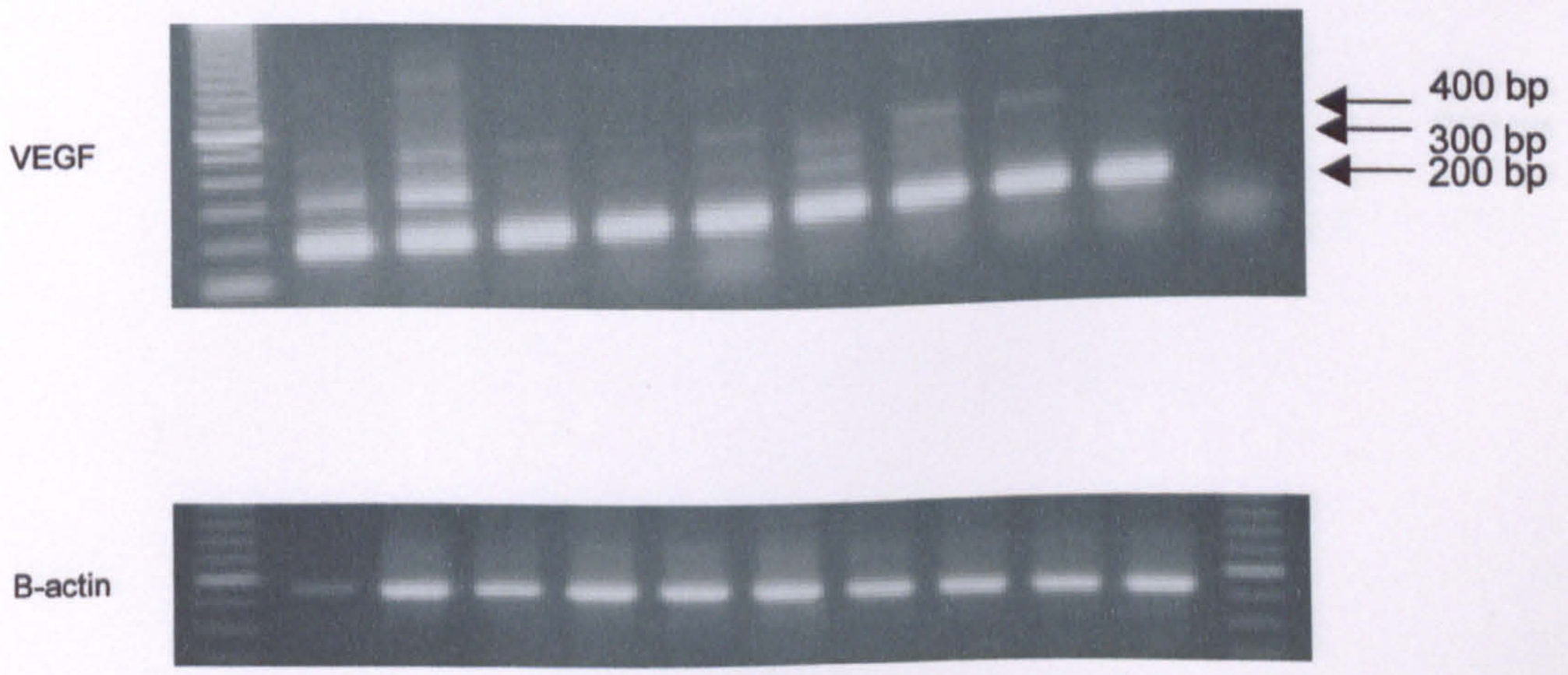


Fig 2.3

RT-PCR of normal pregnant myometrial samples for
VEGF,PlGF, Flt-1, KDR and ß -actin

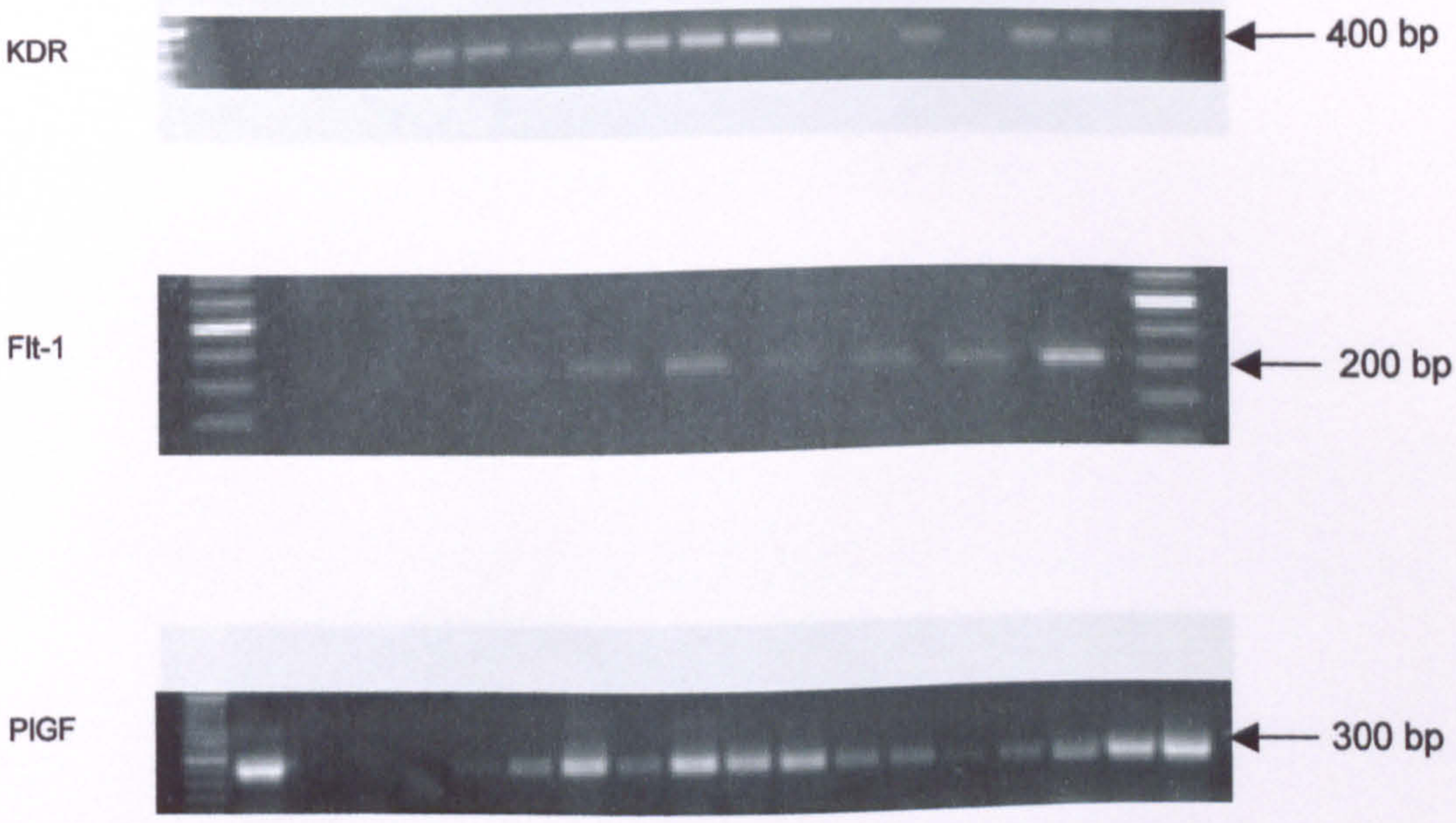
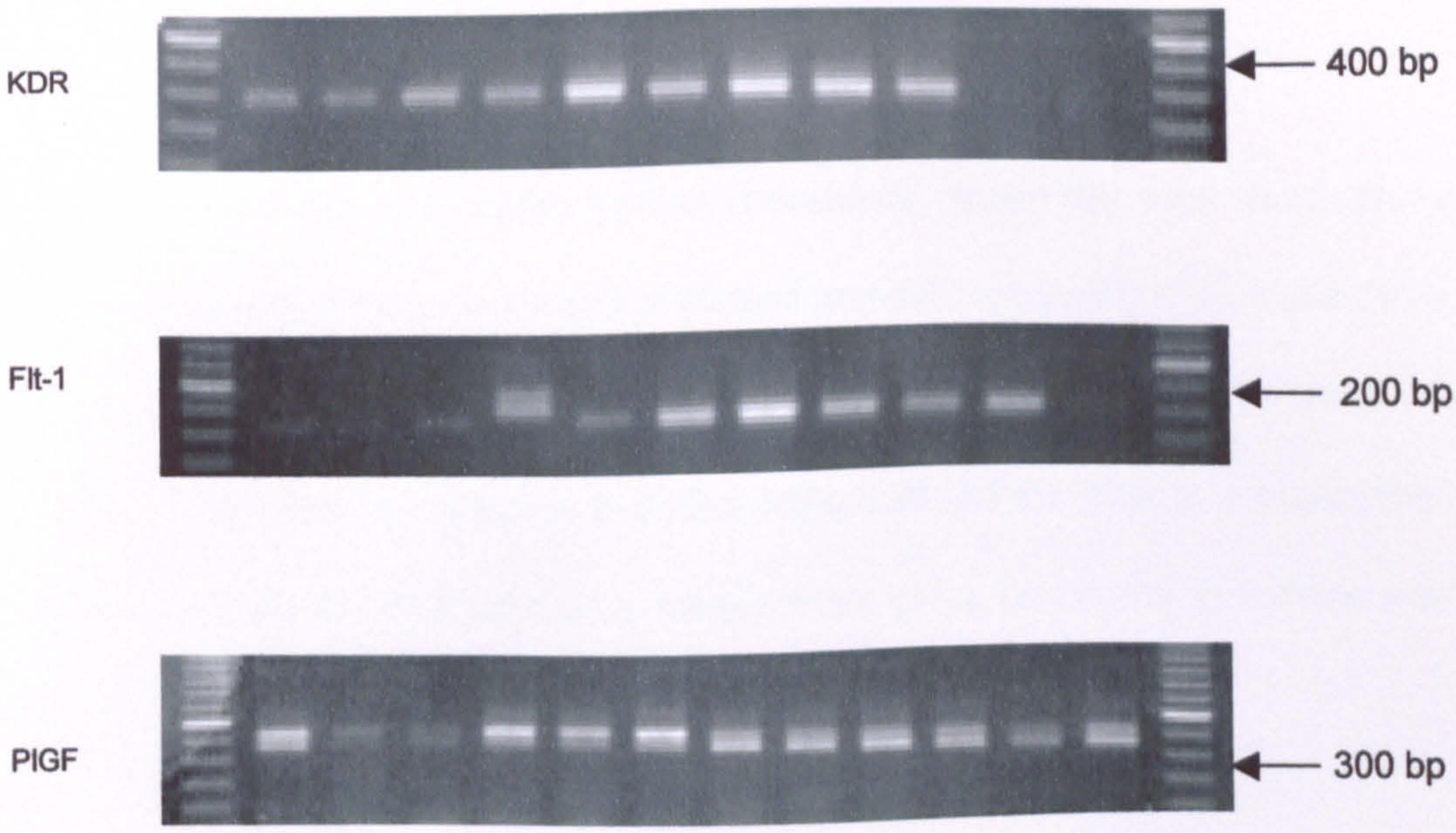


Fig 2.4

RT-PCR of myometrial samples from women with pre-eclampsia for VEGF,PlGF, Flt-1, KDR and ß-actin



for each sample was performed in triplicate and optical densitometry assumed to be not normally distributed, the median of the ratios and the range of the optical densitometry ratios were calculated.

2.5: Isometric myography:

2.5.1 Introduction:

An improved understanding of contractile function of resistance vessels has come about with the technique of myography. Bevan and Osler first detailed isometric myography (Bevan and Osher, 1972), which utilises the scaled down organ bath first described by Dale in 1914. Blood vessels are mounted on two wires, one attached to a force transducer and the other to a displacement device. This set-up allows the simultaneous measurement of the contractility of isolated small arteries to agonist stimulation.

2.5.2 Resistance vessels studied-collection and dissection:

2.5.2a Uterine biopsy samples:

Uterine biopsy samples were obtained from women undergoing elective Caesarean section as described previously (Chapter 2.4.1). Specimens were collected into oxygenated ice-cold physiological salt solution (PSS) (see Appendix II). In a similar manner, biopsies were obtained from women with pre-eclampsia. Uterine biopsies were also collected from non-pregnant women as described previously (Chapter 2.4.1).

Resistance arteries of 200-550μm diameter were dissected from these samples using a stereomicroscope. Dissection was performed in similar cold, oxygenated PSS. Once vessels had

been dissected they were assigned to one of the experimental protocols and unless otherwise stated experiments were performed on fresh tissues.

2.5.2b Omental biopsy samples:

In addition, the results obtained from myometrial vessels were compared with similar sized systemic vessels. Work in our laboratory prior to the commencement of this thesis had demonstrated that omental vessels were the optimum available to use as these had comparable diameters to myometrial vessels (Ashworth, 1998). Biopsies of the omentum were taken at the same time as myometrial vessels. The surgeon would identify a vessel traversing the tissue and them divide the tissue to include this vessel. Vessels were dissected in cold PSS; the temperature was maintained at 4 ^{0}C by placing the dissection dish onto ice. Once a suitable artery had been identified, a coarse dissection was undertaken to remove excess adipose tissue. On completion of this stage the vessel was pinned out and fine dissection was performed. This involved the removal of the vein and as much adipose tissue as possible from the artery. The vessel was then cut at both ends and mounted directly onto the myograph.

Vessels from both vascular beds studied had their problems. One common problem was that biopsies were too small and no suitable vessel could be found. Also some vessels failed to respond to vasopressin; these vessels were abandoned. The success rate of dissection and mounting improved with experience. The overall success rate for myometrial and omental vessels once mounted was 65%. One further problem involved the recruitment of non-pregnant women,

as over the course of this thesis the routine use of hysterectomy for the treatment of menorrhagia was replaced by an alternative medical therapy.

2.5.3 Myography – vessel mounting:

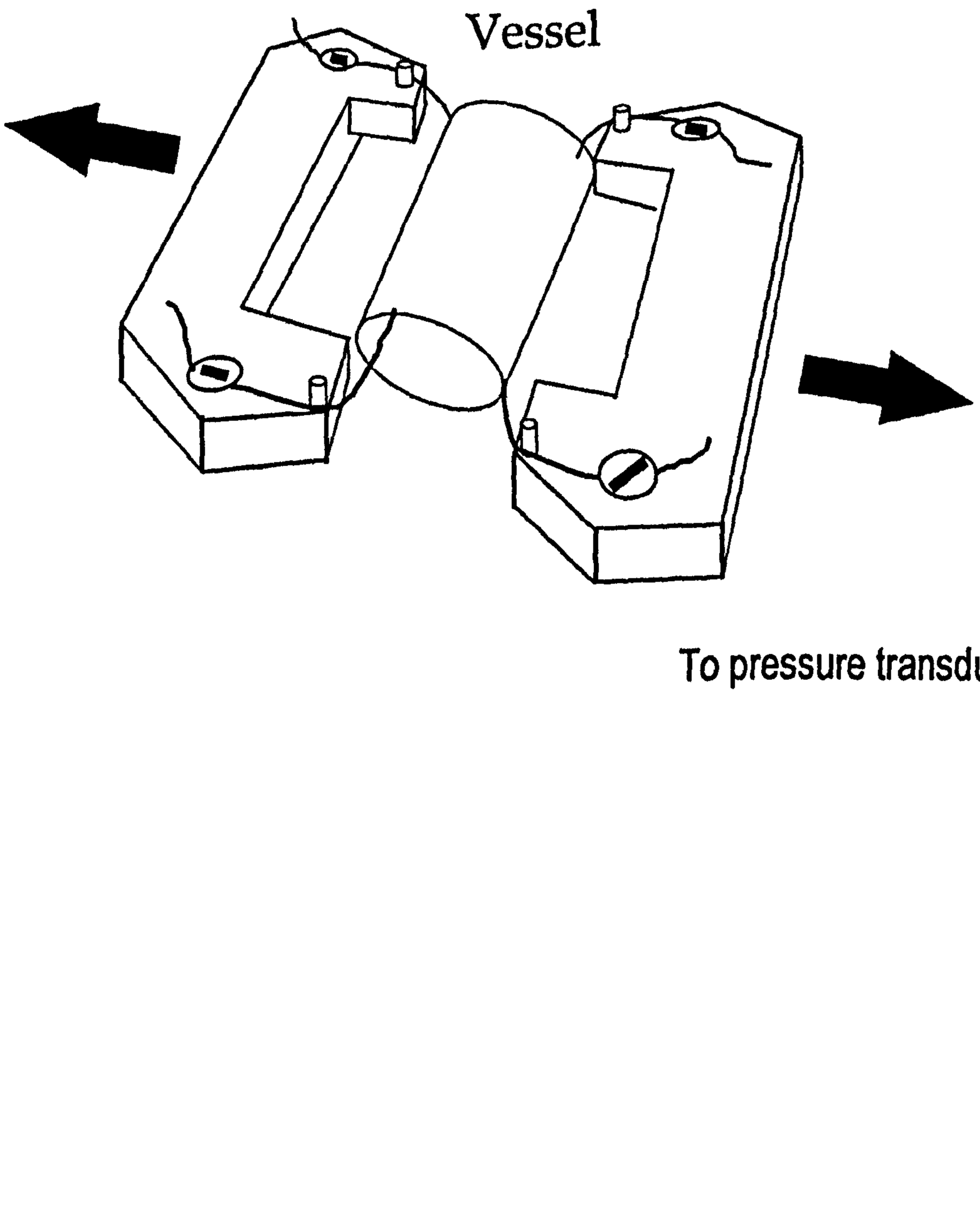
2.5.3a Myometrial arteries:

40μm stainless steel wires, which are used routinely in myography (Mulvany and Halpen, 1976), had been shown to distort when myometrial resistance vessels are stretched. Wire compliance affects the calculation of the vessel diameter and so indirectly, the results obtained. Previous work in the laboratory where this thesis was conducted had demonstrated that 50μm wires were resistant to distortion and as such were utilised.

Once vessels had been dissected they were mounted in oxygenated ice-cold PSS onto the Mulvany Wire myograph (Fig 2.5). A 50μm stainless steel wire was held between the two jaws and attached to one of the screws on the left-hand jaw. The vessel was then threaded along the wire until it lay between the jaws and this wire was attached to the other screw. A second wire was introduced through the lumen and attached to the screws on the right-hand myograph jaw. The two wires were then aligned so that they were parallel: if they are not, errors occur in the normalisation process (see 2.4.1 -normalisation). Once mounted, vessel length was measured with a calibrated eyepiece graticule. The PSS was changed and the organ bath temperature was then raised to 37^{0}C over one hour, via heated chambers adjacent to the organ chamber. During the experiment, constant oxygenation was achieved by passing 95% oxygen and 5% carbon

Fig 2.5

A Mulvany wire myograph



dioxide (BOC, UK) through the PSS. After a constant 37^{0}C temperature was attained, normalisation of the vessels was performed.

2.5.3b Omental arteries:

Omental arteries were mounted in a similar way to that described above. However, 40μm stainless steel wire was substituted for 50μm (see Chapter 2.4.3a).

2.5.4 Myography – normalisation:

The maximal contractile response of a vessel ring depends both on its internal diameter and its length. The wire myograph aims to preserve true isometric conditions by maintaining these parameters, and to maximise the active characteristics (size of contraction and degree of relaxation) whilst minimising the contribution from the resting tone. Normalisation, the process by which these goals are achieved, is a two-stage process.

The first is the determination of the internal diameter of the vessel ring at a transmural pressure of 13.3 KPa (100 mm Hg) under passive conditions (Mulvany and Halpen, 1976). This was achieved by constructing length-tension curves for each vessel by stretching it in small steps and between stretches, the distance between the two parallel wires was measured, and the passive transmural pressure recorded. This allowed the internal diameter for which the effective pressure was 13.3 KPa to be calculated from the Laplace relation (see appendix III). This is termed L_{100}.

The second part of the normalisation process was to set the internal diameter, as a proportion of L_{100} at which a maximum active transmural pressure can be achieved in response to a specific stimulus. This requires experimental investigation for each different type of vessel used. Previous work in our laboratory had determined these characteristics for myometrial and omental arteries of 200 – 550 μm normalised internal diameter from normal pregnant women and non-pregnant women (Ashworth, 1998).

Omental resistance vessels	0.85 L_{100}
Myometrial resistance vessels	0.90 L_{100}

As the same vessels were utilised in the identical system these preliminary experiments were not repeated.

2.5.5. Vasoconstrictor / dilator agonists investigated addition: protocol and concentrations:

2.5.5.1: Vasopressin:

Vasopressin (VP), also known as anti-diuretic hormone, is a hypothalamic hormone, released via the pituitary gland. Although it is a potent vasoconstrictor, it has been assumed to play little role in routine homeostasis of blood-flow, but rather to be of importance in circulatory shock when both its vasoconstrictor and anti-diuretic properties have a significant role (Smith and Kampine, 1990).

However, studies of species other than humans would suggest that the direct vasoconstrictor effects of vasopressin do have a role in the physiological regulation of peripheral resistance (Montani et al., 1980) (Johnson et al., 1981) (Liard et al., 1982) (Woods and Johnston, 1983). Such studies are limited by the difficulty of studying *in vivo* effects of hormones, and the likely inter-species variability, as well as by the varying responses found in different vascular beds. Much of the *in vivo* and *in vitro* work has been conducted in animal models, such as dogs (Montani et al., 1980) (Liard and Spadone, 1984) (Aki et al., 1994) and rabbits (Carter and Gothlin, 1970) and whether this can be extrapolated to humans remains to be determined.

Vasopressin exerts its action through at least three receptor types; V_{1A}, (vascular), V_2 (renal) and V_3 (V_{1B}) (pituitary). These have been characterised by their primary structures (Lolait et al., 1992) (Morel et al., 1992) (Birnbaumer et al., 1992) (de Keyzer et al., 1994) (Thibonnier et al., 1994), their coupling mechanisms (Thibonnier et al., 1994), their tissue distribution and their pharmacological properties (Morel et al., 1992). AVP stimulation of V_2 receptors causes the activation of adenylate cyclase resulting in the production of cyclic AMP. V_2 receptors are present in renal epithelial cell lines, as well as in the medullary parts of the kidney where they have been shown to control free water and urea absorption (Butlen et al., 1978) (Jans et al., 1989).

In contrast, stimulation of the V_{1A} and V_{1B} receptors activates phopholipase A_2, C and D (Thibonnier, 1992). This activation results in the formation of inositol –1,4,5 – triphopshate and 1,2 – diacylglycerol, the mobilisation of intracellular calcium, and the activation of protein kinase C resulting in protein phosphorylation (Michell et al., 1979). V_{1A} receptors have been shown to be present on vascular smooth muscle cells, platelets, hepatocytes, and can effect contraction, proliferation and cellular hypertrophy (Thibonnier and Roberts, 1985) (Jard et al., 1987).

Several groups have identified receptors specific to vasopressin in the human peripheral vasculature. Tagawa et al, Calo et al, and Jovanovic et al have demonstrated the V_{1a} receptor to be present in vessels from human forearm, gastric arteries and uterine vasculature (Tagawa et al., 1995) (Calo et al., 1997) (Jovanovic et al., 1997). More importantly, Bossmar et al demonstrated that in patients with primary dysmenorrhoea, the reduction in uterine blood flow seen in this condition was partially ameliorated by the vasopressin V_{1a} antagonist, SR 49059 (Bossmar et al., 1996). These observations suggest that vasopressin may exert a physiological influence on vascular tone, through specific receptors which are distributed throughout the cardiovascular system.

2.5.5.2: U46619 (Thromboxane mimetic):

Prostaglandins are a complex group of substances demonstrated to be crucial to the interplay between platelets and the vascular wall. Arachidonic acid has been established as the most common fatty acid present in phospholipids (Moncada and Vane, 1979) and is the precursor of all the bisenoic prostaglandins. It is liberated from membrane phospholipids by the phospolipases group of enzymes (Vankeman and Von Dorp, 1968). The importance of prostaglandins in vascular functioning was demonstrated in 1974, with the discovery that one of its non-prostaglandin metabolites, thromboxane, exhibited vasoconstrictor properties (Hamberg and Samuelsson, 1974).

There are three distinct stages in the formation of prostaglandins from their arachidonic acid precursor; first, phospholipid cleavage to mobilise arachidonic acid; second the sequential conversion to the prostaglandin endoperoxides; and third the subsequent isomerisation /reduction of these endoperoxides to produce the biologically active compounds (Moncada and Vane, 1979). The final step in the conversion of arachidonic acid to one of its biological active compounds is dependent on a multitude of product specific enzymes. The predominance of one of these enzymes determines which prostaglandin is the major cellular product. This is best exemplified in the platelet, where the enzyme thromboxane synthetase predominates and leads to the production of thromboxane (Ullrich and Haurand, 1983).

2.5.5.3: Endothelin:

Endothelin is a potent endogenous vasoconstrictor peptide, which was first isolated and sequenced from the cultured supernatant of porcine aortic endothelial cells (Yanagisawa et al., 1988). The EC_{50} value (8.2 –log mol/L) for endothelin is at least one order of magnitude lower than the reported values for angiotensin II, vasopressin or neuropeptide Y, indicating that endothelin is the most potent mammalian vasoconstrictor peptide known to date (Yanagisawa et al., 1988). There are three subtypes of endothelin; endothelin 1,2 and 3. However, only endothelin-1 is produced by endothelial cells (Inoue et al., 1989).

Like many biological active peptides, the three endothelin isopeptides are produced from the corresponding 200 residue prepropolypeptides; each encoded for on three separate genes (Inoue et al., 1989). Endothelial cells produce ET-1 through constitutive and inducible pathways. ET-1 is

released continuously from endothelial cells via the constitutive pathway, occurring at a constant rate and regulated mainly at the level of protein biosynthesis. There are also limited storage sites as ET-1 can be rapidly released in response to external stimuli (Harrison et al., 1995).

In the original study by Yanagisawa et al (1988), an initial dilator phase proceeded the vasoconstrictor effect of ET-1. This suggested the existence of more than one type of ET receptor. ET_A receptors are found on the smooth muscle cells and mediate a major part of the vasoconstrictor effect of ET-1. ET_B receptors are found on the endothelial cells and mediate endothelium-dependent vasodilatation (Bigaud and Pelton, 1992), via production of prostacyclin (PGI_2) and nitric oxide (de Nucci et al., 1988). Stimulation of ET_B receptors by selective agonists can also induce vasoconstriction (Clozel et al., 1992) and this has led to the hypothesis that there are two types of ET_B receptors.

2.5.5.4: Bradykinin:

Bradykinin (BK) is a nonapeptide which is liberated form its substrate kininogen by the action of kallikrein (Graf et al., 1994) (Oza et al., 1990) (Regoli et al., 1990). In the circulation, it has been shown to have differing vasoactive effects depending on the particular species and tissue studied (Toda, 1977). In humans BK is a vasodilator in skeletal muscle vessels, kidney vessels, mesenteric vessels, and in cardiac vessels (Cherry et al., 1982). Bradykinin acts locally through binding to the BK_2 –receptor on the endothelium to stimulate the production of the vasodilators nitric oxide, prostacyclin and the putative EDHF (Cherry et al., 1982) (O'Kane et al., 1994) (Mombouli and Vanhoutte, 1995) (Mombouli et al., 1996).

2.5.5.5: Histamine:

In humans, at least three different histamine receptors have been demonstrated. These include the H_1 receptor on vascular smooth muscle cells which have been shown to mediate contractions in isolated human coronary arterial muscle preparations. The H_2 receptor on smooth muscle has been demonstrated, along with H_1 receptors on the vascular endothelium, to mediate vessel relaxation through both an endothelium-dependent and independent action. The discovery of the H_3 receptor on rabbit cerebral arteries (Ea-Kim and Oudart, 1988) (Ea Kim et al., 1992), prompted the investigation of these receptors in humans. Ortiz et al (1992) demonstrated that human pulmonary arterial constricted to muscle 5-hydroxytryptamine, underwent endothelium dependent relaxation to low dose histamine, whereas higher doses resulted in vasoconstriction (Ortiz et al., 1992). They demonstrated, using a variety of histamine receptor antagonists, that the observed vasodilatation was mediated through the H_1 receptor situated on the endothelium and through a combination of eicosanoid and nitric oxide synthesis.

With regard to human myometrium, H_1 and H_2 receptors have been demonstrated to be both present and functional (Cruz et al., 1989) (Martinez-Mir et al., 1992) (Rudolph et al., 1993) (Gonzalez et al., 1994). Stimulation of the H_1 receptor causes the contraction of isolated myometrial muscle, with stimulation of the H_2 receptor causing relaxation (Martinez-Mir et al., 1992). It has also been shown that H_1 receptor affinity increases in the myometrium of term pregnancies when compared to non-pregnant controls; this increased affinity occurs with a concomitant decrease in the number of receptors available for binding (Gonzalez et al., 1994).

However, the distribution of these receptors and the pharmacological determination of which receptors are responsible for the observed relaxation still remains to be elucidated.

2.5.5.6: Acetylcholine:

Acetylcholine is synthesised from choline and acetyl coenzyme A; catalysed by the action of the enzyme choline acetyltransferase. This synthesis occurs mainly in the axon terminal of the parasympathetic nervous system, were it acts upon muscarinic receptors on the recipient organ. In the peripheral vasculature, the parasympathetic system causes vasodilatation in the vascular beds of the brain, myocardium, salivary glands, sex organs and possibly the intestine.

There is much evidence suggesting that in humans that acetylcholine, acts independently of the nervous system, and is responsible for the automaticity in a number of tissues. Evidence comes from the observations that the enzymes necessary for its synthesis and breakdown are found in these tissues, and that drugs that potentiate or inhibit the action of acetylcholine modify the activity of the tissues. One such tissue is the placenta, which contains more choline acetyltransferase and acetylcholine than are found in cholinergic nerves. It has been postulated that the role of acetylcholine in this location is to cause vasodilatation of the blood vessels of the placenta and adjacent tissues so as to facilitate nutrition of the embryo (Morris, 1966) (Morris and Grewaal, 1971) (Carter and Olin, 1973) (Egund and Carter, 1980).

2.5.6 Addition protocol:

Once the normalisation process was complete, the agonists of interest were added. The concentrations of the agonist to be investigated were determined from literature data and previous data in the laboratory. Each vasoconstrictor was added in 0.5M incremental concentrations at 1-minute intervals. Once a steady state contraction had been obtained the vasodilator was also added in incremental concentrations at 1 minutes intervals. After the addition of the last concentration vessels were washed with PSS at least four times or until the vessel returned to its base line. At the end of each experimental protocol the vessel was constricted to 123nm potassium replaced physiological salt solution (KPSS) (see appendix II) in the presence of noradrenaline.

2.5.6 Concentrations added:

2.4.6.1 Vasopressin:

Vasopressin - $1x10^{-10}$ –$1x10^{-8}$ M (Akerlund et al., 1983) (Ashworth, 1998)

2.5.6.2 U46619:

U46619 - $1x10^{-11}$ –$1x10^{-7}$ M (Hansen et al., 1996) (Kenny et al., 1999b)

2.5.6.3 Endothelin:

Endothelin - $1x10^{-11}$ – $1x10^{-7}$ (Reizebos et al., 1994) (Ashworth, 1998)

2.5.6.4 Bradykinin:

Bradykinin - $1x10^{-10}$-$1x10^{-8}$ M (Knock and Poston, 1996) (Ashworth, 1998)

2.5.6.5 Histamine:

Histamine - $3x10^{-11}$-$1x10^{-7}$ M (Toda, 1990) (Ashworth, 1998)

2.5.6.6 Acetylcholine:

Acetylcholine – $3x10^{-7}$–$1x10^{-3}$ M (McCarthy et al., 1993b) (Ashworth, 1998)

2.5.6 Longitudinal storage of vessels:

Vessels were stored, so that the effect on vessel physiology of prolonged VEGF interaction with the endothelium could be studied (see Chapter 8). Vessels were stored in 10 mls of PSS with 1U/ml heparin at a temperature of 4^0C overnight. Heparin was added to the solution as preliminary experiments had shown that plasma clotted with overnight incubation (Ashworth, 1998). Prior to, and after storage, pH was measured using a 840 series blood gas analyser (Chiron Diagnostics Ltd, Suffolk, UK), and 1ml of PSS was removed and stored at -20^0C for glucose concentration determination (n=6). Glucose concentrations were measured on an Olympus AU 600 analyser by the Department of Clinical Chemistry using a hexokinase method. The inter-assay coefficient of variation was 1.18% .

2.6 Cell Culture:

2.6.1 Basic Cell Culture techniques:

All cells were cultured in an incubator at 37°C, at an atmosphere of 5% CO_2 and 95% oxygen. All plastic cell culture products were from Corning Costar (Corning, NY, USA), unless otherwise stated. All media, solutions and fetal bovine serum for culture of decidual cells and bovine microvascular endothelial cells were supplied by Gibco BRL:UK. Dermal microvascular endothelial cells, culture media and supplements for culture of were supplied by Clonetics; UK as well as the culture media for human umbilical vein endothelial cells. All antibiotics and trypsin/EDTA were supplied by Sigma (Sigma UK) unless otherwise stated.

2.6.1.1 Passaging cells and freezing cells:

Culture media was aspirated from the culture flask. Cells were washed with warmed Hanks Balanced Salt solution (HBSS) and after aspirating this solution, warmed Trypsin/EDTA was added (2ml/T25 flask, 6ml/T75 flask). Cells were visualised at two-minute intervals for signs of separation: rounding and floating. Once separation was noted the flask was struck four times to mechanically dislodge cells. The effects of the trypsin/EDTA were then neutralised by the addition of an equal volume of fetal bovine serum (FBS). Cells were then centrifuged at 1000 rpm for 5 minutes. The supernatant was then aspirated, leaving a pellet of cells. If cells were to be grown they were then resuspended in 1ml of their specific culture medium, counted using a hemocytometer and plated at the density required. However, cells for storage were re-suspended in FBS containing 10% DimethylSulphoxide. These were counted and $1x10^{-6}$ cell aliquots were

produced in 1ml crytotubes. These tubes were then placed in a cryostat chamber containing propan-1-ol and placed in a –80^{0}C freezer from 24 hours. The propan – 1-ol freezes at a constant rate and prevents cell damage from sudden freezing. After 24 hours the cryotubes were transferred into liquid nitrogen.

2.6.1.2 Media changing:

Cell medium was changed on a daily basis. New culture media was warmed to 37^{0}C in a water bath. Culture flask medium was aspirated and new medium pipetted into the flask/well. When cells reached confluence they were either passaged or stored in liquid nitrogen (Chapter 2.5.1.1).

2.6.1.3 Defrosting cells:

Reconstitution of cells stored in liquid nitrogen was achieved as follows. 5 mls of culture medium was pipetted into a T-25 culture flask that was then heated in the culture incubator. The cryopreserved cells were then removed from the liquid nitrogen and thawed by heating in a water bath at 37^{0}C. Cells were then plated at 250,000 cells per flask.

2.6.2 Primary Cell Cultures:

2.6.2.1 Human Decidual Microvascular Endothelial Cells:

Decidual endothelial cells were isolated by a modified method of (Gallery et al., 1991). Myometrium biopsies were obtained as described in Chapter 2.4.1. and placed in ice cold RPMI 1640 containing penicillin (50 U/ml), streptomycin (50µg/ml), and amphotericin B (50ng/ml). The

decidua was dissected from the myometrium and minced finely with scissors. The minced tissue was then incubated in trypsin 1-250 (0.12 g/dL: ICN, UK) and pronase (0.08 g/dL) at 37^{0}C for 10 minutes. The fragments were washed five times with Hanks' balanced salt solution (HBSS) and then a scalpel blade was used to separate cells from the vessels. The cell suspension was then collected, centrifuged at 1000 x g for 5-minutes and resuspended in 480 µl of Hanks balanced salt solution containing 5% fetal calf serum. 20µl lectin europaeus 1 (UEA 1) – coated Dynalbeads ® (4x10^8 beads/ml) (see appendix I) was then added to the cell suspension and incubated with "head over end" rotation for 10 minutes at 4^{0}C. The solution was then washed with 1ml Hanks balanced salt solution plus 5% fetal calf serum in a magnetic particle concentrator for a total of five times. Cells were plated down on 35-mm^2 tissue culture dishes in a growth medium consisting of medium 199, which contained penicillin (50 µg /ml), 0.1 mg/ml endothelial cell growth supplement, 10u/ml heparin, and 40% (v/v) pooled normotensive pregnancy serum. Media was changed daily until cells were grown to confluence. Once confluence was reached, cells were passaged using Trypsin 0.05% -EDTA. After the first passage, once cells reached confluence they were stored as a cryoprecipate in 10% DMSO.

Production of quiescent cells:

Previous work in the department had determined that cells rendered quiescent in medium not containing serum or endothelial growth factor supplement changed their cell morphology, rounded up and floated off the culture plate. In contrast, cells grown in serum depleted media which contained endothelial growth factor supplement appeared normal, with few or no floating cells.

Thus cells that were required to be quiescent prior to the start of an experiment were cultured in this manner for 24 hours beforehand.

2.6.3 Cell Lines:

2.6.3.1 Human Dermal Microvascular Endothelial Cells:

Human dermal microvascular cells (HdMEC) were purchased from Clonetics (USA) as a cryoperserved aliquot of 500,000 cells in passage 3. Cells were cultured in modified endothelial basal medium (EBM) containing human recombinant epidermal growth factor (10 ng/mL), insulin (5 μg/mL), hydrocortisone (1μg/mL), gentamicin (50 μg/mL), amphotericin B (50 ng/mL), and 5% fetal bovine serum (FBS). Cells were passaged with 0.025% trypsin-0.01% EDTA.

Production of quiescent cells:

Quiescent cells were produced by culturing in serum depleted media without insulin and hydrocortisone, but containing 0.05% bovine serum albumin, gentamicin, and amphotericin for 24 hours before commencement of experiments.

2.6.3.2 Human Umbilical Vein Endothelial Cell line:

The HUVEC used in these experiments were transformed cell line, SGHEC-7 and were a generous gift of Dr Guy Whitley of St Georges' Hospital Medical School, London, UK. Cells were supplied as a proliferating T-75 flask at passage 16 and were cultured in modified endothelial

basal medium (EBM) containing human recombinant epidermal growth factor (10 ng/mL), insulin (5 μg /mL), hydrocortisone (1μg/mL), gentamicin (50 μg/mL), amphotericin B (50 ng/mL), and 5% fetal bovine serum (FBS). Cells were passaged with 0.025% trypsin-0.01% EDTA.

Production of quiescent cells:

Quiescent cells were produced by culturing in serum depleted media without insulin and hydrocortisone, but containing 0.05% bovine serum albumin, gentamicin, and amphotericin for 24 hours before commencement of experiments.

2.6.3.3 Bovine Aortic Microvascular Endothelial Cell Line (B88):

The bovine microvascular endothelial (B88) cell line was a gift from Gensia (San Diego, CA) and supplied as a cryoperserved aliquot of 500,000 cells. These cells were used as they had been previously demonstrated to produce significant differences in endothelial products on stimulation with plasma from women with pre-eclampsia (Baker et al., 1995b) (Baker et al., 1996a). The cellular characteristics include growth as a monolayer, a cobblestone morphology at confluence, positive immunostaining for von Willebrand factor, the secretion of prostaglandins, nitric oxide and tissue-type plasminogen activator.

Cells were grown in medium consisting of alpha-modified minimum essential medium (α-MEM: GIBCO) containing 10% horse serum, 2 mmol/L L-glutamine, gentamicin (5μg/mL), kanamycin (20μg / mL) and nystin (10 U/mL). Cells were passaged with 0.025% trypsin-0.01% EDTA.

Production of quiescent cells:

Quiescent cells were produced by culturing in serum depleted media replaced with 0.05% bovine serum albumin, 2 mmol/L L-glutamine, gentamicin (5μg/mL), kanamycin (20μg / mL) and nystin (10 U/mL) for 24 hours before experiments.

2.6.4 Media and protein collection:

2.6.4.1 Collection of media from cultured cells:

After cultured endothelial cells had been exposed to the substance of interest for 24 hours, the culture media were collected and aliquoted into 250μl aliquots and then stored at –80^{0}C until assayed.

2.6.4.2 Deproteinisation of media:

Previous work carried out in the laboratory prior to this thesis had demonstrated that plasma affected the Griess reaction for the determination of nitrite, because the free ammonium groups within proteins react to form azo-dyes and as such a colour change. Therefore, all samples required its removal before the Greiss reaction could be employed. Deproteinization was achieved by the addition of 200μl of medium to 400μl of 0.5M NaOH and 400μl 10% $ZnSO_4$. This mixture was then vortexed for 30 seconds and centrifuged at 14,000 RPM for 5 minutes at 4^{0}C. The supernatant was then removed and stored at –80^{0}C until assayed for nitrite.

2.6.4.3 Extraction of proteins from cultured cells:

After culture media had been collected from the experimental dishes, cells were washed three times with 1 ml HBSS to remove any dead cells. Cellular proteins were extracted by the addition 500µl of protein extraction buffer (see appendix IV) to the culture well. Once cellular proteins were in solution 250µl aliquots were collected and stored at -80°C until use.

2.7 Assays

2.7.1 Griess Reaction:

Nitrite production was determined using the spectrophotometric Griess reaction. The concentration of nitirite in the sample was calculated by comparison to a standard curve of nitrite and absorbance was measured at 550 nm on a spectrophotometer (Labsystems, UK).

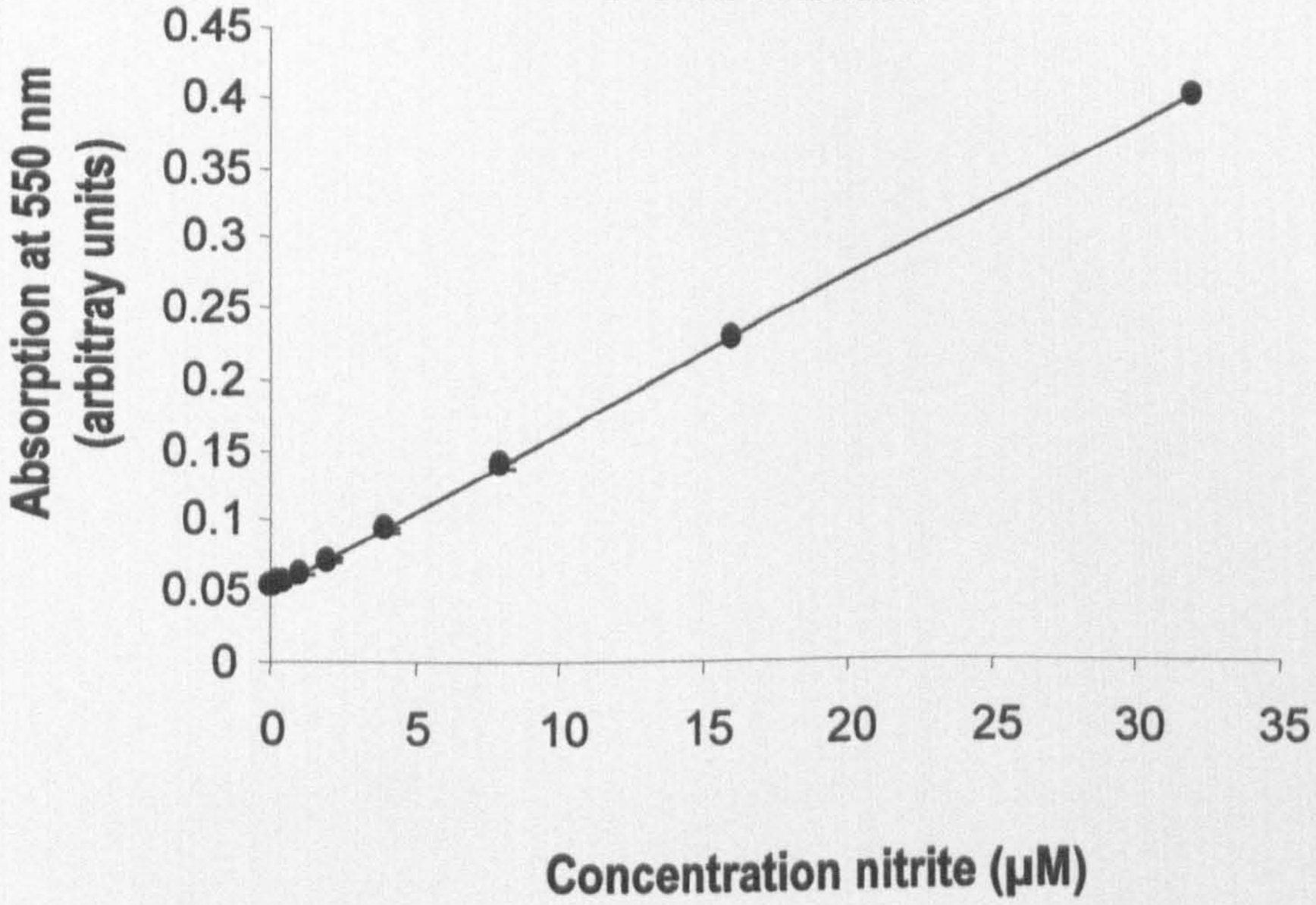
The standard curve was constructed from a stock solution of nitrite. The stock solution was produced by dissolving $NaNO_2$ (0.069g) in pure water (10ml), producing a 0.1 M solution. 10µl of stock solution was added to 990µl water to give a 1000µM nitrite solution. 64µl of this solution was then added to 946µl of culture media to give a 128µM solution. 1:1 dilutions were then carried out to produce the standard curve of 64,32,16,8,4,2,1,0.5,0.25,0.125 µM nitrite (Fig 2.6 A).

100µl of standards and unknown solutions were aliquoted into a 96-well plate. Equal volumes of Greiss reagents #1 and #2 were mixed together (see appendix V) and 100µl of this mixture was added to each well and incubated at room temperature for 10 minutes. The optical density was then determined on a spectrophotometric plate reader (Ladsystems, UK) at 550nm. Each determination was carried out in triplicate and the mean of these used in the final determination.

Fig 2.6

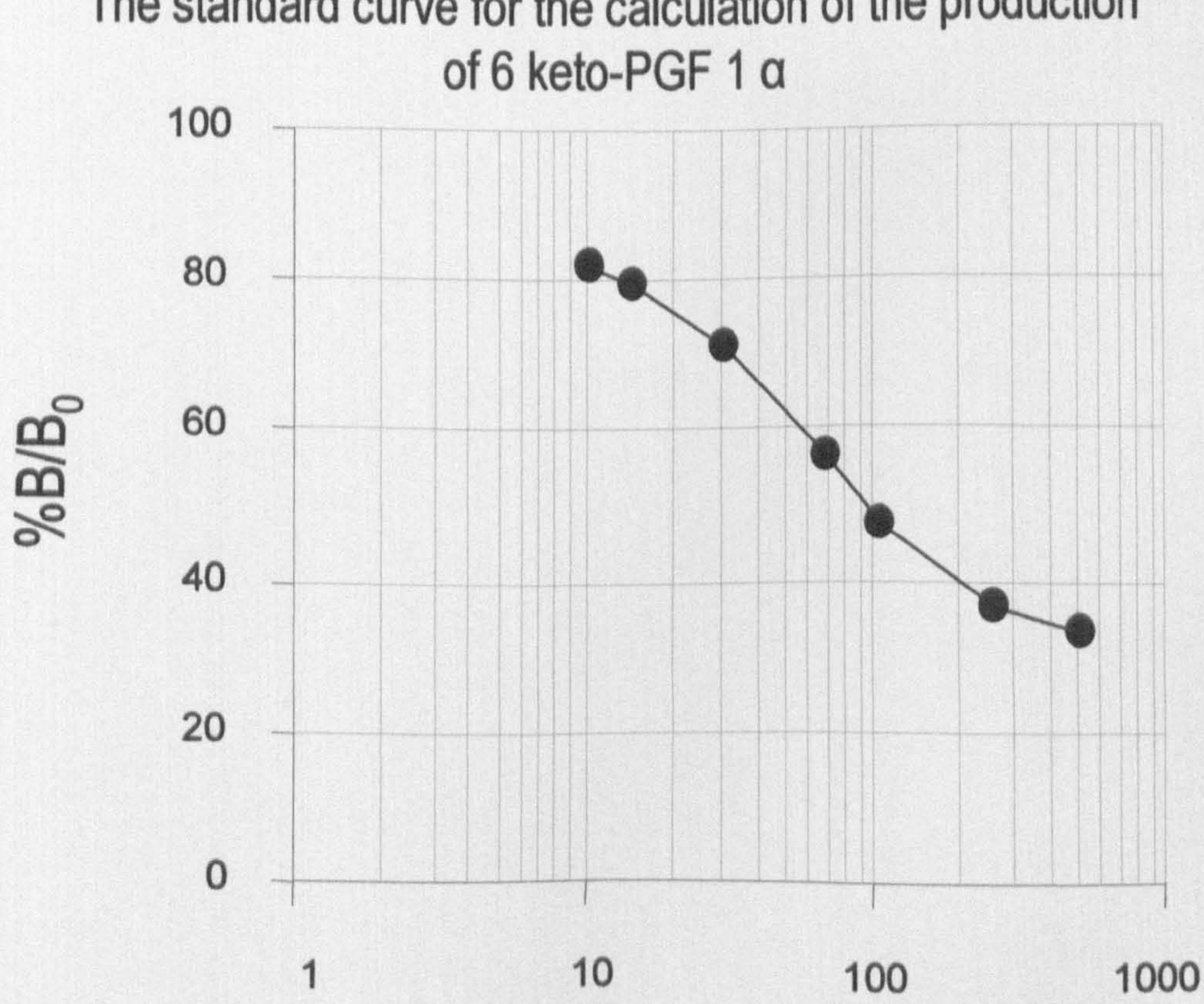
(A)

The standard curve for the measurement of nitrite using the spectrophotometric Greiss reaction



(B)

The standard curve for the calculation of the production of 6 keto-PGF 1 α



2.7.1.1 Reproducibility:

The reproducibility of the assay method was calculated from the measurement of a standard (pooled media from stimulated cells) aliquoted and stored at –80^0 C until required. The inter-assay variation was evaluated from measuring the media standard in six different experiments. The intra-assay variation was calculated from eight measurements in the same experiment.

	Mean (μM) +/- S.D	CV
Intra-assay variation	4.57 +/- 0.25	5.46%
Inter-assay variation	3.95 +/- 0.42	10.80%

2.7.1.2 Sensitivity:

A significant difference was shown between the optical absorbance of the lowest standard (0.25 μg/ml) and the zero optical absorbance levels (t-test, p= 0.033, n=3)

2.7.2 6-keto prostaglandin $F_{1\alpha}$ assay:

Production of the stable metabolite 6-keto prostaglandin $F_{1\alpha}$ was measured using a commercially available enzyme linked immunosorbent assay (ELISA; Cayman Chemicals, Ann Arbor, MI). The rabbit antiserum – 6-keto $PGF_{1\alpha}$ (either free or tracer) complex binds to a mouse monoclonal anti-rabbit antibody that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's Reagent (which contains the substrate to

acetylcholinesterase) is added to the well. The product of this enzymatic reaction has a distinct yellow colour and absorbs strongly at 412 nm. The intensity of this colour is proportional to the amount of free 6-keto-$PGF_{1\alpha}$ present in the well during the incubation. Although this assay was commercially available and the manufacturer produces performance characteristics, several validatory experiments were performed to confirm these characteristics. The standards and unknowns were all measured in triplicate and the means used in the final determination (Fig. 2.6 B).

2.7.2.1 Specificity:

6-keto Prostaglandin $F_{1\alpha}$	100%
2,3-dinor-6-keto Prostaglandin $F_{1\alpha}$	8.70%
Prostaglandin $F_{2\alpha}$	2.10%
Prostaglandin E_2	0.92%
Prostaglandin $F_{1\alpha}$	0.80%

2.7.2.2 Reproducibility:

The inter-assay variation was evaluated from measuring the standard in five different experiments. The intra-assay variation was calculated from eight measurements in the same experiment.

	Mean (ng/ml) +/- S.D	CV
Intra-assay variation	304.30 +/- 18.54	6.09%
Inter-assay variation	273.56 +/- 27.47	10.05%

2.7.2.3 Sensitivity:

A significant difference was shown between the percentage binding of the lowest standard (7.8 ng/ml) and the zero binding levels (t-test, p= 0.001, n=3)

2.7.3 Protein assay:

The concentration of protein was determined using a commercially available spectrophotometric assay (Bio-Rad). This assay is based on the Lowry assay, utilising the reaction of protein with alkaline copper tartrate solution and Folins reagent, producing a characteristic blue colour. The concentration of protein in the sample was calculated by comparison to a standard curve of albumin and absorbance was measured at 750 nm.

Protein standards were constructed by dissolving bovine albumin (0.02g) in deionised distilled water (10mls) to produce a 2 mg/ml stock solution. Standards were prepared containing from 0.5 mg/ml to 2.0 mg/ml protein. All standards were prepared in the same extraction buffer as the sample. 5 µl of the standards were then diluted in 20µl of extraction buffer and aliquoted into a microtitre plate. 25 µl of sample was pipetted into the microplate. Reagent A' (25µl) was then added to each well using a pipette. Folin reagent B (200µl) was then added and the plate gently

agitated for 30 seconds. It was left to stand for 15 minutes and the absorbance measured at 750 nM on a plate reader (Labsystems, UK). All standards and unknowns were measured in triplicate.

3.7.3.1 Reproducibility:

The inter-assay variation was evaluated in nineteen different experiments. The intra-assay variation was calculated from five measurements in the same experiment.

	Mean (mg/ml) +/- S.D	CV
Intra-assay variation	0.272 +/- 0.012	4.63%
Inter-assay variation	0.265 +/- 0.021	7.81%

3.7.3.2 Sensitivity:

A significant difference was shown between the percentage binding of the lowest standard (0.1 g/ml) and the zero binding levels (t-test, p= 0.007, n=3)

3.7.4 Lactate Dehydrogenase determination:

Cellular viability was assessed by measurement of lactate dehydrogenase (LDH) levels. The assay was based on the measurement of reduced nicotinamide adenine dinucleotide formed from nicotinamide adenine dinucleotide plus lactate. Medium (50µl) was added to 1 ml of reagent (Sigma Diagnostics, UK). LDH was measured at 25^{0}C by the change in absorbance at 340nm

using a UNICAM UV/ visible spectrophotometer. For assessment of maximal LDH release, LDH concentrations were also measured after cells had been exposed to 1% Triton X-100 for 24 hours.

2.8 Statistical Analysis:

The placing of a numerical value on the uncertainty of results is one of extreme importance in science, as it allows colleagues to assess the validity of our conclusions. However, of similar importance, is selection of the appropriate statistical test to determine whether a Null hypothesis should be rejected, as an incorrect analysis may give rise to an invalid conclusion. This section will highlight the main statistical tests utilised in this thesis, along with the reasoning behind such analyses.

2.8.1 Basic terminology:

2.8.1.1 Null hypothesis (H0):

This is a hypothesis based on a proposition that there is no difference between one 'control' group and another 'experimental' group.

2.8.1.2 P value:

This is the probability of having the experimental data that supports (H0). Although the value for P is arbitrarily chosen, a $P < 0.05$ is assumed to be statistically significant.

2.8.1.3 Type I error (α):

The attainment of a statistically-significant result when the null hypothesis is in fact correct (a 'false positive' result)

2.8.1.4 Type II error (β):

The attainment of a statistically non-significant result when the null hypothesis is not correct (a 'false negative' result).

2.8.1.5 Power:

For any hypothesis test, the value of α is determined in advance, and is usually set at 5%. The value of β depends upon the size of the effect that one is interested in, and also the sample size. The power of a study is the ability to detect an effect of a specified size, where the power is 1-β or 100*(1-β) %. β may also be fixed in advance by choosing an appropriate sample size.

2.8.1.6 ED_{50}:

The log molar concentration of a drug required to produce a 50 % maximal response.

2.8.1.7 E_{max}:

The term E max in this thesis corresponds to the response obtained from the highest concentration of vasoactive drug used.

2.8.1.7 Normality testing:

Prior to statistical analysis data were examined for normality of distribution; this was achieved by use of the skew test. This test calculates a value for the symmetry of data around the mean, a result of 1 indicating symmetry. In both plasma measurements and cell culture experiments the data obtained showed a high degree of skewness, therefore, distribution-free statistics were employed. However, the myography data showed a normal distribution and as such parametric

statistical analysis were performed. All data were stored on a personal computer, in a form appropriate for use with Stat View for windows, Abacus Concepts, Inc.

2.8.2. Plasma concentrations of Vascular Endothelial Growth Factor:

Where plasma concentrations of Vascular Endothelial Growth Factor (VEGF) were measured, the data were from unpaired groups and had a skewed distribution in both. These were compared using the Mann-Whitney statistical test. Mann-Whitney is a non-parametric test that is equivalent to the Students t-test for comparing data from two independent groups. This test requires all the data points to be ranked as if they were from a single sample. The sum of the ranks of one of the groups is calculated, the P value then being obtained from probability tables. The Mann-Whitney test relies on several assumptions. These are that the two samples have been independently and randomly drawn from their respective populations, the measurement scale is at least ordinal, the variable of interest is at least continuous, and the populations only differ with respect to their medians- if they differ at all (Altman, 1991).

Further analysis of the data was carried out to determine whether levels of Vascular Endothelial Growth Factor could be related to the clinical signs of the disease. Those parameters considered were: proteinuria (g/l), plasma uric acid (μmol/L) concentrations and liver function tests, blood pressure measurements and fetal weight (assessed by individual birth weight ratio). These parameters all displayed skewed distributions and as such non-parametric correlations were employed. The Spearman rank correlation coefficient was used to determine P-values for these data. This test quantifies the strength of any relationship between two variables. It involves the ranking of both (X) and (Y) values. From these values the d_i value (i indicates the i^{th} value) can be

calculated, this is the difference between the ranked individual paired value of X_i and Y_i. From this the r_s value is calculated and used to determine the P-value from Pearson's probability tables. However, if the study number is greater than 30 then a Z-value may be calculated and the appropriate P-value obtained from Z-tables (Altman, 1991).

2.8.3 Myography Experiments:

The technique of wire myography was utilised in this thesis to

1) determine the vascular reactivity changes that occur with pregnancy and pre-eclampsia
2) make comparisons of the effects that incubation with VEGF and plasma had on resistance vessel behaviour.

Detection of a significant result depends upon the power, the level of significance and the size of the sample to be analysed. Then null hypothesis for all the myography experiments stated that:

1) the degree of vessel relaxation to the endothelium dependent vasodilator would be the same in all vessel groups studied.
2) the degree of induced vessel tone would the same in all vessel groups studied.

For each series of myography experiments the power was designated at 80% and a significance level of 5% was felt to be appropriate. In order to calculate the sample size required to fulfil these parameters, the degree at which biological relevance would be achieved was assigned. In pharmacology a halving or doubling in the response would be regarded as biologically significant

(Bowman and Rand, 1984). From pilot data from vessels from six patients, the EC_{50} to bradykinin was calculated to be –7.5 +/- 0.6 $x10^{-10}$ M. A significant pharmacological effect would be a $0.5x10^{-10}$ M shift in the EC_{50}. Therefore the standardised difference was calculated as 0.5/0.6 = 0.83. From these data the total sample size was determined from Altman's nomogram to be 60 i.e. 30 in each group (Altman, 1991). However, both the effect of VEGF and plasma were noted to be significantly greater than a $0.5x10^{-10}$ M shift and as such sample sizes of 6 were required to give an α error of 0.05 and a β of 0.9. The next consideration was how best could the data be statistically analysed.

One suitable method of statistical analysis would have been to determine the ED_{50} values from the dose response curves; these can then be analysed by use of the paired Student t-test. This indicates whether a shift in the dose response curve is statistically significant. However, in several cases this was not possible, as incubation with VEGF made the ED_{50} value unattainable with protocol concentrations of vasodilators. Further increase in vasodilator concentration was deemed not to be physiological as well as too expensive.

The technique of analysis of variance was also utilised to confirm the statistical analysis, as the experimental design required testing to be carried out in relation to six concentrations of vasodilators. The variances of these results were similarly demonstrated to be approximately equal, this being another assumption of the test. The repeated measure analysis of variance was employed as the statistical test, this being an extension of the paired t-test. The strength of the design is that repeated measures are made on the same tissues, and as such the variation between tissues is reduced. Specific hypotheses about the concentration of vasodilators that was

required to produce a significant result were analysed by the use of the post-hoc Bonferroni-Dunn test. This is an application of the Student's t-test with an adjustment made for multiple testing.

2.8.4. Cell Culture Experiments:

Data from cell culture experiments were shown to be of a skewed nature, therefore, the non-parametric Mann-Whitney test was utilised. Experiments were designed, to allow comparison of the production of markers of endothelial cell activation when various cell lines were incubated with the plasma from the women with pre-eclampsia and control plasma from uncomplicated pregnancies. The experimental design allowed for comparison between two independent groups.

Cell culture experiments were likewise designed to assess the effect of incremental doses of the cytokines, VEGF and Placental Growth Factor, on production of the same endothelial markers. These require a different statistical approach as the data have more than two unpaired groups. This can be achieved via a one-way analysis of variance. However, skewed data requires a non-parametric equivalent, this being the Kruskal-Wallis analysis. Analysis of variance may be defined as a technique whereby the total variation present in a set of data is partitioned into two or more components. Associated with each of these components is a specific source of variation, so that in the analysis it is possible to ascertain the magnitude of the contribution of each of these sources to the total variation. Specific hypothesis testing about whether the response to the cytokine follows a dose response was carried out via the use of the Mann- Whitney test between each group with an adjustment made to correct the significance value. The adjustment made was to multiply the obtained P-value by the number of groups under examination.

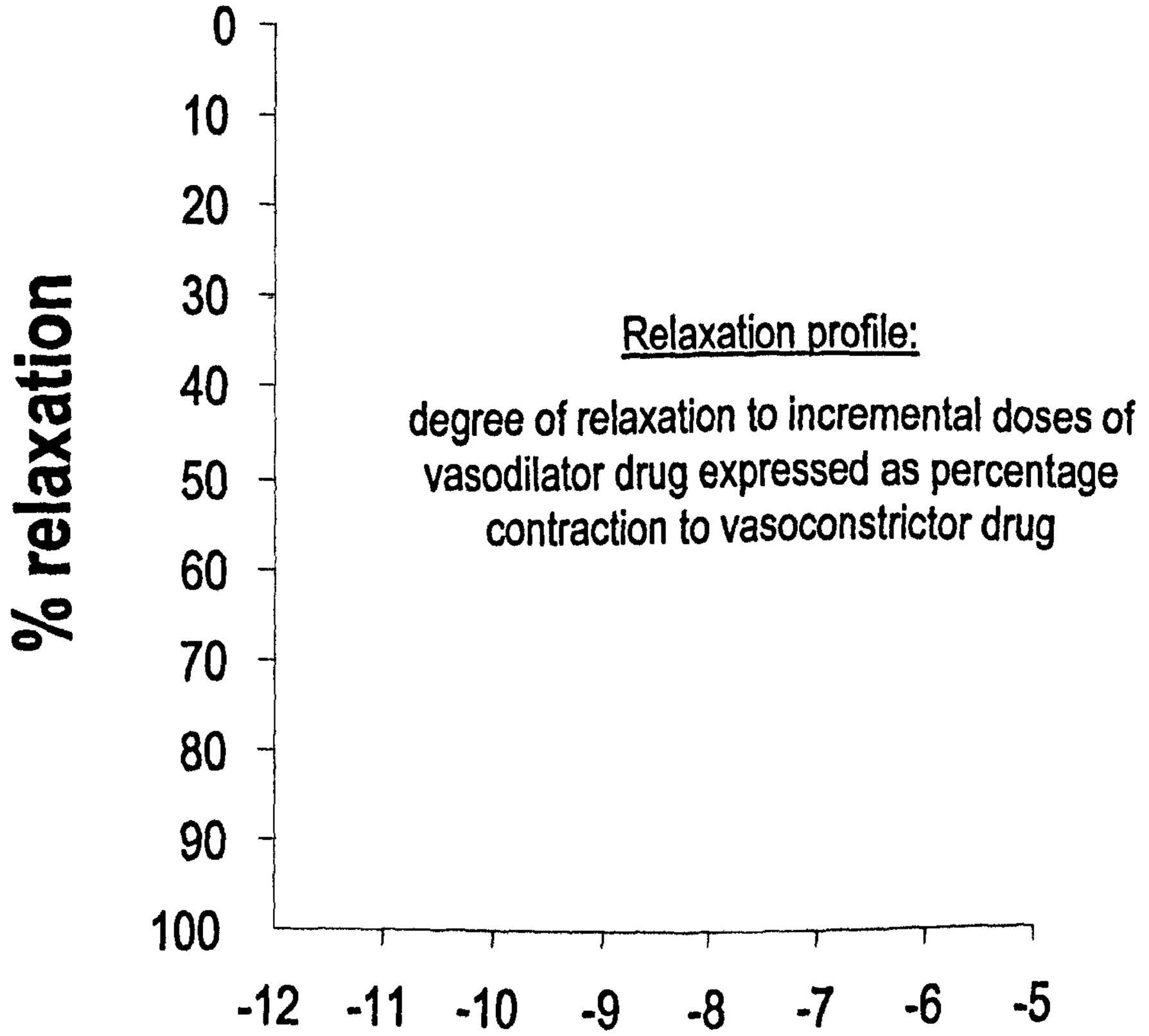
2.9 Presentation of data:

2.9.1 Myography:

All myography data presented in this thesis will follow the same format as that shown for example in (Fig 2.7 and 2.8). The vasoconstrictions were expressed as a percentage of the maximal contraction to KPSS/ noradrenaline. Vasodilator responses are presented as the degree of relaxation expressed as a percentage of the minimal size of the constricted vessel to vasopressin. Data points are expressed as means +/- standard error of the mean. Numerous concentration response curves (CRCs) were generated. Where possible, comparator pairs have been drawn to identical scale and mounted to allow direct visual comparison.

Fig 2.7

Title



Legend:

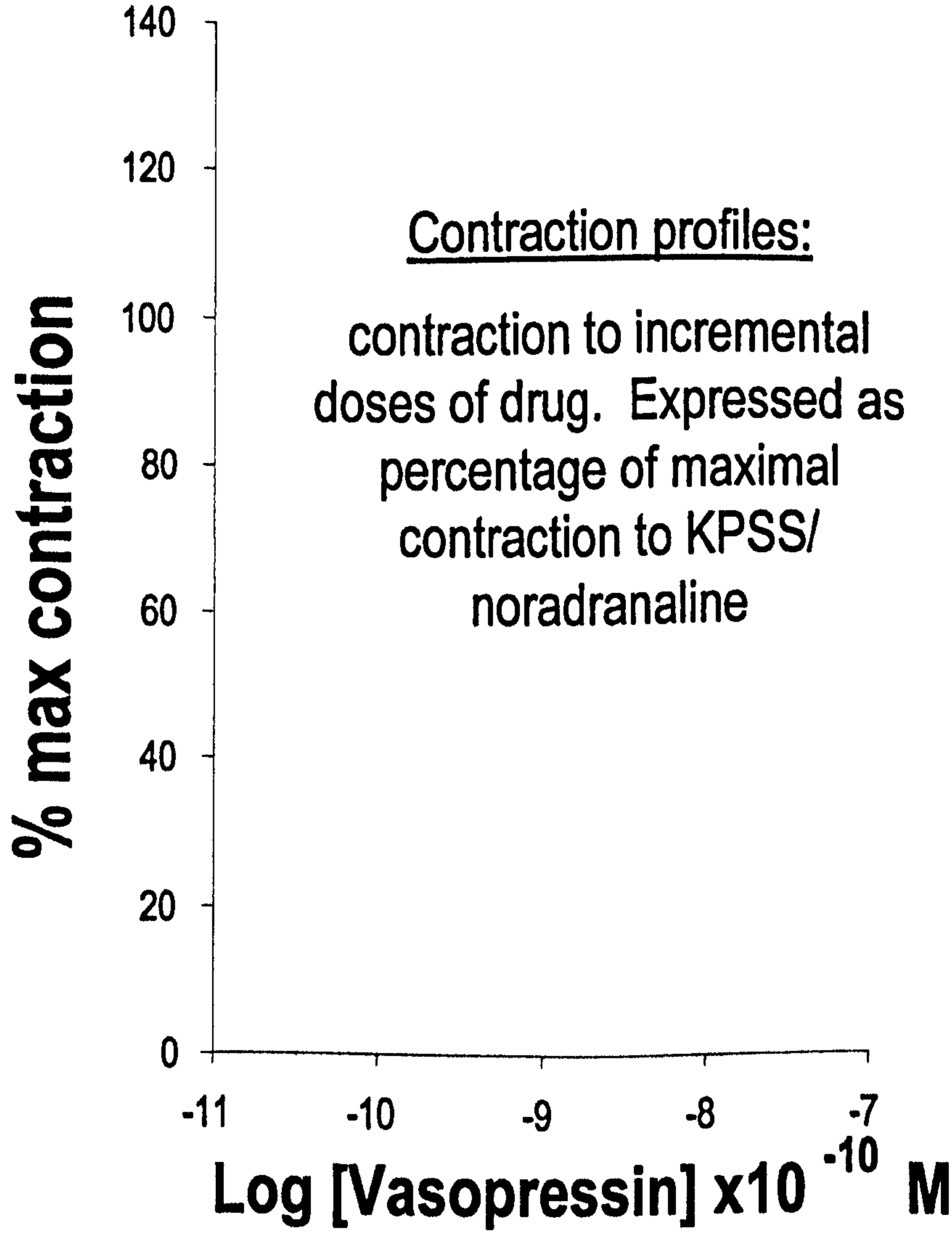
details of the graphs

Statistical comparisons:

Analysis of groups using: repeated measures of analysis of variation (ANOVA) (p), post hoc Bonferoni Dunn (*) , ED_{50} and E max.

Fig 2.8

Title



Contraction profiles:

contraction to incremental doses of drug. Expressed as percentage of maximal contraction to KPSS/ noradranaline

Legend:

details of vessels in experiment

Statistical comparisons:

Analysis of groups using: repeated measures of analysis of variation (ANOVA) (p), post hoc Bonferoni Dunn (*) , ED_{50} and E max.

Chapter Three: Validation, Pilot data and Protocols:

Numerous pilot studies were undertaken before final protocols were devised. These are summarised in the next part of the thesis together with the protocols that were subsequently designed from it for the main studies.

3.1 Pilot data and determination of experimental protocol:

3.1.1 Selection of an appropriate contractile agonist:

3.1.1.1 Aims:

As it was hypothesised that the interaction of VEGF with the endothelium was important in the pathogenesis of pre-eclampsia, it was necessary to determine whether it altered pressor responses. For this reason several vasoconstrictor agents were required to determine whether pressor responses were altered in pre-eclampsia and whether VEGF could initiate these alterations in normal pregnant vessels. Previous work had determined that vasopressin produced reproducible contractions and as such this was taken to compare all other vasoconstrictors examined (Ashworth, 1998).

3.1.1.2 Experimental design:

Previous work in the laboratory where this thesis was conducted had demonstrated that the vasoconstrictors noradrenaline, angiotensin II, 5-hydoxytryptymine were incapable of producing sustained contractions in myometrial vessels (Ashworth, 1998) and such this preliminary work was not repeated. As both endothelin (Taylor et al., 1990b) (Alfredo et al., 1991) (Mastrogiannis

et al., 1991) (Nova et al., 1991) (Clark et al., 1992) (Kraayenbrink et al., 1993) (Wolf et al., 1996) (Maleska et al., 1996) (Bussen et al., 1999) and eicosanoids (Pedersen et al., 1983b) (Pedersen et al., 1983a) (Ylikorkala, 1983) (Makila et al., 1984) (Walsh, 1985) (Yamaguchi and Mori, 1985) (Minuz et al., 1988) (Scafer et al., 1993) (Mills et al., 1999a) have been implicated in the pathogenesis of pre-eclampsia it was considered that these agents were the most relevant to investigate.

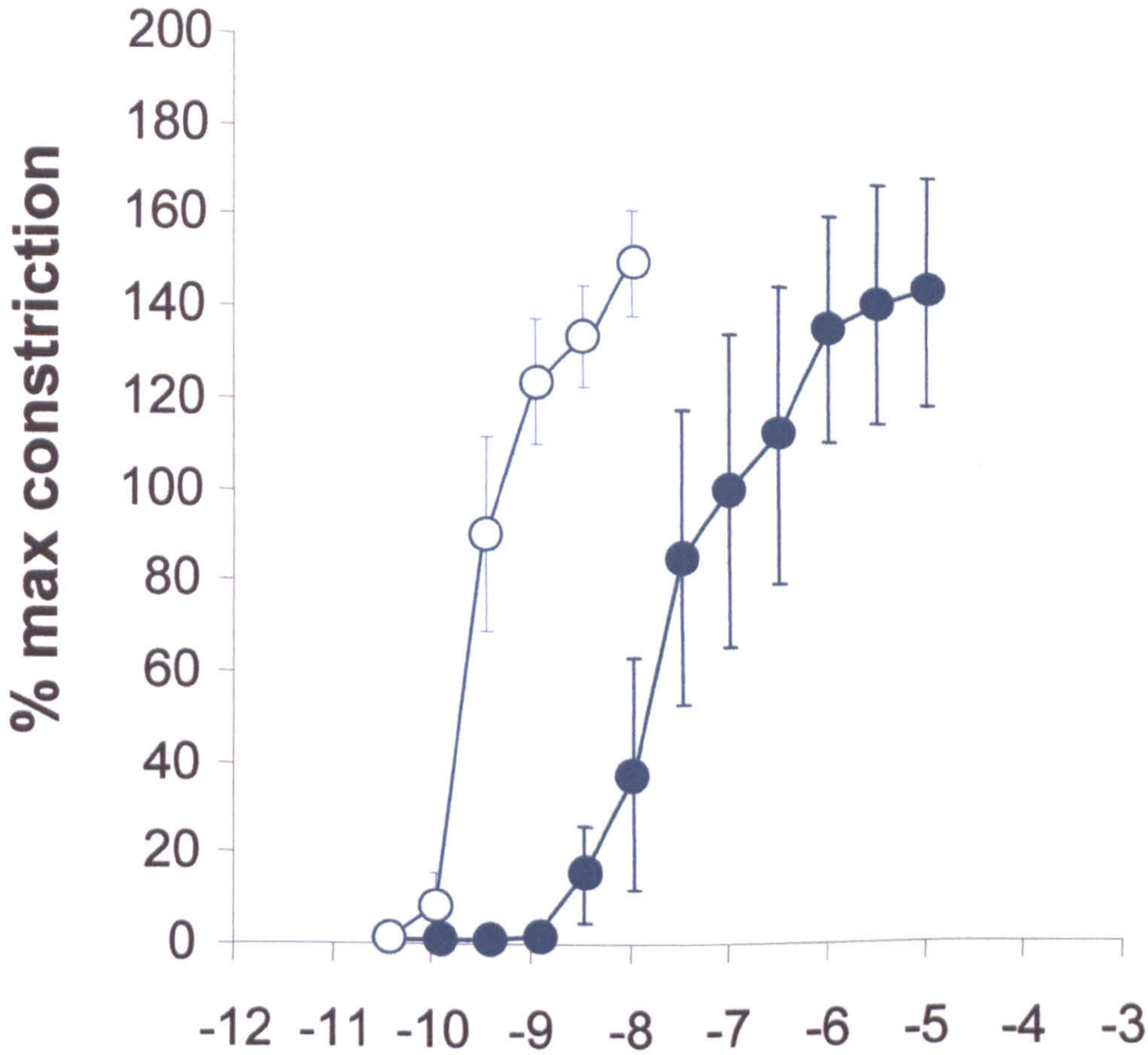
Myometrial specimens were collected as described in Chapter 2.4.2 from pregnant (n=5 each agonist) and non-pregnant women (n=5 each agonist). Two vessels were mounted on two identical myographs as described previously (Chapter 2.4.3). Vessels were randomised to one arm of a Latin square experimental design, one vessel was designated as A and the other as B. Vessel A was exposed to vasopressin as described previously (Chapter 2.4.4). After the vessel had returned to its resting state it was exposed to incremental doses of the agent under examination (U46619 (9,11-dideoxy-11α, 9 α epoxymethano-prostaglandin $F_{2\alpha}$) or Endothelin) (Chapter 2.4.5). Vessel B underwent the opposite protocol. The Latin square design was used to determine if the vasoconstrictor under consideration affected the contractility of the control vasoconstrictor (vasopressin) and vice versa.

3.1.1.3 Results:

Vasopressin, as shown previously, produced sustained contractions in tissues from both non-pregnant and pregnant vessels. The thromboxane mimetic (U46619) produced reproducible and sustained contractions in both the non-pregnant and pregnant groups (Fig 3.1 & 3.2). Prior constriction with vasopressin did not alter a subsequent constriction curve obtained with U46619

Fig 3.1

Comparison between U46619 and vasopressin in non-pregnant myometrial resistance vessels



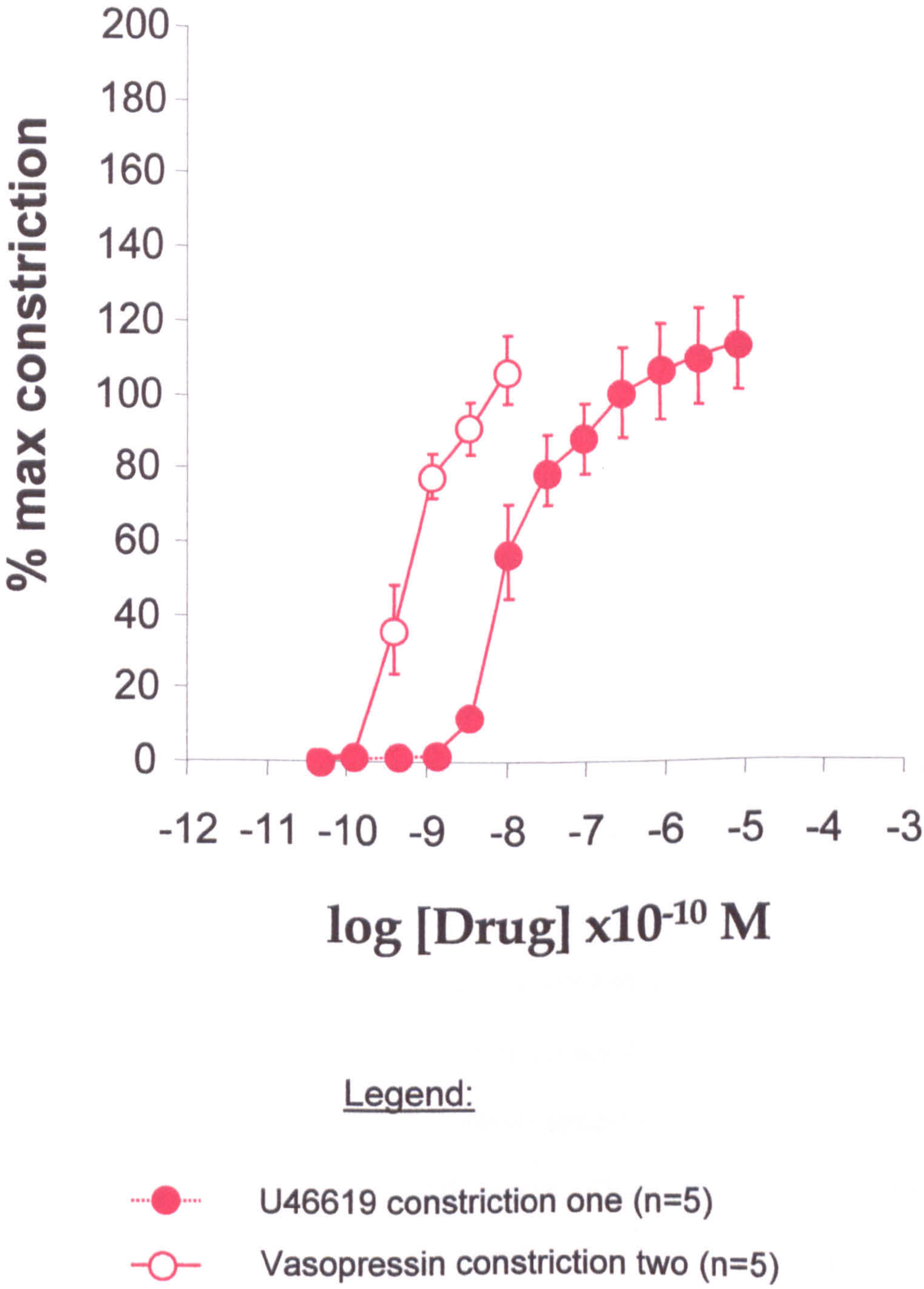
Legend:

U46619 constriction one (n=5)

Vasopressin constriction two (n=5)

Fig 3.2

Comparison between U46619 and vasopressin in pregnant myometrial resistance vessels



(Fig 3.3 & 3.4), this was also observed with the converse (non-pregnant (P= 0.39 (ANOVA)), pregnant (P=0.59 (ANOVA)). Comparison of non-pregnant and pregnant U46619 constriction curves revealed that there was no significant differences between the two groups (P = 0.63, repeated measures analysis of variance (ANOVA)) (E_{max} 144.4+/- 25.7 Vs 112.4+/- 25.3: p= 0.30, unpaired t-test).

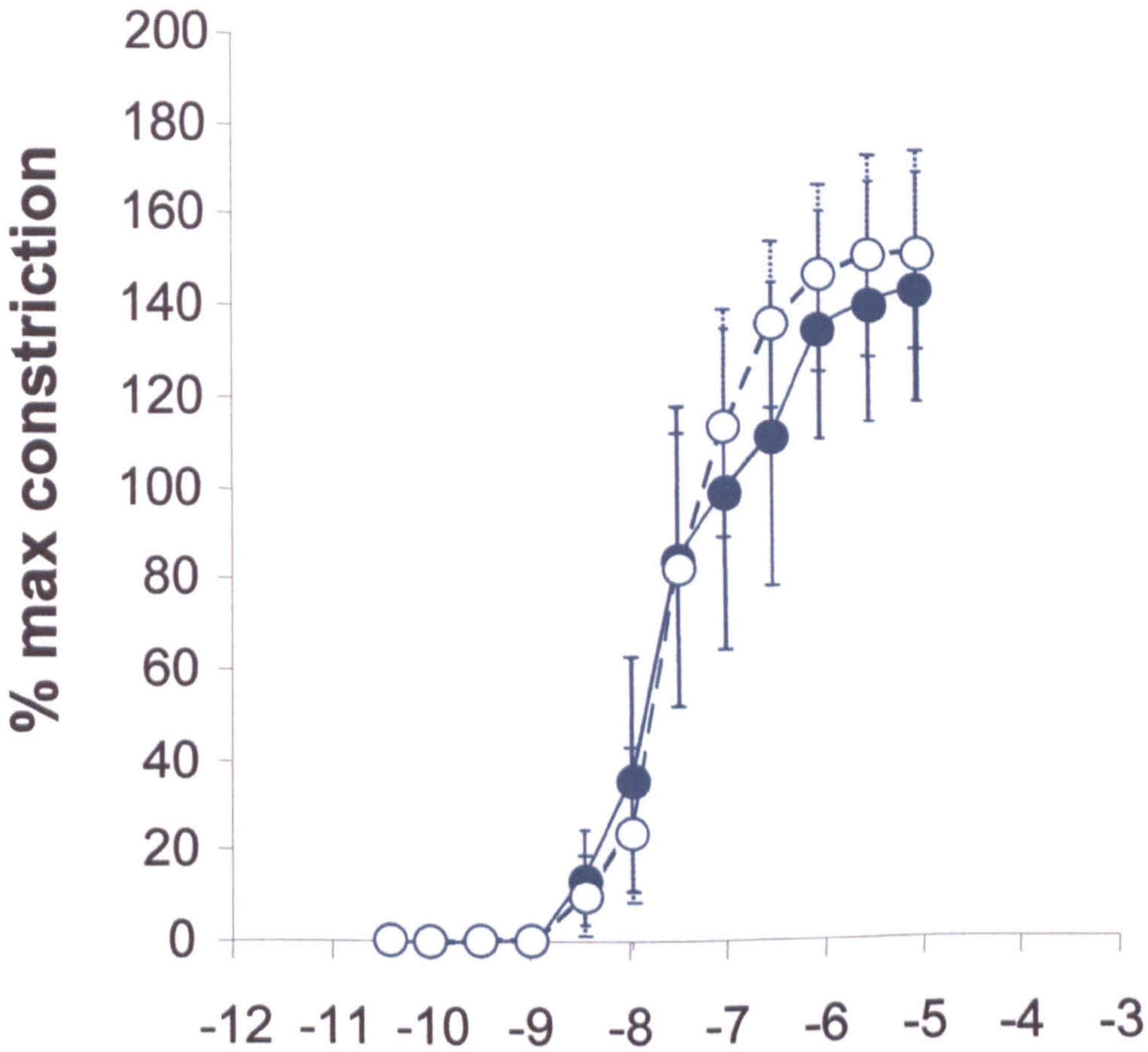
As with U46619, endothelin produced sustainable and reproducible contractions in both study groups (Fig 3.5 & 3.6). The constriction curves to endothelin remained unaltered with a prior constriction to vasopressin. Non-pregnant (P=0.59: ANOVA)(E_{max} 102.3+/- 18.2 Vs 117.2+/- 13.5: P=0.53, unpaired t-test), Pregnant (P=0.17 ANOVA) (E_{max}137.3 +/-11.2 Vs 118.7+/-10.2: P=0.16, unpaired t-test).

However, comparison of the three vasoconstrictors demonstrated a significant difference in their potencies. Non-pregnant EC_{50}: Vasopressin = -9.0+/-0.2, U46619 = -7.5+/-0.4, Endothelin = -7.4+/-0.1 (P=0.003;ANOVA). Pregnant EC_{50}: Vasopressin = -9.4+/-0.1, U46619 = -8.0+/-0.1, Endothelin = -8.5+/-0.2(P=0.001). Post hoc analysis demonstrated that significant differences occurred between the vasopressin and endothelin responses in both non-pregnant (P=0.0004) and pregnant groups (P=0.0007), and also between vasopressin and U46619 in both non-pregnant (P= 0.0008) and pregnant (P=0.0001) vessels. There was no significant difference between endothelin and U46619 in either group (P>0.5).

A comparison the EC_{50} of non-pregnant and pregnant vessels for the vasoconstrictors investigated, demonstrated that there was no significant difference between the responses to vasopressin (EC_{50} –9.0+/-0.2 Vs –9.4+/-0.1: P=0.12, t-test) and U46619 (EC_{50} –7.6+/-0.3 Vs –

Fig 3.3

The effect of vasopressin on the response to U46619 in non-pregnant myometrial resistance vessels



Legend:

U46619 -not exposed (n=5)

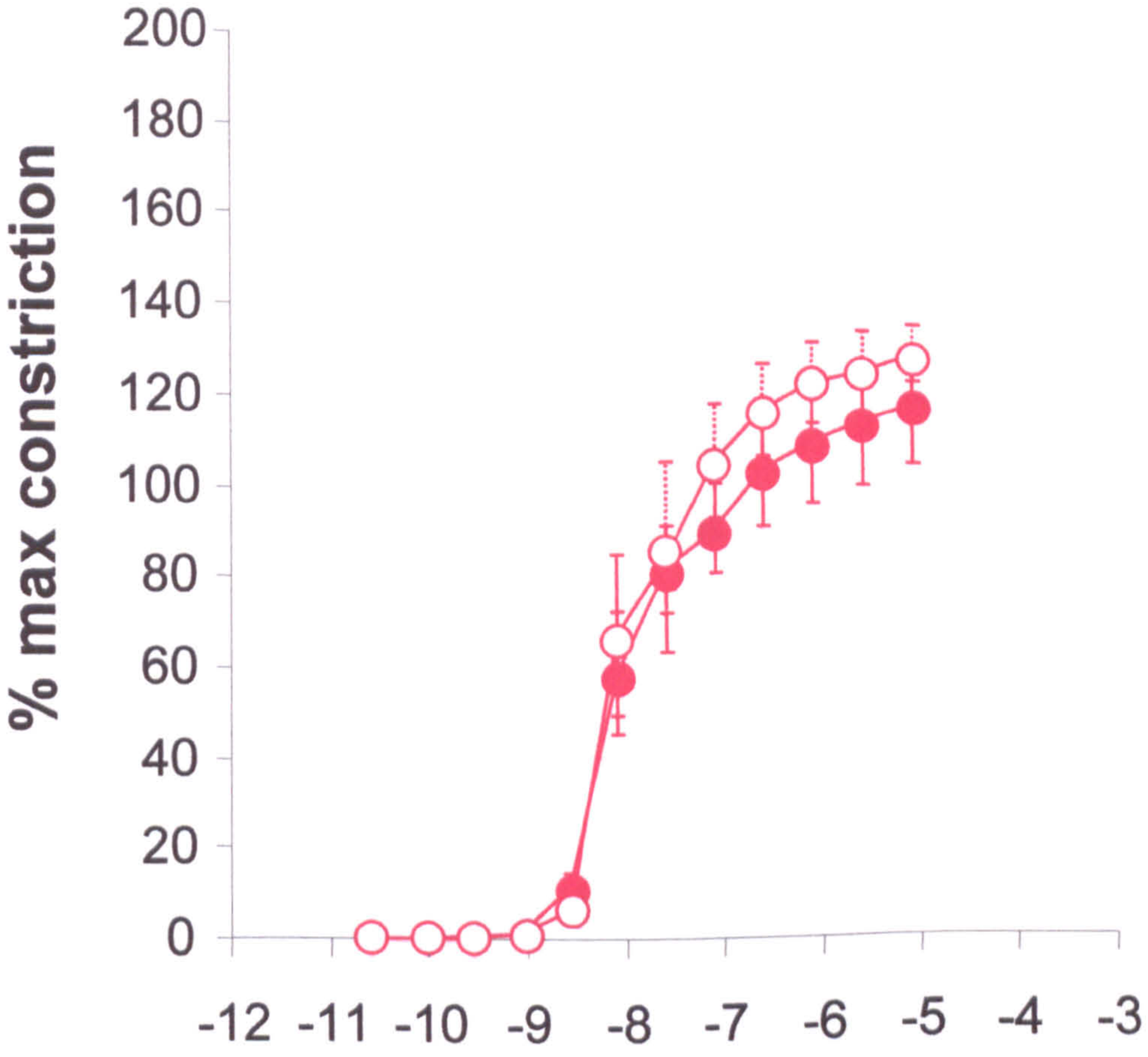
U46619 - exposed(n=5)

Statistical comparisons:

	E_{max}
U46619 -not exposed (n=5)	144.0+/-25.7
U46619 - exposed(n=5)	152.6+/-22.2
t-test (unpaired) P=	0.35
ANOVA (repeated measures)	0.59

Fig 3.4

The effect of vasopressin on the response to U46619 in pregnant myometrial resistance vessels



Legend:

U46619 -not exposed (n=5)

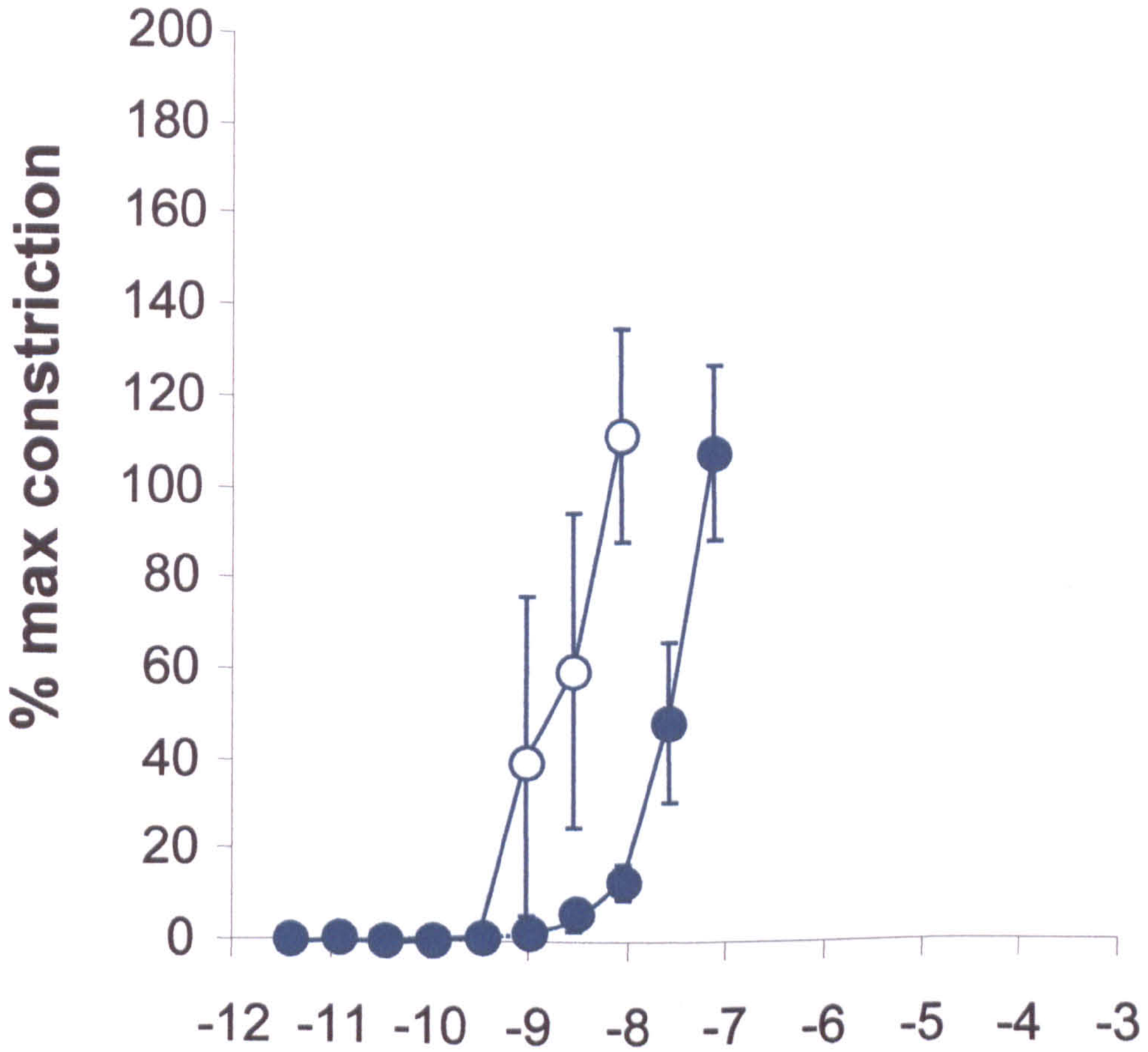
U46619 - exposed(n=5)

Statistical comparisons:

	E_{max}
U46619 -not exposed (n=5)	112.4+/-12.6
U46619 - exposed(n=5)	123.3+/-6.1
t-test (unpaired) P=	0.44
ANOVA (repeated measures)	0.94

Fig 3.5

Comparison between endothelin and vasopressin constriction curves in non-pregnant myometrial resistance vessels



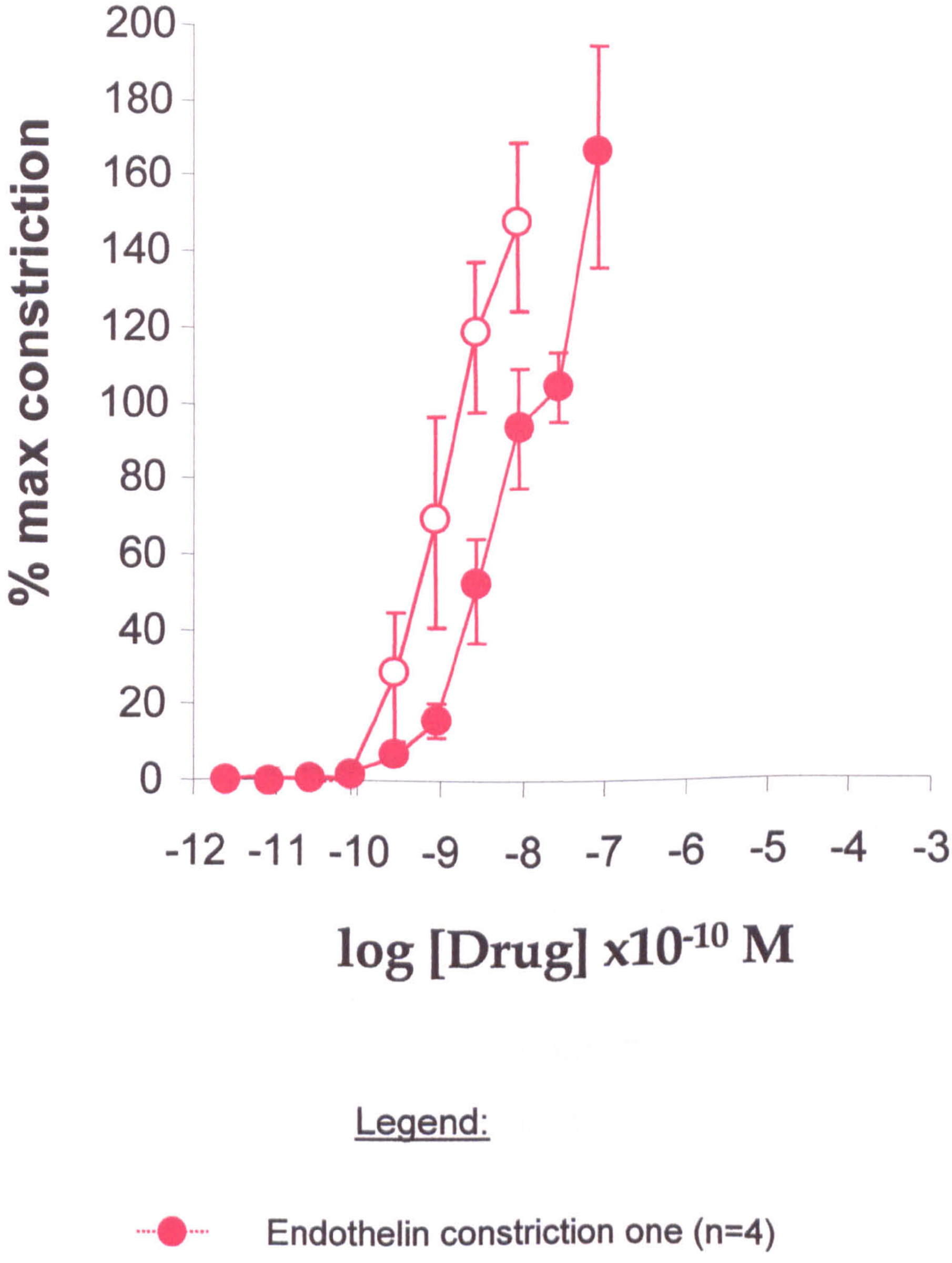
Legend:

Endothelin constriction one (n=4)

Vasopressin constriction two (n=4)

Fig 3.6

Comparison between endothelin and vasopressin constriction curves in pregnant myometrial resistance vessels



Legend:

Endothelin constriction one (n=4)

Vasopressin constriction two (n=4)

7.5+/-0.4: P=0.39, t-test). However, the endothelin responses were enhanced in pregnancy (EC_{50} –7.4+/-0.1 Vs –8.5+/-0.2: P=0.001) (Figs 3.5 & 3.6).

3.1.1.4 Discussion:

It has previously been demonstrated that low concentrations of potassium cause sustained contractions when compared to 123nM potassium, however, the size of contraction varied widely (Ashworth, 1998). It was suggested from these findings that this large variation was due to both the degree of depolarisation and the position along the length / active tension curve between vessels. As these were likely to influence subsequent endothelium-dependent relaxation, this mode of constriction was unsuitable (Ashworth, 1998).

Both endothelin and U46619 produced sustained contractions, which were comparable to both vasopressin and 123mM KPSS / Noradrenaline. U46619 contractions showed no significant differences with pregnancy and this is in accord with several other studies (Skajaa et al., 1990) (Svane et al., 1991) (Kublickiene et al., 1994) (Kublickiene et al., 1995). Comparison of these vasoconstrictor potencies shows that AVP is the most effective vasoconstrictor in human myometrial resistance vessels and further adds evidence to support its role in the regulation of myometrial blood flow.

In this study myometrial vessels from non-pregnant women exposed to vasopressin and U46619 showed significantly higher E_{max} values than normotensive pregnant controls, with no apparent shift in the EC_{50} values. This might have been postulated from the work of Gant et al (1973) which demonstrated that the pressor effects of angiotensin II are decreased in pregnancy (Gant et

al., 1973). In a similar manner, pregnancy has also been shown to alter the vascular reactivity of guinea-pig uterine arteries to norepinephrine, phenylamine, angiotensin II, serotonin, and the thromboxane-memetic U46619 (Weiner et al., 1989b) (Weiner et al., 1991) (Weiner et al., 1992b) (Weiner et al., 1992a) (Jovanovic et al., 1995b). Weiner et al showed that the vasoconstrictor effect of these substances is significantly reduced in pregnant uterine arteries with intact endothelium when compared to that of vessels from the non-pregnant guinea-pig with the same endothelial status (Weiner et al., 1989b) (Weiner et al., 1991) (Weiner et al., 1992b) (Weiner et al., 1992a). They also demonstrated that these effects were mediated through changes in endothelial function, as removal enhanced the responses in the arteries from pregnant animals, returning them toward the non-pregnant state, but responses remained unaltered in non-pregnant arteries (Weiner et al., 1989b) (Jovanovic et al., 1995b).

Steele et al (1993) also implicated the endothelium as pivotal to the vascular adaptations that occur in pregnancy. They demonstrated that, although there were no differences in the contraction responses curves for intact vessels, removal of the endothelium from myometrial vessels from pregnant women significantly enhanced the constriction responses to noradrenaline when compared with non-pregnant controls in a similar state, and postulated that the increased sensitivity is countered by an endothelium-derived relaxing factor (Steele et al., 1993). This would suggest that during pregnancy the endothelium undergoes various alterations that allow the modulation of smooth muscle. This effect may be hypothesised to allow increased control over the local blood flow to the feto-maternal unit.

In contrast to AVP and U46619, which showed blunted responses of myometrial resistance vessels during the third trimester of pregnancy, the concentration response curves to endothelin

were significantly enhanced over non-pregnant vessels. This is in accordance with previous pilot data collected in our laboratory (Ashworth, 1998). The literature and this thesis would suggest that *in vitro* responses to vasoconstrictors are reduced via endothelial modulation. However, enhanced responses have also been reported to vasopressin in guinea-pig uterine vessels (Jovanovic et al., 1995a). Jovanovic et al (1995) showed that these responses were independent of modulation by the endothelium, and suggested that either vasopressin receptor density was increased or that their coupling effect was enhanced. This study did not investigate the role of the endothelium with respect to the enhanced response to endothelin with pregnancy.

There are several possible hypothesises that might be put forward to explain the observation of enhanced responses to endothelin in pregnancy. ET-1, as a vasoconstrictor, may have an increased role in the control of myometrial blood flow during pregnancy. ET-1 is known to contribute to the process of angiogenesis. Stiles et al (1997), while investigating vascularity in human gliomas, have shown that ET-1 is important in the progressive angiogenesis of the human glioma (Stiles et al., 1997). During pregnancy, the uterus supplies the developing placenta with blood, through the formation of new blood vessels. Therefore, it could be postulated with the increased proliferation of the uterine smooth muscle in pregnancy, that ET-1 might contribute to the vascularisation of the increased muscle mass.

Vasopressin produced sustained contractions in comparison to KPSS/ noradrenaline at significantly enhanced potencies to both the other vasoconstrictors and with a pregnancy-related shift in the EC_{50}. As such vasopressin was used as the vasoconstrictor of choice for the investigation of the different vasodilators, however see Chapter 10.

3.1.2.Reproducibility of dose response curves vessels obtained to the vasopressin:

3.1.2.1 Aims:

The vasoconstrictor agent used was required to be capable of being used repeatedly on the same vessel with consistent results. Therefore confirmation was required that vasopressin conformed to this requirement.

3.1.2.2 Experimental design:

Myometrial (M) and omental (O) biopsies were collected from non-pregnant (n=7;M) (n=5;O), and pregnant (n=11;M) (n=6;O) and women with pre-eclampsia (n=6;M) (n=8;O) as described previously (Chapter 2.4.2). Vessels were dissected fresh and mounted within 2 hours of collection.

After normalisation, vessels were constricted with incremental doses of vasopressin as described in Chapter 2.4.5. A total of two constriction profiles were performed for each vessel.

3.1.2.3 Results:

In all groups studied, the two dose response curves were compared with each other by paired t-tests of the EC_{50} and E_{max} responses and by repeated measures analysis of variance. In all the vessels except those from omentum from women with pre-eclampsia there were no significant differences between the first and second constriction curves (Tables 3.1 and 3.2.). In the group of

Table 3.1

Reproducibility of vasopressin contraction curves for non- pregnant, pregnant and pre-eclamptic myometrial vessels

Non-Pregnant		Statistical comparisons:	
		ED_{50}	E_{max}
First constriction curve		-9.1+/-0.1	122.57+/-8.48
Second constrcition curve		-9.0+/-0.05	124.29+/-7.26
t-test (paired)	p=	0.17	0.81
ANOVA	p =	0.24	

Pregnant		Statistical comparisons:	
		ED_{50}	E_{max}
First constriction curve		-8.9+/-0.1	109.32+/-13.63
Second constriction curve		-9.2+/-0.1	128.41+/-16.47
t-test (paired)	p=	0.19	0.12
ANOVA	p =	0.89	

Pre-eclampsia		Statistical comparisons:	
		ED_{50}	E_{max}
First constriction curve		-9.2+/-0.2	115.11+/-12.65
Second constriction curve		-9.1+/-0.1	120.99+/-6.40.
t-test (paired)	p=	0.49	0.55
ANOVA	p =	0.28	

Table 3.2

Reproducibility of vasopressin contraction curves for non- pregnant, pregnant and pre-eclamptic omental vessels

Non-Pregnant		Statistical comparisons:	
		ED_{50}	E_{max}
First constriction curve		-8.5+/-0.0	76.87+/-8.40
Second constriction curve		-8.5+/-0.1	84.23+/-8.98
t-test (paired)	p=	0.99	0.65
ANOVA	p =	0.94	

Pregnant		Statistical comparisons:	
		ED_{50}	E_{max}
First constriction curve		-8.7+/-0.3	70.57+/-5.96
Second constriction curve		-8.5+/-0.2	77.08+/-5.08
t-test (paired)	p=	0.68	0.08
ANOVA	p =	0.13	

Pre-eclampsia		Statistical comparisons:	
		ED_{50}	E_{max}
First constriction curve		-8.8+/-0.1	79.76+/-5.57
Second constriction curve		-8.8+/-0.1	91.57+/-3.52.
t-test (paired)	p=	0.78	0.02
ANOVA	p =	0.54	

omental vessels from women with pre-eclampsia there was a significant difference in the E_{max} values. It was also noted that in all groups the second E_{max} response was greater than the first.

3.1.2.4 Conclusions:

Vasopressin produces a sustained contraction in all groups studied that could be repeated at least twice on the same vessel without signs of fatigue. However, it was noted that in all groups there was a greater response in the second CRC when compared to the first. This reached significance in the group of omental vessels from women with pre-eclampsia. As these differences were considered to be a possible source of a β-error it was considered that time control experiments should be incorporated into the final protocol.

3.1.3 Variability of vessels obtained from the same patient to vasopressin:

3.1.3.1 Aims:

- to determine whether vessels segments from the same experimental sample produced comparable contraction curves to allow time comparisons to be made.

3.1.3.2 Experimental design:

Myometrial (M) and omental (O) vessels were collected, from non-pregnant (n=8:M) (n=5:O), and pregnant women (n=7:M) (n=5:O) and women with pre-eclampsia (n=8:M) (n=5:O). Three vessels were dissected from each biopsy (Chapter 2.4.1) and mounted on separate myographs. The

protocol in Chapter 2.4.5 was then followed and vessels subjected to incremental doses of vasopressin.

3.1.3.3 Results:

In all groups studied, sustained contractions were produced by all vessels exposed to vasopressin (Figs 3.7, 3.8 and 3.9. and Table 3.3). The dose response curves obtained were compared with each other by comparison of their EC_{50} and E_{max} values. No significant differences were noted in any of the groups investigated.

3.1.3.4 Conclusion:

Vasopressin produces comparable contractions in vessels segments from the same patient and thus allowed time comparisons to be incorporated into the final protocol.

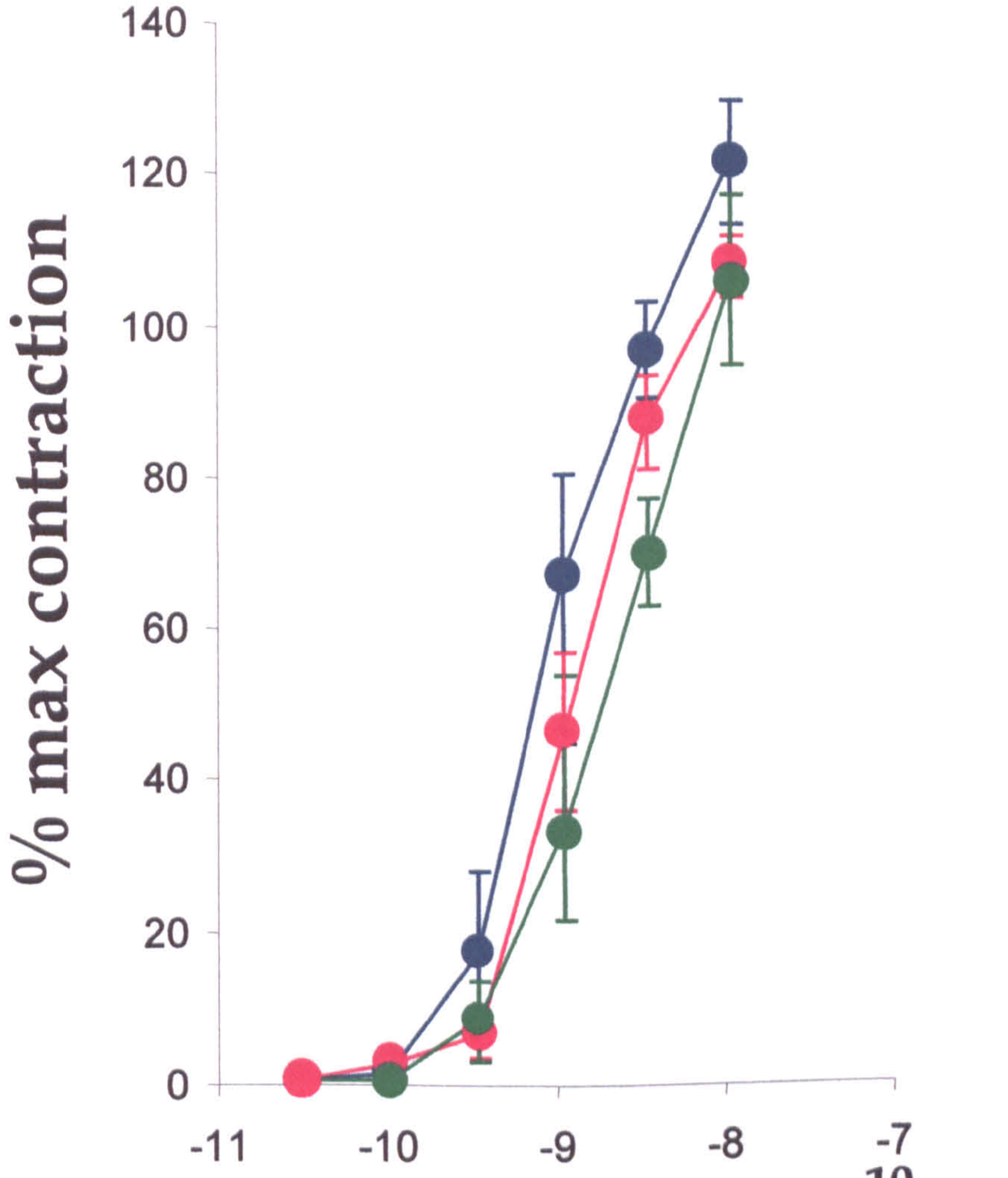
3.1.4. Selection of an endothelium-dependent vasodilator:

3.1.4.1 Aims:

As it was hypothesised that the interaction of VEGF with the endothelium was important in the pathogenesis of pre-eclampsia, it was necessary to determine whether it altered vasodilator responses. As such several endothelium dependent vasodilator agents were required to determine whether their responses were altered in pre-eclampsia and whether VEGF could initiate these alterations in normal pregnant vessels. Previous work had determined that bradykinin, acetylcholine and histamine produced reproducible endothelium dependent concentration

Fig 3.7

Variability of vessels taken from the same sample from non pregnant women



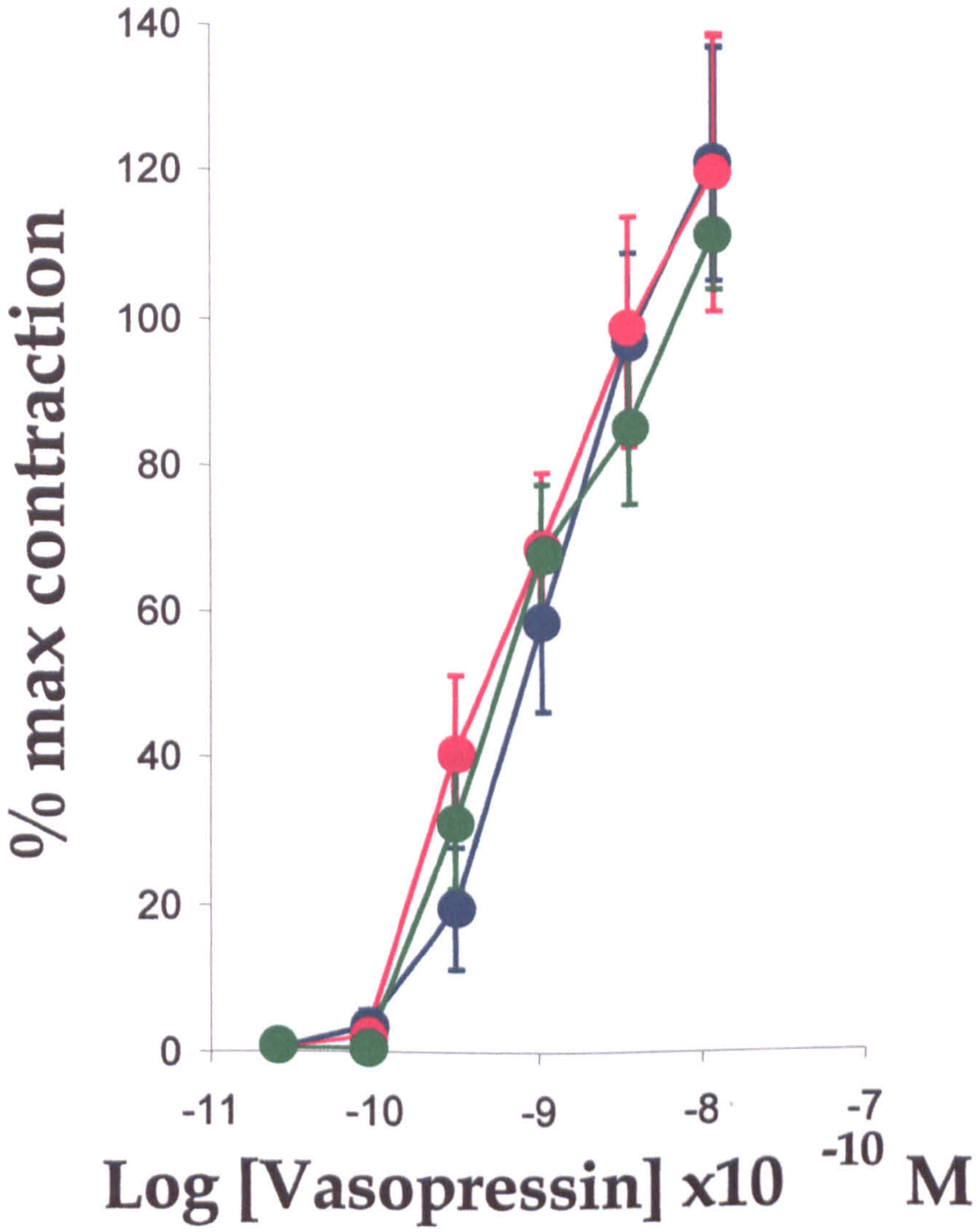
Legend:

	EC_{50}	E_{max}
vessel one	-9.1+/-0.1	122.57+/-8.48
vessel two	-9.0+/-0.1	108.47+/-4.06
vessel three	-8.9+/-0.1	106.47+/-11.41
ANOVA (factorial)	0.30	0.35

Statistical comparisons:

Fig 3.8

Variability of myometrial vessels taken from the same sample from pregnant women



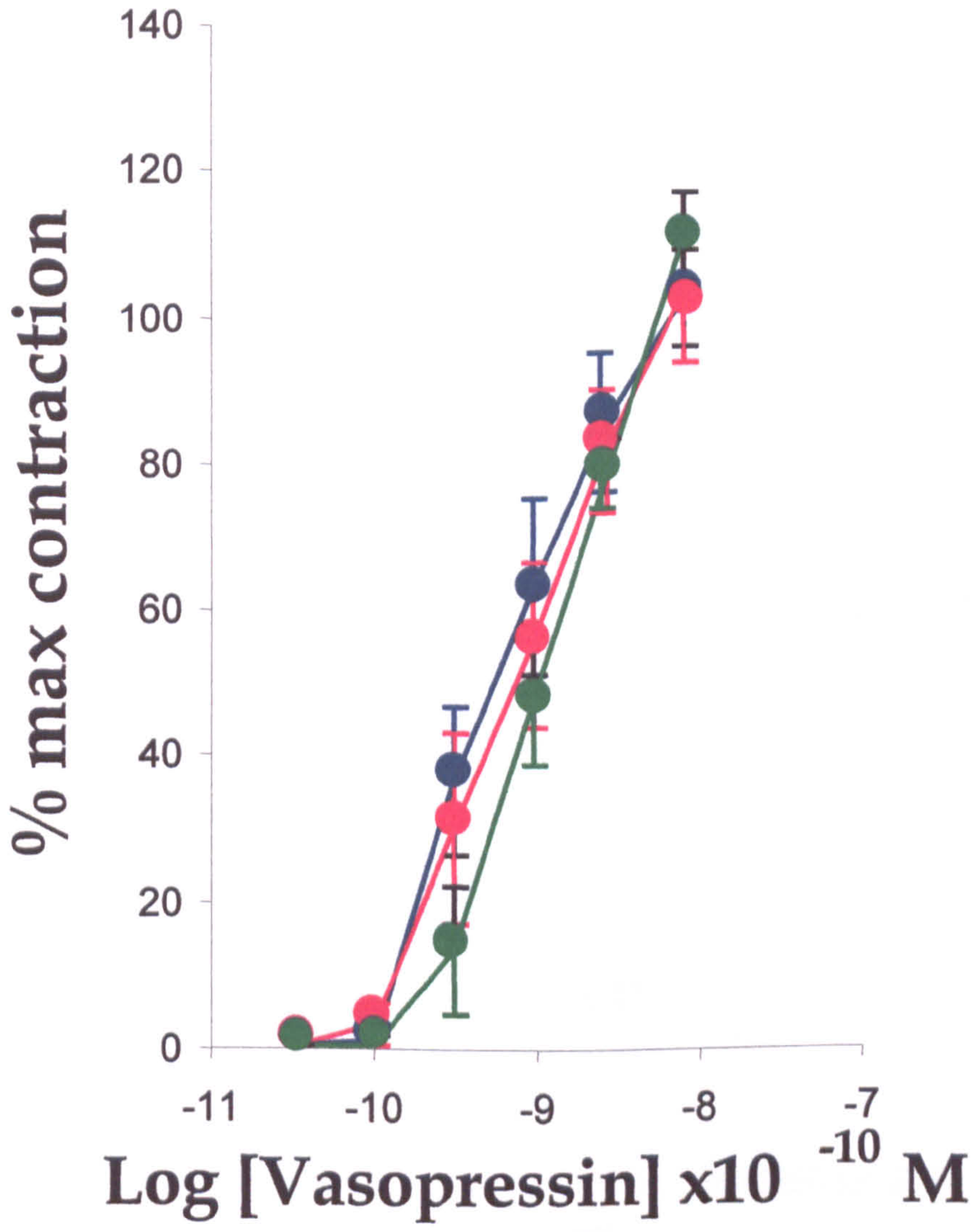
Legend:

Statistical comparisons:

	EC_{50}	E_{max}
vessel one	-9.1+/-0.1	123.21+/-16.57
vessel two	-9.3+/-0.2	121.66+/-19.50
vessel three	-9.2+/-0.2	112.76+/-7.43
ANOVA (factorial)	0.89	0.75

Fig 3.9

Variability of myometrial vessels taken from the same sample from women with pre-eclampsia



Legend:

Statistical comparisons:

	EC_{50}	E_{max}
vessel one	-9.1+/-0.2	104.32+/-6.97
vessel two	-9.1+/-0.2	104.69+/-9.15
vessel three	-9.0+/-0.1	112.21+/-7.29
ANOVA (factorial)	0.77	0.70

Table 3.3

Variability of dose response curves to vasopressin for non- pregnant, pregnant and pre-eclamptic omental vessels

Non-Pregnant (n=5)		Statistical comparisons:	
		ED_{50}	E_{max}
Vessel one curve		-8.5+/-0.0	76.88+/-8.40
Vessel two curve		-8.7+/-0.3	66.76+/-5.80
Vessel three curve		-8.7+/-0.2	72.61+/-7.70
ANOVA (factorial)	p=	0.83	0.66

Pregnant		Statistical comparisons:	
		ED_{50}	E_{max}
Vessel one curve (n=5)		-8.4+/-0.1	67.23+/-10.06
Vessel two curve (n=5)		-8.4+/-0.1	67.36+/-10.19
Vessel three curve (n=5)		-8.6+/-0.2	70.39+/-8.94
ANOVA (factorial)	p=	0.67	0.95

Pre-eclampsia (n=5)		Statistical comparisons:	
		ED_{50}	E_{max}
Vessel one curve		-8.9+/-0.3	83.42+/-14.87
Vessel two curve		-8.8+/-0.2	95.33+/-8.64
Vessel three curve		-8.8+/-0.2	81.23+/-12.66
ANOVA (factorial)	p=	0.90	0.65

response curves in myometrial vessels and as such this was not repeated (Ashworth, 1998). It has also been previously demonstrated that both bradykinin and acetylcholine produced reproducible endothelium dependent relaxation in omental resistance vessels (Ashworth et al., 1996b) (Pascoal and Umans, 1996), however, no such data existed on histamine. Therefore experiments were designed to investigate whether histamine caused endothelium dependent relaxation in omental resistance vessels.

3.1.4.2 Experimental design:

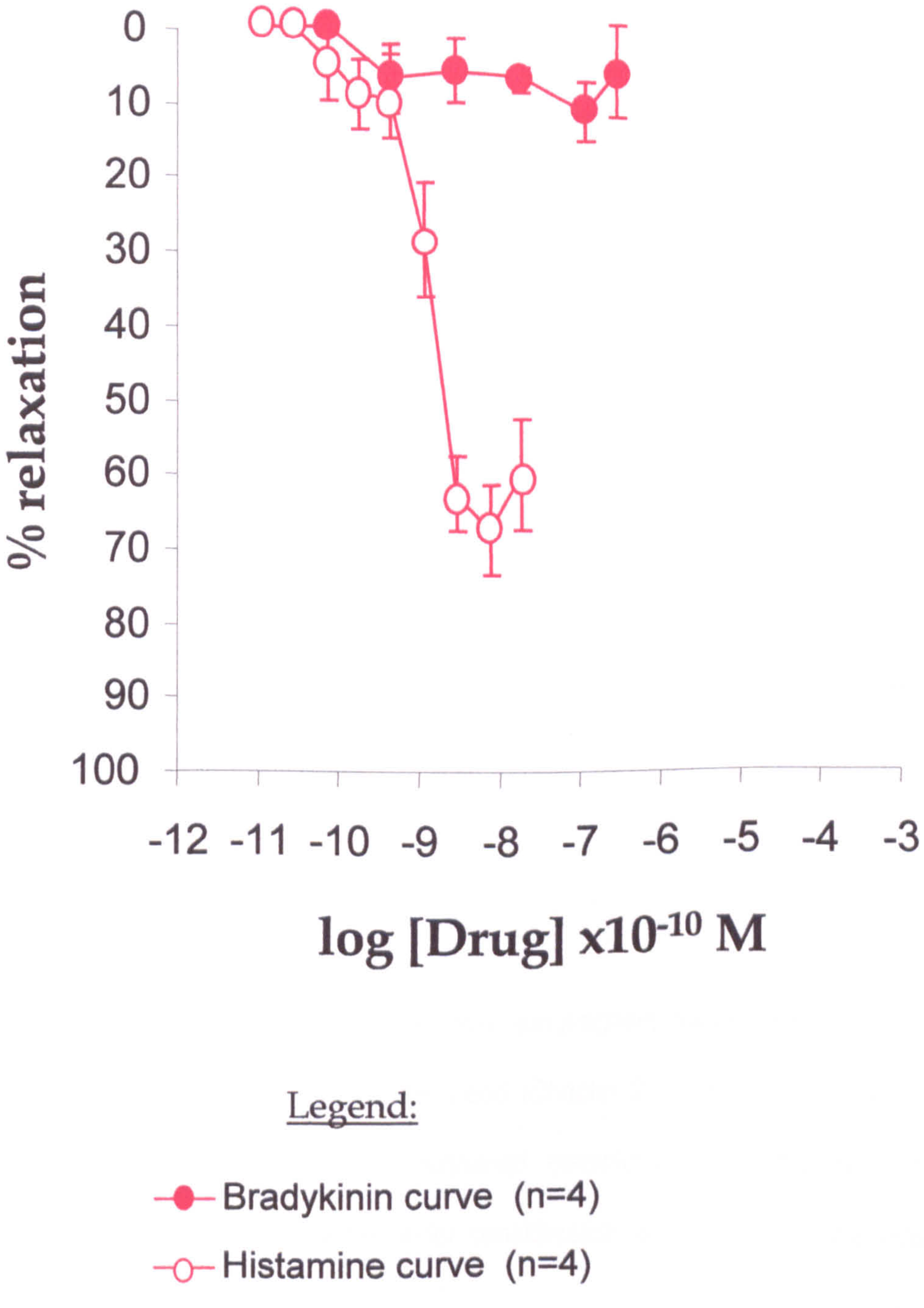
Omental vessels (n=4) were dissected as described previously (Chapter2), and mounted in intact / and endothelium stripped pairs. Following equilibration and normalization, vessels were then constricted with cumulative doses of vasopressin. Once a sustained contraction was attained, cumulative doses of bradykinin were added to the vessel chamber, to confirm endothelial stripping. After conformation that one of the pairs had been stripped of its endothelium a further constriction was initiated with incremental doses of vasopressin, again once a sustained contraction was attained, cumulative doses of histamine were added to the organ bath.

3.1.4.3 Results:

Omental vessels with the endothelium denuded were unable to produce any significant relaxation to bradykinin (Fig 3.10), which served to confirm the absence of the endothelium. However, the addition of histamine to the same vessels produced a significant relaxation response (Fig 3.10).

Fig 3.10

Comparison between bradykinin and histamine relaxation curves in omental resistance vessels stripped of endothelium



3.1.4.4 Conclusions:

Histamine produces non-endothelium dependent relaxations in human omental vessels. As such histamine was not used in experiments that utilised omental vessels.

3.1.5 Reproducibility of dose response curves vessels obtained to the vasodilators:

3.1.5.1 Aims:

- To confirm previous data from our laboratory that the agents to be used produced dose response curves.
- To demonstrate that the dilator agents used were capable of being used repeatedly on the same vessel with consistent results.

3.1.5.2 Experimental design:

Myometrial and omental vessels were obtained from non-pregnant women, pregnant women and women with pre-eclampsia as previously described (Chapter 2.3) and were then exposed to vasopressin (Chapter 2.3.5). Once a sustained constriction was attained, cumulative concentrations of the vasodilator agent under consideration were added to the myography chamber (Chapter 2.2.5).

However, during the course of the preliminary experiments it became apparent that acetylcholine may not cause an endothelium dependent relaxation in pregnant myometrial resistance vessels.

As such a further experimental protocol was designed to test this hypothesis. The experimental work during this set of experiments was carried out under the close supervision of an experienced technician (Mrs A.Y.Warren), who confirmed that the preparation of drugs was correct and that the correct dosages were added to each myography bath.

Two vessels were dissected and mounted on separate myographs, omental vessels were also dissected to act as positive controls (these had been demonstrated to display endothelium dependent relaxation to acetylcholine). Once equilibration and normalisation had been completed vessels were randomised to one arm of a Latin square experiment (see Chapter 3.2.1.2).

3.1.5.3 Results:

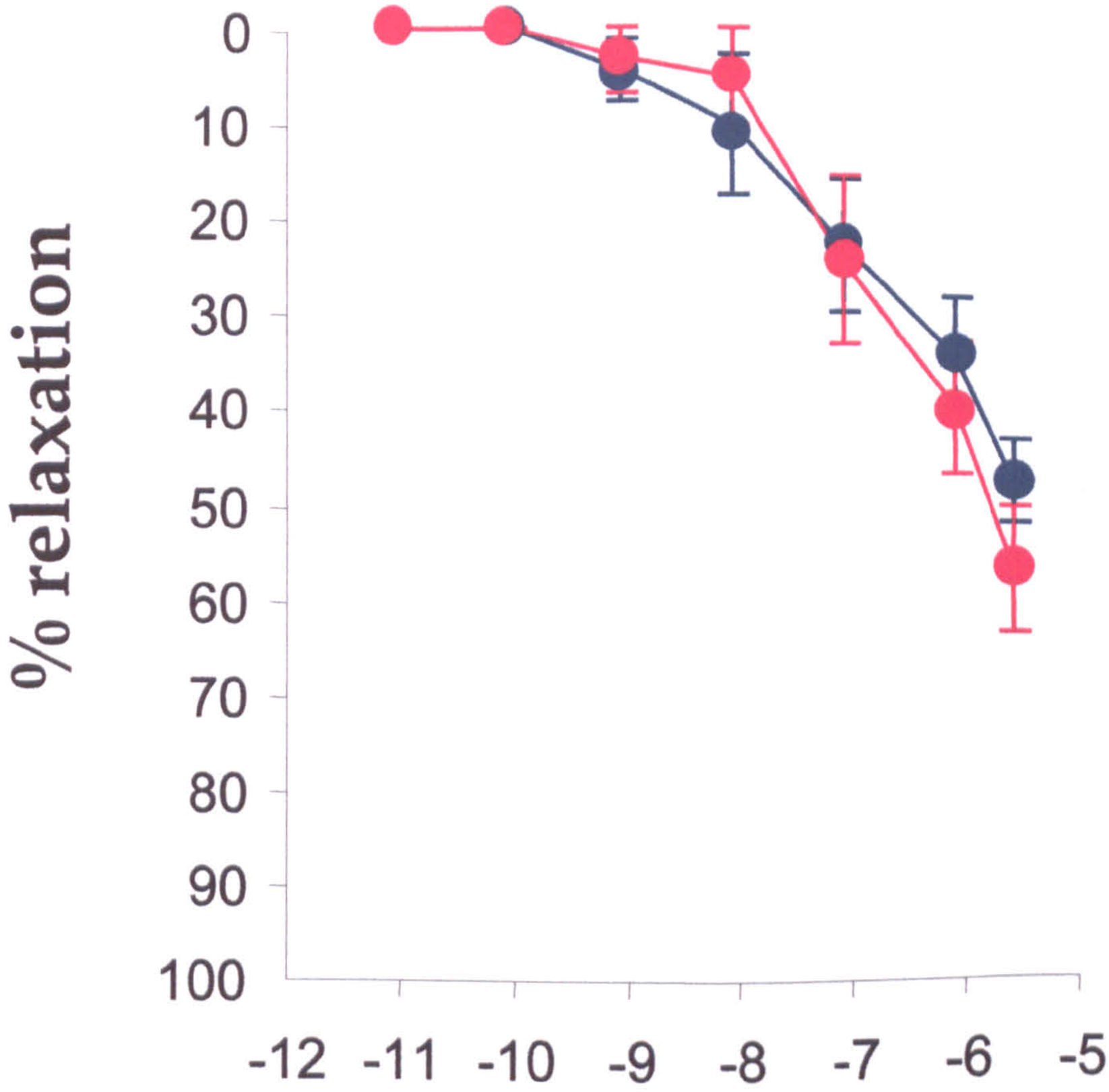
Endothelium dependent relaxation:

Myometrial vessels produced endothelium dependent relaxation to bradykinin and histamine in all groups (see Fig 3.11, 3.12, 3.13 and Table 3.4), although the endothelium dependent relaxation to bradykinin was significantly reduced in the women with pre-eclampsia (see Chapter 6). Omental vessels produced endothelium dependent relaxation in response to bradykinin and acetylcholine (See Table 3.5 & 3.6). The endothelium dependent response to aceylcholine was also significantly attenuated in the women with pre-eclampsia (see Chapter 6).

In preliminary studies, acetylcholine produced no endothelium dependent relaxation in myometrial resistance vessels. The Latin square design was used to demonstrate that the endothelium was present and functional prior to exposure to acetylcholine. Yet in spite of the presence of a

Fig 3.11

Reproducibility of relaxation curves for non-pregnant myometrial vessels



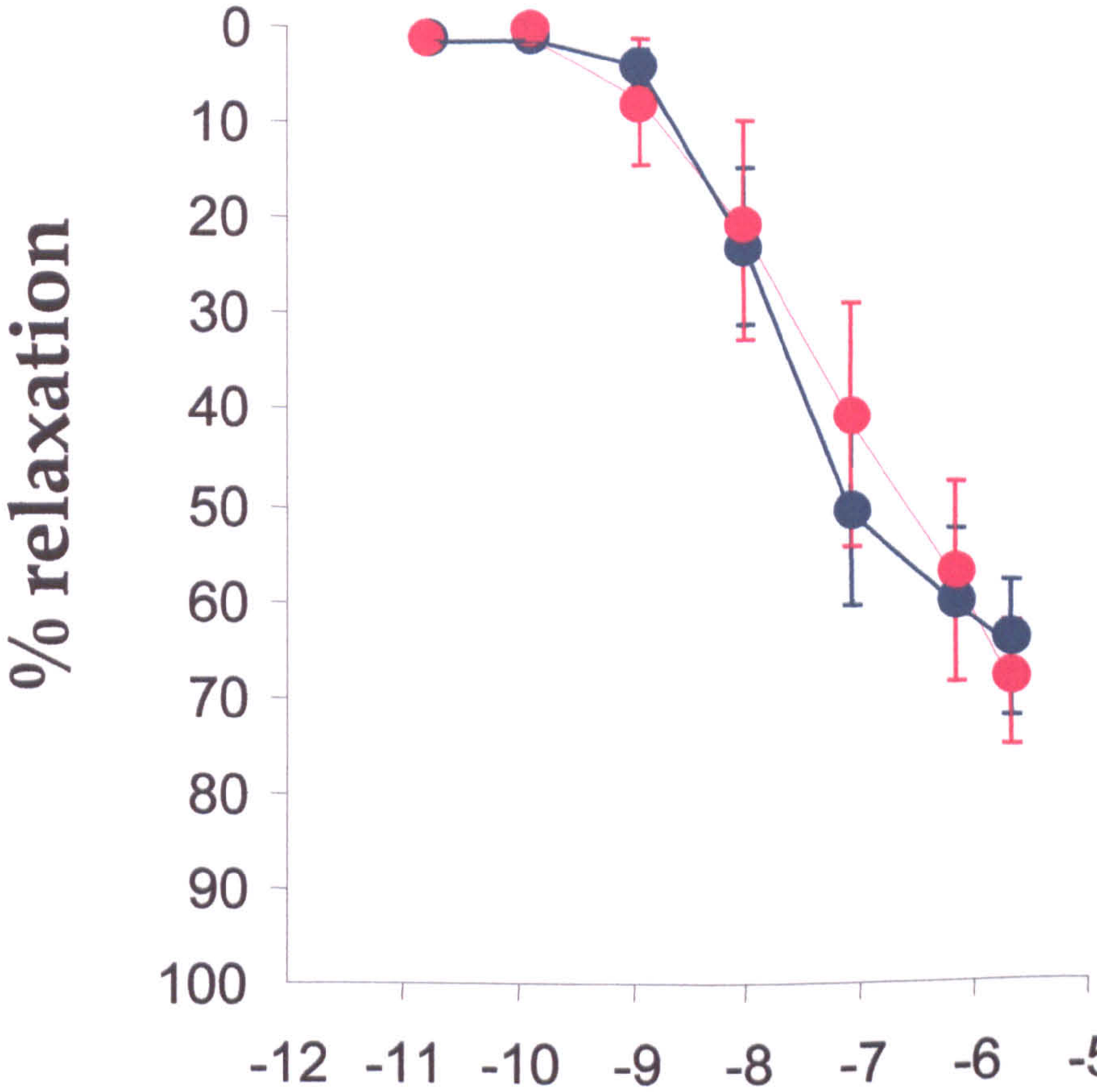
Legend:

Statistical comparisons:	ED_{50}	E_{max}
First relaxation curve	-5.6+/-0.3	46.30+/-4.18
Second relaxation curve	-5.8+/-0.4	55.10+/-6.32
t-test (paired)	0.67	0.22

ANOVA: P=0.61

Fig 3.12

Reproducibility of relaxation curves for pregnant myometrial vessels



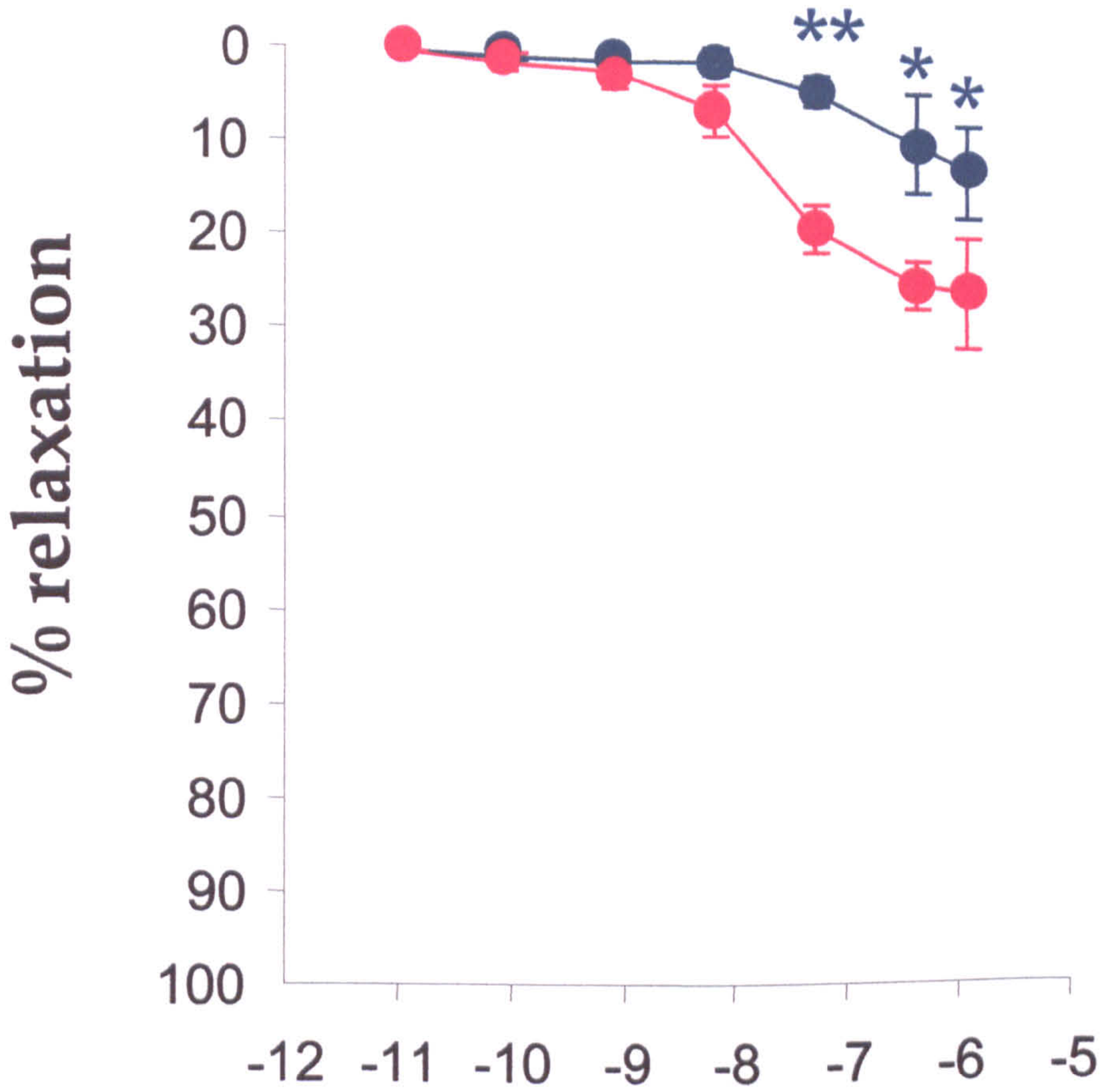
Legend:

Statistical comparisons:	ED_{50}	E_{max}
First relaxation curve	-7.5+/-0.2	66.43+/-7.44
Second relaxation curve	-7.2+/-0.5	70.34+/-6.71
t-test (paired)	0.48	0.52

ANOVA; p=0.91

Fig 3.13

Reproducibility of relaxation curves for myometrial vessels from women with pre-eclampsia



Legend:

	$ED_{50[Mod]}$	E_{max}
First relaxation curve	-6.3+/-0.3	14.44+/-5.76
Second relaxation curve	-7.5+/-0.2	29.03+/-3.61
t-test (paired)	0.02	0.03

Statistical comparisons:

Bonferroni-Dunn

** - P< 0.01

* - p < 0.05:

ANOVA (Repeated measures of analysis); p=0.005

Table 3.4

Reproducibility of histamine relaxation curves for non- pregnant, pregnant and pre-eclamptic myometrial vessels

Non-Pregnant		Statistical comparisons:	
		ED_{50}	E_{max}
First relaxation curve		-9.2+/-0.2	78.27+/-3.54
Second relaxation curve		--9.2+/-0.3	80.04+/-4.88
t-test (paired)	p=	0.83	0.63
ANOVA	p =	0.96	

Pregnant		Statistical comparisons:	
		$ED_{50[mod]}$	E_{max}
First relaxation curve		-8.7+/-0.3	43.27+/-2.57
Second relaxation curve		-8.8+/-0.2	53.18+/-0.87
t-test (paired)	p=	0.93	0.074
ANOVA	p =	0.01	

Pre-eclampsia		Statistical comparisons:	
		$ED_{50[mod]}$	E_{max}
First relaxation curve		-9.7+/-0.3	40.88+/-3.48
Second relaxation curve		-9.3+/-0.2	52.17+/-5.37
t-test (paired)	p=	0.08	0.09
ANOVA	p =	0.07	

Table 3.5

Reproducibility of bradykinin relaxation curves for non- pregnant, pregnant and pre-eclamptic omental vessels

Non-Pregnant (n=6)			Statistical comparisons:	
		ED_{50}		E_{max}
First relaxation curve		-8.3+/-0.3		90.08+/-5.30
Second relaxation curve		-8.1+/-0.1		88.24+/-4.30
	t-test (paired) p=	0.35		0.4
	ANOVA p =	0.84		

Pregnant			Statistical comparisons:	
		ED_{50}		E_{max}
First relaxation curve		-8.9+/-0.1		98.10+/-0.91
Second relaxation curve		-8.7+/-0.4		93.79+/-1.89
	t-test (paired) p=	0.39		0.06
	ANOVA p =	0.32		

Pre-eclampsia			Statistical comparisons:	
		ED_{50}		E_{max}
First relaxation curve		-7.8+/-0.6		81.18+/-9.47
Second relaxation curve		-8.0+/-.5		91.12+/-3.12
	t-test (paired) p=	0.42		0.85
	ANOVA p =	0.85		

Table 3.6

Reproducibility of acetylcholine relaxation curves for non- pregnant, pregnant and pre-eclamptic omental vessels

Non-Pregnant (n=4)		Statistical comparisons:	
		ED_{50}	E_{max}
First relaxation curve		-5.5+/- 0.4	67.9 +/- 4.8
Second relaxation curve		-5.9+/- 0.5	70.4 +/- 5.9
t-test (paired)	P=	0.75	0.72
ANOVA	P =	0.2	

Pregnant		Statistical comparisons:	
		ED_{50}	E_{max}
First relaxation curve		-6.6+/-0.4	69.63+/-6.16
Second relaxation curve		-6.4+/-0.2	73.37+/-5.47
t-test (paired)	p=	0.72	0.18
ANOVA	p =	0.52	

Pre-eclampsia		Statistical comparisons:	
		ED_{50}	E_{max}
First relaxation curve		-5.4+/-0.5	19.86+/-2.15
Second relaxation curve		-6.0+/-0.5	22.57+/-2.19
t-test (paired)	p=	0.29	0.21
ANOVA	p =	0.90	

functional endothelium, acetylcholine was unable to produce either relaxation or constriction in these vessels (Fig.3.14). All the omental vessels studied relaxed to acetylcholine. The Latin square design also showed that this was not a time dependent phenomenon as a bradykinin doses response curves were obtained after exposure to acetylcholine (Fig 3.14 & 15). The omental vessels that acted as positive controls produced endothelium dependent relaxation to both acetylcholine and bradykinin (Table 3.6).

Reproducibility of dose response curves:

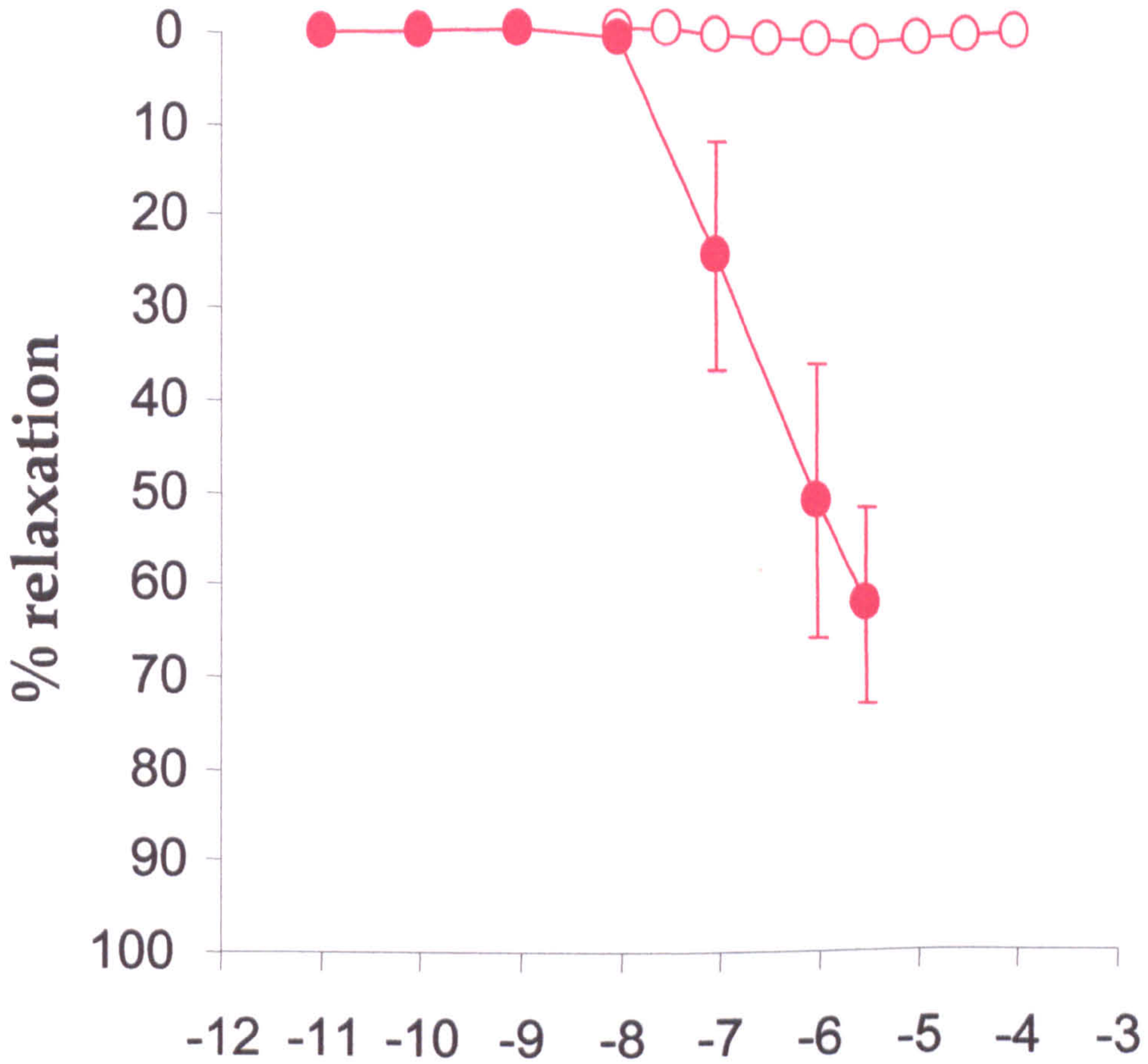
In each group studied, and for each vasodilator, comparisons of the two dose response curves were made by paired t-tests of the EC_{50}, E_{max} responses and by repeated measures analysis of variance. Both myometrial and omental non-pregnant and pregnant vessels produced no significant differences between the first and second dose response curves: myometrial (Fig 3.11, 3.12 and Table 3.4) and omental (Table 3.5 & 3.6). However, in the group of women with pre-eclampsia there was a statistically significant difference between the first dose response curve and the second to bradykinin (Fig 3.13)

3.1.5.4 Discussion:

In the omentum, bradykinin and acetylcholine caused an endothelium dependent relaxation. This is in accord with several other studies which demonstrated relaxation to bradykinin (Ashworth et al., 1996b) (Pascoal and Umans, 1996) (Pascoal et al., 1998) and to acetylcholine (Pascoal and Umans, 1996) (Pascoal et al., 1998). However, see Chapter 7 for a further experimental work and discussion.

Fig 3.14

Comparison between bradykinin and acetylcholine relaxation curves in pregnant myometrial resistance vessels



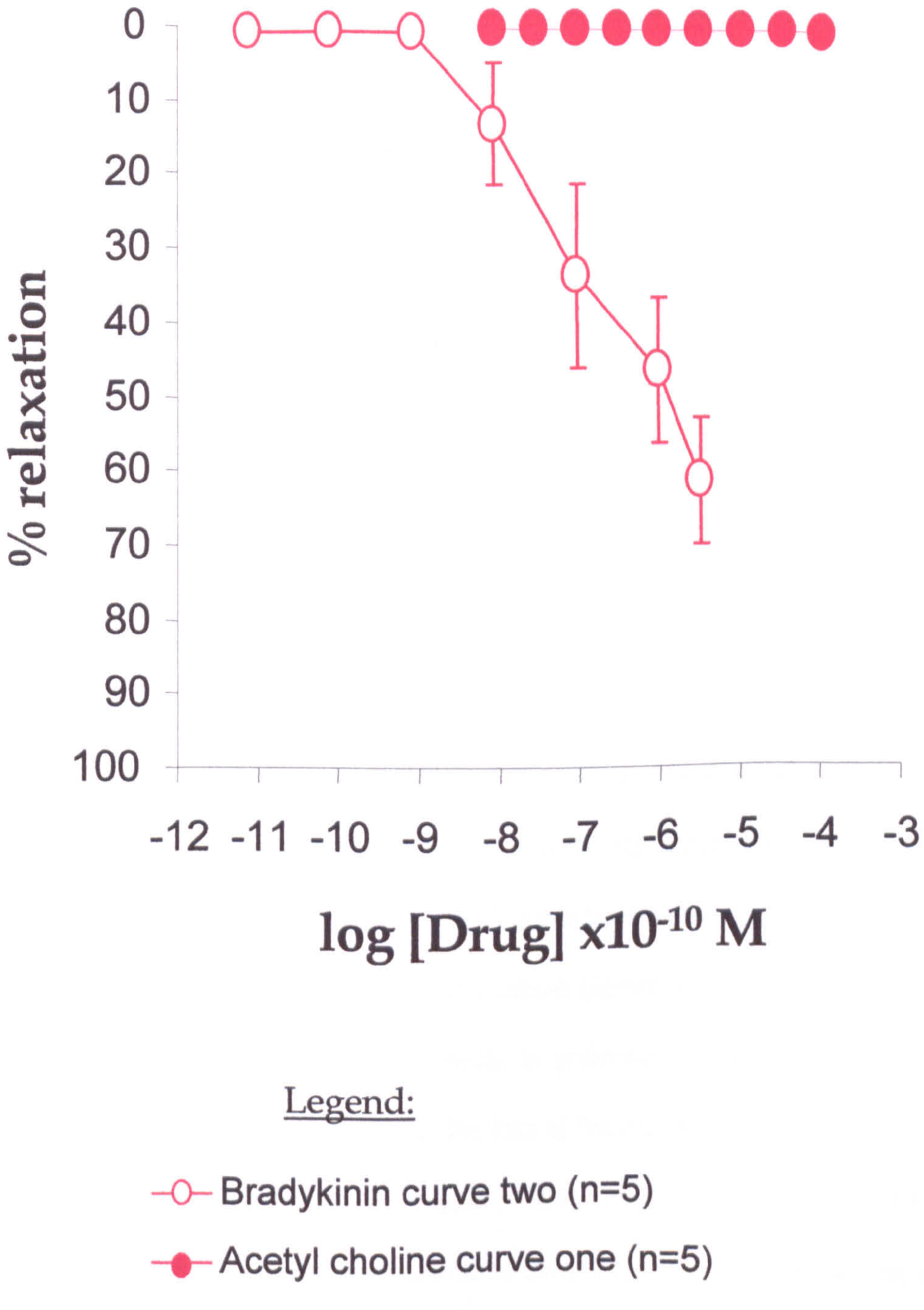
Legend:

Bradykinin curve one (n=5)

Acetyl choline curve two (n=5)

Fig 3.15

Comparison between bradykinin and acetylcholine relaxation curves in pregnant myometrial resistance vessels



The demonstration of endothelium dependent relaxation to bradykinin and histamine in myometrial vessels confirms the previous work of Ashworth et al (Ashworth et al., 1996a) (Ashworth, 1998). However, the lack of response to acetylcholine in these vessels is contradictory to that of Ashworth et al (1996). Ashworth et al (1996) demonstrated that acetylcholine elicited an endothelium dependent relaxation. However, the number of vessels utilised was small (n=3) and the standard error of the means were large. A further caveat to the results of Ashworth et al is the fact that vessels were stored for up to forty-eight hours after dissection. Vessel storage may have affected the results obtained as storage may have increased the density of the acetylcholine receptors on the vessels. This would have the effect of increasing the relaxation to acetylcholine that was obtained. This hypothesis of increased relaxation with storage was not formally tested. However, Kublickiene et al (1997) utilised the more physiological pressure myography system and demonstrated that acetyl choline produces endothelium dependent relaxation in myometrial vessels that have been contracted with noradrenaline (Kublickiene et al., 1997). Noradrenaline has been shown to be unable to produce sustained contractions when wire myography was utilised (Ashworth, 1998), a finding that has also been confirmed by the author of this thesis in preliminary experiments (n=2). These differences have been postulated to be due to the loss of the myogenic component of vascular tone which occurs in isometric studies (Dunn et al., 1994). However, this is in contrast to the study of Steele et al which demonstrated that noradrenaline could induce contractions in both pregnant and non-pregnant myometrial vessels (Steele et al., 1993). Discussion with the Steele et al revealed these responses were obtained with the vessel partially depolarised with potassium (12mM). Preliminary data (n=2) confirmed this finding.

It is known that different endothelium dependent relaxatory agents act in different ways to initiate relaxations even in the same vascular bed (Adeagbo and Triggle, 1993) (Alonso et al., 1991) (Garcia-Pascual et al., 1995) (see Chapter 6 &7) and a logical extension of this would be that the nature of the constrictor agent could further alter the subsequent relaxation. Several studies have demonstrated that omental vessels that are constricted with vasopressin undergo endothelium dependent relaxation to acetylcholine (Vila et al., 1991) (Pascoal and Umans, 1996) and this thesis has confirmed this (see Chapter 7). However, omental vessels constricted with U46619 have been shown to undergo neither contraction nor relaxation to acetylcholine (Wallerstedt and Bodelsson, 1997), a result which may suggest that the choice of vasoconstrictor used is important to the results obtained. Given these differences and discrepancies, the use of acetylcholine as a vasodilator in myometrial resistance vessels was abandoned.

The endothelium dependent vasodilators bradykinin and histamine were used for myometrial studies and acetylcholine and bradykinin were used for omental studies. The resistance vessels studied, were thus constricted to vasopressin, and could be relaxed with bradykinin, histamine and acetylcholine (in the omentum) on two occasions without tachyphylaxis or alteration of the response curves. However, the finding of a alteration in the response curves with consecutive relaxations in the group of women with pre-eclampsia meant that any protocol utilised would have to allow for time control vessels.

3.1.6 Variability of vessels obtained from the same patient to the vasodilators:

3.1.6.1 Aims:

- To determine whether vessel segments from the same experimental sample produced similar comparable relaxation curves that would allow comparisons to be made with respect to time.

3.1.6.2 Methods:

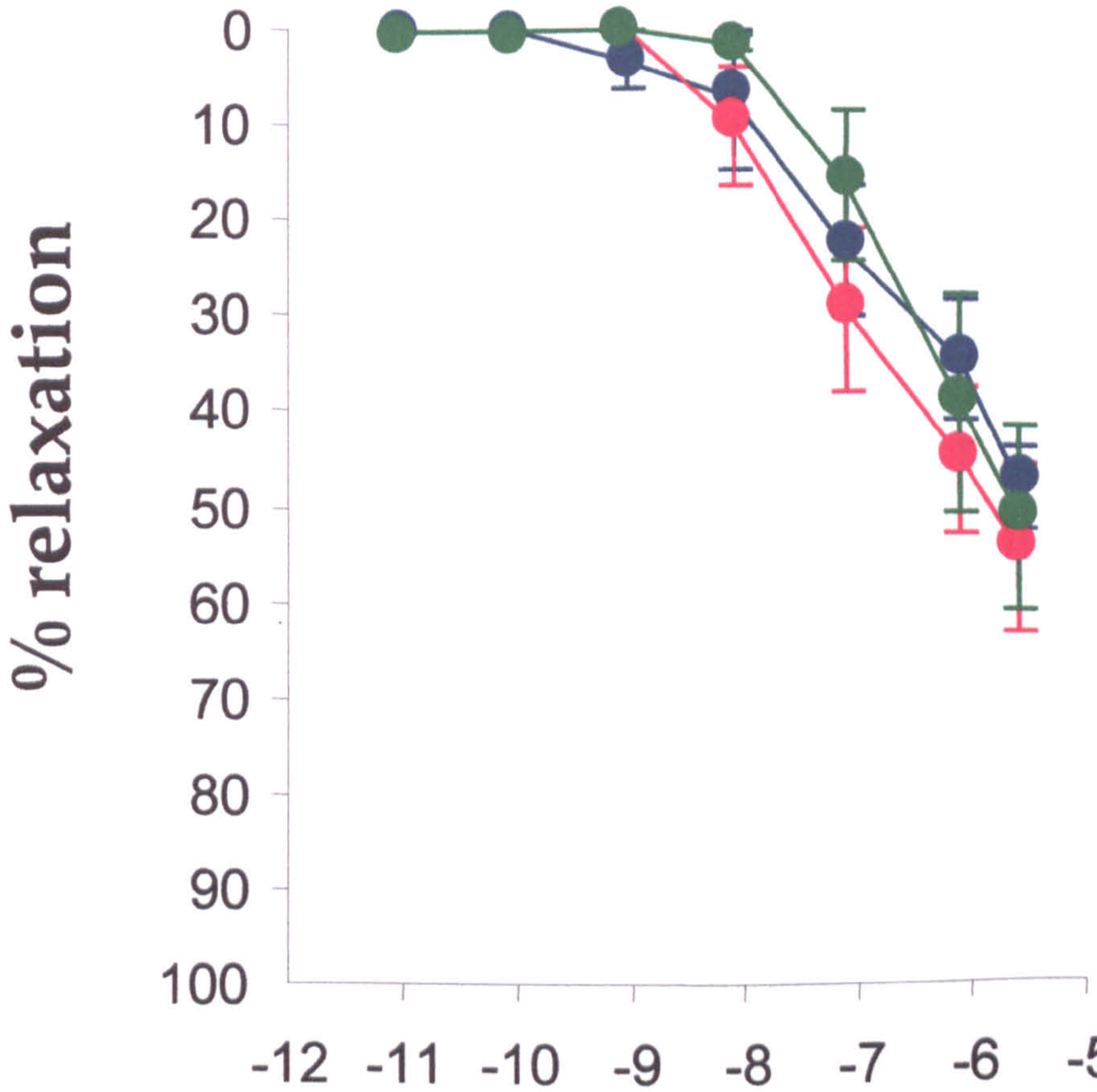
Myometrial and omental vessels were collected, dissected and mounted within 2 hours. Three vessels were then mounted on separate myographs. Vessels were then subjected to incremental doses of vasopressin. Following the addition of the vasoconstrictor, vessels were exposed to an incremental dose of the vasodilator under investigation bradykinin, histamine, and acetylcholine.

3.1.6.3 Results:

In all groups studied, all three vessels exposed to the vasodilator of interest (Fig 3.16,3.17,3.18 and Table 3.7, 3.8 & 3.9) produced dose response curves. The dose response curves obtained were compared with each other by comparison of their EC_{50} and E_{max} values. No significant differences were noted in any of the groups investigated.

Fig 3.16

Variability of myometrial vessels taken from the same sample from non pregnant women



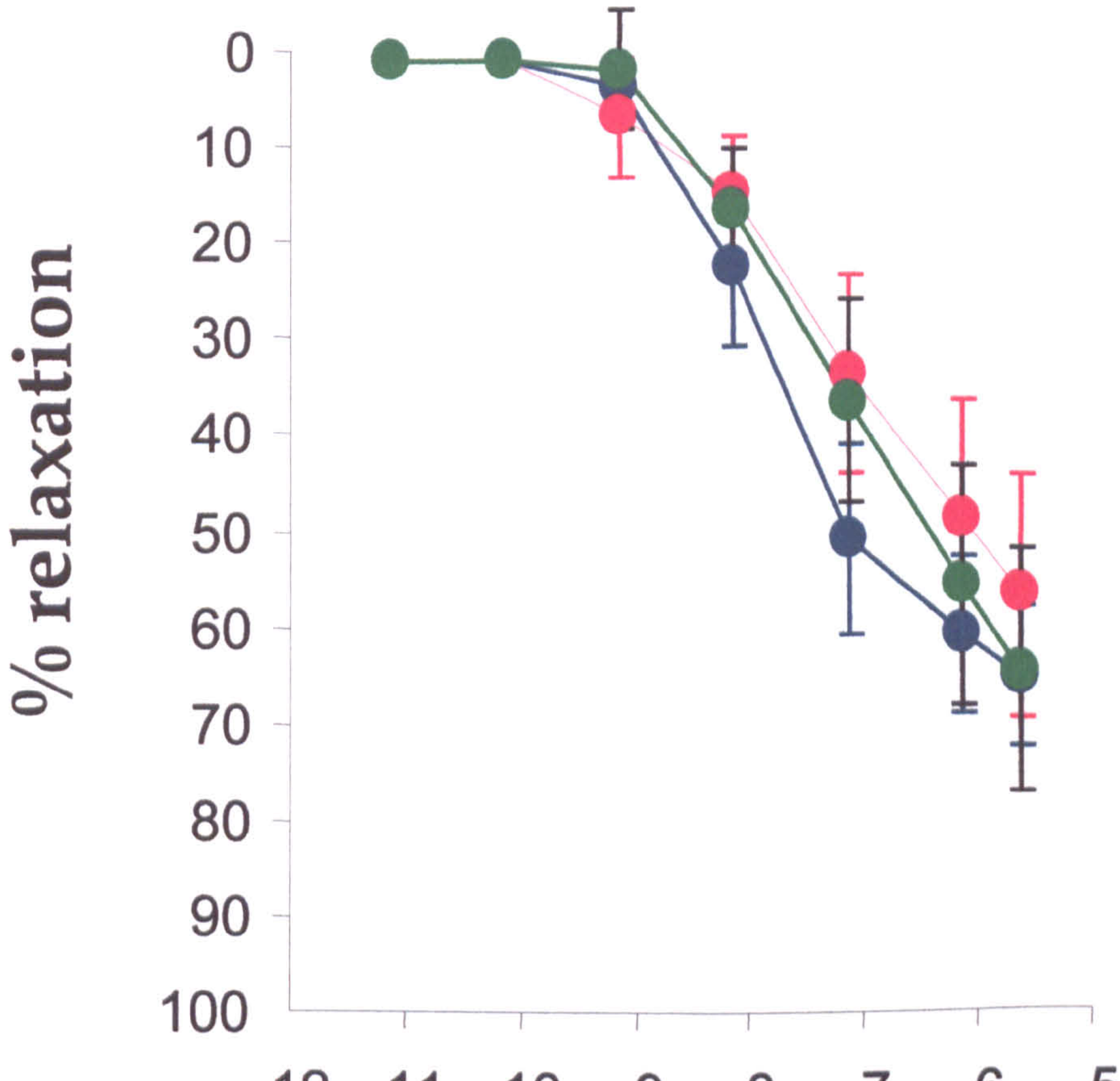
Legend:

Statistical comparisons:

	$ED_{50[mod]}$	E_{max}
vessel one	-7.2+/-0.4	47.07+/-4.12
vessel two	-7.0+/-0.2	53.41+/-8.72
vessel three	-6.6+/-0.2	50.23+/-9.35
ANOVA (factorial)	0.52	0.85

Fig 3.17

Variability of myometrial vessels taken from the same sample from pregnant women



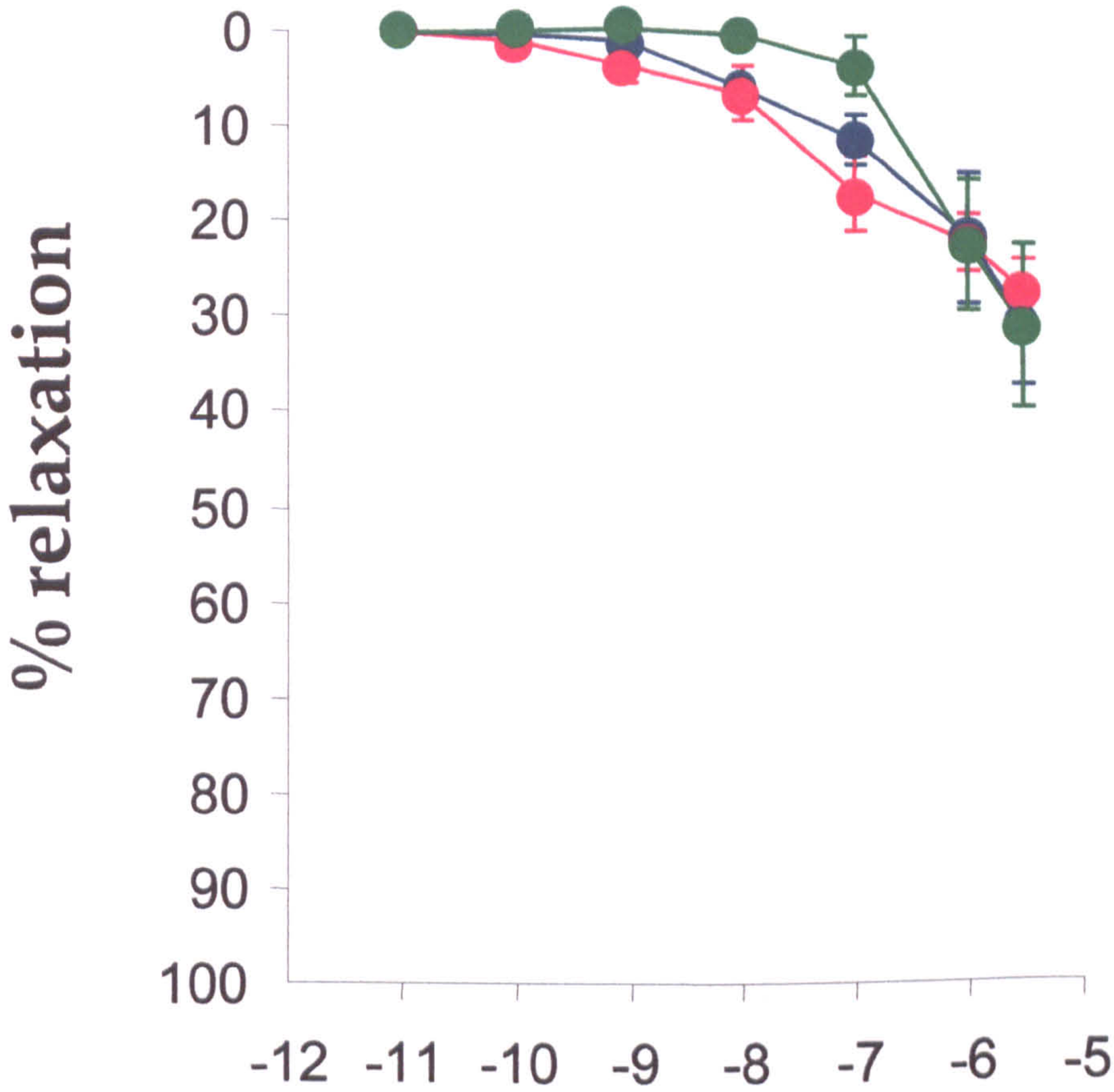
Legend:

Statistical comparisons:

	$ED_{50[mod]}$	E_{max}
vessel one	-7.5+/-0.2	66.19+/-7.48
vessel two	-7.7+/-0.3	57.52+/-13.03
vessel three	-7.2+/-0.4	65.87+/-7.75
ANOVA (factorial)	0.52	0.79

Fig 3.18

Variability of myometrial vessels taken from the same sample from pregnant women with pre-eclampsia



Legend:

Statistical comparisons:

	$ED_{50[mod]}$	E_{max}
vessel one	-6.5+/-0.2	30.51+/-6.82
vessel two	-6.7+/-0.3	29.02+/-3.63
vessel three	-6.2+/-0.4	32.97+/-9.10
ANOVA (factorial)	0.52	0.90

Table 3.7

Variability of histamine relaxation curves for non- pregnant, pregnant and pre-eclamptic myometrial vessels

Non-Pregnant (n=6)		Statistical comparisons:	
		ED_{50}	E_{max}
Vessel one curve		-9.3+/-0.3	77.50+/-4.28
Vessel two curve		-8.7+/-0.3	70.20+/-6.20
Vessel three curve		-8.7+/-0.6	70.31+/-13.64
ANOVA (factorial)	p=	0.44	0.63

Pregnant (n=6)		Statistical comparisons:	
		$ED_{50[mod]}$	E_{max}
Vessel one curve		-8.8+/-0.3	42.90+/-3.30
Vessel two curve		-9.1+/-0.3	39.25+/-3.42
Vessel three curve		-8.7+/-0.4	42.29+/-2.49
ANOVA (factorial)	p=	0.68	0.69

Pre-eclampsia (n=5)		Statistical comparisons:	
		$ED_{50[mod]}$	E_{max}
Vessel one curve		-9.0+/-0.2	42.38+/-2.57
Vessel two curve		-9.0+/-0.2	51.72+/-3.93.
Vessel three curve		-8.7+/-0.3	46.11+/-5.63
ANOVA (factorial)	p=	0.56	0.27

Table 3.8

Variability of braykinin relaxation curves for non- pregnant, pregnant and pre-eclamptic omental vessels

Non-Pregnant (n=6)		Statistical comparisons:	
		ED_{50}	E_{max}
Vessel one curve		-8.3+/-0.3	90.08+/-5.30
Vessel two curve		-8.2+/-0.5	93.09+/-3.12
Vessel three curve		-8.0+/-0.3	93.81+/-1.97
ANOVA (factorial)	p=	0.83	0.76

Pregnant (n=6)		Statistical comparisons:	
		ED_{50}	E_{max}
Vessel one curve		-8.4+/-0.1	98.10+/-0.91
Vessel two curve		-8.5+/-0.3	94.82+/-2.01.
Vessel three curve		-8.2+/-0.3	91.07+/-3.64
ANOVA (factorial)	p=	0.75	0.14

Pre-eclampsia (n=6)		Statistical comparisons:	
		ED_{50}	E_{max}
Vessel one curve (n=3)		-8.3+/-0.3	90.29+/-3.65
Vessel two curve (n=6)		-8.2+/-0.9	90.72+/-4.35.
Vessel three curve (n=6)		-8.6+/-0.6	92.53+/-7.47
ANOVA (factorial)	p=	0.96	0.86

Table 3.9

Variability of acetylcholine relaxation curves for non- pregnant, pregnant and pre-eclamptic omental vessels

Non-Pregnant (n=6)		Statistical comparisons:	
		ED_{50}	E_{max}
Vessel one curve		-5.5+/-0.4	67.89+/-4.83
Vessel two curve		-6.0+/-0.5	69.74+/-5.93
Vessel three curve		-5.7+/-0.1	73.19+/-2.74
ANOVA (factorial)	p=	0.79	0.73

Pregnant (n=6)		Statistical comparisons:	
		ED_{50}	E_{max}
Vessel one curve		-6.0+/-0.6	69.63+/-6.16
Vessel two curve		-5.9+/-0.6	77.38+/-7.970
Vessel three curve		-6.4+/-0.5	77.90+/-7.34
ANOVA (factorial)	p=	0.75	0.67

Pre-eclampsia (n=5)		Statistical comparisons:	
		$ED_{50[mod]}$	E_{max}
Vessel one curve		-5.4+/-0.5	19.86+/-2.15
Vessel two curve		-5.3+/-0.2	22.04+/-2.53
Vessel three curve		-4.9+/-0.4	11.26+/-2.39
ANOVA (factorial)	p=	0.67	0.37

3.1.6.4 Discussion:

All vasodilators investigated produced comparable dose response curves in different vessels from the same patient and thus allowed time control experiments to be incorporated into the protocol.

3.1.7 Determination of vascular function following storage:

3.1.7.1 Aim:

- To determine if resistance vessels could be stored overnight without loss of receptor-mediated vasoreactivity.

3.1.7.2 Experimental design:

Myometrial vessels were obtained from pregnant women (n=16). The vessels were dissected out as described in Chapter 2.4.2. Some vessels were mounted and experimented on immediately while others were stored overnight before experimentation as described in Chapter 2.4.6.

After storage the vessels were mounted and the experimental protocol performed (Chapter 2.5.5); vessels were subjected to incremental doses of vasopressin and then bradykinin (Chapter 2.4.5.1,2 and 5).

3.1.7.3 Results:

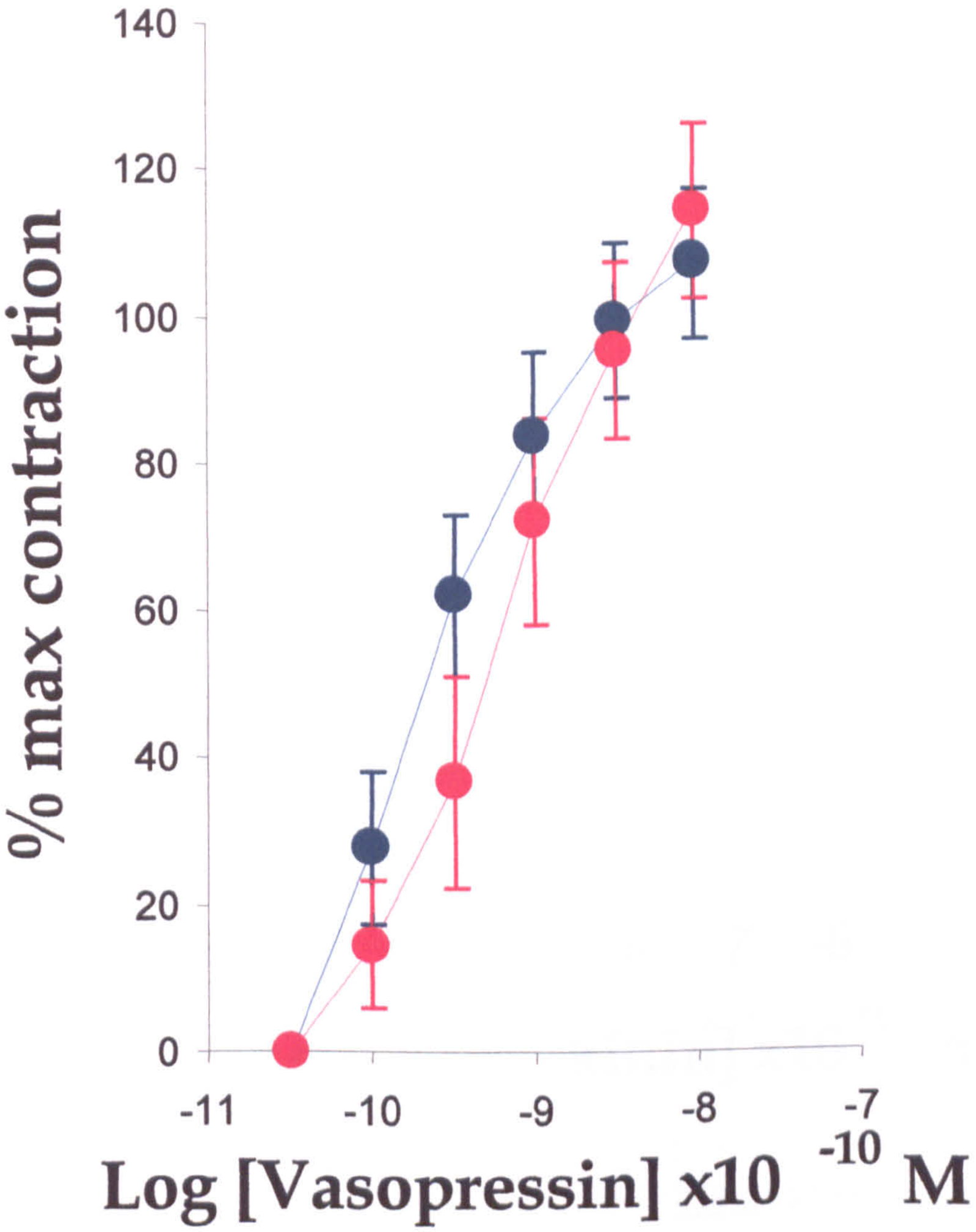
In this study 81% of the vessels stored gave a response to vasopressin. Stored vessels that were capable of responding demonstrated no significant differences in either their vasoconstrictor responses or their endothelium dependent relaxation responses when compared to fresh vessels (ANOVA; p=0.35, Vasopressin, p = 0.10, Bradykinin) (Fig 3.19 & 3.20). There was no-significant increase in the pH of PSS due to storage (pH 7.40 +/- 0.06 Vs 7.54 +/- 0.15 t-test; p=0.089). There was also no significant difference in the glucose concentrations with storage overnight (6.28+/- 0.01 Vs 6.30+/- 0.05: t-test; = 0.67).

3.1.7.4 Conclusions:

The preservation of pH, glucose and response to the pharmacological agents vasopressin and bradykinin over the storage period, meant that longitudinal experiments could be undertaken without detrimental effects on vessels function with respect to vasopressin and bradykinin causing bias to the system.

Fig 3.19

Effect of storage on contraction dose response curves for myometrial vessels



Legend:

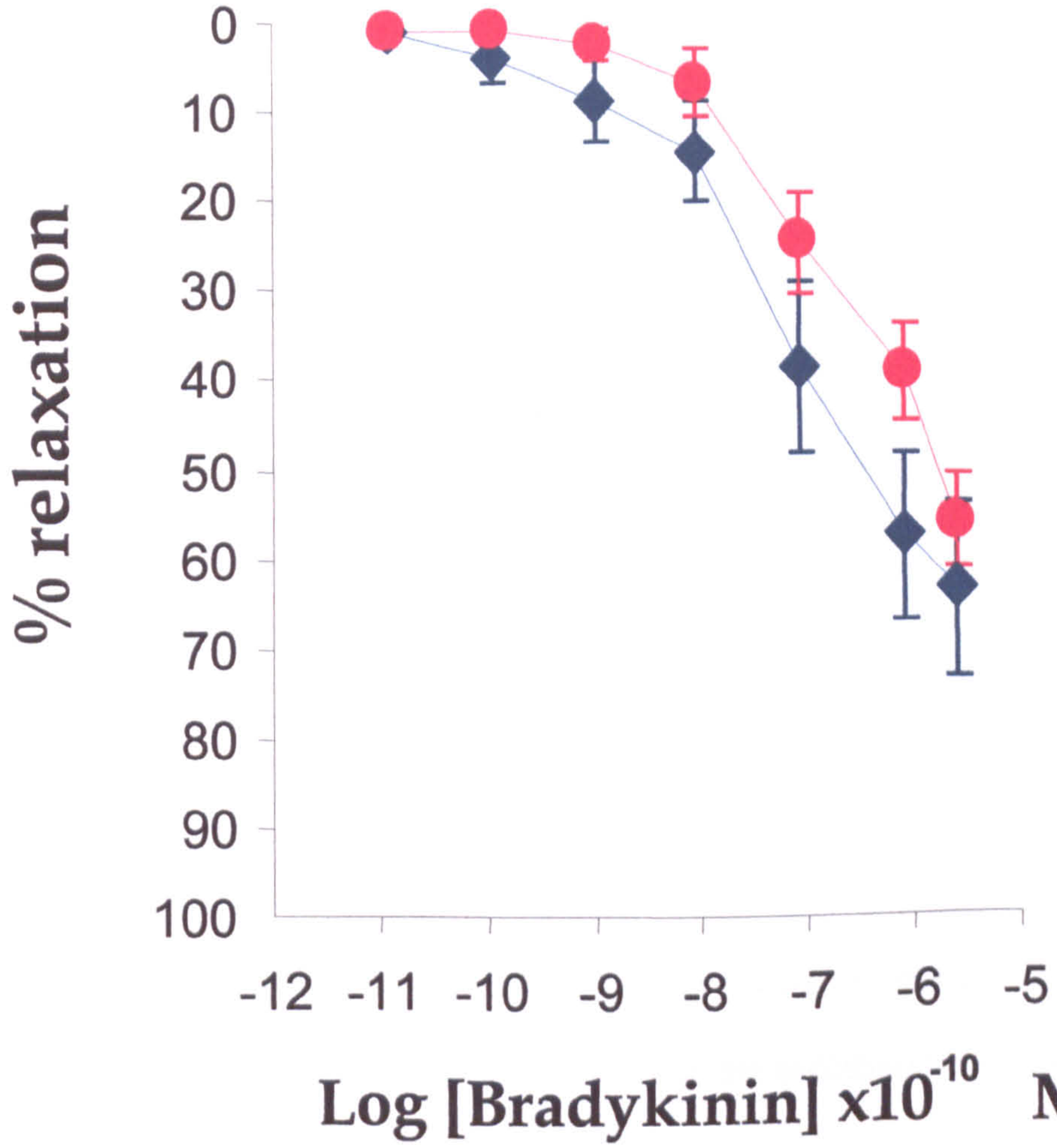
	EC 50	E max
Stored vessels	-9.5+/-0.2	106.5 +/- 10.0
Fresh vessels	-9.6+/-0.2	113.6 +/- 11.8
t-test (paired)	0.98	0.65

Statistical comparisons:

ANOVA; p=0.35

Fig 3.20

Effect of storage for 16 hours on myometrial vessels relaxation dose response curves



Legend:

Statistical comparisons:

	ED_{50}	E_{max}
Stored vessel	-6.1+/-0.8	63.43+/-9.19
Fresh vessel	-6.0+/-0.2	57.78+/-4.07
t-test (paired)	0.91	0.60
		ANOVA; p=0.10

3.2 Definitive Experimental Protocol

The definitive protocol was determined from the pilot data given above and in conjunction with other available literature (McCarthy et al., 1993b) (Ashworth et al., 1996a) (Knock and Poston, 1996) (Ashworth, 1998).

In order to compare contractions of resistance vessels to vasopressin between different groups of women a standard contraction was performed for each vessel. The standard contraction used was a KPSS contraction in the presence of noradrenaline ($1x10^{-5}$ M). However, as it had been suggested that such a contraction might result in tachphylaxis and affect results obtained by altering the membrane potential (personnel communication with Dr W Dunn), this contraction needed to be at the end of the protocol. It was also necessary to demonstrate that vessels exhibiting decreased endothelium-dependent relaxation were not doing so due to a smooth muscle abnormality. This was achieved by the use of the endothelium-independent vasodilator sodium nitroprusside ($1x10^{-9}$ -$1x10^{-5}$ M).

Resistance arteries of 200 - 550 µm were dissected from the uterine and omental biopsy specimens, which had been collected into oxygenated ice-cold PSS as described. In all the study groups, vessels from the same patient, and where possible portions of the same vessel were allocated to each study arm.

3.2.1 Protocol for the investigation of vascular reactivity changes with pregnancy and pre-eclampsia:

All vessels in this protocol were dissected and experiment begun within 2 hours. Vessels were mounted and normalised (Chapter 2) and then left for 15 minutes to attain a steady baseline. Once this was achieved the following protocol was performed. Three vessels were dissected and mounted on different myographs whenever possible.

Myograph one:

1) Contraction to vasopressin ($1x10^{-10}$ -$1x10^{-8}$ M) then relaxation to bradykinin ($1x10^{-10}$– $3.3x10^{-10}$ M).
2) Chamber washed four times with 40 mls of PSS.
3) Contraction to vasopressin ($1x10^{-10}$ -$1x10^{-8}$ M) then relaxation to bradykinin ($1x10^{-10}$– $3.3x10^{-10}$ M).
4) Chamber washed four times with 40 mls of PSS.
5) Contraction to vasopressin ($1x10^{-10}$ -$1x10^{-8}$ M) then relaxation to sodium nitroprusside ($1x10^{-9}$ – $1x10^{-5}$ M).
6) 123mM KPSS with the addition of noradrenaline.

Myograph two:

1) Contraction to vasopressin ($1x10^{-10}$ -$1x10^{-8}$ M) then relaxation to bradykinin ($1x10^{-10}$– $3.3x10^{-10}$ M).
2) Chamber washed four times with 40 mls of PSS.

3) Incubated with indomethacin 10^{-5} M for 40 minutes.

4) Contraction to vasopressin ($1x10^{-10}$–$1x10^{-8}$M) then relaxation to vasodilator.

5) Chamber washed four times with 40 mls of PSS.

6) Contraction to vasopressin ($1x10^{-10}$–$1x10^{-8}$M) then relaxation to sodium nitroprusside ($1x10^{-9}$-$1x10^{-5}$ M).

7) 123mM KPSS with the addition of noradrenaline.

Myograph three:

1) Contraction to vasopressin ($1x10^{-10}$–$1x10^{-8}$M) then relaxation to vasodilator.

2) Chamber washed four times with 40 mls of PSS.

3) Incubated with indomethacin ($1x10^{-5}$M) and L-NAME ($1x10^{-4}$M) for 40 minutes.

4) Contraction to vasopressin ($1x10^{-10}$–$1x10^{-8}$M) then relaxation to vasodilator.

5) Chamber washed four times with 40 mls of PSS.

6) Contraction to vasopressin ($1x10^{-10}$–$1x10^{-8}$M) then relaxation to sodium nitroprusside ($1x10^{-9}$-$1x10^{-5}$ M).

7) 123mM KPSS with the addition of noradrenaline.

3.2.2 Protocol for the interaction of Vascular Endothelial Growth Factor and plasma from women with pre-eclampsia with the endothelium:

Vessels were dissected and incubated overnight (16 hours) in the presence or absence of VEGF or plasma. Once dissected vessels were placed into 10mls of oxygenated cold PSS in a 20mls sterile screw cap tube (Sterilin). 1 iu/ml heparin was required to prevent the formation of a fibrin clot, and was also added to all control vessels. Allocation of each vessel to each experimental arm was performed randomly.

For plasma experiments, a concentration of 2% plasma was chosen as had previously been demonstrated to have an effect in cell culture studies and on vessel behaviour (Baker et al., 1995a) (Baker et al., 1996a) (Ashworth et al., 1998).

After storage vessels were mounted and normalised (Chapter 2). Vessels were then left for 15 minutes to attain a steady baseline. Once this was achieved the following protocol for myograph 1 in Chapter 3.2.1 was performed.

3.3 Cell Culture pilot data:

It has previously been reported that myometrial resistance vessels from women with pre-eclampsia exhibit a loss of endothelium dependent relaxation (Ashworth et al., 1998). Pilot data within the department had suggested that incubation of normal myometrial resistance arteries with the plasma from women with pre-eclampsia caused an alteration in myograph behaviour which was similar to that exhibited by vessels from women with pre-eclampsia (Ashworth et al., 1998). It is also well documented that plasma from women with pre-eclampsia alters the production of nitric oxide and prostacyclin from cultured endothelial cells (Baker et al., 1995a) (Davidge et al., 1996a) (de Groot et al., 1995). Therefore it was felt appropriate that incubation with both VEGF and plasma should be carried out in both the myography and cell culture systems. It was also considered that the subcellular mechanism of action of plasma and VEGF could be studied with the use cell culture.

3.3.1 Determination of optimal plasma concentration for stimulation and viability:

3.3.1.1 Aim:

As the effects of plasma on cultured endothelial cell production of nitric oxide and prostacyclin were to be determined. It was necessary to determine the optimal concentration for maximum cell stimulation with minimum loss of cell viability.

3.3.1.2 Experimental Design:

Bovine microvascular endothelial cells were cultured as described previously (Chapter 2.5.2.3). Cells were grown to confluence in 24-well sterile culture plates (NUNC, Denmark). Once cells were confluent they were made quiescent (Chapter 2.5.2.3). This medium was then aspirated and fresh medium (1 ml) added containing pooled plasma from normotensive women or those with pre-eclampsia at increasing percentages (1%, 2%, 5%, 10%, and 20%). Pooled plasma was used to minimise individual subject variability and ensure optimum use of the plasma available. Heparin was added to the medium to give a final concentration of 10 U/ml. Heparin was added to prevent the plasma clotting in the wells during the experimental time course. Twenty-four hours after the addition of plasma, the media were collected as described previously (Chapter 2.5.3.1). The media were then assayed for nitrite production (stable product nitric oxide), 6-keto prostaglandin F1α (a stable product of prostacyclin), and lactate deydrogenase (an assessment of cell viability) (see Chapter 2.6).

3.3.1.3 Results:

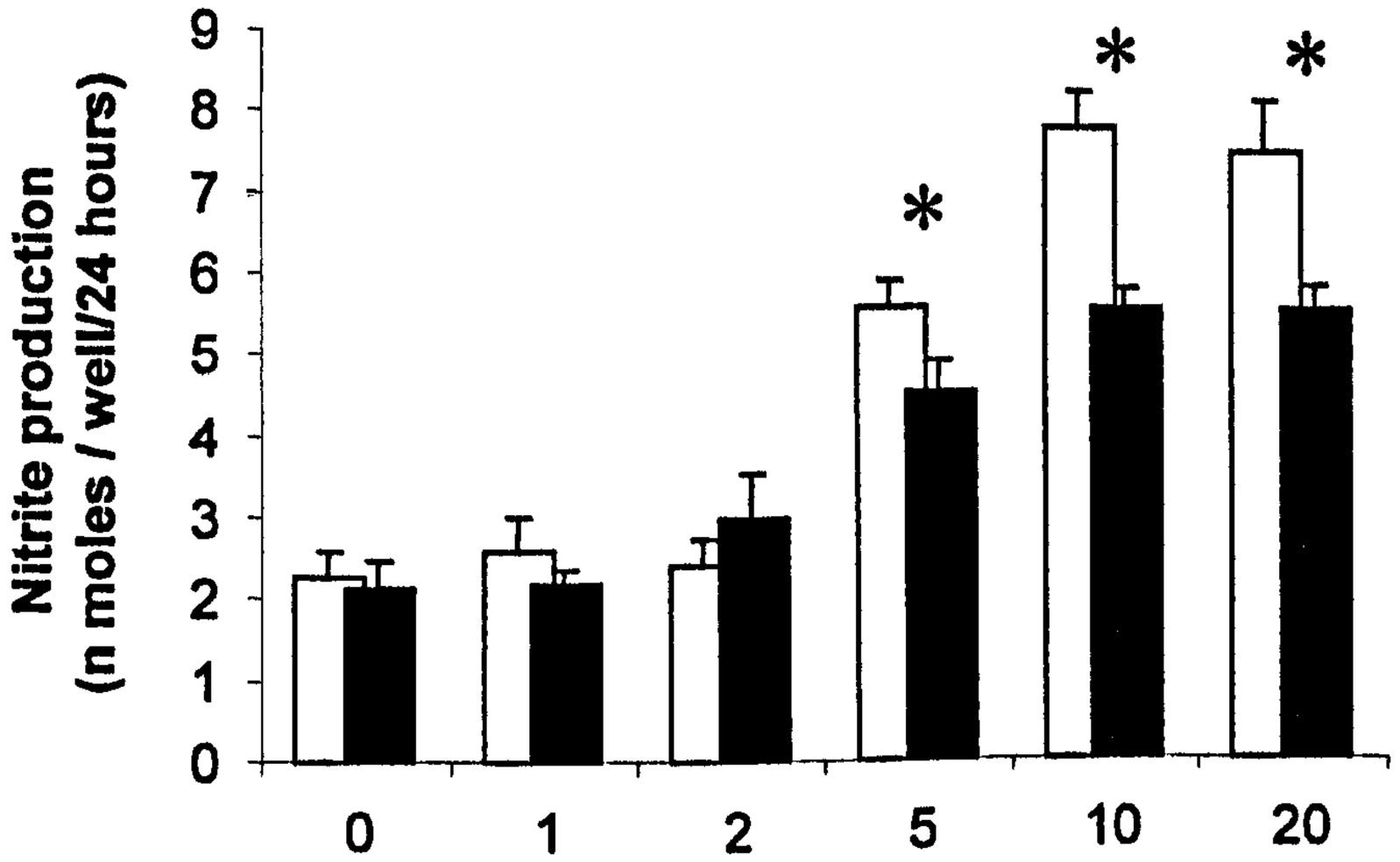
3.3.1.3a Nitric oxide:

Nitric oxide production from stimulated B88 endothelial cells increased as the percentage of pooled plasma rose in the stimulating media ($p = 0.003$; Kruskal-Wallis) (Fig 3.21 a). Post hoc testing showed that a significant difference from the control occurred at 5% added plasma ($p=0.02$: Mann-Whitney-U test).

Fig 3.21

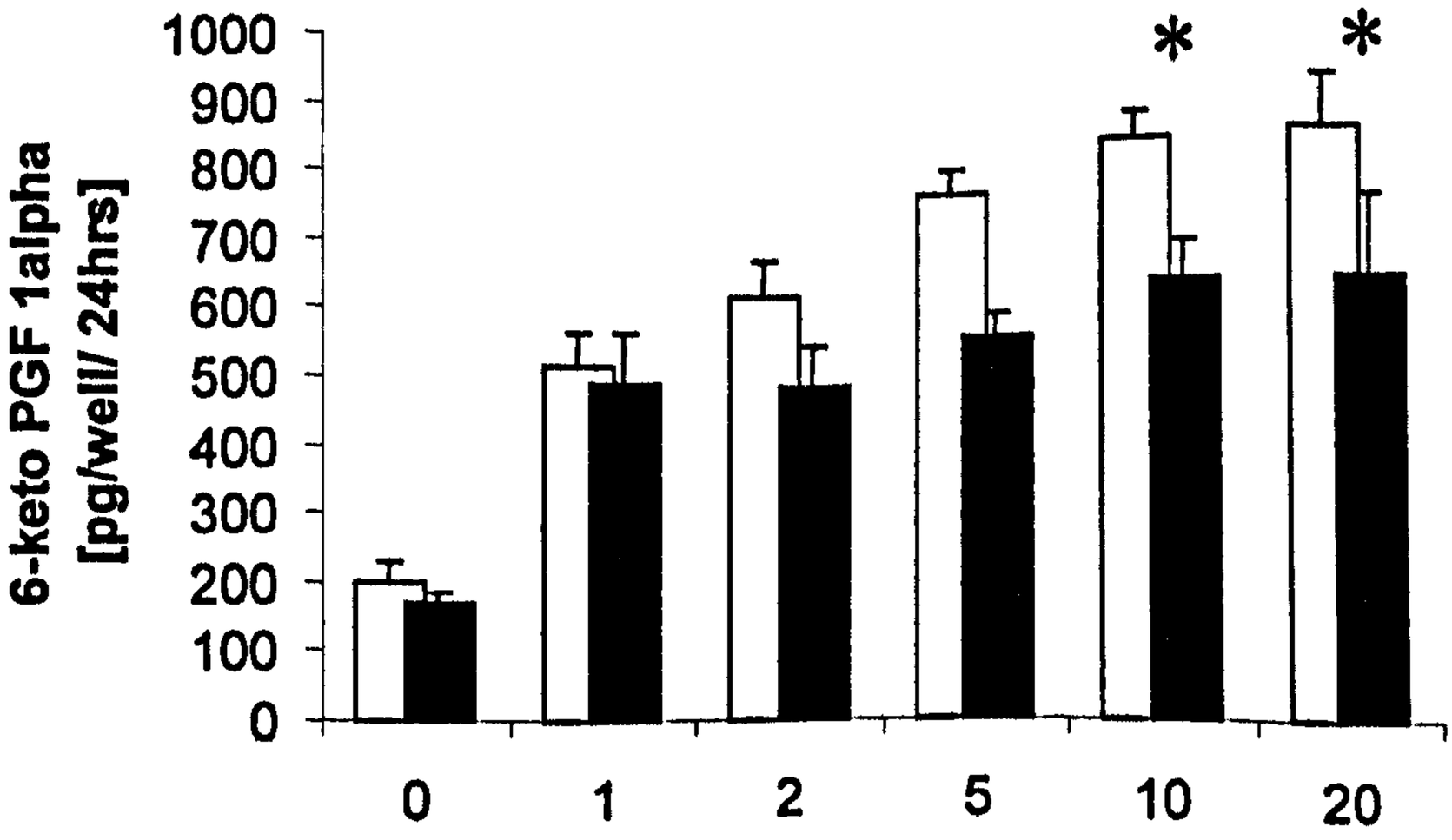
a

B88 nitrite production after stimulation with increasing concentrations of pooled normotensive and pre-eclemptic plasma



b

B88 6-keto PGF 1alpha production after stimulation with increasing concentrations of pooled normotensive and pre-eclamptic plasma



KEY

Data is represented as mean +/- S.E.M
Open boxes represent women with pre-eclampsia. Closed boxes represent control plasma

3.3.1.3b Prostacyclin:

Prostacyclin was determined indirectly from the measurement of its stable metabolite 6 keto-prostaglandin F1α. 6Keto-PGF1α concentrations increased in a concentration dependent manner with increasing concentrations of plasma ($p=0.012$: Kruskal-Wallis) (Fig 3.21 b). Post hoc testing showed that a significant difference from the control occurred with the addition of 2% plasma ($p=0.02$: Mann-Whitney-U Test)

3.3.1.3c Lactate dehydrogenase (LDH):

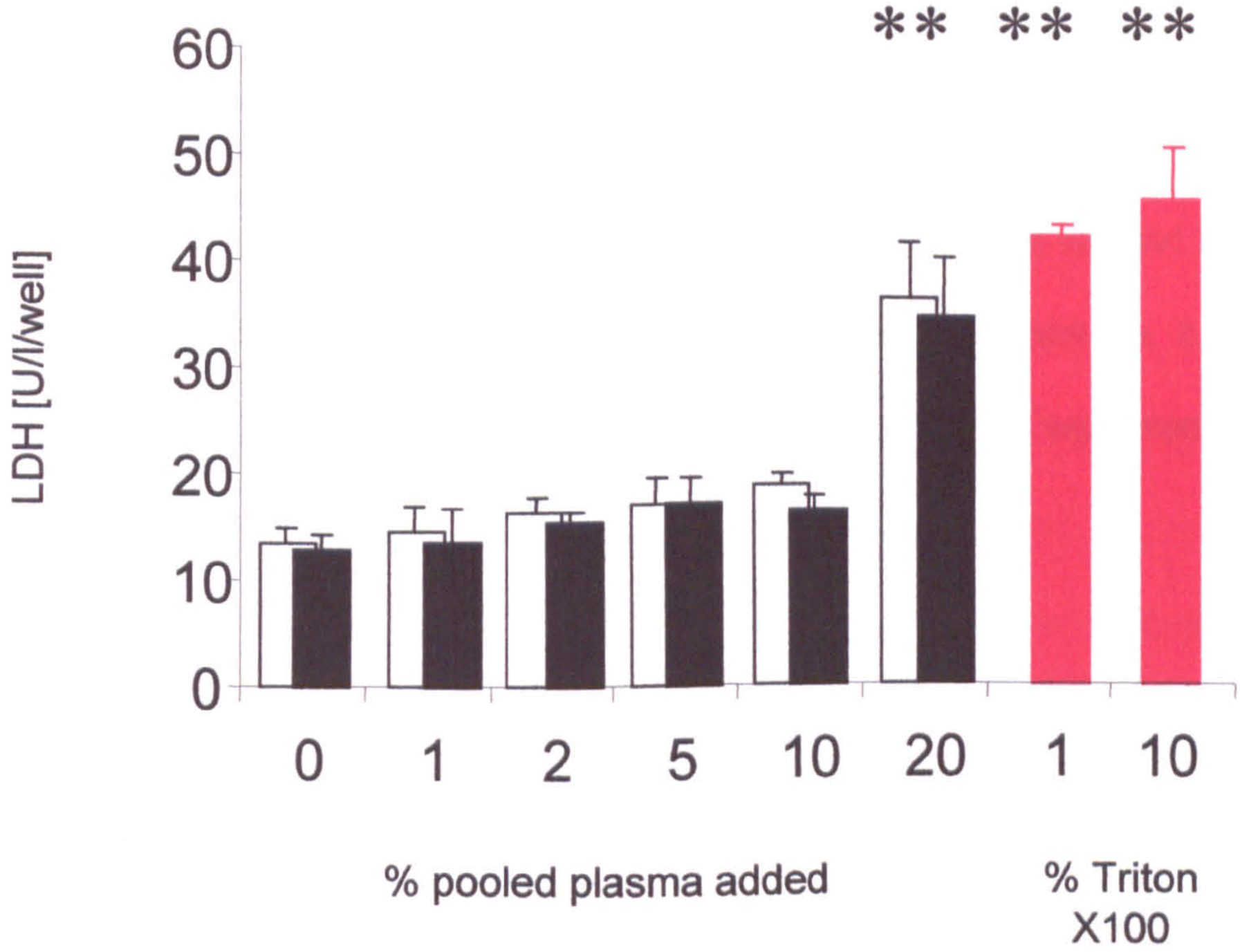
Lactate dehydrogenase levels are significantly increased with incremental concentrations of plasma ($p=0.03$:Kruskal-Wallis) (Fig 3.22). Post hoc testing revealed that the addition of 20% plasma (from women with pre-eclampsia or controls) caused the LDH levels to significantly increase over the control (0% plasma) ($p=0.02$:Mann-Whitney-U test).

3.3.1.4 Discussion:

In contrast to the findings of Baker et al (1995,1996) and Davidge et al (1995,1996) there was no activation with regard to nitrite production of the B88 cell line at a concentration of 2% plasma. These differences may have come from differences in the assays used, however, the prostaglandin F1α assay utilised in both this thesis and the studies of Baker et al and Davidge et al were from the same manufacture (Cayman Chemical, Ann Arbor; USA). The Griess reaction utilised a similar protocol, however, the sources of the chemicals were different and so variations in purity may have affected the chemical reactions. Alternatively, the differences may reflect

Fig 3.22

B88 lactate dehydrogenase production after stimulation with increasing concentrations of pooled normotensive and pre-eclamptic plasma



KEY

Data is represented as mean +/- S.E.M
Open boxes represent women with pre-eclampsia. Closed boxes represent control plasma

differences in the population of patients studied, which is indeed another caveat for testing of the hypothesis that plasma from women with pre-eclampsia activates endothelial cells.

Although differing concentrations of plasma were required to obtain results than those reported previously, the increase in the nitric oxide and prostacylin production from cells that had been stimulated with plasma from women with pre-eclampsia was the same. Therefore, it was considered that a concentration of plasma of 10% should be utilised in cell culture experiments.

Chapter Four: Vascular Endothelial Growth Factor concentrations in pregnancy and pre-eclampsia:

4.1 Introduction:

Roberts et al (1990) proposed that the alteration in endothelial function characteristic of pre-eclampsia was due to (a) factor(s) in the serum / plasma which caused increased vascular permeability, proteinuria, and vasospasm leading to hypertension (Roberts et al., 1989). VEGF is well documented to cause an increase in vascular permeability (Senger et al., 1986) (Connolly et al., 1989b) (Wu et al., 1996) (Wu et al., 1999) (Tilton et al., 1999) and has also been implicated in the renal disease associated proteinuria (Uchida et al., 1994) (Shulman et al., 1996) and promotes vascular coagulation (Ferrara et al., 1992).

The concentration of VEGF in normal and abnormal pregnancies have been studied previously, however results are far from conclusive. Baker et al, (1995b) examined serum concentrations using a sandwich-type immunofluorescencent assay. Although they suggested that VEGF was increased in pre-eclampsia, they obtained measurable concentrations in 13 % (10) of all subjects sampled (78). From this 13% they demonstrated that a higher proportion of women with pre-eclampsia had measurable concentrations of VEGF when compared to the normotensive group (9 Vs 1). However, Baker et al (1995b) presented no validatory data to confirm the sensitivity of the assay used. Two further studies, which utilised commercially available assays, have also produced conflicting results. Sharkey et al (Sharkey et al., 1996) found that both plasma and serum concentrations of VEGF were increased in pre-eclampsia using a competitive enzyme immunoassay assay (CEIA). In contrast Lyall et al (Lyall et al., 1997a), using a sandwich two-site

enzyme-linked immunosorbent assay (ELISA), demonstrated that the serum VEGF immunoreactivity was reduced in pre-eclampsia. Neither Sharkey et al, (1996) or Lyall et al, (1997) present confirmatory data to suggest that their commercially available assays were applicable to pregnancy. It is interesting to note that the assay utilised by Sharkey et al (1996) has been validated for pregnancy plasma and serum samples by the manufacturers, whereas the assay utilised by Lyall et al (1997) has not (R&D systems, UK: VEGF ELISA data sheet). Furthermore, Lyall et al (1997) stated that the median serum concentration for the group of women with pre-eclampsia was 2 pg/ml, whereas the manufacturers data sheet quotes 12 pg/ml as lower limit of detection (R&D systems, UK: VEGF ELISA data sheet). These observations must bring into question the use of this assay in pregnancy.

Recently, Anthony et al have developed a competitive binding radioimmunoassay (RIA) for VEGF using a polyclonal antibody (Anthony et al., 1997). In their pilot study of twenty normotensive pregnant subjects and twenty non-pregnant subjects, serum VEGF was detected in all subjects and levels were raised in comparison to non-pregnant controls. In contrast, the commercial VEGF ELISA (R&D systems: UK) used by Lyall et al was unable to detect VEGF in 19 of the 20 maternal serum samples analyzed. Moreover, the recovery of exogenous VEGF added to pregnancy samples has been shown to be low or unrecordable with the R&D ELISA (Anthony et al., 1997) (Vuorela-Vepsalainen et al., 1999), whereas using the RIA, 82-101% of the added VEGF was recovered. Anthony et al (1997) postulated that these differing results could be explained by the formation of VEGF-protein complexes that are detectable using RIA but undetectable with the ELISA.

Therefore, the measurement of VEGF in pregnancy is still controversial, and as yet there have been no satisfactory assessment of VEGF concentrations in the condition of pre-eclampsia. This study utilised the assay developed and validated for pregnancy of Anthony et al (Chapter 2) to investigate these important questions.

4.2 Aims:

- To investigate the concentrations of VEGF in non-pregnant women, normal pregnant women and women with pre-eclampsia utilising the radioimmunoassay developed by Anthony et al.
- To investigate whether VEGF concentrations correlate with the abnormal parameters of this disease.

4.3 Experimental design:

Plasma samples were collected from 22 subjects diagnosed as having pre-eclampsia (Chapter 1.1). To act as controls, plasma was taken from 22 gestation matched normotensive women without any complications of pregnancy or underlying illness. Samples were also taken from eight non-pregnant women who were not taking the oral contraceptive pill. Samples were collected as described previously in the Chapter 2.2.2. Measurements of the total VEGF immunoreactivity were made by a competitive radioimmunioassay detailed in Chapter 2.3.1 which had previously been validated for pregnancy (Anthony et al., 1997).

4.4 Results:

4.4.1: VEGF concentrations in non-pregnant and pregnant women and those with pre-eclampsia:

Table 4.1 summaries the clinical data of the non-pregnant, pregnant controls and the pre-eclampsia group. As anticipated, systolic and diastolic blood pressures were significantly greater in the group of women with pre-eclampsia than in the control or non-pregnant group (P= 0.001).

All VEGF concentration data is expressed in this thesis as μg/L, this is due to the fact that, although it would be preferable to express concentrations in molar values, this is not appropriate as VEGF exists in several isoforms of differing molecular weights (see Chapter 1). The median plasma concentration in the non-pregnant group was 0.56μg/l (interquartile range 0.49-0.71μg/l) and in the pregnant group 3.4μg/l (interquartile range 2.7-4.1μg/l). Plasma VEGF concentrations were also significantly raised in women with pre-eclampsia when compared to the normotensive controls (Mann-Whitney-U; p=0.001). The median plasma concentration of VEGF in the pre-eclampsia group was 5.7μg/l (interquartile range 4.8-6.9μg/l) (Fig 4.1). Analysis of the VEGF concentrations showed that a significant difference occurred between the three groups (Kruskal – Wallis test; p=0.0001) (Fig 4.1). Further analysis demonstrated VEGF concentrations were increased with pregnancy (Mann-Whitney-U; p=0.0001).

Patient demographic details for plasma samples

Table 4.1

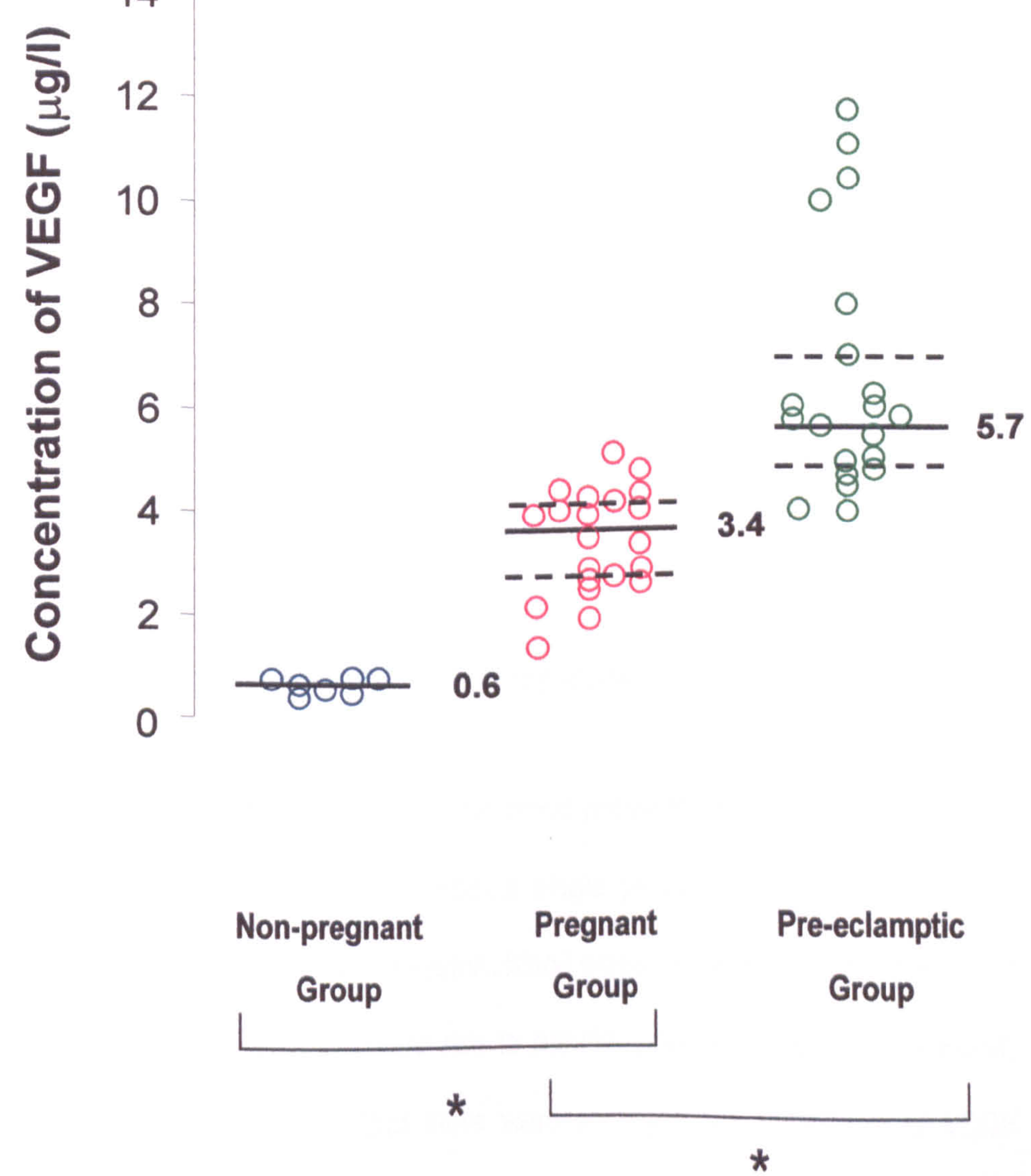
Plasma Type	Age (years)	BMI (kg/m^2)	Parity	Gestation at venepuncture (days)	Gestation at delivery (days)	Mean Arterial Pressure Booking mm Hg	Mean Arterial Pressure Maximum mm Hg	Protein g/dl	Individualised Birth-weight Ratio
Non - pregnant	31.0 (27.5 - 34.7)	26.1 (22.7 - 28.6)	0 (0 - 1.2)			87.5 (84.6 - 94.1)			
Normal pregnant	26 (22 - 30)	23.8 (21.8 - 26.2)	0 (0 - 1)	252 (238 - 266)	271 (263 - 280)	84.9 (73.2 - 88.6)	89.9 (86.6 - 93.3)	0.0	47 (33 - 78)
Pregnant women with pre-eclampsia	28 (24 - 30)	25.1 (23.5 - 26.3)	0 (0 - 0)	252 (230 - 266)	257 (230 - 266)	85.0 (79.9 - 89.9)	114.6 (113.3 - 123.3)	0.74 (0.43 - 1.08)	16 (0 - 28)

Data are summarised as medians (Inter Quartile Ranges)

Fig 4.1

Plasma concentrations of VEGF in non-pregnant women, normotensive pregnant women, and women with pre-eclampsia

Data are summarised as medians (Inter Quartile Ranges)



Statistical comparisons:

Kruskal-Wallis p = 0.0001

Mann Whitney U * p =0.001

4.4.2 Effect of gestation on results:

VEGF concentrations within the normotensive group increased with gestation (Spearman rank correlation: r= 0.45; P = 0.025) (Fig 4.2). However, in the group of women with pre-eclampsia there was no significant correlation of VEGF with gestation (Spearman rank correlation: r= 0.01; P = 0.940). In a similar manner, blood pressure within the normotensive group rose with increasing gestation (Spearman rank correlation: r=0.47; P= 0.001), but no similar correlation could be demonstrated within the pre-eclampsia group (Spearman rank correlation: r=0.37; P= 0.250) (Fig 4.3).

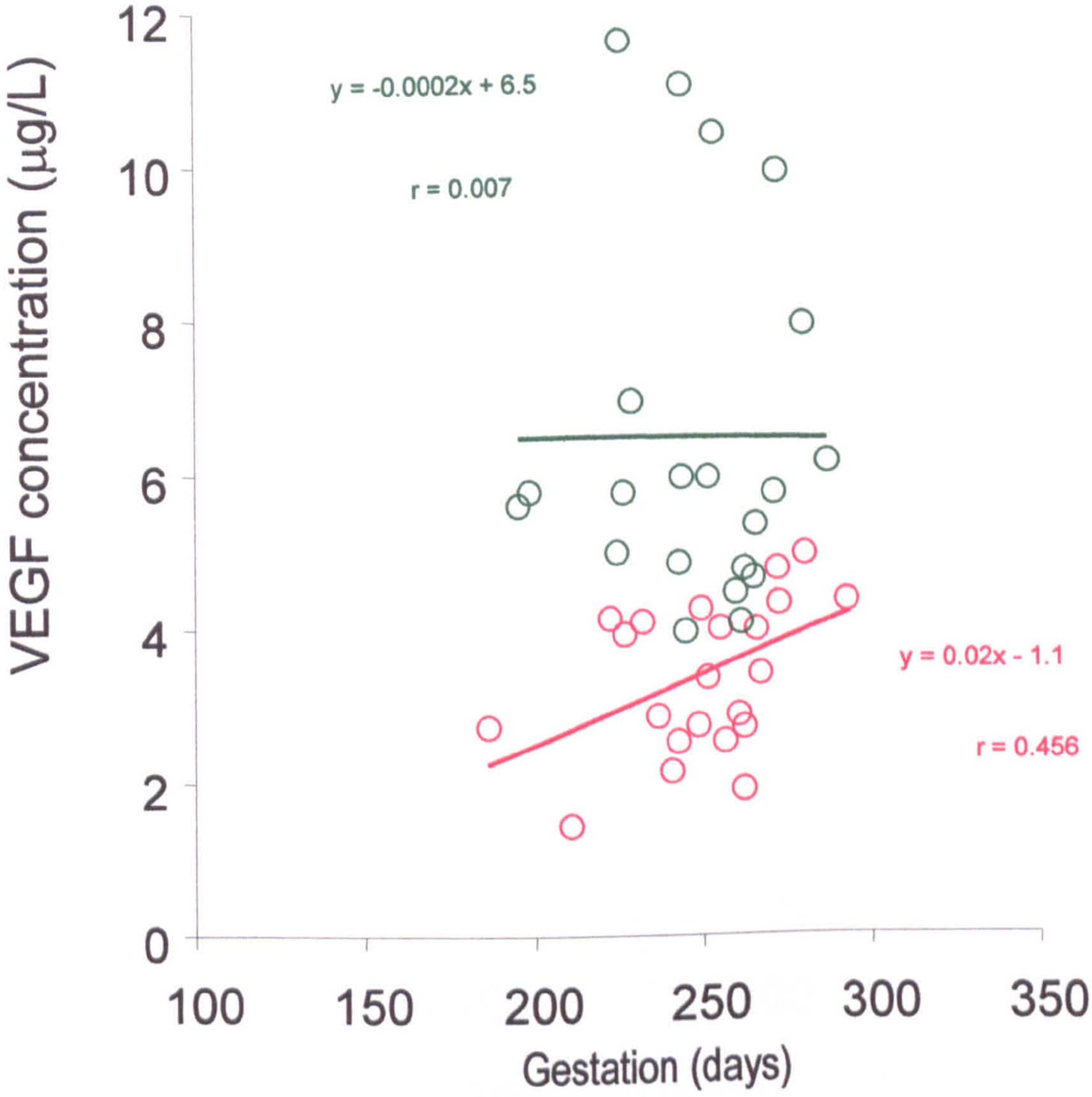
4.4.3 Correlation with disease parameters:

4.4.3.1 VEGF plasma concentrations with blood pressure

There is no evidence for a bimodal distribution of the blood pressure in pregancy and as such data must therefore be considered as coming from a single group. There were significant correlations of VEGF with both the diastolic and systolic blood pressure, demonstrating that VEGF concentrations and the maternal blood pressure rise in parallel (Figure 4.4 & 4.5). However, analysis of the individual groups showed that there were no significant correlation of VEGF concentration with systolic blood pressure in either the normotensive group or women with pre-eclampsia (Figure 4.4 & 4.5). There was also no significant correlation of VEGF concentration with diastolic blood pressure in the normotensive group (Figure 4.4 & 4.5). However, a significant

Fig 4.2

The correlation of plasma VEGF concentrations with gestation in women with pre-eclampsia and normotensive controls



Key:

○ Pre-eclampsia group

○ Normotensive group

Statistical comparisons:

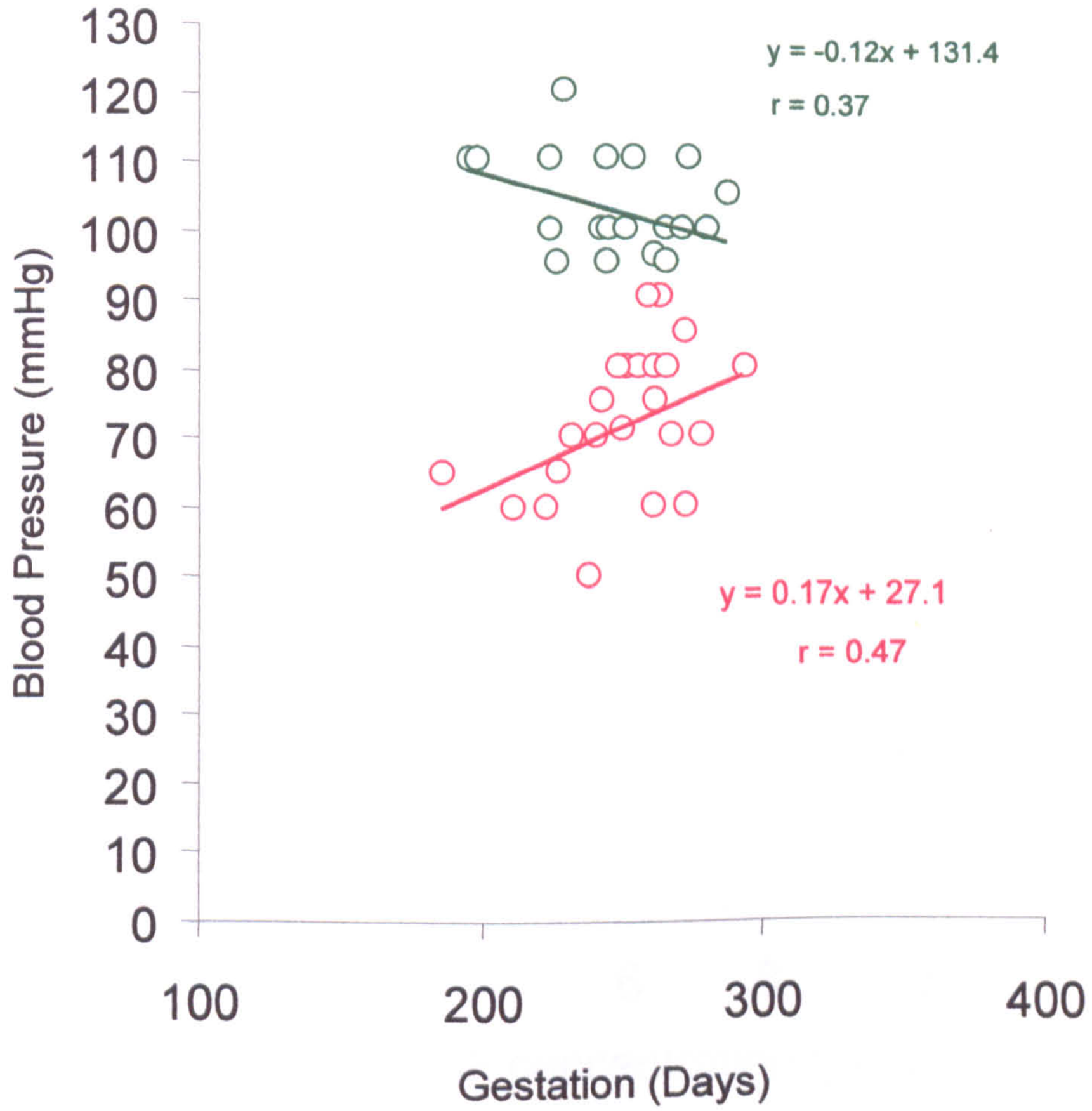
Spearman's rank correlation

p = 0.940

p = 0.025

Fig 4.3

The relationship of maternal blood pressure with gestation in women with pre-eclampsia and normotensive pregnant controls



Key:

○ Pre-eclampsia group

○ Normotensive group

Statistical comparisons:

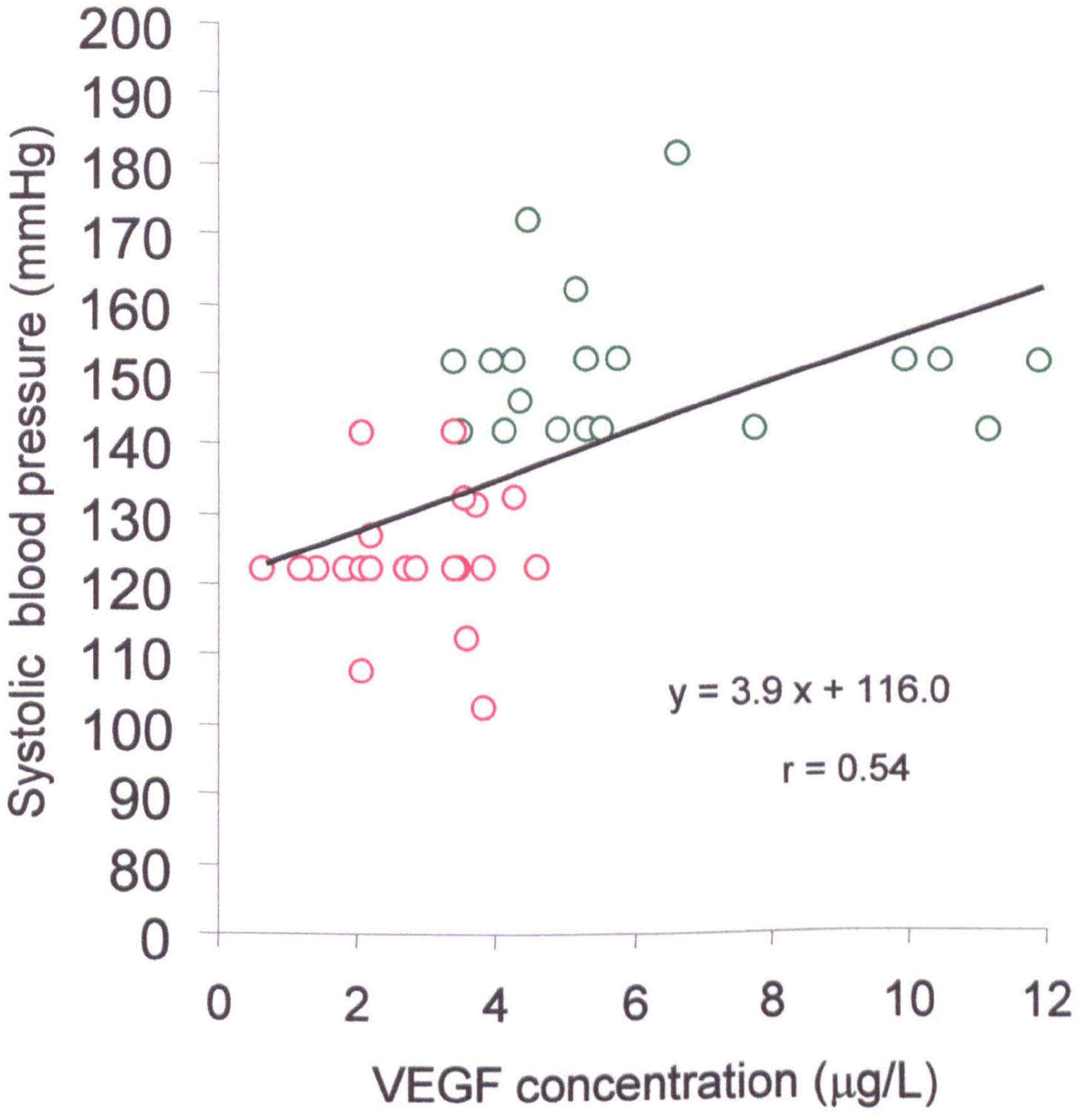
Spearman's rank correlation

p = 0.250

p = 0.001

Fig 4.4

The correlation of systolic blood pressure with plasma VEGF concentrations in women with pre-eclampsia and normotensive pregnant controls



Key:

○ Pre-eclampsia group

○ Normotensive group

Statistical comparisons:

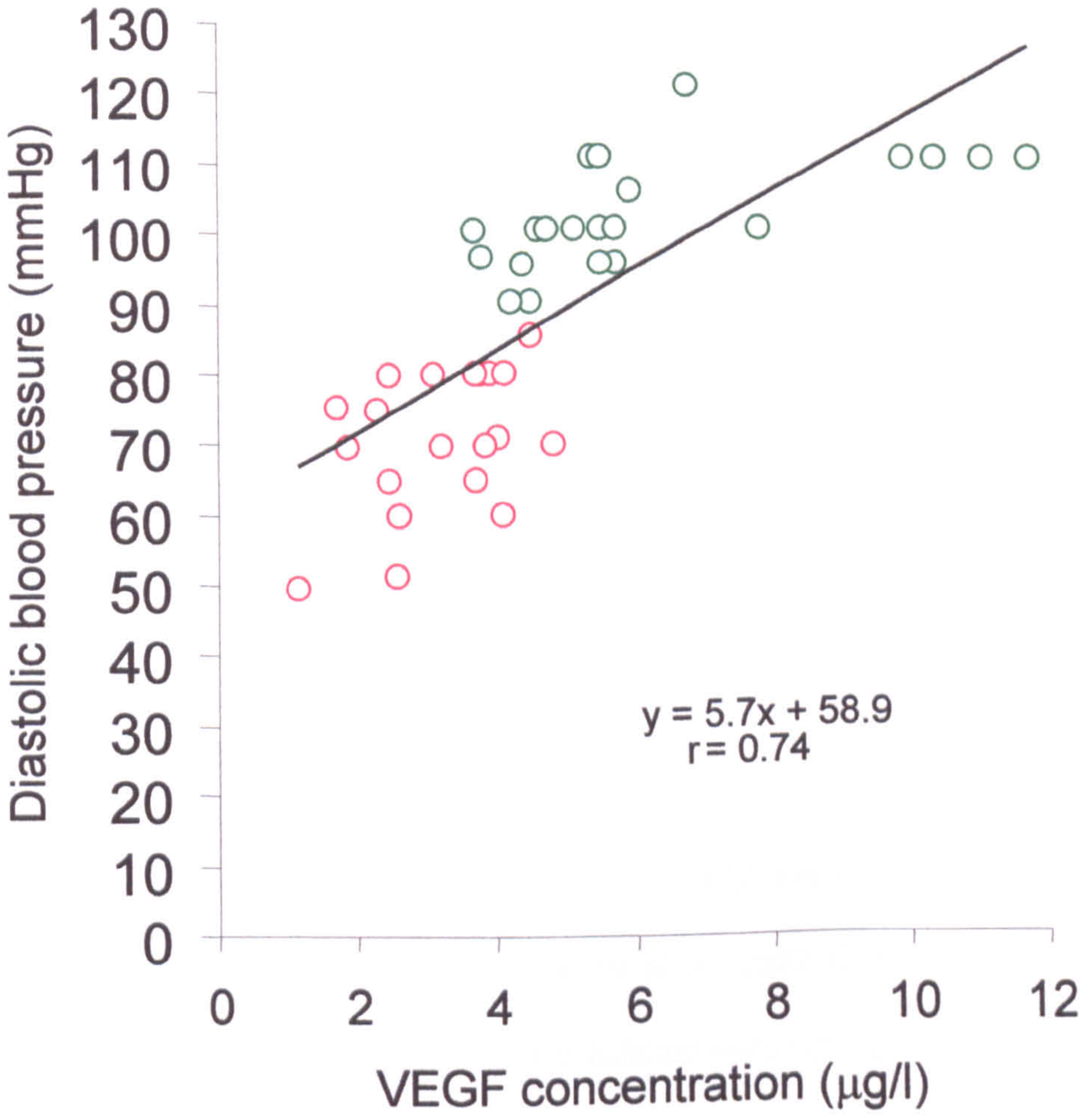
Spearman's rank correlation

p = 0.920

p = 0.720

Fig 4.5

The correlation of diastolic blood pressure with plasma VEGF concentrations in women with pre-eclampsia and normotensive controls



Key:

○ Pre-eclampsia group

○ Normotensive group

Statistical comparisons:

Spearman's rank correlation

p = 0.003

p =0.250

correlation of VEGF concentration with diastolic blood pressure occurred within the group with pre-eclampsia (Spearman rank correlation; p = 0.001) (Fig 4.4).

4.4.3.2 Plasma VEGF concentrations with other parameters:

Plasma VEGF concentrations showed no significant correlation with either urinary protein (Spearman rank: r= 0.14; p = 0.66); plasma uric acid (Spearman rank: r= 0.07; p = 0.22); or platelet count (r= 0.17; p=0.70) (Fig 4.6, 4.7 & 4.8).

4.5 Discussion:

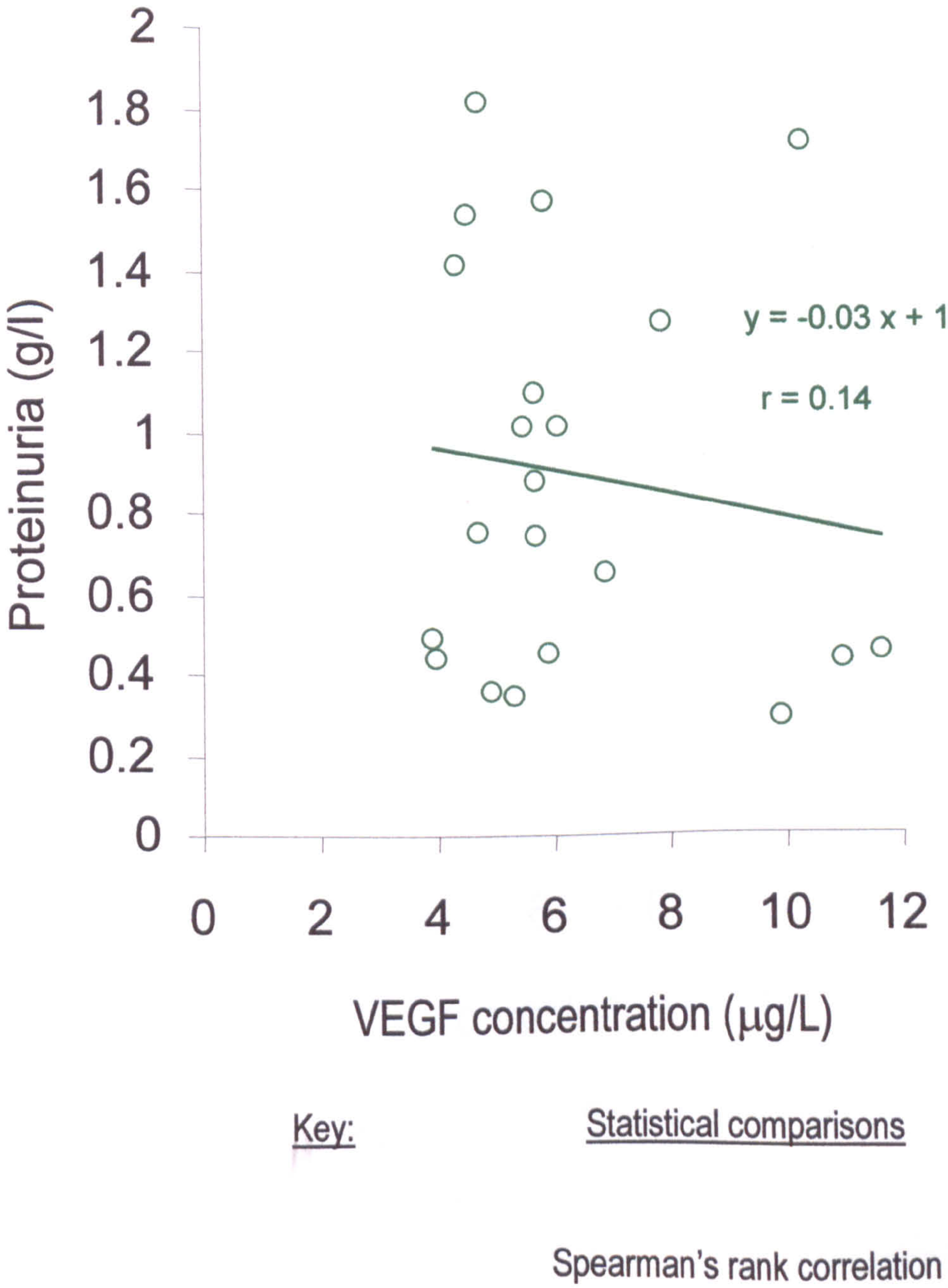
VEGF plasma concentrations:

In accord with previous studies, the plasma concentrations of VEGF are in the picomolar range (Sharkey et al., 1996) (Kupferminc et al., 1997b) (Hunter et al., 2000) (Bosio et al., 2001); a concentration which stimulates biological responses in cultured endothelial cells (Bikfalvi et al., 1991). However, Kupferminic et al report values which are three times higher than other studies. This may be due to their use of serum and not plasma; as higher concentrations of VEGF have been demonstrated in serum due to its release from platelets (Webb et al., 1998).

Although, this study measured total VEGF it did not allow discrimination between separate isoforms (see Chapter 1). VEGF $_{189}$ and VEGF $_{206}$ each contain a highly basic 24 amino acid insert that promotes binding to cell surface heparin sulphates. In contrast VEGF $_{121}$ and VEGF $_{165}$

Fig 4.6

The relationship of urinary protein with plasma VEGF concentrations in women with pre-eclampsia



Key:

○ Pre-eclampsia group

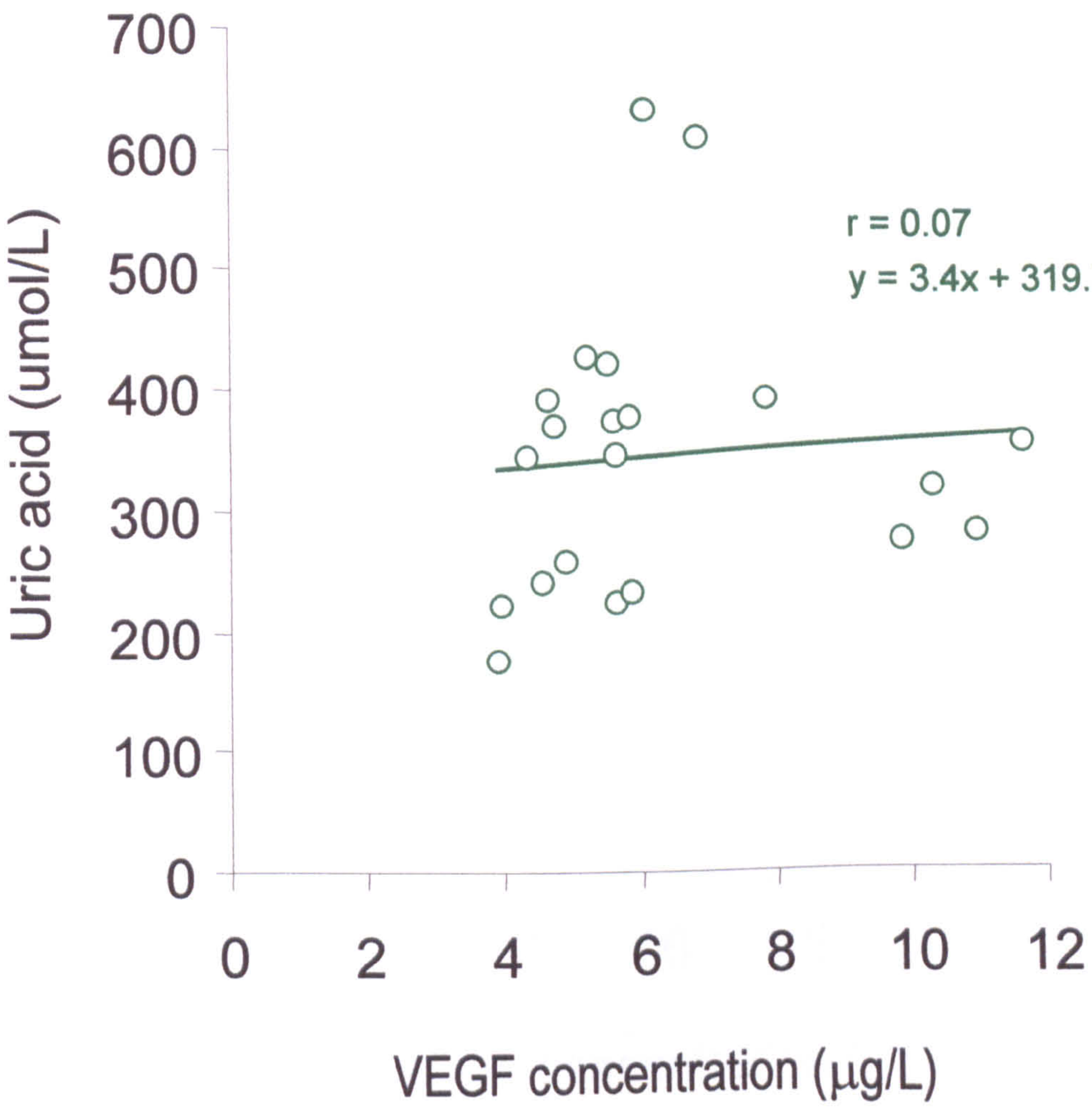
Statistical comparisons

Spearman's rank correlation

p = 0.660

Fig 4.7

The relationship of plasma uric acid concentration with plasma VEGF concentrations in women with pre-eclampsia



Key:

○ Pre-eclampsia group

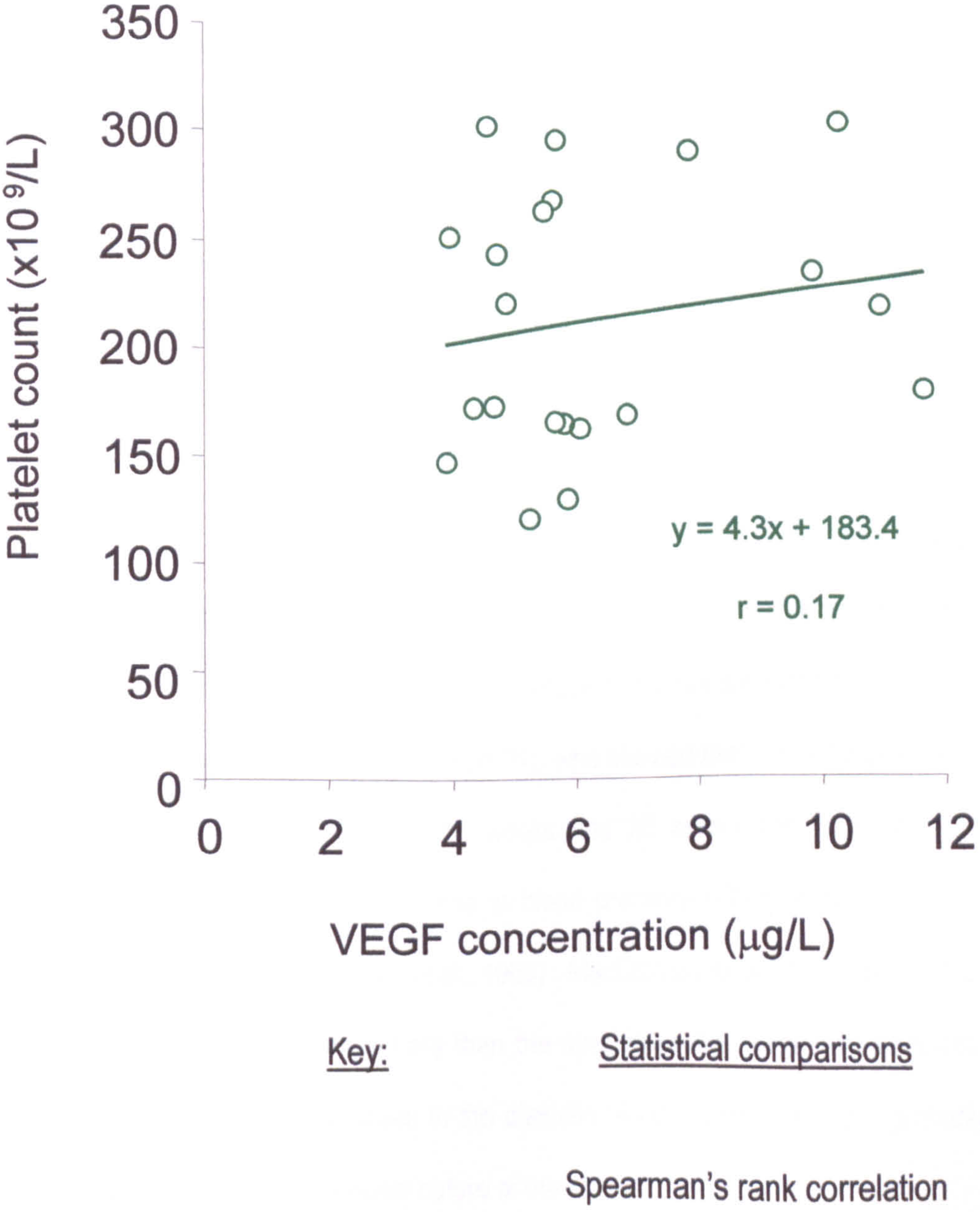
Statistical comparisons

Spearman's rank correlation

p = 0.220

Fig 4.8

The relationship of platelet concentration with plasma VEGF concentrations in women with pre-eclampsia



Key:

○ Pre-eclampsia group

Statistical comparisons

Spearman's rank correlation

p = 0.700

are diffusible and therefore it would seem likely to be these isoforms which are recognised, however, further work using selective antibodies is needed.

VEGF concentrations have previously been reported to increase in the first trimester of pregnancy when compared to non-pregnant controls (Evans et al., 1997) (Evans et al., 1998) (Wheeler et al., 1999). This has been correlated to both gestational age (to 10 weeks) and various hormones reflecting placental function, including β-HCG and progesterone (Evans et al., 1997). The authors speculated that VEGF may be crucial for both the development of the placenta and the adaptations of the cardiovascular system.

In relation to late pregnancy, this thesis shows that plasma concentrations of VEGF are increased in the third trimester when compared to non-pregnant controls. VEGF rose in the third trimester of normal pregnancy with gestation, when diastolic blood pressure, but not systolic blood pressure also rose. This finding confirms that of Bosio et al (2001), who showed that in normal pregnancy VEGF concentrations were constant between 10 weeks and 28 weeks and then rose with gestation to delivery (Bosio et al., 2001). The rise in blood pressure with gestation is also in accord is with numerous studies (MacGillivary et al., 1969). MacGillivary et al (1969) showed that diastolic blood pressure rose more substantially than the systolic as the pregnancy advances. This thesis only showed a significant increase in the diastolic blood pressure and this probably due to the sample size and the cross-sectional nature of the study.

These observations led to the hypothesis that VEGF may be important with regard to the control of maternal blood pressure in the third trimester. In support of this hypothesis is the demonstration that there was a trend for the diastolic blood pressure to rise with VEGF

concentration in the control pregnant group, although this did not reach significance. This result may have not reached significance due to several possible reasons, firstly, a small sample size; secondly the inherent errors in blood pressure measurement with regard to the diastolic blood pressure in pregnancy, thirdly any association may be very weak and finally it may simply be an epiphenomemon. One methodological problem when measuring blood pressure indirectly during pregnancy, is whether the 4th or 5th Korotkov sound should be taken as the cut-off point for the diastolic blood pressure. In the non-pregnant state, it has been demonstrated that the 5th Korotkov sound correlates closely with the diastolic pressure recorded using a direct measurement technique (Rafetery and Ward, 1968). However, in pregnancy, use of the 5th Korotkov sound skews the data, as it has been demonstrated to be present with the cuff pressure at zero (MacGillivray et al., 1969). As such the less accurate 4th sound is used and may produce an increased false positive rate on the diagnosis of hypertension.

Paradoxically, the only studies to date on the *in vivo* effects of VEGF on cardiovascular physiology, have shown that injection of VEGF into non-pregnant rats causes a transient drop in the blood pressure by the stimulation of nitric oxide (Yang et al., 1996) (Webb et al., 1999). Yang et al (1996) also noted that the haematocrit was increased in the experimental animals and hypothesised that this occurred through an increased vascular permeability, which subsequently reduced venous return and indirectly blood pressure (Yang et al., 1996). Although an increase in vascular permeability may be one mechanism, VEGF has also been shown to act in an *in vitro* system, directly on the endothelium to cause vasodilatation via nitric oxide (Ni et al., 1997a). However, it remains to be clarified whether these observations are the same in human vessels, and particularly whether these effects occur in vessels from pregnant women (see Chapter 8).

This study confirms reports that VEGF is increased in pregnancies complicated by pre-eclampsia (Sharkey et al., 1996b) (Kupferminc et al., 1997a) (Bosio et al., 2001) (El-Salahy et al., 2001). Although, Lyall et al (1997) and more recently (Reuvekamp et al., 1999) Helfer et al., (2000) and Livingston et al (2000) have demonstrated that reduced levels of VEGF occur in pregnancies complicated by pre-eclampsia, all these studies utilised the same commercial assay, the deficits of which have been discussed in this Chapter's introduction (Reuvekamp et al., 1999) (Hefler et al., 2000) (Livingston et al., 2000).

The data in this thesis demonstrates that as the maternal VEGF immunoreactivity rises there is a parallel rise in both the systolic and diastolic blood pressure. A similar association between these parameters has been demonstrated previously, with the authors postulating that VEGF was important in the pathogenesis of this disease (Kupferminc et al., 1997a).

The increased concentration of VEGF in pre-eclampsia is consistent with the hypothesis that, VEGF as a circulating factor may be involved in the endothelial dysfunction. Further circumstantial evidence is provided by the work of Davidge et al (Davidge et al., 1996a); they found that fractionated plasma in the molecular weight range 42,000-62,000 kDa stimulated prostacyclin production in endothelial cells. This is in the molecular weight range of VEGF, however, it is also in the molecular weight range of many other substances that have been implicated in the pathogenesis of pre-eclampsia, including renin (Brown et al., 1997c). Therefore, it is important to determine whether VEGF alters endothelial function in a similar manner to plasma from women with pre-eclampsia (Baker et al., 1995a) (de Groot et al., 1995) (Davidge et al., 1995b) (Baker et al., 1996a) (Davidge et al., 1996a) (Ashworth et al., 1998) and whether

inhibition of plasma VEGF with specific antibodies inhibits the observed changes of endothelial cell function (see Chapters 8 and 9).

Hunter et al (2000) have described a longitundinal study of maternal VEGF in normal pregnancies and those complicated by pre-eclampsia. They confirmed the finding that VEGF immunoreactivity was increased in pregnancies complicated by pre-eclampsia, and also demonstrated that VEGF levels are elevated several weels prior to the onset of the clinical disease (Hunter et al., 2000). Bosio et al (2001) have recently confirmed in a similar longitundinal study that plasma VEGF concentrations, measured using the assay of Anthony et al (1997), of women destined to develop pre-eclampsia were elevated at least four weeks prior to the clinical onset of disease (Bosio et al., 2001).

Bosio et al (2001) also examined the relationship between maternal VEGF immunoreactivity and total peripheral resistance. Bosio et al (2001) studied 70 subjects, from an original cohort of 400; 20 of these developed pre-eclampsia, 24 developed gestational hypertension (defined as hypertension without proteinuria) and 26 acted as normotensive controls. Bosio et al (2001) also showed that VEGF concentrations correlated with the increased total peripheral resistance (TPR) associated with disease. Interestingly, the group of women with gestational hypertension alone neither showed increased TPR nor increased VEGF concentrations and this led the authors to postulate that the increased VEGF production occurred by mechanical stress on the endothelium as a result of an increased TPR. However, closer analysis of their data indicates that in some women who developed pre-eclampsia, the VEGF concentrations had risen prior to the increase in TPR, which is not consistent with their hypothesis. An alternative hypothesis for their data is that

increased VEGF concentrations alter endothelial function, with the resultant effect of increasing in the TPR.

The observation that plasma VEGF concentrations are positively correlated with blood pressure and TPR (Kupferminc et al., 1997a) (Bosio et al., 2001) (Chapter 4.4.3) raises the important question: "Does VEGF either directly or indirectly alter vascular reactivity to cause peripheral vasoconstriction" which is addressed in Chapter 8.

Source of VEGF:

This study does not address several important questions relating to the source of the circulating VEGF. Firstly, which tissues produce the increased VEGF concentrations with gestation? Secondly, are these tissues also the same sites for increased production in pre-eclampsia and gestation or do they differ?

VEGF is widely distributed throughout the body's tissues, but in pregnancy, additional sites of importance may include decidual macrophages, placental macrophages, trophoblast, and fibroblasts, all of which have been shown to express VEGF mRNA and protein (Clark et al., 1996) (Wheeler et al., 1995). A number of studies have shown that the placenta and the myometrium are an abundant source of VEGF (Sharkey et al., 1993) (Charnock-Jones et al., 1993) (Jackson et al., 1994) (Greb et al., 1995) (Harrison-Woolrych et al., 1995) (Brown et al., 1997a). Hunter et al (2000) in their longitudinal study demonstrated that the VEGF levels returned to non-pre-eclampsia levels within 24 hours of delivery. Therefore it could be hypothesised that the

maternofetal interface causes the increased circulating concentrations of VEGF in normal pregnancy and pre-eclampsia (see Chapter 5).

VEGF and renal effects:

In the normal adult kidney, only the smallest plasma proteins are able to cross the glomerular barrier. This selectivity is primarily due to characteristics of the basement membrane and the endothelium. VEGF is normally expressed at high levels by podocytes, and the visceral epithelial lining of Bowmans capsule, which support the glomerular capillaries (Brown et al., 1992). This led to the suggestion that, as VEGF causes an increase in vascular permeability (Senger et al., 1983) (Connolly et al., 1989a) (Connolly, 1991) (Wu et al., 1996) (Bates and Curry, 1996) (Bates and Curry, 1997), it may have a physiological role in regulating renal glomerular permeability.

It has also been demonstrated that VEGF may be important in the development of proteinuria in various pathological conditions (Uchida et al., 1994, Shulman et al., 1996) (Horita et al., 1998). As pre-eclampsia is partly defined by proteinuria, and as VEGF has been implicated in renal disease, it would be reasonable to hypothesise that it may be of importance in the development of the renal pathology associated with pre-eclampsia.

As well as proteinuria, hyperuricaemia is well documented as an associated biochemical abnormality of pre-eclampsia (Slemons and Bogert, 1917), which has been correlated with the severity of disease and histological changes in renal biopsies (Pollak and Nettles, 1960). Hyperuricaemia has also been demonstrated to be a sensitive indicator of renal function and blood flow (Breckenridge, 1966) (Messerli et al., 1980a) (Messerli et al., 1980b). Therefore, if

VEGF is involved in the pathological changes that occur in the renal system in pre-eclampsia it may be expected that the maternal plasma levels of VEGF would correlate with both proteinuria and uricaemia as these are markers of renal damage.

Contrary to our hypothesis, VEGF did not correlate to either plasma uric acid concentrations or urinary protein concentrations (Figure 4.6 & 4.7). Although these data does not support the hypothesis, it does not preclude it, as the tissue concentrations are more important than the circulating concentrations. Recently, it has been demonstrated in a Sprague-Dawley rat model of glomerulonephrosis, induced by the intraperitoneal injection of bovine albumin, that both VEGF and its receptors (flt-1 and KDR) are dramatically up-regulated in the glomeruli of experimental animals when compared to controls, and that this is associated with increased proteinuria (Horita et al., 1998). However, the direct injection of VEGF into similar rats causes no increased proteinuria or renal histological changes (Webb et al., 1999). There are a number of reasons why these differences may have occurred; the VEGF may have been neutralised by a factor in the blood, or that the VEGF did not reach the kidney in sufficient concentrations to cause an effect.

Despite the lack of correlation of maternal plasma VEGF concentrations to the markers of renal disease in this thesis, a role for VEGF in the renal pathology of pre-eclampsia has recently been suggested by the work of Hunter and colleagues. They demonstrated that the injection of VEGF in dextran 40 into guinea-pigs causes the development of significant proteinuria, a significant drop in platelet count and an elevation of liver enzymes when compared to the control group (Hunter, 2000). All these results are in accord with the clinical presentation of pre-eclampsia. Hunter and colleagues also injected VEGF into non-pregnant guinea-pigs and demonstrated that these animals did not suffer a similar derangement in blood parameters or develop proteinuria (Hunter,

2000). However, guinea-pigs are not humans and extrapolations should be treated with caution. Therefore, further studies are required of human pregnant kidney to determine if altered expression of VEGF or its receptors may explain the observed increase in proteinuria in pre-eclampsia. Such studies may include in situ hybridisation, immunohistochemical and quantitative RT-PCR for both VEGF and its receptors in human kidney biopsies.

4.6 Summary:

In summary it has been confirmed that circulating concentrations of VEGF are increased in pregnancies complicated by pre-eclampsia and that the increases rise in parallel with the maternal blood pressure. However, several important questions still remain to be addressed. These include the source of the increased VEGF both in normal and abnormal pregnancies, and whether VEGF can directly or indirectly alter vascular reactivity.

Chapter Five - mRNA levels of Angiogenic Growth Factors (VEGF and PlGF) and their receptors (flt-1 and KDR) in placentas and myometrium from pregnant women and women with pre-eclampsia and myometrium from non-pregnant women.

5.1 Introduction:

The origin of these increased maternal plasma / serum concentrations in both normal pregnancies and those complicated by pre-eclampsia remains to be determined. Although the placenta has been studied with respect to the expression of VEGF, no study has examined the other half of the human feto-maternal unit, the uterine myometrium, as a possible source of VEGF in pregnancy and pre-eclampsia.

The cellular secretion of the four isoforms of VEGF is dependent on their physical properties, with the larger isoforms being mainly extracellular matrix associated (Houck et al., 1992). These isoforms display varying specificites to the different VEGF receptors, and this may act to regulate function (Gitay-Goren et al., 1992) (Terman et al., 1994). To date no study has examined the effect of pregnancy or pregnancy complications on the expression of these VEGF isoforms or that of PlGF on either side of the feto-maternal unit.

5.2 Aims:

- To examine semi-quantitatively the mRNA expression of the VEGF isoforms and PlGF in the placentas from normotensive pregnant women and in pregnancies complicated by pre-eclampsia

- To examine semi-quantitatively the mRNA expression of VEGF, and PlGF in the myometrium of non-pregnant women, pregnant women and in women with pre-eclampsia.

5.3 Experimental design:

Placental samples were obtained from normal pregnant women (n=7) and women with pre-eclampsia (n=6) (Chapter 2.4.2). Samples of myometrium were also collected from non-pregnant women of reproductive age (n=6), pregnant women (n=7) and women with pre-eclampsia (n=7) as detailed in Chapter 2.4.1. Samples were then processed (Chapter 2.4.3) and RT-PCR (Chapter 2.4.3-5) was performed on the samples for β-actin, VEGF, PlGF, flt-1 and KDR (Chapter 2.4.5).

5.4 Results:

Table 5.1 and 5.2 summaries the clinical data of the non-pregnant, pregnant controls and the pre-eclampsia group.

5.4.1 mRNA expression of the VEGF isoforms and its receptors in the placentas from pregnant women and women with pre-eclampsia:

In placentas from normotensive control women and women with pre-eclampsia the $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ isoforms were expressed. Analysis of the data showed that the $VEGF_{121}$ isoform in placentas from both normotensive controls (Kruskal-Wallis: P= 0.048) and women with pre-eclampsia (Kruskal-Wallis: P= 0.0013) was the most abundantly expressed. However, no significant differences were noted in the expression of $VEGF_{121}$ (Mann-Whitney-U: P= 0.89), $VEGF_{165}$ (Mann-Whitney-U: P= 0.67), or $VEGF_{189}$ (Mann-Whitney-U: P= 0.39) between the

Patient demographic details for myometrial biopsies

Table 5.1

	Age (years)	BMI (kg/m ^{2})	Parity	Gestation at delivery (days)	Mean Arterial Pressure Booking mm Hg	Mean Arterial Pressure Maximum mm Hg	Protein g/dl	Individualised Birth-weight Ratio
Non - pregnant (n=6)	37 (31 - 40)	22.9 (22.4 - 24.6)	2 (2 - 2.8)		87 (84 - 92)			
Normal pregnant (n=7)	28 (25 - 32)	24.7 (24.1 - 25.4)	1 (0.5 - 1)	267 (266 - 269)	85 (70 - 90)	90 (85 - 95)	0.0 (0.0 - 0.0)	60 (40 - 79)
Pregnant women with pre-eclampsia (n=7)	30 (28 - 33)	25.1 (23.5 - 26.3)	0 (0 - 0)	230 (208 - 250)	84 (84 - 92)	121 (117 - 140)	0.5 (0.39 - 0.8)	3.5 (1.2 - 14.8)

Data are summarised as medians (Inter Quartile Ranges)

Patient demographic details for placental biopsies

Table 5.2

	Age (years)	BMI (kg/m^2)	Parity	Gestation at delivery (days)	Mean Arterial Pressure Booking mm Hg	Mean Arterial Pressure Maximum mm Hg	Protein g/dl	Individualised Birth-weight Ratio
Normal pregnant (n=7)	28 (26 - 29)	26.5 (23.1 - 29.7)	1 (0 - 1)	274 (268 - 282)	83 (78 - 89)	91 (86 - 97)	0 (0 - 0)	68 (66 - 95)
Pregnant women with pre-eclampsia (n=6)	24 (20 - 30)	22.5 (22.3 - 24.4)	0 (0 - 0.75)	246 (224 - 264)	84 (84 - 93)	125 (123 - 130)	0.8 (0.6 - 0.9)	0.5 (0 - 4.8)

Data are summarised as medians (Inter Quartile Ranges)

placentas from the normotensive control group and the group of women with pre-eclampsia (Fig 5.1).

As well as VEGF mRNA, the placentas from the normotensive control women and the women with pre-eclampsia also expressed flt-1 and KDR receptor mRNA (Fig 5.2 & 5.3). Statistical analysis of the data revealed no differences in the expression of either receptor in the normotensive controls when compared to the pre-eclampsia group (flt-1: P= 0.77: Mann Whitney U test; KDR: P= 0.48: Mann Whitney U test).

5.4.2 mRNA expression of PlGF in the placentas from pregnant women and women with pre-eclampsia:

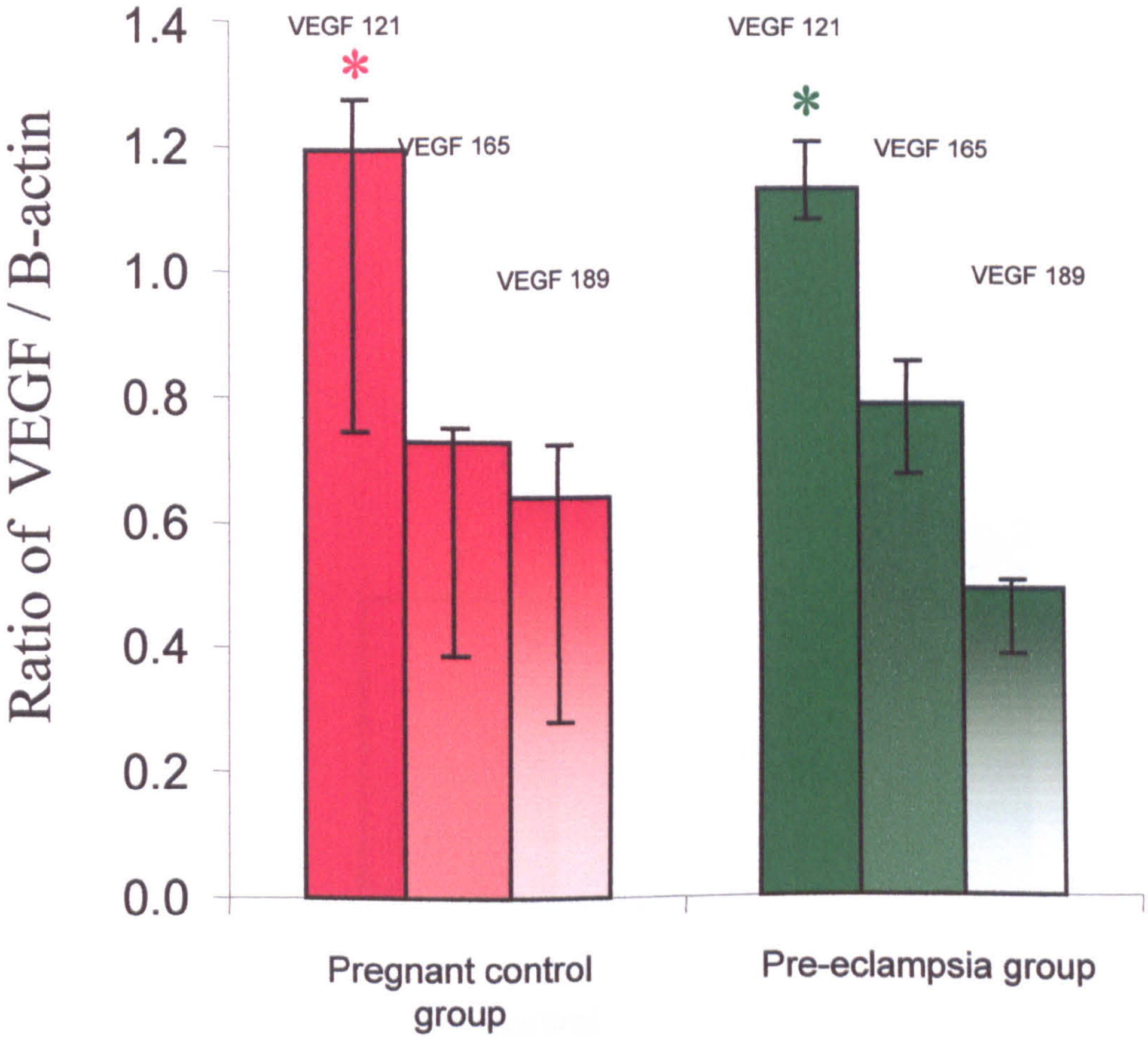
Although PlGF was expressed by all placentas studied there was no significant difference in expression between the placentas from normotensive controls and the placentas from women with pre-eclampsia (Mann-Whitney-U: P=0.09) (Fig 5.4).

5.4.3 mRNA expression of the VEGF isoforms and its receptors in the myometrium of non-pregnant women, pregnant women and women with pre-eclampsia:

In the myometrial biopsies from non-pregnant women, normotensive control women and women with pre-eclampsia the $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ isoforms were all expressed (Fig 5.5). Sub-analysis within the study groups, revealed that in the non-pregnant (Kruskal-Wallis: P= 0.002) and the pre-eclampsia groups (Kruskal-Wallis: P= 0.008) the $VEGF_{121}$ isoform was most

Fig 5.1

The semi-quantitative expression of the VEGF isoforms mRNA in placentas from control women and women with pre-eclampsia

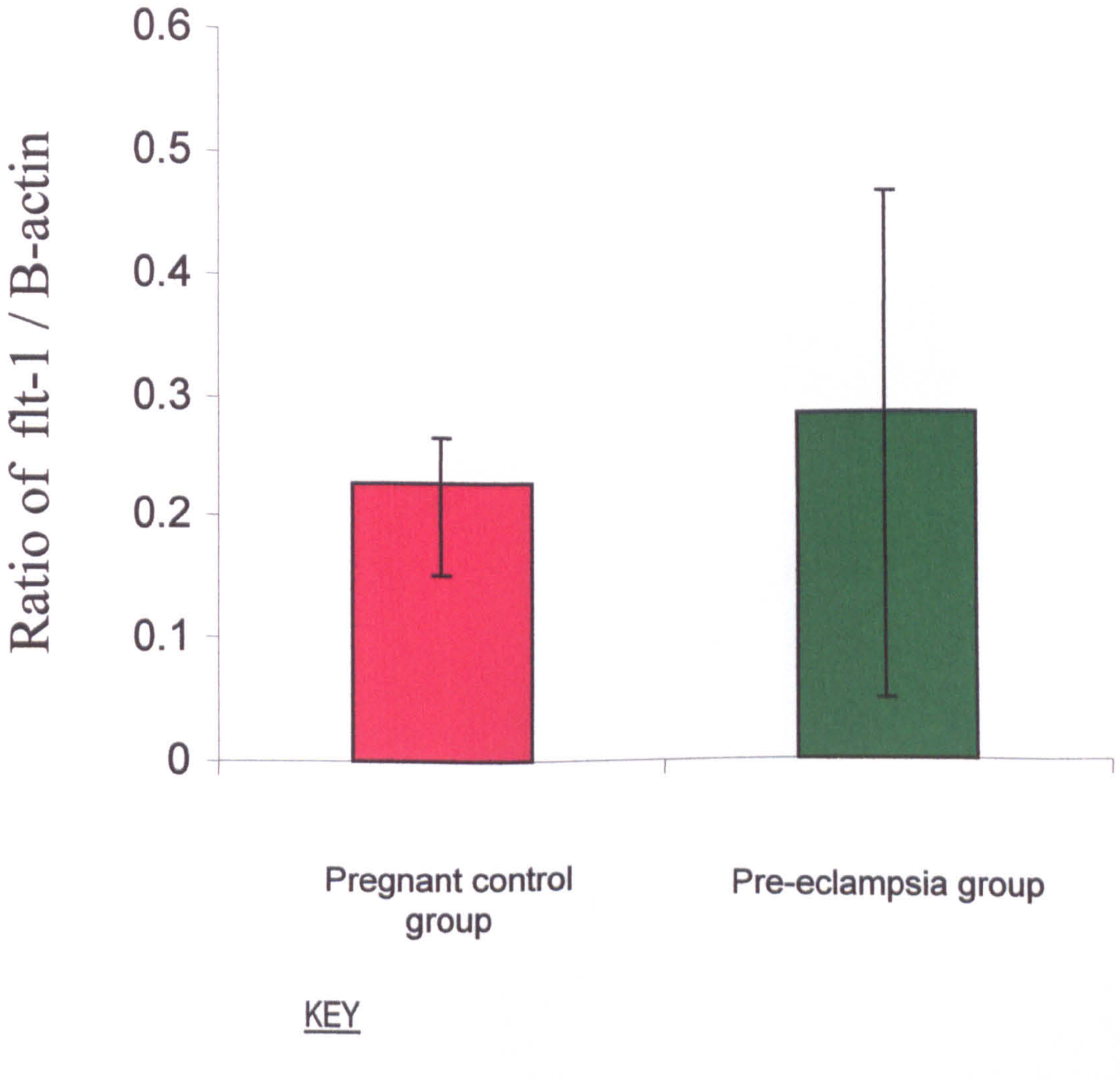


KEY

Data are represented as medians +/- inter-quartile ranges. Green boxes represent women with pre-eclampsia. Red boxes represent control women.

Fig 5.2

The semi-quantitative expression of the flt-1 mRNA in placentas from control women and women with pre-eclampsia

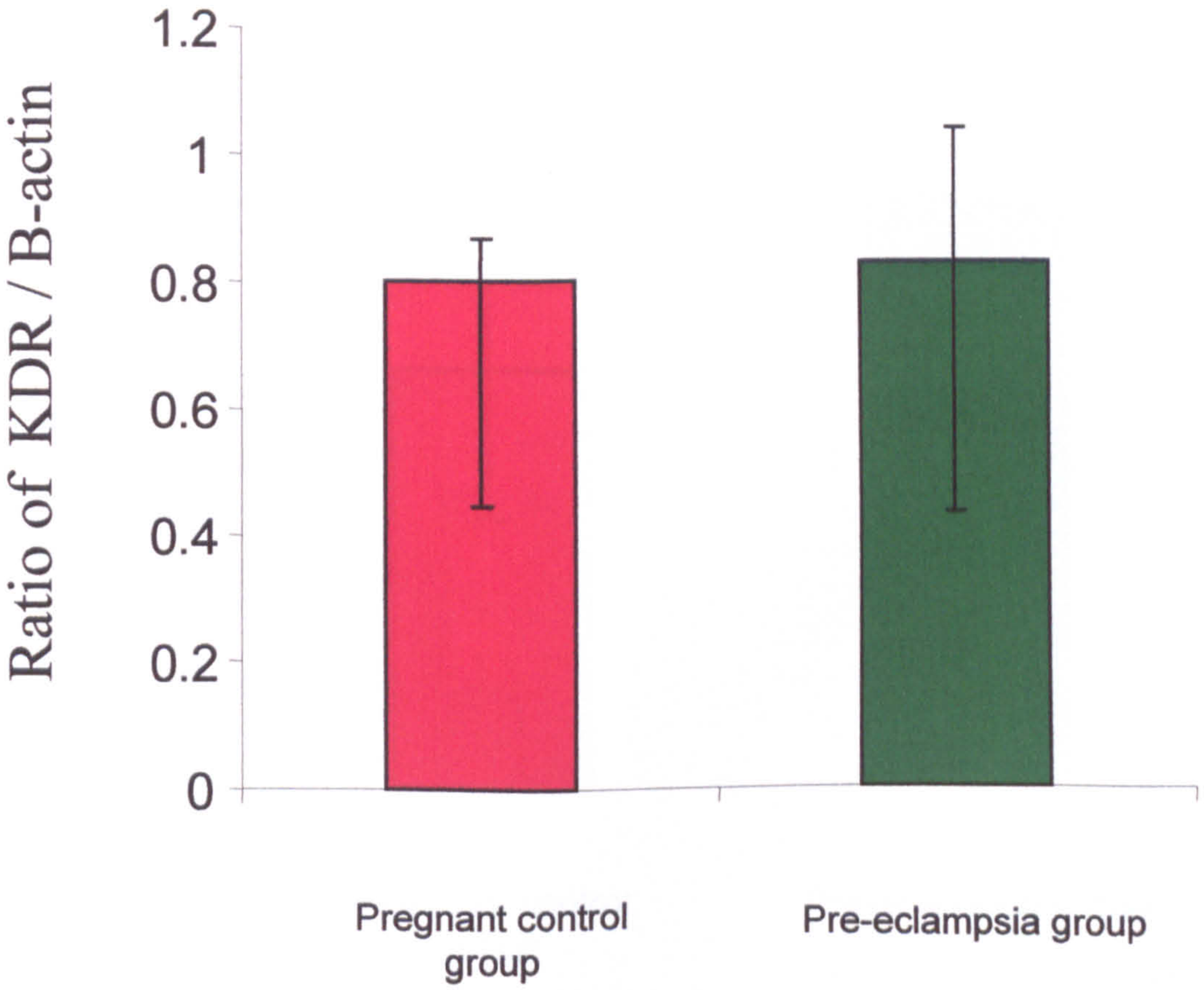


KEY

Data are represented as medians +/- inter-quartile ranges.. Red boxes represent placentas from control women. Green boxes represent placentas from women with pre-eclampsia.

Fig 5.3

The semi-quantitative expression of the KDR mRNA in placentas from control women and women with pre-eclampsia

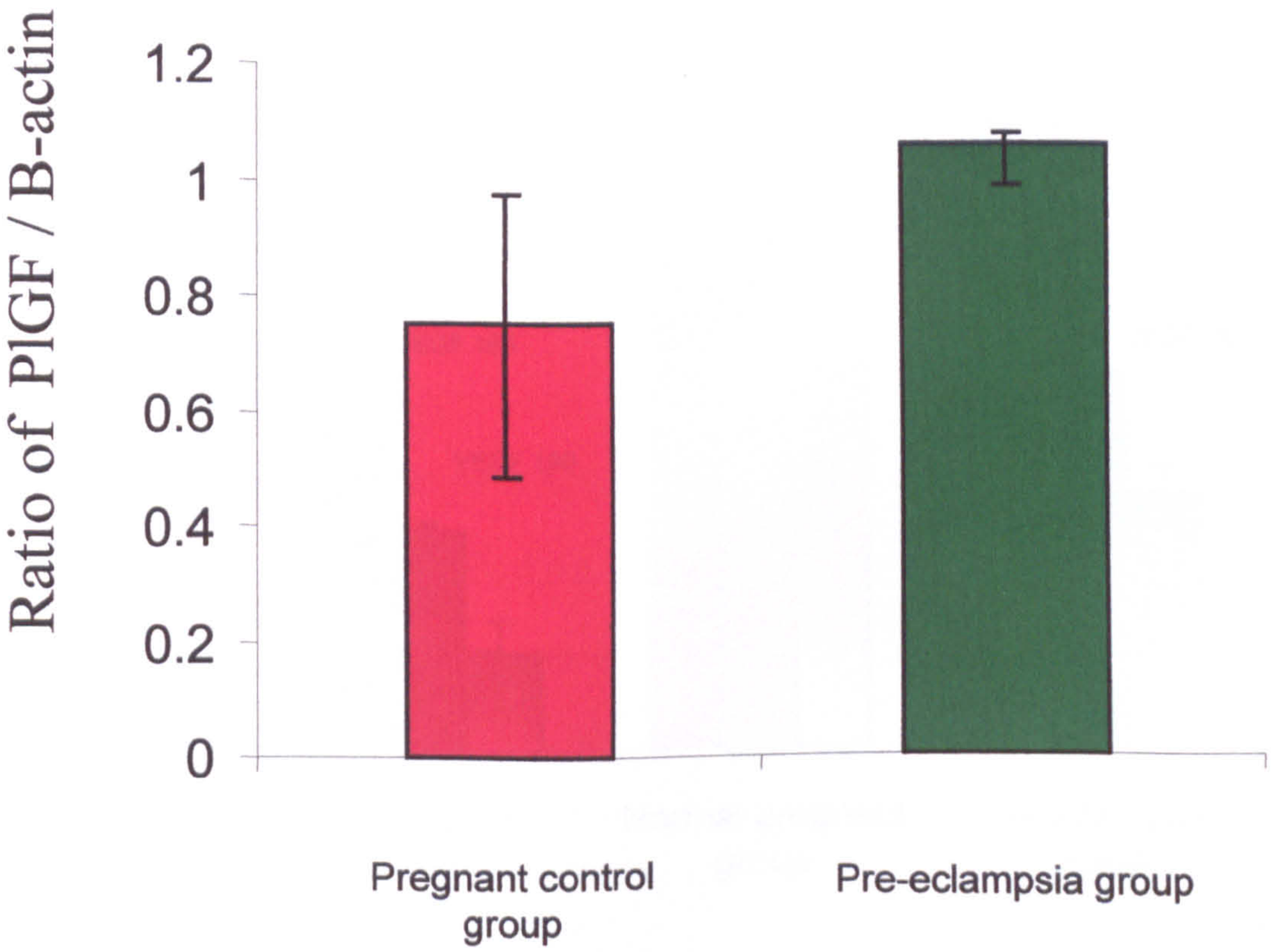


KEY

Data are represented as medians +/- inter-quartile ranges.. Red boxes represent placentas from control women. Green boxes represent placentas from women with pre-eclampsia.

Fig 5.4

The semi-quantitative expression of the PlGF mRNA in placentas from control women and women with pre-eclampsia

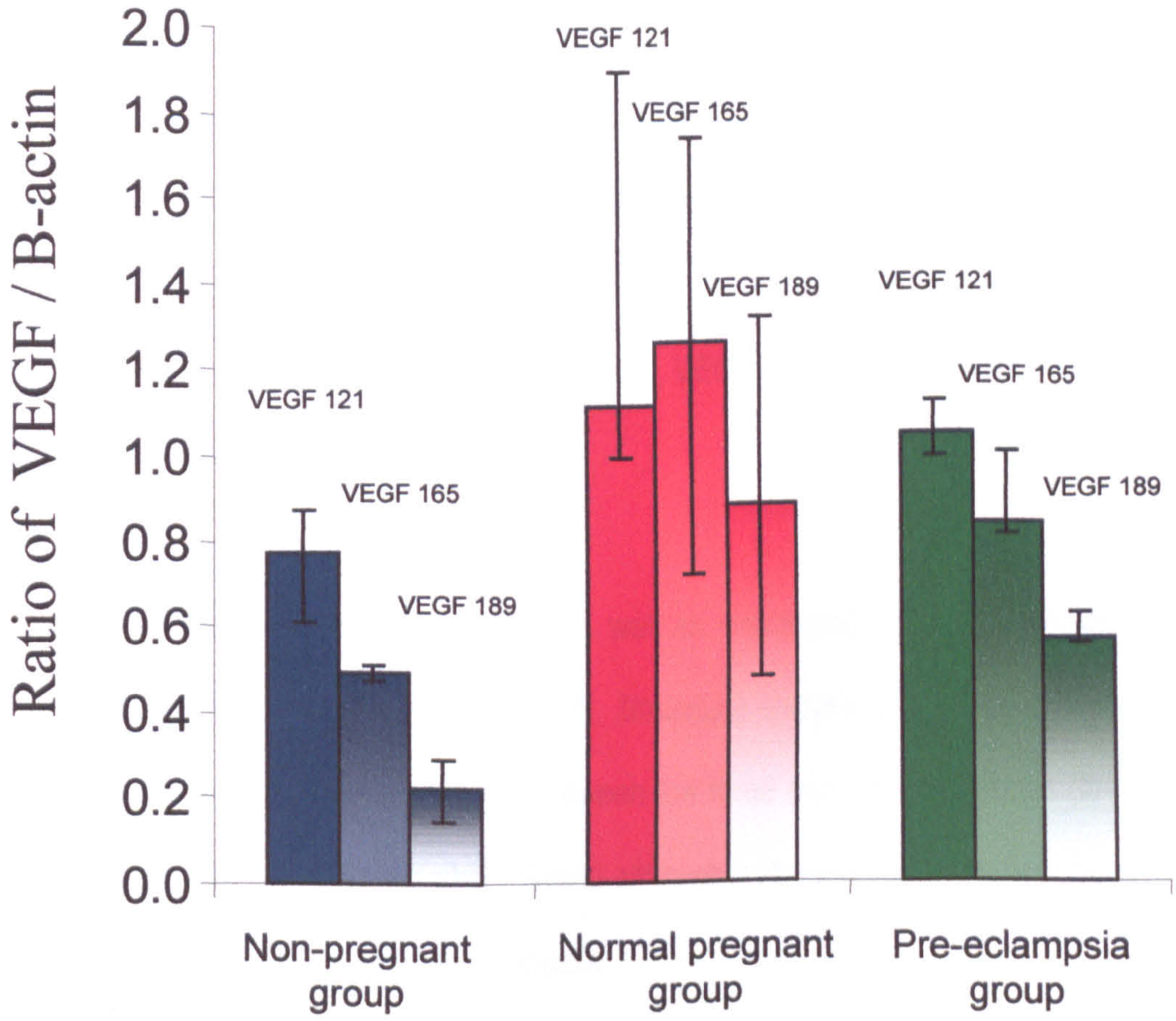


KEY

Data are represented as medians +/- inter-quartile ranges.. Red boxes represent placentas from control women. Green boxes represent placentas from women with pre-eclampsia.

Fig 5.5

The semi-quantitative expression of the VEGF isoforms mRNA in the myometrium from non-pregnant, pregnant women and women with pre-eclampsia



KEY

Data are represented as medians +/- inter-quartile ranges. Blue boxes represent myometrium from non-pregnant women. Red boxes represent myometrium from control women. Green boxes represent myometrium from women with pre-eclampsia.

abundantly expressed, however, in the normal pregnant group the expression of three isoforms were similar (Kruskal-Wallis: P= 0.5).

Analysis of the isoform expression between the study groups showed that there were significant differences in expression: VEGF $_{121}$ (Kruskal-Wallis: P= 0.04) VEGF $_{165}$ (Kruskal-Wallis: P= 0.02) and VEGF $_{189}$ (Kruskal-Wallis: P= 0.004). Post hoc testing demonstrated that all three isoforms were significantly increased in the pregnant group when compared to the non-pregnant group: VEGF $_{121}$ (Mann-Whitney: P= 0.045), VEGF $_{165}$ (Mann-Whitney: P= 0.04) and VEGF 189 (Mann-Whitney: P= 0.01). In contrast no significant differences were noted between the pregnant group and pre-eclampsia group: VEGF $_{121}$ (Mann-Whitney: P= 0.96), VEGF $_{165}$ (Mann-Whitney: P= 0.66) and VEGF 189 (Mann-Whitney: P= 0.54).

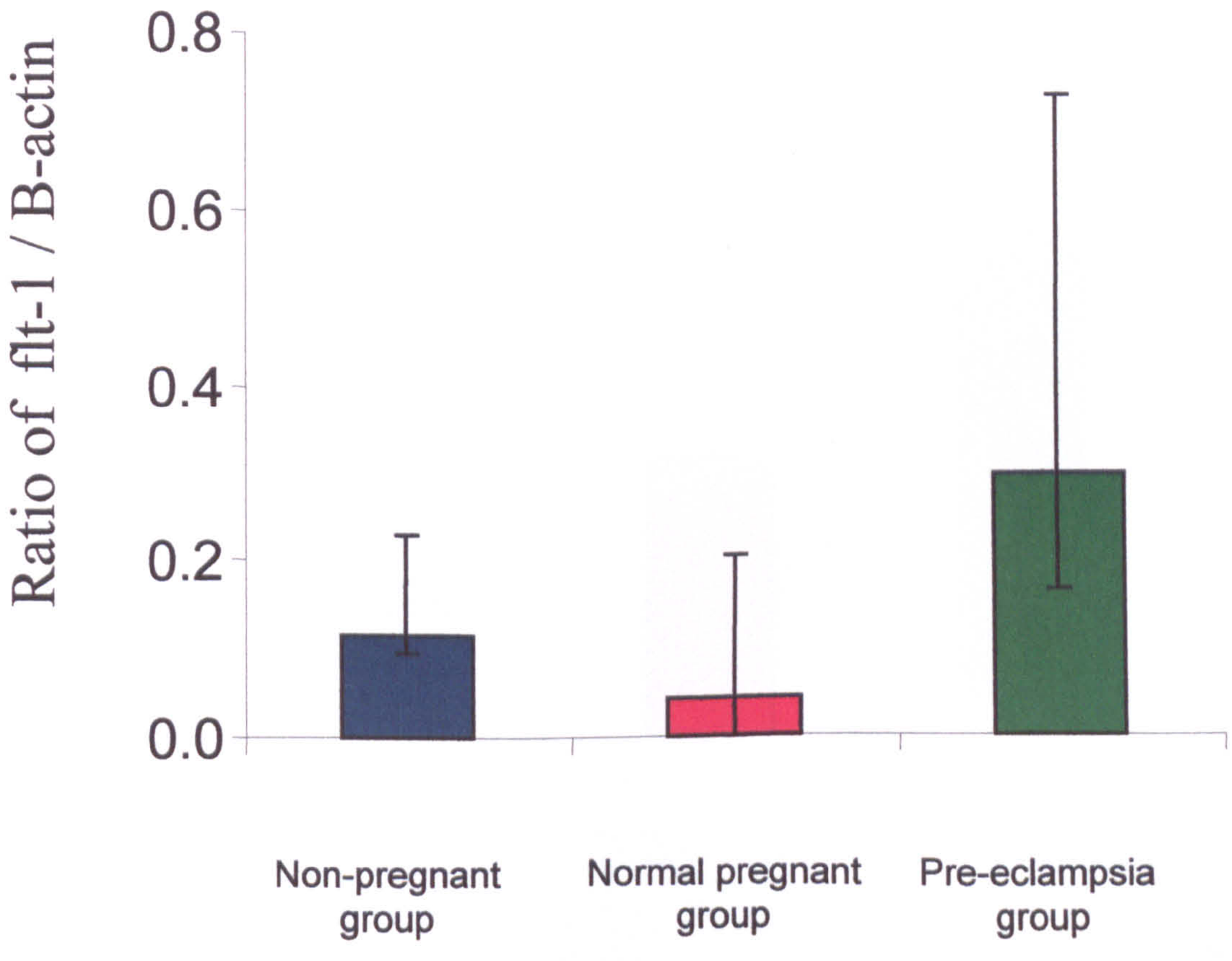
The myometrium of all three experimental groups, as well as expressing VEGF mRNA, also expressed flt-1 and KDR receptor mRNA (Fig 5.6 & 5.7). Statistical analysis of the data revealed no differences in the expression of either receptor between the three experimental groups (flt-1: P= 0.21: Kruskal - Wallis; KDR: P= 0.47: Kruskal - Wallis) although both receptors were present at somewhat higher density in the group with pre-eclampsia.

5.4.4 mRNA expression of PlGF in the myometrium of non-pregnant women, pregnant women and women with pre-eclampsia:

There was a significant difference in the expression of PlGF mRNA between the three study groups (Kruskal – Wallis: P=0.02) (Fig 5.8). However, post-hoc analysis was unable to reach significance when comparisons were made of the non-pregnant *Vs* normal pregnant groups

Fig 5.6

The semi-quantitative expression of the flt-1 mRNA in the myometrium from non-pregnant women, pregnant women and women with pre-eclampsia

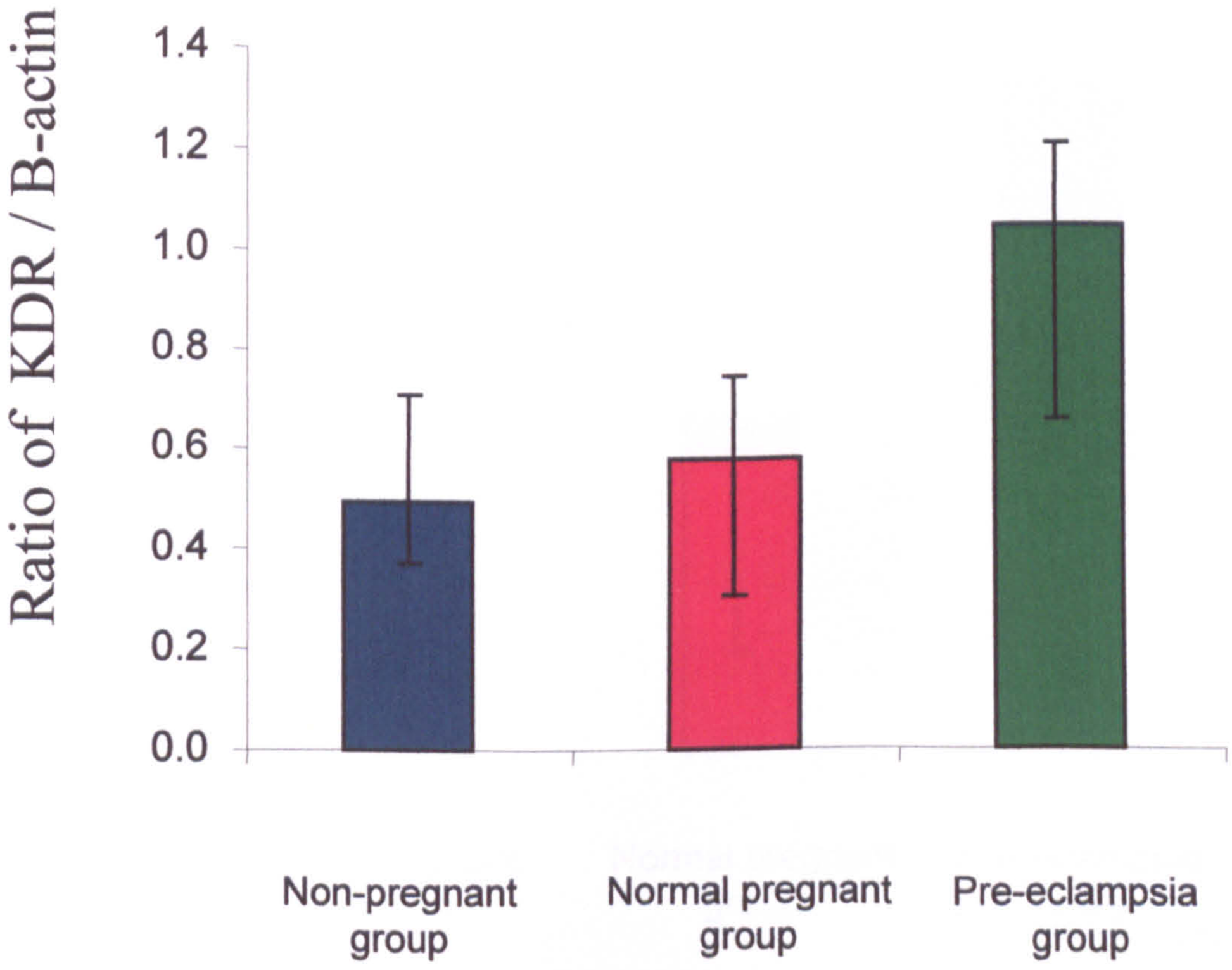


KEY

Data are represented as medians +/- inter-quartile ranges. Blue boxes represent myometrium from non-pregnant women. Red boxes represent myometrium from control women. Green boxes represent myometrium from women with pre-eclampsia.

Fig 5.7

The semi-quantitative expression of the KDR mRNA in the myometrium from non-pregnant women, pregnant women and women with pre-eclampsia

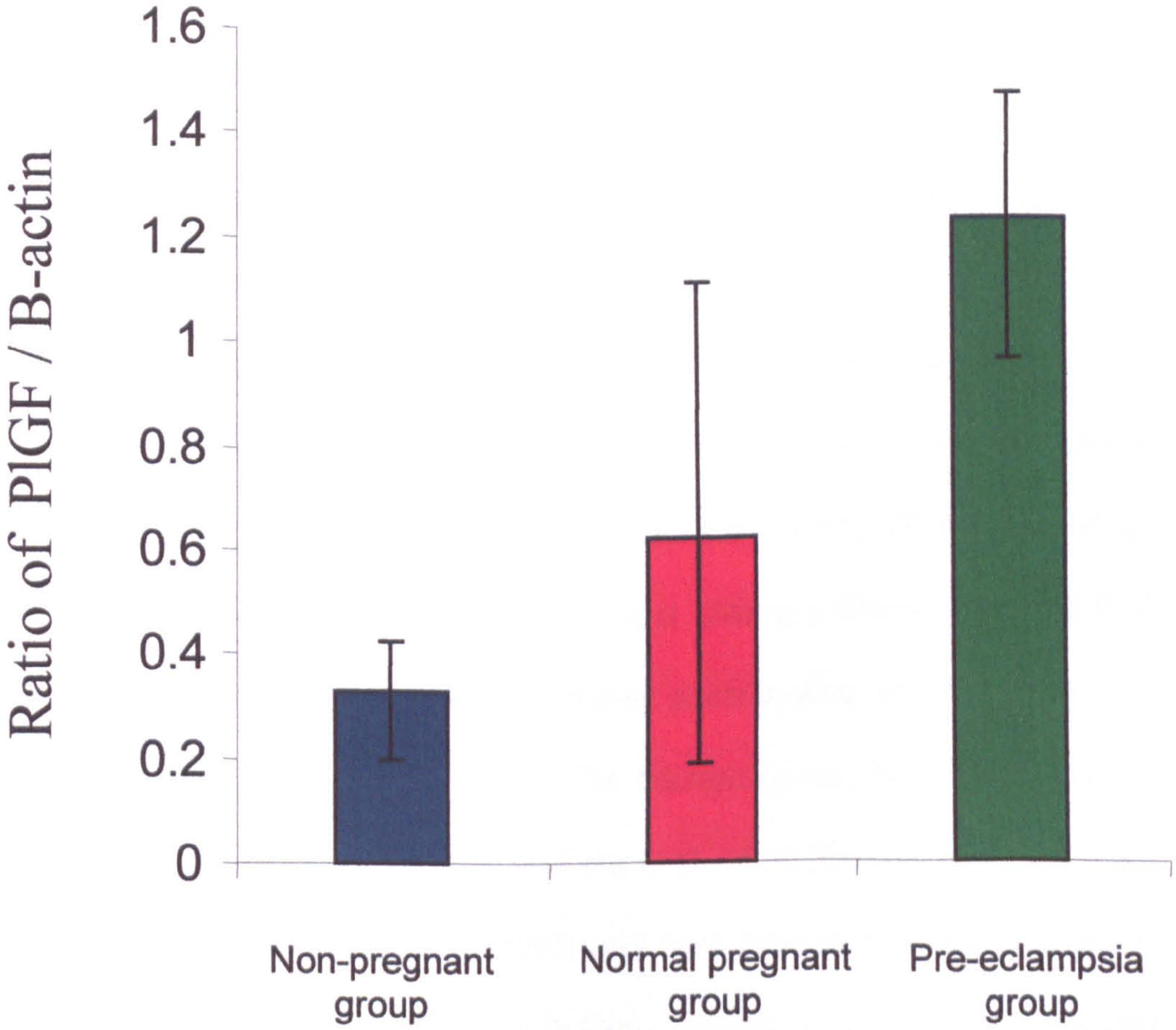


KEY

Data are represented as medians +/- inter-quartile ranges. Blue boxes represent myometrium from non-pregnant women. Red boxes represent myometrium from control women. Green boxes represent myometrium from women with pre-eclampsia.

Fig 5.8

The semi-quantitative expression of the PlGF mRNA in the myometrium from non-pregnant women, pregnant women and women with pre-eclampsia



KEY

Data are represented as medians +/- inter-quartile ranges. Blue boxes represent myometrium from non-pregnant women. Red boxes represent myometrium from control women. Green boxes represent myometrium from women with pre-eclampsia.

(Mann Whitney: P= 0.39) and the normal pregnant *Vs* pre-eclampsia groups (Mann Whitney: P= 0.08). This apparent failure to reach significance may be due to insufficient numbers or to no difference existing between these groups.

5.5 Discussion:

5.5.1 Placental expression:

The data presented in this thesis demonstrates, using a nested semi-quantitative PCR technique, that in third trimester placentas obtained from normal pregnancies at least three isoforms of VEGF are expressed, $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$. Several other studies have shown similar results. Sharkey et al, (1993) demonstrated utilising the same nested RT-PCR protocols that four isoforms of VEGF were present in human first trimester and third trimester placenta, $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{198}$ (Sharkey et al., 1993). Anthony et al, utilising a different two round PCR protocol were able to confirm this finding in first trimester placenta along with the addition of a further $VEGF_{206}$ isoform (Anthony et al., 1994). The discrepancy may have arisen from the different PCR primers and protocols employed (Anthony et al., 1994) (Sharkey et al., 1993) and as such both this study and Sharkey et al (1993) may have required extended protocols or different primers to detect the larger $VEGF_{206}$ isoform. Jackson et al (1994) demonstrated utilising Western blot analysis that the $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$ isoforms were expressed by term placenta (Jackson et al., 1994). It is interesting that the messages for larger VEGF molecules, which do not appear to be secreted in readily soluble form and become bound to the extra-cellular matrix (Houck et al., 1992) are produced at lower copy numbers and require extended PCR protocols. The requirement of extended PCR protocols for

the detection of mRNA from extracellular matrix bound products has also been reported for basic fibroblast growth factor (Watson et al., 1992). This may represent longer half-life times for these molecules, however, there is at present no data to support this suggestion.

This thesis demonstrated in both placentas from normal pregnant women and from women with pre-eclampsia, that the VEGF $_{121}$ isoform is most abundantly expressed. This confirms the finding of Houck et al (1991) who, using Western Blot analysis, also reported VEGF $_{121}$ to be the primary expression product of placenta (Houck et al., 1991). However, Jackson et al (1994) also utilised Western blotting and demonstrated that VEGF $_{206}$ was the most abundant isoform. The data of this thesis and that of Anthony et al (1994) would oppose the observation of Jackson et al (1994), unless the VEGF $_{206}$ isoform has an extended the half-life. However, the *in vivo* half-life of VEGF$_{206}$ still remains to be determined. These differences may also have occurred for several reasons. Firstly, the antibodies sensitivities used in the Western immunoblotting may have differed. Secondly, different techniques were employed; this study utilised semi-quantitative RT-PCR, which quantifies mRNA, whereas the studies of Houck et al (1991) and Jackson et al (1994) used Western blotting, which detects transcribed protein. Thirdly, both Houck et al (1991) and Jackson et al (1994) did not express their results related to housekeeping gene product. Anthony et al (1994) utilising RT-PCR suggested that VEGF $_{165}$ was predominant, however, their results were obtained from cultured first trimester placental tissues. Removal of the placenta from its regulatory hormone milieu may have profound effects on VEGF expression, producing misleading results.

It is evident that the human placenta produces various amounts of all the VEGF isoforms. Although the biological significance of the different isoforms of VEGF is still unclear, VEGF $_{121}$, VEGF $_{145}$ and VEGF $_{165}$ are found to be efficiently secreted and appear to possess angiogenic activity (Houck et al., 1991) (Poltorak et al., 1997), whereas VEGF $_{189}$ and VEGF $_{206}$ contains an insertion encoded by exon 6 and remain predominantly cell-associated. However, the expression of both secreted and cell-associated isoforms of VEGF allows the possibility that VEGF acts in a paracrine manner in those tissues to regulate both vascular angiogenesis and permeability. The functions of the different isoforms within the uteroplacental environment thus remain to be determined.

The localisation and distribution pattern of immunoreactive VEGF and its flt-1 receptor at different gestations have been examined by Ahmed et al (1995). They reported that both proteins were detected in the cytotrophoblast of first and second trimester placental tissue (Ahmed et al., 1995). Cytotrophoblast cells are undifferentiated stem cells which overlays the chorionic villi and display proliferate activity undergoing differentiation into villous and extravillious trophoblast (Yeh et al., 1989). This extravillous trophoblast then migrates towards and into the maternal decidua. In situ hybridisation for VEGF mRNA has shown that in first trimester human placenta, expression is limited to the cytotrophoblast and syncytiotrophoblast surrounding the villi (Sharkey et al., 1993). In contrast, in term placenta; mRNA distribution changed and was confined to the syncytiotrophoblast, extravillous trophoblast and maternal macrophages (Sharkey et al., 1993). Using an immuniohistochemistry technique, these original observations have been confirmed, with the VEGF protein localised in the first trimester to cytotrophoblast and syncytiotrophoblast and as gestation progressed to only syncytiotrophoblast (Barleon et al., 1994). The differing spatio-

temporal observations of VEGF in early and late gestation placentae indicate that VEGF may play a role in both placental development and endothelial maintenance.

Cooper et al 1995 examined the expression of VEGF mRNA in placentas from normotensive control women delivered through the third trimester and showed that expression decreased as gestation increase. A finding recently confirmed by Khaliq et al (1999). However, it could be suggested that the control group they used were not normal, as they were delivered prematurely, and this may have biased the data obtained. The premature deliveries in their normotensive group were for placental abruption, premature labour, and evidence of intra-uterine infection, all of which could affect the placental expression of VEGF to give misleading values. Indeed placental abruption and pre-eclampsia have recently been shown to be associated with genetic mutations for the thrombophila genes; Factor V, methyltetrahydrofolate reductase and prothrombin (Kupferminc et al., 1999). The authors postulated that the result of these mutations leads to abnormal placentation and its subsequent sequelae, either pre-eclampsia or placenta abruption. Whether this alteration in expression with gestation is accompanied by an alteration in the VEGF isoform expression remains to be demonstrated.

Charnock-Jones et al, 1994 and Clark et al, 1996 have utilised in situ hybridisation to reveal strong localisation of VEGF mRNA within the maternal decidual macrophages (Charnock -Jones et al., 1994) (Clark et al., 1996). Ahmed et al, 1995 utilised immunostaining to confirm VEGF protein production by these tissues and also demonstrated that extravillous trophoblast expressed high levels of the flt-1 receptor. From this they postulated that VEGF may act as a chemoattractant to the invading trophoblast. Evidence supporting this comes from the study of Lash et al,1999 who showed in an *in vivo* invasion model, that VEGF acted on an immortalised

trophoblast cell line (expressing the flt-1 receptor only) to increase motility but not invasion (Lash et al., 1999). However, Athanassiades et al, 1998 have recently failed to show a role for VEGF in the migration and invasiveness of an *in vitro* cultured immortalised human extra-villous trophoblast cell line (Athanassiades et al., 1998). The differences in the results obtained from Lash et al, 1999 and Athanassiades et al, 1998 may be due to the cells utilised. However, most evidence would support a role for VEGF as promoting placental development through enhancing migration of trophoblast into the maternal decidua at implantation.

Poor placental development has been associated with intrauterine growth restriction and pre-eclampsia (Brosens et al., 1972) (Fox, 1986) (Meekins et al., 1994) (Chapter 1.1). This thesis shows (Figure 5.1 & 5.2) no differences in the mRNA expression of VEGF in the placentas of pregnancies complicated by pre-eclampsia when compared to normal control placentas. Previous studies of VEGF in pregnancies complicated by pre-eclampsia have demonstrated that VEGF immunoreactivity is decreased in placentas from women with pre-eclampsia (Cooper et al., 1996) (Lyall et al., 1997b). Lyall et al examined immunohistochemically the expression of VEGF in normal placentae and those complicated by pre-eclampsia (Lyall et al., 1997b). They demonstrated that syncytiotrophoblst VEGF staining was decreased in placentae from women with pre-eclampsia (Lyall et al., 1997b). However, in a similar manner to Cooper et al (1996), the control group selection and the selection of placental site may also have affected their study. Ranheim et al (2001) have recently examined utilizing Northern Blott techniques, and a more acceptable control group, the expression of VEGF mRNA in placentae from women with pre-eclampsia and normal controls, demonstrating in line with this study that the mRNA remains unaltered in placentae from women with pre-eclampsia (Ranheim et al., 2001).

Baker et al (1995) examined the placental production of VEGF in complicated pregnancies by the measurement of VEGF concentrations in umbilical vein serum (Baker et al., 1995b). Although, they were unable to demonstrate any differences in the VEGF concentration between normal and pre-eclamptic venous serum, there was a tendency for the vein serum from babies from women with pre-eclampsia to be lower than the control group. This finding has been confirmed by Lyall et al (1997). One problem with this study is that umbilical vein blood as taken to represent placental production. This may not be the case if the placenta secretes the VEGF into the maternal system, in which case a lower VEGF concentration might be expected in the fetal system.

The literature would suggest the hypothesis that trophoblast expression of flt-1 receptor might be increased in pregnancies complicated by pre-eclampsia, as it is this that may aid migration of the extravillous trophoblast into the maternal decidua. Although results of this thesis do not support this hypothesis, they due not exclude it, as placental tissues contain a mixed cell population that may mask any alteration in trophoblast flt-1 production. Further evidence to confirm or refute this hypothesis would require investigation of distribution changes of the flt-1 receptor within placentas from women with pre-eclampsia.

This study found no differences in the expression of the VEGF receptors (flt-1 and KDR) in placental tissue from normal control women and women with pre-eclampsia. Although the expression of both receptor mRNA was not increased in pre-eclampsia, this does not exclude a role for these receptors in the pathophysiology of this disease, as alterations of membrane protein expression may dramatically alter the function of VEGF. However, this was not investigated further due to the limited time allowed for this thesis. Evidence to support a role for the flt-1 receptor in the pathogenesis of pre-eclampsia comes from Helske et al (20001). They have

demonstrated utilizing the techniques of immunohistochemitry and Western Blotting that protein expression of the flt-1, but not KDR receptor are increased in the placenta of women with pre-eclampsia when compared to normal controls (Helske et al., 2001). This data and that presented in this thesis may suggest that the flt-1 receptor undergoes a post-translational modification that reduces its turnover time, with the affect of increasing its cellular expression.

This study demonstrates that PlGF mRNA is expressed by normal placenta and is consistent with other studies (Maglione et al., 1991) (Khaliq et al., 1996) (Clark et al., 1998b). Khaliq et al (1996) used immunohistochemical techniques on term normal placentas and showed that PlGF was localised to the vasculosyncytial membrane and in the media of large blood vessels of the placental villi. Clark et al (1998) utilized in situ hybridization and confirmed that PlGF mRNA was produced in large amounts by villous cytotrophoblast, syncytiotrophoblast and extravillous trophoblast. These results suggest that PlGF may be an important paracrine factor for vascular endothelial cells in placental angiogenesis and as an autocrine mediator of trophoblast function (Khaliq et al., 1996).

Khaliq et al (1999) also demonstrated that placental PlGF mRNA and protein increased with gestation, whilst VEGF decreased (Khaliq et al., 1999). *In vitro* studies on chick chorioallantoic membranes have demonstrated that VEGF mediates branching angiogenesis, whereas PlGF mediates non-branching angiogenesis (Kurz et al., 1998) (Wilting et al., 1996). Reports suggest that in second and third trimester placenta, angiogenesis changes from a predominately branching to non-branching, forming terminal villi, the functional unit of the placenta (Demir et al., 1989) (Demir et al., 1997). These observations led Khaliq et al (1999) to postulate that early

pregnancy placental angiogenesis is mediated by VEGF and that late placental angiogenesis is via PLGF (Khaliq et al., 1999).

In addition Khaliq et al (1999) showed that maternal decidual cells stain strongly for PlGF protein. PlGF initiates its function through the flt-1 receptor (Park et al., 1994). As the placental extravillous trophoblast express flt-1 receptor it might be postulated that PlGF, in a similar manner to VEGF, promotes migration of these cells into the maternal decidua. However, the studies of Athanassiades et al (1998) and Lash et al (1999), which demonstrate that PlGF neither caused migration or invasion, do not support this hypothesis. Both these studies utilized bacterially produced PlGF which has been reported to be devoid of biological activity on human microvascular endothelial cells (Birkenhager et al., 1996) and HUVEC (Cao et al., 1996). In contrast, PlGF –1 produced by human transfected fetal kidney cells is chemotactic, mitogenic and angiogenic to human umbilical vein endothelial cells. Therefore further work is required to ascertain whether non-bacterially produced PlGF affects trophoblast migration and invasion.

Although there was a trend for the PlGF mRNA expression to be increased in pregnancies complicated by pre-eclampsia when compared to normotensive controls this did not reach statistical significance (Mann Whitney: P= 0.09) (Figure 5.4). This finding is consistent with that of Khaliq et al (1999) (Khaliq et al., 1999) who demonstrated utilising both protein Western blotting and RNA Northern blotting that placentas from women with pre-eclampsia do not significantly increase their production of PlGF mRNA or protein. Interesting they demonstrated that placentas from women with inter-uterine growth restriction significantly increased production of PlGF (Khaliq et al., 1999). They also demonstrated that hypoxia decreased and hyperoxia increased production PlGF from cultured trophoblast and suggested that these observations were consistent

with the hypothesis of Kingdom et al (1997), that placenta of fetuses with inter-uterine growth restriction, characterised by absent end-diastolic flow, are in a hyperoxic environment (Kingdom and Kaufmann, 1997).

VEGF and PlGF form functional hetrodimers that initiate their responses through the flt-1 receptor (Cao et al., 1996). The observation of Clark (1998) that the mRNAs encoding VEGF and PlGF were not co-localised, suggests that it is unlikely that any significant production of VEGF/PlGF heterodimer occurs in the placenta (Clark et al., 1998a).

5.5.2 Myometrial expression:

Although the expression of VEGF in human myometrium has been investigated previously (Charnock-Jones et al., 1993) (Harriso-Woolrych et al., 1996), this is the first study to examine the mRNA expression of the VEGF isoforms in the maternal myometrium in non-pregnant women, pregnant women, and women with pre-eclampsia. This thesis demonstrated that at least three VEGF isoforms are expressed by human myometrium, $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and confirmed the findings of Charnock-Jones et al (1993) and Harrison-Woolrych et al (1995). It also showed that all VEGF isoforms are increased in control third trimester myometrium and this raises the possibility hormonally controlled expression. Harrison-Woolrych et al (1995) utilised an RNAse protection to demonstrate that VEGF production was enhanced in the proliferative phase of the oestrous cycle, postulating that the ovarian steroids, oestrogen and progesterone, mediated the observed increased expression (Harriso-Woolrych et al., 1996). Further evidence to support this comes from the observation that in the endometrial cancer cell line HEC-1A, oestadiol stimulates VEGF mRNA production (Charnock-Jones et al., 1993).

Although the ovarian steroids may control VEGF production within the myometrium, myometrial VEGF function still remains to be fully elucidated. In early pregnancy it may enhance the uterine environment, through increased angiogenesis, to aid implantation of the fertilised ovum (Charnock-Jones et al., 1993). It has also been demonstrated that maternal serum concentrations of VEGF are increased in the early gestations of normal pregnancy and this has been postulated to drive the maternal cardiovascular adaptations (Evans et al., 1997) (Evans et al., 1998) (Wheeler et al., 1999). Increased myometrial production may also increase the blood supply to the endometrium through vasodilatation of the uterine and myometrial vessels. It has previously been reported that in a rat model, VEGF causes the vasodilatation of rat uterine arteries (Ni et al., 1997b). It would of interest to examine whether VEGF affects human myometrial resistance arteries in a similar manner; this may enhance our understanding of the early changes that occur in these vessels to allow the implantation and early development of the conceptus (Chapter 8).

This thesis also showed that the myometrial expression of the VEGF isoforms were altered with pregnancy; in non-pregnant myometrium $VEGF_{121}$ predominates, however, in pregnant myometrium all the isoforms are expressed in similar quantities. This may indicate that the VEGF isoforms have varying functional properties dependent on their physical properties, however, the reason for the change with pregnancy remains to be elucidated.

Although myometrium from women with pre-eclampsia showed no overall differences in the expression of the VEGF isoforms when compared to control pregnant myometrium, the isoform expression pattern in the pre-eclampsia group returned toward the non-pregnant pattern, with

VEGF $_{121}$ being most abundant. This may indicate that an alteration in the ratios of VEGF in the myometrium is necessary for a normal pregnancy outcome.

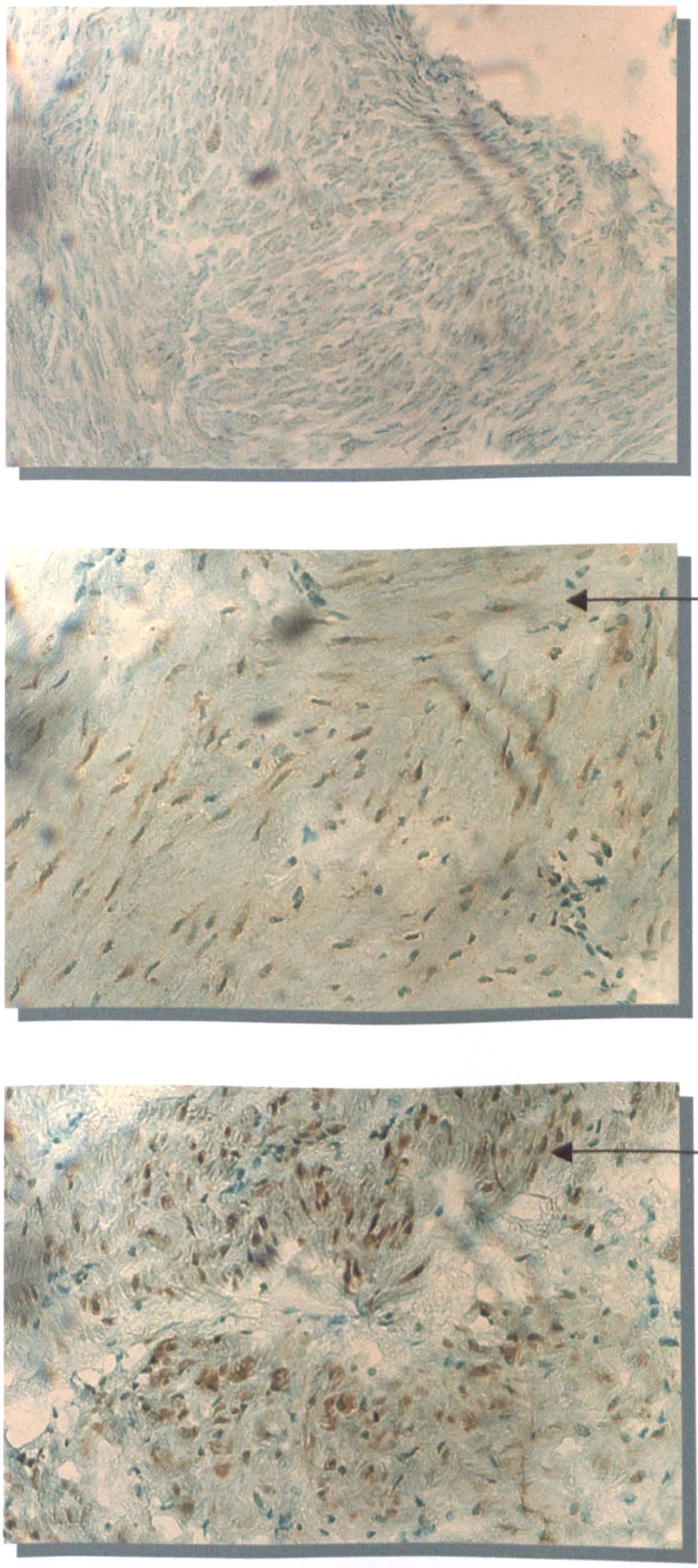
During the time of this thesis, frozen samples of myometrium from non-pregnant women, pregnant women, and women with pre-eclampsia were collected and transported on dry ice to the laboratory of Dr S Davidge (University of Alberta, Canada) and immunohistochemical staining was performed for VEGF (Figures 5.9 & 5.10). This showed that VEGF protein was present in the myometrium of non-pregnant women, pregnant women and women with pre-eclampsia. The protein expression was increased in pregnant women and paradoxically, increased further in the myometrium of pregnancies complicated by pre-eclampsia.

VEGF mRNA has been shown to increase in the myometrium of pregnant women when compared to non-pregnant women. The increased expression may be postulated to explain the increased circulating concentrations that parallel gestation in the third trimester (Chapter 4). Although this is an interesting hypothesis it would require a cross-sectional study of VEGF plasma concentrations determined in uterine artery and vein samples of women undergoing Caesarean sections at varying gestations and correlation with VEGF mRNA and protein expression. However, this would then be subject to sampling basis with the abnormal pregnancies contributing to the early gestations.

VEGF function is also regulated by the bioavailability of VEGF receptors (flt-1 and KDR). This thesis demonstrated that although no significant differences in VEGF receptor expression (flt-1/KDR) occurred between the three study groups there was a trend for increased expression of both flt-1 and KDR receptors in the myometrium of pregnancies complicated by pre-eclampsia.

Fig 5.9

Immunohistochemical staining for VEGF in myometrium from non-pregnant, pregnant, and women with pre-eclampsia (x200 mag)



Non pregnant

Pregnant

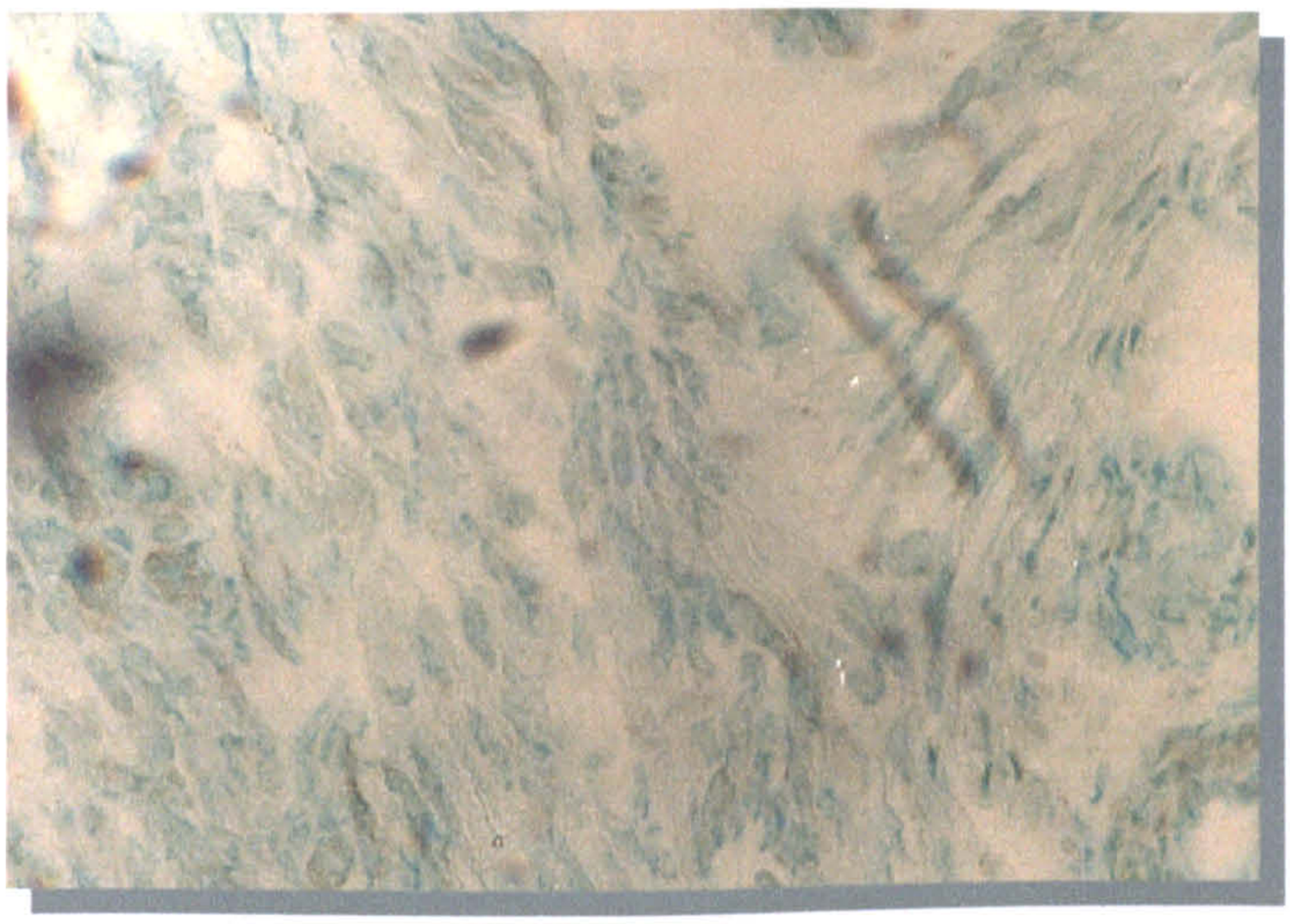
VEGF

Pre-eclampsia

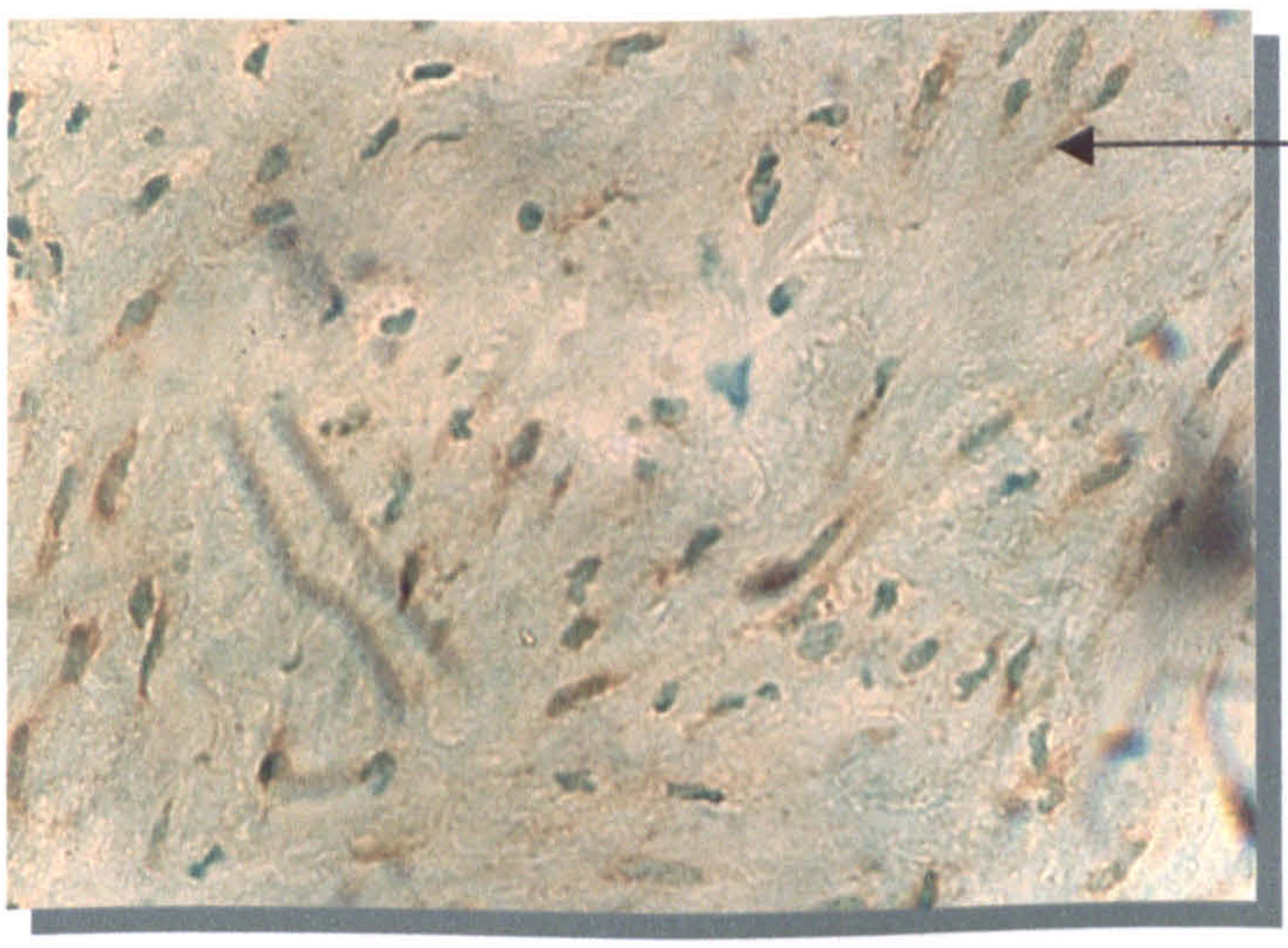
VEGF

Immunohistochemical staining for VEGF in myometrium from non-pregnant, pregnant, and women with pre-eclampsia (x400 magnification)

Fig 5.10

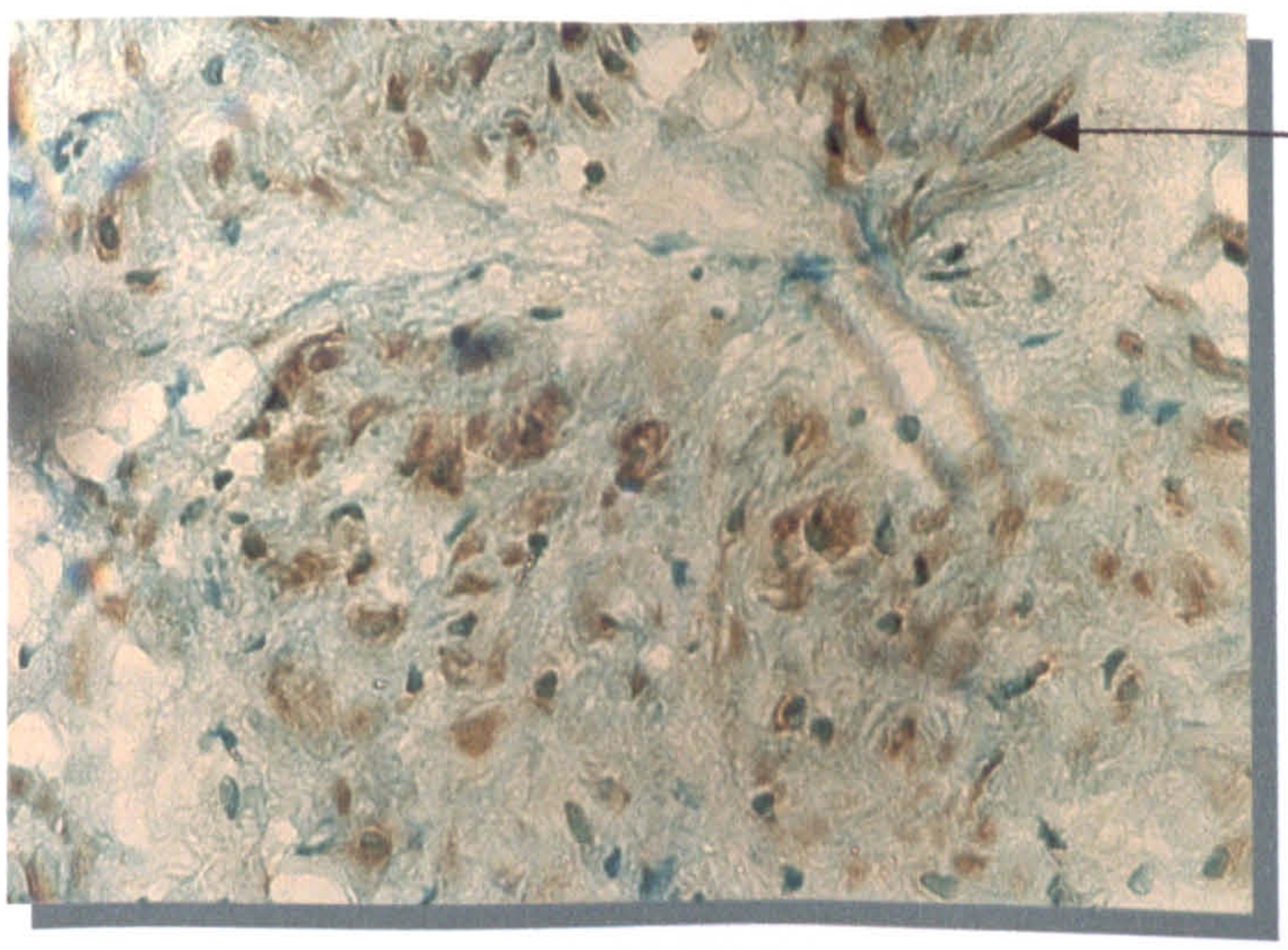


Non pregnant



Pregnant

VEGF



Pre-eclampsia

VEGF

This study confirms the finding of (Brown et al., 1997b) who, utilising in situ hybridisation, demonstrated that uterine smooth muscle from non-pregnant women expressed both flt-1 and KDR receptors. They also demonstrated that isolated myometrial smooth muscle cells proliferated strongly in the presence of VEGF and that this proliferation was in the same order of magnitude as in endothelial cells (Brown et al., 1997b). From this they postulated that VEGF was acting as an autocrine growth factor in these muscle cells and that alterations of the expression of VEGF or its receptors may play a role in the pathogenesis of tumours of the uterus (Brown et al., 1997b).

5.6 Summary:

It has been shown previously that increased circulating concentrations of VEGF occur in both pregnancy and pre-eclampsia (Chapter 4). This chapter sought to test the hypothesis that the observed increased concentrations in pre-eclampsia were due to an altered production of VEGF in the fetomaternal unit. Contrary to the original hypothesis the data presented in this thesis demonstrates that there are no differences in the production of VEGF by the fetomaternal unit in the disease of pre-eclampsia.

Chapter six: The vascular adaptations that occur in myometrial resistance vessels in pregnancy and in pregnancies complicated by pre-eclampsia:

6.1 Introduction:

Normal pregnancy is characterised by a fall in the blood pressure, a reduction in peripheral vascular resistance, and a 40% increase in the cardiac output (Duvekot et al., 1993) (Duvekot and Peeters, 1994) (van Oppen et al., 1996). The vasodilatation of pregnancy is also accompanied by an alteration in the vascular reactivity to the pressor effects of angiotensin II (Gant et al., 1973). In a longitudinal study Gant et al (1973) found a decreased sensitivity to infused angiotenisn II in pregnant women when compared to non-pregnant controls (Gant et al., 1973). Similar findings were reported in women with pre-existing chronic hypertension (Gant et al., 1977).

The mechanism for these changes has been extensively studied and it is clear that the observed decreases are not due to changes in plasma renin concentrations as rapid infusions of fluid do not alter angiotensin II sensitivity (Matsuura et al., 1981). These observations of Gant et al (1977) and Matsuura et al (1981) would indicate that the hypertension in pre-eclampsia is a delayed clinical manifestation of an abnormality at the microvascular level, which is specific to pregnancy. Evidence to support the involvement of the endothelium comes from cell culture studies. Endothelial cells cultured in the presence of plasma from women with pre-eclampsia show altered production of endothelial products, such as nitric oxide and prostacyclin, when compared to plasma from control women (Lorentzen et al., 1991) (Taylor et al., 1991) (Branch et al., 1992) (Roberts et al., 1992) (Baker et al., 1995b) (de Groot et al., 1995) (Baker et al., 1996a) (Kupferminc et al., 1996).

The difficulties with *in vivo* studies in human pregnancy have led researchers to examine the vascular changes that occur in pregnancy with indirect methods. These have included animal models and myography. Myography allows the study of vascular reactivity in *ex vivo* resistance vessels and can be used to determine the contribution of the endothelium to the observed changes.

Uterine resistance vessels are juxtaposed to the developing placental bed and undergo considerable remodelling with pregnancy. As such it is pertinent to study these vessels when examining the changes that occur during pregnancy. However, there is a paucity of data into the effect of pregnancy on myometrial resistance vessels. This is primarily due to the technical difficulties of sample collection. The studies that have investigated endothelium-dependent relaxation in myometrial resistance arteries have utilised different vasoconstrictor (vasopressin, endothelin and U46619) and vasorelaxant agents (bradykinin and acetylcholine) and as such comparison is difficult (Svane et al., 1991) (Ashworth et al., 1996a) (Kublickiene et al., 1997).

6.2 Aims:

- To examine whether pregnancy alters the vasopressin responses of myometrial resistance arteries.
- To examine whether pregnancy or pre-eclampsia alters endothelium dependent relaxation in the uterine circulation.
- To characterise the mechanism by which these changes occur.

- To elucidate which vasodilator changes were to be studied when considering the interaction of the endothelium with Vascular Endothelial Growth Factor.

6.3 Experimental design:

6.3.1 Vasoconstrictors:

6.3.1.1 Vasopressin:

Myometrial resistance vessels were obtained from non-pregnant (n=6), pregnant (n=7) and women with pre-eclampsia (n=6) as described previously in Chapter 2.4.2a. Whenever, possible three vessels from the same patient were mounted on separate myographs. Vessels were normalised as described in Chapter 2.4.4. The myography protocol (Chapter 3.3.1) was then followed and incremental doses of vasopressin were used to cause constriction (Chapter 2.4.5.5).

In a separate set of experiments, myometrial vessels were obtained from pregnant women (n=7). Two vessels were dissected out as described previously, and mounted on the same myograph. After mounting, the endothelium lining the vessels was removed by debridement with a human hair prior to experimentation. Vessels were normalised as described in Chapter 2.4.4. The myography protocol (Chapter 3.3.1) was then followed and incremental doses of vasopressin were used to cause constriction (Chapter 2.4.5.5).

6.3.2 Vasodilators:

6.3.2.1 Bradykinin:

Myometrial resistance vessels were obtained from non-pregnant (n=6), and pregnant women (n=7) and women with pre-eclampsia (n=6) as described previously in Chapter 2.4.2a. Whenever possible three vessels from the same patient were mounted on the three separate myographs. Vessels were normalised as described in Chapter 2.4.4. The myography protocol for vasopressin (Chapter 3.3.1) was then followed and incremental doses of bradykinin were used to cause endothelium dependent relaxation (Chapter 2.4.5.5).

6.3.2.2 Histamine:

Myometrial resistance vessels were obtained from non-pregnant (n=7), and pregnant women (n=8) and women with pre-eclampsia (n=9) and mounted as described previously. The myograph protocol for vasopressin (Chapter 3.3.1) was then followed and incremental doses of histamine were used to cause an endothelium dependent relaxation (Chapter 2.4.5.6).

6.4 Results:

Table 6.1, 6.2 and 6.3 detail the demographics and vessel characteristics of the non-pregnant, pregnant controls and the pre-eclampsia group. As anticipated, systolic and diastolic blood pressure were significantly greater in the group of women with pre-eclampsia than the control pregnant or non-pregnant groups.

Patient demographic details for vessels from non-pregnant women, control women and women with pre-eclampsia

Table 6.1

Vasopressin	Age (years)	BMI (kg/m^2)	Parity	Gestation at delivery (days)	Mean Arterial Pressure Booking mm Hg	Mean Arterial Pressure Maximum mm Hg	Protein g/dl	Individualised Birth-weight Ratio
Non - pregnant	36.0 (33.0 - 41.0)	30.7 (23.9 - 31.8)	2 (2 - 2.5)		88.0 (86.1 - 96.7)			
Normal pregnant	32 (26 - 35)	24.0 (22.8 - 27.2)	1 (1 - 1)	270 (264 - 273)	83.3 (78.1 - 87.8)	89.5 (86.7 - 92.6)	0	88 (57 - 95)
Pregnant women with pre-eclampsia	31 (29 - 36)	28.2 (22.3 - 31.2)	0 (0 - 1.0)	238 (194 - 257)	87.0 (83.3 - 93.7)	123.3 (112.3 - 123.3)	1.50 (0.51 - 4.83)	5 (0.5 - 4.8)

Data are summarised as medians (Inter Quartile Ranges)

Patient demographic details for vessels from non-pregnant women, control women and women with pre-eclampsia

Table 6.2

Bradykinin	Age (years)	BMI (kg/m^2)	Parity	Gestation at delivery (days)	Mean Arterial Pressure Booking mm Hg	Mean Arterial Pressure Maximum mm Hg	Protein g/dl	Individualised Birth-weight Ratio
Non - pregnant	37.0 (34.0 - 39.5)	31.8 (26.9 - 32.7)	2 (2 - 2.5)		86.7 (84.8 - 93.0)			
Normal pregnant	31 (28 - 33)	27.1 (23.0 - 30.2)	1 (1 - 1)	265 (263 - 269)	85.0 (81.7 - 87.3)	90.0 (88.6 - 95.0)	0	92 (73 - 98)
Pregnant women with pre-eclampsia	34 (29 - 36)	22.3 (21.5 - 35.1)	0 (0 - 0.5)	245 (197 - 266.5)	83.3 (83.3 - 91.7)	123.3 (112.3 - 123.3)	1.52 (0.86 - 1.55)	18 (2.5 - 20)

Data are summarised as medians (Inter Quartile Ranges)

Patient demographic details for vessels from non-pregnant women, control women and women with pre-eclampsia Table 6.3

Histamine	Age (years)	BMI (kg/m^2)	Parity	Gestation at delivery (days)	Mean Arterial Pressure Booking mm Hg	Mean Arterial Pressure Maximum mm Hg	Protein g/dl	Individualised Birth-weight Ratio
Non - pregnant	34.5 (33.0 - 40.5)	28.6 (24.4 - 30.9)	2 (1.3 - 2.0)		88.3 (86.7 - 95.8)			
Normal pregnant	32 (23 - 35)	23.7 (22.8 - 24.2)	1 (1 - 1)	272 (271 - 274)	80.0 (76.7 - 86.7)	86.7 (78.3 - 90.3)	0	76 (52 - 92)
Pregnant women with pre-eclampsia	30.5 (29.3 - 33.3)	29.0 (28.0 - 31.0)	0 (0 - 0.8)	245 (197 - 266.5)	89.5 (86.5 - 92.9)	121.6 (117.5 - 130.8)	1.5 (0.6 - 3.0)	1 (1 - 4)

Data are summarised as medians (Inter Quartile Ranges)

6.4.1 Vasoconstrictors:

6.4.1.1 Vasopressin: Comparison of vessels from non-pregnant and pregnant women and women with pre-eclampsia:

There were no significant differences in the dose response curves obtained with vasopressin for vessels from non-pregnant, normal pregnant women and women with pre-eclampsia (Fig 6.1), or the EC_{50} values for the three groups. However, analysis of the E_{max} values revealed a difference between the three groups. Post hoc testing demonstrated that these differences occurred between the non-pregnant and normal pregnant groups (E_{max}; P=0.02) and between the normal pregnant and pre-eclampsia group (E_{max}; P=0.04).

The addition of indomethacin had no significant effect on EC_{50} or E_{max} values for any of the groups studied; non pregnant: (EC_{50} –9.1+/-0.1 Vs –9.0+/-0.1: P=0.59: E_{max}; P=0.12), pregnant (EC_{50} -8.9+/-0.1 Vs –8.9+/-0.1;P=0.45: E_{max}: P=0.45), or women with pre-eclampsia (EC_{50} -9.1+/-0.1 Vs –9.2+/-0.2: P=0.70: E_{max}: P=0.67).

As with indomethacin alone, the addition of Indomethacin and L-NAME caused no shift in either the EC_{50} or E_{max} values: non-pregnant (EC_{50} –9.0+/-0.1 Vs –9.0+/-0.1: P=0.98: E_{max}; P=0.59), normal pregnant (EC_{50} –8.9+/-0.1 Vs –8.9+/-0.1; P=0.96: E_{max}; P=0.87), women with pre-eclampsia (EC_{50} -9.2+/-0.1 Vs –9.2+/-0.1; P=096: E_{max} ; P=0.17).

The removal of the endothelium from the group of vessels from pregnant women caused a non-significant rightward shift of the concentration response curve (EC_{50} ; P=. 0.6). However, analysis of the E_{max} values revealed a difference between the endothelial stripped and intact groups (P=0.05; paired t-test). The group of vessels, which had had the endothelium removed showed an enhanced E_{max} responses when compared to the endothelium intact group (Fig 6.2).

Vasodilators:

6.4.2 Bradykinin:

6.4.2.1 Comparison of endothelium dependent relaxation in non-pregnant and pregnant women and women with pre-eclampsia:

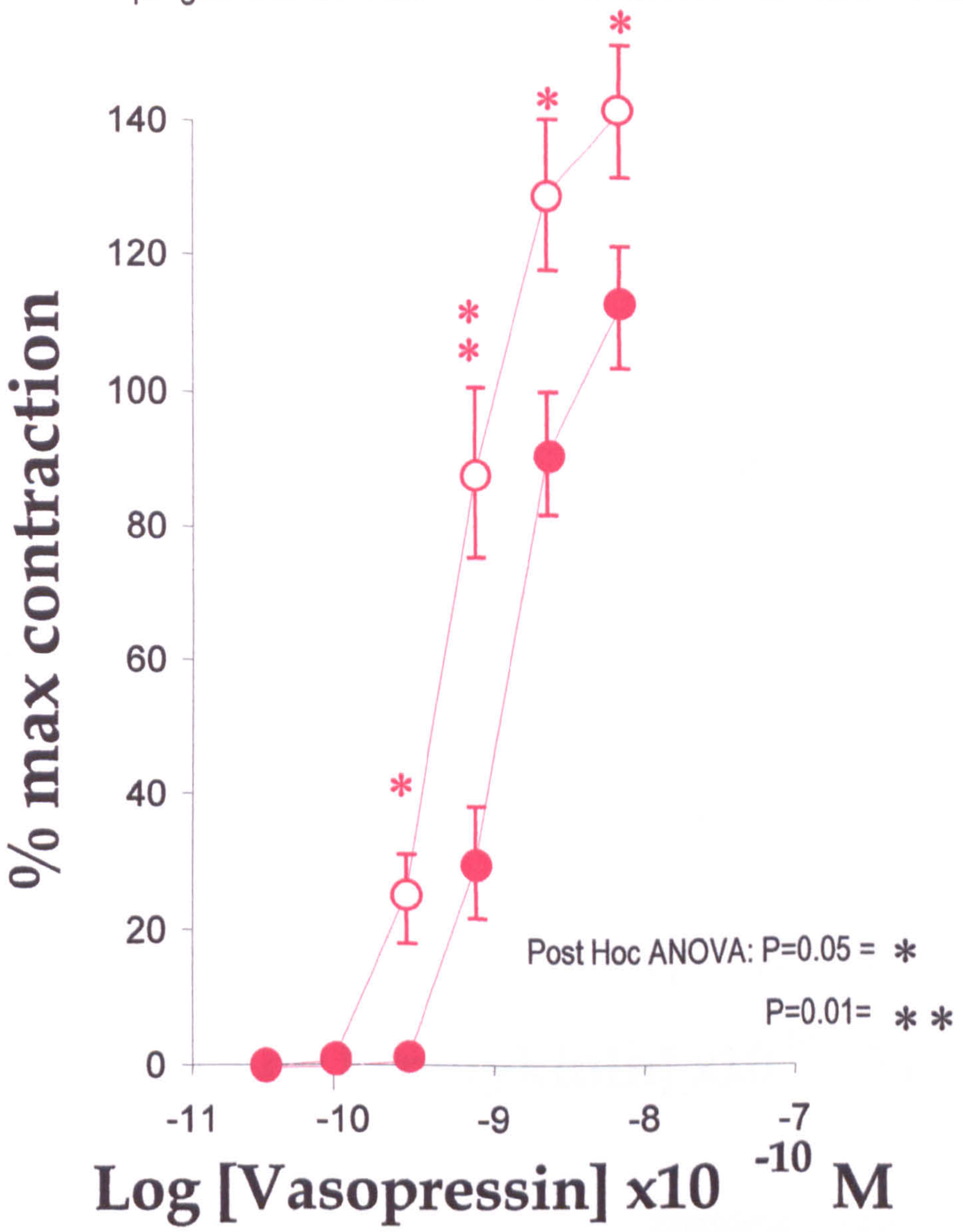
There was no statistical difference in the dose response curves obtained between myometrial vessels from non-pregnant and pregnant women (Fig 6.3) (p = 0.41, repeated measures analysis of variance (ANOVA). However, there was a marked decrease in endothelium-dependent relaxation to bradykinin in myometrial resistance vessels from women with pre-eclampsia when compared to normal pregnant women (Fig 6.3) (p = 0.002, ANOVA) (E_{max}: P = 0.002; unpaired t-test).

6.4.2.2 Effect of incubation of vessels with Indomethacin:

The addition of indomethacin had no significant effect on the relaxation to bradykinin in vessels from non-pregnant (Fig 6.6) (p = 0.73, ANOVA), normal pregnant (Fig 6.7) (p = 0.90, ANOVA) or women with pre-eclampsia (Fig 6.8).

Fig 6.2

Comparison of vasopressin contraction curves in vessels from pregnant women with or without removal of the endothelium



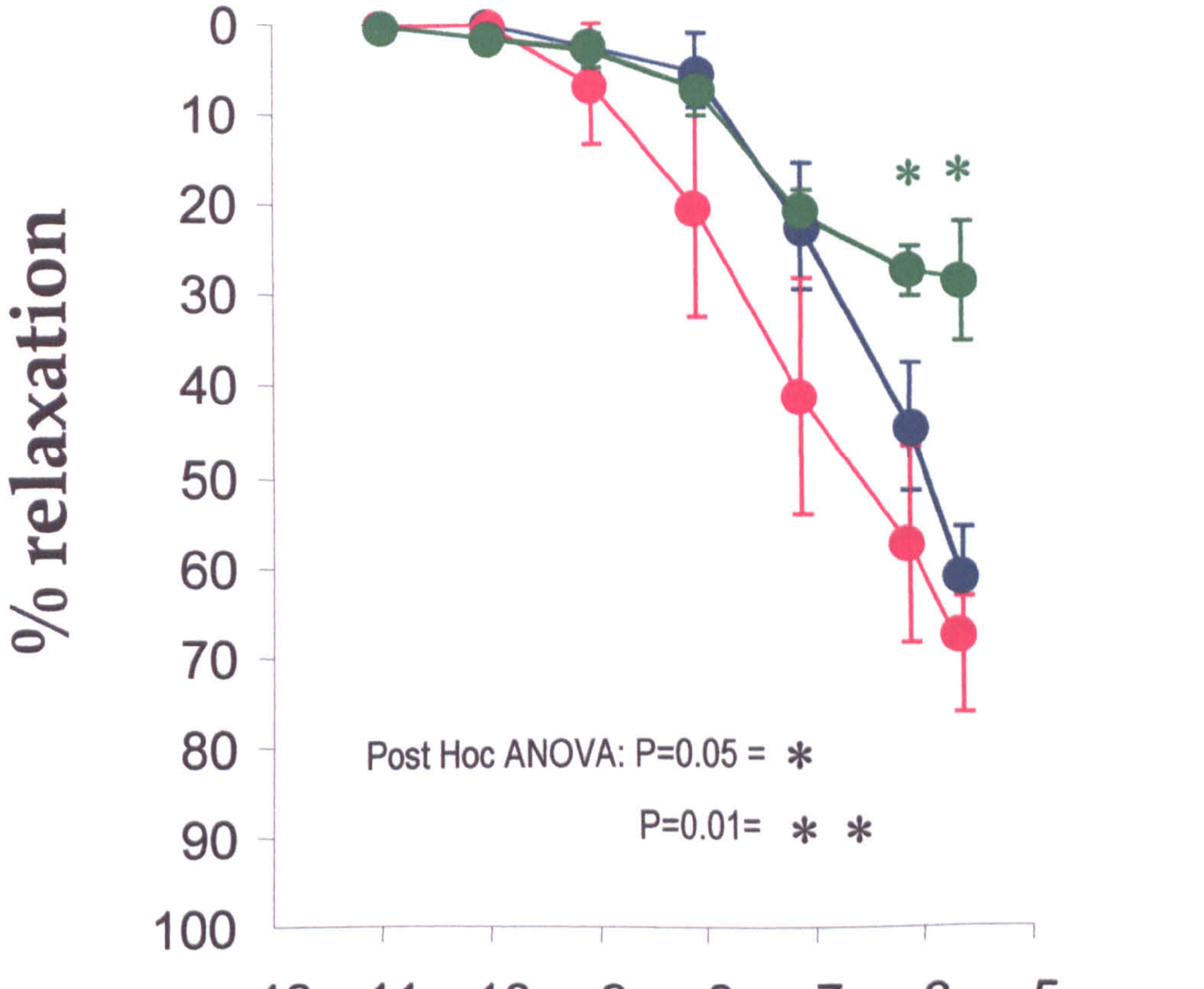
KEY:

Statistical comparisons:

	EC_{50}	E_{max}
Stripped	-9.3+/-0.1	141.1+/-10.1
Intact	-8.8+/-0.1	111.8+/-9.0
ANOVA (factorial)	0.06	0.05

Fig 6.3

Endothelium-dependent relaxations to bradykinin in myometrial vessels from non-pregnant women, pregnant women and women with pre-ecampsia



KEY:

Non-pregnant

Pregnant

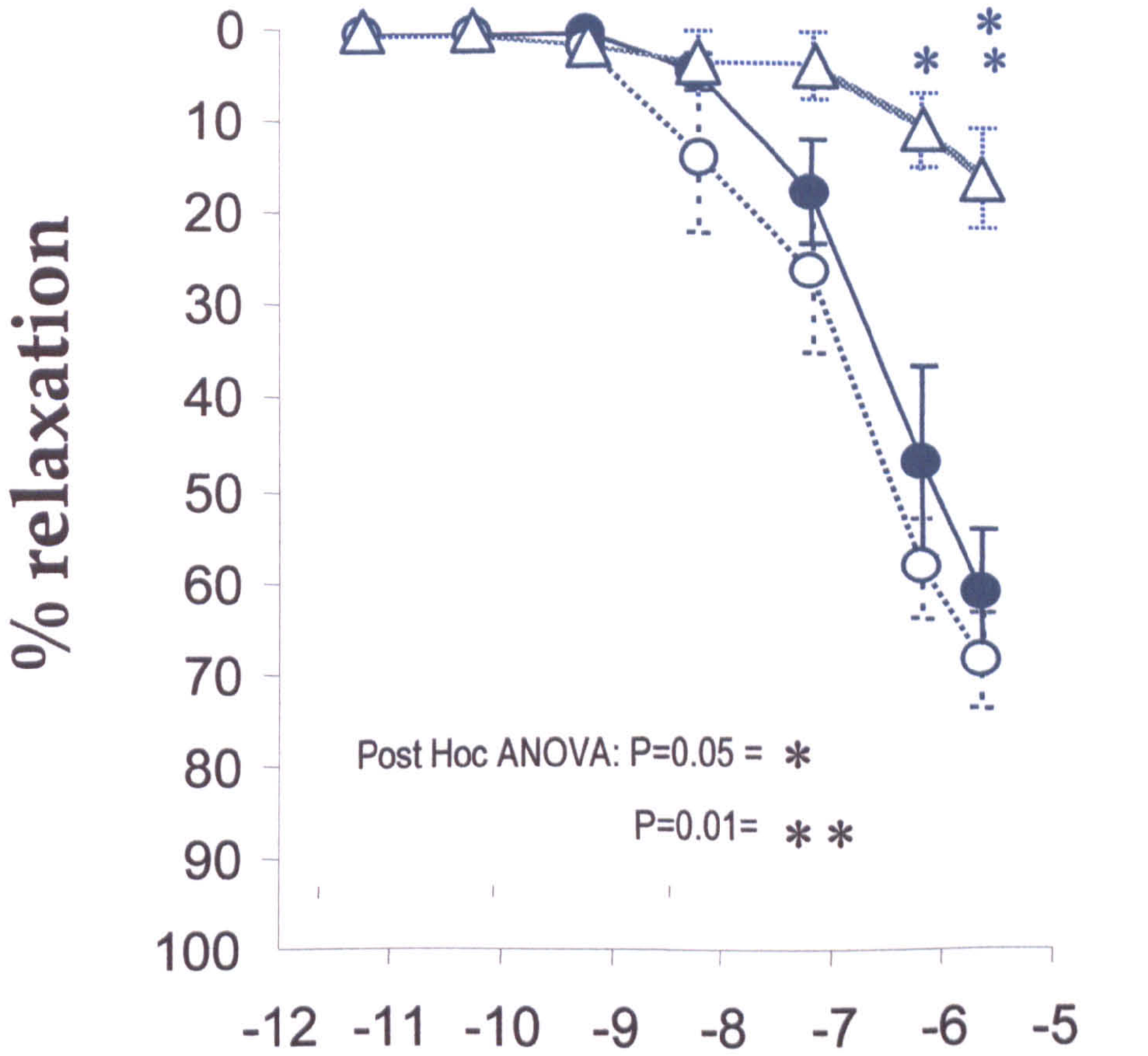
Pre-eclampsia

Statistical comparisons:

		E_{max}
		62.30+/-5.66
		70.81+/-6.66
		29.03+/-3.61
ANOVA (factorial)	p	0.001
ANOVA (repeated measures)	p	0.001

Fig 6.4

The effect of indomethacin and indomethacin and L-NAME on the endothelium-dependent relaxation of vessels from non-pregnant women



KEY:

Control

Indomethacin

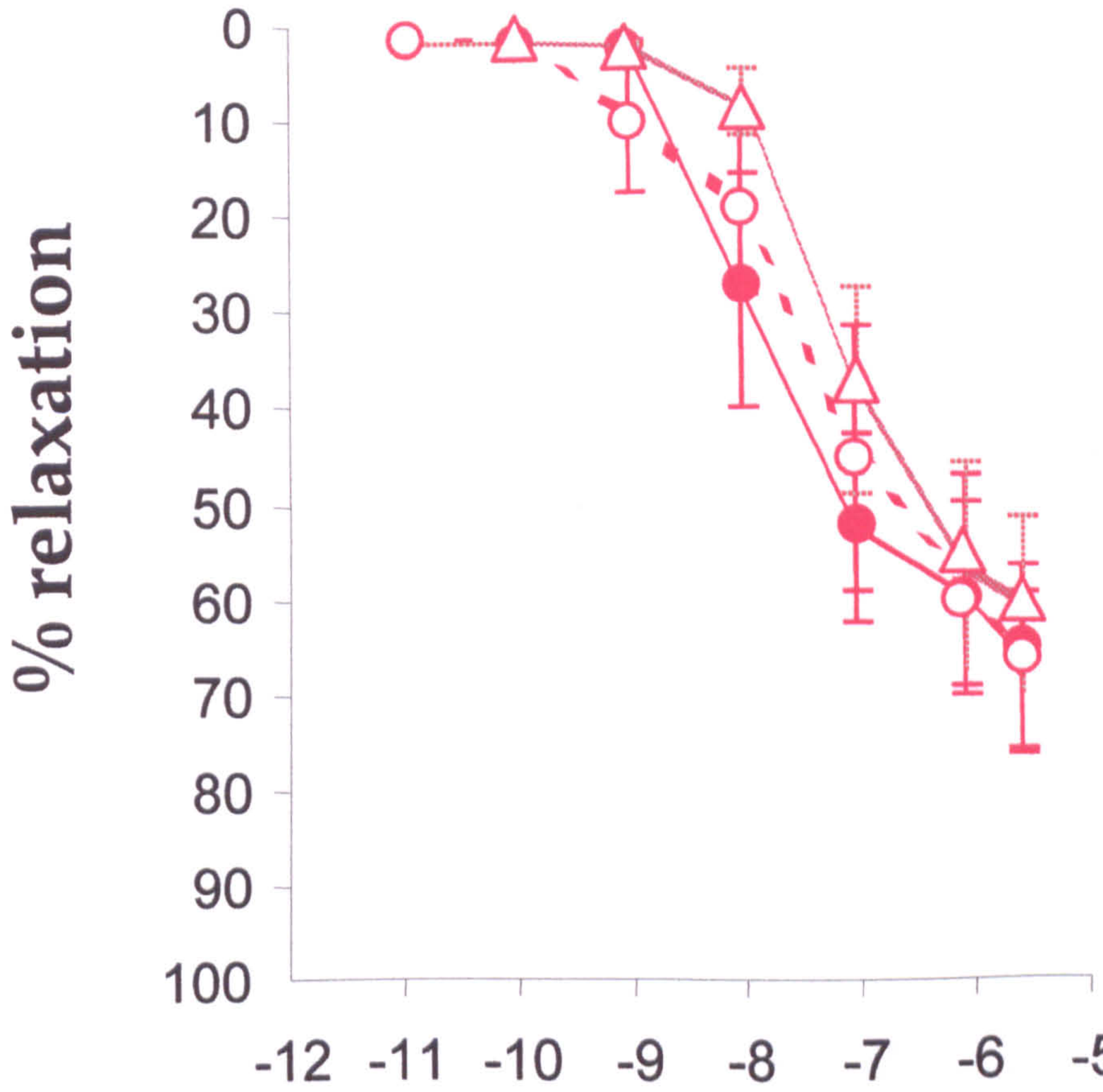
Indo + L-NAME

Statistical comparisons:

		E_{max}
		58.76+/-6.61
		65.97+/-5.07
		15.08+/-5.46
ANOVA (factorial)	p	0.001
ANOVA (repeated measures)	p	0.001

Fig 6.5

The effect of indomethacin and indomethacin and L-NAME on the endothelium-dependent relaxation of vessels from pregnant women



KEY:

Control

Indomethacin

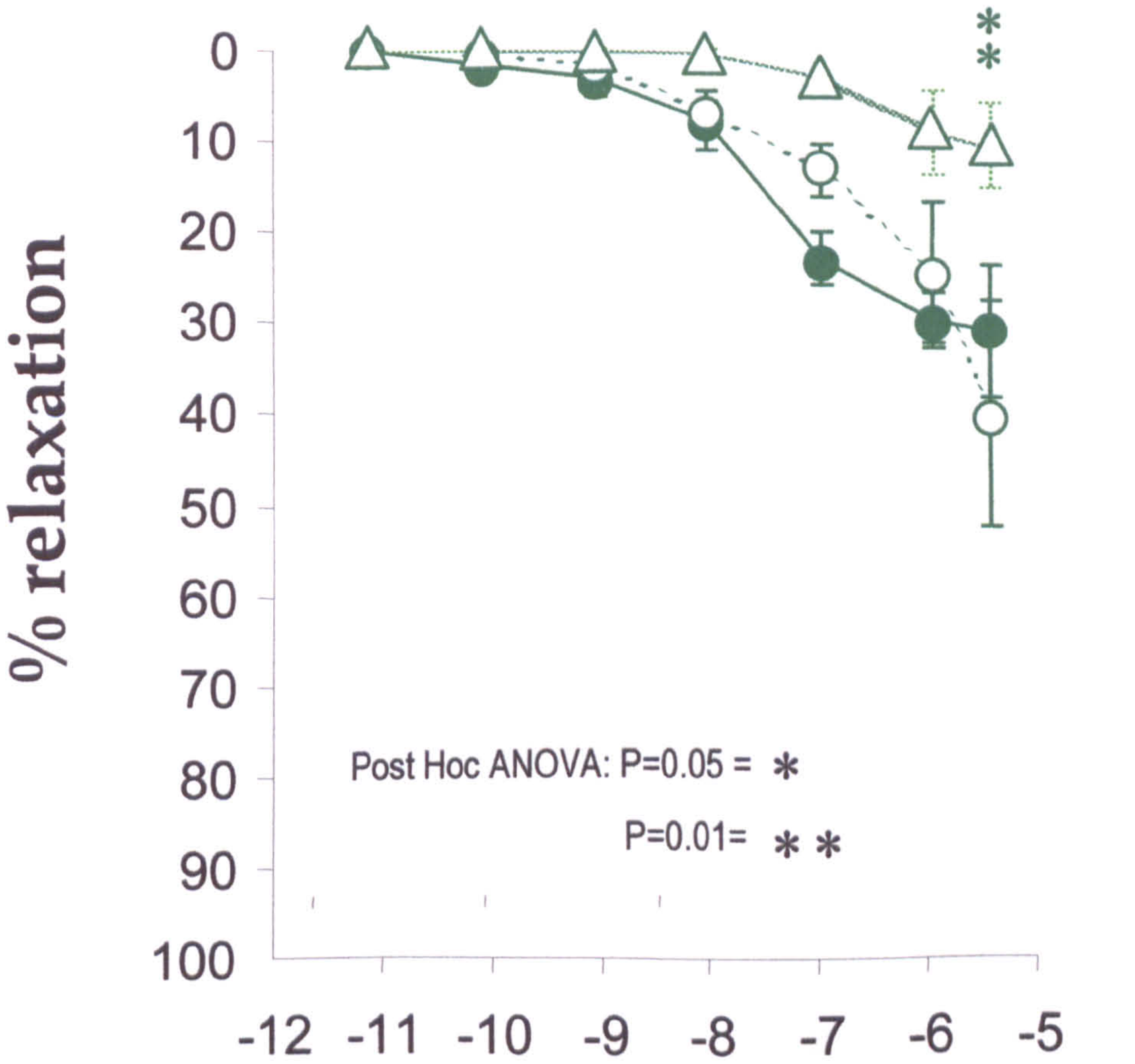
Indo + L-NAME

Statistical comparisons:

		E_{max}
Control		64.92+/-9.73
Indomethacin		66.69+/-8.53
Indo + L-NAME		59.52+/-9.54
ANOVA (factorial)	p	0.87
ANOVA (repeated measures)	p	0.93

Fig 6.6

The effect of indomethacin and indomethacin and L-NAME on the endothelium-dependent relaxation on myometrial vessels from women with pre-eclampsia



Legend:

Control

Indomethacin

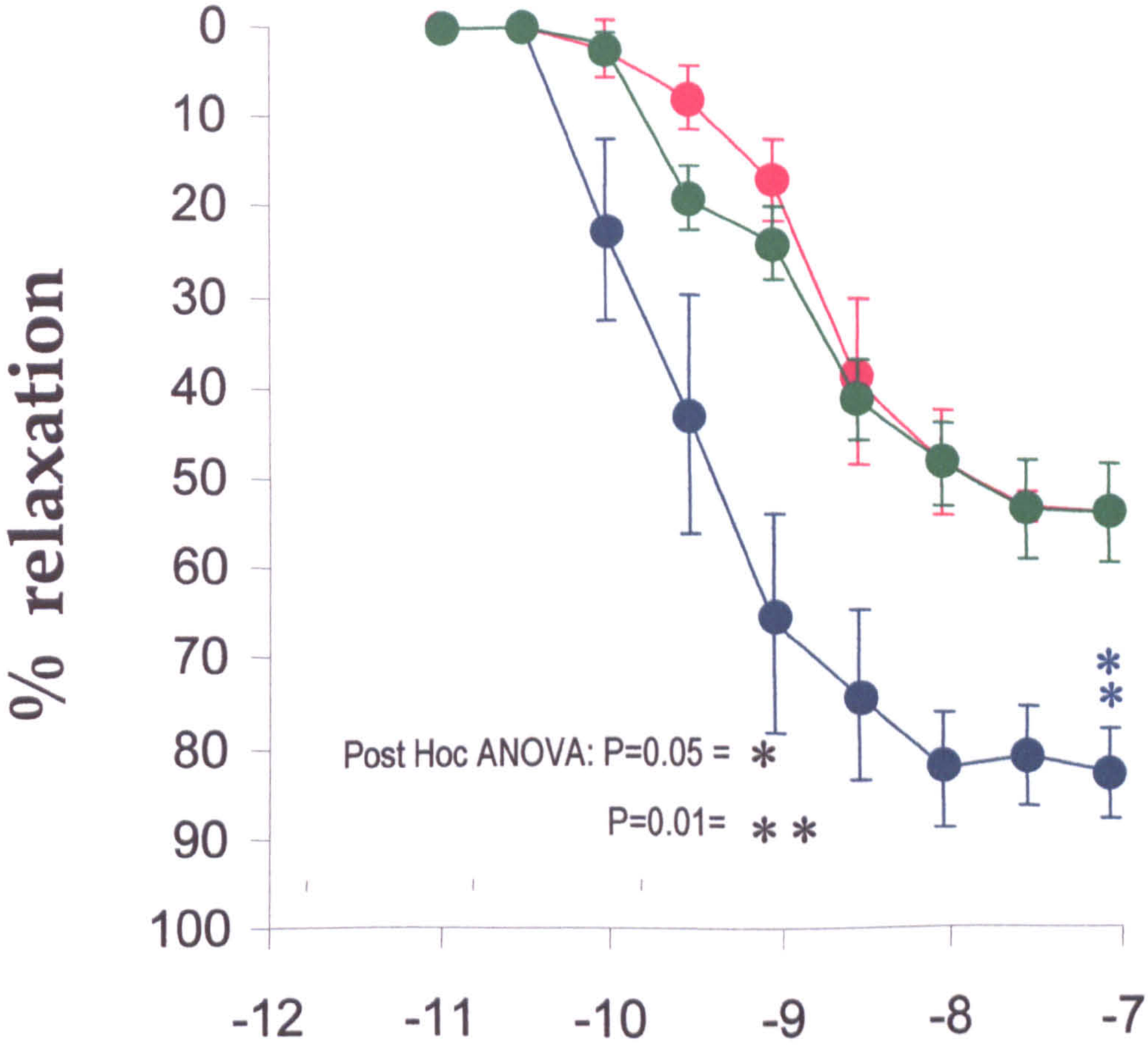
Indo + L-NAME

Statistical comparisons:

		E_{max}
		29.01+/-6.67
		37.51+/-11.57
		10.05+/-4.30
ANOVA (factorial)	p	0.009
ANOVA (repeated measures)	p	0.005

Fig 6.7

Endothelium-dependent relaxations to histamine of myometrial vessels from non-pregnant women, pregnant women and women with pre-eclampsia



KEY:

Non-pregnant

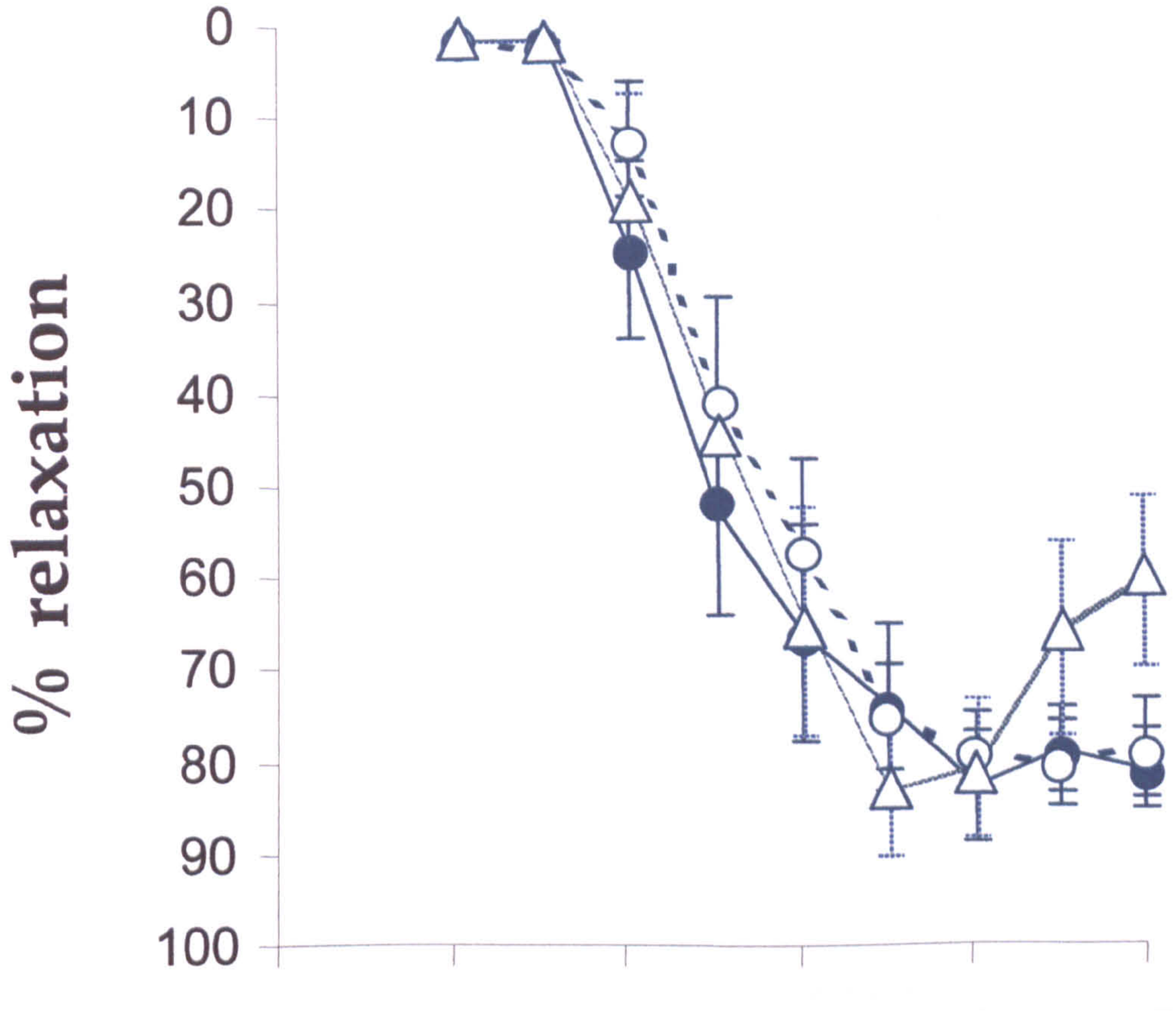
Pregnant

Pre-eclampsia

Statistical comparisons:	EC_{50}	E_{max}
Non-pregnant	-9.2+/-0.3	80.00+/-4.99
Pregnant	-7.7+/-0.4	52.18+/-0.87
Pre-eclampsia	-7.4+/-0.6	47.84+/-5.37
ANOVA (factorial)	0.03	0.006
ANOVA (repeated measures)		0.001

Fig 6.8

The effect of indomethacin and indomethacin and L-NAME on the endothelium-dependent relaxation obtained from vessels from non-pregnant women



KEY:

		EC_{50}	E_{max}
Control		-9.2+/-0.3	80.00+/-4.99
Indomethacin		-9.1+/-0.2	52.18+/-0.87
Indo + L-NAME		-9.2+/-0.3	47.84+/-5.37
ANOVA (factorial)	p	0.98	0.08
ANOVA (repeated measures)	p		0.78

Statistical comparisons:

6.4.2.3 Effect of incubation with Indomethacin and L-NAME:

The addition of indomethacin and L-NAME to non-pregnant vessels caused a marked decrease in the endothelium dependent relaxation when compared to both control vessels and those incubated with indomethacin (Control *Vs* Indomethacin and L-NAME incubated vessels (P= 0.001, ANOVA) (E_{max}: P= 0.004; unpaired T-test). Indomethacin *Vs* Indomthacin and L-NAME incubated vessels (p= 0.001, ANOVA) (E_{max}: P= 0.001, unpaired T-test)). However, the addition of indomethacin and L-NAME to vessels from pregnant women did not significantly affect endothelium dependent relaxation. (Control Vs Indomethacin + L-NAME incubated vessels (p= 0.58, ANOVA): Indomethacin Vs Indomethacin + L-NAME incubated vessels (p= 0.96, ANOVA) (Fig 6.5)). Although women with pre-eclampsia displayed a reduced endothelium dependent relaxation, the addition of Indomethacin and L-NAME further attenuated the observed response when compared to both control vessels and those incubated with indomethacin. (Control *Vs* Indomethacin and L-NAME incubated vessels (P= 0.001, ANOVA) (E_{max}: P= 0.06; unpaired T-test). Indomethacin *Vs* Indomethacin and L-NAME incubated vessels (p= 0.006, ANOVA) (E_{max}: P= 0.012, unpaired T-test) (Fig 6.6)).

6.4.3 Histamine:

6.4.3.1 Comparison of endothelium dependent relaxation in non-pregnant and pregnant women and women with pre-eclampsia

There was a statistical difference in the dose response curves obtained between myometrial vessels from non-pregnant and pregnant women (Fig 6.7) (P = 0.001, ANOVA) (E_{max}: P= 0.001,

unpaired t-test). However, there were no differences in the endothelium-dependent relaxation from women with pre-eclampsia when compared to normal pregnant women (Fig 6.7) (P = 0.66, ANOVA).

6.4.2.2 Effect of incubation of vessels with Indomethacin:

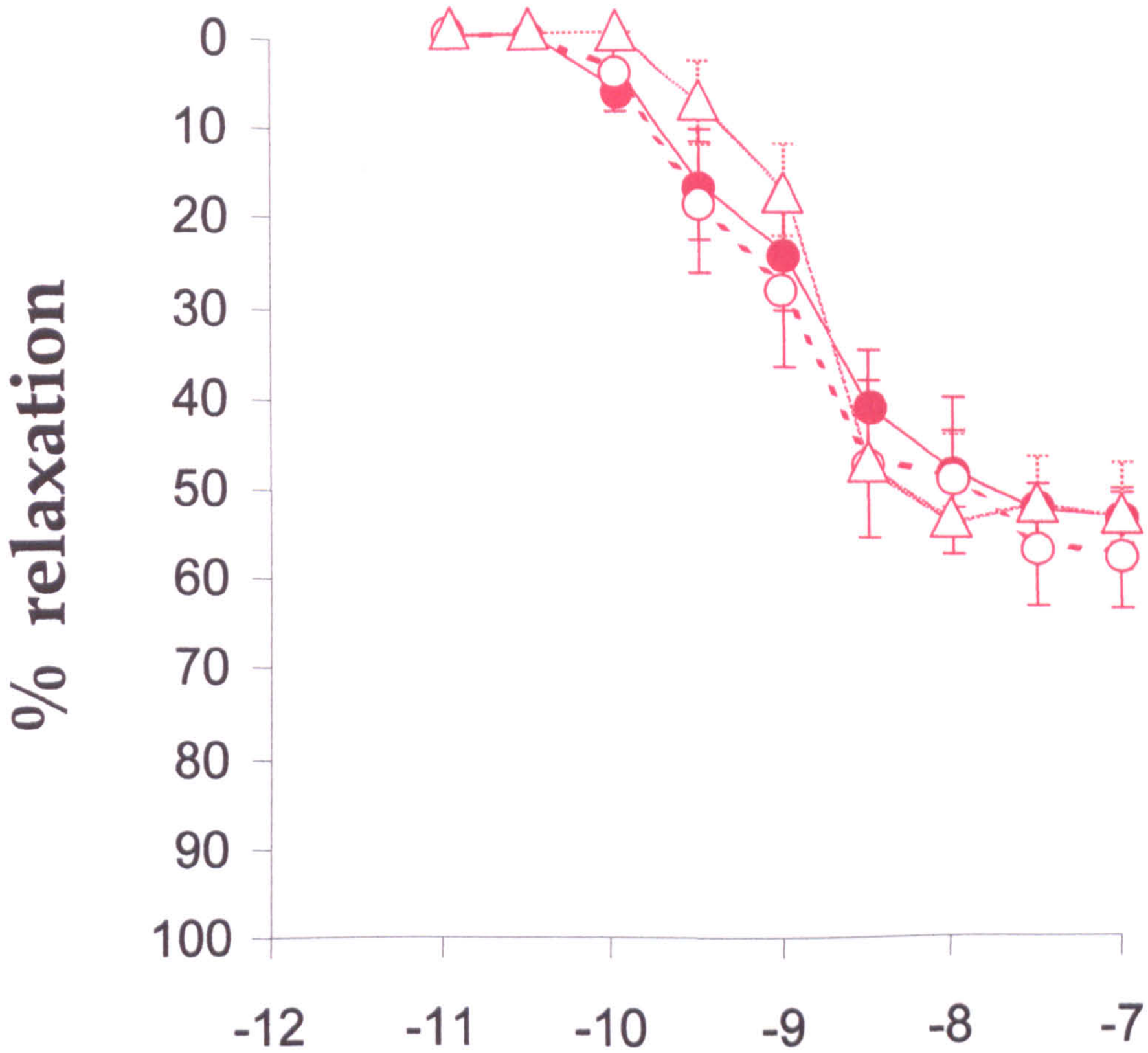
The addition of indomethacin had no significant effect on the relaxation curve to histamine in the non-pregnant (Fig 6.8) (P = 0.79, ANOVA) or pregnant women (Fig 6.9) (P = 0.99, ANOVA) or women with pre-eclampsia (Fig 6.10) (P = 0.91, ANOVA).

6.4.2.3 The effect of incubation with Indomethacin and L-NAME:

The addition of indomethacin and L-NAME had no effect on the endothelium dependent relaxation to histamine in the non-pregnant group of women when compared to both control vessels and those incubated with indomethacin alone (Control *Vs* Indomethacin + L-NAME incubated vessels (P = 0.79, ANOVA). Indomethacin *Vs* Indomethacin + L-NAME incubated vessels (P = 0.52, ANOVA) (E_{max}: P = 0.09; unpaired T-test) (Fig 6.10)). Likewise, the addition of indomethacin and L-NAME to pregnant vessels did not significantly effect the endothelium dependent relaxation when compared to both control vessels and those incubated with indomethacin (Control *Vs* Indomethacin + L-NAME incubated vessels (P= 0.57, ANOVA). Indomethacin *Vs* Indomethacin + L-NAME incubated vessels (P= 0.68, ANOVA) (Fig 6.9)). However, the addition of Indomethacin and L-NAME to vessels from women with pre-eclampsia caused a significant diminution of the endothelium dependent relaxation when compared to both control vessels and those incubated

Fig 6.9

The effect of indomethacin and indomethacin and L-NAME on the endothelium-dependent relaxation obtained from vessels from pregnant women



KEY:

Control

Indomethacin

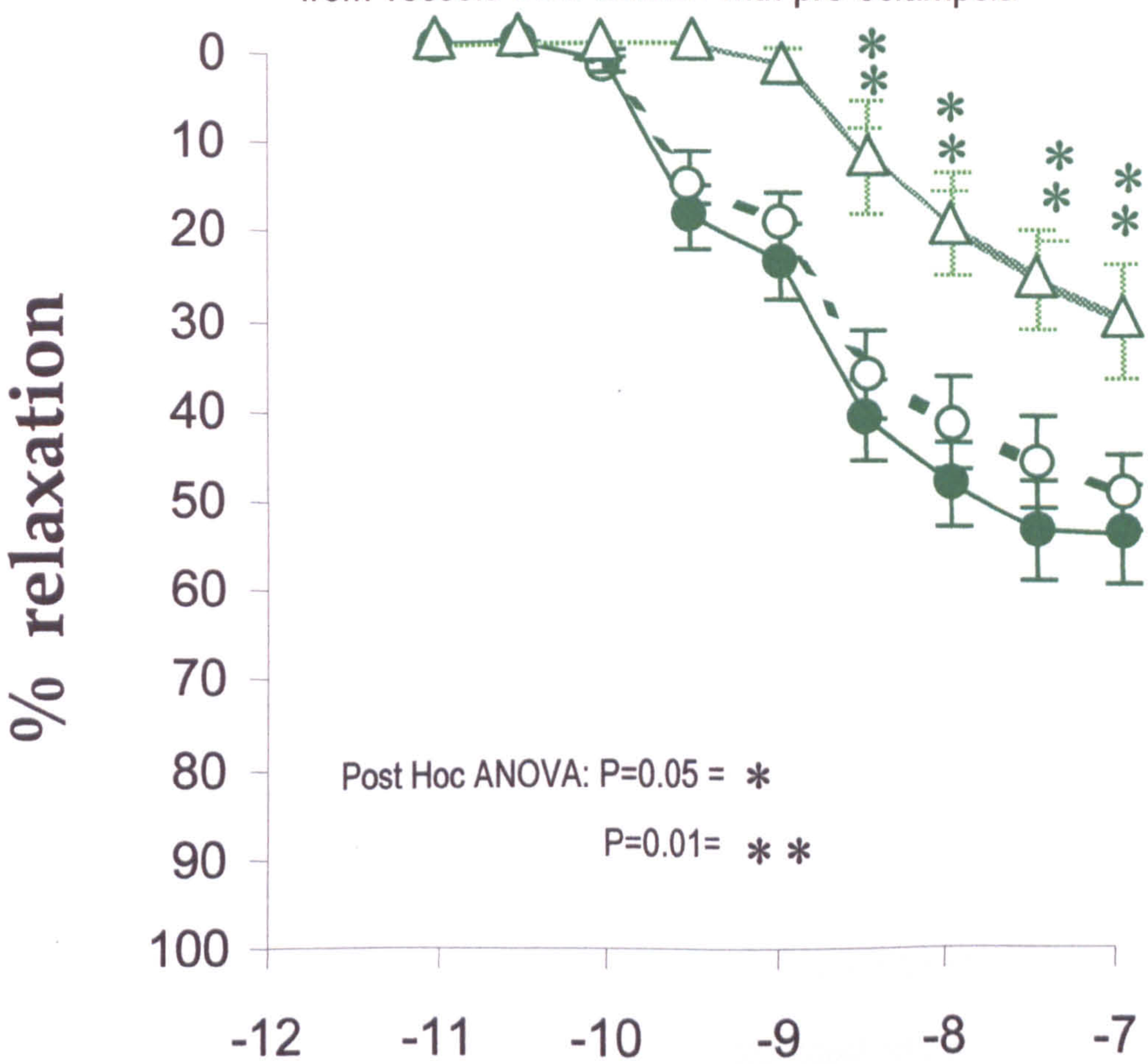
Indo + L-NAME

Statistical comparisons:

		E_{max}
		49.82+/-2.88
		53.44+/-6.00
		49.90+/-5.41
ANOVA (factorial)	p	0.85
ANOVA (repeated measures)	p	0.92

Fig 6.10

The effect of indomethacin and indomethacin and L-NAME on the endothelium-dependent relaxation obtained from vessels from women with pre-eclampsia



KEY:

- Control
- Indomethacin
- Indo + L-NAME

Statistical comparisons:

		E_{max}
		52.17+/-5.37
		47.80+/-11.91
		29.57+/-6.05
ANOVA (factorial)	p	0.021
ANOVA (repeated measures)	p	0.001

with indomethacin. Control *Vs* Indomethacin + L-NAME incubated vessels (p= 0.001, ANOVA) (E_{max}: P= 0.021; unpaired T-test). Indomethacin *Vs* Indomethacin + L-NAME incubated vessels (p= 0.002, ANOVA) (E_{max}: P= 0.022, unpaired T-test) (Fig 6.10).

6.4.3 Sodium Nitroprusside:

6.4.3.1 Comparison of non- endothelium dependent relaxation in pregnant and women with pre-eclampsia:

The formal comparison of non-endothelium dependent relaxation of vessels to sodium nitroprusside from the three study groups had been done prior to the author commencing this study; and as no significant differences were shown it was felt that this should not be repeated (Ashworth, 1998). However, comparison of non-endothelium dependent relaxation between vessels from pregnant women and women with pre-eclampsia was undertaken. This was used to demonstrate that vessels exhibiting loss of endothelium dependent relaxation were capable of smooth muscle relaxation. Comparison between these two study groups showed no significant differences (EC_{50} –6.3+/-0.3 *Vs* –6.6+/-0.4;P=0.62: E_{max} 82.14+/-5.02 *Vs* 83.33+/-2.27;P=0.85).

6.5 Discussion:

This chapter will focus on the vascular alterations that occur in the myometrial resistance vessels with pregnancy and pre-eclampsia. The differences between the myometrial and systemic circulation will be discussed in Chapter 10.

6.5.1:Vasoconstrictors:

This thesis confirmed previous studies that, in contrast to other arteries (Katusic et al., 1984) (Vanhoutte et al., 1984) (Evora et al., 1993), in which relaxant responses to AVP have been observed, AVP induces contraction of uterine resistance vessels (Svane et al., 1991) (Kostrzewska et al., 1997).

In this study myometrial vessels from non-pregnant women exposed to vasopressin showed significantly higher E_{max} values than normotensive pregnant controls, with no apparent shift in the EC_{50} values. This might have been postulated from the work of Gant et al (1973) which demonstrated the pressor effects of angiotensin II are decreased in pregnancy (Gant et al., 1973). As discussed previously pregnancy has also been shown to alter the vascular reactivity of guinea-pig uterine arteries to norepinephrine, phenylamine, angiotensin II, serotonin, and the thromboxane-memetic U46619 (Weiner et al., 1989b) (Weiner et al., 1991) (Weiner et al., 1992b) (Jovanovic et al., 1995c).

This study confirms in a human *in vitro* system, the importance of the endothelium in the regulation of pressor responses, as the removal of the endothelium from pregnant vessels returned their E_{max} value of constriction curves to vasopressin to a similar level to that of the non-pregnant state (Fig 6.3). Although, this thesis did not examine whether removal of the endothelium from non-pregnant vessels effects vasopressin responses, several previous studies have investigated this in both myometrial resistance vessels and human uterine arteries, and demonstrated that removal has no significant affect on the responses obtained (Jovanovic et al., 1995c) (Ashworth, 1998). It is also well documented that, in certain blood vessels, removal of the

endothelium can potentiate the responses of vascular smooth muscle to vasopressin (Katusic and Krstic, 1987) (Randall et al., 1988), as opposed to some other arteries which lack endothelial modulation (Katusic and Krstic, 1987) (Conde et al., 1991).

Steele et al (1993) also implicated the endothelium as pivotal to the vascular adaptations that occur in pregnancy. They demonstrated that, although there were no differences in the CRC for intact vessels, removal of the endothelium from myometrial vessels from pregnant women significantly enhanced the constriction responses to noradrenaline when compared with non-pregnant controls in a similar state, and postulated that the increased sensitivity is countered by an endothelium-derived relaxing factor (Steele et al., 1993). This literature and the data in this thesis would suggest that during pregnancy the endothelium undergoes various alterations that allow the modulation of smooth muscle. This effect may be hypothesised to allow increased control over the local blood flow to the feto-maternal unit.

Although removal of the endothelium from pregnant vessels enhanced constrictor responses, these effects were not mediated through either the endothelial nitric oxide or the eicosanoid systems, as inhibition of these systems had no effect on the responses obtained in endothelium intact vessels. This is in accord with the observations of Steele et al who also demonstrated that the observed differences between endothelial denuded and intact pregnant myometrial vessels were also independent of nitric oxide (Steele et al., 1993). These results imply that the observed decreased in systemic vascular resistance in pregnancy may be mediated at least in part by a non-nitric oxide, non-eicosanoid pathway and implicates a role for the putative Endothelial Derived Hypoerpolarisation Factor (EDHF) in both pregnancy and pre-eclampsia.

EDHF, although it remains to be formally identified, has recently been demonstrated to act through gap junctions (Kuhberger et al., 1994) (Griffith and Taylor, 1999) (Fukuta et al., 1999) (Edwards et al., 1999). It is well documented that gap-junction concentrations increase in the myometrium of pregnant women through increased oestrogen drive (Neulen and Breckwoldt, 1994). Moreover, it has been shown that non-pregnant myometrial vessels, which have been treated with oestradiol or diethylstilbestrol show reduced sensitivity to vasopressin (Kostrzewska et al., 1993). The effect of oestrogen may be postulated to be through alterations in the vascular gap-junction expression, which leads to an increase in the transport of EDHF, however, this remains to be established.

Although pregnancy caused myometrial vessels to display a decreased sensitivity to vasopressin; vessels from women with pre-eclampsia exhibited the reverse with responses shifted towards the non-pregnant state (Fig 6.1). These vessels were characterised by the enhancement of the E_{max} value without any significant change in the EC_{50}. This is in accord with the work of Allen et al (1989), who demonstrated increased contractile responses to vasopressin in similar vessels from women with pre-eclampsia when compared to normal controls (Allen et al., 1989). Allen et al (1989) also showed that there was an enhanced response to U46619 in vessels from women with pre-eclampsia. However, responses in myometrial resistance vessels from women with pre-eclampsia have been demonstrated to be blunted to endothelin (Wolff et al., 1996). This suggests that in pre-eclampsia, the responses of myometrial vessels to vasoconstrictor agents are similar to those of vessels from non-pregnant women. It could therefore be hypothesised that in pre-eclampsia either the endothelium fails to adapt to pregnancy or function returns toward the non-pregnant state. As such it would be of interest and importance to determine whether exposure to

VEGF enhances the responses to vasopressin from normal pregnant vessels, in a similar manner to vessels from women with pre-eclampsia.

6.5.2 Bradykinin:

In this study of myometrial resistance vessels there was no significant increase in endothelium-dependent relaxation to bradykinin when vessels from pregnant women were compared to non-pregnant controls (Fig 6.3). This confirms the findings of Ashworth et al (1999).

The addition of indomethacin to the vessels from non-pregnant, pregnant and women with pre-eclampsia did not significantly alter the concentration responses curves of these vessels to bradykinin. However, Ashworth et al demonstrated an increase in the relative production of vasoconstrictor to vasodilator prostanoids in vessels from women with pre-eclampsia, although they were unable to characterise the nature of the change (Ashworth, 1998) (Ashworth et al., 1999). In Chapter 3 of this thesis it was demonstrated that these results might be due to the protocol utilised by Ashworth et al (1998). The inhibition of eicosanoid synthesis with indomethacin does not only inhibit the vasodilator eicosanoids such as prostacyclin but also inhibits any vasoconstrictor eicosanoids originating from these blood vessels. Such eicosanoids could include thromboxane A_2 or prostaglandin E_1, both of which are produced by the endothelium (Wetzka et al., 1996). Therefore, it can not be simply concluded from this data that prostacyclin has no role in the relaxatory response of these vessels to bradykinin.

Indomethacin inhibits both the isoforms of cyclo-oxygenase, the rate-limiting enzyme responsible for the conversion of arachadonic acid into prostaglandins. It is possible that the use of this drug

masked subtle alterations in the balance of the eicosanoids within vessels. Recent studies have demonstrated that the inducible form, COX-2, is the major isoenzyme involved in the pathological situations (Lora et al., 1998), it is possible that experimentation with a specific COX-2 inhibitor would provide different results to those observed in this study. Such a methodological approach may help to unravel the complex balance that exists between the various eicosanoids in normal pregnancy.

When vessels from normal pregnant women were studied, the addition of both indomethacin and L-NAME had no significant effect on the concentration response curves to bradykinin. However, the addition of L-NAME and indomethacin to non-pregnant vessels significantly attenuated the vasodilator response. Although, the effect of L-NAME on its own was not studied (See Chapter 10), it can be concluded that myometrial resistance vessels undergo specific adaptations during pregnancy, which appear to be mediated through the endothelium. This adaptation appears to be mediated through a switch in the mechanism of vasodilatation from a predominantly nitric oxide system, to a non-nitric oxide, non-prostaglandin-mediated system. This adds further support to the hypothesis of an altered endothelial-derived factor being important in the normal pregnancy adaptation. The suggestion of adaptation of the resistance vessels of the myometrium to a non-nitric oxide, non-prostanoid mediated relaxation has recently been confirmed by Kenny et al (Kenny et al., 1999a). Moreover, Gerber et al have recently shown that EDHF has an enhanced role in the relaxation of mesenteric vessels from pregnant rats when compared to non-pregnant controls (Gerber et al., 1998).

Previous work on agonist induced vasodilatation in the same vascular bed has been unable to demonstrate similar differences (Ashworth et al., 1996a). There are two possible reasons for

these observed differences, Ashworth et al (1996) utilised L-NNMA as their nitric oxide inhibitor and vessels were stored for up to 48 hours prior to use. In pilot data (n=2) L-NNMA had no significant effect on endothelium-dependent relaxation in non-pregnant vessels. Therefore it may be postulated that the nitric oxide synthase inhibitor used by Ashworth et al (1996) was not effective in these vessels with this protocol.

The stimulus for the alteration in the vascular function during pregnancy remains to be described; however, possible candidates include the hormones; progesterone and oestrogen. Both hormones are known to have effects on the vascular endothelium and oestrogen has been shown to cause endothelial changes with respect to vasoconstrictor agents (Kostrzewska et al., 1993).

Endothelium dependent relaxation to bradykinin of myometrial resistance vessels from women with pre-eclampsia was attenuated in comparison to similar vessels from pregnant control women (Fig 6.3) and this confirms the observation of Ashworth et al (1997). Losses of endothelium dependent relaxation have also been demonstrated, with other vasodilators (acetylcholine, histamine, and bradykinin) in other vascular beds from women with pre-eclampsia, including omental vessels, and subcutaneous vessels (McCarthy et al., 1993a) (Oguogho et al., 1996) (Pascoal et al., 1998). However, this finding appears to be specific for bradykinin in myometrial vessels, as the degree of endothelium-dependent relaxation to histamine remained unaltered in pre-eclampsia (see section 4.2). The observed generalised loss of endothelium dependent relaxation would suggest that this process is not mediated through a loss of receptors, but through a more fundamental cellular transduction system.

The addition of indomethacin to vessels from women with pre-eclampsia had no significant effect on the relaxation to bradykinin. This differs from previous data, which has demonstrated that indomethacin caused enhancement of endothelium-dependent relaxation in vessels from women with pre-eclampsia (Ashworth et al., 1999). The differences between these observations have been discussed previously (see Page 191).

The addition of indomethacin and L-NAME attenuated the concentration response curves to bradykinin in the vessels from women with pre-eclampsia, which would suggest that this residual vasodilatory capacity was due to the synthesis of nitric oxide. This is in accord with the observations of Ashworth et al (1999) and Kenny et al (2000), who demonstrated a similar finding in myometrial vessels from women with pre-eclampsia.

Myometrial vessels, at present, appear to be unique in their responses to pregnancy and pre-eclampsia, when compared to other vascular beds. Unlike other vascular beds (McCarthy et al., 1993a) (Pascoal et al., 1998), myometrial vessels undergo adaptations to pregnancy mediated through the endothelium. Moreover, myometrial vessels from women with pre-eclampsia appear to exhibit two distinct responses, firstly a loss of endothelium-dependent relaxation and secondly a lack of adaptation of the mechanism of vasodilatation. Therefore, it could be hypothesised that these changes occur, either through a failure of adaptation with a superimposed loss of endothelium dependent relaxation, or that there is a loss of endothelium dependent relaxation consequent on the return of nitric oxide mediated relaxation. However, the hypothesis of a failure of adaptation is not supported by the data obtained for histamine concentration response curves, as this demonstrates that in vessels from women with pre-eclampsia the pregnancy specific adaptation is not lost (Fig.6.4). Furthermore, the loss of EDHF relaxation mediated through an

increase in nitric oxide is supported by the observation that there appears to be a reciprocal relationship between nitric oxide and EDHF (Bauersachs et al., 1996) (Bauersachs et al., 1997) (Randall and March, 1998).

In view of these observations it would be of interest to determine whether exposure to VEGF causes normal pregnant vessels to behave in a similar manner to vessels from women with pre-eclampsia when exposed to bradykinin, and this is addressed in Chapter 8.

6.5.3 Histamine

In the myometrial resistance vessels there was a significant decrease in the degree of endothelium-dependent relaxation to histamine from pregnant women when compared to non-pregnant controls (Fig 6.7). These differences did not appear to be due to the reduction of either eicosanoid or nitric oxide systems (Fig 6.8). Therefore, it might be postulated that these differences occurred due to either the reduction in the available histamine receptors, or a reduction in the efficacy of the second messenger system. It has been shown that H_1 receptor affinity increases in the myometrium of term pregnancies when compared to non-pregnant controls; this increased affinity occurs with a concomitant decrease in the number of receptors available for binding (Gonzalez et al., 1994).

However, histamine mediated endothelium dependent relaxation was not reduced in vessels from women with pre-eclampsia. It was also intriguing to note that the addition of indomethacin and L-NAME attenuated the relaxatory responses to both bradykinin and histamine in the vessels from women with pre-eclampsia when these were compared to control pregnant vessels (Fig 6.6 &

6.10). This would suggest that the residual vasodilatory capacity in vessels from women with pre-eclampsia is due to the increased synthesis of nitric oxide.

The role of endothelial-derived nitric oxide in pre-eclampsia is far from certain. Evidence for decreased production is weak (reviewed in Chapter one) and there is accumulating evidence that nitric oxide may actually be increased in this condition (Smarason et al., 1997). It has recently been proposed that increased nitric oxide in the presence of oxidative stress could lead to peroxynitrite formation and subsequent endothelial cell dysfunction (Roggensack et al., 1999).

Peroxynitrite is an oxidant formed by the reaction of nitric oxide and superoxide anions (produced under conditions of oxidative stress) (Radi et al., 1991). This reaction is approximately three times faster than the reaction rate of superoxide anions with superoxide dismutase, therefore there is a greater propensity for the reaction to favour peroxynitrite formation even if superoxide dismutase is present (McBride and Brown, 1997). The mechanism of peroxynitrite formation could either: reduce the availability of nitric oxide to act as a vasorelaxant, or result in peroxynitrite acting on the vasculature as a pro-oxidant

In addition, peroxynitrite reacts with tyrosine residues in proteins, a process that could affect tyrosine phosphorylation/dephosphorylation reactions involved in signal transduction (Darley-Usmar and Halliwell, 1996). The formation and action of peroxynitite can be detected by immunohistochemical localisation of nitrotyrosine residues in tissues. Moreover, increased nitrotyrosine immunostaining in the placental terminal and stem villous vessels of women with pregnancies affected by pre-eclampsia and/or IUGR as compared with normal pregnant controls has been described (Myatt et al., 1996). Peroxynitrite may have additional direct damaging

effects on cells. Peroxynitrite has been shown to mediate apoptosis, along with cell necrosis (Sandau et al., 1997). Changes in cell viability either by necrosis or apoptosis could then result in increased permeability of the endothelium and thus the loss of fluid from the intravascular space that is observed in some women with pre-eclampsia.

In an experimental model of oxidative stress, such as the cholesterol-fed rabbit, oxidative inactivation of nitric oxide contributes to the vascular pathology (Palombo et al., 1999) (Gerber et al., 1999). Whether peroxynitrite, and thus inactivation of nitric oxide, contributes to the maternal vascular pathologies of pre-eclampsia is not known. However, placental production of nitric oxide is increased in pregnancies complicated by pre-eclampsia. This has been shown by various method including indirect immunostaining of nitric oxide synthetase enzyme (Rutherford et al., 1995), and the direct measurement nitric oxide from umbilical arterial blood sampled after delivery (Lyall et al., 1996). These increases in nitric oxide synthesis have been shown to be through increased production of endothelial nitric oxide synthetase (Nasiell et al., 1998), and that increased production occurs in the terminal and stem villous vessels (Myatt et al., 1997).

Similarly, Roggensack et al have recently reported the presence of increased nitrotyrosine staining of the peripheral vascular endothelium of women with pre-eclampsia as compared with pregnant controls (Roggensack et al., 1999). In addition, they also found an increased level of eNOS together with decreased levels of superoxide dismutase in the vasculature, suggesting that peroxynitrite is involved in endothelial dysfunction in pre-eclampsia. Furthermore, during the time of this thesis frozen samples of myometrium from non-pregnant women, pregnant women, and women with pre-eclampsia were collected and transported on dry ice to the laboratory of Dr S Davidge (University of Alberta, Canada) where immunohistochemical staining was performed for

nitrotyrosine residues (Fig 6.11 & 6.12). This again shows that in women with pre-eclampsia there is increased staining for nitrotyrosine residues, further implicating nitric oxide in the pathophysiology of this disease.

6.6 Summary:

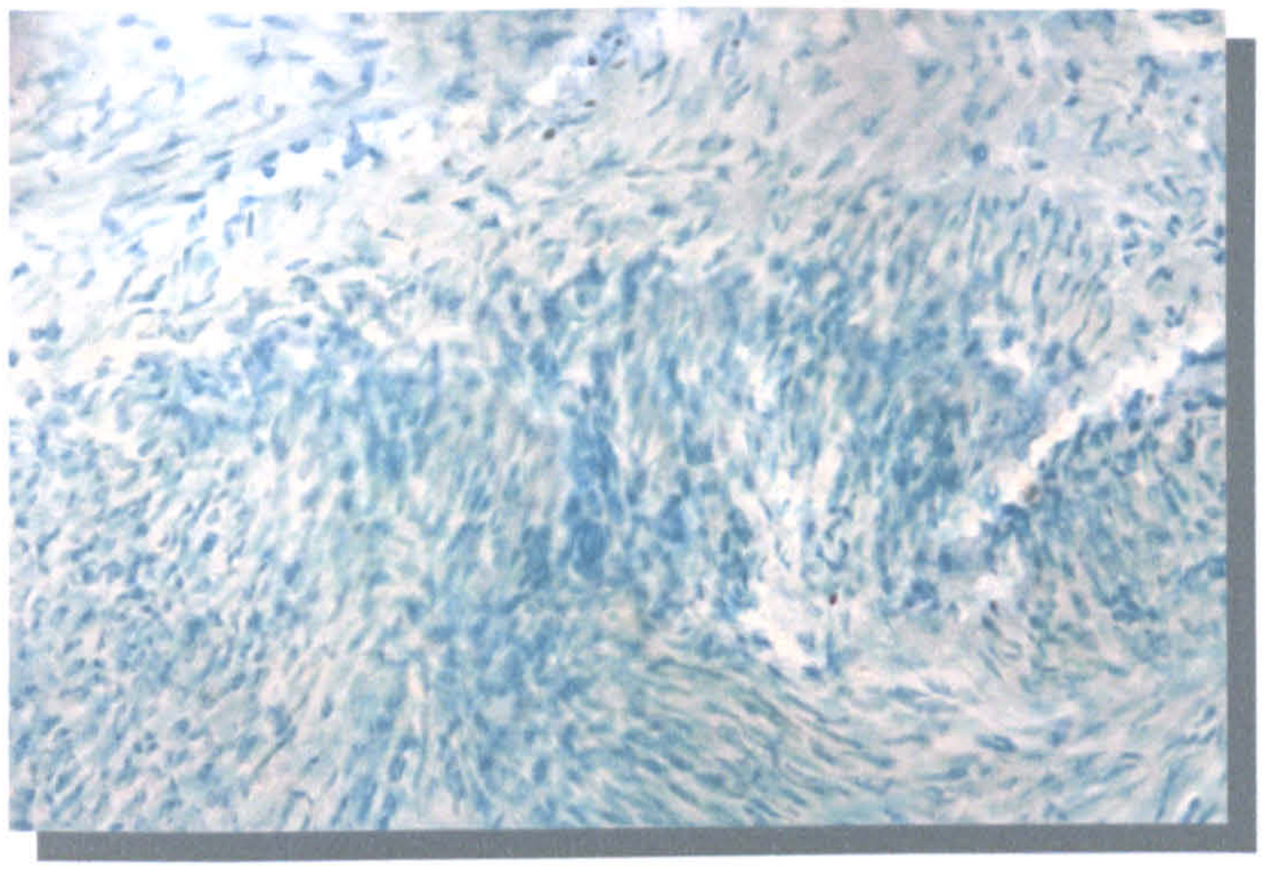
In this chapter the vasoconstrictor responses to vasopressin in non-pregnant, pregnant and vessels from women with pre-eclampsia have been characterised. It has been shown that in pregnant myometrial vessels there is blunted response to vasopressin and that in vessels from women with pre-eclampsia this adaptation either does not occur or is lost with the onset of disease. It also demonstrates that this effect is independent of both eicosanoids and nitric oxide, implicating indirectly a role of endothelial derived hyperpolarizing factor.

Data presented in this thesis clearly demonstrates that pregnancy and pre-eclampsia are associated with alterations in endothelial dependent relaxation. It also demonstrates that these changes may be mediated through the alteration in the reciprocal relationship between NO and EDHF.

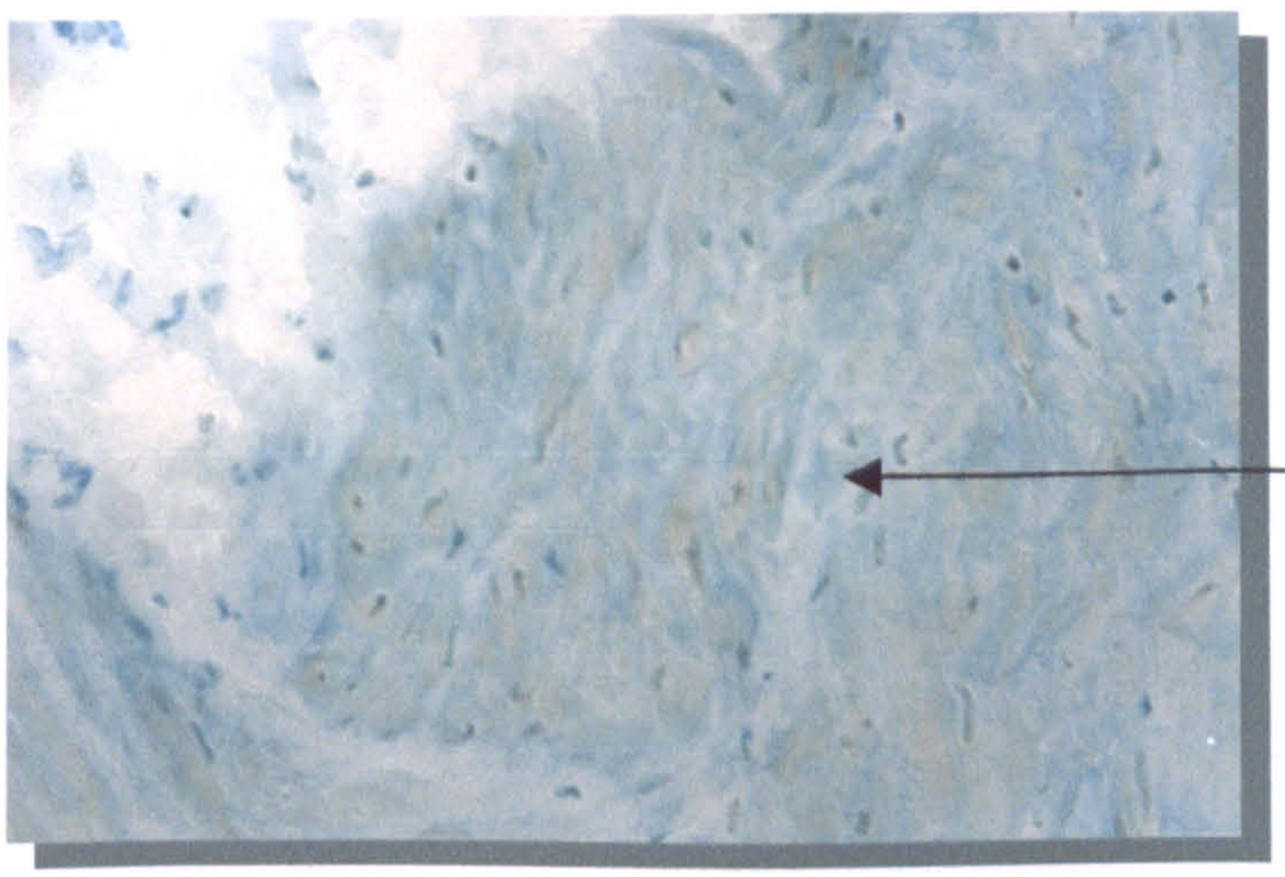
Data presented in this chapter also show that when the interaction of the endothelium with Vascular Endothelial Growth Factor and plasma (Chapter 8) are investigated, the effect that these have with the endothelium on the bradykinin response should be considered.

Immunohistochemical staining for nitrotyrosine residues in myometrium from non-pregnant, pregnant, and women with pre-eclampsia (x200 mag)

Fig 6.11

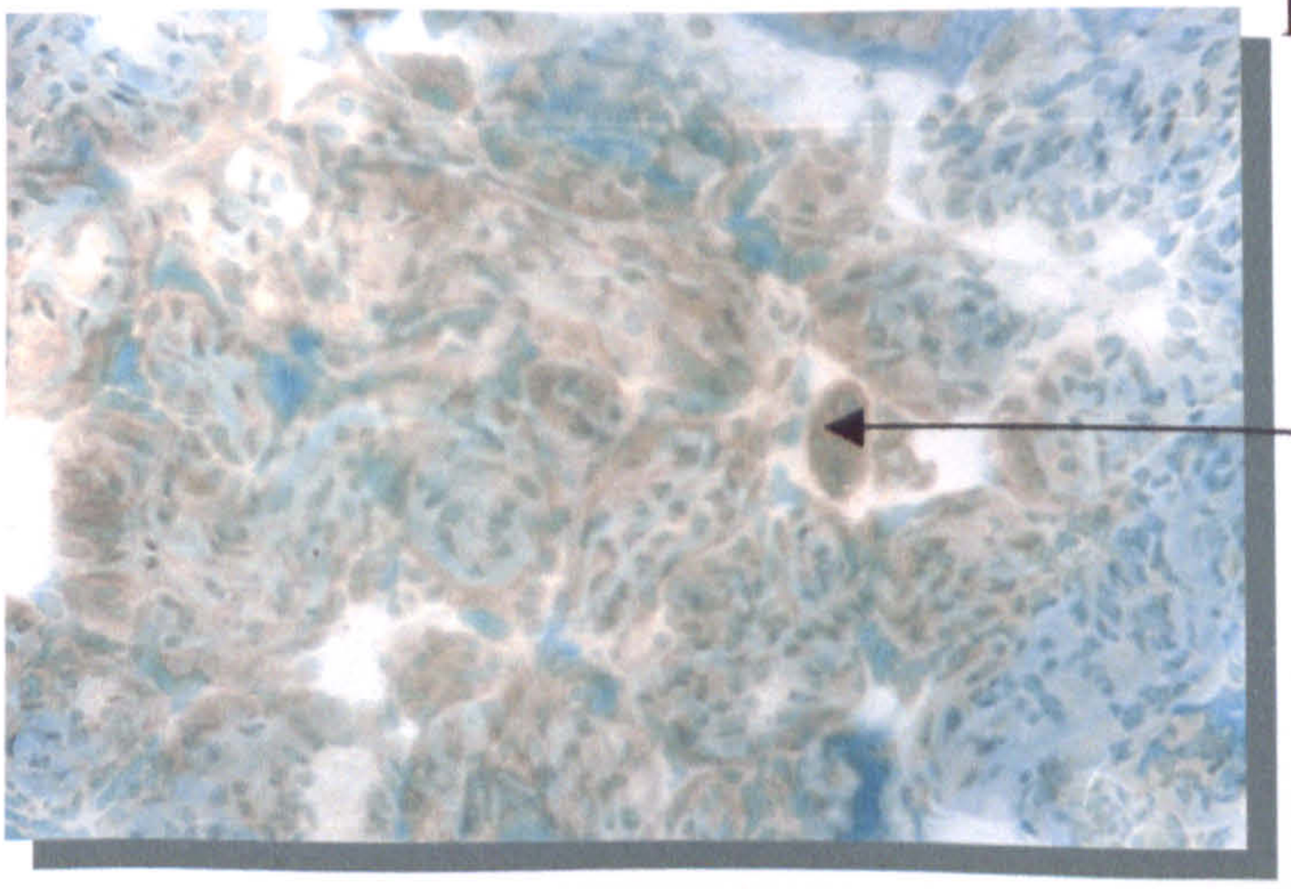


Non pregnant



Pregnant

Nitrotyrosine staining

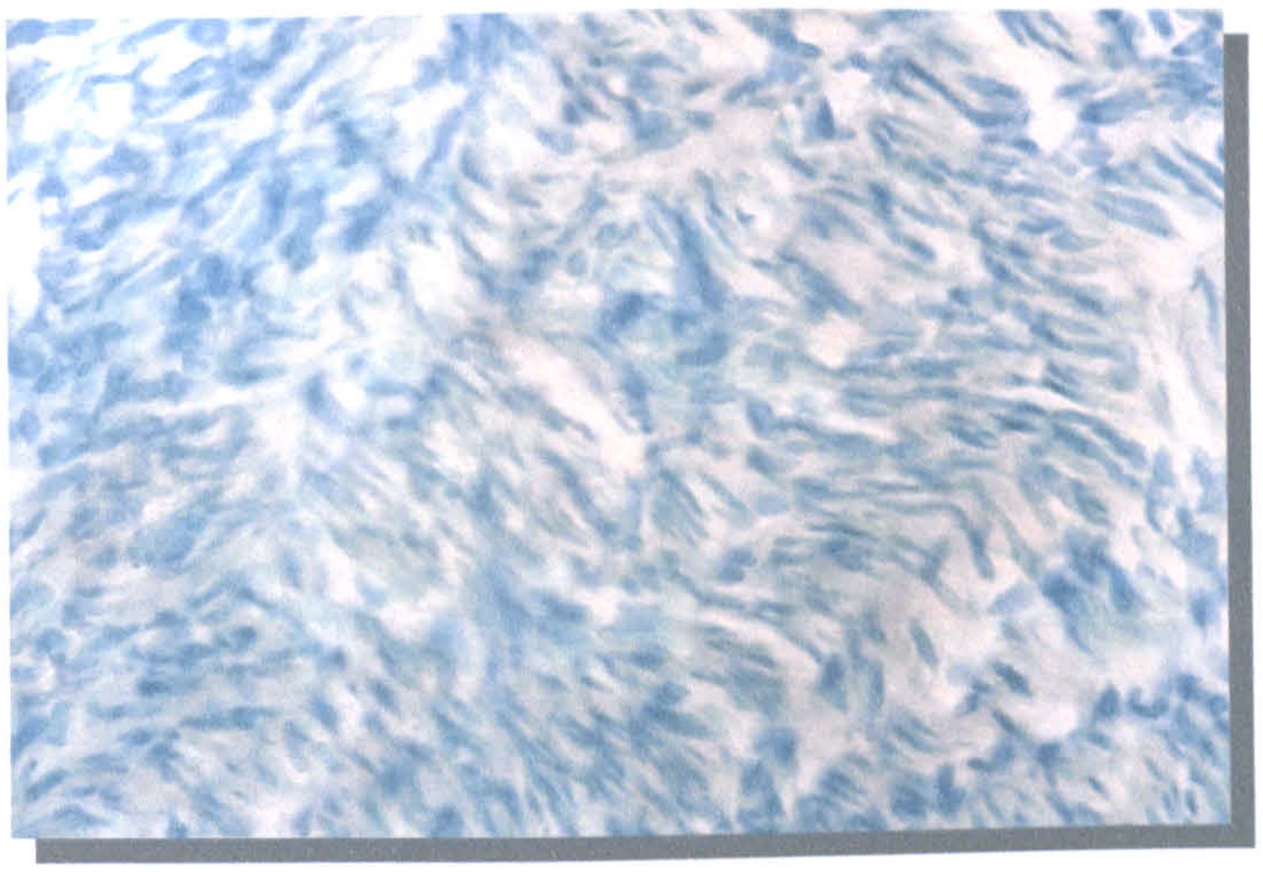


Pre-eclampsia

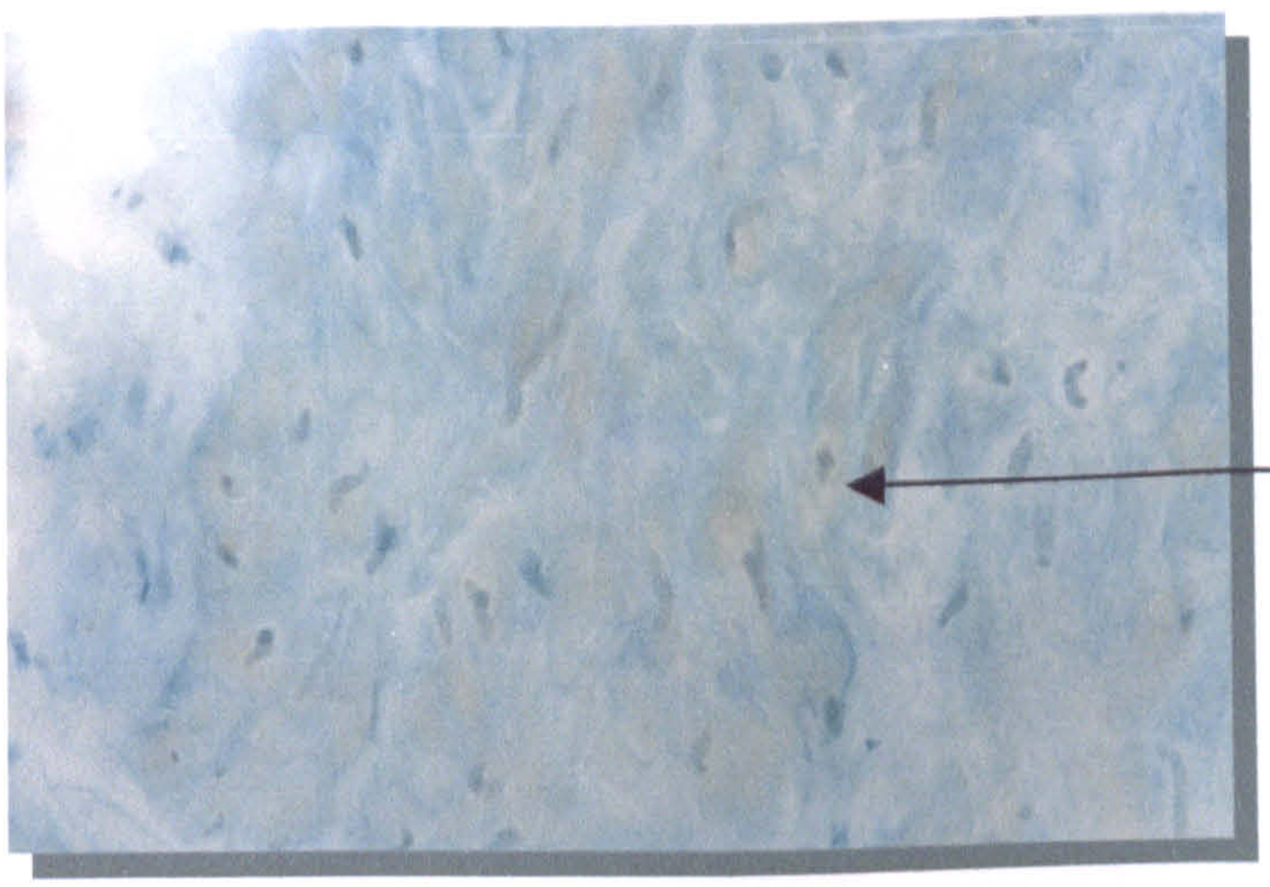
Nitrotyrosine staining

Immunohistochemical staining for nitrotyrosine residues in myometrium from non-pregnant, pregnant, and women with pre-eclampsia (x400 mag)

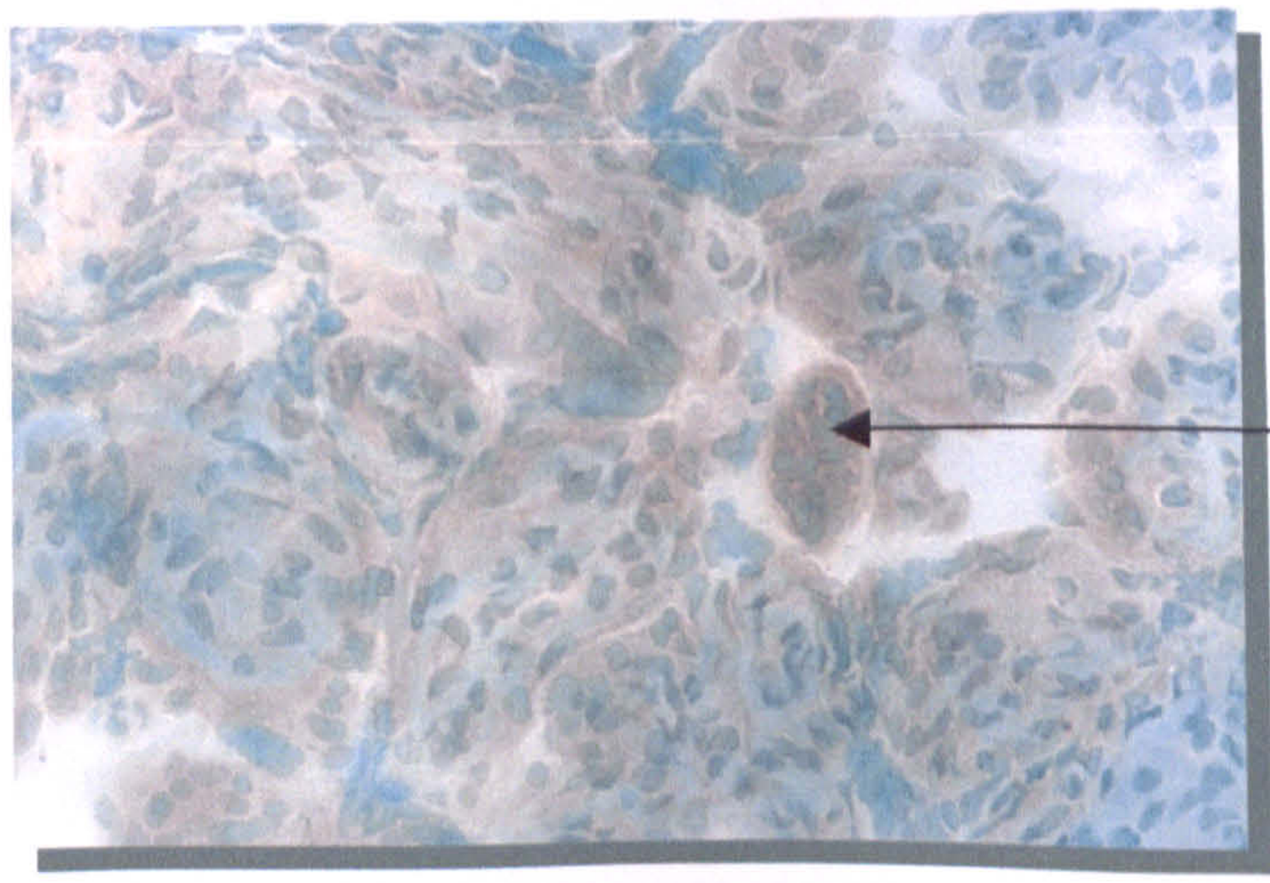
Fig 6.12



Non pregnant



Pregnant



Pre-eclampsia

Chapter Seven The vascular reactivity adaptations that occur in omental resistance vessels in normal pregnancy and pregnancies complicated by pre-eclampsia:

7.1 Introduction:

Several studies have demonstrated that systemic small vessel physiology is altered in pre-eclampsia. However, there is no consensus as to how these changes occur. Recent evidence may partially explain this with the suggestion that the vasoconstrictor used to induce vessel tone affects the mechanism of vasodilatation (Pascoal and Umans, 1996) (Wallerstedt and Bodelsson, 1997). It is also evident that the vascular bed studied alters the results obtained (Ashworth et al., 1996b). To date no study has established whether there are differences in the vasodilator responses in which the same agent induces tone in systemic vessels in normal pregnancy and pre-eclampsia.

7.2 Aims:

- To characterise the vasoconstrictor responses from omental resistance vessels from non-pregnant and pregnant women and women with pre-eclampsia.
- To characterise the mechanism by which the different vasodilators cause relaxation in human omental vessels.
- To examine whether normal pregnancy or pre-eclampsia alters endothelium dependent relaxation in the systemic circulation.
- To elucidate which of these changes should be studied when considering the interaction of the endothelium with Vascular Endothelial Growth Factor and plasma.

7.3 Experimental design:

7.3.1 Bradykinin:

Omental resistance vessels were obtained from non-pregnant (n=6) and pregnant women (n=10) and women with pre-eclampsia (n=6) as described previously in Chapter 2.5.2 b (Table 7.1). When ever possible three vessels from the same patient were mounted on separate myographs. Vessels were normalised as described in Chapter 2.5.4. The myography protocol (Chapter 3.3.1) was then followed and incremental doses of bradykinin were used to cause endothelium dependent relaxation (Chapter 2.5.5.5). In a separate set of experiments omental vessels were obtained from pregnant women (n=4). Four vessels were mounted on separate myographs, one of these vessels acted as both a time and internal control, while the other three were incubated with varying doses of L-NAME ($1x10^{-4}$ M, $3x10^{-4}$ M, and $1x10^{-3}$ M).

7.3.2 Acetylcholine:

Omental resistance vessels were obtained from non-pregnant (n=4) and pregnant women (n=7), and women with pre-eclampsia (n=6) (Table 7.2), and mounted as described previously. The myograph protocol (Chapter 3.3.1) was then followed and incremental doses of acetylcholine were used to cause an endothelium dependent relaxation (Chapter 2.5.5.6).

Patient demographic details for vessels from non-pregnant women, control women and women with pre-eclampsia Table 7.1

Bradykinin	Age (years)	BMI (kg/m^2)	Parity	Gestation at delivery (days)	Mean Arterial Pressure Booking mm Hg	Mean Arterial Pressure Maximum mm Hg	Protein g/dl	Individualised Birth-weight Ratio
Non - pregnant	37.0 (33.0 - 42.0)	24.6 (23.5 - 25.9)	2 (1.3- 2.8)		81.7 (77.5 - 85.8)			
Normal pregnant	31 (27.5 - 36.5)	28.3 (24.2 - 31.1)	1 (0.8 - 1.3)	271.5 (269.5 - 273.8)	83.1 (76.7 - 83.8)	89.5 (76.7 - 94.2)	0	84.5 (66.8 - 91.3)
Pregnant women with pre-eclampsia	27.5 (24.5- 32.5)	26.7 (22.1 - 32.5)	0 (0 - 0.8)	235 (219 - 246.8)	85.3 (83.5 - 87.6)	114.0 (110.3 - 130.4)	1.5 (0.6 - 3.0)	18 (2.5 - 20)

Data are summarised as medians (Inter Quartile Ranges)

Patient demographic details for vessels from non-pregnant women, control women and women with pre-eclampsia Table 7.2

Acetylcholine	Age (years)	BMI (kg/m ^{2})	Parity	Gestation at delivery (days)	Mean Arterial Pressure Booking mm Hg	Mean Arterial Pressure Maximum mm Hg	Protein g/dl	Individualised Birth-weight Ratio
Non - pregnant	32.5 (31.8 - 35.8)	23.5 (23.2 - 24.8)	2 (1.5 - 2.3)		88.3 (83.3 - 91.6)			
Normal pregnant	33 (27 - 34)	26.0 (25.9 - 28.7)	1 (1 - 1)	270 (266.0 - 270.0)	83.3 (80.6 - 84.3)	90.0 (85.3 - 90.0)	0	57 (16 - 62)
Pregnant women with pre-eclampsia	28.0 (23.0 - 28.0)	25.2 (24.9 - 28.2)	0.0 (0.0 - 0.0)	248 (216.0 - 252.0)	80.0 (76.0 - 86.7)	116.7 (116.7 - 123.3)	1.52 (0.86 - 1.55)	4 (0 - 18)

Data are summarised as medians (Inter Quartile Ranges)

7.4 Results:

7.4.1 Vasopressin

7.4.1 Vasopressin: Comparison non-pregnant, pregnant and women with pre-eclampsia

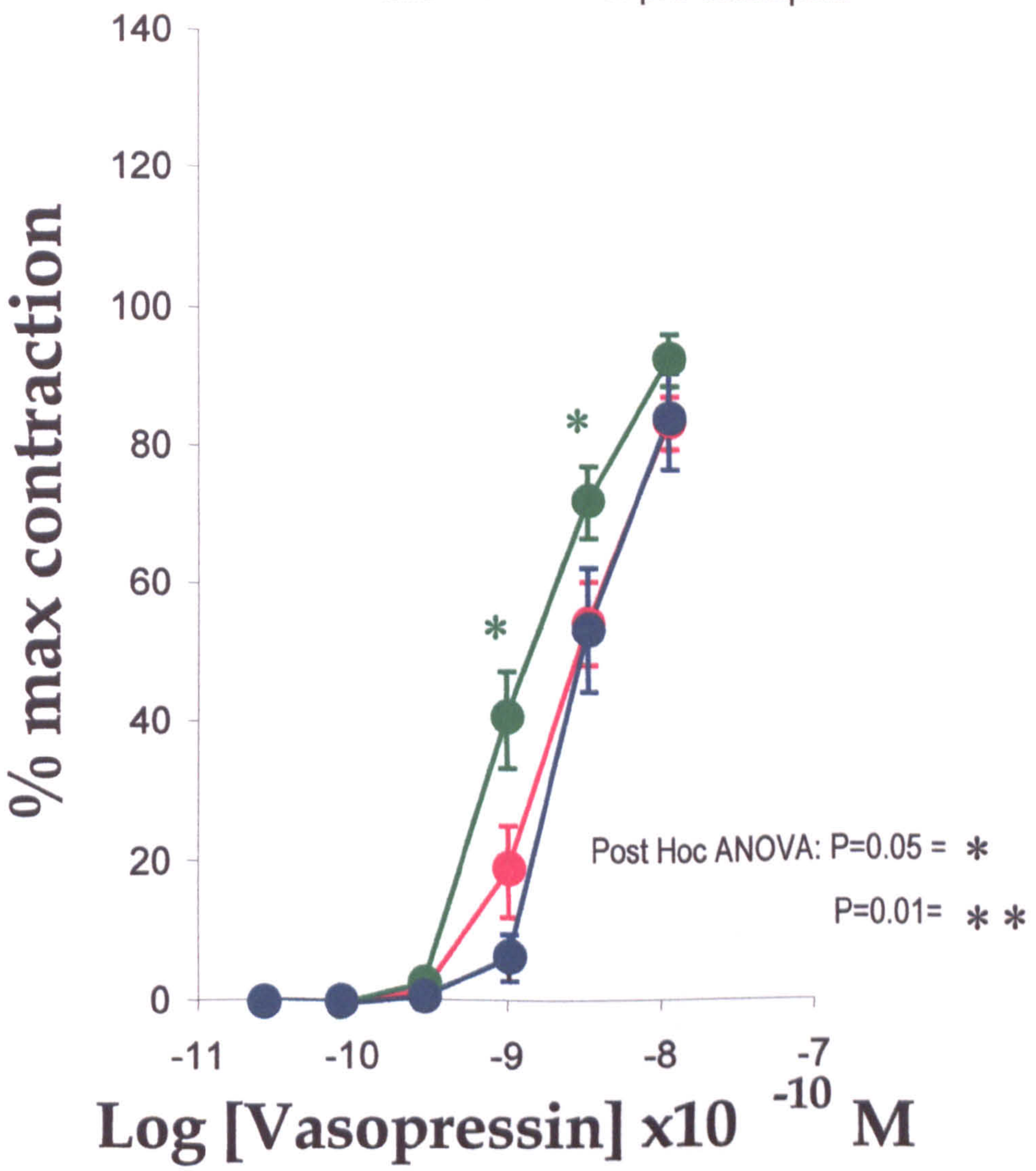
Analysis of the vasopressin dose response curves obtained from non-pregnant, pregnant and women with pre-eclampsia revealed a significant difference (P=0.03; repeated measure ANOVA) (Fig 7.1). ANOVA Post hoc testing demonstrated that there was a significant difference between vessels from normal pregnant women and women with pre-eclampsia (P=0.001) and between vessels from non-pregnant women and women with pre-eclampsia (P=0.003), however there was no difference between non-pregnant women and pregnant women. Further analysis of the EC_{50} values found significant differences (non-pregnant (–8.5+/-0.1) *Vs* pregnant (–8.5+/-0.1) *Vs* pre-eclampsia (-8.8+/-0.1); P=0.004), while no differences in the E_{max} were noted (non-pregnant (80.65+/-6.55) *Vs* pregnant (80.41+/-3.76) *Vs* pre-eclampsia (89.43+/-3.81); P=0.30). Post hoc analysis of the EC_{50} values demonstrated differences between the normal pregnant and pre-eclampsia groups (P=0.009) and the non-pregnant and pre-eclampsia group (P=0.011).

7.4.1.2 Effect of incubating with Indomethacin and indomethacin/L-NAME:

The addition of indomethacin to the vessels had no significant effect on the concentration response curves for the normal pregnant women and the women with pre-eclampsia (Figs 7.3

Fig 7.1

Comparison of contraction response curves for vasopressin in omental vessels from non-pregnant and pregnant women, and women with pre-eclampsia



KEY:

Non-pregnant

Pregnant

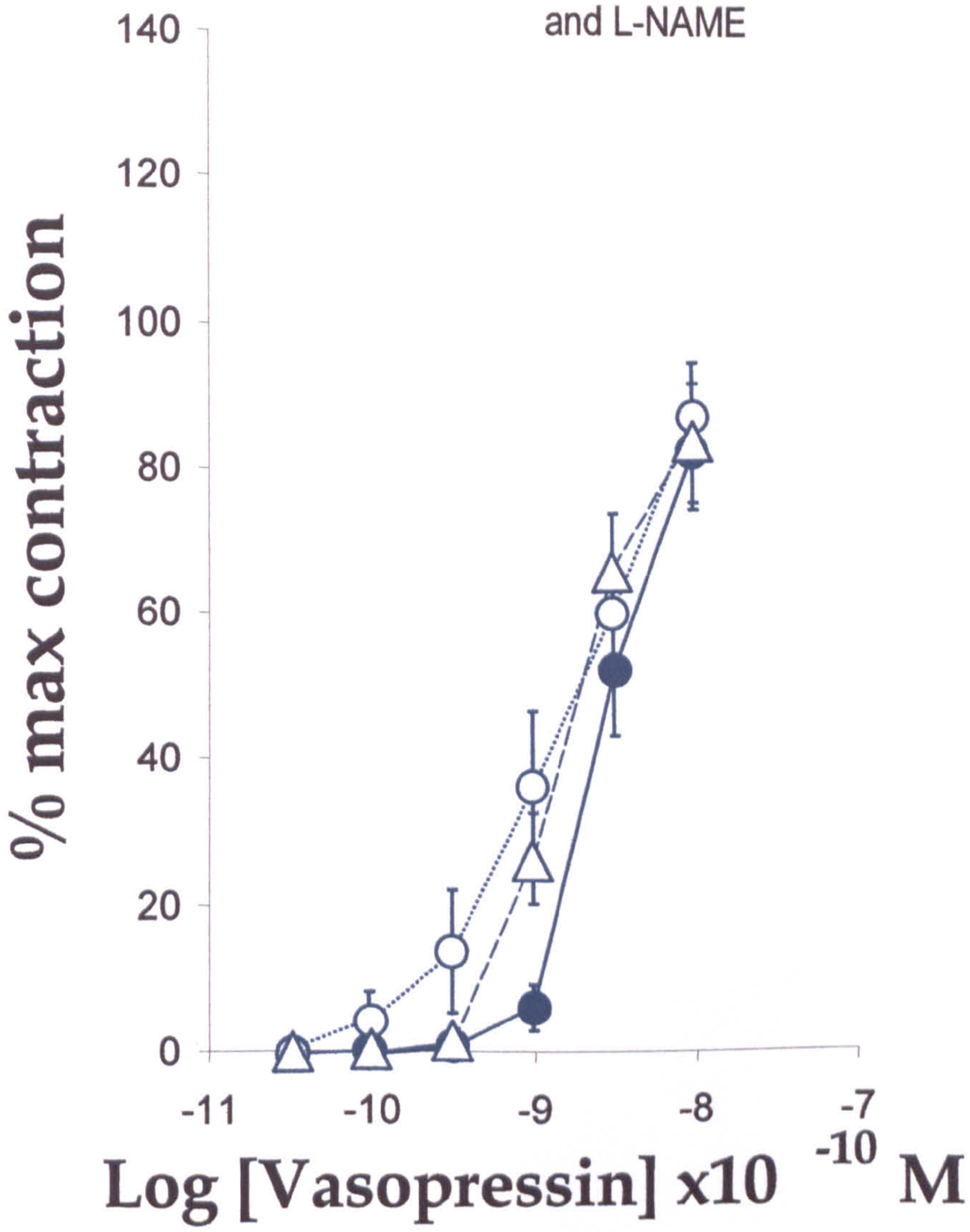
Pre-eclampsia

Statistical comparisons:

		EC_{50}	E_{max}
		-8.5+/-0.1	80.85+/-6.55
		-8.5+/-0.1	80.41+/-3.76
		-8.8+/-0.1	89.43+/-3.81
ANOVA (factorial)	p	0.004	0.30
ANOVA (repeated measures)	p	0.0004	

Fig 7.2

Comparison of CRC for vasopressin in non-pregnant omental vessels in the presence of indomethacin and indomethacin and L-NAME



KEY:

Control

Indomethacin

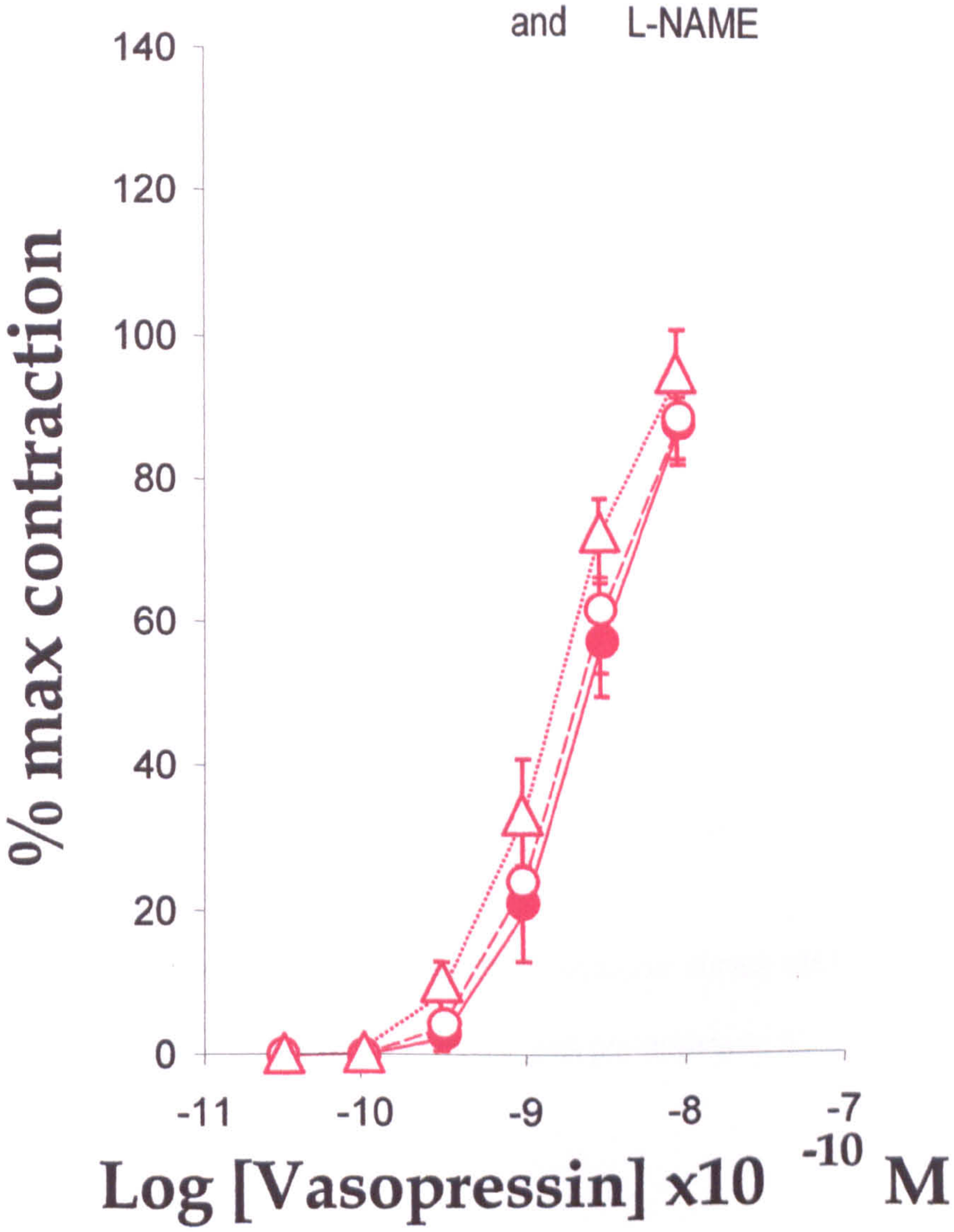
Indo + L-NAME

Statistical comparisons:

		EC_{50}	E_{max}
		-8.5+/-0.1	80.85+/-6.55
		-8.8+/-0.2	85.38+/-4.55
		-8.6+/-0.2	83.23+/-9.91
ANOVA (factorial)	p	0.34	0.87
ANOVA (repeated measures)	p	0.09	

Fig 7.3

Comparison of CRC for vasopressin for pregnant omental vessels in the presence of indomethacin and indomethacin and L-NAME



KEY:

Control

Indomethacin

Indo + L-NAME

Statistical comparisons:

		EC_{50}	E_{max}
		-8.6+/-0.1	83.93+/-3.76
		-8.7+/-0.1	81.26+/-3.97
		-8.8+/-0.1	87.40+/-6.84
ANOVA (factorial)	p	0.18	0.57
ANOVA (repeated measures)	p	0.85	

& 7.4). However, its addition to vessels from non-pregnant women caused a leftward shift in the response curves (ANOVA; P=0.09), without an alteration the EC_{50} or the E_{max} values (Fig 7.2).

The addition of indomethacin and L-NAME significantly effected the CRC of vessels from women with pre-eclampsia, shifting the EC_{50} (-8.7+/-0.2 *Vs* –9.1 +/-0.1;P=0.019) to the left without altering the E_{max} (91.52+/-3.93 Vs 97.28+/-5.43;P=0.4) (Fig 7.4). Although the addition of Indomethacin/L-NAME affected the pre-eclampsia group it had no significant effect on the other two groups (see Figs 7.2 & 7.3).

7.4.1 Bradykinin:

7.4.1.1 Comparison of endothelium dependent relaxation from vessels from non-pregnant and pregnant women and women with pre-eclampsia:

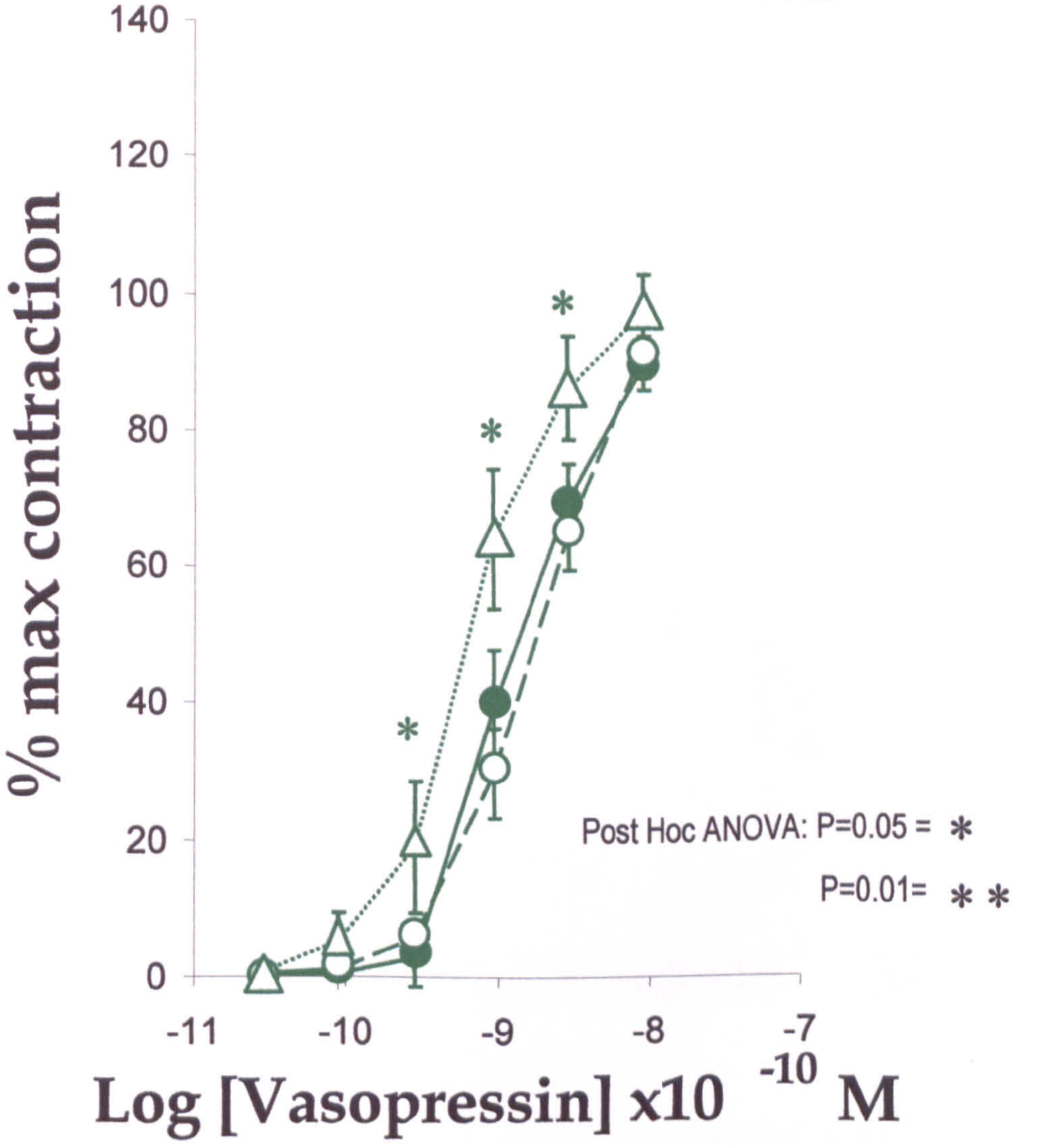
There was no statistical difference in the dose response curves obtained between omental vessels from non-pregnant, pregnant and women with pre-eclampsia (P=0.96) (Fig 7.5).

7.4.1.2 Effect of incubating with indomethacin:

The addition of indomethacin had no significant effect on the relaxation to bradykinin in the non-pregnant group (Fig 7.6) (p = 0.98, ANOVA), the pregnant group (Fig 7.7) (p = 0.71, ANOVA), the pre-eclampsia group (Fig 7.8) (p = 0.48, ANOVA).

Fig 7.4

Comparison of CRC for vasopressin for omental vessels from women with pre-eclampsia in the presence of indomethacin and indomethacin and L-NAME



KEY:

Control

Indomethacin

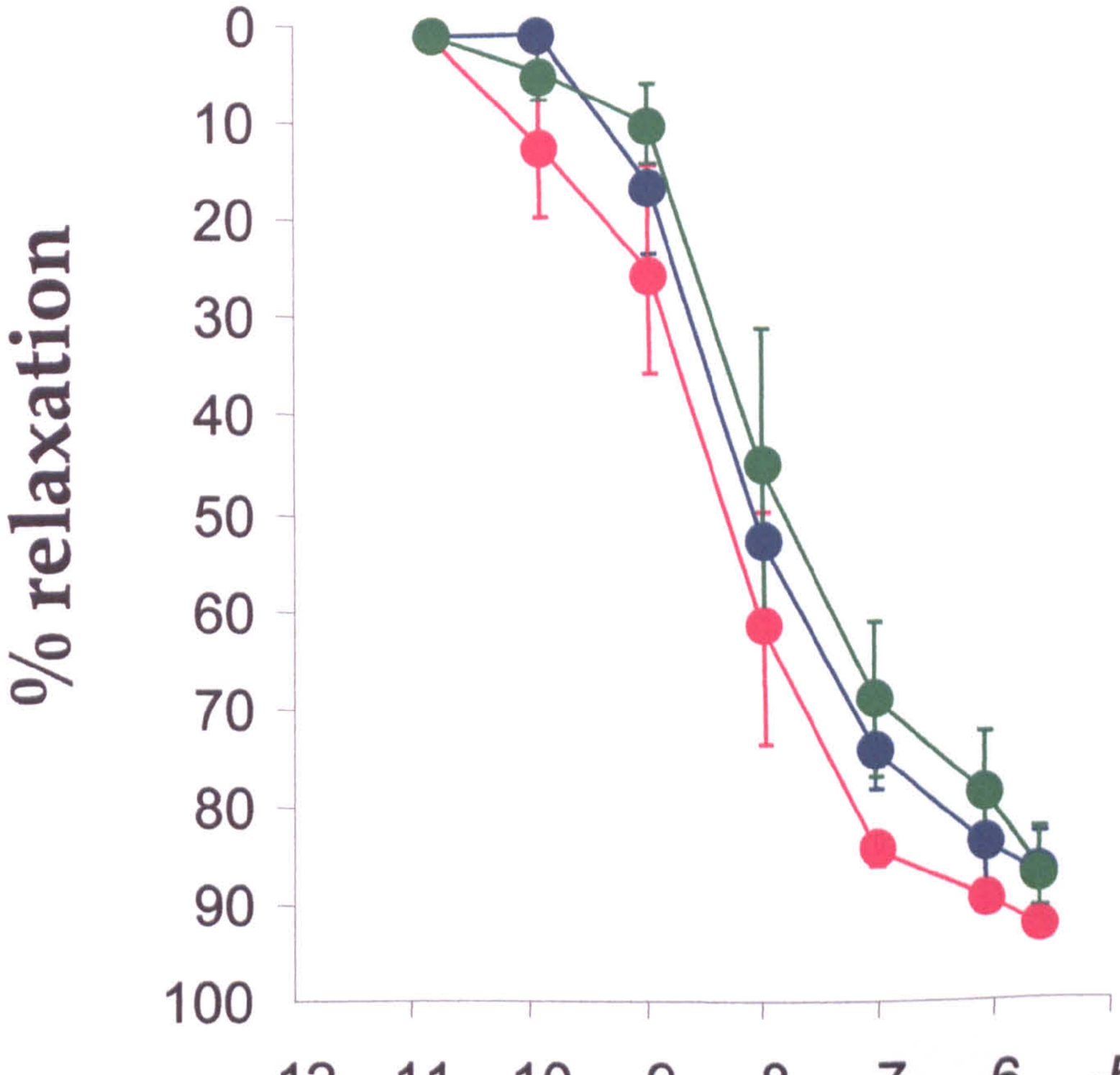
Indo + L-NAME

Statistical comparisons:

		EC_{50}	E_{max}
		-8.8+/-0.1	89.43+/-3.33
		-8.7+/-0.2	91.52+/-3.93
		-9.1+/-0.1	97.28+/-5.43
ANOVA (factorial)	p	0.049	0.46
ANOVA (repeated measures)	p	0.019	

Fig 7.5

Comparison of CRC for bradykinin in omental vessels from non-pregnant women,and pregnant women and women with pre-eclampsia



KEY:

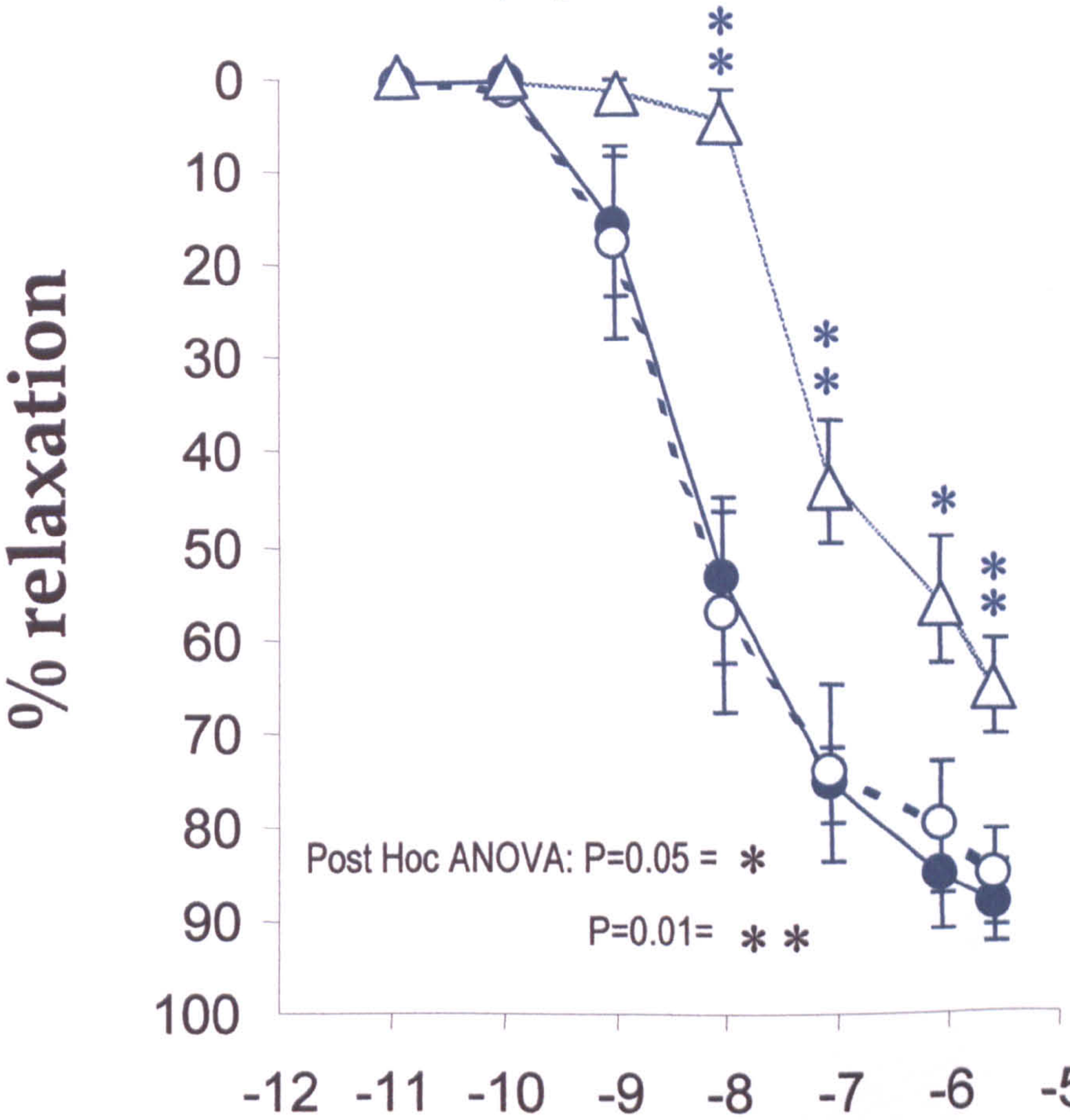
- Non-pregnant
- Pregnant
- Pre-eclampsia

Statistical comparisons:

		EC_{50}	E_{max}
		-8.1+/-0.2	88.24+/-4.29
		-8.5+/-0.3	93.79+/-1.39
		-7.7+/-0.3	87.73+/-4.16
ANOVA (factorial)	p	0.20	0.36
ANOVA (repeated measures)	p	0.96	

Fig 7.6

The effect of indomethacin alone and indomethacin and L-NAME on the endothelium-dependent relaxation of non-pregnant omental vessels



KEY:

Control

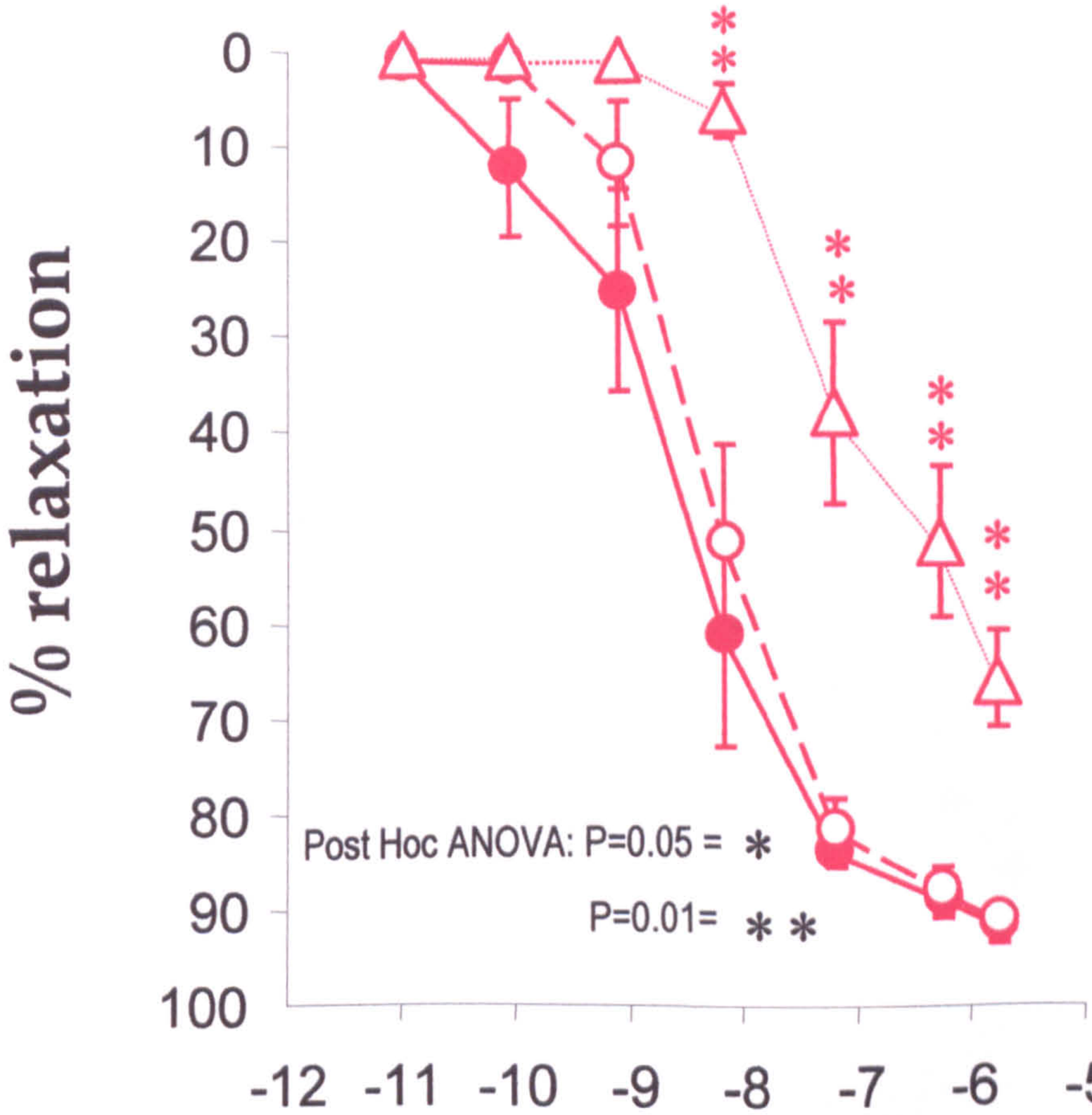
Indomethacin

Indo + L-NAME

Statistical comparisons:		EC_{50}	E_{max}
		-7.9+/-0.3	88.24+/-4.29
		-8.0+/-0.5	85.32+/-5.07
		-6.5+/-0.3	65.13+/-5.09
ANOVA (factorial)	p	0.01	0.008
ANOVA (repeated measures)	p	0.001	

Fig 7.7

The effect of indomethacin alone and indomethacin and L-NAME on the endothelium-dependent relaxation of pregnant omental vessels



KEY:

Control

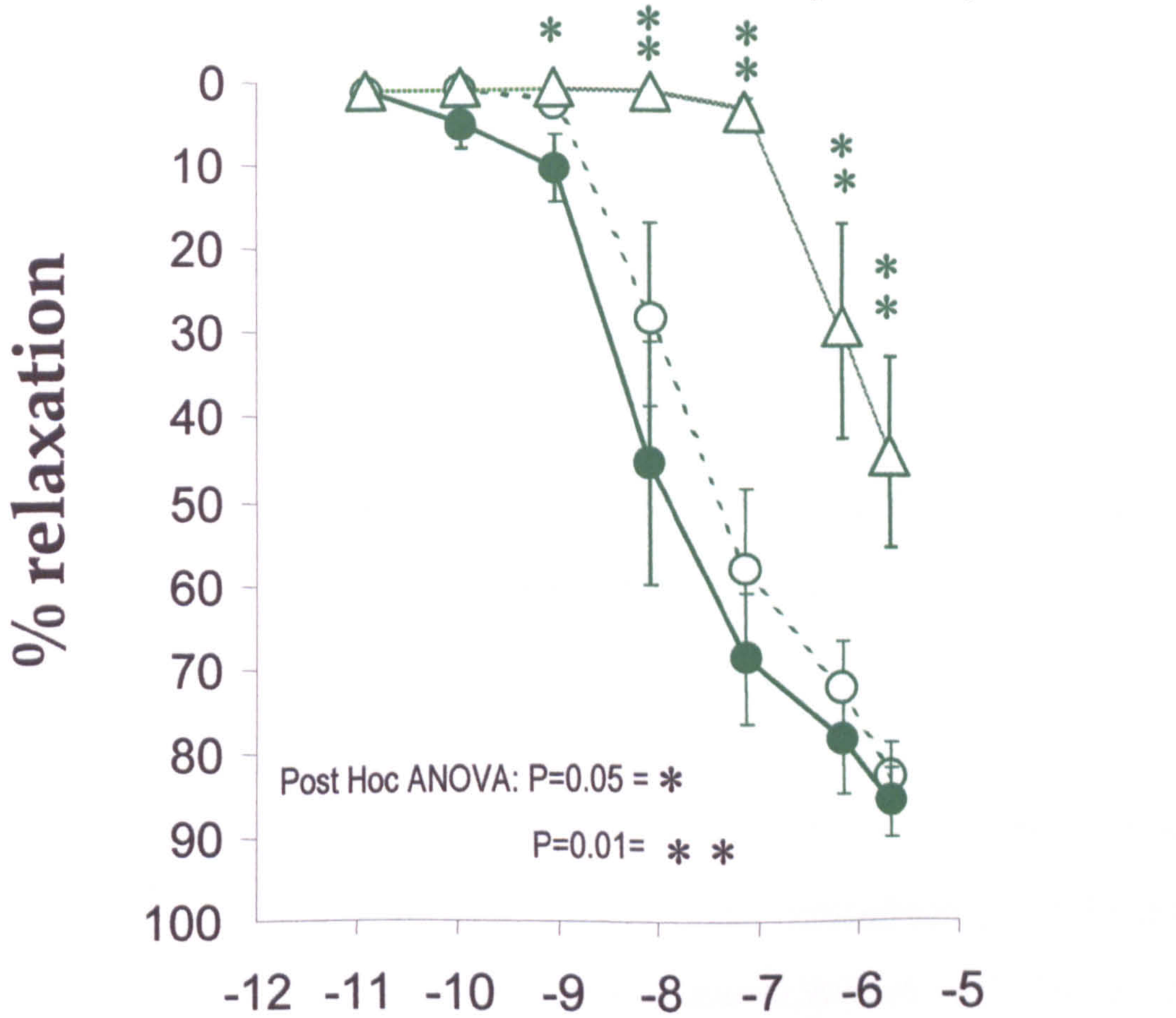
Indomethacin

Indo + L-NAME

Statistical comparisons:		EC_{50}	E_{max}
		-8.6+/-0.1	93.79+/-1.39
		-8.7+/-0.1	93.36+/-2.08
		-8.8+/-0.1	67.13+/-5.27
ANOVA (factorial)	p	0.004	0.001
ANOVA (repeated measures)	p	0.001	

Fig 7.8

The effect indomethacin alone and indomethacin and L-NAME on the endothelium-dependent relaxation on omental vessels from women with pre-eclampsia



KEY:

Control

Indomethacin

Indo + L-NAME

Statistical comparisons:		EC_{50}	E_{max}
		-7.7+/-0.3	87.73+/-4.16
		-7.3+/-0.4	84.39+/-4.18
		-5.8+/-0.4	55.29+/-11.6
ANOVA (factorial)	p	0.008	0.001
ANOVA (repeated measures)	p	0.002	

7.4.1.3.Effect of incubating with indomethacin and L-NAME:

The addition of L-NAME and indomethacin caused a marked decrease in the endothelium dependent relaxation in all three groups studied, when compared to both control vessels and those incubated with indomethacin.

Non-pregnant: Control *Vs* L-NAME and indomethacin incubated vessels (p= 0.008, ANOVA) (E_{max} 88.24+/- 4.29 Vs 65.13+/- 5.09: p= 0.006; unpaired T-test). Indomethacin *Vs* L-NAME and indomethacin incubated vessels (p= 0.009, ANOVA) (E max 85.32+/-5.07 Vs 65.13+/-5.09: p= 0.02, unpaired T-test).

Pregnant: Control *Vs* L-NAME and indomethacin incubated vessels (p= 0.001, ANOVA) (E_{max} .93.79+/- 1.39 *Vs* 67.13+/- 5.21: p= 0.009; unpaired T-test). Indomethacin *Vs* L-NAME and indomethacin incubated vessels (p= 0.001, ANOVA) (E max 93.36+/-2.08 *Vs* 67.13+/-5.27: p= 0.02, unpaired T-test).

Pre-eclampsia: Control *Vs* L-NAME and indomethacin incubated vessels (p= 0.001, ANOVA) (E_{max} 29.01+/- 6.67 Vs 10.05+/- 4.30: p= 0.06; unpaired T-test). Indomethacin *Vs* L-NAME and indomethacin incubated vessels (p= 0.006, ANOVA) (E_{max} 37.51+/-11.57 *Vs* 10.05+/-4.30: p= 0.012, unpaired T-test).

7.4.1.4 The dose response of vessels from pregnant women to L-NAME in the presence of indomethacin:

All three concentrations of L-NAME utilised caused a significant reduction in the endothelium dependent relaxation to bradykinin (Fig 7.9) (p= 0.006; ANOVA), although, there were no demonstrable differences between vessels that were incubated with the incremental concentrations of L-NAME (p= 0.93; ANOVA).

7.4.2 Acetylcholine:

7.4.2.1 Comparison of endothelium dependent relaxation in omental vessels from non-pregnant and pregnant women and women with pre-eclampsia:

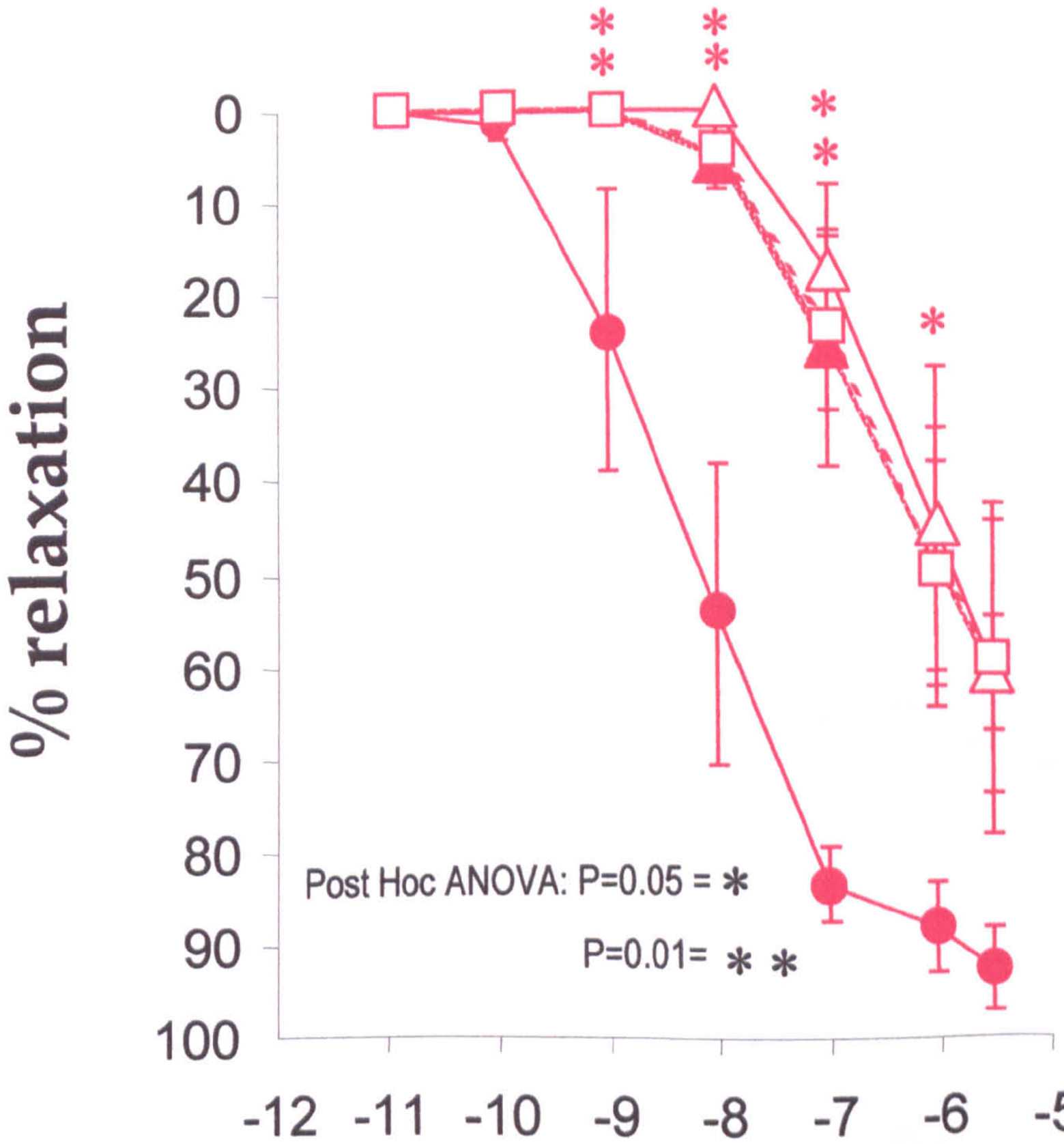
A comparison of the three groups demonstrated significant differences between the dose response curves obtained with acetylcholine ((Fig 7.10) (p = 0.001), repeated measures analysis of variance (ANOVA)). Post hoc testing revealed these differences to be between the pre-eclampsia group and the other two study groups (pregnant *Vs* pre-eclampsia: E_{max}; P=0.001).

7.4.2.2 Effect of incubation with Indomethacin:

The addition of indomethacin had no significant effect on the relaxation to acetylcholine in the non-pregnant group (Fig 7.11) (P = 0.62, ANOVA), the normal pregnant group (Fig 7.12) (P = 0.75, ANOVA), or the pre-eclampsia group (Fig 7.13) (P = 0.99, ANOVA).

Fig 7.9

The effect of incremental doses of L-NAME on the endothelium-dependent relaxation of pregnant omental vessels



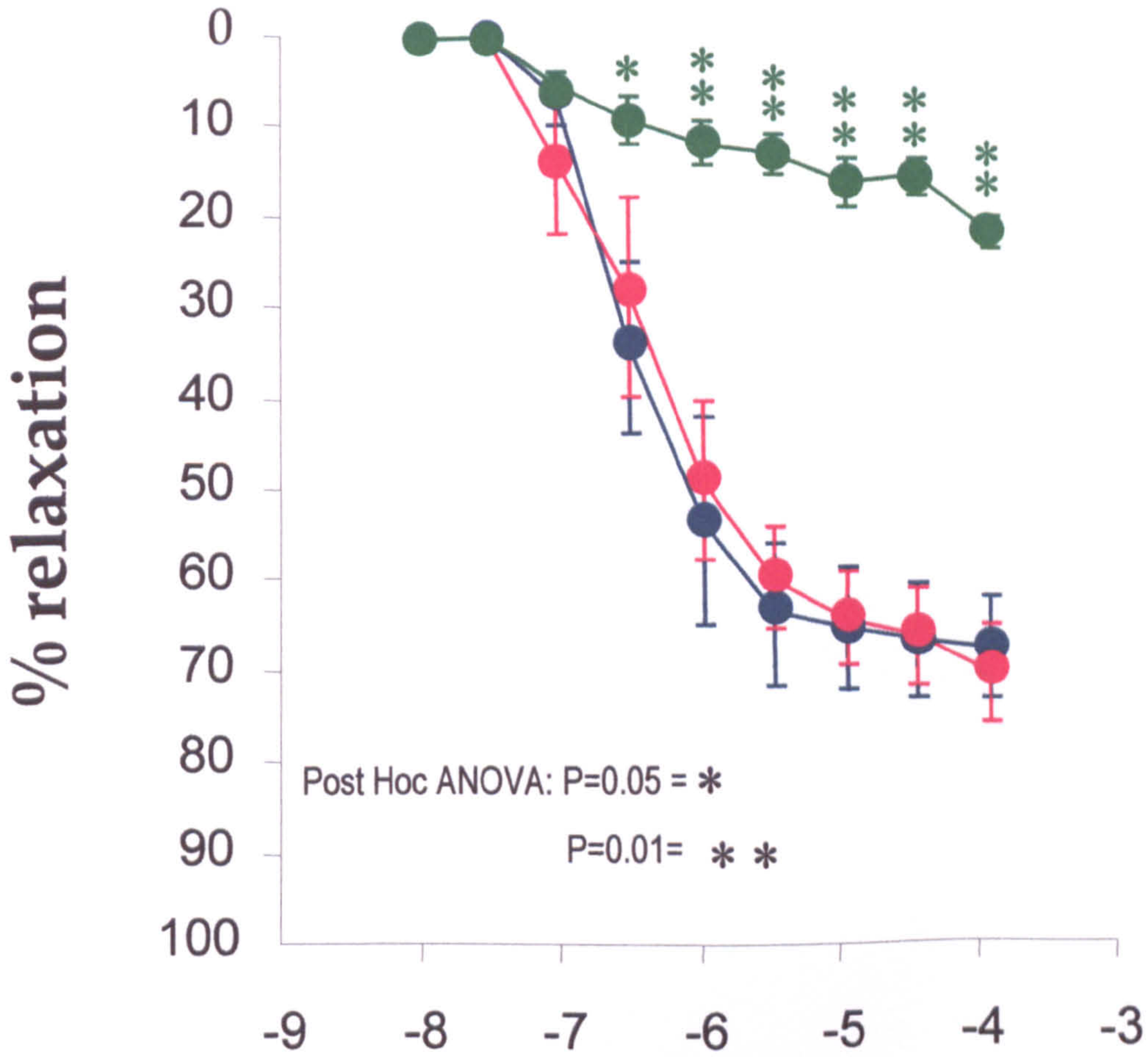
KEY:

		EC 50	Emax
Control		-8.4+/-0.4	91.67+/-4.45
L-NAME (10-4)		-6.2+/-0.4	60.25+/-6.23
L-NAME(3*10-4)		-5.8+/-0.5	59.89+/-17.68
L-NAME(10-3)		-5.6+/-0.7	58.50+/-14.45
ANOVA (factorial)	p	0.008	0.20
ANOVA (repeated measures)	p		0.006

Statistical comparisons:

Fig 7.10

Comparison of CRC for acetylcholine in omental vessels from non-pregnant women,and pregnant women and women with pre-eclampsia



KEY:

Non-pregnant

Pregnant

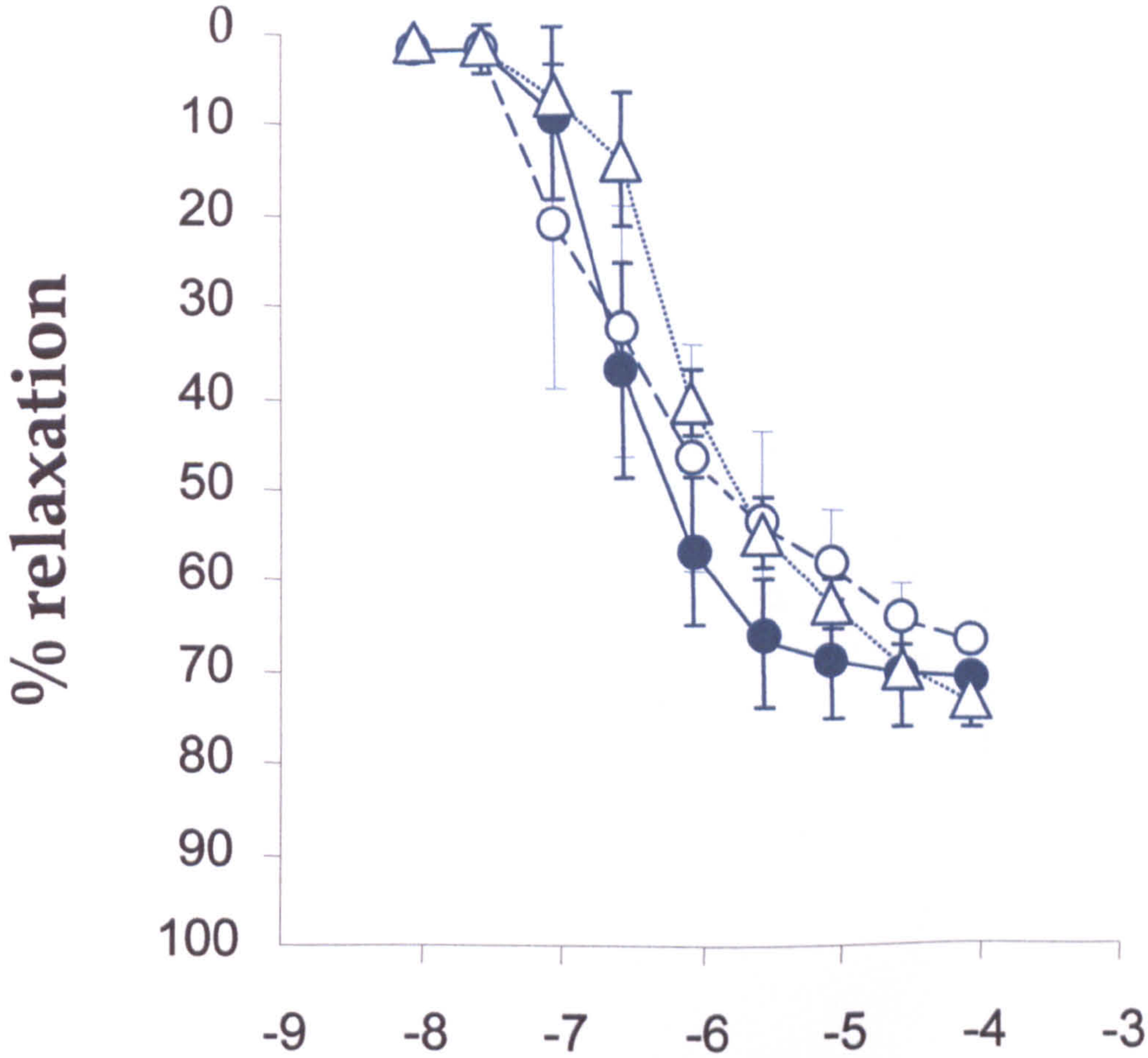
Pre-eclampsia

Statistical comparisons:

		E_{max}
		70.44+/-5.93
		73.36+/-5.47
		22.57+/-1.95
ANOVA (factorial)	p	0.006
ANOVA (repeated measures)	p	0.001

Fig 7.11

The effect of indomethacin and indomethacin and L-NAME on the endothelium-dependent relaxation of omental vessels from non-pregnant women



KEY:

Control

Indomethacin

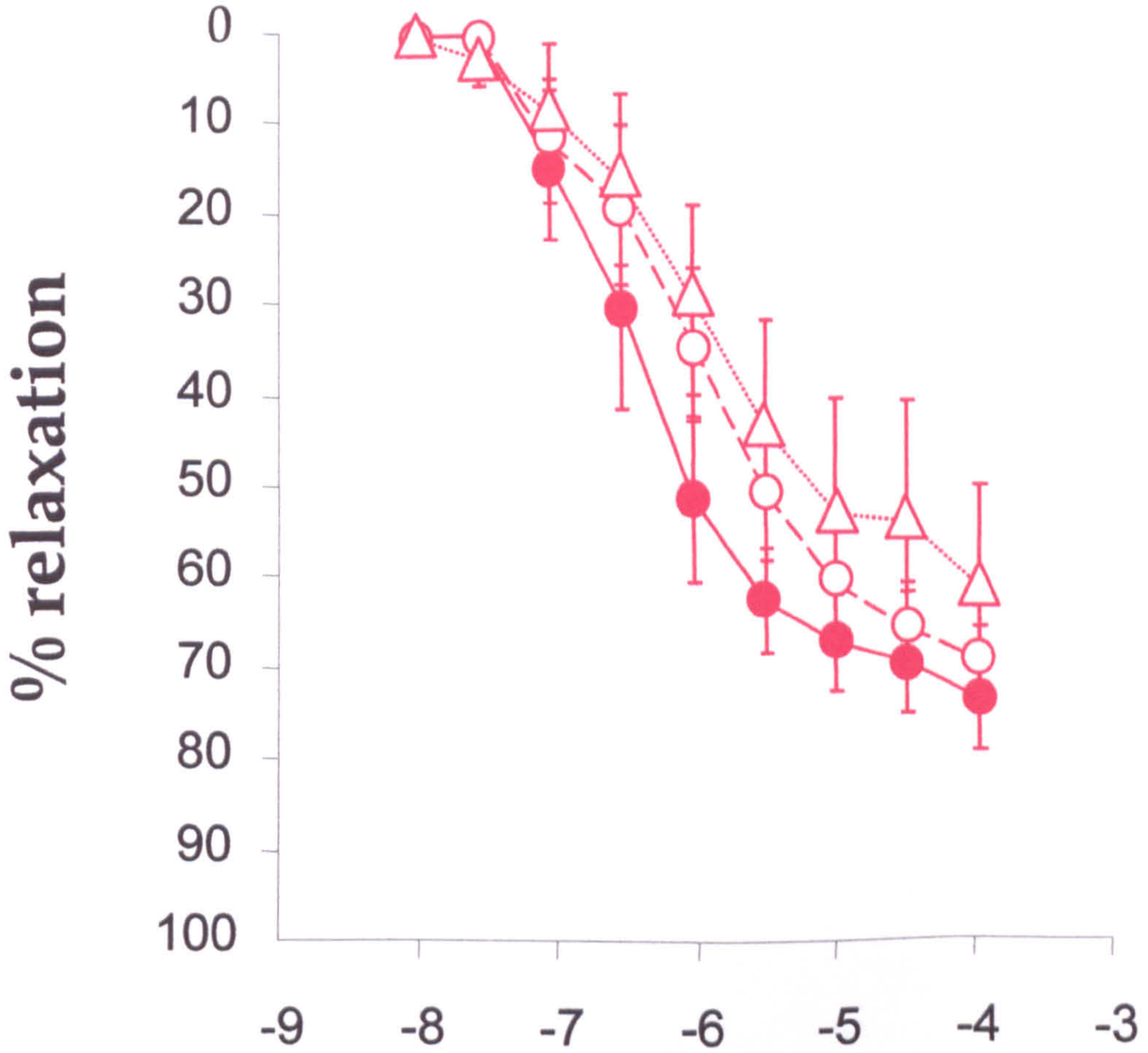
Indo + L-NAME

Statistical comparisons:

		EC_{50}	E_{max}
Control		-5.6+/-0.4	70.44+/-5.93
Indomethacin		-5.8+/-0.6	66.09+/-4.42
Indo + L-NAME		-5.7+/-0.1	73.18+/-2.73
ANOVA (factorial)	p	0.92	0.56
ANOVA (repeated measures)	p	0.23	

Fig 7.12

The effect of indomethacin alone and indomethacin and L-NAME on the endothelium-dependent relaxation of omental vessels from pregnant women



KEY:

Control

Indomethacin

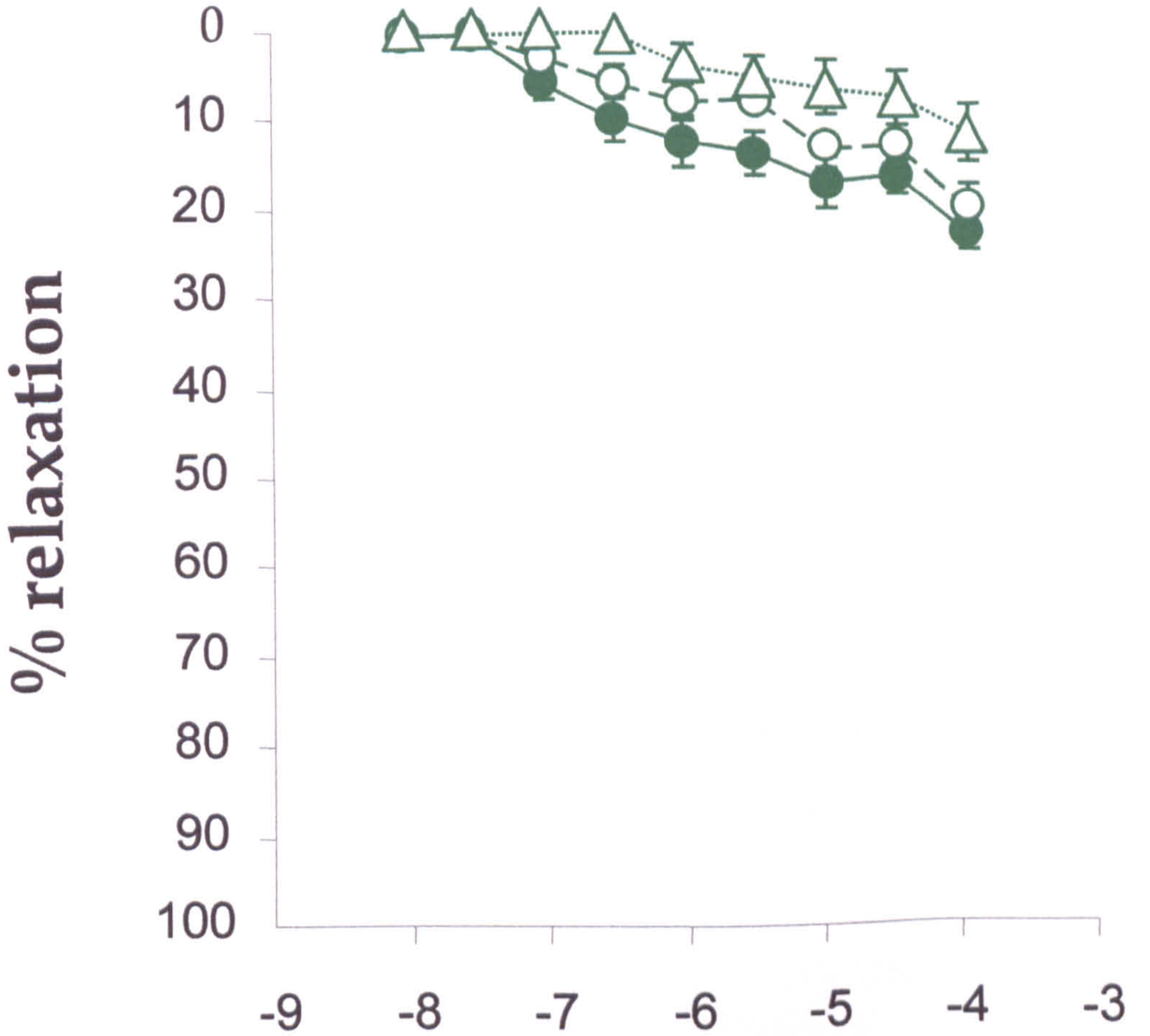
Indo + L-NAME

Statistical comparisons:

		EC_{50}	E_{max}
		-6.0+/-0.4	73.36+/-5.47
		-5.8+/-0.2	71.35+/-2.95
		-5.3+/-0.5	61.62+/-11.6
ANOVA (factorial)	p	0.57	0.57
ANOVA (repeated measures)	p	0.91	

Fig 7.13

The effect of indomethacin and indomethacin and L-NAME on the endothelium-dependent relaxation of omental vessels from women with pre-eclampsia



KEY:

Control

Indomethacin

Indo + L-NAME

Statistical comparisons:

		E_{max}
		22.57+/-1.95
		19.66+/-2.92
		11.26+/-3.25
ANOVA (factorial)	p	0.33
ANOVA (repeated measures)	p	0.83

7.4.2.3 Effect of incubating with Indomethacin and L-NAME:

In a similar manner to the effect of indomethacin, the addition of indomethacin and L-NAME caused no change in the relaxation profiles in non-pregnant (P=0.23), pregnant (P= 0.92), and women with pre-eclampsia (P=0.83)

7.4.4 Sodium Nitroprusside:

7.4.4.1 1 Comparison of endothelium dependent relaxation in non-pregnant, pregnant and women with pre-eclampsia

Comparison of non-endothelium dependent relaxation between vessels from normal pregnant women and women with pre-eclampsia showed no significant differences (P=0.93, ANOVA).

7.5 Discussion:

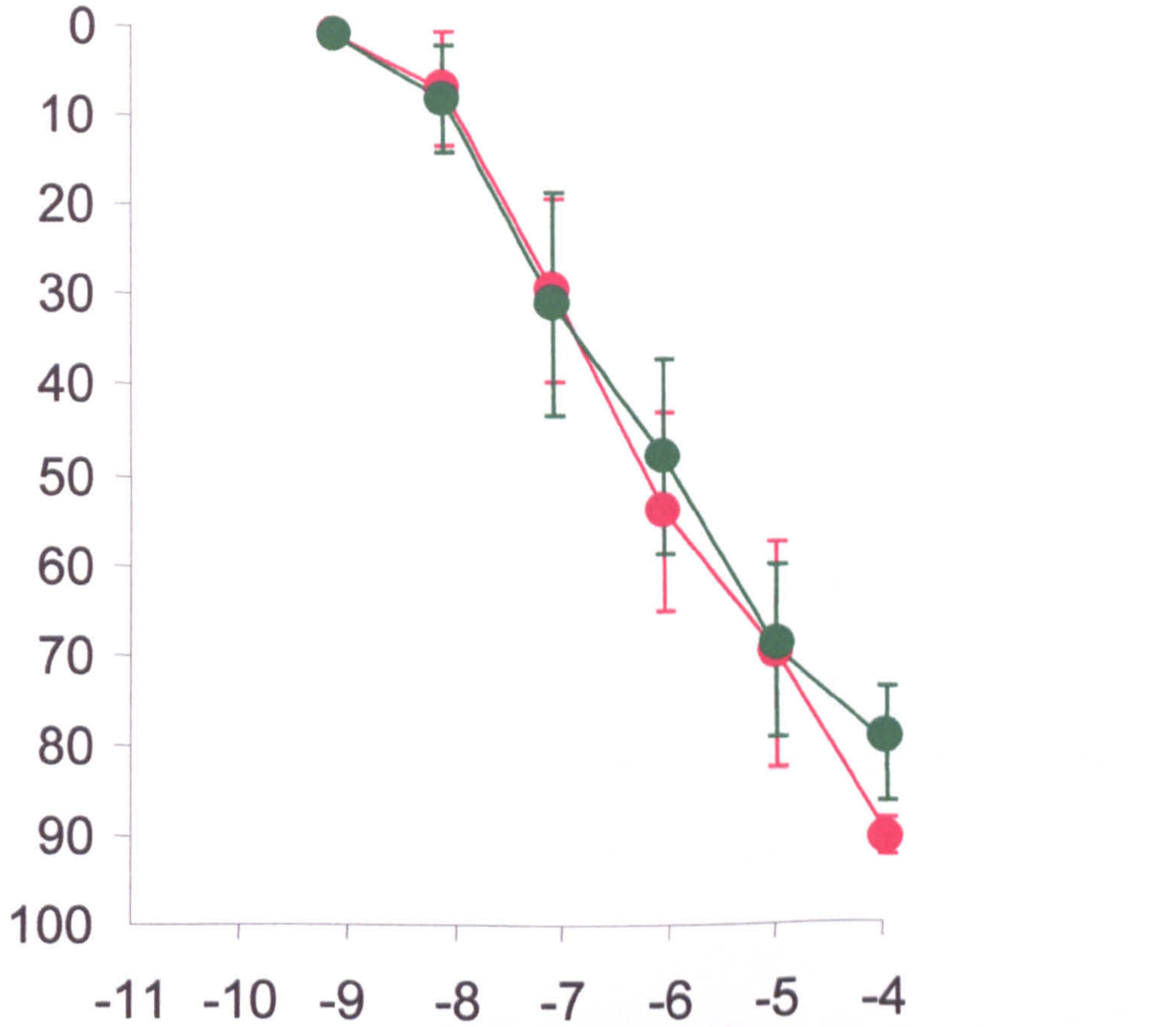
7.5.1 Vasoconstrictors:

7.5.1.1 Vasopressin:

Pressor responses to angiotensin II in pregnancy are reduced both in humans (Gant et al., 1973), and sheep (Rosenfeld and Gant, 1981), a finding that appears to be universal to all animals studied (Joels and Humphreys, 1985) (Jansakul et al., 1990) (Ahokas et al., 1991) (Ahokas and Sibai, 1992) (Ramsay et al., 1992) (Ramsay et al., 1993) (Allen et al., 1994) (Hines et al., 1994). Responses in pregnant rats to vasopressin have also been shown to be decreased when

Fig 7.14

Non-endothelium-dependent relaxations to sodium nitroprusside in, pregnant and pre-ecamptic omental vessels



KEY:

Pregnant

Pre-eclampsia

Statistical comparisons:

		EC_{50}	E_{max}
ANOVA (factorial)	p	0.49	0.14
ANOVA (repeated measures)	p		0.89

compared to non-pregnant animals (Hines et al., 1994), however the responses to vasopressin of human pregnant women remains to be fully elucidated.

This thesis confirms the findings of Pascoal et al (1998) that during pregnancy, human omental vessels undergo no alteration in their response to vasopressin when compared to non-pregnant vessels (Pascoal and Umans, 1996). Similar findings have been also been reported in both animal and human studies of different systemic vascular beds. Norepinepherine induced contractions of human pregnant subcutaneous resistance vessels also remain unchanged with pregnancy (McCarthy et al., 1993b) (Knock and Poston, 1996) (Van de Voorde et al., 1997). This is in sharp contrast to the data from vessels obtained from pregnant myometrium which undergo dramatic changes in endothelial response to various vasoconstrictor agents; these differences have already been discussed in Chapter 6 and will not be dwelt on here.

The addition of indomethacin, or indomethacin and L-NAME, to the non-pregnant and pregnant vessels had no effect on the concentration response curves to vasopressin. This is similar to previously reported findings in human myometrial vessels constricted to vasopressin (Chapter 6) and noradrenaline (Steele et al., 1993), and systemic subcutaneous vessels constricted to noradrenaline (McCarthy et al., 1993b).

Human and animal studies suggest that pregnancy is clearly associated with an alteration in the basal release of endothelium-derived products. These effects are not due to alterations in either nitric oxide or eicosanoid synthesis, as inhibition of these pathways caused no differences between pregnant and non-pregnant vessels in either animal or human vessels (Fig 7.1) (Chapter 6) (Davidge and McLaughlin, 1992) (Steele et al., 1993) (Kim et al., 1994). As such, these

observations further support a significant role for the yet to be identified endothelial derived relaxing factor in the vascular changes associated with pregnancy.

Omental vessels from women with pre-eclampsia showed enhanced constrictor responses to vasopressin when compared to normal pregnant controls (Fig 7.1), an observation that has also been reported by Pascoal et al (1998). Similar findings have been reported in myometrial vessels (Chapter 6) (Allen et al., 1989), subcutaneous vessels (McCarthy et al., 1993b) and inferior epigastric arteries (Oguogho et al., 1996). These observations from various non-uterine vascular beds of human pregnant women with pre-eclampsia support the hypothesis that there is an alteration in endothelial function that causes the clinical manifestation of this disease. The generality of this observation throughout the human system would also support the hypothesis of Roberts et al (1989), that a blood borne factor(s) causes the alteration in endothelial function (Roberts et al., 1989) (Roberts et al., 1991).

The addition of the eicosanoid inhibitor indomethacin to omental vessels from pregnant women and women with pre-eclampsia caused no significant alterations in the concentration response curves to vasopressin (Fig 7.3 and 7.4). However, the addition of both indomethacin and the nitric oxide inhibitor, L-NAME, to the vessels from women with pre-eclampsia caused a significant leftward shift in their response, a shift that was not observed in vessels from the normal control group (Fig 7.3 and Fig 7.4). This suggests that there is an increased basal release of nitric oxide in vessels from women with pre-eclampsia. Smarason et al (1997) have recently reported that plasma levels of nitric oxide are increased in pre-eclampsia (Smarason et al., 1997) and several studies including this thesis have suggested that peroxynitrites are increased in various tissues in

women with pre-eclampsia (Myatt et al., 1996) (Roggensack et al., 1999) (Kossenjans et al., 2000) (Chapter 6).

7.5.2 Vasodilators:

7.5.2.1 Bradykinin:

This study is in accord with several other studies that have demonstrated relaxation to bradykinin (Ashworth et al., 1996b) (Pascoal and Umans, 1996) (Ashworth et al., 1996c) (Pascoal et al., 1998) and acetylcholine in omental vessels (Pascoal and Umans, 1996) (Pascoal et al., 1998). However, the maximal response obtained to bradykinin differs between studies. This study obtained relaxations of approximately 70-80%, which is similar to those obtained by Ashworth et al, whereas Pascoal et al reported 100% relaxations. Moreover, analysis of the EC_{50} values between the various studies demonstrates marked differences, Pascoal et al did not calculate EC_{50} values, however analysis of their presented data suggests an EC_{50} value of approximately (-) 7 x10 $^{-10}$ M. However, this study and that of Ashworth et al report EC_{50} values of (–) 9 x10 $^{-10}$M. Discrepancies in these studies may have come from differences in the passive diameter of the vessels; this study and that of Ashworth et al (1996) used passive diameters that corresponded to 90% of the calculated diameter at 100mmHg, whereas Pascoal et al (1998) used a passive diameter that corresponded to the diameter at 100mmHg. McPherson et al have demonstrated that increasing of the passive diameter causes a rightward shift in the EC_{50} values to the contractile agent methaxamine in rat mesenteric vessels (McPherson, 1992). Therefore, it could be postulated that the tension that the vessel is under might affect the vasodilator responses in a similar manner.

Data presented in this thesis show that relaxation to bradykinin is similar in vessels from normal pregnant and non-pregnant women (Fig 7.5). These observations confirm those published by Pascoal et al (1998) (Pascoal and Umans, 1996) (Ashworth et al., 1996b). Human uterine resistance vessels have been demonstrated to show no differences in bradykinin mediated relaxations between pregnant and non-pregnant women (Ashworth et al., 1997) (Chapter 6). However, studies from human subcutaneous vessels are more controversial; Knock et al (1996) reported enhanced relaxation to bradykinin from vessels obtained from pregnant women when compared to those from non-pregnant women (Knock and Poston, 1996), but, in a similar study Van de Voorde et al (1996) were unable to demonstrate any significant differences in relaxation between the two study groups (Van de Voorde et al., 1997). The observed differences in these studies may be due to differences in protocol. Van de Voorde et al (1997) utilised norepinephrine in depolarised [30 mmol K+] conditions to induce contractions. It is now well documented that varying extra-cellular potassium concentrations effectively separates nitric oxide and EDHF mediated relaxation (Adeagbo and Triggle, 1993); in such conditions EDHF relaxation would be abolished. Knock et al (1996) utilised the same vasoconstrictor agent without partial depolarisation and demonstrated an increase in relaxation in pregnant vessels.

Pascoal et al demonstrated that responses were independent of both eicosanoids and nitric oxide (Pascoal and Umans, 1996) (Pascoal et al., 1998). This is in contrast to the results of this thesis where the three vessel groups studied displayed a varying degree of nitric oxide mediated relaxation (Fig 7.6, 7.7, 7.8, 7.9). These differences may have occurred for several reasons. Firstly, vessel size: it is well documented that smaller diameter vessels display proportionally greater EDHF mediated relaxation (Woolfson and Poston, 1990) (Hasunuma et al., 1991) (Mugge

et al., 1991) (Nagao et al., 1992) and this study utilised vessels that were of a two fold greater diameter than those used by Pascoal et al (412 *Vs* 210μm). Secondly, the passive diameter of the vessel during experimentation may effect the results as stated previously. The EC_{50} values of the L-NAME exposed pregnant and non-pregnant vessels were in the order of (-) 6.6 x 10^{-10}M (Fig 7.6 and 7.7) and in the study of Pascoal et al the EC_{50} values with and without a nitric oxide synthase inhibitor were approximately (-) 7.0 x 10^{-10} M. From this it could be hypothesised that vessels at a higher passive diameter (under greater tension) require greater concentrations of bradykinin to initiate relaxation, and therefore the bradykinin concentrations are above those that mediate relaxation through nitric oxide. This hypothesis was not formally tested.

With regard to pregnancy, unlike myometrial vessels, omental vessels displayed no significant increase in the proportion of non-eicosanoid, non-nitric oxide mediated relaxation (EDHF) (P=0.88). However, it might be postulated that in these vessels the nitric oxide synthetase enzyme had not been adequately inhibited. This hypothesis was tested through the dose response to varying concentrations of L-NAME. The results demonstrated that doubling the concentration of L-NAME had no further effect the rightward shift of the CRC and suggest further that EDHF is important in the relaxation of human vessels (Fig 7.8).

Although data from animal research suggests that the decrease in peripheral resistance in normal pregnancy might be mediated be enhanced nitric oxide mediated relaxation (Hull et al., 1992) (Matsumoto et al., 1992) (Chu and Beilin, 1993), the observations of this thesis on omental and myometrial (Chapter 6) vessels provides no evidence to support this hypothesis. On the contrary the observations of myometrial resistance vessels suggest that there is in fact a reduction in the nitric oxide mediated relaxation during pregnancy (Chapter 6). Similar resistance to nitric oxide

blockade has also been reported in subcutaneous resistance vessels (Knock and Poston, 1996) (Van de Voorde et al., 1997). The observations of this thesis and the observed differences between the reports of Knock et al (1996) and Van de Voorde et al (1997) would imply that increased EDHF, not nitric oxide, mediated relaxation is important in human pregnancy adaptation.

Omental vessels, unlike myometrial vessels, displayed no adaptations to pregnancy through an increase in EDHF mediated relaxation. Furthermore, omental vessels displayed no significant loss of endothelium dependent relaxation in pre-eclampsia, a finding in keeping with that of Pascoal et al (1996). Omental vessels from women with pre-eclampsia, in a similar manner to vessels from the myometrium, displayed an increase in the proportion of nitric oxide mediated relaxation when compared to normal control vessels (Fig 7.9) (P=0.028). This again suggests that in pre-eclampsia there is a compensatory up-regulation of nitric oxide mediated relaxation. This finding of an increased release of nitric oxide on stimulation is in keeping with the observation that there is an increase in the basal release of nitric oxide in vessels from women with pre-eclampsia (Fig 7.4). Further support for this comes from Roggensack et al (1999) who reported increased nitrotyrosine residues and eNOS in the vascular endothelium of women with pre-eclampsia (Roggensack et al., 1999) (see Chapter 6). Whether this increased nitric oxide component is detrimental to vessel function remains to be elucidated.

7.5.2.2 Acetylcholine:

This study demonstrates that omental vessels constricted with vasopressin displayed endothelium-dependent relaxation to acetylcholine (Fig 7.10). This is in accord with others studies that have examined acetylcholine responses in the omentum (Pascoal and Umans, 1996) (Pascoal et al., 1998). However, omental vessels constricted with U46619 have been shown to undergo neither contraction nor relaxation to the same doses of acetylcholine [$1x10^{-9}$ – $1x10^{-4}$ M] (Wallerstedt and Bodelsson, 1997), a result which may suggest that the choice of vasoconstrictor used is important to the results obtained. Evidence to support this comes from this thesis where myometrial vessels contracted with vasopressin also showed no endothelial dependent relaxation to acetylcholine despite the presence of a functioning endothelium (Chapter 3) whereas vessels constricted to noradrenaline showed a 30% endothelial dependent relaxation to acetylcholine (Kublickiene, 1998). However, the vessel size may prove to be a determinant to the amount of relaxation obtained. Pascoal et al (1996) obtained 100 % relaxation in vessels that were in the region of 200μm, this thesis obtained 70% in vessels that were in the region of 400μm, and Wallerstedt et al (1997) obtained no relaxation in vessels that were greater than 600μm. This suggests that as vessels diverge down the vascular tree they become more responsive to acetylcholine due to an increase in available receptors.

Although omental vessel endothelial dependent relaxation to acetylcholine has been demonstrated in this thesis, as with the responses to bradykinin, concentration response curves were unaltered with pregnancy (Fig 7.10). Moreover, both non-pregnant and pregnant vessels relaxation is independent of eicosanoids and nitric oxide, implicating a possible role for EDHF (Fig

7.11 & 7.12). Pascoal et al (1996) also showed similar results with acetylcholine when using a similar method to that used in this thesis.

Omental vessels are utilised in humans as representative of mesenteric vascular system, however, animal studies of mesenteric vessels have produced very different results. In pregnancy, animal models have demonstrated enhanced endothelium dependent relaxation to the agonists acetylcholine and bradykinin in small resistance arteries from rats (Davidge and McLaughlin, 1992) (Pascoal et al., 1995) (Learmont et al., 1996). In common with the non-pregnant state (Garland and McPherson, 1992) small arteries from pregnant rats (Pascoal et al., 1995) usually show persistent relaxation to acetylcholine and bradykinin when nitric oxide and prostacyclin synthesis are inhibited. In non-pregnant animals this relaxation has been attributed to EDHF (Garland and McPherson, 1992). Although, studies have suggested that EDHF is increased in pregnancy (Pascoal and Umans, 1996) (Bobadilla et al., 1997) neither of these directly demonstrated the presence of EDHF by inhibition through partial depolarisation or measurement of smooth muscle hyperpolarision. Recently, Gerber et al demonstrated that the increased response to acetylcholine in pregnant mesenteric vessels is mediated through EDHF production. They demonstrated, utilising the technique of wire myography, that pregnant mesenteric vessels had enhanced relaxation over non-pregnant controls in the presence of an eicosanoid inhibitor, a nitric oxide synthetase inhibitor, and the soluble guanylate cyclase inhibit oxidiazole quinoxalin (ODQ). However, when both vessels from non-pregnant and pregnant animals were partially depolarised with 25 mmol K+ the relaxation was attenuated. This demonstrates that in mesenteric vessels, the enhanced relaxation of pregnancy may be mediated through increased EDHF (Gerber et al., 1998).

Experimental evidence from both intact tissues and cell culture support a role for gap junctions in EDHF cell-cell communication and control of arterial function (Little et al., 1995) (Chapter 1). With regard to human pregnancy Kenny et al have recently demonstrated that isolated myometrial vessels contracted with vasopressin and relaxed to bradykinin are resistant to eicosanoid and nitric oxide blockade, but that the additional presence of 18-alpha glycyrrhetinic acid, a gap junction uncoupler (Taylor et al., 1998), completely abolishes the relaxation. This would imply that in myometrial vessels that EDHF responses are of considerable importance (Kenny et al., 1999a). This thesis (Chapter 6) has also demonstrates that myometrial vessels alter their endothelium dependent relaxation with pregnancy in favour of EDHF. Therefore, this would concur with the data of Dantas et al (1999) that gap junction play an integral role in the vascular pregnancy adaptation response (Dantas et al., 1999). Whether, this increase in gap junction mediated relaxation occurs in vessels in the systemic circulation remains to be elucidated.

Acetylcholine also displayed a loss of endothelium dependent relaxation in vessels from women with pre-eclampsia and confirms the results of Pascoal et al (1996). The addition of eicosanoid or nitric oxide synthase inhibitors had no significant effect on the concentration response curves and demonstrates that in this vascular bed the acetylcholine-mediated relaxation may be mediated exclusively through EDHF. Evidence to support this comes from Pascoal et al (1996) who also demonstrated that vessels constricted with vasopressin were unaffected by nitric oxide or eicosanoid synthetase inhibitors when relaxation occurs with acetylcholine.

The gross mechanisms of vasodilatation have been characterised in omental vessels to bradykinin and acetylcholine. Bradykinin appears to mediate its relaxation predominately through

nitric oxide with a small EDHF component. In contrast acetylcholine mediates its relaxation exclusively through EDHF.

Omental vessels show no endothelial-mediated alterations with pregnancy; this may in part be due to their anatomical placement. It could be hypothesised that vessels that are required to accommodate and supply the growing conceptus would undergo greater adaptations to pregnancy than those that have little to due with peripheral vascular resistance. Another important vascular bed that receives a large increase in the blood flow during pregnancy is the kidneys, however, the inaccessibility of these vessels in the human patients precludes then from *in vitro* study.

Although omental vessels show no adaptations to pregnancy they undergo alteration in endothelial function with pre-eclampsia which may be mediated through an imbalance in nitric oxide and EDHF. These include an increased pressor response to vasopressin that is independent of eicosanoids, but is dependent unpon increased nitric oxide (Fig 7.2, 7.3 & 7.4). Endothelium-dependent relaxation is also altered in omental vessels in women with pre-eclampsia; a loss of endothelium-dependent relaxation to acetylcholine was found through a decrease in presumed-EDHF mediated relaxation. In vessels exposed to bradykinin, there is a decrease in EDHF mediated relaxation and an increase in nitric oxide mediated relaxation. This reciprocal decrease in EDHF and increase in nitric oxide mediated relaxation has been well documented (Randall and March, 1998). It might be postulated that the loss of endothelium-dependent relaxation to acetylcholine is through an increase in the production of nitric oxide, which attenuates the EDHF response, but these vessels are incapable of vasodilation through nitric oxide when stimulated with these agonists. Evidence for increased nitric oxide in these

vessels in the disease of pre-eclampsia comes from Roggensack et al who demonstrated a increase in endothelial staining for peroxynitrite and eNOS in these vessels (Roggensack et al., 1999).

The lack of adaptation to pregnancy in these vessels and the observed alteration in endothelial function in pre-eclampsia further enhance the hypothesis that there is a circulating factor in the blood of women with pre-eclampsia that causes this alteration and it is not a failure of maternal adaptation. The nature of this circulating factor remains to be elucidated, however, it has been recently demonstrated that chronic exposure to the pro-inflammatory cytokine TNFα causes rabbit carotid and porcine coronary vessels to lose EDHF mediated relaxation through the upregulation of nitric oxide (Kessler et al., 1999). Whether this is also the case in humans or whether other cytokines affect vascular function remains to be studied (see Chapter 8).

7.6 Summary:

In this chapter the vasoconstrictor responses to vasopressin in non-pregnant, pregnant and vessels from women with pre-eclampsia have been characterised. It has been shown that in pregnancy there were no blunted responses to vasopressin. This thesis has also demonstrated an enhanced response to vasopressin in vessels from women with pre-eclampsia and that basal release of nitric oxide appears to be increased as a method of off-setting this response. The data presented here suggest that in human pregnancy less adaptation occurs in human omental vessels than in myometrial vessels. This may represent the fact that these vessels have less involvement in the physiology of pregnancy than myometrial vessels.

Data presented in this thesis clearly demonstrate that pregnancy is not associated with alterations in endothelial dependent relaxation, but that pre-eclampsia dramatically alters endothelium dependent relaxation in the systemic circulation. It also demonstrates that these changes may be mediated through the alteration in the reciprocal relationship between NO and EDHF, in favour of nitric oxide that reduces that EDHF response.

Data presented in this chapter also show that when the interaction of the endothelium with Vascular Endothelial Growth Factor and plasma [Chapter 8] are investigated, the effect that these have with the endothelium on the acetylcholine response should be considered.

Chapter eight: The interaction of vascular endothelial growth factor (VEGF) and plasma from women with pre-eclampsia with the endothelium in an in vitro model. Evidence that VEGF is implicated in the pathogenesis of pre-eclampsia:

Graphs 8.14,8.15, 8.16 and 8.20 were obtained by collaboration with Dr Richard Hayman.

8.1 Introduction:

There is accumulating evidence for a pathogenic model of pre-eclampsia, whereby a deficiency in the trophoblast invasion of the placental bed spiral arteries leads to a poorly perfused feto-placental unit. This results in secretion of a factor(s) into the maternal circulation which causes 'activation' of the vascular endothelium; the clinical syndrome results from widespread changes in endothelial cell function (see Chapter 1). However, the nature of the circulating factor(s) and the mechanism by which the endothelium is activated, are as yet undetermined.

There is much circumstantial evidence to support the hypothesis that placental-derived VEGF mediates the endothelial activation of pre-eclampsia. A detailed review of VEGF has been carried out in Chapter 1 and its biological functions will not be discussed further. However, VEGF is increased in the plasma of women with pre-eclampsia when compared to normotensive pregnant controls (Chapter 4). In chapter 6 it was demonstrated that myometrial resistance vessels from women with pre-eclampsia had an increased response to vasopressin and an attenuated response to bradykinin. It has also been previously demonstrated that normal myometrial vessels' responses to bradykinin are altered after incubation with plasma from women with pre-eclampsia in a manner which is similar to vessels obtained from women with pre-eclampsia (Ashworth et al.,

1998). In Chapter 7 omental vessels (as representative of the peripheral vascular system) were also shown to have a specific loss of endothelium dependent relaxation to acetylcholine. Therefore it was considered interesting to investigate whether plasma and VEGF could initiate similar responses to those obtained from myometrial and omental vessels from women with pre-eclampsia.

8.2 Aims:

- To investigate whether VEGF causes a constrictor response to myometrial and omental resistance vessels.
- To use an *ex vivo* myographic model to investigate whether VEGF and plasma were capable of altering the behaviour of normal myometrial vessels to vasopressin and bradykinin in a similar manner to pre-eclampsia.
- To use an *ex vivo* myographic model to investigate whether VEGF and plasma were capable of altering the behaviour of normal omental vessels to vasopressin and acetylcholine in a similar manner to vessels from women with pre-eclampsia.
- To investigate the mechanism of action of VEGF on myometrial and omental resistance vessels

8.3 Experimental design:

A 16-hour incubation period (overnight) was used for the majority of the interactions investigated in this study. This was chosen as previous work in our laboratory had demonstrated this to be an effective time period for reproducible results. As a consequence, 1iu heparin/ml was added to all

the tubes in order to prevent the formation of a fibrin clot in those mixtures to which plasma had been added.

In all the studies where plasma was required individual patient's plasma was used except in the experiment where VEGF was removal from the plasma with the use of Dynal beads. In this experiment pooled plasma was utilised as the cost of removing the VEGF from individual samples was deemed to be too expensive. Pooled plasma had been demonstrated to have a comparable effect as individual sample (Hayman, 1999).

8.3.1: The direct effect of VEGF on myometrial resistance vessels:

Myometrial resistance vessels were obtained from pregnant women (n=4) as described previously in Chapter 2.5.2a. Vessels were mounted and normalised as described in Chapter 2.5.4. The myography protocol (Chapter 3.3.1) was then followed and incremental doses of vasopressin were used to confirm that the vessel was capable of constriction (Chapter 2.5.5.5). The vessel was then washed as previously described. Once it had returned to the base line incremental doses of VEGF (0.1nM –5nM) were added.

In separate experiments myometrial vessels were obtained from non-pregnant (n=3) and pregnant (n=2) women as described previously in Chapter 2.5.2a. Vessels were mounted and normalised as described in Chapter 2.5.4. The myography protocol (Chapter 3.3.1) was then followed and incremental doses of vasopressin were used to cause a constriction (Chapter 2.5.5.5); once a steady a state constriction had been obtained vessels were exposed to incremental doses of bradykinin to confirm presence of the endothelium. Vessels were then washed with PSS (Chapter

2.5.5.5) and allowed to return to a base line. Once a base line was maintained, vessels were again contracted with vasopressin and this time VEGF in incremental doses was added to the chamber solution (0.1nM –5nM).

8.3.2: The effect of incubation with vascular endothelial growth factor and placental growth factor on endothelium dependent resistance artery behaviour from non-pregnant and pregnant women.

Myometrial vessels from normal pregnant (n=10) and non-pregnant women (n=6) were incubated overnight with 5 nmol VEGF and compared to control vessels. In separate experiments vessels were obtained from normal pregnant (n= 5) and non-pregnant women (n= 10) and incubated overnight in the presence or absence of 5nmol PlGF.

8.3.3: The effect of concentration of vascular endothelial growth factor on endothelium dependent resistance artery behaviour.

Myometrial vessels from normal pregnant women (n=6) were collected as described previously (Chapter 2). At least four vessels were dissected from each sample; vessels were then randomly divided into four groups and placed in cold oxygenated PSS for overnight storage (Chapter 2). VEGF (5nM, 0.5nM, 0.05nM) was then added to the PSS solution in three of the groups by a technician, so that the investigator was blinded to which VEGF concentration had been added to each vessel; one vessels remained without VEGF to act as an internal control. After the experimental protocol (Chapter 3.2.1) was completed the results were analysed and the VEGF concentrations revealed.

8.3.4: The effect of time of incubation with vascular endothelial growth factor on endothelium dependent resistance artery behaviour.

Myometrial vessels were obtained from a total of 18 normal pregnant women as described in Chapter 2. Myometrial vessels were incubated with VEGF (0.5nM) for three different time periods; one-hour, two-hour and sixteen-hours (overnight). Those vessels in the one hour protocol (n=6) were dissected, mounted and normalised as described in Chapter 2. VEGF (0.5nM) was then added to the myography chamber and the vessels incubated as the temperature was raised to 37^0C over a 1 hour period. Vessels in the two-hour protocol were also dissected and mounted as in Chapter 2. VEGF (0.5nM) was added to the myography chamber and the vessels incubated as the temperature was raised over 1 hour, after equilibration the PSS was replaced with fresh heated PSS containing VEGF and the vessels were left for a further 1 hour before commencing the experimental protocol (Chapter 3). Vessels in the sixteen-hour protocol were dissected and randomly divided into two groups and placed in cold oxygenated PSS for storage overnight (Chapter 2). VEGF was then added to the PSS solution as described in Chapter 8.3.3.

8.3.5: The effect of parity on vascular endothelial growth factor's effect on endothelium dependent resistance artery behaviour.

To examine whether parity affected the endothelial response to VEGF, vessels from primiparous (n=8) and multiparous (n=7) women were obtained from myometrial biopsies. These were incubated overnight (Chapter 3) in the presence or absence of VEGF (0.5nM).

8.3.6: The effect of repeated concentration response curves (CRC) to bradykinin on the endothelial interaction with vascular endothelial growth factor.

Vessels were obtained from myometrial biopsies from pregnant women (n=6) as described in Chapter 2. Vessels were than allocated into two groups and incubated overnight in the presence or absence of VEGF (0.5nM) (Chapter 3). After incubation vessels were mounted and normalised (Chapter2). These were then constricted with vasopressin and concentration response curves for bradykinin obtained; a total of four CRCs were produced for each vessel.

8.3.7: The effect of plasma from women with pre-eclampsia on endothelium-dependent resistance artery behaviour.

At least three myometrial resistance vessels were obtained from pregnant (n=13) as described previously in Chapter 2.5.2a. Vessels were randomly allocated into three groups and stored overnight (Chapter 2.5.6) in either the presence of 2% normotensive plasma, 2% plasma from women with pre-eclampsia, or no plasma (Ashworth et al., 1998). Following incubation, vessels were mounted and normalised as described in Chapter 2.5.4. The myography protocol (Chapter 3.3.1) was then followed in which incremental doses of vasopressin were used to cause constriction and incremental doses of bradykinin were used to cause relaxation (Chapter 2.5.5.5).

8.3.8: The effect of antibody to VEGF on endothelium dependent resistance artery behaviour.

To determine whether VEGF in the plasma of women with pre-eclampsia was responsible for the alterations in endothelial function seen using this bioassay technique, vessels from 8 patients were incubated overnight with either 0.5 nmol VEGF, 2% plasma from women with pre-eclampsia or 2% plasma from normal pregnant women, each in the presence or absence of 250 iu monoclonal antibody to VEGF (R&D, UK, Abingdon). Similar sized myometrial resistance arteries from normal pregnant women (n=8) were divided into 6 groups, thus ensuring comparable vessels within each group.

8.3.9: The effect of anti-Flt-1 and anti-KDR antogonists on endothelium dependent resistance artery behaviour produced by VEGF and plasma.

We next sought to determine which VEGF receptor was responsible for the alteration in endothelial function seen using our bioassay technique. Pregnant vessels (n=6) were incubated in the presence of VEGF (1.0 nM), with either anti-Flt-1 (10ng/ml) or anti-KDR (10ng/ml) antagonists (Generously supplied by Professor A Ahmed, University of Birmingham). In separate experiments, vessels were incubated with normal pregnant plasma and plasma from women with pre-eclampsia in the presence or absence of ant-Flt-1 antibody (10ng/ml).

8.3.10: The effect of Dynabead® removal of VEGF from plasma from women with pre-eclampsia on endothelium dependent resistance artery behaviour.

In order to establish whether VEGF exerts a direct effect upon vessel behaviour or whether the uncharacterised factor(s) exert their effect by inducing endogenous production /or release of VEGF, plasma from women with pre-eclampsia was treated with anti-VEGF coated Dynabeads® (Dynal® treated plasma) (Chapter 2).

Similar sized myometrial resistance arteries from normal pregnant women (n=6) were divided into 3 groups, thus ensuring comparable vessels within each group. These vessels were then incubated overnight with either 2% plasma from women with pre-eclampsia, 2% plasma from normal pregnant women, or 2% Dynal® treated plasma (Chapter 3).

8.3.11: Effect of Indomethacin and L-NAME on vessels incubated with VEGF and appropriate time controls:

As described in Chapter 3, the altered concentration response curves of myometrial vessels from women with pre-eclampsia returned towards the response curves of control vessels with repeated exposures to vasopressin and bradykinin accompanied by washing with PSS. Protocols were thus designed to allow for this when the interaction of VEGF with the endothelium were investigated (Chapter 3.2.2). Vessels from normotensive pregnant women (n=6) at term were obtained as in Chapter 2. Vessels were divided into three groups and all were exposed to VEGF (0.5 nM) for sixteen hours (Chapter 2). After sixteen hours incubation with VEGF vessels were

mounted and normalised on separate Mulavny wire myographs. One-concentration response curve for vasopressin and bradykinin was obtained for each vessel. After this vessels were allocated to one of the three experimental groups: indomethacin alone, indomethacin and L-NAME or control.

8.3.12: Effect of plasma incubation on the CRC for acetylcholine obtained from omental resistance vessels:

In order to confirm that the observed effects of plasma and VEGF were not limited to the myometrium, experiments were initially designed to investigate the effect of plasma on omental vessels. It was felt that if plasma demonstrated a response, then VEGF responses would be investigated. In Chapter 7 it was demonstrated that omental vessels from women with pre-eclampsia lost endothelium dependent relaxation to both acetylcholine. Omental vessels were collected from normal pregnant women (n=6) as described in Chapter 2. One vessel was dissected out in cold PSS, this was then divided into three separate sections. These were then stored for 16 hours at 4^0C in separate sterilin tubes (Chapter 2). A member of the technical staff then added 2% plasma from either normal pregnant women or women with pre-eclampsia to two of the sterilin tubes; the third vessel section was also stored over 16 hours without plasma. After incubation vessels were mounted and experimented on as described in Chapter 3.

8.4 Results:

Table 8.1and 8.3 detail the demographics and vessel characteristics of the non-pregnant, pregnant controls and the pre-eclampsia group. Table 8.2 and 8.4 detail the patient demographics for the plasma samples used. As anticipated, systolic and diastolic blood pressure were significantly greater in the group of women with pre-eclampsia than the control pregnant or non-pregnant groups.

8.4.1: The direct effect of VEGF on myometrial resistance vessels:

The addition of vasopressin to myometrial vessels produced a dose dependent contraction. Although the vessels utilised had demonstrated a constrictor response, the incremental addition of VEGF to these vessels produced no demonstrable response (Fig 8.1).

The addition of incremental doses of VEGF to pregnant vessels caused non significant relaxation in vasopressin constricted vessels. However, the addition of VEGF to non-pregnant vessels caused a vasodilatation of these vessels (Fig 8.2).

8.4.2: The effect of incubation with vascular endothelial growth factor and placental growth factor on endothelium dependent resistance artery behaviour from non-pregnant and pregnant women.

Myometrial vessels from normal pregnant women which were incubated with 5 nmol VEGF showed significantly increased responses to vasopressin over control vessels (ANOVA: P=0.02)

Patient demographic details for myometrial vessels from non-pregnant and normal pregnant women

Table 8.1

	Age (years)	BMI (kg/m ^{2})	Parity	Gestation at delivery (days)	Mean Arterial Pressure Booking mm Hg	Mean Arterial Pressure Maximum mm Hg	Protein g/dl	Individualised Birth-weight Ratio
Non - pregnant	36.0 (35.0 - 40.0)	25.4 (22.8 - 30.0)	2 (1.0- 2.0)		87.0 (83.5 - 90.8)			
Normal pregnant	30 (28.0 - 32.0)	25.4 (22.8 - 30.0)	1 (1.0 - 2.0)	269.5 (267.8 - 273.0)	83.6 (79.9 - 90.0)	87.5 (82.7 - 94.0)	0	54.0 (33.0 - 81.0)

Data are summarised as medians (Inter Quartile Ranges)

Patient demographic details for plasma from normal pregnant women and women with pre-eclampsia

Table 8.2

Study Details	Plasma Type	Age (years)	Gestation at delivery (days)	Mean Arterial Pressure Booking mm Hg	Mean Arterial Pressure Maximum mm Hg	Protein g/dl	Individualised Birth-weight Ratio
Effects of incubation with plasma	normal pregnant	25 (24 - 34)	264 (256 - 273)	88 (86.5 - 88)	96.3 (89.2 - 98.3)	0 (0.0 - 0.11)	12 (11 - 76)
	pre-eclamptic	29 (25.5 - 30.7)	253 (245.7 - 262.3)	83.5 (77.8 - 89.7)	115 (113 - 130)	1.43 (1.1 - 1.5)	24 (2.7 - 39.3)
Effects of incubation with antibodies to VEGF	normal pregnant	19.5 (16.5 - 21.8)	246 (239.2 - 249)	88.6 (78 - 91)	86.0 (79.4 - 91.1)	0.0 (0.0 - 0.0)	46.5 (41.2 - 63)
	pre-eclamptic	29 (27.5 - 29)	243 (238 - 255.5)	75 (73.5 - 86.7)	111.5 (108.3 - 113.3)	1.1 (0.7 - 1.7)	1.3 (0 - 3.5)
Effect of incubation with Flt-1 antagonist	normal pregnant	26.9 (22 - 31.3)	246 (227.3 - 261.8)	81.7 (74.2 - 86.7)	90 (80 - 93.3)	0	57 (41 - 84)
	pre-eclamptic	28.5 (23.3 - 30)	249 (234.8 - 261.8)	82.83 (76.7 - 95)	113.33 (110.8 - 123.3)	1.1 (0.9 - 1.6)	13 (2.25 - 24)

Data are summarised as medians (Inter Quartile Ranges)

Patient demographic details for omental vessels from normal pregnant women

Table 8.3

	Age (years)	BMI (kg/m ^{2})	Parity	Gestation at delivery (days)	Mean Arterial Pressure Booking mm Hg	Mean Arterial Pressure Maximum mm Hg	Protein g/dl	Individualised Birth-weight Ratio
Normal pregnant	20 (30.5 - 38.5)	26.4 (24.0 - 32.3)	1 (0.5 - 1.0)	271.0 (268.5 - 272.5)	87.6 (80.0 - 89.3)	90.0 (82.1 - 95.3)	0	56.0 (29.0 - 64.0)

Data are summarised as medians (Inter Quartile Ranges)

Patient demographic details for plasma from normal pregnant women and women with pre-eclampsia

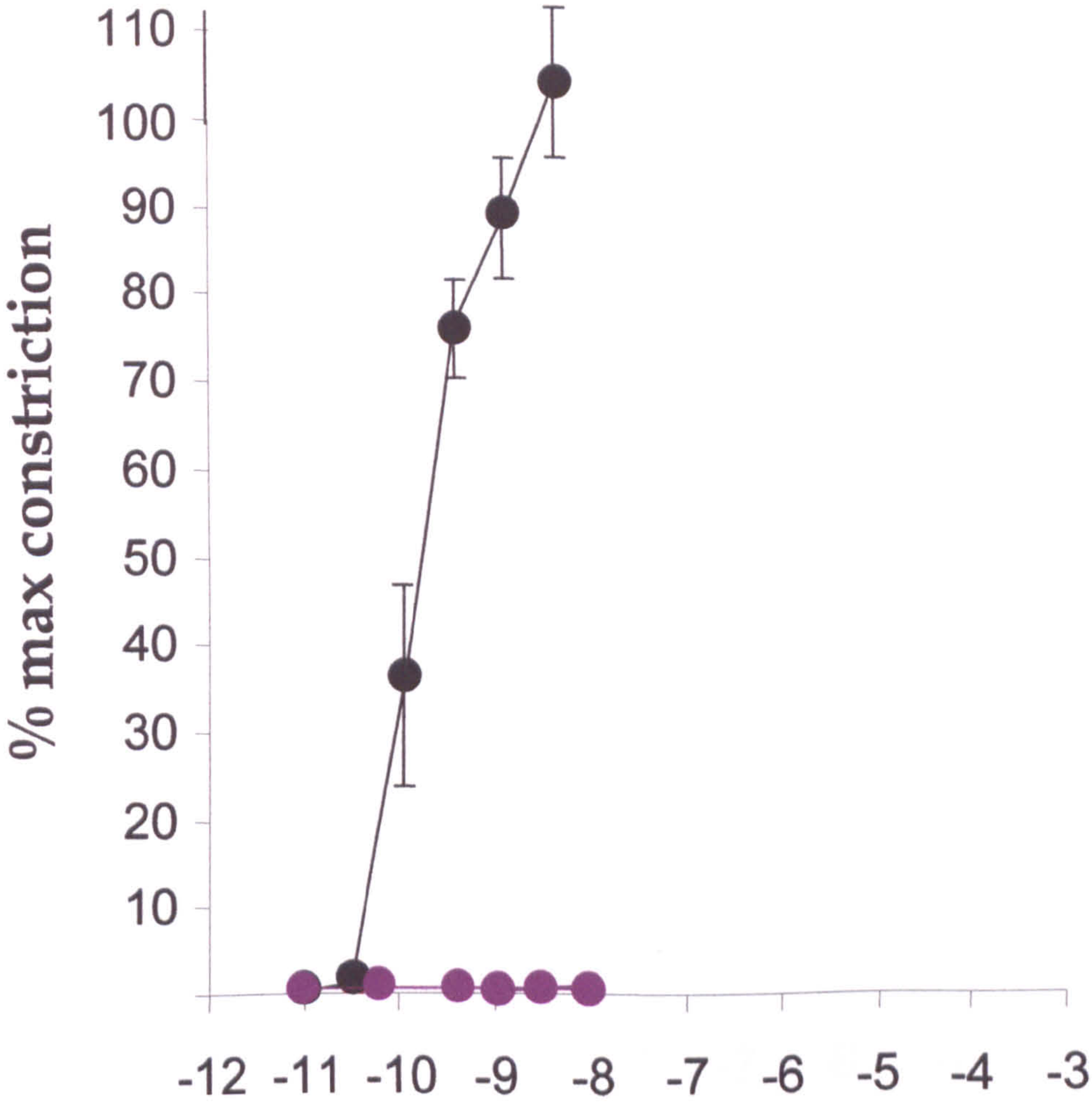
Table 8.4

Study Details	Plasma Type	Age (years)	Gestation at delivery (days)	Mean Arterial Pressure Booking mm Hg	Mean Arterial Pressure Maximum mm Hg	Protein g/dl	Individualised Birth-weight Ratio
Effect of plasma on vessel behaviour following incubation n = 8	normal pregnant	25.0 (24.7 – 34.0)	264.0 (256.2 – 273.5)	88.1 (86.5 - 88)	96.3 (89.2 - 98.3)	0.0 (0.0 - 0.11)	12.3 (11.0 – 76.9)
	pre-eclamptic	29 (25.5 - 30.7)	253.0 (245.7 - 262.3)	83.5 (77.8 - 89.7)	115.1 (113.7 – 130.2)	1.43 (1.05 - 1.55)	24 (2.75 - 39.3)

Data are summarised as medians (Inter Quartile Ranges)

Fig 8.1

The direct constrictor effect of VEGF on human pregnant myometrial resistance vessels.



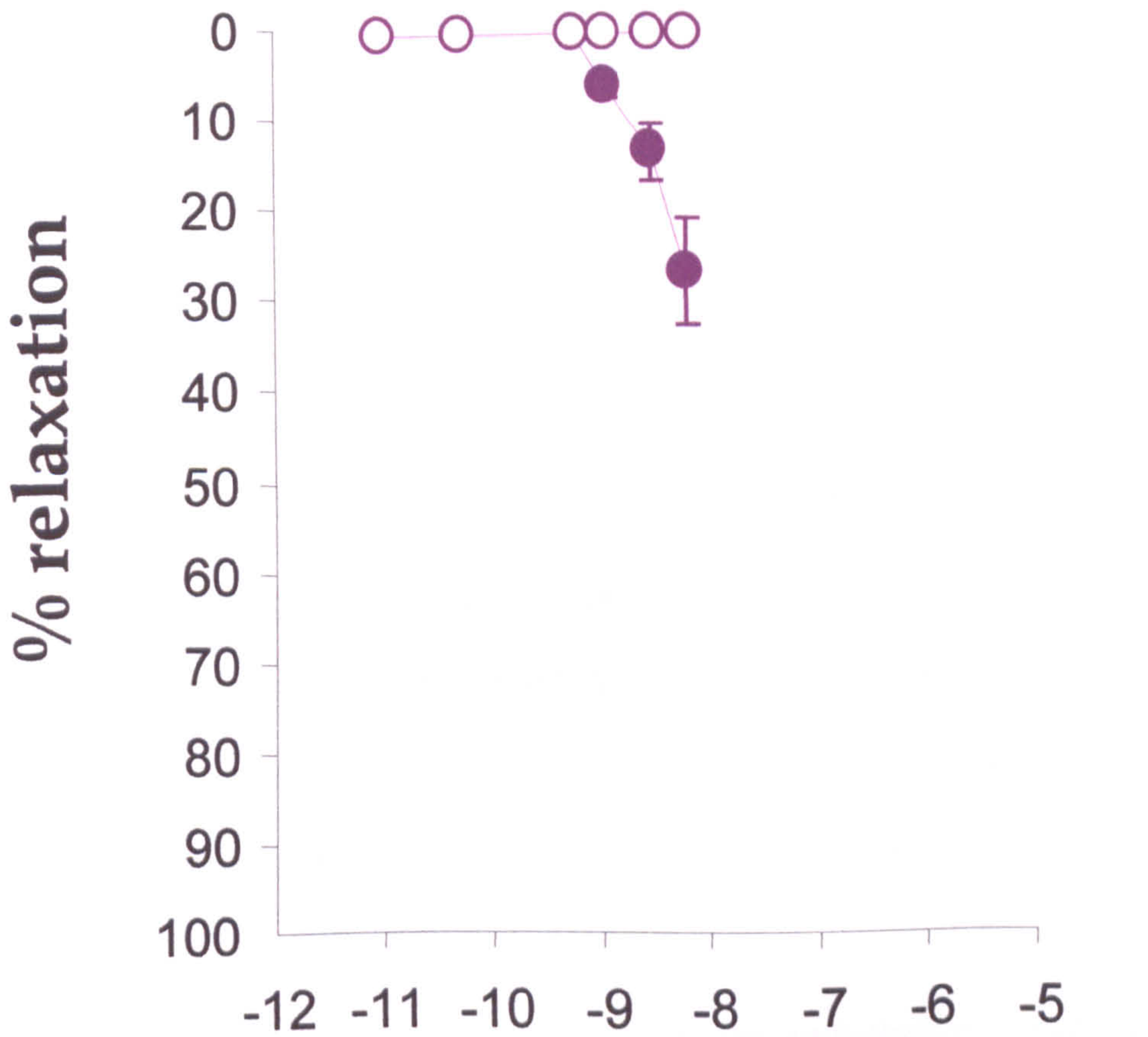
KEY:

VEGF curve

Vasopressin curve

Fig 8.2

The CRC curves obtained to incremental doses of VEGF on myometrial vessels, from non-pregnant women and pregnant women, contracted with vasopressin



KEY:

Pregnant

Non-pregnant

Statistical comparisons:

	E_{max}
Pregnant	0.0+/-0.0
Non-pregnant	24.6+/-5.3

t-Test (unpaired) P= 0.0001

(Figure 8.3). Sub-analysis of the data, revealed that the observed increase, occurred only in the maximum contraction obtained (E_{max} P=0.04) and there was no shift in the EC_{50} value (-9.5+/-0.1 Vs –9.3+/-0.2: P=0.4). In contrast, vessels from non-pregnant women, which were exposed to VEGF exhibited no differences in their contractile responses when compared to control vessels (P=0.92) (Figure 8.4).

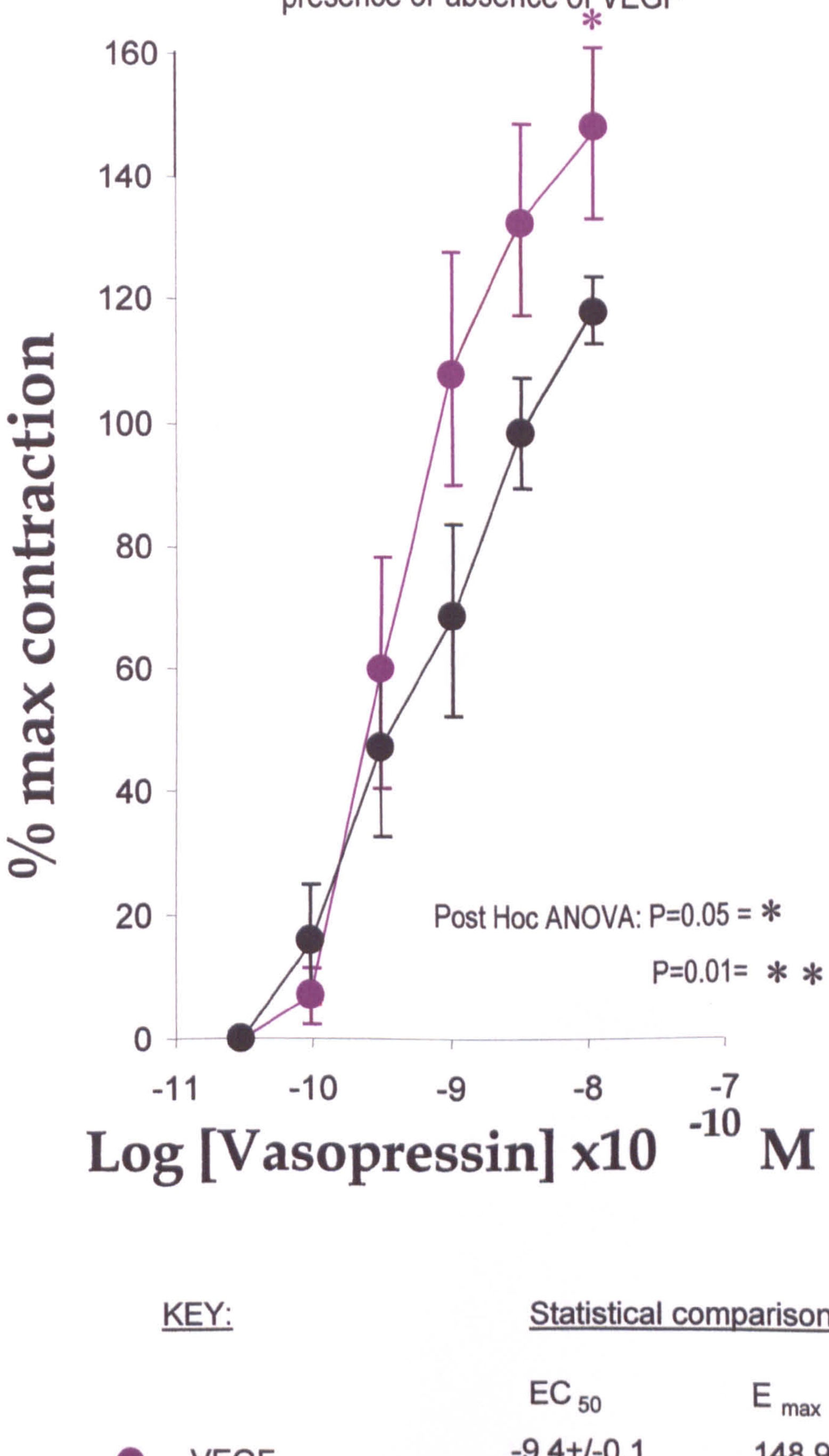
A significant reduction was also noted in bradykinin-induced endothelium dependent relaxation in vessels incubated with 5 nmol VEGF ($p < 0.0001$ Figure 8.5). Once again the observed effect of VEGF was demonstrated to be pregnancy specific; no differences were found between non-pregnant myometrial vessels incubated with VEGF and controls (P=0.85) (Figure 8.6). The addition of PlGF to either pregnant or non-pregnant vessels produced no differences in bradykinin-induced endothelium dependent relaxation (Figure 8.7 and 8.8).

8.4.3: The effect of concentration of vascular endothelial growth factor on endothelium dependent resistance artery behaviour:

VEGF was found to act in a dose dependent manner; a significant reduction in bradykinin-induced endothelium dependent relaxation was found in vessels incubated with 5 nmol and 0.5 nmol VEGF when compared with vessels incubated in 0.05 nmol VEGF and without VEGF ($p< 0.01$). There was no significant difference between the effects of the 5 nmol and 0.5 nmol concentrations, nor differences between the effects of 0.05 nmol concentration and no VEGF (Figure 8.9).

The effect on the vasopressin CRC from pregnant myometrial vessels after overnight incubation in the presence or absence of VEGF

Fig 8.3



KEY:

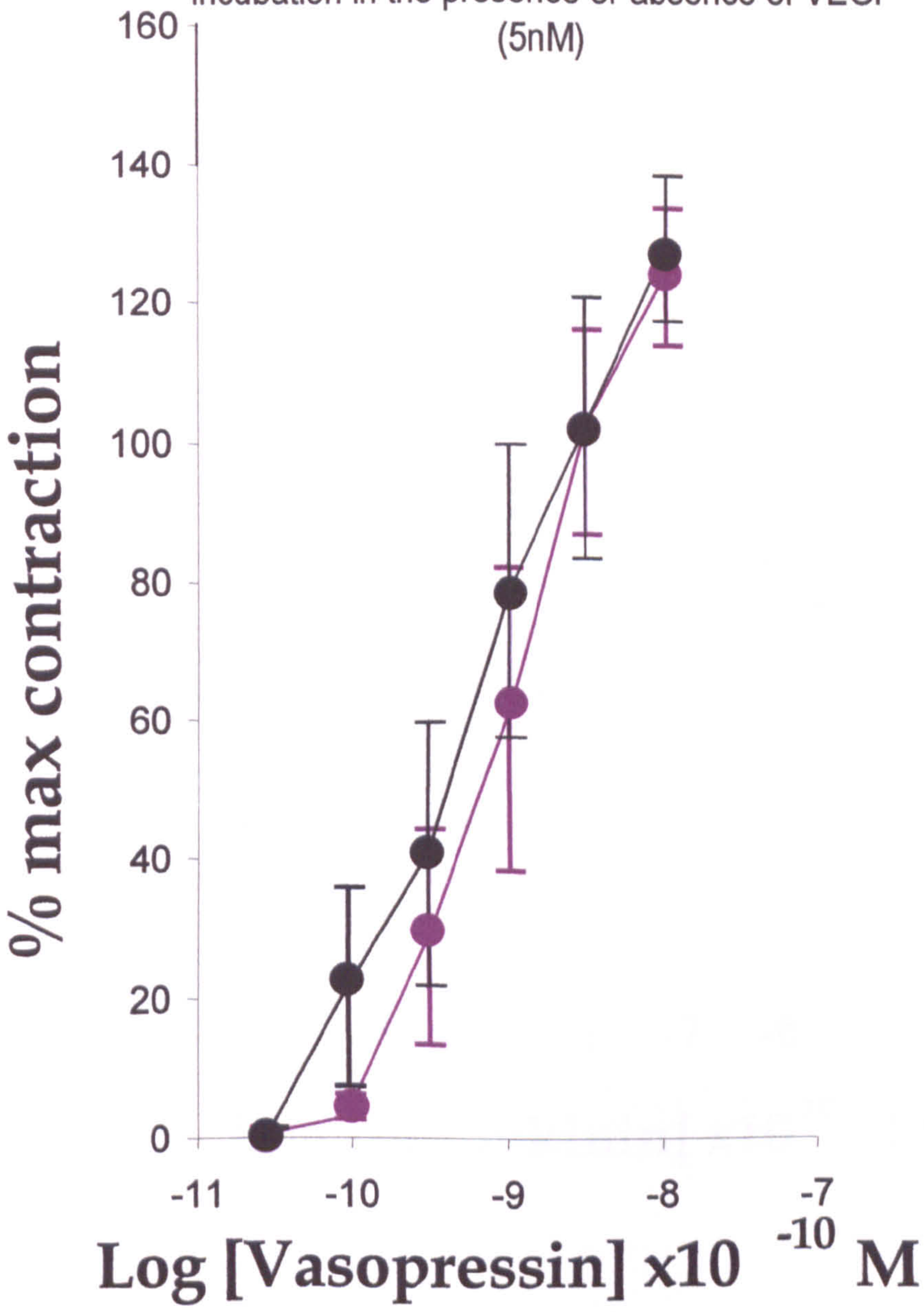
- VEGF
- Control

Statistical comparisons:

	EC_{50}	E_{max}
VEGF	-9.4+/-0.1	148.9+/-14.3
Control	-9.2+/-0.2	117.2+/-5.5
t-Test (unpaired) P=	0.41	0.04
ANOVA (repeated measures) P=		0.02

Fig 8.4

The effect on the vasopressin CRC from non-pregnant myometrial vessels after overnight incubation in the presence or absence of VEGF (5nM)



KEY:

VEGF

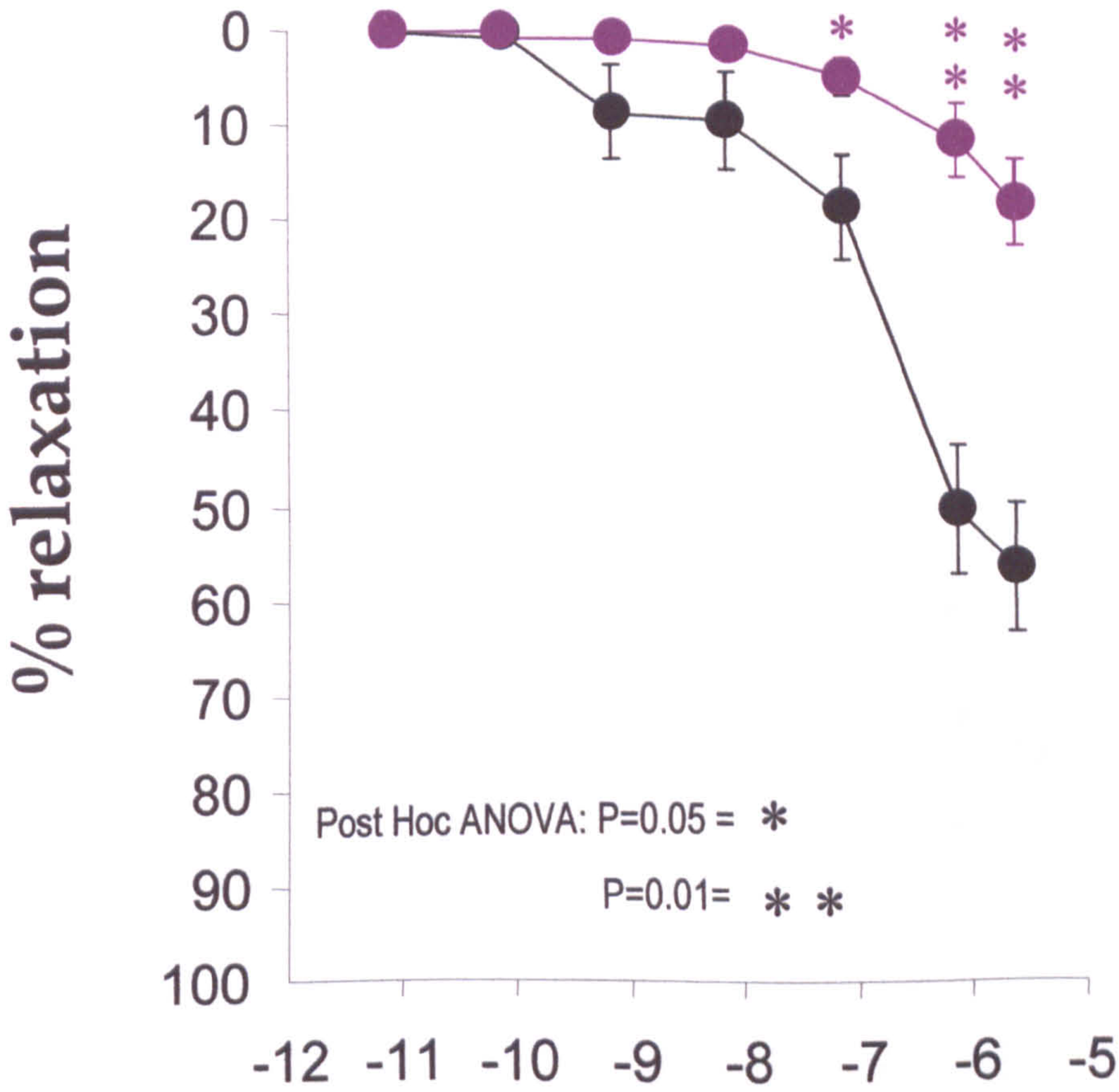
Control

Statistical comparisons:

	EC_{50}	E_{max}
VEGF	-9.2+/-0.2	122.8+/-9.7
Control	-9.2+/-0.3	126.9+/-10.3
t-Test (unpaired) P=	0.89	0.78
ANOVA (repeated measures) P=		0.92

Fig 8.5

The effect on the bradykinin CRC from pregnant myometrial vessels after overnight incubation in the presence or absence of VEGF (5nM)



KEY:

VEGF

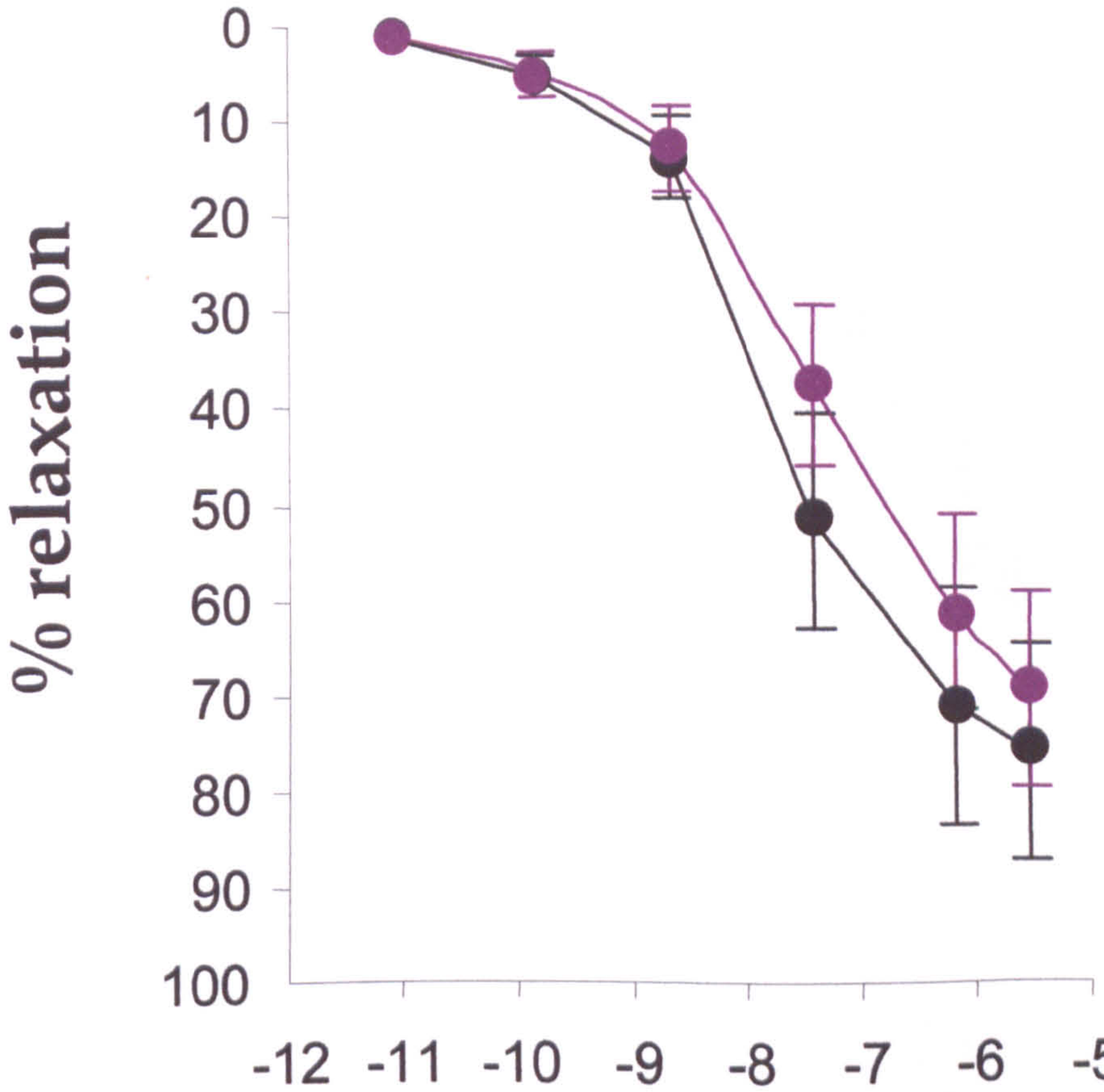
Control

Statistical comparisons:

	E_{max}
	18.4+/-4.5
	56.1+/-6.5
t-Test (unpaired) P=	0.0001
ANOVA (repeated measures) P=	0.0001

Fig 8.6

The effect on the bradykinin CRC from non-pregnant myometrial vessels after overnight incubation in the presence or absence of VEGF (5nM)



KEY:

VEGF

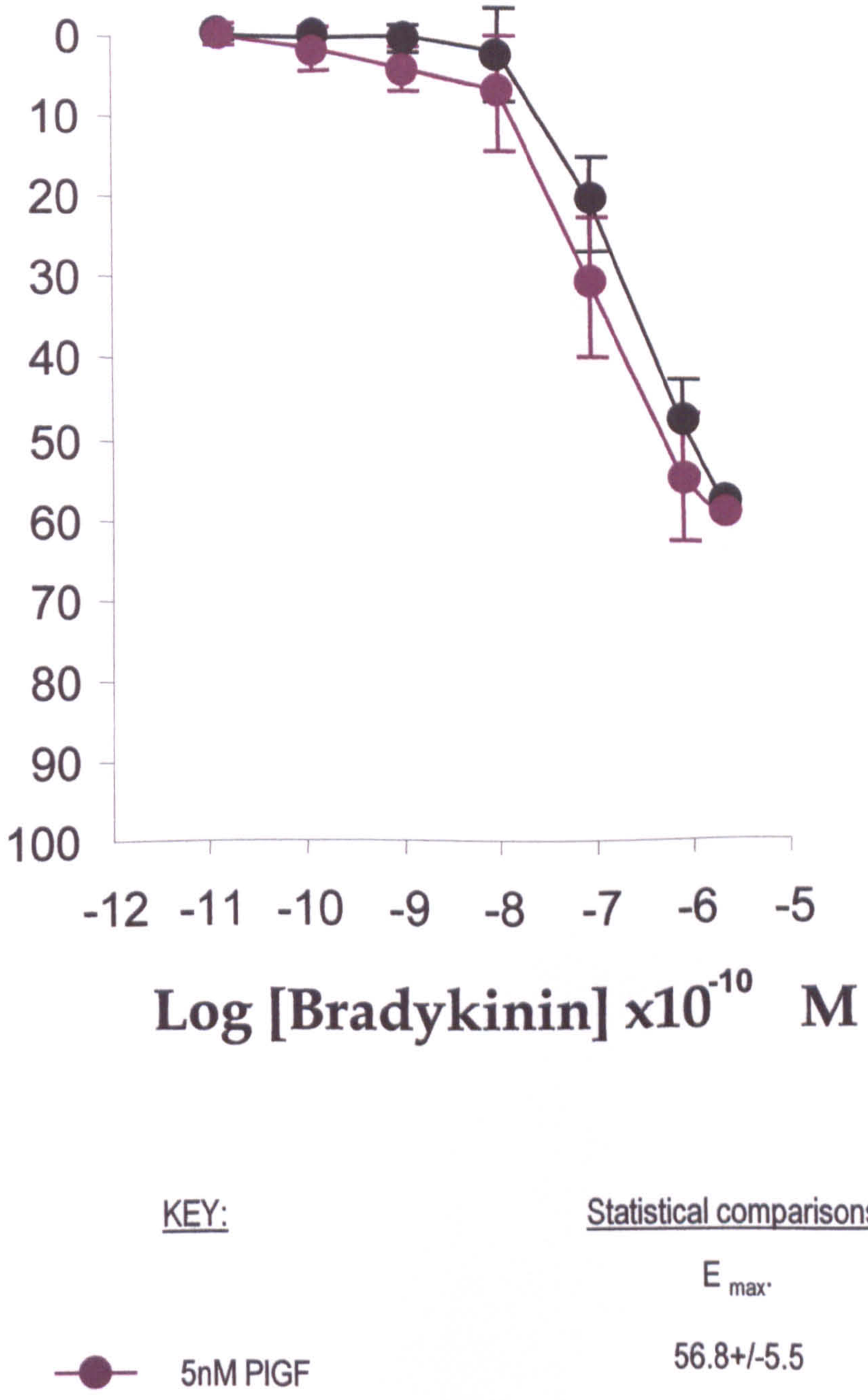
Control

Statistical comparisons:

	E_{max}
	74.7+/-11.2
	68.3+/-10.2
t-Test (unpaired) P=	0.68
ANOVA (repeated measures) P=	0.85

Fig 8.7

Effect on the bradykinin CRC obtained from pregnant myometrial resistance vessels after overnight incubation in the presence or absence of PLGF(5nM) .



KEY:

5nM PIGF

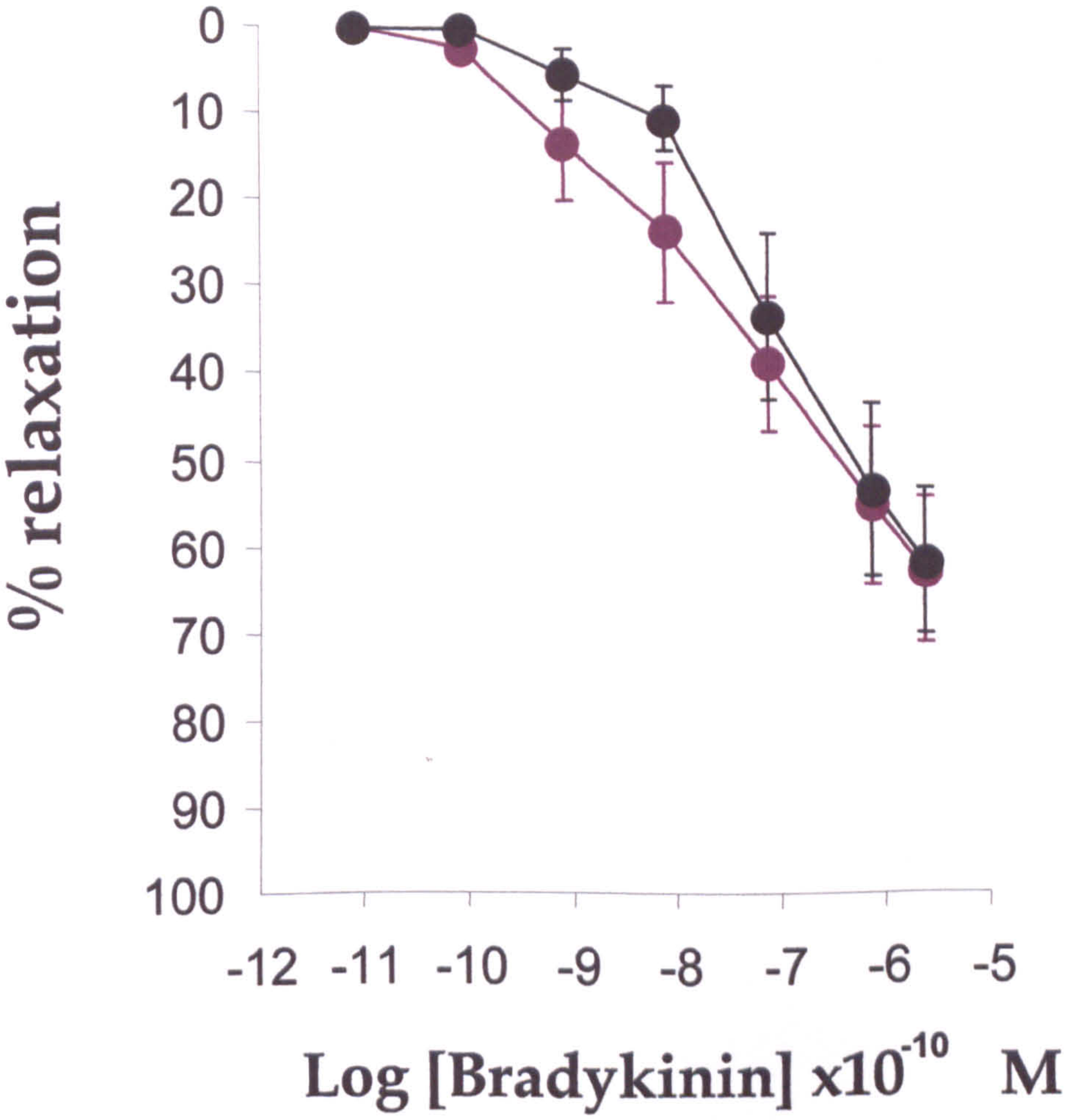
Control

Statistical comparisons:

	E_{max}
	56.8+/-5.5
	58.3+/-7.6
t-Test (unpaired) P=	0.87
ANOVA (repeated measures) P=	0.86

Fig 8.8

Effect on the bradykinin CRC obtained from non-pregnant myometrial resistance vessels after overnight incubation in the presence or absence of PLGF (5nM).



KEY:

5nM PlGF

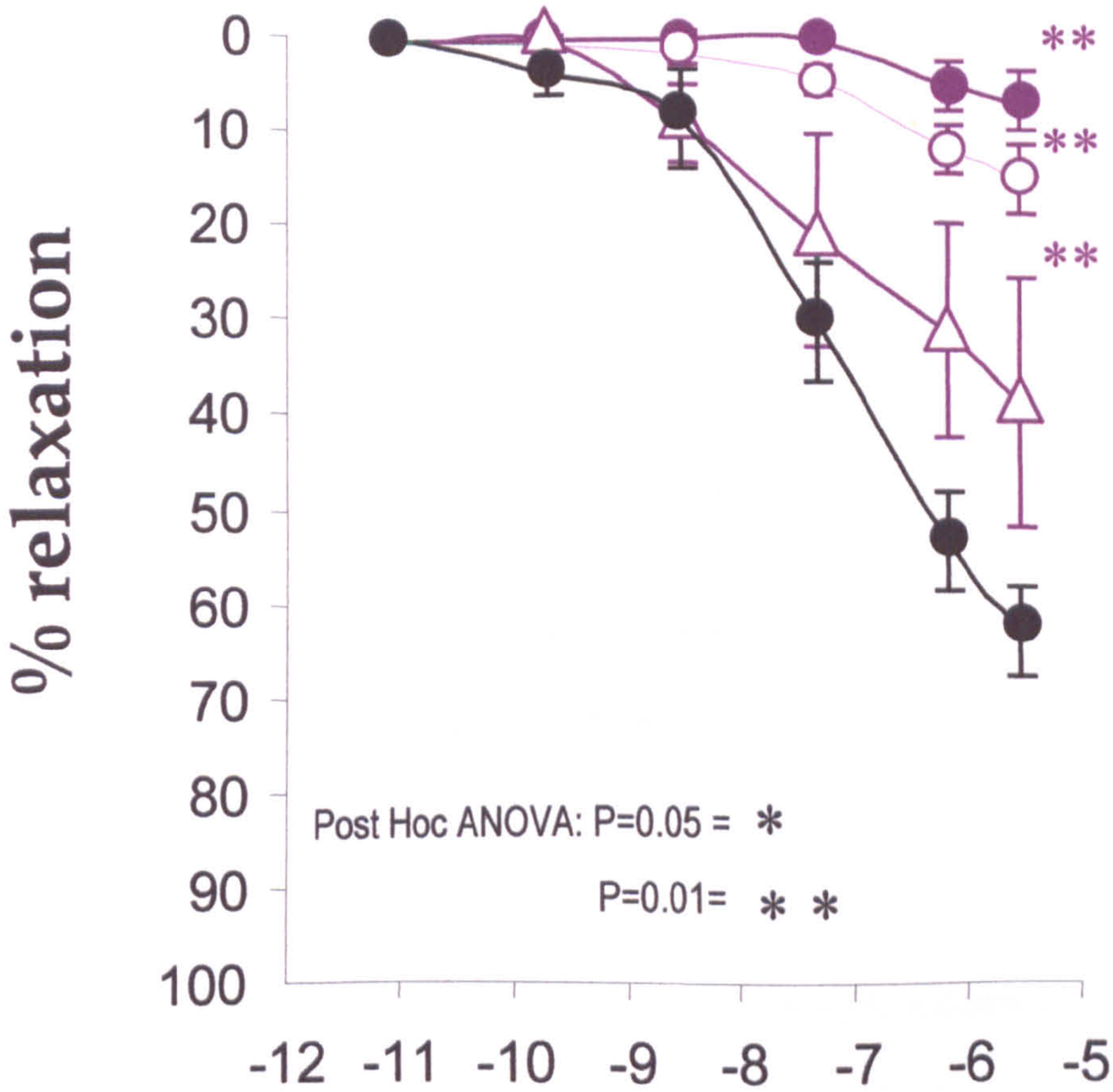
Control

Statistical comparisons:

	E_{max}
	63.8+/-8.5
	62.8+/-8.5
t-Test (unpaired) P=	0.94
ANOVA (repeated measures) P=	0.47

Fig 8.9

The effect of different concentrations of VEGF on bradykinin CRC from myometrial vessels from pregnant women after overnight incubation with VEGF.



KEY:

VEGF 5 nM

VEGF 0.5 nM

VEGF 0.05 nM

Control

Statistical comparisons:

	E_{max}
	6.3+/-2.0
	13.1+/-3.2
	34.3+/-11.6
	55.7+/-4.2
t-Test (unpaired) P=	0.0001
ANOVA (repeated measures) P=	0.0001

8.4.4: The effect of time of incubation on vascular endothelial growth factor on endothelium dependent resistance artery behaviour:

VEGF was found to act in a time dependent manner, with a significant reduction in endothelial dependent relaxation only occurring after sixteen-hour periods of incubation (Figure 8.10).

8.4.5: The effect of parity on vascular endothelial growth factors' effect on endothelium dependent resistance artery behaviour.

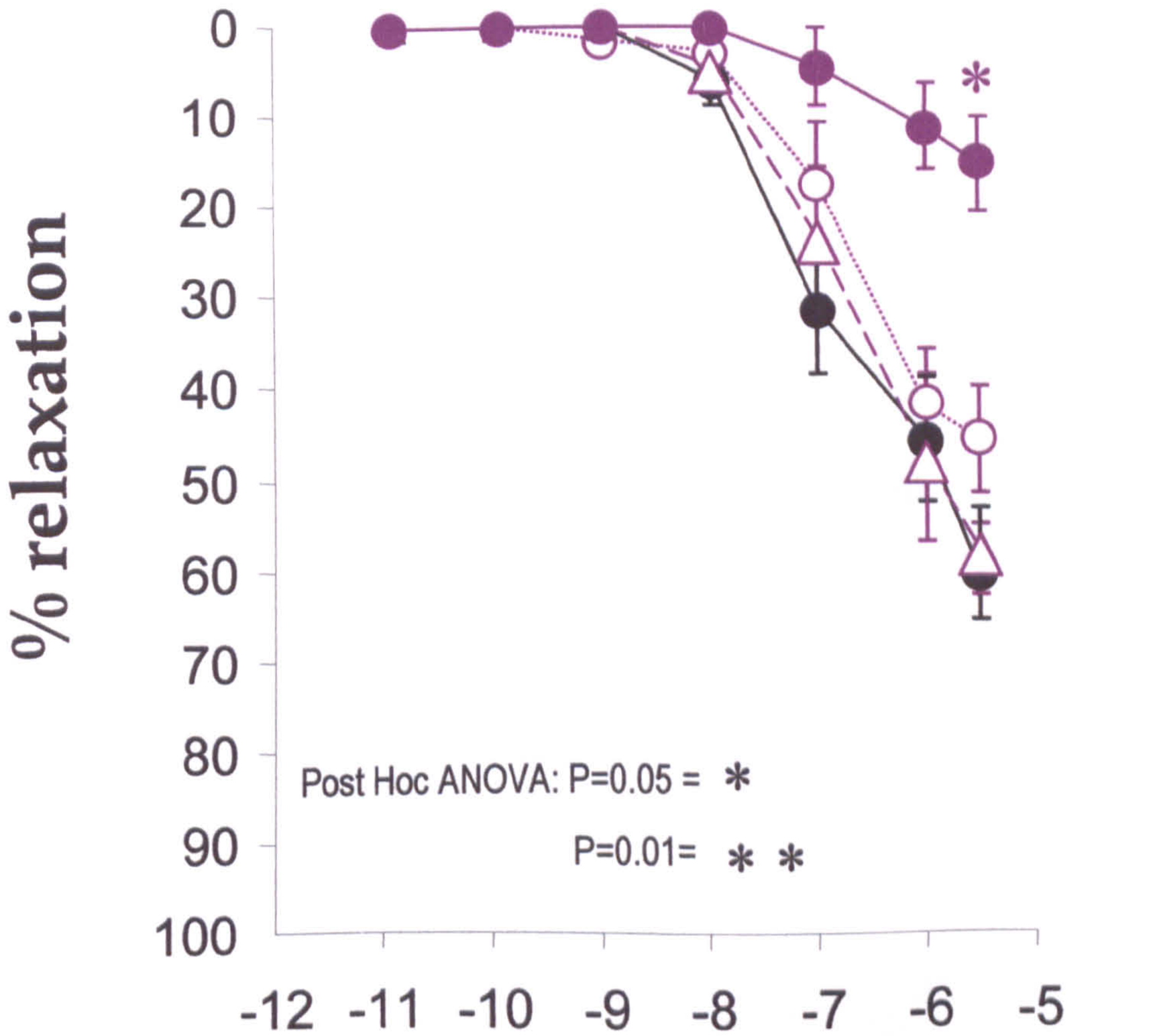
The observed effect of VEGF on pregnant myometrial resistance vessel behaviour was independent of the parity of the patients from whom the vessels were taken (Figure 8.11 and 8.12).

8.4.6 The effect of repeated washing of vessels after incubation with vascular endothelial growth factor:

The effect of incubation of vessels with VEGF was reversible; the endothelium-dependent relaxation to bradykinin being restored following sequential washing of VEGF from the vessels and repeated concentration response curves to bradykinin (Fig 8.13).

Fig 8.10

The effect of different VEGF incubation times on the bradykinin CRC for myometrial vessels from pregnant women.



KEY:

VEGF -16 hours

VEGF - 2 hours

VEGF - 1 hour

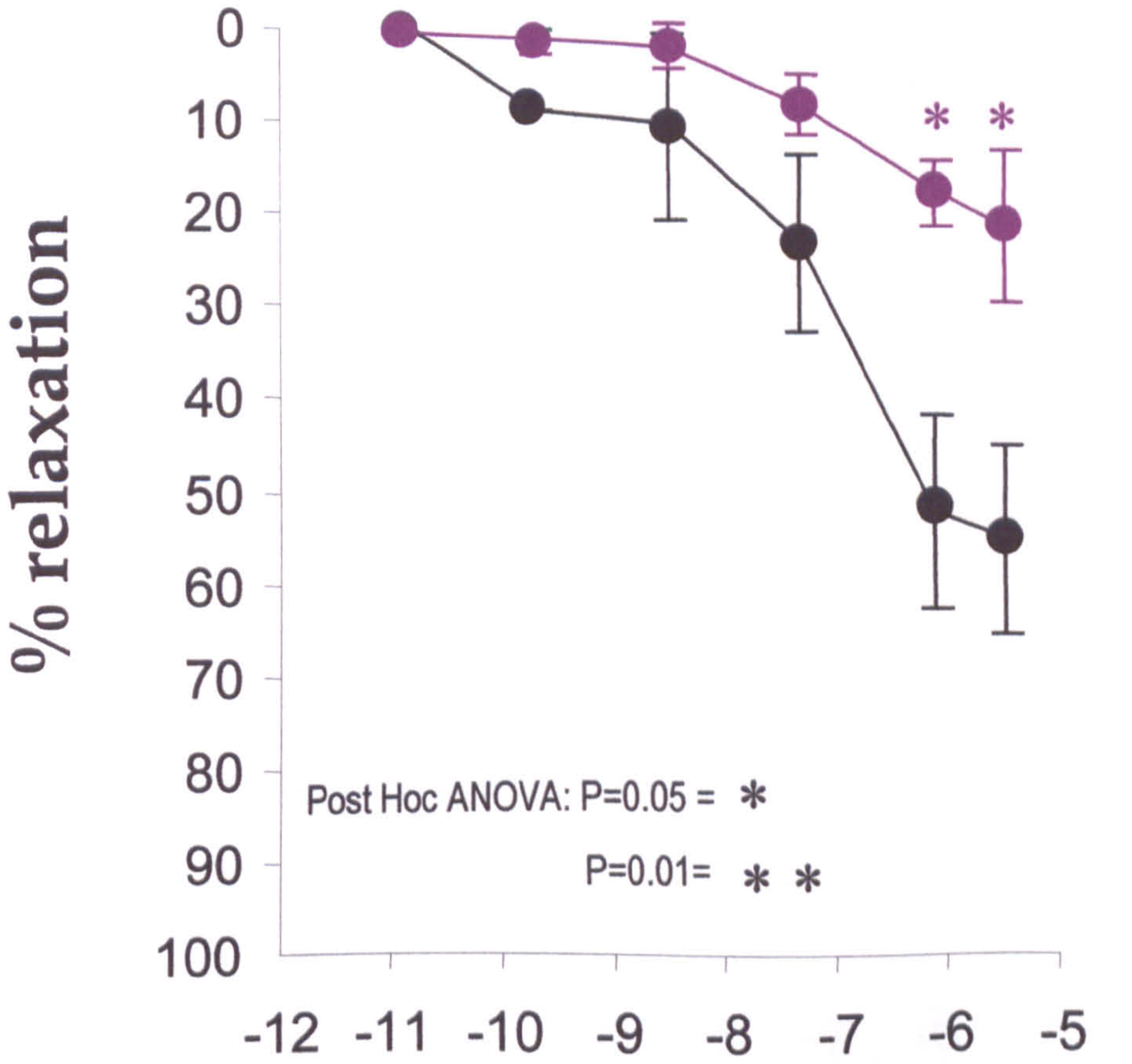
Control

Statistical comparisons:

	E_{max}	
	16.8+/-5.2	
	56.3+/-4.7	
	44.3+/-5.6	
	58.6+/-5.2	
t-Test (unpaired) P=		0.0001
ANOVA (repeated measures) P=		0.0001

Fig 8.11

The effect on the bradykinin CRC from myometrial vessels from multiparous pregnant after overnight incubation in the presence or absence of VEGF (5nM)



KEY:

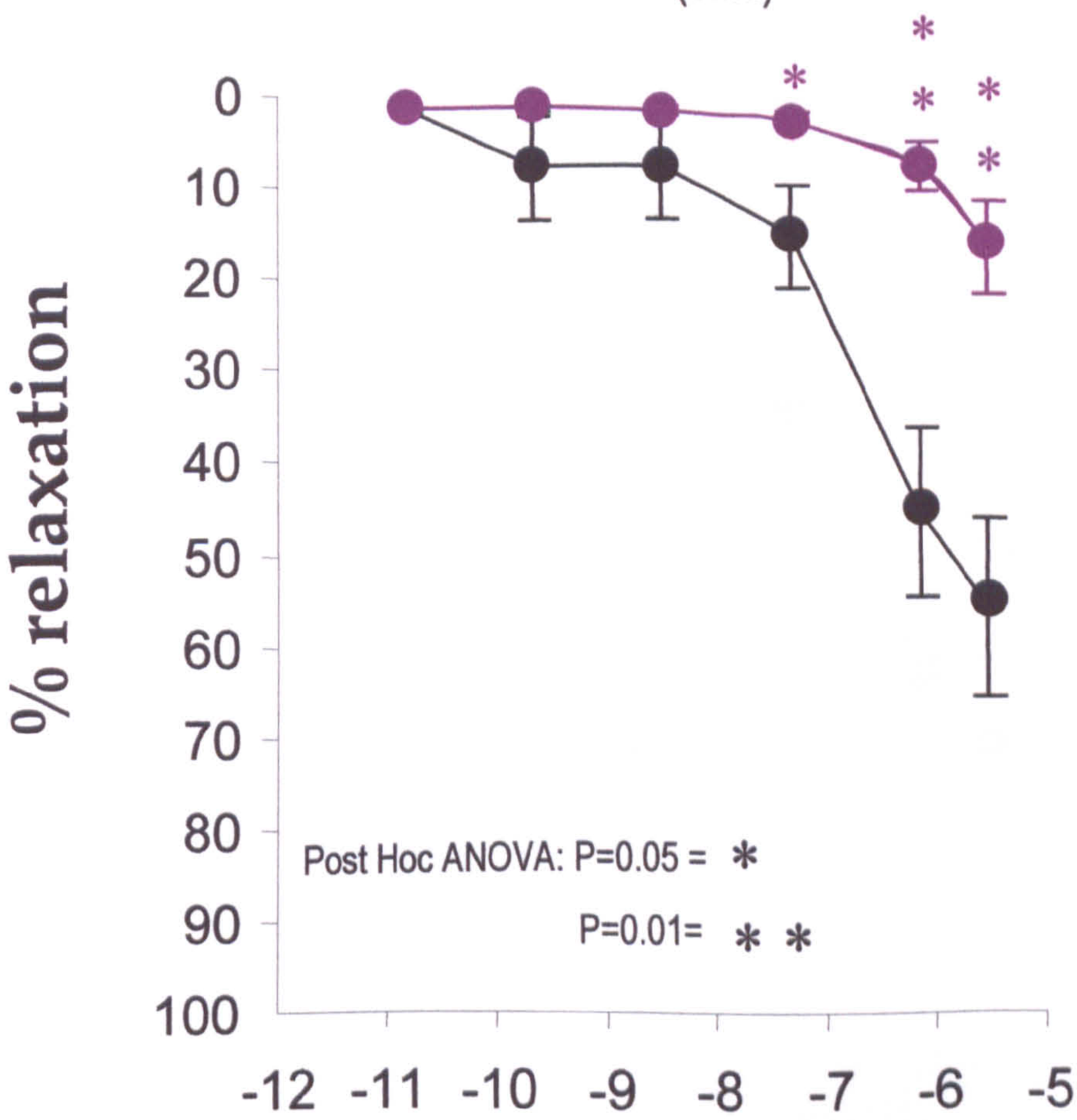
	E_{max}
VEGF	23.7+/-7.6
Control	56.3+/-9.8

Statistical comparisons:

t-Test (unpaired) P=	0.02
ANOVA (repeated measures) P=	0.004

The effect on the bradykinin CRC from myometrial vessels from nulliparous pregnant women after overnight incubation in the presence or absence of VEGF (5nM)

Fig 8.12



KEY:

VEGF

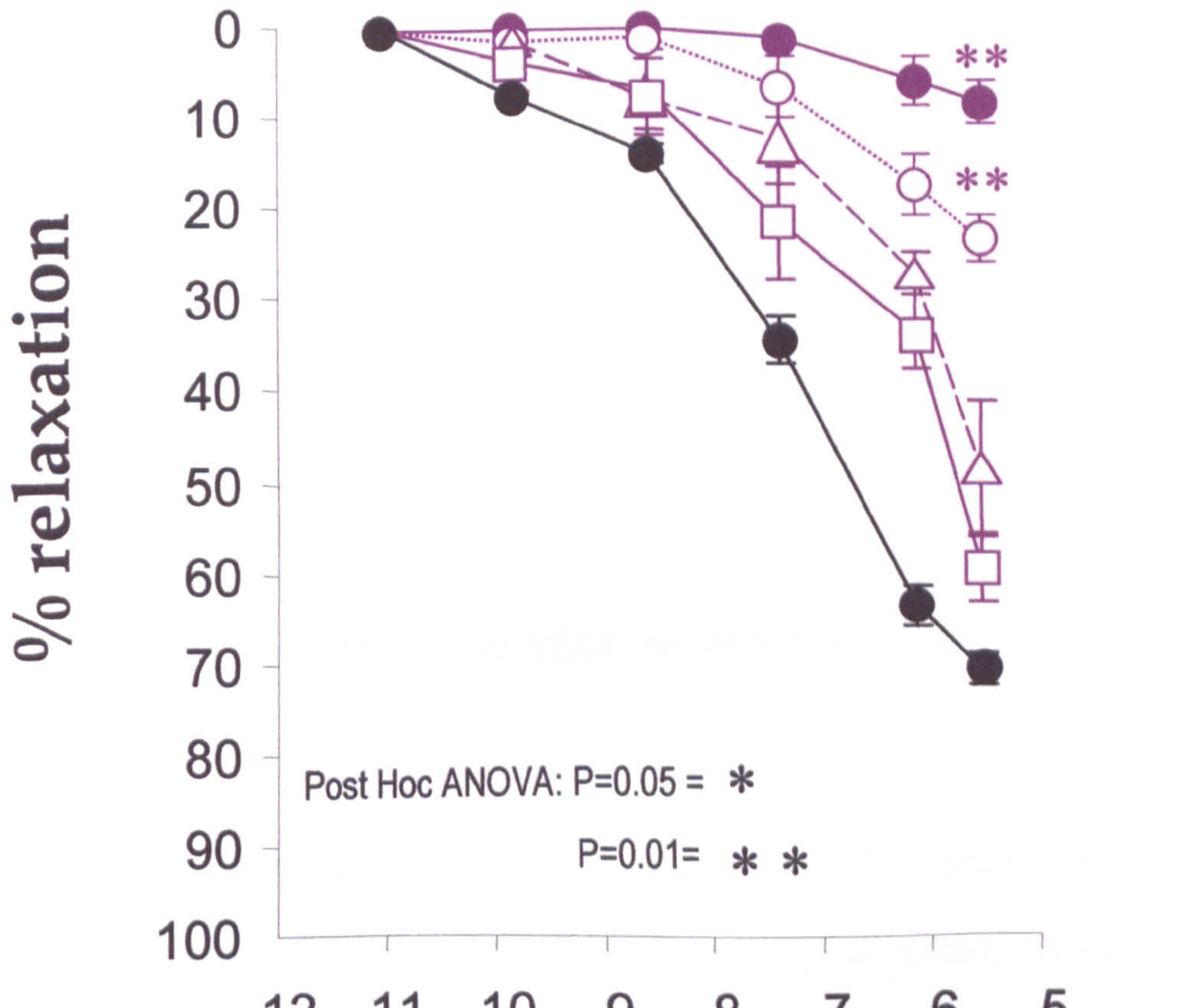
Control

Statistical comparisons:

	E_{max}
	15.4+/-5.20
	54.9+/-10.0
t-Test (unpaired) P=	0.004
ANOVA (repeated measures) P=	0.0001

Fig 8.13

The effect of repeated CRC on the bradykinin mediated relaxation after incubation of myometrial vessels from pregnant women with VEGF (5nM) compared to controls



KEY:

- Relaxation curve one
- Relaxation curve two
- Relaxation curve three
- Relaxation curve four
- Control

Statistical comparisons:

	$E_{max.}$
	8.00+/2.3
	23.0+2.5
	47.8+/-3.7
	58.0+/-3.7
	69.6+/-1.5
t-Test (unpaired) P=	0.0001
ANOVA (repeated measures) P=	0.0001

8.4.7: The effect of incubation with plasma from women with pre-eclampsia on endothelium dependent resistance artery behaviour.

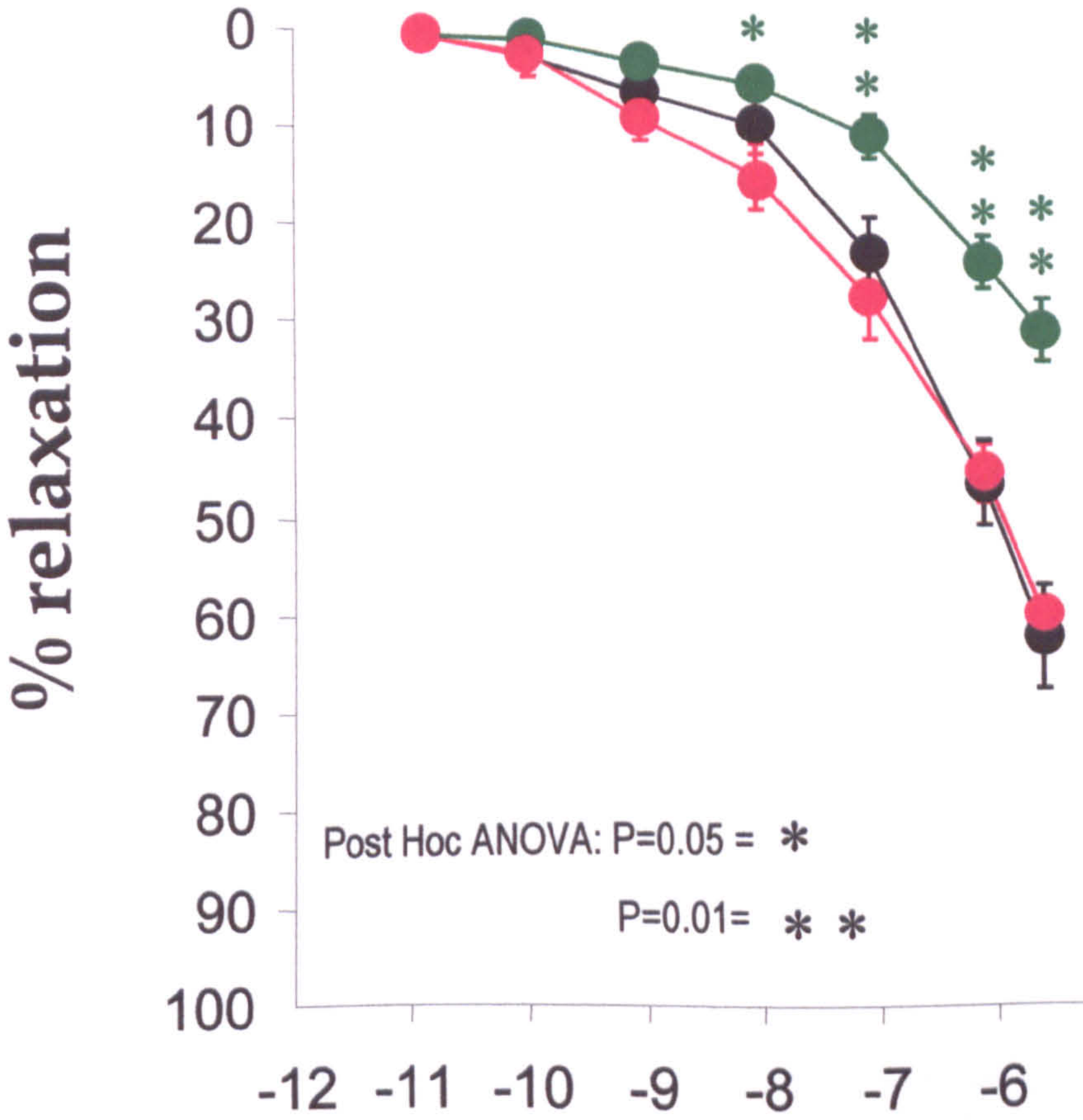
As noted previously (Ashworth et al., 1998), there was a significant reduction in endothelium-dependent relaxation when vessels were incubated with plasma from women with pre-eclampsia as compared with vessels incubated in 2% plasma from normal pregnant women or without plasma ($P < 0.0001$) (Fig 8.14).

8.4.8: The effect of antibody to VEGF on endothelium dependent resistance artery behaviour:

Incubation of vessels with both 0.5 nmol VEGF and 2% plasma from women with pre-eclampsia resulted in a marked attenuation of relaxation when compared to control vessels and those incubated with 2% plasma from normal pregnant women ($p<0.0001$) (Fig 8.15 and 8.16). There was no significant difference between the relaxation noted in those vessels incubated in 2% plasma from women with pre-eclampsia and those incubated in 0.5 nmol VEGF. The effect of VEGF / plasma from women with pre-eclampsia was significantly altered following the addition of VEGF antibody, such that vessel incubated with 2% plasma from women with pre-eclampsia and those incubated with 0.5 nmol VEGF exhibited a degree of endothelium dependent relaxation that approached that seen in vessel incubated in 2% plasma from normal pregnant women (Fig 8.15 and 8.16).

Fig 8.14

The effect on the bradykinin CRC for myometrial vessels from pregnant women incubated overnight with plasma from controls and women with pre-eclampsia



KEY:

- 2% plasma from women with pre-eclampsia
- 2% plasma from control women
- Control : no plasma

Statistical comparisons:

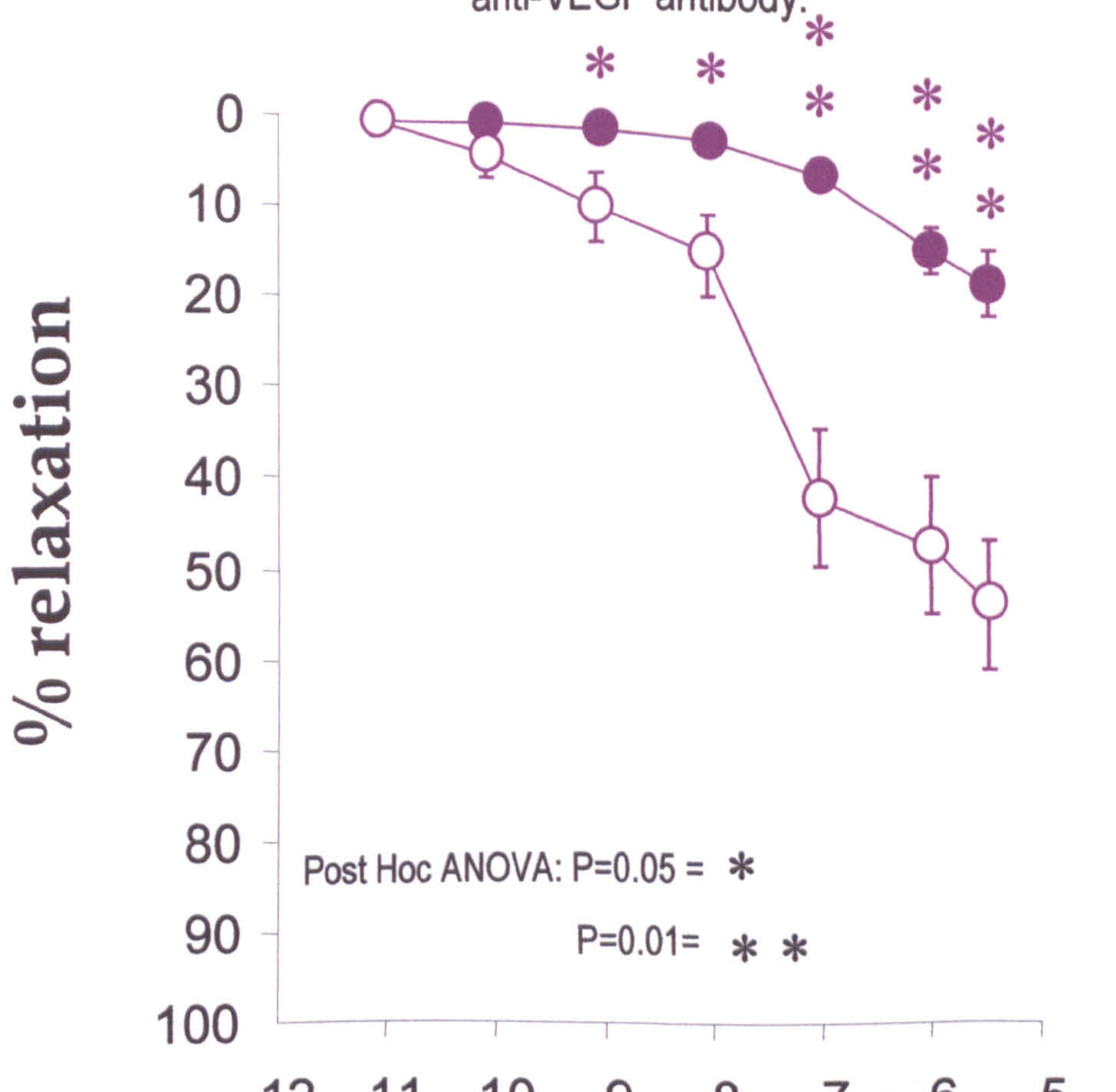
	E_{max}
2% plasma from women with pre-eclampsia	31.3+/-3.4
2% plasma from control women	60.0+/-3.0
Control : no plasma	63.0 +/-5.2

ANOVA (factorial) P= 0.0001

ANOVA (repeated measures) P= 0.0001

Fig 8.15

The effect on the bradykinin CRC after overnight incubation of myometrial vessels from pregnant women with VEGF in the presence or absence of anti-VEGF antibody.



KEY:

0.5nM VEGF

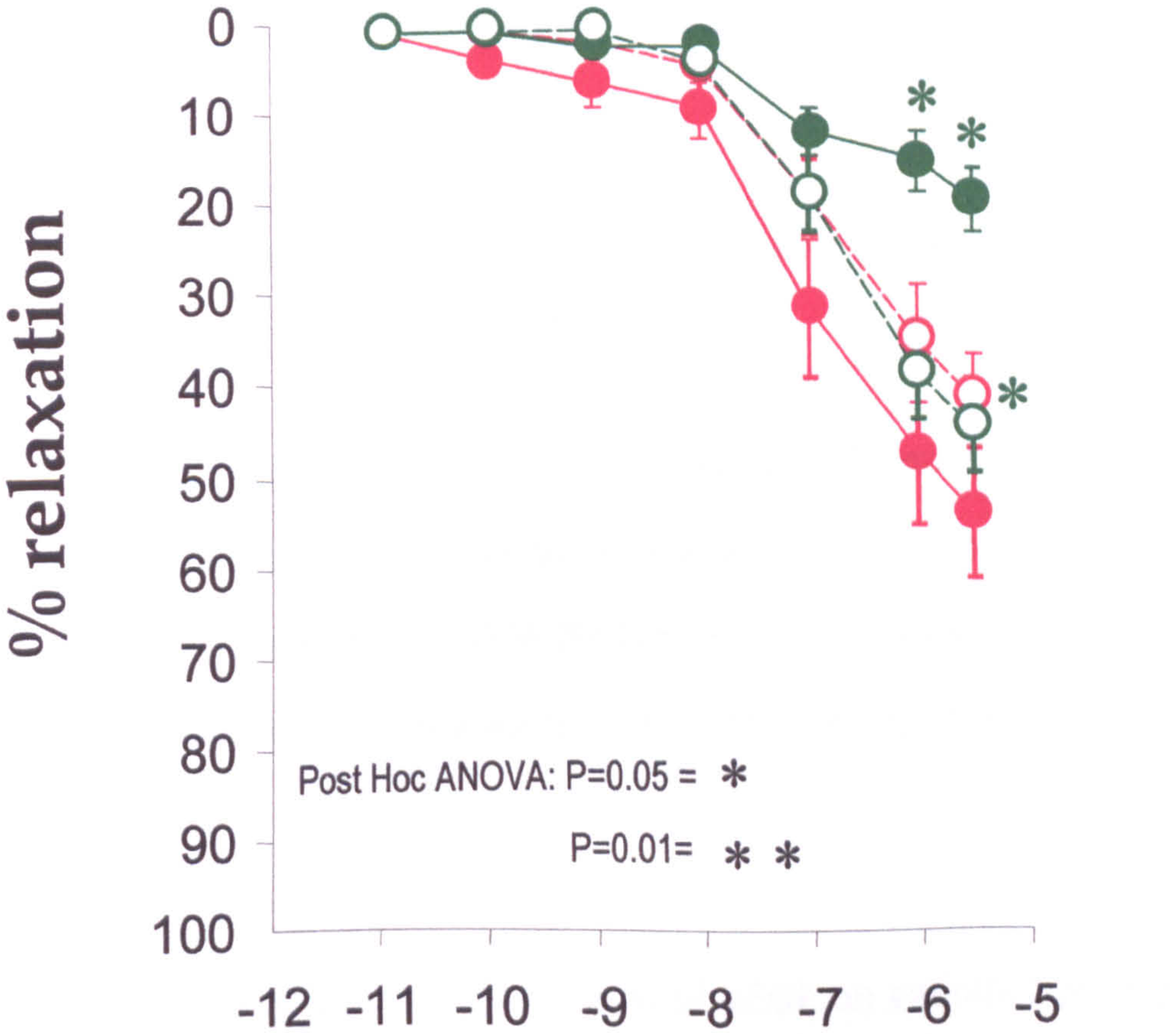
0.5nM VEGF + antibodies to VEGF

Statistical comparisons:

	E_{max}
	14.5+/-5.7
	56.9+/-11.9
t-Test (unpaired) P=	0.01
ANOVA (repeated measures) P=	0.0001

Fig 8.16

The effect of overnight incubation of myometrial vessels from pregnant women with plasma from women with pre-eclampsia and controls in the presence and absence of a anti-VEGF antibody.



KEY:

- 2% normotensive plasma
- 2% normotensive plasma + VEGF antibody
- 2% pre-eclamptic plasma
- 2% pre-eclamptic plasma + VEGF antibody

Statistical comparisons:

	E_{max}
	53.8+/-7.4
	41.3+/-5.2
	19.0+/-3.8
	44.5+/-4.8
ANOVA(factorial) P=	0.0004
ANOVA (repeated measures) P=	0.0001

There was no significant alteration in the degree of endothelium dependent relaxation in vessels incubated in 2% plasma from normal pregnant women, either with or without the addition of antibody to VEGF (Figure 8.16).

8.4.9: The effect of anti-Flt-1 and anti-KDR antogonists on endothelium dependent resistance artery behaviour.

The presence of KDR receptor antibody had no effect on the VEGF response ($P>0.05$) (Fig 8.17). The presence of the anti-Flt-1 receptor antagonist returned the endothelium-dependent relaxation to normal, in spite of the presence of VEGF ($P< 0.05$) (Fig 8.18). Vessels exposed to the plasma of women with pre-eclampsia in the presence of the anti-flt-1 antibody exhibited normal relaxation (Fig 8.19).

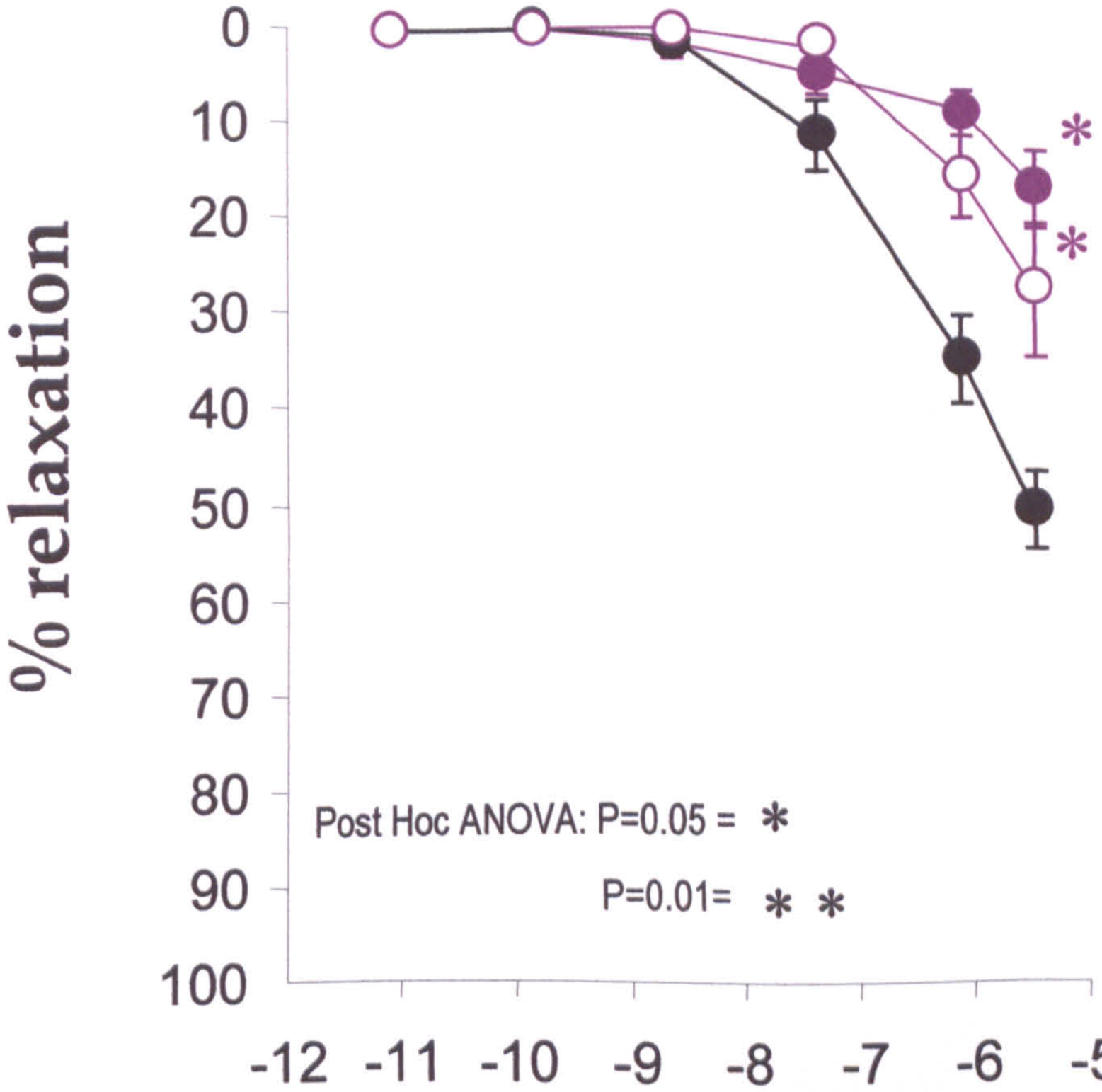
8.4.10: The effect of Dynabead® removal of VEGF on endothelium dependent resistance artery behaviour:

Incubation of vessels with both Dynal® treated plasma and 2% plasma from women with pre-eclampsia resulted in a marked attenuation of relaxation when compared to vessels incubated with 2% plasma from normal pregnant women ($p<0.0001$). There was no significant difference between the relaxation noted in those vessels incubated in 2% plasma from women with pre-eclampsia and those incubated in Dynal® treated plasma (Fig 8.20).

Dr F Anthony measured the concentration of VEGF in our samples using a radio-immuno-assay technique (RIA) (Chapter 2). Levels of VEGF were 0.16 nmol in the pooled normal pregnant

Fig 8.17

The effect of overnight incubation of myometrial vessels from pregnant women with VEGF in the presence and absence of an antibody to the KDR receptor.



KEY:

VEGF

VEGF + KDR antibody

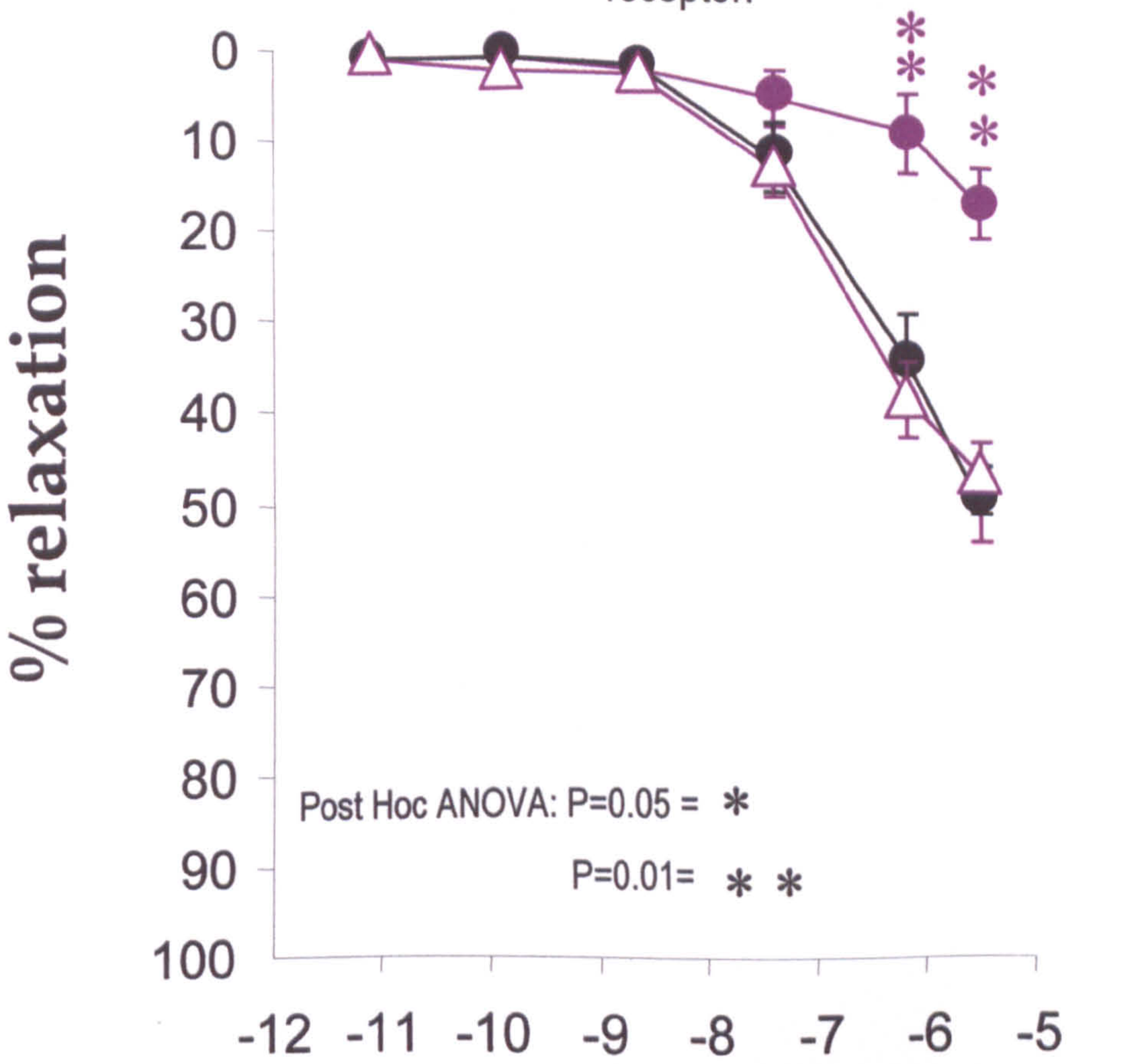
Control

Statistical comparisons:

	E_{max}
	13.0+/-2.5
	23.3+/-9.3
	58.0+/-3.7
ANOVA (factorial) P=	0.0001
ANOVA (repeated measures) P=	0.0001

Fig 8.18

The effect of overnight incubation of myometrial vessels from pregnant women with VEGF in the presence and absence of an antibody to the flt-1 receptor.



KEY:

VEGF

VEGF + flt antibody

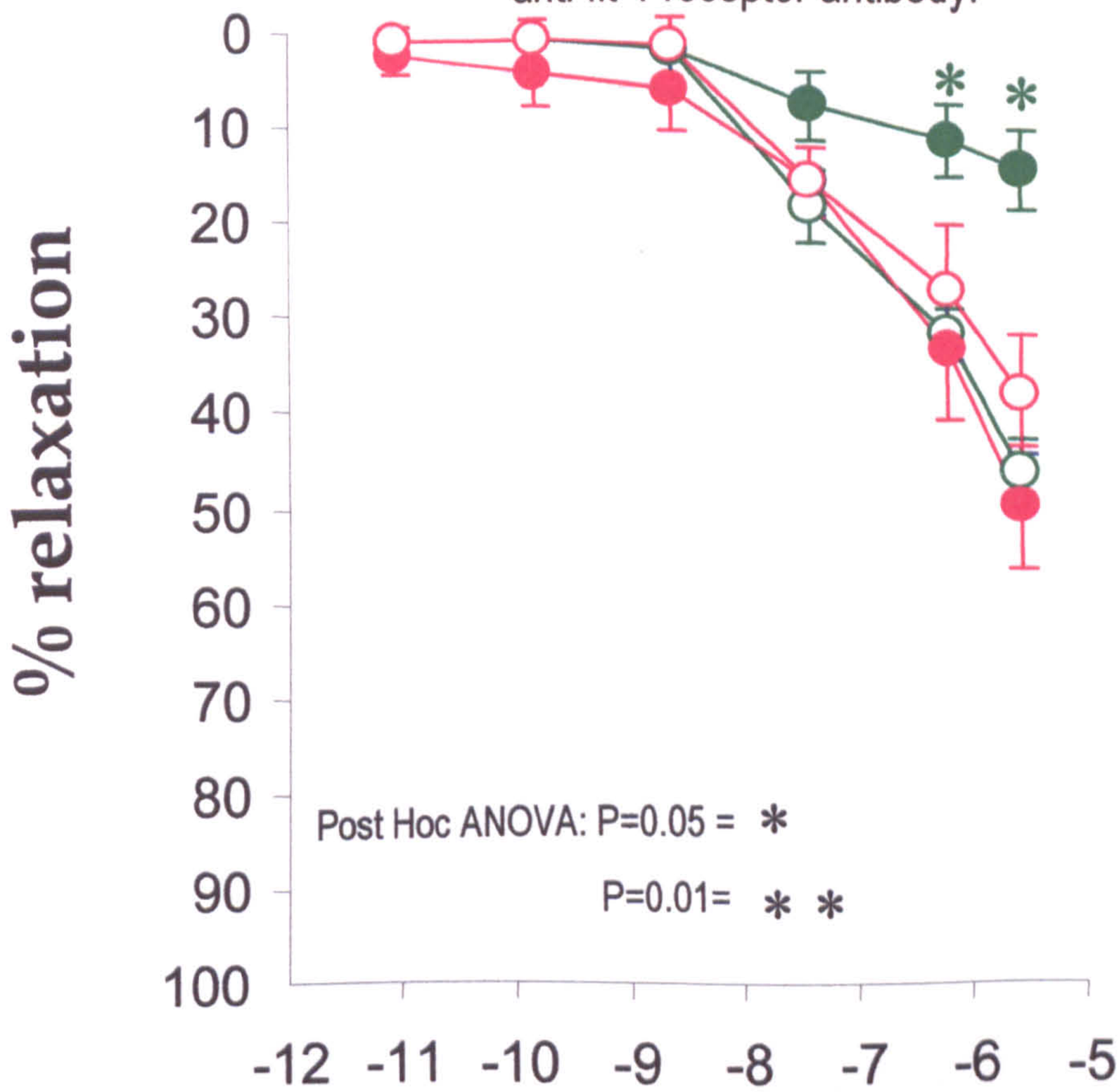
Control

Statistical comparisons:

	E_{max}
	13.0+/-2.5
	47.8+/-3.7
	58.0+/-3.7
ANOVA (factorial) P=	0.0001
ANOVA (repeated measures) P=	0.0001

Fig 8.19

The effect of overnight incubation of myometrial vessels from pregnant women with plasma from women with pre-eclampsia and controls in the presence and absence of a anti-flt-1 receptor antibody.



KEY:

PET

PET + Flt

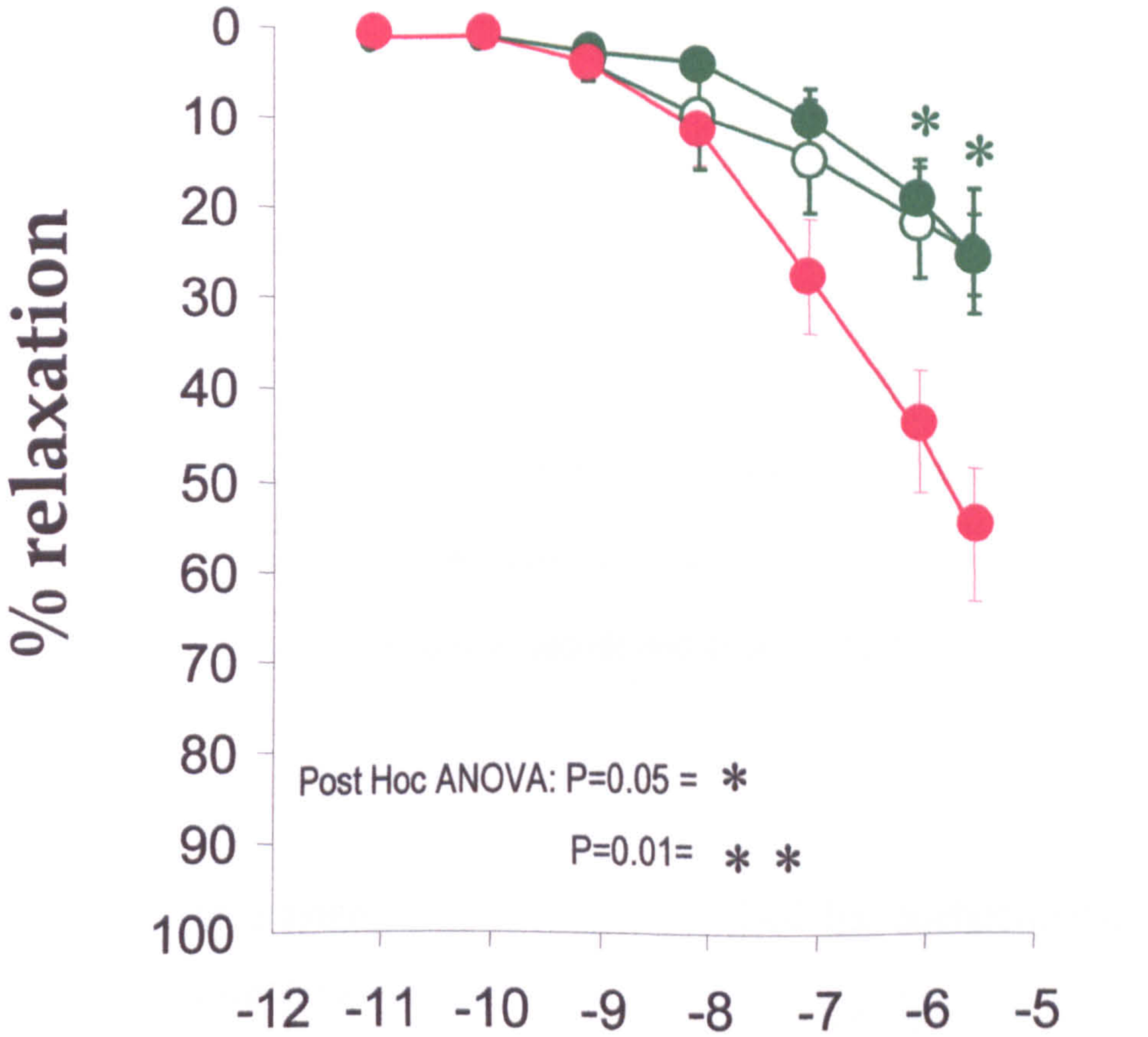
NPP

NPP + Flt

Statistical comparisons:	E_{max}
	13.3+/-4.1
	44.1+/-3.4
	47.8+/-6.0
	36.4+/-4.5
ANOVA (factorial) P=	0.0001
ANOVA (repeated measures) P=	0.0001

Fig 8.20

The effect on the bradykinin CRC from myometrial resistance from pregnant women after incubation with plasma from women with pre-eclampsia treated with Dynal beads to remove VEGF



KEY:

Dynabead PET plasma

PET

NPP

Statistical comparisons:

	E_{max}
Dynabead PET plasma	25.5+/-7.4
PET	26.0+/-4.7
NPP	58.7+/-7.6
ANOVA (factorial) P=	0.0001
ANOVA (repeated measures) P=	0.0001

plasma, 0.22 nmol in the pooled plasma from women with pre-eclampsia and 0.16 nmol in the Dynal® treated plasma.

8.3.11: Effect of Indomethacin and L-NAME on vessels incubated with VEGF and appropriate time controls:

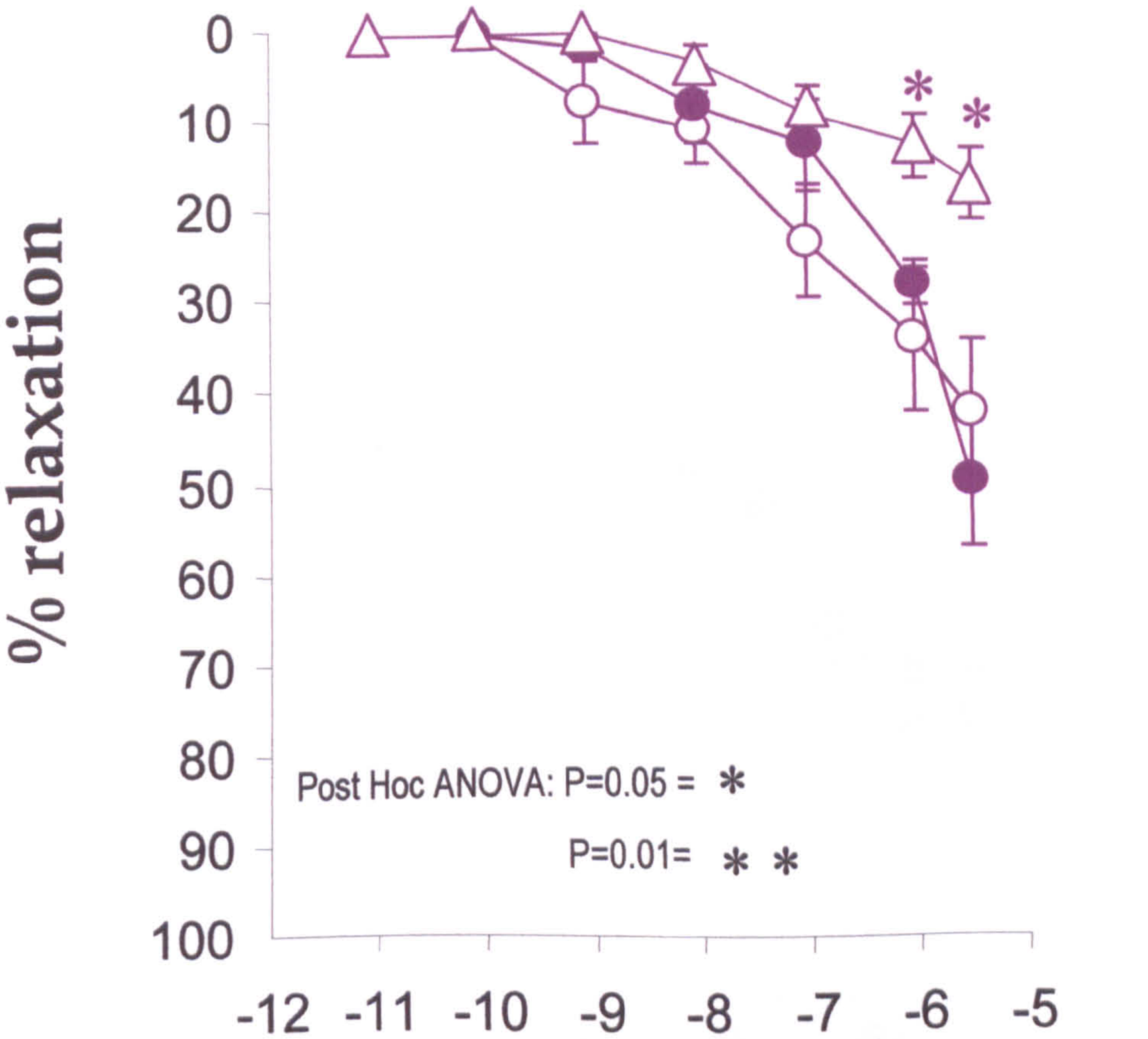
The addition of indomethacin had no significant effect on the bradykinin concentration response curve of vessels incubated with VEGF (Figure 8.21). However, the addition of indomethacin and L-NAME to vessels exposed to VEGF caused a marked decrease in the endothelium dependent relaxation when compared to both control vessels and those incubated with indomethacin (Figure 8.21)

8.3.12: Effect of plasma incubation on the CRC for acetylcholine obtained from omental resistance vessels:

When omental vessels from normal pregnant women which were incubated with 2% plasma from control pregnant women, 2% plasma from women with pre-eclampsia and no plasma were studied, there were no significant differences in theS endothelium dependent relaxation to acetylcholine between the three groups (ANOVA: P=0.83) (Fig 8.22).

Fig 8.21

The effect on the bradykinin CRC from myometrial vessels from pregnant women after overnight incubation with VEGF an then exposed to indomethacin or indomethacin and L-NAME



KEY:

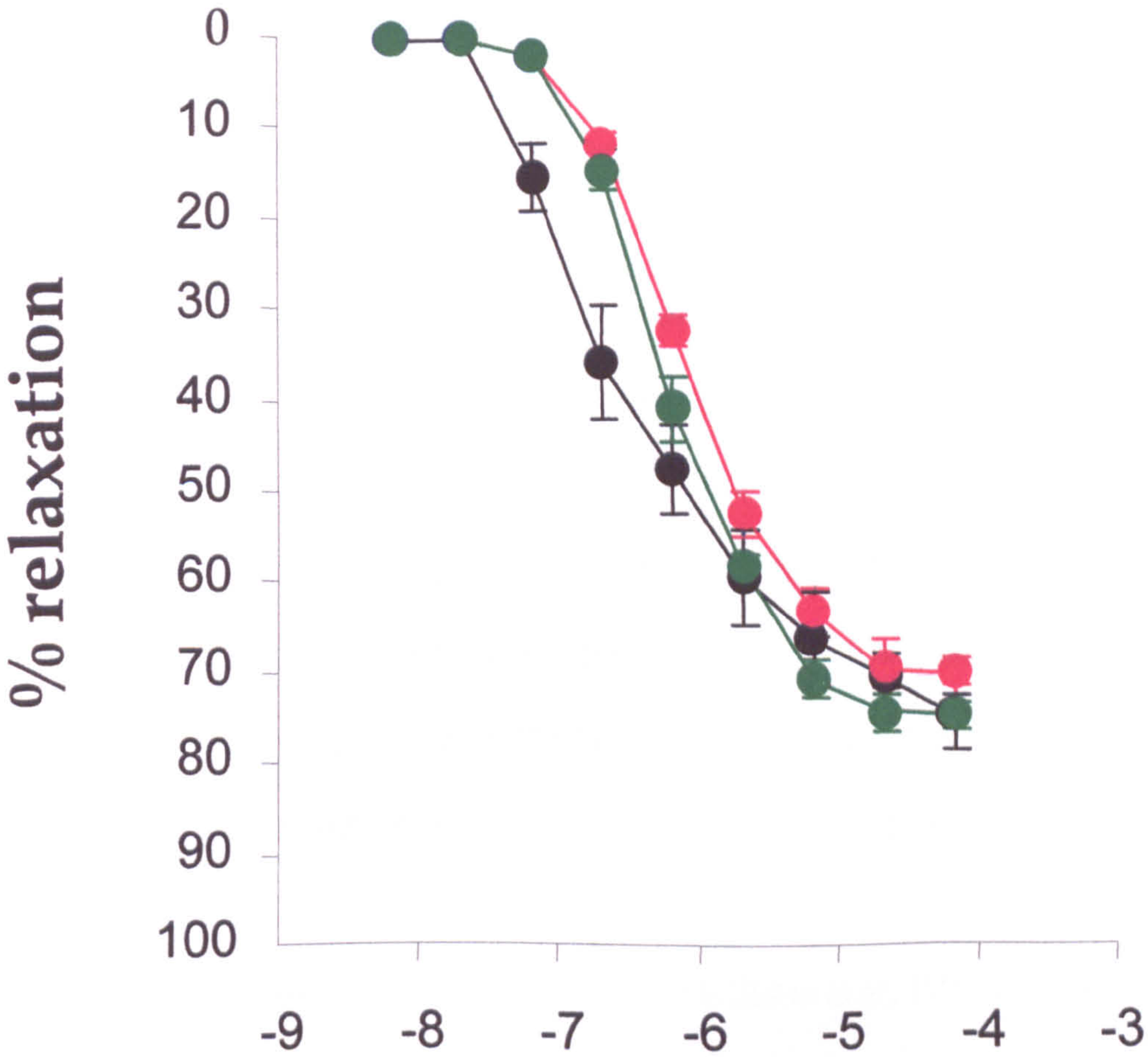
- VEGF
- VEGF+Indo
- VEGF + Indo + L-NAME

Statistical comparisons:

	E_{max}
VEGF	47.8+/-7.3
VEGF+Indo	40.5+/-7.6
VEGF + Indo + L-NAME	16.0+/-3.7
ANOVA (factorial) P=	0.001
ANOVA (repeated measures) P=	0.0001

Fig 8.22

The effect on the Acetylcholine CRC for omental vessels from pregnant women after incubating with 2% plasma for sixteen hours



KEY:

	Statistical comparisons: E_{max}
Plasma from women with pre-eclampsia	76.1+/-3.5
Plasma from pregnant control women	71.5+/-2.0
Control: No plasma	75.9+/-1.4
ANOVA (factorial) P=	0.83
ANOVA (repeated measures) P=	0.30

8.5 Discussion:

Maternal hypertension and proteinuria define pre-eclampsia. Maternal diastolic and systolic blood pressure (Kupferminc et al., 1997b) (Chapter 4), and the increased total peripheral resistance associated with pre-eclampsia (Bosio et al., 2001), have all been shown to correlate with plasma VEGF concentrations. VEGF is also well documented to cause an increase in vascular permeability (Senger et al., 1986) (Connolly et al., 1989a) (Wu et al., 1996) (Wu et al., 1999) (Tilton et al., 1999), and has been implicated in the renal disease proteinuria (Uchida et al., 1994) (Shulman et al., 1996), both of which are also associated with pre-eclampsia. The literature data and the data shown in Chapter 4 led to the hypothesis that "VEGF may act directly upon the endothelium or vascular smooth muscle to cause vasoconstriction of vessels".

Although VEGF is increased in pre-eclampsia (Chapter 4) (Baker et al., 1995a) (Kupferminc et al., 1997b) (Bosio et al., 2001), contrary to the original hypothesis no direct vasoconstrictor effect was evident upon its addition to either human myometrial or omental vessels (Figure 8.1 and 8.20). Neither did VEGF cause a vasodilator response in pregnant myometrial vessels; however, it appeared to produce a vasodilator response in non-pregnant vessels (Fig 8.2). This study thus demonstrated conclusively that VEGF is incapable of causing an endothelial or non-endothelial dependent contraction in either human myometrial or systemic vessels. In view of this data, an alternative hypothesis was formulated to explain the observation that plasma VEGF concentrations correlation with blood pressure (Chapter4) and increased peripheral resistance (Bosio et al., 2001).

Data from this thesis (Chapter 6 & 7) and the previous studies suggest that a significant proportion of human myometrial and omental resistance vessel relaxation, during pregnancy, is mediated through EDHF (discussed in Chapter 6 and 7) (McCarthy et al., 1993a) (McCarthy et al., 1993b) (Knock and Poston, 1996). Endothelial production of nitric oxide and EDHF have been shown, *in vitro*, to exhibit a reciprocal relationship (Bauersachs et al., 1996) (Randall and March, 1998). Recently, VEGF has been shown to increase the production of nitric oxide from various cultured endothelial cells (Ku et al., 1993) (Ahmed et al., 1997) (Tsurumi et al., 1997) (Ni et al., 1997a) (Kroll and Waltenberger, 1998) (Hood et al., 1998) (Tilton et al., 1999) (He et al., 1999). The reciprocal relationship of nitric oxide and EDHF, and the observation that VEGF increases nitric oxide production led to the development of an alterative hypothesis: "that VEGF by its direct action upon the endothelium cause an alteration in endothelial nitric oxide production, which leads to the altered vascular reactivity that is characteristic of pre-eclampsia (Chapter 6 and 7)".

In Chapter 6 it was demonstrated that myometrial vessels from women with pre-eclampsia displayed enhanced responses vasopressin. This study shows that extended incubation of myometrial vessels with VEGF enhances the contractile responses to vasopressin, and in a similar manner to the behaviour of vessels from women with pre-eclampsia, the enhancement of the E_{max} value was not accompanied by a significant shift in the EC_{50} value (Fig 8.3). These observations implicate a possible alteration in endothelial function, and although this was not formally tested, it may also be postulated that this occurs through an alteration in endothelial EDHF release. The unaltered EC_{50} suggests that these observations are not mediated through any increases in the receptor density, as this would cause a shift in the EC_{50}, and are more likely to be due to either increases in downstream secondary messengers or a decrease in an reciprocal vasodilator.

This study confirms the finding of Ashworth et al (1998) that plasma from women with pre-eclampsia, on extended incubation with normal pregnant myometrial vessels, alters their vessel function in a direction that is similar to vessels from women with pre-eclampsia (Ashworth et al., 1998). The results in this thesis also demonstrate that extended incubation with VEGF causes a loss of endothelium dependent relaxation and this mimics the previously reported effect of plasma from women with pre-eclampsia on myometrial vessel behaviour (Ashworth et al., 1998). VEGF was also shown to produce its effect in dose dependent manner (Fig 8.10), which was reversible upon washing with PSS (Fig 8.12).

It was demonstrated VEGF acted in a dose dependent manner at concentrations of 500 pmol or greater, but exerted no significant effect at 50 pmol or lower. This represents an effect at concentrations of VEGF 50 times that found in 2% pre-eclamptic plasma. *In vivo*, the mean circulating level of VEGF has been estimated to vary between 10 pmol (Baker et al., 1995a) and 720 pmol (Sharkey et al., 1996). In the pool utilised in this study we measured the levels of VEGF to be between 157 pmol and 220 pmol (Chapter 4). Although the concentration of VEGF in our 2% plasma samples is significantly less than utilised in our VEGF experiments, it is possible that dilution of plasma results in the release from its binding protein(s) of the free / bioactive component, the consequence of this could be the maintenance of the level of the active factor in the same range as in undiluted plasma. Evidence to support this comes from Davidge et al (1996) who fractionated plasma from women with pre-eclampsia and showed that it contained a factor that produced higher prostacyclin levels in cell culture experiments than unfractionated plasma (Davidge et al., 1996a). Moreover, the circulating level of VEGF remains a contentious

issue, depending upon the assay utilised (discussed in Chapter 4) and may be higher than previously cited values.

Alternatively our results can be explained by assuming that it is the concentration of VEGF at the endothelial surface that determines effect. If factor(s) within the plasma, such as IL-1α (Ben-Av et al., 1995), IL-6 (Cohen et al., 1996), TNF-α (Ryuto et al., 1996) and nitric oxide, which are documented to be increased in pre-eclampsia, induce the release of VEGF from the endothelium, local concentrations may well achieve parity with the concentrations of VEGF alone that we employed in our bioassay.

Evidence to support the hypothesis that VEGF is pivotal to the pathogenesis of pre-eclampsia comes from a recent report that the intravenous injection of VEGF into guinea-pigs causes a pre-eclampsia-like syndrome (Hunter, 2000) (see Chapter 4). This shows, in accord with the *in vitro* data of this thesis, that both guinea pig and human myometrial vessels have to be primed by pregnancy to react to VEGF in such a deleterious fashion (See Chapter 4).

The hypothesis that cytokines may act in this manner is also supported by reports that blunted endothelium-dependent relaxation to various receptor dependent agonists, including acetylcholine, bradykinin and substance P, occurs on exposure to the cytokines TNF α and Il –1 (Aoki et al., 1989) (Greenberg et al., 1993). TNF α and Il-1 have also recently been shown to cause a reduction in EDHF mediated relaxation in both rabbit and porcine coronary arteries through the up-regulation of nitric oxide (Kessler et al., 1999). In the study, of Kessler et al, (1999) the interaction of TNFα with the endothelium was time dependent requiring incubation

times of up to sixteen hours (Kessler et al., 1999). In a similar manner, the effect of VEGF on myometrial vessel function was also shown to be time dependent with alterations in endothelial function occurring at sixteen-hours. Shorter VEGF incubation times left endothelial cell function effectively unaltered (Figure 8.9). The lack of effect of the short-term treatment suggests that the effect is unlikely to be caused by the direct effect of VEGF receptor dependent signal transduction pathway on the agonist stimulated biosynthesis of EDHF. Although VEGF required extended incubation times, Hayman et al, 2000 have demonstrated that 2% plasma from women with pre-eclampsia is capable of altering myometrial endothelial function within one hour (Hayman et al., 2000). If VEGF is one factor in the plasma of women with pre-eclampsia that causes alterations in endothelial function, then it may be hypothesised that there are increased concentrations of chemicals that act synergistically with VEGF, or enhance the expression of VEGF and its receptors, and thus reduce the exposure time required of plasma.

This study has shown that when vessels are incubated with VEGF, the attenuated endothelial dependent relaxation is reversible, as the degree of vessel relaxation increased with sequential washing of the vessel with physiological salt solution (Fig 8.10). This indicates that the endothelium is undamaged by exposure to VEGF. The study of Kessler et al (1997) also demonstrated that once the cytokine response was withdrawn endothelium dependent relaxation returned to normal (Kessler et al., 1997).

Whether the endothelium dependent effect of VEGF is generalised or is specific to the vasodilator bradykinin remains to be elucidated and this requires further investigation. Determination of its generality may well enhance our understanding of the mechanism of action of VEGF.

The pregnancy-specific nature of pre-eclampsia suggests that any factor involved in the pathogenesis should have an effect that is also pregnancy specific. VEGF fits with this observation; the alteration in myometrial resistance vessel function was only demonstrable on incubation with vessels from normal pregnant women (Fig 8.4 & 8.5). The pregnancy specific nature of VEGF is further supported by the observation by Hunter et al (2000). These differences probably represent a pregnancy-associated change in the vessel physiology, however, the precise mechanism for this change remains to be determined.

It is clear from the data presented in this thesis that VEGF itself does not cause the endothelial changes that are associated with pregnancy (Fig 8.3). However, it has been shown that non-pregnant myometrial vessels, which have been treated with oestradiol or diethylstilbestrol show reduced sensitivity to vasopressin, in a manner that resembles pregnant vessels (Kostrzewska et al., 1993). Therefore it might be postulated that oestrogens sensitise the endothelium to the adverse effects of VEGF. This could also explain the apparent lack of effect that VEGF has on non-pregnant myometrial vascular function (Fig 8.3 & 8.5) and in other pathological conditions where circulating concentrations are increased (Hyodo et al., 1998) (Jinno et al., 1998) (Duque et al., 1999).

It has also been demonstrated that the loss of endothelium dependent relaxation observed on incubation with plasma from women with pre-eclampsia can be inhibited with the addition of antibodies to VEGF and a specific anti-flt-1 receptor antibody. This provides the strongest support for our hypothesis that VEGF is integral to the pathogenesis of pre-eclampsia. It is important to note that although the addition of VEGF antibody significantly increased the vessel relaxation in those vessels incubated in 2% plasma from women with pre-eclampsia, vessel behaviour was not

completely restored to normal. There remained a significant attenuation of endothelium dependent relaxation in these vessels when compared to those with 0.5 nmol VEGF + antibody, and those incubated with 2% plasma from normal pregnant women. These results may have occurred due be the monoclonal antibody used only binding to certain of the VEGF isoforms. An alternative explanation is that there was a contribution of factor(s) other than VEGF to the effect of plasma from women with pre-eclampsia on the vessel behaviour. Moreover, the results of our Dynabead® treatment of plasma from women with pre-eclampsia suggest that the circulating factor(s) may partially exert its effects through VEGF produced within the locality of the vascular endothelium.

The RIA measurement of VEGF in our samples indicates that treatment of plasma from women with pre-eclampsia with anti-VEGF coated Dynabeads® only removed a small amount of VEGF. It was significant that treatment reduced the levels of VEGF to those seen in the plasma of normal pregnant women. However, further extraction of VEGF from the plasma is likely to be hampered by highly complex interactions with binding proteins (such as soluble flt), and as a consequence, complete denudation of VEGF from plasma by immunoadsorption would be extremely difficult. It is possible that not only does VEGF exert its effects locally in the vascular endothelium, but that VEGF exists in a "free" bioactive form, and in a "bound" inactive reservoir. Published evidence to support this hypothesis comes from Anthony et al who have shown that there may be binding proteins that affects measurement of VEGF (Anthony et al., 1997). The flt-1 receptor antagonist completely inhibited the effect of the plasma from women with pre-eclampsia strongly suggesting that the flt-1 receptor is the final common mediator in this pathological process.

Human pregnancy is marked by increased amounts of soluble flt-1 receptor (Charnock-Jones et al., 1997) (Clark et al., 1998a) (Banks et al., 1998). Clark et al have demonstrated that trophoblast cells contain mRNA which encodes this soluble form of the VEGF receptor (soluble flt) and that this protein is a potent antagonist of VEGF (Clark et al., 1998a). It can be hypothesised that an alteration in the equilibrium of soluble flt receptor and VEGF in pre-eclampsia would diminish the endothelium-dependent response to bradykinin. It will therefore be interesting and important to investigate the interaction of VEGF with the soluble flt-1 receptor in pregnancy and pregnancies complicated by pre-eclampsia.

The VEGF family includes five VEGF isoforms and the homologous placental growth factor (PlGF) which shares the VEGF receptor system. The $VEGF_{165}$ isoform used in our studies binds to both receptors, whereas $VEGF_{121}$ has a greater affinity to KDR and PlGF to flt-1 (Masabum, 1993). Kendel et al (1994) demonstrated that like VEGF, PlGF was capable of binding to the flt-1 receptor but was unable to bind the KDR receptor system (Kendall et al., 1994).

Although VEGF appears to have an affect on myometrial vessel function through the flt-1 receptor, incubation with PlGF caused no reduction in endothelium dependent relaxation in either pregnant or non-pregnant vessels. Park et al (1994) showed that PlGF, although capable of binding to the flt-1 receptor, was unable to initiate a stimulus via tyrosine phosphorylation (Park et al., 1994). However, non- bacterial PlGF has been demonstrated to have a high mitogenic effect on cultured endothelial cells through the flt-1 receptor system (Ziche et al., 1997). The apparent lack of PlGF activity on myometrial vessels may well be due to the use of human recombinant PlGF in these experiments, however, PlGF with carbohydrate moieties is not available commercially and the author was unable to obtain any from Ziche et al. Although possibly

artefactual, it would be interesting and important to examine the possible interactions of VEGF and PlGF in this myometrial vessel model.

In this study, the addition of indomethacin did not significantly alter the endothelium dependent relaxation of vessels from normal pregnant women incubated with VEGF (Fig. 8.21). However, the presence of an inhibitor of nitric oxide synthetase (L-NAME), in addition to an inhibitor of cyclooxygenase, significantly attenuated the endothelium dependent relaxation in normal pregnant vessels that were incubated with VEGF (Fig 8.21). As observed in Chapter 6, normal pregnant vessel responses to bradykinin are resistant to the addition of an nitric oxide synthetase and cyclooxygenase inhibitor. The observed attenuation of relaxation in vessels incubated with VEGF suggests that the relaxation in these vessels is primarily dependent on the release of nitric oxide, with little contribution from EDHF. In this respect the theses observations mimic those seen in myometrial vessels from women with pre-eclampsia (Chapter 6) (Ashworth et al., 1997). Therefore, it could be postulated that these changes occur, either through a disruption of EDHF mediated responses with the compensatory increase in nitric oxide mediated relaxation, or that there is a loss of endothelium dependent relaxation consequent to the upregulation of endothelial nitric oxide. These hypothesises were not formally tested, but could be achieved via similar methodology to this thesis.

8.6 Summary:

This study demonstrated that VEGF is incapable of causing a constrictor response to myometrial and omental resistance vessels, but paralleled the effects of plasma from women with pre-eclampsia on vessel behaviour, at a concentration similar to those found *in vivo* (0.5 nmol). This

study also shows that in a similar manner to vessels from women with pre-eclampsia, VEGF causes a compensatory increase in nitric oxide mediated relaxation in myometrial vessels. However, antibodies to VEGF only partially block the interaction of plasma from women with pre-eclampsia with the vascular endothelium, this suggests that there are additional factors involved in the endothelial dysfunction seen in the bioassay technique.

Chapter Nine: The effects of vascular endothelial growth factor on four endothelial cell types:

9.1 Introduction:

A number of studies have demonstrated that serum or plasma from women with pre-eclampsia causes activation of endothelial cells *in vitro* (Lorentzen et al., 1991) (Branch et al., 1991) (Baker et al., 1995a) (Davidge et al., 1995b) (Davidge et al., 1996a). The majority of the studies that have investigated the effects of plasma on endothelial cell function have used human umbilical vein endothelial cells (HUVECs) (Lorentzen et al., 1991) (Branch et al., 1991) (Silver et al., 1996) because this type of cell is relatively easily isolated and the tissue is readily available. However, there are a number of important caveats to be considered when using human umbilical vein endothelial cells as representative of the endothelium per se. First, HUVECs are from large conduit vessels and therefore will not reflect the endothelium derived from the microvasculature. As pre-eclampsia is believed to be a disease with dysfunction of the microvascular endothelium, this type of endothelial cell may not prove to be the most appropriate. Second, HUVECs are fetally derived cells and pre-eclampsia involves dysfunction of the maternal endothelium.

Several studies have also utilised bovine aortic microvascular endothelial cells (B-88) as a model of the human microvasculature, however, these are not without their limitations (Baker et al., 1995b) (Davidge et al., 1995b) (Baker et al., 1996b) (Baker et al., 1996a) (Davidge et al., 1996a). Bovine cells, although microvascular, are from a different species and it has been demonstrated that microvascular endothelium derived from different organs displays distinctive tissue-specific

characteristics (Fajardo, 1989). Therefore, it must be questioned as to the relevance of findings that have utilised these cells to the *in vivo* situation.

9.2 Aims:

- To ascertain whether human microvascular cells (from the uterus and systemic circulation) and human macrovascular cells (umbilical) cells were stimulated in a similar manner to B-88 cells.
- To compare the abilities of VEGF, plasma from women with pre-eclampsia and plasma from normal pregnant women, to stimulate the different human endothelial cell types and B-88 cells.
- To investigate whether VEGF produces any of the observed alterations produced by plasma.
- To investigate whether human cells (uterine) could be used to investigate further the sub-cellular mechanism of action of VEGF and plasma on myometrial vessels.

9.3 Experimental design:

9.3.1 Subjects:

Plasma samples were obtained from 11 patients with pre-eclampsia as described in Chapter 2.2. In addition, plasma was collected from 11 normal pregnant patients who had no complication of pregnancy or underlying illness. After the initial experiments there was insufficient plasma from the original patients, therefore, in a further experiments to examine the effects of VEGF and

plasma; new plasma samples were collected from 12 further patients diagnosed as having pre-eclampsia and 12 normal pregnant patients (Chapter 2.2)

9.3.2 Cell culture:

The addition of plasma:

The four types of endothelial cell were grown as described in Chapter 2.4, and passaged into 24 well plates. B-88 cells were initially used as a positive control for endothelial activation, as preliminary data had confirmed that these cells were activated by plasma from women with pre-eclampsia (Chapter 3.3). Cells were made quiescent by culturing cells in serum depleted media as described in Chapter 2.4. After cells had been made quiescent for 24 hours medium was aspirated and fresh media added containing plasma from each subject at a final concentration of 10%. A plasma concentration of 10% was utilised, as previous data, in the laboratory were this thesis was undertaken, had demonstrated that 2% plasma was unable to show differences between the two study groups of interest (Wellings et al., 1998). Each patient's plasma was studied in triplicate. Heparin was added to each well to give a final concentration of 10 U/ml. Twenty-four hours after addition of plasma, medium was collected as described in Chapter 2.5.3. Nitrite, 6-keto-prostaglandin $F_{1\alpha}$ and LDH (Chapter 2.6) were then measured in the media.

The addition of VEGF:

After cells had been made quiescient (Chapter 2.5), they were stimulated for 24 hours with increasing concentrations of VEGF (0.01-1.0nM). Effects were studied in quadruplicate wells. The

VEGF experiments were repeated at least twice and the results displayed are for a typical experiment. Heparin was added to each well to give a final concentration of 10 U/ml. In a further set of experiments it was determined in the B-88 cell type whether the addition of an anti-VEGF antibody (10 ng/ml: R&D system, UK) could block the observed effect of VEGF on these endothelial cells. After stimulation, media were collected for determination of 6-keto prostaglandin $F_{1\alpha}$ and nitrite (stable metabolites of prostacyclin and nitric oxide).

The addition of plasma from women with pre-eclampsia and from normal pregnant women with the addition of anti-VEGF antibody:

In a further set of experiments, B-88 cells (as these were the only cells to be stimulated by plasma) were stimulated for 24 hours in serum free media containing 0.05% bovine albumin and 10% plasma. The VEGF concentrations of the plasma samples used had been determined previously by radioimmunoassay (Chapter 2.1). After stimulation, media was collected and the concentrations of prostaglandin F1α, nitric oxide, lactate dehydrogenase (LDH) and cellular protein were determined. Experiments were repeated in the presence of anti-VEGF antibody (10ng/ml; R&D Systems: UK).

9.4 Results:

9.4.1: Patient data:

Patient demographics are summarised in Tables 9.1& 2. As anticipated, the pre-eclampsia patient group had blood pressures that were significantly higher than those of the normal pregnant

Demographic details for samples used to stimulate four endothelial cell types at 10 % plasma concentration

Table 9.1

Plasma Type	Age (years)	BMI (kg/m ^{2})	Gestation at venepuncture (days)	Gestation at delivery (days)	Mean Arterial Pressure Booking mm Hg	Mean Arterial Pressure Maximum mm Hg	Protein g/dl	Individualised Birth-weight Ratio
Normal pregnant n=11	26 (22 - 30)	23.8 (21.8 - 26.2)	252 (238 - 266)	271 (263 - 280)	84.9 (73.2 - 88.6)	89.9 (86.6 - 93.3)	0.0	47 (33 - 78)
Pregnant women with pre-eclampsia n=11	28 (24 - 30)	25.1 (23.5 - 26.3)	252 (230 - 266)	257 (230 - 266)	85.0 (79.9 - 89.9)	114.6 (113.3 - 123.3)	0.74 (0.43 - 1.08)	16 (0 - 28)

Data are summarised as medians (Inter Quartile Ranges)

Demographic details for samples used to stimulate endothelial cells at 10% plasma concentration Table 9.2

Plasma Type	Age (years)	BMI (kg/m ^{2})	Plasma VEGF concentration (ng/ml)	Gestation at venepuncture (days)	Gestation at delivery (days)	Mean Arterial Pressure Booking mm Hg	Mean Arterial Pressure Maximum mm Hg	Protein g/dl	Individualised Birth-weight Ratio
Normal pregnant n=12	26 (22 - 29)	22.3 (19.6 - 26.2)	3.9 (3.1 - 4.1)	254 (242 - 266)	278 (260 - 280)	86.7 (79.8 - 90.8)	90.2 (89.2 - 93.3)	0.0	66 (46 - 85)
Pregnant women with pre-eclampsia n=12	25 (21 - 29)	24.1 (21.0 - 30.1)	5.2 (4.6 - 5.3)	252 (245 - 264)	260 (245 - 266)	87.5 (83.3 - 93.3)	113.3 (110.0 - 119.2)	0.73 (0.51 - 1.64)	16 (0 - 28)

Data are summarised as medians (Inter Quartile Ranges)

controls. In addition, significant proteinuria was only observed in the pre-eclampsia group. Table 9.2 summarises the demographic data and the VEGF concentrations in the cohorts of women with pre-eclampsia and the normotensive pregnant controls used in the set of experiments that examined the effect of the VEGF antibody. As anticipated, the plasma VEGF levels were significantly higher in the women with pre-eclampsia (5.2 *Vs* 3.9: P>0.01, Mann-Whitney-U test).

9.4.2: Nitrite production in response to plasma and VEGF:

Plasma:

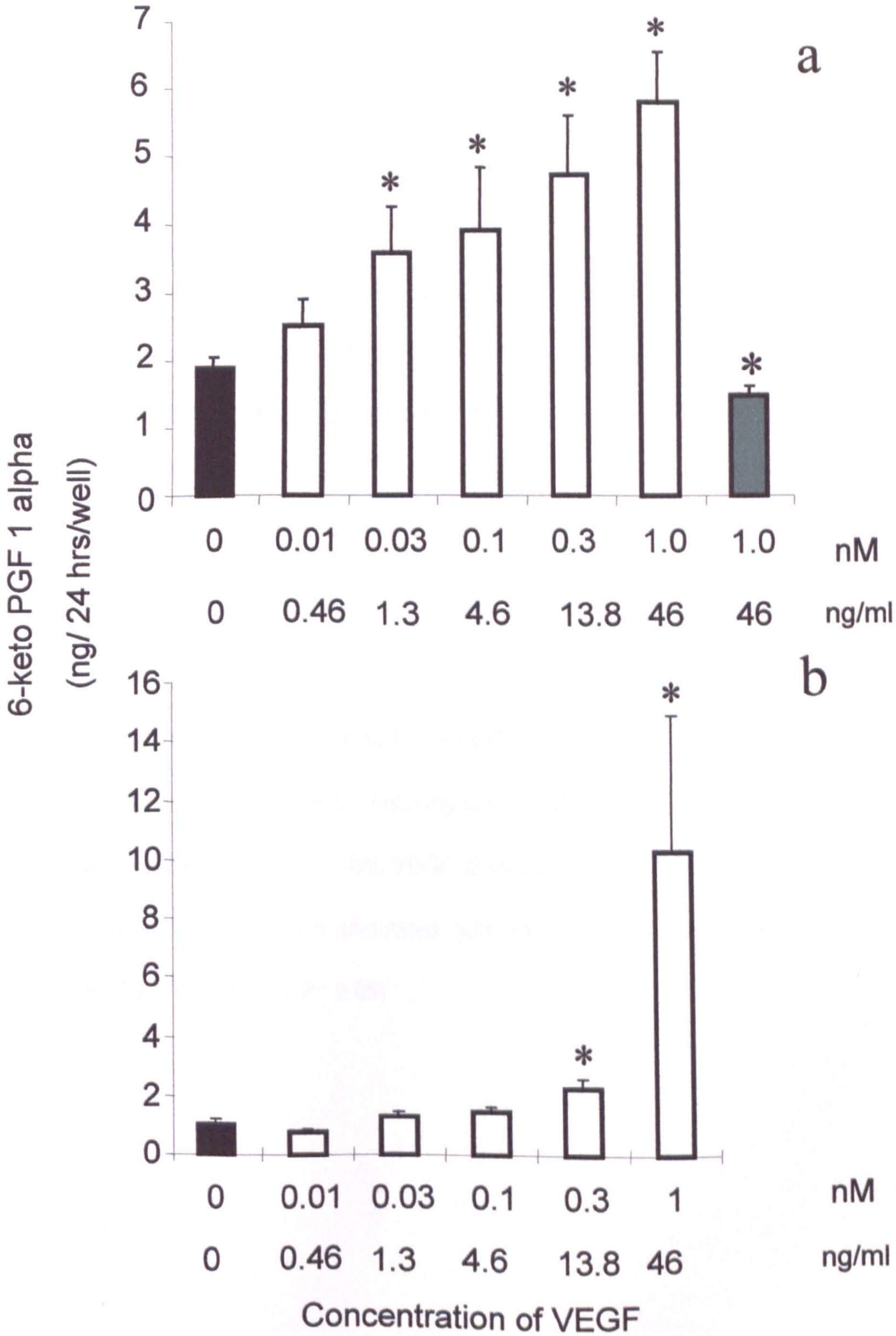
Nitrite production by the HUVEC and the HdMEC was undetectable in most wells, whereas there was a significant production of nitrite by both the decidual and B-88 cells (Table 9.3). In the decidual cells there was no significant difference in nitrite production by cell exposed to plasma from the women with pre-eclampsia when compared to the normal pregnant group. In contrast, production of nitrite by B-88 cells was significantly higher in the supernatants from cells exposed to plasma from women with pre-eclampsia when compared with that from the control pregnant group (Fig 9.3; P= 0.048, Mann-Whitney-U test).

VEGF:

In response to VEGF nitrite was undetectable in the media from HUVECs, and although production was detected in all other cells types, only HdMEC showed an increased production on stimulation with VEGF (P<0.05) (Fig 9.1).

Fig 9.1

Dose response of 6-ketoPGF 1α production by B88 (a) & HUVEC (b) cells stimulated with incremental concentrations of VEGF. Bars represent means +/- SEM. The effect of a monoclonal antibody for VEGF (10ng/ml) is represented by the grey bar



9.4.3: Prostacyclin production in response to plasma and VEGF:

Plasma:

Concentrations of prostacyclin production were markedly different according to cell type (Table 9.3). Prostacyclin production was similar in response to stimulation by plasma from both normal pregnant and women with pre-eclampsia in the HUVEC, HdMEC, and decidual endothelial cell types at a concentration of plasma of 10%. In contrast, prostacyclin concentrations in media from B88 cells in response to plasma from women with pre-eclampsia were significantly higher than in B-88 cells exposed to plasma from normal pregnant women (Fig 9.3; P= 0.046, Mann-Whitney-U test).

VEGF:

The production of prostacyclin was detected in all four endothelial cell types. However, only HUVEC and B-88 cell production increased with increasing concentrations of VEGF (Fig. 9.1a & 9.1b, repeated measures ANOVA, $P < 0.05$). Anti-VEGF antibody (10ng/ml) had the effect of inhibiting the prostacyclin production of B-88 stimulated cells back to the observed base line production (Figure 9.1a, Mann-Whitney –U, $P< 0.05$)

6 keto-prostaglandin F 1 α (ng /10 ^{6} cells / 24 hours) in the four different endothelial cell types stimulated by 10 % plasma

Table 9.3

Cell type	Normal pregnant group	Pre-eclampsia group
HUVEC	0.65 (0.53- 0.77)	0.67 (0.57- 0.81)
HdMEC	0.84 (0.80- 0.94)	0.90 (0.76- 0.10)
Decidual	0.15 (0.15- 0.17)	0.16 (0.13- 0.18)
B-88	3.26 (2.79- 3.60)	4.35 (3.17- 6.44)*

Nitrite production (nmoles/ 10 ^{6} cells /24 hours) in the four different endothelial cell types stimulated by 10 % plasma

HUVEC	nd	nd
HdMEC	nd	nd
Decidual	1.72 (1.28-1.95)	1.50 (1.32-1.84)
B-88	2.61 (2.22-3.66)	4.72 (3.87-5.26)*

nd=not detectable

Mann-Whitney-U test: * P= 0.05

9.4.4: Correlation of plasma VEGF levels with prostacyclin and nitric oxide production:

There was no significant correlation between plasma VEGF concentrations and nitric oxide production in the media from the stimulated B88 cell line when either plasma from women with pre eclampsia (r= 0.180, P>0.05, Spearmans Rank correlation) or normal pregnant controls were studied, (r= 0.223, P>0.05, Spearmans Rank correlation). However, in the pre-eclampsia group there was significant correlation between prostacyclin production and plasma VEGF levels, (Fig 9.2, r= 0.64, P=0.02, Spearmans Rank correlation). This correlation was not present when the normal pregnant group was studied. (Fig 2. r= 0.339, P >0.05 Spearmans Rank Correlation).

9.4.5: Effect of anti-VEGF antibody on plasma induced endothelial cell prostacyclin and nitrite production:

When B-88 endothelial cells were stimulated with plasma there was a significant increase in the nitric oxide and prostacyclin production by cells that had been stimulated with plasma from women with pre-eclampsia as compared to normal pregnant women (Fig 9.3 P=0.01). When VEGF antibody was added to both groups' plasma, there was no effect on the nitrite production (Mann-Whitney-U P> 0.47). However, prostacyclin production by cells stimulated with plasma from women with pre-eclampsia was significantly inhibited (P= 0.01), and under these circumstances there was no significant differential effect of plasma from the two groups (P>0.05).

Fig 9.2

B88 6-ketoPGF1α and nitric oxide production of cells stimulated with plasma from women with pre-eclampsia and normotensive controls

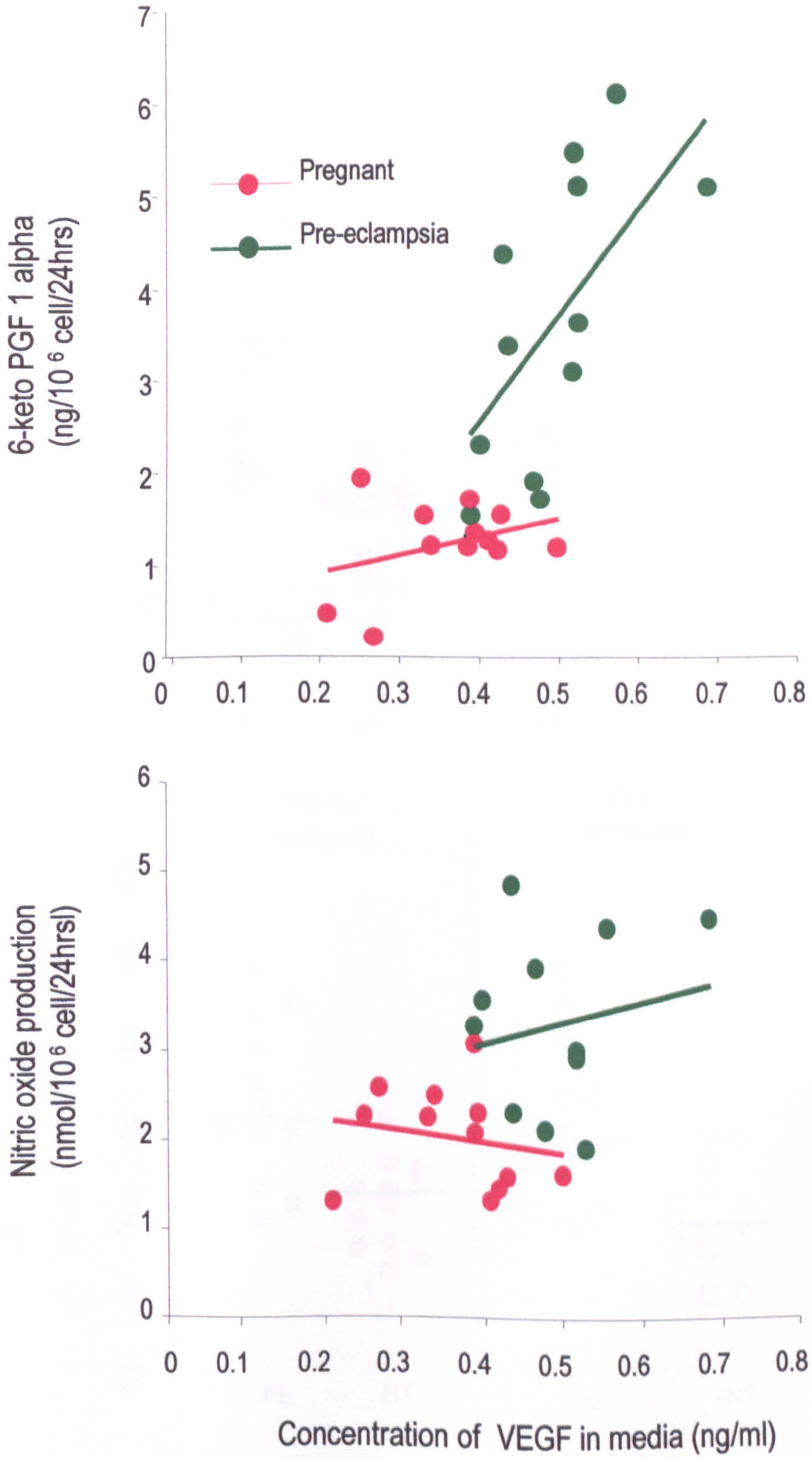
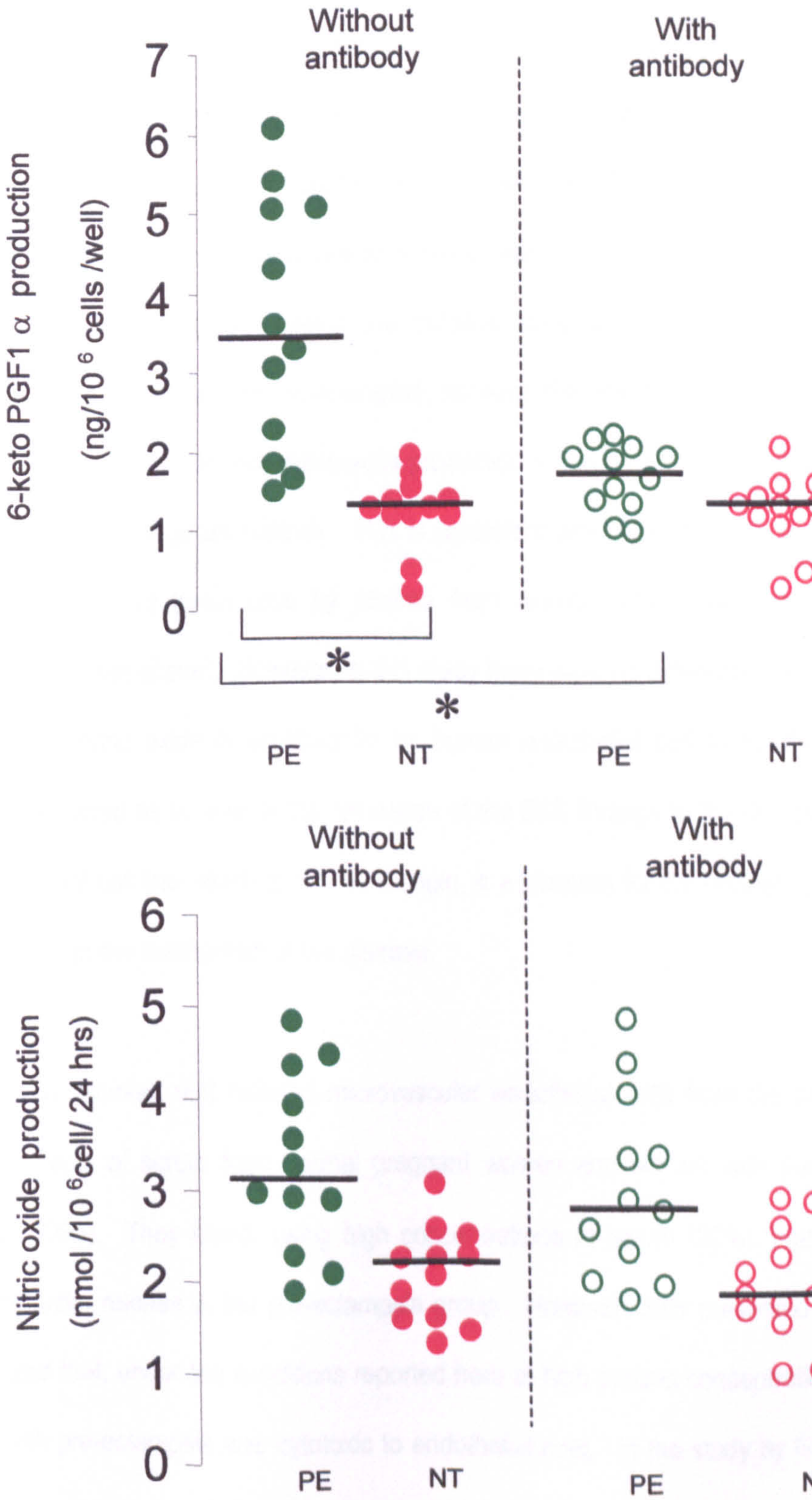


Fig 9.3

B-88 6-keto PGF 1 α and nitric oxide production after stimulation with plasma from 12 normotensive and 12 preeclamptic women +/- anti VEGF antibody. Horizontal lines represent medians.



9.5 Discussion:

It is now well documented that plasma or serum from patients with pre-eclampsia contains factor(s) that are capable of altering endothelial cells (Lorentzen et al., 1991) (Branch et al., 1992) (Baker et al., 1995a) (Davidge et al., 1995a) (de Groot et al., 1995) (Davidge et al., 1996a) (Zammit et al., 1996). To date, most studies have investigated the effects of plasma on either the B-88 or freshly cultured human umbilical vein endothelial cells. This study confirmed that plasma from women with pre-eclampsia contains the putative circulating factor(s) believed to be associated with the pathogenesis of pre-eclampsia, because the plasma from patients with pre-eclampsia increased nitric oxide and prostacyclin production in the B-88 cells in comparison with the plasma from normal pregnant controls. This is consistent with other literature reports which have shown activation of these cells by plasma from women with pre-eclampsia or from fractionated plasma (see above). However, in this study there were no differences in secretion of the metabolites of nitric oxide or prostacyclin by human endothelial cell types, therefore the question must be asked as to what is the relevance of the B88 findings to the disease state. It may be that the B-88 cell line, which is of bovine origin, is a bioassay for the circulating factor and has little relevance to the mechanism of the disease.

Gallery et al have isolated and cultured microvascular endothelial cells from the decidua and assessed the effects of serum from normal pregnant women and women with pre-eclampsia (Gallery et al., 1995). They found, using high concentrations of serum (20%), that there was increased prostcyclin release in the pre-eclampsia group. However, data presented in Chapter 3.3 demonstrated that, under the conditions reported here at high plasma concentrations plasma from women with pre-eclampsia was cytotoxic to endothelial cells. In the study by Gallery et al,

markers of cellular viability were not measured to assess this possibility, and this may account for the differences observed. The study of Gallery et al also utilised serum and not plasma, which may add another confounding variable, as it is well known that cellular products are released into the serum on coagulation and these may produce *in vitro* alterations that are not relevant to the *in vivo* situation (Fox and DiCorleto, 1984).

Nitric oxide concentrations in plasma-stimulated media collected from HdMEC cells were not detectable in any of the wells. This suggests that the microvascular endothelial cells derived from the skin behave differently with respect to the production of nitric oxide, which may reflect different local control of vascular tone by the endothelium in different tissues. Indeed, it has been shown that microvascular endothelium derived from different organs displays distinctive tissue-specific characteristics (Fajardo, 1989).

With regard to VEGF stimulation of these endothelial cells, nitric oxide was only detectable in the HdMEC type. In this cell type VEGF caused a dose-dependent increase in nitric oxide cell production. Analysis of the dose response by post hoc testing revealed that the concentrations required to produce an effect were relatively high (13.8ng/ml: 0.3nM). This finding that nitric oxide was stimulated by VEGF is consistent with other *in vitro* and *in vivo* data (Ahmed et al., 1997) (Horowitz et al., 1997) (Ni et al., 1997a) (Papapetropoulos et al., 1997) (Tsurumi et al., 1997) (Hood et al., 1998) (Murohara et al., 1998) (Tilton et al., 1999). Ahmed et al demonstrated that 10ng/ml VEGF produced 2.1nmol/ml nitric oxide; this is in accord with the production in this study (13.8ng/ml produced 1.5 nmol/ml). This study did not address question of which receptor system was involved in the increased production from HdME cells. However, in Chapter 8 it was

demonstrated that the effect of VEGF on myometrial resistance vessel endothelium was via the flt-1 receptor and this affected appeared to be mediated through nitric oxide.

Although plasma from women with pre-eclampsia stimulated only B-88 endothelial cell prostacyclin production, VEGF stimulated significant production in both B-88 cells and HUVECs. Further analysis of the data concluded that concentration of VEGF required to produce an effect in the HUVECs was (13.6ng/ml: 0.3nM) ten times that required in the B-88 (1.36ng/ml: 0.03nM).

Why do these endothelial cell types respond differently to the stimulatory effects of plasma from women with pre-eclampsia? It is tempting to postulate that the circulating factor(s) described by Davidge et al activated specific receptors on endothelial cells that are present on B-88 cells but have been lost or down-regulated in the human endothelial cell types (Davidge et al., 1996a). If VEGF was one of the circulating factors then the differential effects of plasma might be explained by the fact that the concentration of VEGF in the plasma (0.52ng/ml) used to stimulate HUVE cells was too low, yet was sufficient to stimulate B-88. This is borne out by the fact that B-88 cells were stimulated with VEGF concentrations that were ten fold lower than that of HUVECs.

This study has demonstrated that VEGF is capable of increasing the endothelial cell production of prostacyclin and that VEGF antibody is capable of blocking this effect in a bovine cell line. It has also shown that plasma VEGF concentrations correlate with the increased production of prostacyclin induced by plasma from patients with pre-eclampsia. Davidge et al (1996) demonstrated that fractionated plasma produced two distinct components; a low molecular weight faction that increased prostacyclin and high molecular weight faction that increased nitric oxide production. The low molecular weight fraction was in the range 42-62 Kda and this corresponds

to that of the VEGF family. The effects of VEGF on cell permeability are well documented (Ferrara et al., 1992) and appear to mediated through the eicosanoid messenger system (Fujii et al., 1997) (Murohara et al., 1998). Recently, Haller et al (1998) have added further circumstantial evidence to our hypothesis that VEGF may be one of the circulating factors in pre-eclampsia by their observation that plasma from women with pre-eclampsia increases the permeability of cultured endothelial cells via the eicosanoid system (Haller et al., 1998). In sharp contrast this does not concur with the data obtained on myometrial resistance vessels which indicated that VEGF in this system may be increasing nitric oxide (Chapter 8). However, as stated previously B-88 cells may only serve as a bioassay and have no relevance in the *in vivo* situation. Furthermore, pre-eclampsia is associated with a decreased prostacyclin production *in vivo*.

This study showed that the plasma VEGF concentrations correlate with increased production of prostacyclin only in the plasma from women with pre-eclampsia. However, if the two groups are combined there is a strong correlation of increasing prostacyclin production with increasing VEGF levels. The fact that individually only the group with pre-eclampsia correlates may well represent the fact that VEGF has a critical concentration above which is has an effect or that there are insufficient numbers in the study groups to demonstrate a correlation.

One further caveat to be considered is the finding that the effects of VEGF which were demonstrated occurred at concentrations above the reported circulating levels (Baker et al., 1995b) (Sharkey et al., 1996). However, many cytokines have been demonstrated to enhance the effect of VEGF *in vitro* and this may explain the discrepancies in these levels. Further work is required to fully elucidate the possible spectrum of cytokine interactions for this observed increase in prostacyclin.

It has also been reported that nitric oxide production was significantly increased in the media collected from endothelial cells (Baker et al., 1995a). Interestingly this study did not demonstrate any correlation between VEGF and nitric oxide production, although nitric oxide was expected to be involved in VEGF action. It is important to note that VEGF has been reported to increase nitric oxide production from human umbilical vein endothelial cells (Hood et al., 1998). Our inability to demonstrate this probably related to the B88 cells utilised and the sensitivity of our assay.

9.6 Summary:

This study demonstrated that VEGF has a broadly comparable effect to that of plasma from women with pregnancies complicated by pre-eclampsia on the production of prostacyclin in cultured B88 endothelial cells and the plasma effects of women with pre-eclampsia can be inhibited by a VEGF antibody. All these observations are consistent with the original hypothesis that VEGF may be one of the circulating factors that causes the alteration in endothelial function that is characteristic of this disease (Roberts et al., 1989).

Chapter Ten: General Discussion

This chapter will be divided into sections; the first will deal with the strengths and weaknesses of this thesis. The second part will aim to place the results obtained in each individual chapter into the more general global context of the disease. It will also highlight where future work is possible and the techniques that could be applied to answer the hypotheses set up from the data.

10.1: Strengthens and weakness:

10.1.1: Myography:

The anatomical inaccessibility of resistance vessels in mammals has proved to be the limiting factor in the study of their behaviour *in vivo*. *In vivo* animal experiments have produced large amounts of information on the accessible vascular beds, however, recent work has demonstrated that individual vascular beds within species behave very differently to each other. As such the study of animal models yields limited information, as extrapolation into the human is potentially unreliable. Initial *ex vivo* studies in humans examined larger size arteries with inherent limitation.

Wire myography has been used extensively in the investigation of small artery function. It is a relatively robust tool that produces consistent and reproducible results. However, it has well recognised limitations. The vessel geometry is significantly altered as it is held in a flat plane by two wires rather than in the more physiological cylindrical shape. Thus the endothelial surface is exposed to the wire pressure and the vessel wall is subject to distension at both ends, rather than the effects of pressure evenly distributed along the entire endothelial surface. Chemical agents have to be applied to the external surface rather than through the lumen. The absence of flow

through the lumen is a further major limitation and has assumed appreciation in recent years as the importance of flow-mediated vasodilatory responses have become increasingly recognised (Mulvany and Aaljaer, 1990). In addition it is almost impossible to investigate the myogenic response using this system. A further constant criticism of this system is of the potential damage on the vessel by the introduction of wires down the lumen. In truth, the effect of this damage is minimal when other limitations are considered and the presence of the endothelium can be confirmed by histological examination.

Clearly the comparison of myometrial vessels from pregnant and non-pregnant groups is compounded by an alteration in the uterine anatomy with pregnancy. This is particularly relevant in the third trimester, when the formation of the lower segment occurs. Therefore it was attempted to minimise the anatomical differences in the source of our vessels by taking biopsies from the isthmus in non-pregnant women, and from the upper part of the lower segment in pregnant women. However, it can not be determined from this study whether the vessels sampled were similar, with pregnancy being the only differentiating factor, or whether the vessels from the pregnant and non-pregnant group were in fact different in origin.

<u>Vasopressin:</u>

Although the hormone vasopressin may have a role in the physiological control of blood pressure through its actions on the V1a receptor, this physiological effect is far from certain. However, as discussed previously evidence for its role the control of uterine circulation is increasing (Chapter 3). Comparison of the potencies of the vasoconstrictors; vasopressin, U46619 and endothelin,

shows that vasopressin is the most effective vasoconstrictors in human myometrial resistance vessels and further adds evidence to support its role in the regulation of myometrial blood flow.

It should also be noted that the initial aim of this project was to perform preliminary characterisation studies of the interaction of VEGF with the endothelium, using several bioassay techniques. Vasopressin provided a sustained, repeatable contraction in the myometrial vessels under investigation, and was therefore chosen as the contractile agonist. Whilst other agents have been shown to be suitable subsequently, the choice of vasopressin was made on the observation of previous pilot studies and experimental work (Ashworth, 1998). This was felt to be acceptable initially, as this study started as an DM thesis, and with the limited time available, protocols were constructed from experimental work carried out prior to the commencement of this work. If this thesis was to be undertaken again it would be advantageous to repeat the pilot data and choose two vasoconstrictors to study as these may have produced differing results.

Sodium nitroprusside:

The observation was made that endothelium dependent relaxation from vessels from non-pregnant, pregnant women and women with pre-eclampsia showed no differences in non-endothelium-dependent relaxation when sodium nitroprusside was used. Sodium nitroprusside has been criticised as it leads to the production of nitrovasodilators other than nitric oxide (Feelisch and Kelm, 1991). Spermine NONOate is considered to be a purer donor, releasing NO on an almost equimolar basis (Ramamurthi and Lewis, 1997). It is possible that the assesement of nitric oxide synthesis in these vessels would have been better served by the utilization of a

guanylate cyclase inhibitor, blocking the action of nitric oxide via its second messenger, or by the use of a combination of nitric oxide synthesis inhibitors (McCarthy et al., 1993b).

Heparin:

The addition of heparin during vessel incubation with plasma could be criticised for its potential to interact with both the circulating factor(s) and the endothelium. However, its presence was required to prevent the formation of a fibrin clot which, when present, prevented further experimentation. Heparin was added in equal concentrations to both the control and pre-eclamptic groups in order to reduce the potential for experimental bias.

10.1.2: Systemic *Vs* uterine:

The use of omental vessels in the study of pregnancy and pre-eclampsia must be questioned as they may not be representative of the peripheral vascular system. It may be argued that it is more pertinent to study vessels from organs that are know to undergo dramatic changes in blood flow with pregnancy; such organs include uterus, skin and kidney. However, these vessels are also not without their deficits, renal vessels which undergo a similar increase in blood flow as the uterus with pregnancy would be of most value (Dunlop, 1976) (Ezimokhai et al., 1981), however these are not available for *in vitro* study. Skin vessels are easily obtained, but whether gluteal biopsies, which are most commonly obtained, are representative of the whole skin surface remains to be elucidated. Therefore myometrial vessels may represent the best option to study vascular adaptation to human pregnancy; are situated juxtaposed to the developing placenta; are

an easily obtainable source of vessels which undergo dramatic blood flow changes during pregnancy, and show no variability depending on the site of biopsy (Svane et al., 1991).

In this study myometrial vessels from non-pregnant women exposed to vasopressin and U46619 showed significant differences to the normal pregnant controls. In a similar manner, pregnancy has also been shown to alter the vascular reactivity of guinea-pig uterine arteries to various vasoconstrictors (Weiner et al., 1989a) (Weiner et al., 1991) (Weiner et al., 1992b) (Weiner et al., 1992a) (Jovanovic et al., 1995a). This thesis and other studies of human and animals have demonstrated that these effects are mediated through changes in endothelial function (Weiner et al., 1989a) (Jovanovic et al., 1995a).

However, the effect that pregnancy has on systemic vessels of other species is far from conclusive. Isolated mesenteric resistance arteries from pregnant rats, assessed utilising wire myography, show attenuated responses to phenylephrine when compared to similar sized non-pregnant vessels (Davidge and McLaughlin, 1992). In a similar manner, isolated guinea pig aorta preparations also show blunted responses to both phenylephrine and norepinepherine in pregnant animals (Harrison and Moore, 1989). Although, resistance vessels from guinea pig kidney and mesentery show no attenuated responses to the thromboxane memetic U46619, removal of the endothelium, from non-pregnant and pregnant vessels, caused enhanced responses of pregnant over non-pregnant vessels (Kim et al., 1994). This finding has also been demonstrated in larger guinea pig carotid vessels during pregnancy contracted to serotonin (Weiner et al., 1992b). Weiner et al hypothesed that although pregnancy caused no detectable alteration in vasoconstrictor agents responses, there was an alteration in vascular endothelium which led to increase basal release of endothelial derived substance relaxing factors. However, removal of the

endothelium from omental and subcutaneous resistance vessels does not cause altered responses with pregnancy (McCarthy et al., 1993b) (Ashworth, 1998). This would suggest that different organs adapt differently to pregnancy and might depend on their function during pregnancy.

All vasodilators studied showed significantly greater vasodilatation in omental arteries than myometrial arteries. The differences are unlikely to be artefactual consequences of the differing vessel wall thickness of tension, as relaxation is the parameter being measured, and is expressed as a percentage of the individual contraction from which relaxation is being induced. Therefore, it may be hypothesised that in myometrial vessels the reduced agonist-mediated responses are substituted by enhanced myogenic or flow-mediated responses. In support of this hypothesis, Kublickiene et al have demonstrated that in pregnant myometrial vessels the contribution of myogenic tone is greater than in omental vessels (Kublickiene et al., 1997). Also flow-mediated responses, which have only been studied in human skin resistance vessels, showed enhanced responses in pregnant vessels when compared to non-pregnant vessels (Cockell and Poston, 1996). Whether these responses show the same pattern in the myometrial vessels remains to be elucidated.

10.1.3: Cell culture Vs Myography:

Comparison of the cell culture and the myography studies undertaken in this thesis show striking similarities, but also striking contradictions. This thesis, in accord with other studies, show that plasma from women with pre-eclampsia is capable of altering endothelial cell function, both in the myography and cell culture system (Baker et al., 1995a) (Davidge et al., 1995b) (Ashworth et al.,

1998). It also demonstrates that VEGF causes similar endothelial alterations in both myography and cell culture experiments, and that inhibition of VEGF with a specific antibody was capable of returning plasma from women with pre-eclampsia toward the control group in both the myography and cell culture systems.

The mechanisms for these changes in the two experimental systems appears to be contradictory. In the cell culture system, both VEGF and plasma increase cellular prostacyclin production, an effect which is inhibited by the addition of VEGF antibody. However, in the myography system the effect appears to be mediated through an alteration in the nitric oxide pathway. This may represent the use of endothelial cells from a different species, as it has been shown that microvascular endothelium derived from different organs displays distinctive tissue-specific characteristics (Fajardo, 1989). When this project was initiated it was planned to utilise human microvascular endothelial cells, however, as Chapter 9 demonstrates this was not possible is human microvascular cells were unresponsive to the effects of plasma.

The majority of such studies (including this one) have utilised either bovine endothelial cells (B88) or human umbilical vein endothelial cells, however, neither of these represent the human pregnant microvascular endothelium where the pathology of this disease is postulated to occurs (Roberts et al., 1989). Therefore, the extrapolation of these *in vitro* results to the *in vivo* situation must be treated with caution. The results presented serve to demonstrate that plasma from women with pre-eclampsia causes an acute in vitro effect on endothelial cell prostacyclin production and that this effect can be inhibited by anti-VEGF antibody. Further work is required to confirm that this observed *in vitro* effect is relevant to the in vivo situation. Studies of 'pre-eclampsia in a dish' may be fraught with danger due to inappropriate extrapolation into the human system.

10.2: Maternal vascular adaptations to pregnancy and the role of VEGF:

Evans et al (1997) have shown a positive and highly significant correlation between maternal serum levels of VEGF and hormones reflecting placental function (hCG, progesterone). They speculated that VEGF had a positive influence on trophoblast development and that it may also be involved in the initiation of the maternal cardiovascular adaptation to pregnancy (Evans et al., 1997). Wheeler et al (1999) have also shown that VEGF measured at 16-20 weeks gestation correlates with placental and fetal weight at birth, suggesting that VEGF is important in the utero-placental adaptations to pregnancy, during the second wave of trophoblast invasion (Wheeler et al., 1999). This thesis demonstrated that VEGF causes a vasodilatation in non-pregnant myometrial vessels, whereas pregnant vessels show no response. This further enhanced the hypothesis that the vascular endothelium undergoes specific changes in response to pregnancy. It is possible to speculate that VEGF may cause vasodilatation in early pregnancy to enhance utero-placental blood flow and the early development of the placenta and that as pregnancy progresses this effect is lost. It would be interesting to determine how VEGF causes vasodilatation in non-pregnant vessels and at what gestation this effect is lost. However, without an animal model this is not possible, unless technology advances to allow non-invasive experiments to be carried out on human myometrial vessels. One possible candidate animal to study would be the guinea pig, as it shares a similar placental development to the human placenta. It has also been recently shown that when pregnant guinea pigs are injected with VEGF they develop a syndrome similar to pre-eclampsia, however, injection into non-pregnant animals does not produce these abnormalities (Hunter, 2000). Therefore, it would be interesting and

potentially useful to ascertain whether guinea pig uterine artery display similar effects to human non-pregnant and pregnant myometrial arteries on exposure to VEGF.

10.3 Maternal vascular adaptations to pre-eclampsia and the role of VEGF:

Although this thesis does not appear to shed any light on the origin of this increased VEGF, It is clear from this thesis and the literature that VEGF is increased in pregnancies complicated by pre-eclampsia (Chapter 4) and that levels return to normal soon after delivery. This rapid return to normal would suggest that the fetal-maternal unit was implicated in the increased levels observed. One methodological problem of this study and others that have examined the placenta as a possible site (Cooper et al., 1997) (Lyall et al., 1997b) (Chapter 5) is the differences in the gestational ages that placental biospies are obtained from each study group. Simmons et al (2000) have recently tried to address this discrepancy by the use of women with pre-eclampsia in the late third trimester and comparison with appropriate gestation matched controls. They demonstrated that placental VEGF immunostaining was significantly increased in women with pre-eclampsia; and that the increased VEGF expression positively correlated to the uterine artery resistance as assessed by Doppler ultrasound. From this they postulated that decreased uteroplacental blood supply causes a relative hypoxia within the placenta and increased production of VEGF (Simmons et al., 2000). Ranheim et al (2001) also using a more appropriate control group have recently demonstrated a non-signifiicant increase in the mRNA expression of placentae from women with pre-eclampsia when compared to normotensive controls. One reason they may have failed to demonstrate any significant differences was the small numbers of placentae used and it may be useful to readdress this question with more appropriate numbers.

It has also been recently demonstrated that VEGF correlates with the increase in total peripheral resistance that occurs in this disease (Bosio et al., 2000). This thesis demonstrates that VEGF is capable of modulating vascular endothelial function in such a way as to enhance the response to the vasoconstrictor vasopressin and inhibit the endothelium dependent relaxation to bradykinin in myometrial vessels. In a manner that is similar to that in vessels from women with pre-eclampsia (Chapter 6) (Allen et al., 1989) (Ashworth et al., 1999) and to normal pregnant vessels incubated with plasma from women with pre-eclampsia (Ashworth et al., 1998).

The contribution of the eicosanoids to the mechanism of the interaction between VEGF and the endothelium was examined by the addition of indomethacin to the organ bath. The inhibition of eicosinoids failed to alter the concentration response curves to vasopressin or bradykinin. These observations suggest that there is no preferential release of dilator or constrictor prostanoids in the vessels from normal pregnant woman, women with pre-eclampsia, or when vessels were incubated with VEGF. This is consistent with the observations of Hayman (1999), who demonstrated that the addition of indomethacin to normal pregnant vessels, which had been exposed to plasma from women with pre-eclampsia, also failed show alterations in their concentration response curves. Although these findings were consistent with previous work (McCarthy et al., 1994), they differ from other observations seen in myometrial vessels from women with established disease (Ashworth et al., 1999). However, in this thesis it has been demonstrated that results of Ashworth et al (1999) may be artifactual due to an increased relaxation in repeated concentration response curves (Chapter 3).

The contribution of nitric oxide to the mechanism of resistance vessel relaxation; from non-pregnant women; and normal pregnant women; and women with pre-eclampsia in both

myometrial and omental vessels; and the interaction between VEGF and the endothelium was studied by the addition of indomethacin and L-NAME. It was observed that omental vessel relaxation in pregnancy and pre-eclampsia remained unaltered by the addition of indomethacin and L-NAME. Vessels obtained from the myometrium of non-pregnant and pregnant women showed dramatic differences, with an alteration in the contribution of nitric oxide to the relaxation. Both vessels from non-pregnant women and women with pre-eclampsia show considerable dependence on nitric oxide mediated relaxation, whereas vessels from pregnant women showed no nitric oxide mediated relaxation. This would suggest that pregnancy is associated with an increase dependence on EDHF mediated relaxation, and that in pre-eclampsia this EDHF mediated relaxation is lost.

McCarthy et al reported that in subcutaneous arteries, the inhibition of both cyclo-oxygenase and nitric oxide synthase attenuated, but did not abolish the endothelium-dependent relaxation to acetylcholine (McCarthy et al., 1994). Pascoal and Umans studied resistance arteries from the omentum and demonstrated, in a similar manner to this thesis, that vessels from normal pregnant women completely relaxed to acetycholine in an endothelium-dependent, but nitric oxide independent manner (Pascoal and Umans, 1996).

Although these results suggest the presence of EDHF, this 'compound' has yet to be identified, thus the importance of its role in physiology is speculative. However, as discussed previously recent experimental evidence supports a role for gap junction communication in the control of arterial function (Chapter1), and it seems that endothelial dependent relaxation, mediated through both nitric oxide and EDHF, partly relies on gap junctions (Javid et al., 1996) (Taylor et al., 1998). As such, the study of gap junctions in pregnancy and pregnancies complicated by pre-eclampsia

would be of interest and may have significant clinical implications. These studies could initially be carried out using the technique of myography with the use of gap junction inhibitors. The definitive investigation of EDHF would require electrophysiology to confirm the loss of the membrane hyperpolarisation with gap junction inhibition.

This thesis showed that VEGF, in a similar manner to plasma from women with pre-eclampsia incubated with normal pregnant vessels, and vessels obtained from women with pre-eclampsia, caused a loss of endothelium-dependent relaxation. This loss of endothelium-dependent relaxation appeared to be associated with in increase in nitric oxide mediated relaxation. From this thesis it could be speculated that VEGF alters gap junction communication. It would be interesting to investigate this further by utilising vessel electrophysiology to establish whether VEGF inhibits hyperpolarisation on stimulation. If this was proven to be the case then this would then stimulate investigation of the mechanism of this response.

Summary:

Pre-eclampsia is a multi-system disease characterised by maternal hypertension and proteinuria. This disease has been the leading cause of maternal death in the last decade. The disease is also responsible for considerable perinatal morbidity and mortality. There is evidence to suggest that the maternal endothelium is pivotal in the pathophysiology. There is also increasing evidence to support the hypothesis that a placentally derived circulating factor causes alterations in endothelial function in pre-eclampsia. The evidence for this comes from cell culture experiments. These have demonstrated that endothelial cells cultured in the presence of plasma from women with pre-eclampsia produces increased amounts of the vasoactive compounds nitric oxide and prostacyclin. It has been demonstrated that normal myometrial vessels incubated with plasma from women with pre-eclampsia exhibit a reduction in endothelium dependent relaxation. However, the nature of this circulating factor remains to be elucidated.

During pregnancy the placenta produces many cytokines that can pass into the maternal circulation. It is well documented that cytokines alter endothelial cell functions and as such are obvious candidates for the circulating factor. Vascular Endothelial Growth Factor (VEGF) is such a cytokine and there is much circumstantial evidence to support the hypothesis that placental-derived VEGF mediates the endothelial activation of pre-eclampsia. VEGF is produced by the placenta and unlike other cyokines contains a signal sequence that allows it to be secreted into the maternal circulation. *In vitro* levels of VEGF are dramatically increased within a few hours of exposure to hypoxia (Shweiki et al., 1992). VEGF exerts its biological activity by binding to one of two receptors: flt-1 and KDR receptors. Different members of the VEGF family have different binding characteristics, this may have important implications for the control and functioning.

The thesis hypothesis was that placentally produced VEGF was important in the altered endothelial function observed in pre-eclampsia

Plasma samples were obtained from primigrivid control normotensive women and primigravid women diagnosed with pre-eclampsia matched for age, gestation and race. Plasma VEGF levels were then measured using a radioimmunoassay validated for use in pregnancy plasma. Utilising this assay it was demonstrated in accord with other studies that levels of VEGF increase in women with pre-eclampsia when compared to normal pregnant controls. We also showed that the maternal serum concentrations of VEGF correlate with the observed increase in both diastolic and systolic blood pressure that occurs with this disease.

Utilising myometrial resistance vessels of a diameter of 200-500μm from biopsies taken at the time of Caesarean section and the technique of wire myography we have confirmed the finding that these vessels from women pre-eclampsia exhibit a loss of endothelium dependent relaxation to bradykinin when contracted with vasopressin. It was also confirmed that normal pregnant myometrial vessels incubated with 2% plasma from women with pre-eclampsia for 16 hours exhibit a similar loss of endothelium dependent relaxation. These studies were thus extende to examine the interaction of VEGF with the endothelium in this vascular bed. It was shown that myometrial vessels when incubated with VEGF exhibit a reduced endothelium-dependent relaxation to bradykinin, which is analogous to that observed in vessels from women with pre-eclampsia and normal vessels exposed to the plasma of women with pre-eclampsia. This response was concentration and time dependent; requiring a minimum concentration of 0.5 μM

and an incubation time of 16 hours. The lengthy incubation time suggests that an alteration in cellular biochemistry may be required for VEGF to have its observed alteration in endothelial function.

This myometrial vessel model of pre-eclampsia was further utilised to characterise the endothelial interactions of VEGF and plasma from women with pre-eclampsia. The observed interaction of VEGF with the endothelium was abolished upon the addition of an anti-VEGF antibody (R&D, UK) to the media prior to incubation. Similarly the addition of anti-VEGF antibody to plasma from women with pre-eclampsia caused these vessels to return towards a normal relaxatory profile. The removal of VEGF from the plasma of women with pre-eclampsia utilising anti-VEGF antibody coated Dynal beads had no significant effect on the loss of endothelium dependent relaxation. These results would suggest that plasma borne factor causes the blood vessels to increase production of VEGF which then affects the endothelial function of the vessel.

VEGF functions through at least two receptors; the flt-1 and KDR receptors. Therefore experiments were designed to elucidate which endothelial receptor VEGF was acting through. The effect of VEGF was unaffected by incubation with a specific anti-KDR receptor antibody. However, the addition of a specific anti-flt-1 receptor antibody to both plasma and VEGF returned endothelial vessel function toward normal. This implies that plasma contains a factor that causes the release of VEGF from the blood vessel, which then acts through the flt-1 receptor to cause the observed alteration in endothelial function.

It has been previously demonstrated that endothelial cells cultured in the presence of plasma showed increased production of the vasoactive compounds prostcyclin and nitric oxide when

compared to normal pregnant control plasma. Therefore to validate the myometrial vessel model results an endothelial cell culture model was utilised. Monlayer cultures of Bovine microvascular endothelial cells were prepared. Cells were then exposed to plasma from women with pre-eclampsia and normal control plasma in the presence or absence of anti-VEGF antibody. The presence of anti-VEGF antibody significantly reduced the production of prostacyclin to a level comparable to that produced by normal pregnant plasma. The production of prostacyclin stimulated by normal pregnant plasma was unaffected by the antibody. These results demonstrate that VEGF may be pivitol to the pathogenesis of pre-eclampsia.

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Appendix I:

Dynabeads were coated with antibody according to manufacturers instructions. Briefly a 1ml suspension of the tosylated Dynabeads (DM280) was aliquoted into a 1.5 ml eppendorff tube (Anachem-Scotlab;Luton,UK)) this was placed in a Dynal® MPC (Dynal® Magnetic Particle Concentrator) for 2 minutes. Following removal of the supernatant the Dynabeads were then re-suspended in 740µl of phosphate buffered saline (PBS). 360µl of the antibody was then dissolved in a borate buffer (pH 9.5), mixed with the Dynabead® suspension (to give 30µg of antibody per 10^8 Dynabeads) in 1m PBS. This mixture was then incubated for 24 hours at 37°C with continuous slow tilt rotation. After incubation, the tube was placed in the Dynal® MPC for 2-3 minutes and the supernatant removed. The coated Dynabeads were washed four times: twice in PBS/BSA (pH 7.4 for 5 minutes at 4°C), once in Tris/BSA (pH 8.0 for 4 hours at 37°C), and once in PBS/BSA (pH 7.4 for 5 minutes at 4°C).

Appendix II

Physiological Salt Solution:

To make up 1 litre of PSS or potassium-replaced PSS:

	PSS		KPSS	
Compound	Weight(g)	mM	Weight(g)	mM
NaCl	6.954	119	0	0
KCL	0.35	5.9	9.223	123
$CaCl_2.2H2O$	0.368	2.5	0.368	2.5
$MgSO_4.7H20$	0.289		0.289	
$NaHCO_3$	2.1		2.1	
KH_2PO_4	0.161		0.161	
EDTA	0.01	0.1	0.01	0.1
Glucose	1.091	6.0	1.091	6.0

The above compounds with the exception of the calcium chloride ($CaCl_2$), are dissolved in distilled water to make up 1 litre. The solution is then carbonated with 5% CO2 and 95% O2 for approximately 15 minutes. The CaCl 2 is then added to the solution. The solution requires re-carbonation prior to usage following any storage. The pHs of the solutions are 7.4.

Appendix III

$$\text{Transmural pressure (Pi)} = \frac{\text{Wall tension}}{(\text{internal circumference}/2\pi)}$$

Appendix IV:

Protein Extraction Buffer:

NaCl 0.1 M

Tris HCL 0.01M (pH 7.6)

EDTA 0.001M (pH 8.0)

Aprotinin 1µg/ml

PMSF100µg/ml

100µl Triton X100

Appendix V

Griess solution #1:

Napthylenediamine dihydrchloride 0.01g/ml

Griess solution#2:

Sulphanilamide 10g were dissolved in 11.8 mls of 85% H_3PO_4 and this was then made up to 100mls.

Appendix VI

DC Protein Assay :

Reagent A – alkaline copper tartrate solution

Reagent B – dilute Folin reagent

Reagent A'- Reagent A with Reagent S (20µl reagent S to 1ml reagentA)

Appendix VI

Buffer Solution for Indomethacin:

KH_2PO_4 0.02M

$NaH_2PO_4.2H_2O$ 0.12M

pH balanced to 7.8