THE ROLE OF NITRIC OXIDE IN CERVICAL RIPENING

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Declaration

The contents of this thesis have not been submitted elsewhere for any other degree, diploma or professional qualification.

This thesis has been composed by myself, and I have been responsible for patient recruitment, tissue collection, clinical management and laboratory studies unless otherwise acknowledged.

Marie Anne Ledingham

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This work is dedicated to my husband, Malcolm.

Abbreviations

ADP	adenosine diphosphate
ANOVA	analysis of variance
AP-1	activator protein-1
bNOS	neuronal (brain) nitric oxide synthase
Ca	calcium
COX	cyclo-oxygenase
CRH	corticotrophin releasing hormone
CRH-BP	corticotrophin releasing hormone binding protein
DAB	diaminobenzidine tetrahydrochloride
DEPC	diethyl pyrocarbonate
DETA-NO	diethylenetriamine-nitric oxide
DMSO	dimethylsuphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol

ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	diaminoethanetetra-acetic acid
ELISA	enzyme linked immunosorbent assay
eNOS	endothelial nitric oxide
ERK	extracellular signal related kinase
FAD	flavin adenine dinucleotide
FCS	fetal calf serum
FMN	flavin mononuceotide
GAG	glycosaminoglycan
GAPDH	glutaraldehyde phosphate dehydrogenase
GTN	glyceryl trinitrate
h	hour
HRP	horse radish peroxidase
HUVECS	human umbilical vein epithelial cells
ICAM	intercellular adhesion molecule
IFN	interferon

IL	interleukin
IMN	isosorbide mononitrate
iNOS	inducible nitric oxide
kDa	kilodaltons
L-NAME	N^G-nitro-L-arginine-methyl-ester
L-NMMA	N^G-monomethyl-L-arginine
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
MCP	monocyte chemoattractant protein
MIP	macrophage inhibitory peptide
MMP	matrix metalloproteinase
MPA	medroxypogesterone acetate
mRNA	messenger ribonucleic acid
N	newtons
NAD	nicotinamide adenine dinuleotide
NF-κB	Nuclear Factor kappa B
NO	nitric oxide

NOS	nitric oxide synthase
OTR	oxytocin receptor
PAF	platelet activating factor
PBS	phosphate buffered saline
PECAM	platelet endothelial adhesion molecule
PGDH	prostaglandin dehydrogenase
PGE_2	prostaglandin E_2
PGEM	prostaglandin E metabolite
PGF_2	prostaglandin F_2
PGFM	prostaglandin F metabolite
PMA	phorbol myristal acetate
RT-PCR	reverse transcriptase polymerase chain reaction
SDS-PAGE	sodium dodecyl sulphate
SEM	standard error of the mean
SNAP	S-nitroso-N-acetyl-D,L-penicillamine
SNP	sodium nitroprusside
SOD	superoxide dismutase

TBS-T	Tween-Tris buffered saline
TIMP	Tissue inhibitor of matrix metalloproteinases
TMB	tetramethyl benzidine
TNF	tumour necrosis factor
TX	thromboxane
UTI	urinary trypsin inhibitor
UV	ultraviolet
V	volts
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
XO	xanthine oxidase

Abstract

Cervical ripening is fundamental to the process of parturition. Recent animal studies have suggested that the inflammatory mediator nitric oxide is involved in this process. The aim of the studies reported in this thesis was to investigate the potential role of the L-arginine-nitric oxide system in ripening the cervix in humans.

The nitric oxide donor isosorbide mononitrate (IMN) administered per vaginam has previously been shown to ripen the human cervix. The studies reported in chapter 2 were performed in order to compare the effectiveness and side effect profile of the nitric oxide donor (IMN) with the prostaglandin analogue gemeprost. 66 primigravid women in the first trimester of pregnancy were therefore recruited to this randomised controlled trial. A significantly greater percentage of women remained asymptomatic following IMN (64%) than following gemeprost (14%). Pre-treatment with gemeprost resulted in abdominal pain in 73% of women and vaginal bleeding in 32% compared with 3% and 0% respectively following IMN. These data suggested that IMN could be used as an alternative to gemeprost for this indication.

A further randomised controlled trial is reported in chapter 3. This explored the hypothesis that combined therapy with IMN (40mg) and the prostaglandin analogue misoprostol (400μg) for preoperative cervical ripening in the first trimester would result in improved clinical effectiveness and fewer side effects compared to each agent used alone. There was no difference in the number of women remaining asymptomatic following either IMN or misoprostol or combination therapy (14/22 [64%] vs 11/21 [52%] vs 11/22 [50%], Fisher's exact test). Pre-treatment with misoprostol used alone resulted in lower cervical resistance than IMN (18.5 N (Newtons) vs 39 N, p=0.04, Mann-Whitney U). The cervical resistance following combination therapy with IMN and misoprostol was not significantly lower than following misoprostol alone (24.5 N vs 18.5 N). These data suggest that the nitric oxide

donor IMN alone or in combination with misoprostol has no advantages over misoprostol alone for preoperative cervical ripening in the first trimester.

The studies reported in chapter 4 were performed to investigate whether an increase in nitric oxide production occurs in the human cervix in conjunction with cervical ripening. Using Western blotting and immunohistochemistry, the expression and localisation of the enzymes responsible for the production of nitric oxide, inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (bNOS) were determined in the human uterine cervix during pregnancy and parturition. The expression of each of the NOS isoforms was greater in cervical tissue from pregnant women compared to non-pregnant. iNOS and bNOS protein expression increased in the first trimester of pregnancy compared to non-pregnant samples ($p<0.005$ for iNOS and bNOS respectively). iNOS was further upregulated in the third trimester of pregnancy prior to the onset of labour ($p<0.01$). These data indicate that expression of NOS is increased in the cervix during pregnancy and that iNOS in particular may play a role in spontaneous cervical ripening during human pregnancy.

The mechanism by which nitric oxide ripens the cervix is unclear. Data presented in chapter 5 demonstrate the effect of nitric oxide donors administered in the first trimester of pregnancy on the secretion of MMPs (Matrix Metalloproteinases) –2 and –9 and their tissue inhibitors (TIMPs) in the human cervix. MMP-2 and MMP-9 were expressed in conditioned explants of cervical tissue from non-pregnant women. MMP-9 secretion was only detected in explants from pregnant women. Treatment with the nitric oxide donors spermine nonoate *in vitro* (non-pregnant cervical biopsies) and isosorbide mononitrate *in vivo* (pregnant cervical biopsies) had no effect on the secretion of MMPs and TIMPs studied. The mechanism of action of nitric oxide donors in inducing cervical ripening must therefore be attributable to other mechanisms.

The mechanism of action of nitric oxide donors was further investigated in chapter 6. Cervical biopsies were obtained from pregnant women randomised to receive IMN or no treatment prior to suction termination. ELISAs were performed on culture supernatant for interleukin (IL)-1, IL-6, IL-8, IL-10, IL-15, TNFα, MCP-1 and prostaglandin metabolites. Biopsies treated with IMN produced significantly greater amounts of $PGF_{2}\alpha$ in culture and lower amounts of thromboxane B_2 than controls (572.8 pg/ml vs 34.9 pg/ml, $p<0.05$: 53.3 pg/ml vs 530.9 pg/ml, $p<0.01$ respectively). Treatment with nitric oxide had no effect on the release of cytokines or other prostaglandin metabolites. These data suggest that the mechanism of action of NO induced cervical ripening during pregnancy may be mediated in part via increased prostaglandin synthesis.

Cervical ripening is an inflammatory process involving leukocyte recruitment. These leukocytes appear to be the source of nitric oxide involved in the cervical ripening process, as demonstrated in the studies described in chapter 4. The factors controlling the infiltration of leukocytes in the cervix are poorly understood. However in other tissues leukocyte infiltration from the vasculature is controlled by the expresssion of a number of cell adhesion molecules. In chapter 7 the expression and localisation of the cell adhesion molecules intracellular adhesion molecule-1 (ICAM-1), E-selectin, platelet endothelial cell adhesion molecule (PECAM) and vascular cell adhesion molecule (VCAM) in the cervix and myometrium during pregnancy and labour were investigated. Biopsies of cervix and myometrium were obtained from non-pregnant women and from pregnant women before and after the onset of labour at term. Expression of cell adhesion molecule mRNA was determined using Northern blotting and localisation of cell adhesion molecule protein was achieved using immunohistochemistry. ICAM-1 mRNA was upregulated in the cervix ($p<0.01$) and myometrium ($p<0.01$) during labour. VCAM mRNA was upregulated in the cervix during pregnancy with no further change with labour. PECAM mRNA was upregulated in myometrium with pregnancy but did not change with labour. Changes in the

expression of these cell adhesion molecules are likely to control the infiltration of leukocytes into these tissues during pregnancy and parturition.

In summary, these data suggest that nitric oxide is fundamentally involved in the process of cervical ripening in human pregnancy. There is an upregulation of the L-arginine-nitric oxide system in the third trimester of pregnancy at the time when cervical ripening takes place. Further evidence for the importance of nitric oxide in this process is generated from the studies presented here demonstrating that nitric oxide donors ripen the cervix in the first trimester of pregnancy with fewer side effects than prostaglandins. Combining nitric oxide donors with prostaglandin analogues however does not seem to improve the clinical effectiveness of these agents. Further insights into the involvement of the nitric oxide system in pregnancy and parturition may improve our treatment of preterm and post-dates pregnancy.

Chapter 1

Literature Review

"If a man will begin with certainties, he shall end in doubts; but if he will be content to begin with doubts, he shall end in certainties."

Francis Bacon, *The Advancement of Learning* (1605).

Introduction

The uterine cervix plays a fundamental role in the process of human pregnancy maintenance and parturition. Its unique structure allows the fetus to remain within the uterine cavity until maturity is complete, whilst facilitating changes at the time of labour to effect the passage of the infant vaginally. The mechanisms which control the changes in the cervix and which trigger the onset of labour in humans remain poorly understood. However an understanding of these events is clearly important in clinical practice where premature and post-dates deliveries have important effects on neonatal outcome.

Premature labour (labour occurring prior to 37 weeks completed gestation) remains the leading cause of infant morbidity and mortality (Pitman M.C. and P.J., 1996). The incidence of premature delivery has remained relatively stable over the past 20 years at around 5-10% of all pregnancies, despite the widespread use of tocolytic agents (Danielian and Hall, 1996). Further, despite improvements in neonatal intensive care, survival is associated with considerable morbidity. Short-term outcomes include respiratory distress, persistent fetal circulation, necrotising enterocolitis, intraventricular haemorrhage and hypoxic ischaemic haemorrhage. In long term survivors, bronchopulmonary dysplasia, retinopathy, sensorineural deafness and neurological handicap are all common (Johnson, et

al., 1993; MacGillivary and Campbell, 1995; McCormick, 1985). A UK study recently reported that 39.1% of cases of cerebral palsy occurred in children delivered prematurely and 23% of infants delivered before 29 weeks had significant morbidity (MacGillivary and Campbell, 1995).

Preterm birth can be iatrogenic associated with such complications as pre-eclampsia, antepartum haemorrhage, fetal growth retardation and isoimmunisation. However up to 38% of preterm deliveries are associated with spontaneous labour without associated pregnancy related problems (Olah and Gee, 1992). Factors such as intrauterine infection, maternal and fetal stress, low socio-economic class, previous cervical damage and anatomical abnormality of the uterine cavity are clearly associated with prematurity. However in the majority of patients presenting with this problem there is no such defined underlying cause and as a result the condition remains poorly understood and inadequately treated.

It is now evident that prolonged gestation is also associated with an increase in neonatal and post neonatal mortality (Crowley, 1989; Hilder, et al., 1998). When induction of labour is considered it can be mediated with a variety of agents, prostaglandins being the most commonly used (MacKenzie and Embrey, 1979). The Royal College of Obstetricians and Gynaecologists recently issued a recommendation that induction of labour should be offered routinely to all women after 41 weeks gestation (The Royal College of Obstetricians and Gynaecologists, 1998).

Improvements in the management of both preterm and post dates pregnancy are hampered by our limited understanding of the events controlling the onset of parturition in the human. Both term and preterm labour share a final pathway of cervical ripening, activation

of the fetal membranes and the onset of regular uterine activity. Previous research examining the pathophysiology of labour has largely concentrated on the mechanics and control of uterine activity, with the role of the cervix being largely ignored (Olah and Gee, 1992). However, the active biological changes occurring within the cervix during pregnancy would suggest that this organ plays an active role in the labouring process. Improvements in the management of both preterm and post dates pregnancy will not occur until we develop a better understanding of the events occurring in both the uterus and the cervix at this time.

Part I: Control of the onset of labour

" Nothing begins, and nothing ends,

That is not paid with moan;

For we are born in other's pain,

And perish in our own."

Francis Thompson, *Daisy* (1913)

The timing of parturition is a crucial event in the survival of any species, and it seems unlikely therefore to be controlled by a single biochemical pathway. It has been proposed that labour occurs due to the transition of the myometrium from an inactive to an active muscle either due to the withdrawal of inhibitory agents or by the addition of uterotonins. A number of agents have been thought to be involved in maintaining the uterus in its state of quiescence during pregnancy including progesterone, prostacyclin, relaxin, nitric oxide, parathyroid hormone related peptide, human placental lactogen, corticotrophin releasing hormone, calcitonin gene related peptide and vasoactive intestinal peptide (Challis, 1998; Lopez, et al., 1995). It is thought that prior to the onset of labour the uterus becomes activated in response to uterotrophins such as oestrogen. This results in increased formation of gap junctions, activation of voltage dependent calcium ion channels, and an increase in expression of myometrial contraction related proteins (including receptors for prostaglandins and oxytocin) (Garfield, et al., 1988; Garfield, et al., 1980). Following activation, the uterus then becomes responsive to uterotonins such as oxytocin, prostaglandin E_2 and prostaglandin $F_2\alpha$ (Norwitz, et al., 1999) which stimulate uterine activity in the responsive myometrium.

In human pregnancy, the specific triggering mechanisms for the onset of labour and the means by which these factors are integrated remains unclear. However, in most viviparous

animals it is the fetus that seems to control the onset of parturition. From an evolutionary viewpoint this would seem appropriate, allowing labour to occur at a time when fetal maturity was adequate for extra-uterine survival. This is certainly the case in the sheep where the trigger for the onset of labour is dependent on activation of the hypothalamic-pituitary-adrenal axis resulting in increased cortisol production. Fetal cortisol increases the activity of placental enzymes including 17-alpha hydroxylase and P450 C-17,20 Lyase, producing increased synthesis of oestrogen and decreased synthesis of progesterone (Flint, et al., 1975; Liggins, 1989; Nathanielsz, 1998). The alteration in oestrogen to progesterone ratio in turn causes gap junction formation in the myometrium, stimulation of oxytocin receptors and activation of the phospholipase A_2 pathway, leading to the onset of labour (Challis and Olson 1988; Garfield, et al., 1979).

In man, there is also a rise in fetal cortisol and placental oestrogen production with the onset of labour, however there is no measurable change in plasma progesterone levels. This is because the target enzyme for cortisol action, 17-alpha hydroxylase is absent in the human placenta (Flint, et al., 1986). Furthermore, in anencephalic fetuses, the mean gestational length is 40 weeks (Honnebier and Swaab, 1973). This would suggest that a functioning hypothalamic-pituitary-adrenal axis is not necessary for the onset of labour in man. Cortisol may have a role in the onset of parturition in the human, however the available evidence would suggest that the means by which it exerts its effects is more complicated than in the sheep model.

It has also been suggested that the endocrine control of parturition may stem from signals produced within the fetal lung. Platelet Activating Factor (PAF) is produced in human fetal lung explants and *in vitro* has been shown to increase production of prostaglandin E_2 in human amnion (Johnston, et al., 1987). In rabbits, increased surfactant production in the lung is associated with an increase in PAF. Thus is would seem reasonable that PAF is involved in the initiation of parturition once fetal lung maturity is complete. However in

cases of congenital bilateral pulmonary agenesis, where lung tissue is obviously absent, there is no associated increase in the incidence of premature labour or prolonged pregnancy (Claireux and Ferreira, 1958). This would suggest that while factors produced by the mature fetal lung may be supportive, they are not sufficient to control labour in man.

While it is unclear whether the fetus triggers the onset of labour in man, there is good evidence that prostaglandins play a role in the control of parturition. Prostaglandins are capable of inducing labour (Calder, 1990) and prostaglandin synthase inhibitors have been used in clinical practice as tocolytic agents due their inhibitory effects on myometrial contractility (Wiqvist, 1979). Following the onset of labour, there is an increased production of prostaglandin E_2 and prostaglandin $F_{2\alpha}$ in the amnion and chorio-decidua (Keirse, 1979; Mitchell, 1984; Skinner and Challis, 1985). Prostaglandin production also increases in the cervix during the period of cervical ripening which occurs prior to the onset of uterine activity (Ellwood, et al., 1980; MacKenzie, 1981). Taken together, these findings suggest that prostglandins are intimately involved in the initiation and progression of labour.

Prostaglandins are synthesised from arachidonic acid by the enzyme cyclo-oxygenase (COX). Two isofoms of this enzyme have been identified, COX-1, the constitutively expressed form and COX-2, the inducible isoform. COX-2 expression is induced in mammalian tissues by growth factors and various cytokines and inhibited by glucocorticoids (Masferrer and Seibert, 1994; Xie, et al., 1991). Recent work has shown that there is an increase in expression of COX-2 in the amnion and chorio-decidua with the onset of labour at term (Slater, et al., 1998; Slater, et al., 1995). Premature prostaglandin release has been implicated in the aetiology of preterm labour. This is thought to occur secondary to intrauterine infection (Romero, et al., 1989) and activation of fetal cortisol-

placental corticotrophin releasing hormone (CRH) under fetal compromise (McLean, et al., 1995). This has prompted suggestions that specific inhibitors of COX-2 may be of future value in the treatment of preterm labour.

While it has been shown that there is no fall in serum progesterone levels in humans with the onset of labour, there remains good evidence that progesterone is fundamentally involved in maintaining uterine quiescence at this time. Progesterone exerts an overall quiescent effect by (i) suppressing genes in the myometrium necessary for uterine contractility (connexin 43, calcium channels, oxytocin receptors), (ii) upregulating relaxation mechanisms and (iii) suppressing release of pro-inflammatory cytokines and decreasing the availability of prostaglandins (Garfield, Radideau et al. 1979; Chwalisz and Garfield 1994; Chwalisz 1994). The administration of antiprogesterones such as RU486 therefore results in activation of uterine activity by increasing myometrial responsiveness to uterotonic agents and in addition causes cervical ripening in humans (Chwalisz, 1994). Labour has been likened to an inflammatory process (Kelly, 1994) and progesterone is a powerful anti-inflammatory agent. Hence it would appear that progesterone is involved in the maintenance of human pregnancy in a similar manner to that of the sheep. Various attempts have been made to explain the observed lack of fall in progesterone levels in the case of the human. Theories include local modulation of oestrogen and progesterone biosynthesis in fetal membranes at levels which would be undetectable in the maternal circulation (Mitchell and Challis, 1988), progesterone inactivation by specific binding proteins (Westphal, et al., 1977) and changes in the expression of the progesterone receptors (Allport, et al., 2000; Pieber, et al., 2000; Winkler, 2000). While further evidence is needed to support the theory of local progesterone withdrawal, it remains an attractive theory, which would link parturition in the human to that of other species.

Corticotrophin releasing hormone (CRH) has also been proposed as the initiator of labour in man (Challis, 1998; Jones and Challis, 1990). CRH is a 41 amino acid peptide hormone, which has similar structure to hypothalamic releasing factor. It was originally thought to be a product of the placental cytotrophoblast but is now known to be expressed in the syncytiotrophoblast where it is stored in granules and released into the maternal compartment (Keelan, et al., 1997; Riley, et al., 1991). Recently the endometrium has also been identified as a source of CRH (DiBlasio, et al., 1996). CRH receptors are present in the human myometrium and in late pregnancy the affinity of these receptors for CRH increases (Grammatopolous, et al., 1995; Hillhouse, et al., 1993). At term, in the presence of oxytocin, activation of these receptors by CRH causes a decrease in myometrial cAMP levels resulting in an increase in contractility (McLean, et al., 1994). The bioavailability of CRH is affected by binding to a specific binding protein (CRH-BP). This is present in relatively stable concentrations in maternal plasma during pregnancy until about 4 weeks prior to the onset of labour when the levels of CRH-BP decline, probably as a result of increased hepatic clearance (Linton, et al., 1993; McLean, et al., 1995). Hence in the final few weeks of pregnancy, rising CRH concentrations accompanied by a fall in CRH-BP levels may both promote final fetal organ maturation through stimulation of fetal adrenal cortisol production and also stimulate the onset of regular uterine activity (Challis, 1998).

Historically oxytocin has been recognised as a primary factor involved in the control of parturition (Hirst, et al., 1993). However, its exact role remains controversial due to the failure to observe any increase in oxytocin levels in the maternal plasma with the onset of labour (Dawood and Khan-Dawood, 1985). The role of oxytocin may therefore be to intensify labour once contractions have started, since agents such as CRH are known to sensitise the uterus to the uterotonic effects of oxytocin (McLean, et al., 1994).

Alternatively, increased myometrial sensitivity to oxytocin through the activation of specific oxytocin receptors (OTRs) could provide another mechanism whereby oxytocin could influence parturition. OTR mRNA is expressed in human decidual cells, chorionic trophoblasts and myometrium and levels increase during labour (Fuchs, et al., 1984; Fuchs, et al., 1991; Kimura, et al., 1996). Furthermore oxytocin can stimulate prostaglandin production in fetal membranes (Fuchs, et al., 1984). It is therefore possible that oxytocin may be important in stimulating intrauterine prostaglandin production at term, amplifying uterine contractile activity.

It is now known that labour is an inflammatory process involving leukocytic influx of the myometrium, cervix and fetal membranes with the subsequent release of various cytokines (Bokstrom, et al., 1997; Kelly, 1994; Liggins, 1981; Rosenberg, et al., 1996; Thomson, et al., 1999). As discussed already, there is also evidence that inflammatory cytokines may have a pivotal role in infection induced preterm labour (Baumann and Romero, 1995; Romero, et al., 1994; Romero, et al., 1994). Levels of interleukin-1 (IL-1), IL-6 and tumour necrosis factor-α (TNF-α) are increased in the amniotic fluid of women with infection induced preterm labour and may act synergistically with prostaglandins to stimulate parturition (Romero, et al., 1991; Romero, et al., 1989; Romero, et al., 1989). The role of cytokines in the process of cervical ripening is discussed later in this review.

Thus, human labour is a physiological process involving the integration of a series of changes within the uterus occurring over a period of days or even weeks. Numerous factors have been implicated in controlling the switch to active labour and it is likely that complex endocrine, paracrine and autocrine mechanisms are involved. The uterus is more than a mere sac, which functions to contain the growing conceptus, and allows it to be expelled at the time of parturition. Furthermore, the active physiological processes that occur within

the uterus at parturition are dependent on changes, which take place in the uterine cervix in the last few weeks of gestation.

Part II: The cervix in pregnancy and parturition

Cervical ripening, the process whereby the cervix undergoes softening, effacement and dilatation, is a gradual process occurring in the 4-6 weeks prior to the onset of labour. The exact mechanisms involved and the factors which control this process remain poorly understood. As clinicians, much of our interest in this process has focused on the therapeutic manipulation of cervical ripening to induce labour at term. Although preterm labour, as in normal labour at term, involves complex changes in cervical structure prior to the onset of uterine activity, research and therapeutic strategies for the management of this condition have largely ignored the important role played by the cervix (Olah and Gee, 1992). Current tocolytic therapies for preterm labour, involving beta sympathomimetics and calcium antagonists, have been shown to be ineffective at prolonging gestation for >48hours and are associated with substantial risks for both mother and fetus (Higby K et al 1993). The failure of these agents is perhaps due to their inability to influence cervical compliance, as changes in the cervix take place at least concurrently with, if not prior to, the onset of uterine activity. A better understanding of the events involved in cervical ripening may therefore allow us to develop better strategies for the management of preterm labour.

Structure of the cervix

The uterus is divided anatomically into 2 basic parts-the uterine body, or corpus, and the cylindrical neck, or cervix. It is said to have the shape of a flattened pear and in the nulliparous woman measures 8x5x3cm (3x2x1in) (McMinn, 1990) and weighs in the region of 50-70g (Leppert, 1995). In multiparous women, the uterus has an average length of 9-10 cm and weighs about 80g. Age as well as parity influences the relative dimensions of the uterus and cervix. In the infant, the cervix is long and comprises 2/3 of the total size,

whereas in the multiparous female, the cervix makes up only 1/3 of the length (Calder, 1994).

The cervix extends into the vagina and classical teaching states that it is joined to the body of the uterus via a narrow central constricted area traditionally known as the isthmus (Vellacott, 1992). The concept of an isthmic portion has been long challenged however since the publication of Danforth's work in 1947. At this time he stated "it is believed that the concept of the isthmic uteri as a separate distinct entity should be eliminated and that, rather, the uterus should be considered as being composed of 2 major parts, corpus and cervix, according to whether the fundamental structure is chiefly muscular or chiefly fibrous." (Danforth, 1947). The upper part of the cervix, or supravaginal portion, lies deep to the vesicouterine pouch and is connected to the bladder above the level of the trigone by dense connective tissue. Posteriorly, the cervix forms the anterior wall of the pouch of Douglas and is covered by peritoneum. The lower part extends into the vagina and is surrounded by the vaginal fornices on all sides, the deepest lying posteriorly. The endocervical canal, which opens into the vagina, is bound by the internal os cephalically and the external os caudally. It is lined by a tall columnar mucus secreting epithelium with highly branched glands. At the external os the epithelium becomes stratified squamous and is in continuity with that of the vagina.

Beneath the epithelium lies a dense connective tissue stroma composed of mainly extracellular matrix. The matrix consists chiefly of type I (66 percent) and type III (33 percent) collagen, with a small amount of type IV present in the basement membrane (Miramoto, et al., 1982). The collagen fibres within the cervix are arranged in dense bundles and play a role in the maintenance of pregnancy by means of their functional rigidity. Elastin fibres are also present and seem to be important due to their recoil

properties in returning the cervix to its original shape after delivery. The fibrous component of the extracellular matrix is embedded in ground substance, made up principally of proteoglycans containing a number of glycosaminoglycans (GAGs). The GAGs are negatively charged large molecular weight mucopolysaccharides with repeating dissacharide subunits. These contain either one hexosamine (glucosamine or galactosamine) or one uronic acid (glucuronic or iduronic) repeating unit. The main GAGs in the cervix are chondroitin and and its epimer dermatan sulphate, but there are a number of others including heparan sulphate and hyaluronic acid. The GAGs vary in size and in the proportion of the various subunits present. Except in the case of hyaluronic acid, they bind to a protein core to form proteoglycans.

Collagen fibres within the cervix are bound by the proteoglycan protein cores. The GAG side chains of the proteoglycan can also bind to collagen and to one another. The binding of the glycosaminoglycan molecules to collagen varies with their chain length and charge density. Hence it is possible to vary the strength of the matrix by altering the glycosaminoglycans present. Hyaluronic acid binds loosely to collagen and hence will destabilise the matrix if present in large quantities. Other molecules such as dermatin sulphate bind strongly to collagen and provide structural rigidity.

The major cell type present in the connective tissue stroma is the fibroblast, which is responsible for the continual turnover of the extracellular matrix. It is postulated that these cells may also have a role in the reduction of cervical circumference after delivery by means of their contractile properties (Liggins, 1981). Smooth muscle fibres make up approximately 10 percent of the cervical tissue's composition (Liggins, 1981). These fibres may contribute to the spatial organisation of collagen fibres and be in some way responsible for the control of cervical rigidity, as cervices from women with incompetent cervices have been shown to have an increased smooth muscle component (Leppert, 1995).

The cervical smooth muscle has some contractile properties (Hillier and Karim, 1970) but it is unlikely that it functions primarily as a sphincter during pregnancy. Recently it has been suggested that the smooth muscle may in fact play an active role in the process of cervical ripening, by means of apoptosis, the process of programmed cell death (Leppert, 1998). Further investigators have suggested that smooth muscle contractions may actively influence the course of labour, with the circular muscle fibres contributing to constriction of the cervical canal and the longitudinal fibres contributing to cervical dilatation (Rudel, 2000).

Changes in the Cervix during Pregnancy.

During gestation there are profound changes which take place within the cervix. In the non-pregnant woman the cervical canal is fusiform and firm, with the consistency of nasal cartilage. During pregnancy, the cervix gradually changes its consistency, becoming soft and compliant as pregnancy advances. These changes occur as a result of alterations in the biochemical composition of the cervix and the rearrangement and realignment of collagen fibres.

The water content of the cervix increases from 80 percent in early pregnancy to around 87 percent at term (Uldbjerg, et al., 1981). As GAGs are highly hydrophilic, increased binding of water to these molecules may destabilise the connective tissue matrix and hence the cervix may be physically altered. There does not seem, however, to be a change in the water content of the cervix in humans immediately before delivery or in the postpartum period (Leppert, 1998). The elastin concentration does not change despite there being an increase in messenger RNA for tropoelastin in pregnancy (Leppert, 1998). There is also an increase in the smooth muscle component of the cervix with these cell becoming more

prominent and presumably as a result also affecting the arrangement of collagen fibres within the tissue.

Collagen concentration is markedly altered during pregnancy. At 10 weeks gestation, the collagen concentration is 70 percent and this drops to around 30 percent at term (Ekman, et al., 1983; Rath, et al., 1994; Uldbjerg, et al., 1983). The collagen fibrils become dissociated and the spaces between them become dilated from about 8-14 weeks gestation (Kokenyesi and Woessner, 1990). Although the collagen concentration is decreased, there is an overall increase in the amount of collagen present, as the other components of the cervix such as water and glycosaminoglycans increase in relatively greater amounts. The fall in collagen concentration during pregnancy may also be attributable to increased turnover of collagen.

Enzymatic degradation of collagen is brought about by the action of proteolytic enzymes. These include several collagenases (MMP-1, MMP-8 and MMP-13) which have the ability to cleave fibrillar as well as non-fibrillar collagen and are all members of the matrix metalloprotease (MMP) family (Hulboy, et al., 1997). The enzymes are secreted in a latent pro form and are activated by other MMPs or in some cases plasmin to form the active enzyme. Enzyme activity can be inhibited by either tissue or plasma inhibitors. Tissue inhibitors of the matrix metalloproteases (TIMPS) are found bound to either the 'active' or the 'pro' forms of the enzyme. The plasma inhibitors, known as the plasma α macroglobulins, are cleaved by MMPs and the resulting covalently bound complex is then subject to intracellular degradation. Tissue and plasma inhibitors play a fundamental role in controlling MMP activity. During pregnancy, increased activity of MMP-1 (tissue and macrophage collagenase) and MMP-8 (neutrophil collagenase) results in hydrolysis of peptide bonds and subsequent dissociation of the collagen fibrils (Leppert, 1995). These enzymes are released by polymorphonuclear leukocytes which infiltrate the cervix from

the blood vessels during the later stages of pregnancy and also by fibroblasts (Bokstrom, et al., 1997; Junquiera, et al., 1980; Osmers, et al., 1995).

Collagen breakdown can also occur due the action of elastase, an enzyme produced by neutrophils, macrophages and eosinophils. This enzyme can also break down elastin and proteoglycans and thus can markedly contribute to changes in biochemical composition of the cervix. Elastase breaks down collagen by its action on the teleopeptide non-helical domains. There is an increase in cervical collagenase and elastase activities in the cervix during pregnancy as the cervical collagen content decreases (Uldbjerg, et al., 1983).

There are marked changes in the structure of the collagen that is produced during pregnancy. Histochemical studies have shown that the concentration of collagen arranged in fibrils postpartum is only 8 percent of that in comparison to samples from non pregnant women (Junquiera, et al., 1980). During pregnancy, the amount of soluble collagen, which represents partly degraded collagen, in the tissue increases (Uldbjerg, et al., 1983; Uldbjerg, et al., 1983). The collagen produced during pregnancy seems to be of an immature nature with fewer crosslinks, making it more amenable to breakdown by proteolytic enzymes (Ito, et al., 1979). There is also an alteration in the GAGs in the cervix as pregnancy advances. The total amount of GAGs increases, indicating active synthesis. However, there is also a change in the specific GAGs present, which has an effect on the tissue's properties. The amount of hyaluronic acid present may increase, remain unchanged or decrease (Leppert, 1995; Uldbjerg, et al., 1991; Von Maillot, et al., 1979). Hyaluronic acid interacts weakly with collagen and fibronectin and the increase in this glycosaminoglycan can help loosen the collagenous network of the cervix (Uldbjerg, et al., 1991).

In the non-pregnant cervix, the predominant proteoglycan is dermatan sulphate (DPSG II or decorin). Decorin both coats the collagen fibres, thus maintaining the collagen at small diameter, and helps to separate the fibrils, by coming between the fibres that make up their fibrils. There are three dermatan sulphates, decorin, biglycan (DSPG I), (which has two dermatan sulphate side chains) and PGL, which is a larger molecule with chondroitin/dermatan sulphate side chains. As pregnancy advances the concentration of decorin increases (Leppert, 1995; Uldbjerg, et al., 1991), presumably contributing to the ripening process. Cervical dilation is also associated with changes in the amounts of the other proteoglycans present (Norman, et al., 1991).

Changes involved in cervical ripening.

In the final few weeks prior to the onset of labour and delivery, the cervix undergoes final maturational change. These changes are a prerequisite for spontaneous labour and normal vaginal delivery. Clinically the cervix is said to 'ripen', becoming softened, effaced and undergoing the early stages of dilatation. These changes occur secondary to further changes in the extracellular matrix at this time. There is an increase in the water content, a reduction in collagen and a change in the constituent GAGs (Calder, 1986; Greer, 1992). The collagen fibres become realigned and dissociated and the amount of ground substance present increases. At this time there is also an influx of inflammatory cells into the connective tissue stroma with the release and activation of degradative enzymes (Bokstrom, et al., 1997; Junquiera, et al., 1980; Liggins, 1981; Osmers, et al., 1995).

Whilst we are aware that these biochemical changes occur in the cervix at this time, we have an incomplete understanding of the mechanisms which control them. Studies, which have been conducted on the human cervix to address this issue, have a number of

methodological problems. Firstly, we are unable to study intact cervical tissue during pregnancy at a biochemical level except in the rare cases where hysterectomy is performed due to intractable bleeding with removal of the intact uterine organ. Secondly, small biopsy samples obtained during pregnancy and labour may not represent changes occuring in the cervix as a whole and thirdly, conflicting study results may arise from sampling different parts of the cervix, especially when lower segment myometrium is confused with cervical tissue. Also, while tissue culture studies provide us with invaluable information, they are no substitute for *in vivo* findings, especially since the conditions in which these experiments are performed are somewhat different to that found in human tissue. However, information from various sources, in both animal and human studies, have allowed some understanding of the factors involved.

Factors involved in cervical ripening.

There are a number of factors that may be involved in cervical ripening. The means by which these factors interact and the relative importance of each is poorly understood. However it is interesting to note that cervical ripening can occur in the cervix which has been physically separated from the uterus (Stys, et al., 1980). Improved understanding of the mechanisms controlling cervical change in the later part of pregnancy will undoubtedly improve our management of both preterm and post-dates pregnancy.

Prostaglandins

Prostaglandins are the most comprehensively studied factors involved in cervical ripening and seem to be produced physiologically during the ripening process (Ellwood, et al., 1979). These agents are widely used pharmacologically to cause cervical ripening prior to

induction of labour (Calder, 1997) and for first trimester suction termination of pregnancy (El Refaey, et al., 1994). The prostaglandins PGE_2, PGI_2 and $PGF_{2\alpha}$ are produced in the cervix during pregnancy and parturition (Ellwood, et al., 1979), but it seems that physiologically PGE_2 is the most important of these. Receptors for prostaglandins are present in the human cervix (Crankshaw, et al., 1979) and amniotic fluid concentrations of PGE_2 and $PGF_{2\alpha}$ correlate directly with cervical ripeness in women at term, not in labour (Calder, 1980). The means by which prostaglandins cause cervical ripening has been the subject of much research. Prostaglandins may effect ripening by inducing collagen breakdown or by modifying the GAG composition of the cervix. In inflammatory situations, PG is thought to act as a powerful vasoactive agent, facilitating inflammatory cell influx.

Although prostaglandins have been for a long time thought of as the key mediators of the ripening process in humans, other agents also appear to be involved. In the physiological setting, cervical ripening occurs independently of uterine activity, i.e. prior to the onset of labour and prostaglandin release. Studies in rats and guinea pigs (Chwalisz, et al., 1991) (Shi, et al., 1996) have demonstrated that the cervix softens in mid to late pregnancy long before the onset of labour. In addition, neither the non selective COX nor specific COX-2 inhibitors are able to inhibit antiprogesterone induced cervical ripening in humans, rats and guinea pigs (Chwalisz, 1994) suggesting that other agents must be important in addition to prostaglandins in the ripening process.

Steroid hormones

Both oestrogen and progesterone have an effect on the ripening mechanism. Oestrogens have been shown to be capable of ripening the unfavourable cervix when applied locally

(Allen, et al., 1989; Gordon and Calder, 1977) and their action may in part be mediated by prostaglandin release (Fitzpatrick and Dobson, 1981). Oestrogens may also mediate cervical ripening by means of programmed cell death or apoptosis (Leppert and Yu, 1994) due to the down regulation of oestrogen receptors.

Progesterone appears to have an inhibitory effect on cervical ripening and parturition in animals where a fall in progesterone at term results in the onset of labour. In humans, this fall is not present, but progesterone must also have some effect on the control of cervical ripening, as demonstrated by the action of various antiprogesterones in the cervix and by the observation that progesterone receptors are down regulated in the human cervix at term (Chwalisz and Garfield, 1994; Chwalisz, et al., 1991; Garfield and Baulieu, 1987; Radestad and Bygdeman, 1992; Stjernholm, et al., 1997; Winkler, et al., 2000). In fact, antiprogestins are effective agents in mediating cervical ripening in all species studied to date (Chwalisz and Garfield, 1994). In guinea pigs, the progesterone antagonists have been shown to decrease cervical collagen and glycosaminoglycan concentration via an effect on the progesterone receptor (Leppert, 1995). Studies in humans have also shown that anti-progestins stimulate collagenolysis within the cervix after their local application (Radestad, et al., 1993). Progesterone, as already stated, is a powerful anti-inflammatory agent and could be important in the cervix during pregnancy in preventing neutrophil influx into the tissue (Jeffrey and Koob, 1980). Furthermore, the effects of anti-progestins in the cervix may in part relate to an increase in prostaglandin production as they can stimulate prostaglandin synthesis and decrease prostaglandin catabolism *in vitro* (Kelly and Bukman, 1990). This may reflect an interaction with the transcription factor NF-kappa B, as progesterone can act through NF-kappa B to inhibit COX-2 expression (Kalkhoven, et al.,

1996). However, this finding has not been consistently reported in other studies (Norman, 1993).

Cytokines

Consistent with the hypothesis that cervical ripening represents an inflammatory reaction, there is evidence that various cytokines are also involved. IL-8, a C-X-C chemokine which promotes neutrophil chemotaxis, has been shown *in vivo* (Sennstrom, et al., 1997) and *in vitro* (Barclay, et al., 1993) to be produced in the cervix and to be capable of causing ripening when artificially applied to the cervix. IL-8 may have a synergistic role with prostaglandins in inducing cervical ripening (Colditz, 1990). IL-1 can induce cervical ripening in animal models (El Maradny, et al., 1995) and its mechanism of action may involve the co-induction of IL-8 (Uchiyama, et al., 1992; Winkler, et al., 2000). Other cytokines, such as TNFα have also been shown to be involved, the local application of this cytokine in guinea pigs causing a ripening effect (Chwalisz, et al., 1994). In this situation TNFα may act in concert with IL-6 to facilitate neutrophil chemotaxis, IL-1 gene expression and endothelial adhesion molecule upregulation (Rees, 1992). Recent studies have suggested that the release of pro-inflammatory cytokines in the cervix may cause cervical smooth muscle cells to induce the expression of degradative enzymes including MMP-1, MMP-3, MMP-9 and cathespin-S (Watari, et al., 1999).

Cytokine production within the cervix and uterus seems to be regulated by progesterone. Progesterone inhibits and antiprogesterones stimulate IL-8 release in choriodecidual cells *in vitro* (Kelly, et al., 1994). Hence during pregnancy progesterone may act as an immunosuppressant in the cervix and decidua. Antiprogestin treatment may therefore

promote cytokine release, prostaglandin release, as well as promoting neutrophil influx into the tissue.

Relaxin

Relaxin is another agent which may have a role in ripening (Evans, et al., 1983; MacLennan, et al., 1986; Weiss, 1995). Relaxin is a peptide hormone which is an insulin derivative. It is synthesised as precursor molecule and undergoes metabolic cleavage to the active form. Expression of relaxin is controlled by two human relaxin genes, which reside on chromosome 9. Relaxin has two biological effects during pregnancy: it causes uterine quiescence and promotes remodelling of the extracellular matrix of the reproductive tract to accommodate pregnancy (Sherwood, 1988).

In rats, relaxin is clearly needed for cervical dilatation, as animals deprived of their relaxin secreting ovaries but given progesterone will go into labour but cannot deliver as the cervix remains rigidly closed (Sherwood, 1988). There is a close correlation in the rat between cervical softening and serum relaxin levels from day 12 until term (Sherwood, 1988). Animal studies have also shown that the cervix can be ripened pharmacologically using this agent (MacLennan, et al., 1986). The human cervix has also been ripened using porcine relaxin (Evans, et al., 1983) although recombinant human relaxin seems to have little effect (Brennand, et al., 1997; Petersen, et al.; Weiss, 1995).

Receptors for relaxin have been demonstrated in the human female reproductive tissues, including the cervix, during pregnancy. The effect of relaxin on the cervix may be secondary to an increase in collagenase activity, as fibroblasts are known to exhibit relaxin receptors (McMurty, et al., 1980) and relaxin has been shown in rats to promote the

widespread reorganisation of collagen fibres in the cervix (Luque, et al., 1998). Of further interest is the fact that relaxin has the potential to induce gap junction formation (Liggins, 1981). Gap junctions have been described in the cervix between the cytoplasmic processes of neighbouring fibroblasts (Ferenczy and Richart, 1974), although their function is unknown. In the porcine cervix, relaxin has been shown to increase the formation of gap junction proteins in pregnancy (Lenhart, et al., 1999). Several recent studies have also indicated that relaxin stimulates nitric oxide synthesis in a number of tissues including the cervix (Shi, et al., 2000). Our understanding of the mechanism of action of relaxin in the human cervix remains unclear however.

Inhibitors of cervical ripening

More recent studies have shown that various inhibitors of cervical ripening may be present in the cervix during pregnancy and this opens further avenues for therapeutic intervention. The anti-inflammatory mediator, secretory leukocyte protease inhibitor, is present in cervical mucus during normal pregnancy (Helmig, et al., 1995) and may be important in the inhibition of cervical ripening brought about by PGE_2 (Denison, et al., 1999). Aminopeptidase N/CD 13 is present on tissue fibroblasts and has been shown to cleave and inactivate IL-8 *in vivo* (Kanayama, et al., 1995), and the combination of IL-8 with the aminopeptidase inhibitor, bestatin, potentiates the effect of IL-8 on cervical ripening in rabbits (Elmaradny, et al., 1995).

Urinary trypsin inhibitor (UTI) has also been implicated in the control of ripening. This enzyme is capable of inhibiting various enzymes including elastase, hyaluronidase and plasmin. It is also capable of inhibiting cytokines, including IL-1 and IL-8. Increased

levels of this enzyme have been found in the cervix and myometrium during pregnancy and studies in rabbits have shown that UTI is capable of suppressing premature cervical ripening (Kanayama, et al., 1995).

Apoptosis

It has also been postulated that the biological changes in the uterine cervix prior to labour result from the proliferation of fibroblasts and smooth muscle cells followed by their programmed cell death, or apoptosis (Leppert and Yu, 1994). Apoptosis is highly organised and follows a universal pattern characterised by chromatin condensation, DNA cleavage by endonucleases, cell shrinkage, membrane blebbing and redistribution of phospatidylserine from the inner to the outer leaflet of the cell membrane, an important mechanism for the recognition of apoptotic cells by surrounding phagocytes. It is induced by stimuli such as oestrogens and other steroid hormones as well as by inflammatory cytokines (Cohen and Duke, 1984; Sandford, et al., 1984) and involves accumulation of p53 and activation of various caspases (Mebmer, et al., 1998). It has been hypothesised that in response to apoptosis within the smooth muscle cells and fibroblasts of the cervix, biochemical pathways could be induced, such as activation of cytokines and MMPs, resulting in alterations in the collagen structure and changes in the proteoglycan composition of the cervix (Leppert, 1998). Recent studies in the human cervix before and after the onset of labour have demonstrated an increase in apoptosis in cervical stromal cells in pregnancy and following labour (Allaire, et al., 2000). Apoptosis is a genetically timed event and this may explain why the onset of labour is time specific for different species.

Nitric oxide

Recently the inflammatory mediator nitric oxide has been implicated in the ripening process. The involvement of nitric oxide in pregnancy and ripening will be discussed in more detail in the following sections of this thesis.

Part III: Nitric oxide

In 1987, Moncada and his colleagues showed that nitric oxide (NO) accounted for the biological activity of endothelium-derived relaxing factor and was responsible for the control of vascular tone (Palmer, et al., 1987; Palmer, et al., 1988). Since its discovery, NO has been identified as a crucial biological mediator, involved in diverse activities such as smooth muscle relaxation, neurotransmission, inflammation and regulation of the immune response (Nathan, 1992). In 1998, Furchgott, Ignarro and Murad were awarded the Nobel Prize for their contribution to science in the field of NO research.

NO is known to play a crucial role in several aspects of female reproductive physiology [for review see Chwalisz 1996 (Chwalisz, et al., 1996) and Rosselli 1997 (Rosselli, 1997)]. These include menstruation (Cicinelli, et al., 1996; Telfer, et al., 1995; Tschugguel, et al., 1998;Tshugguel et al., 1999) and conception by means of its effects on LHRH release (McCann, et al., 1998) sperm motility (Lewis, et al., 1996) ovarian function (Ellman, et al., 1993; Jablonka-Shariff and Olson, 1998; Rosselli, et al., 1998) and implantation (Chwalisz, et al., 1999; Purcell, et al., 1998). Increased NO production may also be inherently involved in the normal haemodynamic changes of pregnancy, in the maintenance of the uteroplacental circulation and the control of maternal vascular tone, while a deficit of NO may contribute to the pathophysiology of intrauterine growth restriction and pre-eclampsia (Izumi, et al., 1993; Sladek, et al., 1997).

Biosynthesis and release of NO

NO is a reactive gas with a very short physiological half-life. It is synthesised from L-arginine by the enzyme nitric oxide synthase (NOS), of which three isoforms have been identified at present (Figure 1.1). These isoenzymes have common features with the

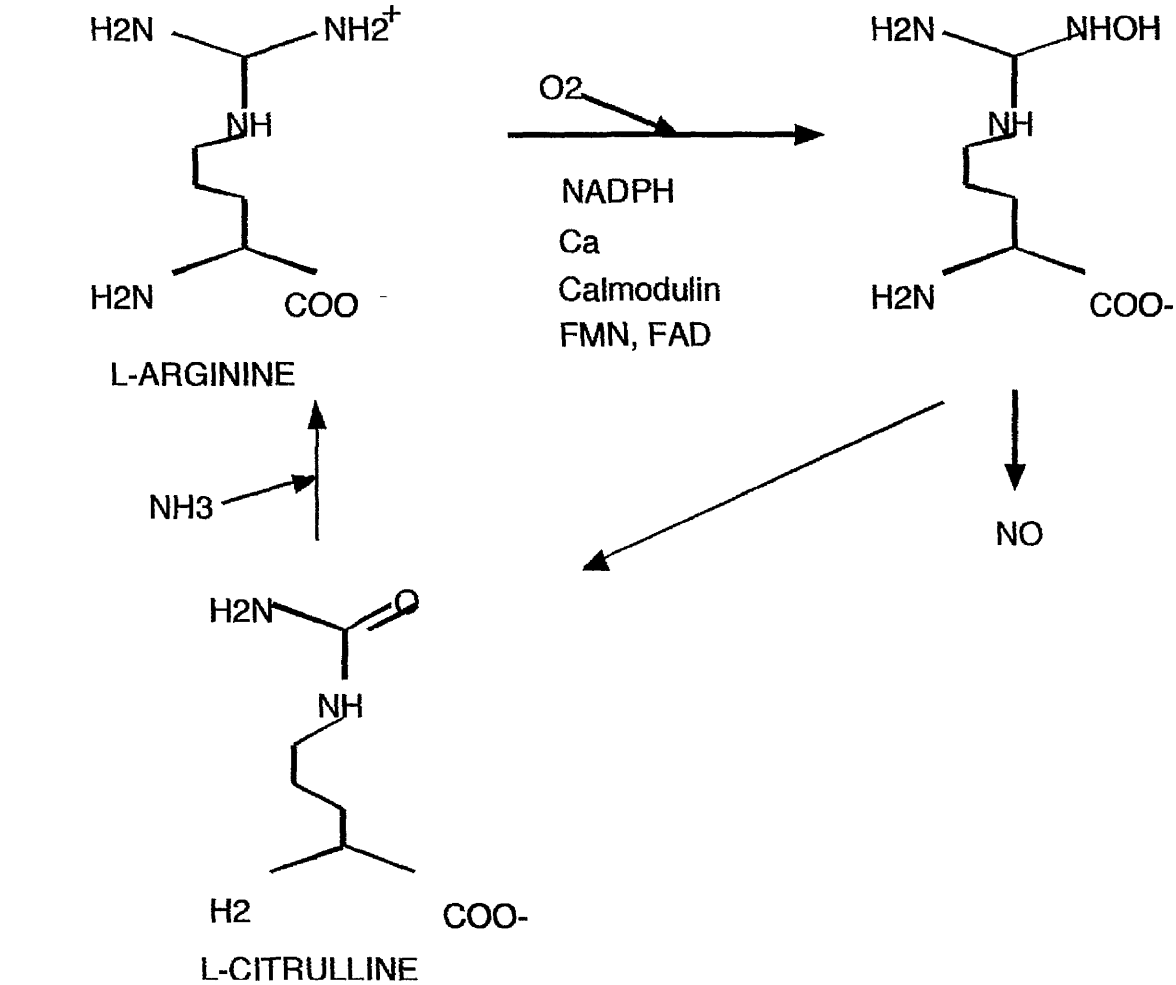
respiratory chain enzyme cytochrome P450 reductase and contain oxidative and reductive domains. The three isoforms of NOS are known as endothelial NOS, (eNOS; NOS III), neuronal or brain NOS, (bNOS; NOS I) and macrophage or inducible NOS, (iNOS; NOS II), and the genes for each of these enzymes has been localised to chromosome 7, 12 and 17 respectively. There is significant homology between the amino acid sequences of NOS from different species and between each of the different isoforms. Whilst the isoforms of NOS are named after the cell type in which they were first identified, it is now recognised that the three isoforms are widely distributed in a variety of tissues. bNOS was originally purified and cloned from neuronal tissues but is now known to have an important level of expression in skeletal muscle, neutrophils, pancreatic cells and respiratory and gastrointestinal epithelia (Nathan and Xie, 1994). iNOS was originally purified and cloned from an immunoactivated macrophage cell line, and has since been identified in neutrophils, mast cells, endothelial cells, vascular smooth muscle, cardiac muscle and hepatocytes (Nathan, 1997). eNOS, the last of the three mammalian NOS isoforms to be isolated, was originally purified and cloned from vascular endothelium, but has since been identified in cardiac myocytes, blood platelets, brain and elsewhere (Michel and Feron, 1997).

For all three NOS isoforms, NO synthesis depends upon the enzyme's binding of the calcium regulatory protein, calmodulin. For eNOS and bNOS, increases in intracellular calcium concentrations are required for their binding to calmodulin, and consequently for their full activation. By contrast, iNOS appears able to bind calmodulin with extremely high affinity, even at the low intracellular concentrations characteristic of resting cells. Thus, in contrast to iNOS, the activity of eNOS and bNOS may be closely regulated by changes in intracellular calcium. eNOS and bNOS are constitutively expressed by cells although their expression can be induced or up regulated. When cells containing

constitutive NOS (cNOS), that is eNOS and bNOS, are stimulated by for example, acetylcholine, bradykinin, glutamate, thrombin, ADP, physical pressure and shear stress, activation leads to an increase in cytosolic calcium, which activates the cNOS to produce NO. In general, expression of iNOS occurs after activation of cells with stimuli, including bacterial endotoxin or exotoxin, and inflammatory mediators, including the cytokines tumour necrosis factor (TNF) and interleukin (IL)-1. However, iNOS may also function as a "constitutive" enzyme under physiological conditions in some cells (Guo, et al., 1995). iNOS produces larger amounts of NO than the other NOS isoforms (Anggard, 1994).

The initial step in NO biosynthesis is hydroxylation of the nitrogen in the guanidino group of L-arginine (Figure 1.1). This step incorporates molecular oxygen into NO and citrulline and requires cofactors including reduced pyridine nucleotides, reduced biopteridines and calmodulin. Whilst levels of L-arginine are normally sufficient for continuous secretory NO biosynthesis, under certain conditions the local cellular L-arginine levels may be insufficient (Anggard, 1994). Furthermore, the limited availability of cofactors *in vivo* can attenuate the production of NO (Sladek and Roberts, 1996; Werner-Felmayer, et al., 1993).

Figure 1.1 Biosynthesis of nitric oxide



NO is a free radical having an unpaired electron, and has a very short physiological half life of a few seconds, since it readily combines with other free radicals (Beckman and Crow, 1993). NO is rapidly converted in biological systems to nitrate and nitrite, a reaction that is catalysed by transition metals including iron. Under anaerobic conditions, haemoglobin inactivates NO by binding it to form nitrosohaemoglobin whilst methaemoglobin is formed by this reaction in the presence of oxygen. The breakdown of NO to nitrate and nitrite may represent separate oxidative reactions, the metabolite ratio being dependent on the redox conditions in the cell microenvironment (Kelm, 1999). The

very short half life of NO and its reactivity mean that it is most likely to act as a local messenger transferring messages within and between individual cells.

Sites of action of NO

NO has several targets of action at a cellular level. The effects of NO on smooth muscle relaxation, inhibition of platelet aggregation and signal transduction within the central nervous system are mediated by activation of the enzyme guanylate cyclase and subsequent formation of cyclic GMP (Ignarro, 1992). Cyclic GMP activates protein kinases and leads in turn to the dephosphorylation of myosin light chains and smooth muscle relaxation.

NO can also mediate its effects through a number of cGMP independent mechanisms. One of the most important of these is termed "nitrosative stress" and involves the nitrosylation of other target proteins. In this way NO can influence other enzymes such as cytochrome oxidase (Ignarro, 1992), ribonucleotide reductase (Lepoivre, et al., 1990) and cyclooxygenase (COX) (Salvemini, 1997) and hence influence cellular respiration, DNA synthesis and inflammatory and immune responses. NO is also able to promote the direct ADP-ribosylation of proteins, i.e. the direct covalent binding of an ADP-ribose to a number of amino-acids. ADP-ribosylation may be an important mechanism whereby NO regulates the state of actin polymerisation and thereby regulates cell adhesion, signalling from the extracellular matrix, cell migration and phagocytosis (Clancy, et al., 1995; Clancy, et al., 1997; Frenkel, et al., 1996). NO may also affect smooth muscle relaxation, cell signalling and phagocytosis by the direct activation of gene transcription factors

(Nathan, 1992; Umansky, et al., 1988). Increasing evidence suggests that NO preferentially affects transcription factors that are sensitive to changes in cellular redox status, particularly NF-κB and activator protein-1 (AP-1) (Sen and Packer, 1996). These transcription factors are intimately involved in inflammatory responses and regulate the expression of chemoattractants, cytokines, cytokine receptors and cell adhesion molecules (Baeuerle and Henkel, 1994; Chen, et al., 1995; Lafayatis, et al., 1990; Siebenlist, et al., 1994).

Finally, NO can interact with free radicals such as superoxide anion (O_2^-) and free thiols to mediate its effects. The reaction with O_2^- results in the formation of toxic hydroxyl radicals ($HO^{\cdot}$) and peroxynitrite ($ONOO^-$) which are involved in host defence responses. Interactions between thiols and NO result in the formation of S-nitrosothiol derivatives that are more stable than their parent compounds and prolong the effects of NO *in vivo* (Clancy, et al., 1998; Clancy, et al., 1994; Jia, et al., 1996).

Detection of NO

The short half-life of NO makes direct measurement difficult in experimental situations; therefore techniques have been developed to measure NO activity indirectly. These include the Greiss reaction, which measures nitrite, the oxidation product of NO (Green, et al., 1982). The protein and mRNA for each of the three isoforms of NOS can be localised by immunocytochemistry and *in situ* hybridisation, respectively. Quantification of tissue NOS mRNA and protein can be achieved by Northern and Western blots respectively. The identification of the gene sequences of each of the NOS isoforms has permitted the

detection of NOS mRNA using the reverse transcription polymerase chain reaction (RT-PCR). The total activity of NOS in a tissue can be quantified by measuring the conversion of radio-labelled L-arginine to L-citrulline, in the presence of added co-factors. Identification of NADPH diaphorase activity *in vitro* has been used to identify NOS activity within a tissue since, certainly in neuronal tissue, NADPH diaphorase activity co-localises with NOS (Hope, et al., 1991). However, it is now recognised that not all NADPH diaphorase activity is NOS, so casting doubt on the validity of this technique (Tracey, et al., 1993). More recent techniques based on chemiluminescence have been developed for the measurement of NO gas produced either directly e.g. in lung tissue or following enzymatic conversion of nitrate and nitrite, but expense currently precludes their widespread use (Persson, et al., 1994).

The role of nitric oxide in inflammation

Inflammation was first described by Celsus in the first century AD as demonstrating the clinical signs of rubor, tumor, calor and dolor. The cellular events involved in these responses have been extensively studied and nitric oxide has recently been shown to be involved. Inflammation is associated with an increase in vascular flow, alterations in the endothelium and extravasation of proteins and leukocytes at the site of injury with the activation of various effector mechanisms. There is increasing evidence that nitric oxide is involved in each of these stages of the inflammatory response.

While it has long been recognised that NO is involved in homeostatic vascular smooth muscle relaxation (Furchgott, et al., 1990), it is now recognised that a number of inflammatory mediators including IL-1 induce cNOS activity and vasodilatation in the early stages of inflammation (Lyons, 1995). Studies suggest that these mediators increase

Ca^{2+} influx into cells, resulting in kinase activation and serine phosphorylation of cNOS protein which is then translocated from its membrane bound position into the cytosol (Michel, et al., 1993). There it interacts with the haem moiety of guanylate cyclase and mediates cGMP-dependent smooth muscle relaxation. Once increased blood flow is initiated, vasodilatation is maintained by an autocrine effect on endothelial NO production (Cooke, et al., 1991). The mechanisms whereby nitric oxide is shut off in this process are unclear. However, since cNOS activation is controlled by phosphorylation and calmodulin binding, there is the potential for kinases and local alterations in the availability of calcium to modulate nitric oxide production.

In severe inflammation, high levels of inflammatory mediators act on smooth muscle cells, which in turn transcribe iNOS protein. Once initiated, iNOS mediated production of NO lasts for several hours with the attendant risk of prolonged profound hypotension. It has been suggested that NO inhibitors may have a therapeutic effect in the management of this condition (Thiermermann, et al., 1993; Wright, et al., 1992). However the decrease in nitric oxide production in other organs may limit the clinical effectiveness of this therapy. Indeed in experimental models, treatment of severe sepsis with NOS inhibitors has been associated with severe liver and intestinal damage with no effect on subsequent survival rates (Kilbourn, et al., 1997).

There is widespread debate as to the role of nitric oxide in the control of vascular permeability. The conflicting evidence however suggests that eNOS produced in low amounts in the early stages of inflammation inhibits plasma protein and leukocyte leakage into the tissues, while iNOS is responsible for enhancing vascular permeability as the inflammatory process progresses. In the rat intestine, it has been shown that during the first two hours of endotoxin-induced increased vascular injury, vascular permeability is increased by inhibitors of NOS, while after three hours, when iNOS activity is present,

LPS-induced vascular permeability is reduced by such agents (Laszlo, et al., 1994). Similarly, NOS inhibitors have been shown to inhibit carrageenan or dextran induced paw oedema in rats (Ialenti, et al., 1992). However in human studies, the involvement of nitric oxide in vascular leak is less clear.

While excessive NO production in the vasculature may promote tissue injury, cNOS appears to be intimately involved in protecting the integrity of the endothelium. NO has been shown to inhibit platelet aggregation, the adhesion of neutrophils to endothelial monolayers and to inhibit the production of leukocytic superoxide anion (Clancy and Abramson, 1995; Clancy, et al., 1992; Kubes, et al., 1991). Although the precise biological relevance of these findings remains to be determined, the vascular defensive properties of NO have been demonstrated by its capacity to protect against tissue injury in myocardial ischaemia-reperfusion injury and the adult respiratory distress syndrome (Bloomfield, et al., 1997; Lefer, 1992). Although NO appears to inhibit leukocyte adherence in normal homeostasis, further studies have shown that neutrophil produced oxidants promote leukocyte adhesion to endothelium (Kurose, et al., 1994; Salvemini, et al., 1991). Thus in inflammation, excess superoxide generation may overwhelm the available scavenging NO, promoting mast cell degranulation and release of mediators which upregulate adhesion molecules and therefore enhance inflammatory cell infiltration of target tissues.

Lastly, NO seems to have an important functional role as an effector molecule in the immune response. In eukaryotic cells, NO inhibits mitochondrial respiration (Brown, 1997) and interferes with DNA synthesis through inhibition of ribonucleotide reductase (Lepoivre, et al., 1990). NO has also been shown to activate all three parallel mitogen-activated protein kinase (MAPK) cascades (Lander, et al., 1996) in Jurkat T cells (the stress activated protein kinase/c-Jun N-terminal kinase), the stress-activated p38 MAPK

cascade and the classical ERK/ MAPK cascade as well as the JAK/signal transducer and activator of transcription (STAT) pathway (Duhe, et al., 1998). These complex pathways then trigger the phosphorylation of nuclear proteins involved in the inflammatory response. NO has critically been shown to control the transcription of the chemokines IL-8, TNF-α and MIP-1(Muhl and Dinarello, 1997; VanDervort, et al., 1994; Villarete and Remick, 1997), to regulate the expression of vascular endothelial growth factor (VEGF), a growth factor which mediates angiogenesis and controls vascular permeability (Tsurumi, et al., 1997) and to initiate apoptotic cell death, all fundamental processes in inflammatory responses.

Clinical uses of NO

Nitrovasodilators, such as glyceryl trinitrate and sodium nitroprusside (SNP), which have been employed in the treatment of angina pectoris for over 100 years, are now known to produce their clinical effects by releasing NO. These drugs, known as NO donors, release NO either spontaneously, (e.g. SNP) or after metabolic conversion, (e.g. glyceryl trinitrate). The clinical effects of other drugs may also be related to the L-arginine-NO pathway. Angiotensin converting enzyme inhibitors inhibit the breakdown of bradykinin, which in turn results in an increased production of NO from endothelial cells. The administration of inhaled NO itself has recently been shown to be of therapeutic benefit in the treatment of pulmonary hypertension and adult and neonatal respiratory distress syndromes (Kinsella and Abman, 1997).

Endogenous NO levels can be raised by administration of the NO substrate L-arginine. Analogues of L-arginine, such as N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-monomethyl-L-arginine (L-NMMA), attenuate the effects of NOS by competing with L-

arginine for the active site of the NOS (Furchgott, et al., 1990). In experimental systems, NO may be inhibited by the addition of oxyhaemoglobin, or the effects of NO on the second messenger system, guanylate cyclase, may be blocked by methylene-blue. Aminoguanidine is a selective iNOS inhibitor that has been used to investigate the role of NO in various inflammatory pathologies (Furchgott, et al., 1990). Inhibitors of NO may have important future therapeutic implications in the treatment of states attributable to NO overproduction such as septic shock and cerebral ischaemia (Vallance and Collier, 1994).

Pregnancy and the role of nitric oxide

NO was first identified in the human reproductive system by Ignarro *et al.*, (1990) (Ignarro, et al., 1990) who demonstrated that NO was generated in response to non-adrenergic/non-cholinergic neurotransmission-mediated penile erection. Since then, the L-arginine-NO system and each of the three isoforms of NOS, have been identified in the female reproductive system, suggesting that locally synthesised NO is directly involved in the physiology of reproduction. A decrease in the production and/or alteration in the uterine responsiveness to NO within the uterus may precede the onset of labour. This mechanism may be important in both the pathophysiologies of term as well as preterm labour. Furthermore, NO may play a role in ripening the uterine cervix prior to the onset of parturition. The remainder of this review will focus on these two specific areas of investigation.

Nitric oxide and the uterus

A number of experimental studies suggest that NO is generated within uterine tissues and provide us with evidence that NO has a role in the control of uterine contractility during

pregnancy. These studies have concentrated on the evidence for the presence of the NO-cGMP system within the uterus and on the effects of NO administration on uterine contractility both *in vivo* and *in vitro*.

NOS enzyme activity within the uterus

Animal studies, using the Greiss reaction, have shown that rat and rabbit uterus produce large quantities of NO (Dong and Yallampalli, 1996; Yallampalli, et al., 1993). NOS activity in the pregnant rat and rabbit uterus has been detected using the radiolabelled arginine to citrulline conversion assay (Natuzzi, et al., 1993; Sladek, et al., 1993; Yallampalli, et al., 1994). This technique has also shown NOS activity within the pregnant guinea-pig uterus (Weiner, et al., 1994). In all these studies, both calcium-insensitive (iNOS) and calcium-sensitive (eNOS and/or bNOS) activity were identified.

Pregnant and non-pregnant human myometrium can generate nitrate/nitrite in culture (Buhimschi, et al., 1995). NOS activity is present in human pregnant myometrium (Buhimschi, et al., 1995; Thomson, et al., 1997), placental villous trophoblast (Di Iulio, et al., 1996; Ramsay, et al., 1996) and fetal membranes (Di Iulio, et al., 1996; Thomson, et al., 1997). This expression and distribution of NOS isoforms within the uterus and the fetal membranes suggests that NO is involved in the regulation of uterine contractility.

Localisation of uterine NOS

NADPH diaphorase staining has been used to localise NOS within the pregnant and non-pregnant uterus of various species and essentially identical patterns of staining have been found, with myometrial and vascular smooth muscle nerve bundles, vascular endothelium,

endometrial epithelium and leukocytes staining positively (Ekerhovd, et al., 1998; Schmidt, et al., 1992; Telfer, et al., 1995; Yallampalli, et al., 1994).

Using immunocytochemistry, bNOS has been localised to the endometrial epithelium (Schmidt, et al., 1992), perivascular neurones (Grozdanovic, et al., 1994; Majewski, et al., 1995), myometrial neurones (Grozdanovic, et al., 1994; Majewski, et al., 1995; Papka, et al., 1995), myocytes, the vascular endothelium and smooth muscle of blood vessels (Thomson, et al., 1997) with confirmation in myocytes by Western blotting (Norman, et al., 1999).

eNOS protein has been immunolocalised to vascular endothelium, endometrial glandular epithelium and endometrial stromal cells but has not been consistently detected in myometrial smooth muscle cells (Campa, et al., 1998; Myatt, et al., 1998; Norman, et al., 1999; Telfer, et al., 1997; Thomson, et al., 1997). The expression of eNOS protein within these tissues has been confirmed by Western blotting and *in situ* hybridisation (Gangula, et al., 1996; Norman, et al., 1999; Telfer, et al., 1997). Using RT-PCR, e/c NOS mRNA has recently been identified in human myometrium (Dennes, et al., 1999).

iNOS has been identified in stromal macrophages and myometrial mast cells (Huang, et al., 1995), glandular epithelial cells, in areas between myometrial cell bundles (Dong, et al., 1998) and the vascular smooth muscle of the myometrium and endometrium (Telfer, et al., 1997; Thomson, et al., 1997). Although there is some debate as to whether human myometrial smooth muscle cells contain NOS (Sladek, et al., 1997), iNOS has been immunolocalised to human myocytes by several investigators (Bansal, et al., 1997;

Thomson, et al., 1997). Western blotting and RT-PCR have also recently been used to confirm the presence of iNOS protein and mRNA respectively in human myometrium (Bansal, et al., 1997; Dennes, et al., 1999).

The expression of each of the three isoforms of NOS has also been described in human placenta and fetal membranes using immunohistochemistry (Myatt, et al., 1993; Norman, et al., 1999; Schonfelder, et al., 1996; Thomson, et al., 1997) Western blotting (Norman, et al., 1999; Schonfelder, et al., 1996; Zarlingo, et al., 1997) and *in situ* hybridization (Conrad, et al., 1993), while NOS enzyme activity has been confirmed using the L-arginine-citrulline conversion assay (Thomson, et al., 1997), and by measuring NADPH-diaphorase activity (Eis, et al., 1995). eNOS and iNOS mRNA have been identified by RT-PCR in amnion and chorio-decidua (Dennes, et al., 1997).

Thus, all three isoforms of NOS have been identified in the uterus and placentae of various species including humans (Table 1.1). This supports a role for NO not only in the regulation of myometrial contractility but also in the regulation of placental blood flow and the inhibition of platelet aggregation at the interface between the maternal and fetal circulations.

Relaxation of myometrium by nitric oxide

In vitro studies

NO is a powerful smooth muscle relaxant. Various *in vitro* studies have shown that it is capable of relaxing the myometrium during pregnancy and may be responsible for uterine quiescence during this period (Buhimschi, et al., 1995; Izumi, et al., 1993; Lee and Chang,

1995; Longo, et al., 1999; Norman, 1996; Sladek, et al., 1997; Yallampalli, et al., 1993). Studies carried out on isolated human and animal myometrial smooth muscle strips have shown that uterine relaxation occurs with NO, delivered as NO gas, NO substrates and NO donors (Buhimschi, et al., 1995; Izumi, et al., 1993; Lee and Chang, 1995; Longo, et al., 1999; Norman, et al., 1997). Furthermore, contractility can be increased by competitive inhibitors of NO synthesis such as L-NAME and N-nitro-L-arginine (Franchi, et al., 1994; Lee and Chang, 1995; Yallampalli, et al., 1993; Yallampalli, et al., 1994). These studies raise the possibility that endogenous NO inhibits contractility *in vivo* (Buhimschi, et al., 1995; Lee and Chang, 1995).

The effects of NO releasing agents on the frequency of myometrial contractions appears complex. A study using the NO donors, sodium nitroprusside (SNP) and glyceryl trinitrate (GTN), found that the magnitude of contractions was reduced by around 50% at the highest concentrations of the drugs used (Norman, et al., 1997). Paradoxically the frequency of myometrial contractions was increased by SNP at concentrations above 30 μl, although the reason for this is unclear.

Table 1.1. Detection of the nitric oxide system within the uterus

Reference	Method used	Species	Tissue used
Yallampalli *et al.*, 1993[44]	Greiss reaction	rat	whole uterus
Yallampalli *et al.*, 1994[48]	arginine to citrulline Greiss reaction NADPH diaphorase	rat	whole uterus
Sladek *et al.*, 1993[46]	arginine to citrulline Greiss reaction	rabbit	decidua
Natuzzi *et al.*, 1993[47]	arginine to citrulline	rat	whole uterus
Weiner *et al.*, 1994[49]	arginine to citrulline	guinea-pig	whole uterus
Buhimschi *et al.*, 1995[50]	Greiss reaction arginine to citrulline	human	cultured myometrium
Thomson *et al.*, 1997[51]	arginine to citrulline immunocytochemistry	human	myometrium placenta fetal membranes
Ramsay *et al.*, 1996[52]	arginine to citrulline	human	myometrium placenta
Di Iulio *et al.*, 1996[53]	arginine to citrulline	human	fetal membranes placenta
Telfer *et al.*, 1995[6]	NADPH diaphorase immunocytochemistry *in situ* hybridisation	human	non-pregnant endometrium non-pregnant myometrium
Schmidt *et al.*, 1992[54]	NADPH diaphorase immunocytochemistry	rat	endometrium/ myometrium

Ekerhovd *et al.*, 1998[55]	NADPH diaphorase	human	cervix
Grozdanovic *et al.*, 1994[56]	immunocytochemistry	mouse	uterus and cervix
Majewski *et al.*, 1995[57]	immunocytochemistry	cow pig	uterus and cervix
Papka *et al.*, 1995[58]	immunocytochemistry	rat	uterus and cervix
Norman *et al.*, 1999[59]	immunocytochemistry Western blotting	human	myometrium
Myatt *et al.*, 1998[60]	immunocytochemistry	human	myometrium (placental bed biopsies)
Campa *et al.*, 1998[61]	immunocytochemistry	human	myometrial vascular endothelium
Gangula *et al.*, 1996[62]	Western blotting Griess reaction	rat	cultured rat myometrial cells
Dennes *et al.*, 1999[63]	RT-PCR	human	myometrium
Huang *et al.*, 1995[64]	immunocytochemistry	mouse	non-pregnant uterus
Telfer *et al.*, 1997[66]	immunocytochemistry	human	non-pregnant endo/myometrium
Bansal *et al.*, 1997[67]	immunocytochemistry Western blotting	human	myometrium
Myatt *et al.*, 1993[68]	immunocytochemistry NADPH diaphorase	human	placenta
Schonfelder *et al.*, 1996[69]	immunocytochemistry Western blotting	human	placenta

Zarlingo *et al.*, 1997[70]	Western blotting	human, monkey, baboon, guinea pig, rat, sheep	placenta
Conrad *et al.*, 1993[71]	*in situ* hybridisation NADPH diaphorase	human	placenta
Eis *et al.*, 1995[72]	immunocytochemistry NAPDH diaphorase	human	placenta
Dennes *et al.*, 1997[73]	RT-PCR	human	fetal membranes

In contrast to previous studies (Buhimschi, et al., 1995; Lee and Chang, 1995), Norman *et al.*, (1997) (Norman, et al., 1997) found no effect on myometrial contractions in response to the NO inhibitor L-NAME, suggesting that there is no endogenous NO activity in the myometrium *in vitro*. This data was further supported by the work of other investigators (Jones and Poston, 1997). The conflicting results from these studies may be attributable to a number of factors. For example, in the studies demonstrating an increase in myometrial contractility in response to NO inhibitors, the muscle strips being tested may not have been free of fetal membranes, decidua or endometrium which generate NO. Buhimschi *et al.*, (1997) (Buhimschi, et al., 1997) have shown that the spontaneously releasing NO donor, diethylenetriamine-NO (DETA-NO), has contrasting effects *in vivo* and *in vitro*. *In vitro* studies of rat myometrial contractility showed decreased inhibitory responses to DETA-NO in tissues collected during spontaneous labour compared with tissues collected before the onset of labour. In contrast, studies *in vivo* showed that the NO donor could decrease uterine contractility even more effectively during delivery, suggesting that the feto-

placental unit increases the availability of NO *in vivo*. This concept is further supported by Segal *et al.*, (1997) (Segal, et al., 1997) who demonstrated in an organ bath system that the addition of placental tissue markedly increased the sensitivity of rat myometrium to the relaxant effects of GTN. A further explanation for these conflicting results is differences in myometrial sampling sites, as the production of NO within uterine tissue is not uniform. Despite these findings the role of NO in the endogenous control of myometrial contractility *in vivo* has not been established.

Growing evidence suggests that the effect of NO on myometrial contractions is dependent on whether the tissue is collected before or after the onset of labour. The same dose of L-arginine was found to completely inhibit spontaneous contractions of rat myometrium for longer when the tissue was obtained before the onset of labour, compared with tissue collected during labour (Yallampalli, et al., 1993). Furthermore, L-arginine completely inhibited contractions induced by the muscarinic agonist carbachol when myometrium was collected at mid-gestation, but had no effect when myometrium was removed during delivery (Izumi, et al., 1993). Similarly, one concentration of 8-bromo-cGMP, a cell permeable analogue of cGMP, had a profound inhibitory effect on contractions of rat myometrium removed at mid-gestation, but concentrations of 1000 times higher were required to produce a similar inhibitory effect when myometrium was removed from labouring animals at term (Izumi, et al., 1993). Buhimschi *et al.*, (1995) (Buhimschi, et al., 1995), have demonstrated a reduction in sensitivity to the tocolytic effect of NO in human myometrium collected following the onset of labour and Okawa *et al.*, (1998) (Okawa, et al., 1998) have demonstrated similar effects in rats. Therefore, NO donors used for the treatment of preterm labour would theoretically have maximal effect at gestations where they would be of most therapeutic benefit. However it would appear that the

concentrations of NO donors required to arrest labour are much greater than those required to prevent the onset of labour.

In vivo studies

It has long been recognised that in the clinical arena, NO donors relax the human uterus. As early as 1882, Barnes reported the use of amyl nitrite to facilitate manual removal of a retained placenta. More recently, intravenous glyceryl trinitrate (GTN) has been reported in uncontrolled case reports to allow correction of uterine inversion (Altabef, et al., 1992; Dayan and Schwalbe, 1996), to facilitate intrapartum external cephalic version (Belfort, 1993), and to allow internal podalic version of a second twin (Wessen, et al., 1995). The use of intravenous nitroglycerin has also been reported to facilitate delivery of a retained 20-week dead fetus following abruptio placentae and uterine hypertonus (Dessard, et al., 1998). In a prospective observational study, intravenous GTN produced relief of intrapartum fetal distress related to uterine hyperactivity (Mercier, et al., 1997). This agent has also been used to facilitate fetal delivery during Caesarean section (Mayer and Weeks, 1992), although a recent randomised trial found that administration of GTN led to no clinically relevant effect on fetal extraction (David, et al., 1998).

Two uncontrolled, observational studies and one randomised controlled pilot study have investigated the effects of GTN patches in women with a diagnosis of preterm labour (Lees, et al., 1994; Rowlands, et al., 1996; Smith, et al., 1999). Although these studies concluded that GTN can arrest preterm labour, further controlled trials and side effect data are required before these agents are adopted as tocolytic agents (Dudley and Elbourne, 1994; Norman, 1996; Sladek, et al., 1997). A multicenter world-wide randomised controlled trial was commenced in 1994 to investigate the efficacy of GTN in comparison

with ritodrine for the management of preterm labour (Lees, et al., 1997). These data suggest that GTN is as effective as ritodrine on contractility but just as with β-mimetics, no direct benefit in terms of improvement in neonatal morbidity or mortality has been shown (Danti, et al., 1997; Lees, et al., 1999).

One randomised controlled trial conducted in the United States has examined the tocolytic effects of intravenous GTN in the treatment of preterm labour (El-Sayed, et al., 1999). In this study however, GTN was found to have a higher tocolytic failure rate than magnesium sulphate. Furthermore, the high incidence of persistent hypotension in the GTN treated group may limit the use of this therapy for this purpose although previous studies examining the fetal and maternal effects of GTN administration have reported no adverse effects (Lees, et al., 1998; Ramsay, et al., 1994).

The role of nitric oxide in parturition

Animal studies suggest that NO 'withdrawal' may be involved in the timing of parturition. Several studies have demonstrated an up-regulation of uterine NO production during pregnancy, and down-regulation during term and preterm labour (Dong and Yallampalli, 1996; Sladek, et al., 1993; Yallampalli and Garfield, 1994; Yallampalli, et al., 1993). Hence, there is a consensus for a decrease in uterine NOS activity at term, although the precise timing of this is not clear. NOS activity is high during rabbit pregnancy and progressively decreases, (by 80%), in decidua during the last 4 days of gestation (Sladek, et al., 1993). Other studies, comparing 16 (Natuzzi, et al., 1993) and 18 days' gestation (Yallampalli, et al., 1993) with labouring rats (22 days), have reported that NOS activity decreases with advancing gestation in whole rat uterus, decidua and myometrium.

However, these studies leave a 4- to 6-day window in which the decline in rat uterine NOS activity takes place. Examining this window, Sladek and Roberts, (1996) (Sladek and Roberts, 1996) found that NOS activity decreased between days 15 and 21 of gestation and did not decrease further at term (day 22). These workers propose that the decrease in pregnant rat uterine NOS activity coincides with the preparation of the uterus for parturition i.e. prelabour rather than the final activation of labour. Data from gene knockout animals suggests that an absence of any single isoform of NOS has no effect on the normal gestational period (Huang, et al., 1995; Huang, et al., 1993; Wei, et al., 1995).

Changes in NOS activity appear to be less important during pregnancy in the guinea pig (Weiner, et al., 1994). In this species, there was no significant difference in NOS activity, measured by the arginine to citrulline conversion assay, between pregnant and non-pregnant animals. Despite this, there was a 200-fold increase in cGMP content from mid- to late-pregnancy, suggesting that factors other than NO are responsible for the changes in cGMP.

In human pregnancy, the L-arginine-NO system has been identified within myometrium, placenta, and fetal membranes. NO derived from each of these tissues may regulate myometrial contractions. However, because of its short half-life of a few seconds and its reactivity, it seems likely that NO produced within the myometrium itself, or tissues in close apposition, will influence myometrial contractility. Several studies have attempted to determine whether a change in NOS activity occurs at the onset of human parturition (Di Iulio, et al., 1996; Ramsay, et al., 1996). Examining tissues collected at Caesarean section, ('before' labour and 'during' labour), and assessing NOS activity using the arginine to citrulline conversion assay, these studies concluded that there was no significant fall in NOS activity during human parturition. Indeed, Ramsay *et al.*, (1996) (Ramsay, et al.,

1996) found a slight increase in NOS activity in myometrium collected during labour compared with that collected before labour. Further studies performed on tissue obtained immediately before and after the onset of labour at term found no difference in the expression (as assessed using immunocytochemistry) or activity (as measured by the L-arginine to citrulline conversion assay) of NO within the uterus or placenta (Thomson, et al., 1997). However, *in vitro* techniques of assessing of enzyme activity, may not reflect the *in vivo* situation (Sladek and Roberts, 1996) where cofactor availability and paracrine activation mechanisms may be important. It is feasible that while NOS activity or expression are not reduced during human parturition, NO may still play a role in the onset of labour by means of a decrease in sensitivity of the myometrium to NO's tocolytic effects.

Other biological mediators, such as superoxide dismutase (SOD), may be important in governing the activity of NO within the uterus at the time of parturition. SOD is responsible for scavenging superoxide anions and hence prolongs the biological effects of NO. At the onset of labour, a decrease in the activity of SOD would reduce the availability of NO within the uterus and lead to an increase in uterine contractility. Xanthine oxidase (XO), the enzyme responsible for superoxide synthesis, may attenuate the biological activity of NO as O_2^- combines with NO to form perioxynitrite. However, Telfer *et al.*, (1997) have failed to show any difference in the activity or expression of SOD in the uterus, placenta or fetal membranes before or after the onset of labour (Telfer, et al., 1997). Similarly, XO expression was not altered in these tissues as assessed using immunohistochemistry. Therefore, whilst SOD and XO do not seem to alter during parturition, their presence within the fetomaternal system suggests that they may be important in controlling the effects of NO during pregnancy in its maintenance of uterine quiescence.

While there does not seem to be any dramatic decrease in production of NO at the onset of labour, there would appear to be a down regulation of constitutive NOS (cNOS) and iNOS in the myometrium during the third trimester of pregnancy. Bansal *et al.*, (1997) (Bansal, et al., 1997) reported that myometrial iNOS expression, assessed by immunohistochemistry and Western blotting was decreased in the late third trimester (37-41 week's gestation) in non-labouring myometrium compared to samples obtained in the early third trimester (26-34 weeks gestation) or in the non-pregnant state. These data suggest that an increase in myometrial iNOS expression might contribute to the maintenance of uterine quiescence during pregnancy. In contrast to this, Dennes *et al.*, (Dennes, et al., 1999) recently demonstrated no change in cNOS and iNOS expression using semi-quantatative RT-PCR during pregnancy or at the onset of parturition. Further studies however, using immunohistochemistry and Western blotting, support the work of Bansal *et al.* and it would appear that eNOS and bNOS are upregulated in the early third trimester myometrium compared to the non-pregnant state. eNOS, but not bNOS, is reduced at term compared to preterm (Norman, et al., 1999). It would seem that a decrease in NOS activity within the uterus occurs in the third trimester as part of a conditioning phase for the onset of labour. This conditioning phase, which seems to occur in the third trimester of pregnancy, encompasses changes within the myometrium, such as up-regulation of receptors and the formation of ion channels, which allow the uterus to become more responsive to uterotonic agents at the onset of labour (Garfield, et al., 1999). These data suggest that agents devised to affect preterm labour would therefore be of benefit at this stage, prior to the onset of active labour.

Control of uterine NO activity

Factors controlling the up-regulation in uterine NOS expression during pregnancy remain uncertain. In animals both oestrogen and progesterone have been proposed as regulators of NOS *in vivo*. Oestrogen upregulates guinea pig constitutive NOS expression (Weiner, et al., 1994). In sheep uterine artery, oestrogen enhanced endothelial NOS activity, and in rat hypothalamus, oestrogen increased bNOS expression (Ceccatelli, et al., 1996; Veille, et al., 1996). In studies of women treated with a Gonadotrophin hormone Releasing Hormone analogue together with either oestrogen replacement or placebo, fasting plasma nitrate concentrations were higher in the oestrogen treatment group, suggesting that oestrogen stimulated NO production (Ramsay, et al., 1995). In human pregnancy, increased oestradiol concentrations may contribute to uterine quiescence through an increase in NOS expression. However, as oestrogen levels are maintained or increase in humans at term, other factors must be responsible for the control of myometrial synthesis of NO. In animal studies, progesterone has been shown to increase iNOS production within the uterus (Buhimschi, et al., 1996). Treatment with antiprogestins has been shown to decrease uterine NO production and hence cause an increase in myometrial contractions. Although human parturition is not associated with a decline in progesterone concentration it is clear from the role of antiprogestins that a more subtle mechanism may be present such as changes in progesterone receptor expression or competition from an endogenous antiprogestagen. Thus progesterone withdrawal may be a regulatory factor in the onset of parturition.

Nitric oxide and cervical ripening

Cervical ripening, the process whereby the cervix undergoes softening, effacement and dilatation during pregnancy is crucial to the control of events in normal labour. At this

time, changes in cervical structure are believed to be due to alterations in the underlying connective tissue composition. Changes in collagen concentration, an increase in tissue water content and an alteration in matrix glycosaminoglycans result in dissociation of the connective tissue structure (Calder and Greer, 1992). Evidence is accumulating that this process is mediated by an inflammatory mechanism involving vasodilatation, altered vascular permeability and neutrophil influx into the tissue (Junquiera, et al., 1980; Leppert, 1992; Liggins, 1981). Various agents may be involved in the control of this process including prostaglandins, cytokines, steroid hormones and relaxin.

NO and cervical ripening in animals

The results of recent animal studies on NO production and NOS expression in the cervix indicate that NO may play a role in cervical ripening. The NO generating system is present in the rat cervix and in contrast to the body of the uterus, it is down-regulated during pregnancy, but up-regulated during term and preterm labour (Buhimschi, et al., 1996). The expression of iNOS was upregulated in both term and preterm labouring cervices, while bNOS was only upregulated in term labouring samples. This may reflect the presumed infective/inflammatory aetiology of preterm labour. Treatment of pregnant guinea-pigs with the NOS inhibitor L-NAME induced preterm labour but delayed physiological cervical ripening resulting in prolonged deliveries (Chwalisz, et al., 1994). Furthermore, L-NAME treatment of pregnant rats significantly prolonged the duration of labour, suggestive of cervical dystocia, whilst a decrease in cervical extensibility was observed after *in vitro* incubation with L-NAME (Buhimschi, et al., 1996). Chwalisz *et al.*, (1997) (Chwalisz, et al., 1997) demonstrated that the local application of the NO donor sodium nitroprusside, produced effective ripening of the guinea-pig cervix, assessed by both force resistance measurements and morphological evaluation. It seems that, at least in

animal pregnancy, NO represents a final metabolic pathway of cervical ripening. It has been suggested previously that prostaglandins, particularly those of the E series, are the final common mediators of cervical ripening. However, cervical ripening brought about by anti-progestins cannot be blocked by the action of specific cyclooxygenase inhibitors (Qing, et al., 1996; Radestad and Bygdeman, 1992). This suggests that other agents such as NO must be inherently involved in this process.

The proposed mechanism of NO induced cervical ripening remains unclear. Induction of iNOS at the sites of inflammation results in the production of large quantities of NO which are responsible for vasodilatation and changes in vascular permeability. As cervical ripening is essentially an inflammatory response, this may be the mechanism by which NO operates. Administration of SNP to guinea pigs results in lymphocyte, granulocyte and macrophage accumulation within cervical tissue associated with stromal oedema and arterial dilatation (Qing, et al., 1996).

NO may also exert its effects through stimulation of matrix metalloproteinases (MMPs). While NO has not yet been shown to influence the activity of MMPs in cervical tissue, in both human and animal studies in other tissues NO has been shown to be capable of stimulating the activity of a number of these MMPs (Murrell, et al., 1995; Sasaki, et al., 1998; Trachtman, et al., 1996).

There is an alteration in the glycosaminoglycan composition of the cervix during pregnancy and this may be important in the ripening process. The amount of hyaluronic acid present in the cervix increases and the chondroitin sulphate and dermatan sulphate composition falls. These changes bring about an alteration in the binding affinity to collagen thus altering tissue hydration and hence cervical extensibility (Rechberger, et al.,

1996). NO has the potential to suppress proteoglycan synthesis (Hauselmann, et al., 1994; Hauselmann, et al., 1998), and its mechanism of action within the cervix may reflect its ability to alter the composition of proteoglycan molecules within this tissue.

The process of programmed cell death or apoptosis has been implicated in cervical ripening (Leppert, 1995). Recent studies in rats have shown that apoptosis occurs in cervical smooth muscle cells (Romero, et al., 1990) and fibroblasts (Leppert, 1998) during ripening and evidence from other tissues suggests that this process may be stimulated by NO (Brune, et al., 1998; Nicotera, et al., 1997).

The process of cervical ripening has been shown to involve other inflammatory mediators including prostaglandins and cytokines. In particular IL-1, IL-8, IL-6 and TNFα have been shown to be produced in the cervix and to be involved in the ripening process (Sennstrom, et al., 1997). There appears to be considerable interaction between NO and these inflammatory cytokines (Chwalisz, et al., 1994; Liew, 1995), suggesting that self-regulatory paracrine mechanisms between such inflammatory mediators may be important in providing overall control.

NO has also been shown to induce the enzyme cyclooxygenase (COX)-2 (Salvemini, et al., 1993; Sautebin, et al., 1994) and hence elevate PG levels in various models of inflammation. Interestingly, both COX-2 and iNOS can be upregulated by similar inflammatory mediators and may act in concert during the ripening process under steroid control.

NO and cervical ripening in humans

There are few published studies examining the effect of NO on human cervical ripening. The effects of the NO donor glyceryl trinitrate (GTN), administered sublingually, on the non-pregnant cervix have been assessed. Yadava, (1990) (Yadava, 1990) reported that GTN facilitated the transcervical introduction of intra-uterine contraceptive devices. Shaker *et al.*, (1993) (Shaker, et al., 1993) found that GTN had no significant effects on the ease of embryo transfer after in-vitro fertilization.

Studies in our department have concentrated on the effects of the NO donors GTN and isosorbide mononitrate (IMN) on the first trimester human cervix (Thomson, et al., 1997; Thomson, et al., 1998). The first of these was a randomised controlled trial performed on forty eight primigravid women at 8-12 weeks gestation undergoing suction termination of pregnancy (Thomson, et al., 1997). The cervical ripening effects of the NO donors IMN (40mg) and GTN (500μg) were compared with that of the prostaglandin analogue gemeprost (1mg) and a control group given no treatment. The ability of these agents to effect cervical ripening was objectively measured using a force sensing apparatus coupled to cervical dilators (Anthony, et al., 1982) and the cervical diameter prior to dilatation measured. The cumulative force required to dilate the cervix to 8mm was significantly less in the IMN group compared to controls given no treatment and a higher cervical diameter before dilatation was recorded. Further studies are presently in progress to determine whether NO is involved in endogenous cervical ripening at term.

Ekerhovd *et. al.*, (1998) (Ekerhovd, et al., 1998) have identified the presence of the NO system within the first trimester cervix by NADPH diaphorase staining and have suggested that NO may have a role in inhibiting smooth muscle relaxation within the cervix although the exact physiological significance of this is uncertain. Tschugguel *et al.*, (1999)

(Tschugguel, et al., 1999) have recently shown that iNOS is upregulated in the cervices of postpartum patients compared to non-pregnant controls, providing further evidence for the involvement of NO in the process of human cervical ripening.

Despite the superiority of prostaglandins in effecting cervical ripening NO donors may have a significant role in clinical practice, both for cervical ripening in the first trimester and at term. Prostaglandins are associated with a high incidence of side effects including nausea and vomiting, diarrhoea, abdominal pain and bleeding and an abnormal fetal heart rate pattern in 7% of women due to increased myometrial activity when used for induction of labour at term (Wing, et al., 1995). Studies presented in chapter 2 of this thesis suggest the use of NO donors may result in a more acceptable side effect profile while still being clinically effective (Thomson, et al., 1998). Following the administration of IMN in the first trimester 70% of women remained asymptomatic compared with only 14% in the prostaglandin treated subjects. While more women in the NO treated group experienced headache, there were no adverse effects recorded on blood pressure or intra-operative blood loss. Further studies presented in chapter 3 examine whether combination therapy with a prostaglandin analogue and a nitric oxide donor produces a more clinically effective regimen for cervical ripening in the first trimester of pregnancy. Clearly further studies need to be performed to confirm the safety of NO donors for cervical ripening but the results of these preliminary studies suggest that NO may have a role in this process in the future.

The mechanism of action of NO donors in the process of cervical ripening has been addressed by a number of *in vivo* and *in vitro* studies. While it is possible that ripening of the cervix may be achieved by the interaction of NO with various MMPs, studies presented in chapter 5 of this thesis have suggested that MMPs-2 and –9 are not involved. However, the effects of NO may be mediated via cross talk with COX and an increase in prostaglandin synthesis which in turn would stimulate other inflammatory mediators such as IL-8 and secretory leukocyte protease inhibitor (Denison, et al., 1999). This is the subject of investigations presented in chapter 6 of this thesis.

Despite such research, the role of NO in the process of endogenous cervical ripening in humans remains unclear. This is compounded by the obvious difficulty in obtaining human cervical tissue at this time for research. However, it would seem from the various *in vitro* and *in vivo* studies already performed that NO is involved in this process, acting in concert with a variety of other mediators such as cytokines and prostaglandins. Clearly, further research in this field will improve our understanding of these key events in the initiation of labour and will provide novel therapeutic strategies for inhibiting and initiating the process of cervical ripening.

Conclusion

Pregnancy maintenance appears to depend upon a functioning NO-cGMP system within the myometrium. Conversely, the onset of labour is dependent upon the increased activity of this system within the cervix. The management of preterm and post-dates pregnancy remain significant challenges for today's obstetrician, and a greater understanding of the role of NO in the pathophysiology of these conditions may contribute to future therapeutic advances. Therapies aimed at the management of preterm and post dates pregnancy must

account for the paradoxical opposing effects which NO has on the uterus and cervix and be targeted for maximum efficiency in a site specific manner.

In summary, the studies presented in this thesis investigate the role of nitric oxide in human cervical ripening and its potential therapeutic role in the clinical manipulation of this process.

A randomised controlled trial of nitric oxide donors for ripening the human uterine cervix in the first trimester of pregnancy: a comparison of the side effect profile with a prostaglandin analogue

Introduction

Cervical ripening before first trimester surgical termination of pregnancy facilitates the procedure whilst reducing the operative morbidity (Grimes, et al., 1984; MacKenzie and Fry, 1981; Schultz, et al., 1983). Pharmacological agents commonly used for this indication include laminaria in North America and prostaglandin analogues in the United Kingdom. Whilst prostaglandins produce more effective cervical ripening and are more convenient to administer than laminaria (Helm, et al., 1988), they are associated with a number of adverse effects including abdominal pain, nausea, vomiting and diarrhoea (Henshaw and Templeton, 1991). The ideal cervical ripening agent should be effective, easy to administer and have a low incidence of side effects.

The inflammatory mediator nitric oxide, which has been implicated in a wide variety of physiological processes (Anggard, 1994), is a fundamental mediator of cervical ripening in animals (Ali, et al., 1997; Buhimschi, et al., 1996). The nitric oxide donor, sodium nitroprusside produces effective ripening when applied locally to the guinea pig cervix (Chwalisz, et al., 1997). In a randomised controlled trial, it has previously been shown that the nitric oxide donors isosorbide-5-mononitrate and glyceryl trinitrate can induce effective cervical ripening compared to no treatment in primigravid women, when administered per vaginam in the first trimester of pregnancy (Thomson, et al., 1997). Since these agents are smooth muscle relaxants (Norman, 1996), they should induce cervical ripening without causing abdominal pain due to myometrial contractions, the most commonly reported side

effect in women receiving prostaglandins in early pregnancy (El Refaey, et al., 1994; Helm, et al., 1988; Henshaw and Templeton, 1991).

We hypothesised that nitric oxide donors can effectively ripen the cervix before first trimester surgical termination of pregnancy with fewer side effects than prostaglandin analogues. We therefore performed a three-group randomised trial to compare the clinical effects of the nitric oxide donor, isosorbide mononitrate (IMN, 40 mg and 80 mg) and the prostaglandin analogue, gemeprost before first trimester surgical termination of pregnancy. In addition, we performed a concurrent observational study to determine whether the cervical ripening effects of IMN are clinically sufficient.

Methods

(a) Randomised study to compare the clinical effects of IMN (40 and 80 mg) and gemeprost.

Between January and November 1997, 67 primigravid women scheduled for surgical termination of pregnancy by vacuum aspiration in the first trimester were recruited to this randomised trial; one woman was excluded before randomisation when she was found to be multigravid (Figure 2.1). The study was approved by the local research ethics committee and informed consent was obtained from each woman on the morning before the operation. Exclusion criteria from the study included: any previous pregnancy, previous cervical surgery, signs of threatened miscarriage or concurrent maternal disease. The gestation of each pregnancy was determined from both the menstrual history and clinical evaluation of uterine size. The women were randomly allocated to one of three treatment groups: the nitric oxide donor IMN 40 mg (Elantan® 40, Schwarz Pharma Ltd., Bucks., UK), IMN 80 mg, or the prostaglandin analogue gemeprost 1mg (16, 16-dimethyl-trans delta 2 PGE_1 methyl ester, Farillon, Essex, UK). Block randomisation was

performed using sequentially numbered, sealed, opaque envelopes prepared using random number tables and 22 women were allocated to each treatment group. The envelopes were opened and the treatment allocated by one investigator after consent was obtained. Each medication was administered by the same investigator to the posterior vaginal fornix three hours prior to surgery. Treatment allocation was concealed from patients until the end of the study period.

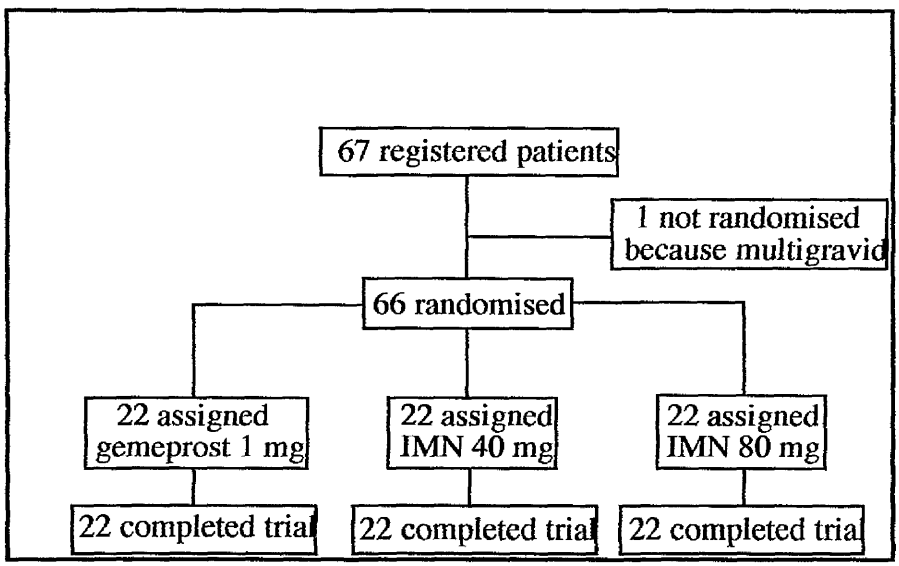


Figure 2.1 Flow chart showing the women's progress through the randomised study. IMN = isosorbide-5-mononitrate.

The primary outcome measures were the development of adverse symptoms for each active treatment group. These were assessed using a symptom questionnaire administered before the medication was given and again immediately prior to surgery. The same investigator administered the symptom questionnaire on each occasion. At these time points, peripheral arterial pulse rates and mean arterial blood pressure recordings were obtained using an automated electronic device, (Dinamap Plus®, Critikon, Florida, USA). For all women, general anaesthesia was induced using an intravenous injection of propofol,

(Diprivan®, Zeneca Pharma, Cheshire, UK), and alfentanil, (Rapifen®, Janssen, Bucks., UK), and maintained with intermittent repeat doses as required. Secondary outcome measures were the cervical diameter before surgical dilatation and the force required to dilate the cervix to 8mm measured with a force sensing apparatus coupled to cervical dilators, as previously described (Anthony, et al., 1982; Richardson, et al., 1989). All the procedures and recordings were performed by two experienced operators who were blind to the treatment given. Each of the treatments dissolved completely in the vagina, further ensuring that the operators were blinded to the treatment group. Suction evacuation of the uterus was then undertaken and the total volume lost, including both blood and products of conception, was measured from a calibrated suction jar.

Using Altman's nomogram (Altman, 1982), we calculated that by studying 66 women (22 in each active treatment group), the study has a power of 85%, at the 5% significance level, to show a reduction of two thirds in the incidence of abdominal pain between the active treatment groups. This calculation is based upon a 70% incidence of abdominal pain occurring in the gemeprost group (Helm, et al., 1988). We considered that such a reduction in the frequency of abdominal pain would be clinically significant.

(b) Observational study to determine whether the cervical ripening effects of IMN are clinically sufficient.

It is current practice to omit preoperative cervical ripening in parous women undergoing first trimester termination of pregnancy (Royal College of Obstetricians and Gynaecologists, *Guideline no. 11,* 1997), since the force required to dilate the parous cervix is significantly less than that required for the primigravid cervix (Anthony, et al., 1982). We considered that for a cervical ripening agent to be considered clinically effective in primigravid women, it should reduce the cervical resistance to a level similar to that measured in parous women. We therefore recruited 22 consecutive parous women

scheduled for surgical termination in the first trimester as a reference group for cervical resistance. The study was approved by the local research ethics committee and informed consent was obtained from each woman on the morning before operation. Each woman had had at least one normal vaginal delivery and exclusion criteria included previous cervical surgery and signs of threatened miscarriage. In these women, the primary outcome measures were the cervical diameter before surgical dilatation, the cumulative force required to dilate the cervix and the intraoperative blood loss, which were measured as described above.

Statistics

One factor ANOVA was used to compare the patient characteristics (age, gestation and interval between treatment and surgery), pulse and blood pressure data between the treatment groups. Data on intraoperative blood loss, cervical diameter and forces required during cervical dilatation were analysed using the Kruskal-Wallis test or the Mann-Whitney U test. The incidence of pre-operative side-effects was compared using Fisher's exact test.

Results

(a) Randomised study to compare the clinical effects of IMN (40 and 80 mg) and gemeprost.

The mean age, gestation and interval between pretreatment and surgical dilatation, in each active treatment group are shown in Table 2.1.

Table 2.1 Clinical details of the women participating in the study.

Data are given as mean (standard deviation).

	Gemeprost (n = 22)	IMN 40 mg (n = 22)	IMN 80 mg (n = 22)	Parous group (n = 22)
Age (years)	21.8 (4.1)	22.6 (6.6)	21.9 (6.2)	27.0 (4.9)
Gestation (weeks)	9.0 (1.2)	9.3 (1.1)	9.3 (1.0)	9.1 (1.0)
Interval between pre-treatment and surgical dilatation (min)	169 (24)	169 (37)	170 (42)	-

IMN = isosorbide-5-mononitrate.

Table 2.2 shows the incidence of new symptoms, which occurred after the administration of the ripening agent. Sixty-eight per cent of women in the IMN 40 mg group and 59% in the IMN 80 mg group remained asymptotic, significantly more than the 14% of women in the gemeprost group ($p<0.001$ and $p<0.005$ respectively). Significantly more women who received the prostaglandin analogue gemeprost experienced abdominal pain ($p<0.0001$) and vaginal bleeding ($p<0.01$) when compared with either of the IMN groups. More women in each of the IMN groups developed a headache ($p<0.05$) when compared with the gemeprost group. No significant difference was found between the IMN 40 mg and 80 mg groups in reported symptoms. No significant difference in the change in mean arterial blood pressure or pulse rate was detected among the groups after treatment.

The efficacy of each agent to dilate the cervix and reduce the cervical resistance is shown in Table 2.3. Pretreatment with gemeprost was more effective at ripening the cervix than either dose of IMN. Gemeprost increased the diameter of the cervical os before surgical dilatation compared with the 40 mg IMN group ($p<0.05$), and was associated with a lower cervical resistance than either dose of IMN ($p<0.02$). There were no significant

differences in the cervical resistance between the two IMN groups. Pretreatment with gemeprost resulted in significantly less ($p<0.0005$) measured intraoperative blood loss compared with either dose of IMN (Table 2.3).

Table 2.2 Incidence of new symptoms occurring after administration of the cervical ripening agent.

	Gemeprost (n = 22)	IMN 40 mg (n = 22)	IMN 80 mg (n = 22)
	n (%)	*n (%)*	*n (%)*
Asymptomatic	3 (14)	15 (68) [a]	13 (59) [a]
Abdominal pain	16 (73)	1 (5) [b]	0 (-) [b]
Diarrhoea	2 (9)	0 (-)	0 (-)
Dizziness	0 (-)	3 (14)	1 (5)
Headache	0 (-)	5 (23) [c]	7 (32) [d]
Nausea/vomiting	4 (18)	1 (5)	2 (9)
Palpitation	0 (-)	1 (5)	3 (14)
Vaginal bleeding	7 (32)	0 (-) [e]	0 (-) [e]

a. significantly greater than gemeprost group, $p<0.005$

b. significantly less than gemeprost group, $p<0.0001$

c. significantly greater than gemeprost group, $p<0.05$

d. significantly greater than gemeprost group, $p<0.01$

e. significantly less than gemeprost group, $p<0.01$

Table 2.3 Results of the intra-operative recordings for both the randomised comparison and the observational study.

Data are given as the median (interquartile range). IMN = isosorbide mononitrate.

	Gemeprost (n = 22)	IMN 40 mg (n = 22)	IMN 80 mg (n = 22)	Parous group (n = 22)
Cervical diameter before surgical dilatation (mm)	6 (5 - 7)*	5 (4 - 5)	5 (5 - 5)	5 (3 - 6)
Cumulative force to dilate cervix to 8 mm (N)	21 (7 - 38)**	40 (23 - 54)	36 (27 - 60)	34 (18 - 61)
Intra-operative blood loss (ml)	58 (45 - 90)***	143 (90 - 220)	160 (105 - 295)	135 (50 - 225)

(*) significantly greater than IMN 40 mg and parous groups, $p<0.03$

(**) significantly less than IMN 40 mg, IMN 80 mg and parous groups, $p<0.02$

(***) significantly less than IMN 40 mg, IMN 80 mg and parous groups, $p<0.0005$

(b) Observational study to determine whether the cervical ripening effects of IMN are clinically sufficient.

Pretreatment of primigravid women with IMN resulted in a cervical resistance similar to that in the parous group (Table 2.3). There was no significant difference in the measured intraoperative blood loss between each IMN group and the parous group of women (Table 2.3). Furthermore, no woman in any group required plasma volume expansion, blood transfusion or reoperation for vaginal bleeding and no woman in the study had a blood loss greater than 500 ml.

Discussion

This randomised study demonstrates that the nitric oxide donor IMN has fewer side effects than the prostaglandin analogue gemeprost when used to ripen the cervix before first trimester surgical termination of pregnancy. The nitric oxide donor is associated with a lower incidence of abdominal pain and pre-operative vaginal bleeding and a higher proportion of patients remaining asymptomatic. However, more women receiving the nitric oxide donor experienced a headache. The prostaglandin analogue produced a greater reduction in force and was associated with a lower intraoperative blood loss than either dose of the nitric oxide donor. The concurrent observational study demonstrated that pretreatment of primigravid women with IMN results in a cervical resistance similar to that of the parous group, whilst the intraoperative blood loss was similar in the IMN and parous groups.

The side effect profile of prostaglandin analogues administered in the first trimester of pregnancy is well characterised and includes nausea, vomiting, diarrhoea, abdominal pain and vaginal bleeding (Helm et al., 1988; Henshaw and Templeton, 1991; Lawrie, et al., 1996). These effects are thought to arise because the prostaglandins stimulate contractions

of uterine and gastrointestinal smooth muscle. Whilst it has been suggested that misoprostol, an orally active synthetic analogue of prostaglandin E_1, is associated with fewer side effects than gemeprost (El Refaey, et al., 1994), a more recent study has reported a high incidence of vaginal bleeding (50%), abdominal pain (47%) and vomiting (22%) in women given misoprostol in the first trimester of pregnancy (Ngai, et al., 1996). It has been suggested that the antiprogestogen mifepristone represents an acceptable and effective cervical ripening agent (World Health Organisation, 1990; Henshaw and Templeton, 1991). Whilst this agent has the advantage of being effective after oral administration, it is also associated with side effects, (including nausea, vomiting, vaginal bleeding and abdominal pain) and requires a latent period of 36-48 hours for the drug to act (Ngai, et al., 1996).

The side effect profile of nitric oxide donors administered vaginally has not been described. Their recognised side effects when administered by other routes (oral, sublingual, transdermal and intravenous), include headache, dizziness, postural hypotension and tachycardia (Robertson and Robertson, 1995). Two studies have assessed the effects of nitroglycerin, a nitric oxide donor, administered either as transdermal patches or as an intravenous infusion (20 μg/min), during pregnancy (Lees, et al., 1994; Ramsay, et al., 1994). No significant changes in maternal heart rate or systemic blood pressure were detected, although headache was reported to Lees et al. (1994) by one third of the women. Other groups have found that larger doses of nitric oxide donors administered during pregnancy have significant adverse effects on the maternal cardiovascular system. Intravenous nitroglycerin, up to 5 μg/kg per minute (Cotton, et al., 1986; Grunewald, et al., 1995) and a single 5mg dose of sublingual isosorbide dinitrate increased maternal pulse rate and decreased mean arterial blood pressure (Thaler, et al., 1996).

In our study, when IMN was administered locally to the cervix, 60 - 70 % of women remained asymptomatic and there were no significant changes in mean arterial blood pressure or pulse rate. This low incidence of side effects may reflect the first uterine pass effect whereby vaginal drug administration results in high uterine and low serum concentrations (Bulletti, et al., 1997). It has previously been shown that vaginal administration of IMN allows effective ripening of the uterine cervix (Thomson, et al., 1997), and the study reported here indicates that such an approach is associated with minimal systemic side effects.

An accumulation of nitric oxide donor in the uterine smooth muscle would be expected to relax the myometrium (Norman, 1996) thereby minimising preoperative abdominal pain and vaginal bleeding. One potential disadvantage of this effect is that myometrial relaxation, or lack of stimulation to contract during surgical evacuation might result in an increased intraoperative blood loss. Haemostasis in the placental bed depends not on the coagulation system, but rather myometrial contraction, the so-called living ligature, in the first instance. Prostaglandin analogues such as gemeprost, which cause myometrial contractions, are associated with a lower intraoperative blood loss when compared with placebo (Greer, et al., 1992). We found that the measured loss in each IMN group was not significantly greater than in a parous group of women receiving no treatment. Blood loss was lower in the gemeprost group, as would be expected with an agent provoking uterine contractility, than either the parous group or the groups receiving IMN. These data suggest that although the uterotonic effects of prostaglandins are associated with a reduction in measured blood loss, the myometrial relaxant effects of nitric oxide donors do not result in an increased blood loss compared with the parous control group. Moreover, no woman in any of the groups had a clinically significant intraoperative blood loss as assessed by the need for plasma volume expansion, blood transfusion or reoperation for blood loss.

The efficacy of the prostaglandin analogue gemeprost to ripen the cervix is significantly better than either dose of IMN. Our results suggest that the ability of IMN to ripen the cervix is not dose dependent, although we have assessed only two doses of the nitric oxide donor. Previous work, which included a primigravid, control group given no treatment, has shown that IMN 40 mg and gemeprost 1 mg reduced the cervical resistance by 40 % and 70 % respectively (Thomson, et al., 1997). Whilst it is widely accepted that a reduction in the force required to dilate the cervix reduces both operative morbidity and long term effects of cervical damage (Grimes, et al., 1984; MacKenzie and Fry, 1981; Schultz, et al., 1983), there are no data on how much force is considered acceptable. In the absence of such data, we propose that the force required to dilate the parous cervix is a clinically acceptable target for ripening agents since it is current practice to omit preoperative cervical ripening in these women (Royal College of Obstetricians and Gynaecologists, *Guideline no. 11*, 1997). We have shown no significant differences in the cervical resistance of primigravid women pretreated with IMN and untreated parous women, suggesting that the cervical ripening effects of IMN are clinically sufficient. One caveat to this conclusion is that the comparison between the IMN groups and the parous group was made in an observational study since randomisation was not possible for the group of parous women. It seems essential that any cervical ripening agent used, should have minimal side effects. As we demonstrate in this paper, the nitric oxide donor IMN appears to be such an agent.

This study has demonstrated that the nitric oxide donor IMN has fewer side effects than gemeprost when used to ripen the cervix in the first trimester of pregnancy. Later in pregnancy, cervical ripening is commonly employed to facilitate induction of labour. Whilst prostaglandins are the agents most commonly used to prepare the cervix at term, they are associated with excessive myometrial activity and an abnormal fetal heart rate pattern in 7% of women (Wing, et al., 1995). Nitric oxide donors relax the myometrium,

and these agents may have advantages over the prostaglandins to ripen the cervix later in pregnancy (Norman, et al., 1998). However, the safety of nitric oxide donors in the third trimester must be established before their efficacy for this indication is determined.

Chapter 3

A randomised controlled trial to compare isosorbide mononitrate, misoprostol and combination therapy for first trimester preoperative cervical ripening.

Introduction

We have demonstrated in the previous chapter that cervical ripening in the first trimester of pregnancy can be effected by the nitric oxide (NO) donors isosorbide mononitrate (IMN) and glyceryl trinitrate (Thomson, et al., 1998). Further, pretreatment with IMN is associated with fewer side effects than gemeprost treatment, although the cervical ripening effects of the prostaglandin analogue are significantly better. Clearly, the ideal agent for preoperative cervical ripening should be clinically effective but have a low incidence of side effects.

We hypothesised that the cervical ripening effects of NO donors and of prostaglandins might be additive. If this hypothesis were correct, this would allow a small dose of NO donor to be given in combination with a small dose of prostaglandin to effect cervical ripening. Such a strategy might reduce the side effects associated with larger doses of each agent used alone. Combination therapy may therefore produce an optimal therapy for first trimester preoperative cervical ripening. Gemeprost is the only prostaglandin currently licensed for preoperative cervical ripening in the United Kingdom, but reports indicate that misoprostol, a synthetic 15-deoxy-16-hydroxyl-16-methyl prostaglandin E1 analogue, represents an inexpensive and effective alternative (Baird, et al., 1995; El Refaey, et al., 1994; Jain and Mishell, 1994). Furthermore, misoprostol is associated with fewer side effects than gemeprost (Henry and Haukkamaa, 1999). We therefore performed a three

group randomised controlled trial comparing the clinical effects of misoprostol (400μg), the NO donor IMN (40mg) and combination therapy with these two agents before first trimester surgical termination of pregnancy.

Methods

66 primigravid women undergoing surgical termination by vacuum aspiration in the first trimester of pregnancy (≤12 weeks gestation) were recruited to the study over a seventeen-month period (December 1997 to April 1999). Gestational age was established on the basis of menstrual history and confirmed by vaginal examination or pelvic ultrasound examination. The local research ethics committee approved the study and written informed consent was obtained from each woman prior to inclusion in the study. A history of any previous cervical surgery or threatened miscarriage excluded women from participation in the trial. Women were recruited to one of three treatment groups as follows;

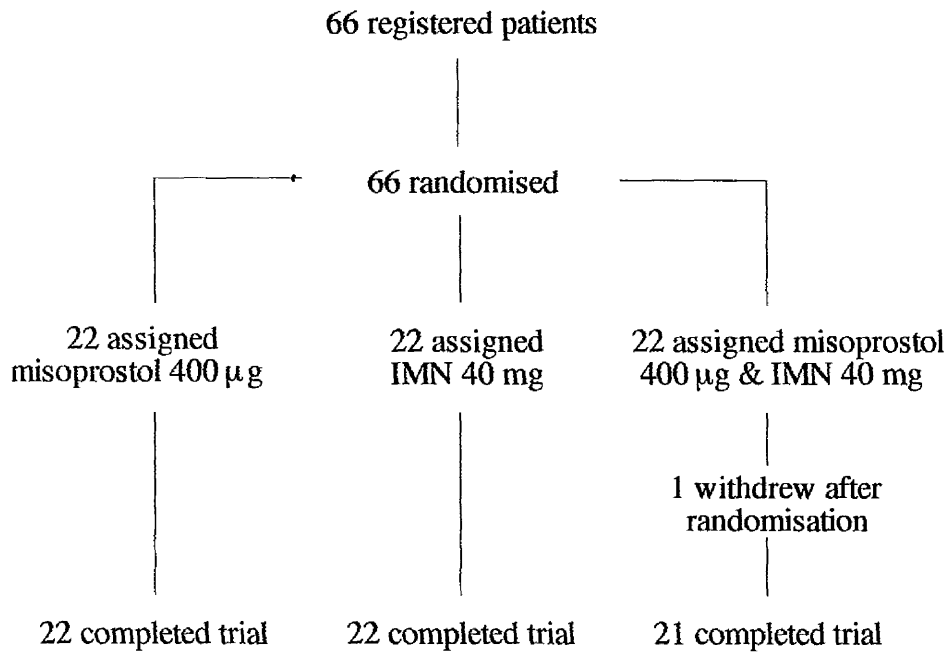
(i) The nitric oxide donor, isosorbide mononitrate (IMN) (Elantan®, Schwarz Pharma Ltd., Bucks, UK), 40 mg

(ii) Misoprostol (Cytotec®, Searle, High Wycombe, Bucks, UK), a prostaglandin E_1 analogue methylated at C-16, 400 μg

(iii) IMN 40 mg in combination with misoprostol 400 μg

Figure 3.1. Flow chart showing the women's progress through the randomised study.

IMN = isosorbide-5-mononitrate



Women were randomised to each of the three treatment groups using sequentially numbered sealed opaque envelopes prepared using random number tables and 22 women were allocated to each group. The envelopes were opened and treatment allocated on the morning of surgery by myself. Each treatment was administered *per vaginam*, to the posterior vaginal fornix approximately 3h prior to surgery. One patient withdrew from the study after administration of treatment having decided not to proceed with termination (Figure 3.1). Treatment allocation was concealed from the patients until completion of the study. The development of adverse symptoms was recorded for each treatment group using a symptom questionnaire. The symptom questionnaire involved a structured series of questions regarding the recognised side effects of each agent. The questions were read from a script before medication was given, and again prior to induction of anaesthesia. Women were asked to respond "Yes" or "No" to the questions, and their responses were recorded. Mean arterial blood pressure and peripheral heart rate recordings were also made

on admission and again immediately prior to surgery using an automated electronic device (Dinamap Plus®, Critikon, Florida, USA). For all women, the procedures were performed under general anaesthesia induced with intravenous propofol (Diprivan®, Zeneca Pharma, Cheshire, UK) and alfentanil (Rapifen®, Janssen, Bucks., UK) and maintained with intermittent repeated doses as required. Gestation was confirmed by bimanual examination under general anaesthetic. The procedures were performed by experienced operators who were blind to the treatment given. The force required to dilate the cervix to 8 mm was recorded using a force sensing apparatus coupled to cervical dilators, as described previously (Anthony, et al., 1982; Richardson, et al., 1989). Suction evacuation of the uterus was performed and the total volume lost, including blood and products of conception, measured using a calibrated suction jar. For the purposes of this study, blood loss was taken as the total volume lost, and compared between the three groups.

Using Altman's normogram (Altman, 1982), we calculated that by studying 66 women (22 in each active treatment group) the study had a power of 80% at the 5% significance level to show a reduction in cervical resistance of 43% when IMN 40mg was combined with misoprostol 400μg, compared with misoprostol 400μg alone. This calculation is based on cervical resistance of 30N in the group treated with misoprostol alone (El Refaey, et al., 1994), a reduction in cervical resistance to ≤ 17N in the group treated with IMN and misoprostol (reduction in cervical resistance of 43% (Thomson, et al., 1998)), and a standard deviation in cervical resistance of 15N.

Statistics

One factor ANOVA was used to compare the patient characteristics (age, gestation, and interval between treatment and surgery), pulse and blood pressure. Intraoperative blood loss and force required to dilate the cervix were analysed using Kruskal-Wallis tests and

significance determined using Mann-Whitney U tests. The incidence of preoperative side effects was compared using Fisher's exact test.

Results

The mean age, duration of gestation and interval between pretreatment and onset of surgery are shown for each of the three treatment groups (Table 3.1.). There was no significant difference in these variables for each of the treatments given.

Table 3.1: Clinical details of the women participating in the study.

Data are given as mean (standard deviation). *IMN = isosorbide-5-mononitrate.*

	Misoprostol (n=21)	IMN (n=22)	IMN & Misoprostol (n=21)
Age (years)	22.4 (5.9)	19.8 (2.7)	21.4 (5.1)
Gestation (weeks)	9.2 (1.1)	9.5 (1.1)	9.5 (1.3)
Interval between pre-treatment and surgical dilatation (min)	169 (29)	150 (28)	157 (22)

The incidences of new symptoms after the administration of each treatment are shown in Table 3.2. There was no significant difference in the number of women remaining asymptomatic after treatment among the three patient groups. A significantly greater number of women given misoprostol experienced abdominal pain when compared to treatment with IMN (p=0.006). The proportion of women who complained of headache was significantly higher in the IMN than the misoprostol treated group (p=0.01). There was no difference in the incidence of abdominal pain between the misoprostol and the combination groups. There was no difference in the incidence of headache between the IMN and combination groups. Women who received combination therapy experienced the

side effects (headache and abdominal pain) of each agent used alone (Table 3.2). There was no significant difference in the change in mean arterial blood pressure or in pulse rate among the three groups.

Table 3.2: Incidence of new symptoms occurring after administration of the cervical ripening agent. *IMN = isosorbide-5-mononitrate.*

	Misoprostol (n=21)	IMN (n=22)	IMN & Misoprostol (n=22)
Symptom free	11(52%)	14 (64%)	11(50%)
Abdominal pain	9 (43%)*	1(5%)	7 (32%)†
Headache	0	7 (32%)**	8 (36%)‡
Dizziness	0	0	1 (5%)
Palpitation	0	0	1(5%)
Diarrhoea	0	0	0
Vaginal bleeding	2 (10%)	0	0
Nausea/vomiting	2 (10%)	0	0

For differences between misoprostol and IMN groups: *p=0.006, **p=0.01

For differences between IMN and IMN & misoprostol groups: †p=0.04,

For differences between misoprostol and IMN & misoprostol groups: ‡p=0.002

The forces required to dilate the cervix to 8 mm following each of the three treatments are shown in Table 3.3. Pretreatment with misoprostol resulted in lower cervical resistance than pretreatment with IMN. Combination therapy with IMN and misoprostol significantly lowered the cervical resistance when compared to IMN alone. However, the combination of these two therapies was no better in achieving a reduction in cervical resistance than misoprostol used alone. There was no significant difference in intraoperative blood loss

between the three groups.

Table 3.3. Intraoperative recordings of force required to dilate the cervix and intraoperative blood loss. Data are given as the median (interquartile range). *IMN = isosorbide mononitrate.*

	Misoprostol (n=21)	IMN (n=22)	Misoprostol & IMN (n=22)
Cumulative force to dilate cervix to 8 mm (N)	18.5 (5-52)	39 (17-54)*	24.5 (10-36)†
Intraoperative blood loss (ml)	175 (100-250)	200 (150-350)	250 (90-400)

*Significantly more than misoprostol group, p=0.04.

†Significantly less than IMN group, p=0.035.

Discussion

Prostaglandins are well-established agents for the purpose of cervical ripening in the first trimester. However, their administration is associated with side effects such as abdominal pain, nausea and vomiting and vaginal bleeding in a significant proportion of women (Fong, et al., 1998; Singh, et al., 1998). The prostaglandin analogue misoprostol is associated with fewer side effects than gemeprost (Henry and Haukkamaa, 1999). The efficacy of each of misoprostol (400μg) and IMN (40mg) for preoperative cervical ripening has previously been shown in randomised controlled trials (El Refaey, et al., 1994, Henry and Haukkamaa, 1999; Thomson, et al., 1997; Thomson et al., 1998; Fong et al., 1998; Singh et al., 1998). In this study, we have shown that combined therapy using

IMN and misoprostol at doses of 40 mg and 400 μg was no more effective in ripening the cervix than misoprostol alone. In addition, misoprostol is a more effective cervical ripening agent in the first trimester than the NO donor IMN. This latter finding is in agreement with previous studies where gemeprost, a PGE_1 methyl prostaglandin analogue, was found to be a more effective cervical ripening agent than the NO donors IMN (40mg) or glyceryl trinitrate (500μg).

The proportion of women remaining asymptomatic was slightly lower in the IMN and misoprostol group compared to the IMN alone group although these differences were not significant. A much greater sample size of around 200 patients in each group would be required for the study to have 80% power to detect such differences at the 5% significance level (Altman, 1982). The reported incidence of abdominal pain in women treated with prostaglandins is up to 70% (El Refaey, et al., 1994; Helm, et al., 1988). This side effect is likely to be caused by myometrial contractility induced by the prostaglandin analogue (Norman, et al., 1991). Clearly the ideal agent for cervical ripening would induce cervical softening with limited or no stimulation of uterine activity. We have previously shown that 70% of women undergoing surgical termination in the first trimester remained asymptomatic following the administration of IMN compared with 14% of women receiving the prostaglandin analogue gemeprost. Pretreatment with gemeprost resulted in a higher incidence of abdominal pain (73%) and vaginal bleeding (32%) presumably due to the contractile effect of this agent on uterine smooth muscle (Norman, et al., 1991; Williams, et al., 1985). We hypothesised combination therapy might reduce side effects, since the smooth muscle relaxant properties of IMN may result in a reduced incidence of the side effects attributable to myometrial contractions. However, we have found that the addition of IMN does not reduce the incidence of side effects associated with misoprostol. In particular, there was no significant difference in the incidence of abdominal pain

between the misoprostol and combination therapy groups. Moreover, the side effects of these two agents appear to be additive, with combined therapy resulting in a greater proportion of women complaining of headache in addition to their abdominal pain. These findings may be attributable to the mechanism of action of NO donors in the process of cervical ripening. NO has been shown to increase prostaglandin production via upregulation of the enzyme cyclooxygenase (Salvemini, et al., 1993; Sautebin, et al., 1994). Preliminary studies *in vivo* discussed in chapter 6 of this thesis have demonstrated an increase in activity of $PGF_{2\alpha}$ in the cervix after administration of IMN (Ledingham, et al., 1999). Denison *et al* (1999) (Denison, et al., 1999) have also recently demonstrated a stimulatory effect of an NO donor on prostaglandin production *in vitro* in non-pregnant cervical tissue. Combination therapy with these two agents could therefore enhance prostaglandin mediated side effects rather than exploit the smooth muscle relaxant properties of NO donors.

One advantage of prostaglandins such as gemeprost for pre-operative cervical ripening is that their use is associated with a reduction in operative blood loss compared to no treatment, presumably due to the uterotonic effects of this drug (El Refaey, et al., 1994; Ngai, et al., 1996; Thomson, et al., 1998). NO donors do not have this advantage. In previous studies, the blood loss associated with the use of IMN was similar to that of no treatment, but greater than that of gemeprost (Thomson, et al., 1998). In the study here, we have found that there was no difference in blood loss between any of the treatment groups, although a trend towards greater blood loss following IMN either alone, or following misoprostol was observed. However, the differences are small, compared to the range of blood loss. A power calculation indicates that a much larger sample size of around 80 per group would be required for the study to have 80% power to detect a difference in blood loss of 75ml (that between the misoprostol and the IMN & misoprostol group) at the 5%

significance level. Our study is therefore insufficiently large to draw firm conclusions about blood loss.

The cervical ripening effects of NO donors may still have a role in clinical practice in women for whom prostaglandins are contraindicated, for example asthmatics with severe bronchospasm or women with known hypersensitivity to prostaglandins (Dollery, 1999). In the third trimester of pregnancy, the uterotonic side effects of prostaglandins may result in fetal distress during cervical ripening particularly in situations such as intrauterine growth restriction (Wing, et al., 1995). The use of agents, like NO donors, which do not stimulate uterine contractility, may be more appropriate in such cases. Randomised controlled trials are required to determine the efficacy and side effect profile of NO donors for preinduction cervical ripening at term.

Conclusions

These data show that misoprostol (400μg) has the best combination of efficacy and side effects when used for the purpose of first trimester cervical ripening. There does not appear to be any advantage in combining misoprostol with the NO donor IMN, either in terms of improvement in efficacy or in the production of a more acceptable side effect profile. IMN does not have a better side effect profile than misoprostol when these agents are used alone.

Chapter 4

The expression of nitric oxide synthase in the human uterine cervix during pregnancy and parturition

Introduction

In the human, cervical ripening is an inflammatory reaction involving leukocytic infiltration, changes in the water content of the extracellular matrix and rearrangement of collagen fibres (Junquiera, et al., 1980; Leppert, 1992; Liggins, 1981; Owiny, et al., 1995). The control of this process remains poorly understood although a number of mediators have been implicated, including progesterone withdrawal, prostaglandins, relaxin, and various inflammatory cytokines (Barclay, et al., 1993; Chwalisz, et al., 1994).

The inflammatory mediator nitric oxide (NO) has recently been implicated in cervical ripening. Previous studies, in both animals and humans, have shown that NO donors can artificially induce cervical ripening following their local application (Chwalisz, et al., 1997; Thomson, et al., 1997; Thomson, et al., 1998). In studies using rat models, nitrate production along with iNOS and bNOS mRNA expression increases in the cervix during labour (Buhimschi, et al., 1996). Tschugguel *et al*, (Tschugguel, et al., 1999) have also suggested a role for NO in human cervical ripening. iNOS expression was increased in the cervices of women in the postpartum period compared to the non-pregnant state. No alteration in the expression of bNOS or eNOS was observed. However, the design of the study was such that effects of cervical ripening on NOS expression could not be separated from the possible effects of pregnancy, labour or the delivery of the term infant through the cervix.

We hypothesised that each of the three isoforms of NOS is expressed in the human cervix and that NOS expression is increased during pregnancy, consistent with the involvement of NO in physiological cervical ripening. To test this hypothesis we examined the changes in expression of each of the three NOS isoforms in the human cervix throughout pregnancy using Western blotting. Further, each of the three isoforms was localised in tissue specimens using immunohistochemistry.

Materials and methods

Cervical tissue biopsies were obtained from women in four different groups:

(i) women undergoing hysterectomy for benign indications (n=8)

(ii) women undergoing suction termination of pregnancy in the first trimester of pregnancy (< 12 weeks gestation) (n=8)

(iii) pregnant women delivered at term (>37 weeks gestation) by elective caesarean section prior to the onset of labour (n=8)

(iv) pregnant women delivered at term (> 37 weeks gestation) undergoing emergency caesarean section after the onset of labour (cervical dilatation > 4cm) (n=8)

The study conformed to the Declaration of Helsinki and was approved by the local research ethics committee. Written informed consent was obtained from each woman prior to recruitment. Pregnant women were excluded from the study if they had a multiple pregnancy or evidence of intrauterine infection, as determined by temperature >38°C. The indication for caesarean section in the labouring group was fetal distress. Women with dysfunctional labour or who had received prostaglandin or oxytocin were excluded. Cervical tissue specimens were divided in two, one half was formalin fixed and paraffin

embedded for immunohistochemistry and the other half was flash frozen in liquid nitrogen and stored at -70°C for subsequent Western blotting.

In the non-pregnant group, cervical biopsies were obtained from the anterior cervical lip using a scalpel within 10 minutes of removal of the uterus. Cervical biopsies from the first trimester subjects were taken from the anterior lip of the cervix using a 6mm biopsy needle (Stifle Laboratories, Wooburn Green, Bucks, UK) under general anaesthetic after evacuation of the uterus. In the pregnant women at term not in labour, biopsies were taken from the anterior lip of the cervix prior to delivery of the infant. Biopsies from the pregnant women in labour were obtained from the same site in the cervix following delivery of the infant.

Immunohistochemistry for eNOS, bNOS and iNOS

Immunohistochemistry was performed on paraffin embedded cervical tissue sections as previously described (Thomson, et al., 1997) using antibodies against eNOS, bNOS and iNOS as detailed in Table 4.1. Briefly, tissue was fixed at the time of collection in 10% neutral buffered formalin and paraffin embedded. Sections were cut to 5μm thickness, dewaxed, rehydrated and endogenous peroxide activity blocked in 0.5% H_2O_2 (Sigma) in methanol for 30 min at room temperature. Sections were washed in Phosphate Buffered Saline (PBS) and antigen was retrieved by microwaving in a pressure cooker (Lakeland Plastics Ltd, Cumbria, UK) if required (Table 4.1). Tissue sections were then blocked in either 20% normal horse (SAPU, Carluke, UK) /human serum (eNOS and iNOS) or 20% goat/human serum (bNOS) for 30 mins at room temperature and incubated for 16 hr at 4°C with the primary antibody diluted in 2% normal horse serum or 2% normal goat serum as appropriate (Table 1). Sections were washed in PBS then incubated for 30 mins with biotinylated horse anti-mouse (Vector Laboratories) diluted 1:200 in 2% normal horse with 5% normal human serum added (eNOS, iNOS) or with biotinylated goat anti-rabbit (Vector Laboratories) at 1:200 in 2% goat serum with 5% human serum added. Sections

were washed in PBS, then incubated with avidin DH/ biotinylated horseradish peroxidase H reagent (Vector Laboratories, UK) in PBS for 30 mins before final washing. Antigen was localised using 1mg/ml diaminobenzidene tetrahydrochloride (Sigma, UK), 0.02% H_2O_2 in 50mM Tris-Cl, pH 7.6 and appeared as a brown end product.

Table 4.1. NOS antibodies for immunohistochemistry

Antibody	Type	Immunogen	Dilution	Pretreatment	Source
eNOS (N30020)	mouse monoclonal IgG1	aa 1030-1209 of human eNOS	1:10,000	microwave	Transduction
bNOS (AHP477)	rabbit polyclonal	aa 1414-1434 of human brain synthetic peptide	1:25	none	Serotec
iNOS (N39120)	mouse monoclonal IgG2	aa 961-1144 of murine iNOS	1:50	microwave	Transduction

Specificities of each of the antibodies was demonstrated by western blotting on lysates of rat cerebellum, human umbilical vein endothelial cells (HUVECS) and LPS/IFNγ stimulated mouse macrophages using the primary antibodies as tabulated above. The positive controls for iNOS, eNOS and bNOS were sections of small bowel from a patient with ulcerative colitis (Guslandi, 1998; Kimura, et al., 1997), umbilical cord (Myatt, et al.,

1997) and rat brain respectively. Negative control slides were set up for all three antibodies with omission of the primary antibody. Negative controls were also set up for eNOS and iNOS with IgG1 and IgG2a immunoglobulins respectively replacing the primary antibody.

Immunohistochemistry for CD45

In order to localise leukocytes in serial sections of each of the tissues, immunohistochemistry was performed on paraffin embedded cervical biopsies using antibody directed against the common leukocyte antigen, CD45 (Dako; M 0701). Briefly, biopsies of cervix were prepared and microwaved as above. Sections were blocked in 20% normal horse (SAPU, Carluke, UK) /human serum for 30 mins at room temperature and incubated for 16 hr at 4°C with anti-CD45 at 1:100 dilution. Sections were incubated for 30 mins with biotinylated horse anti-mouse (Vector Laboratories) at 1:200 dilution and then incubated with avidin DH/ biotinylated horseradish peroxidase H reagent (Vector Laboratories, UK). Antigen was localised using diaminobenzidene tetrahydrochloride as before and counterstained with Harris haematoxylin. Negative controls included slides incubated without the primary antibody.

Western blot analysis for eNOS, iNOS and bNOS

Total protein was extracted from the cervical tissue biopsies using the TRIZOL™ method according to the manufacturers instructions (Life Technologies, Paisley, UK). Protein was quantified using the BCA protein assay reagent (Pierce, Illinois, USA) and UV spectrophotometry at 562nm. Samples containing 60 μg protein were prepared in equal volumes of sample application buffer, separated by SDS-PAGE and transferred to a nitrocellulose membrane (pore size 450μm) (Hybond, Amersham Life Sciences, Little Chalfont, Buckinghamshire, England) by wet blotting (100V for 1 h 50 mins). Gels were stained with Coomassie Blue to check protein transfer. Membranes were blocked in 5%

Marvel™ in 0.05% v/v Tween-Tris buffered saline (TBS-T) for at least 1hr prior to antibody application. The antibodies were as used for immunohistochemistry at the following concentrations: iNOS at 1:10,000, eNOS at 1:2,500 and bNOS at 1:10,000. Lysates of IFNγ/LPS treated mouse macrophages (Transduction Laboratories), HUVECS (Transduction Laboratories) and rat cerebellum were used as the controls for iNOS, eNOS and bNOS respectively. Immunoreactivity was visualised using HRP linked secondary antibodies against the appropriate species and the ECL detection system as per the manufacturer's instructions (Amersham). Stained molecular weight markers (Biorad) were transferred to the nitrocellulose membrane and used to identify and characterise the molecular weights of the NOS isoforms examined.

Data analysis

Transmission densitometry was used to quantify NOS activity in the westerns (Bio-Rad Multi-Analyst'/PC Version 1.1.). Parallel background readings of equal area were obtained in order to calculate relative intensities from the autorads and calculated using dedicated software. Readings were only compared between samples run in parallel under exactly the same conditions (i.e. same electrophoresis run, same buffers, stains and incubation periods). All densitometric assessments were performed within the optimal range of sensitivity. Statistical analysis was performed using Kruskal-Wallis Test with Mann Whitney-U as a post-hoc test. $P<0.05$ was regarded as significant.

For immunohistochemical analysis, the slides were examined using light microscopy by two independent observers. Staining was localised but there was no attempt to quantify staining in the different groups.

Results

Immunohistochemistry

iNOS

In each of the cervical tissues sampled from pregnant and non-pregnant women, iNOS protein was localised to the vascular endothelium. Immunostaining for iNOS was absent from the cervical glands. In biopsies obtained from pregnant women at term, (in labour and not in labour), iNOS was identified in cells within the cervical stroma. Co-localisation with the common leukocyte antigen CD45, identified these cells as leukocytes. The positive control sections (small bowel in ulcerative colitis) showed appropriate localisation for iNOS whilst the negative control sections showed no staining, (Figure 4.1).

eNOS

There was no difference in the immunolocalisation of eNOS among the 4 groups of women. Immunostaining was identified in the vascular endothelium, the parabasal cells of the surface epithelium and the cervical glandular epithelial cells in each of the sections. The positive control sections (term placenta) showed appropriate localisation for eNOS and the negative control sections showed no staining, (Figure 4.2).

bNOS

bNOS was localised in each of the cervical biopsies from non-pregnant and pregnant women. Immunostaining for bNOS was identified in the intermediate and superficial cells of the surface epithelium. Staining for bNOS was absent in the cervical glands and on the vascular endothelium. In cervical biopsies collected from pregnant women at term, bNOS staining was identified in leukocytes in the cervical stroma. The positive control sections (rat brain) showed appropriate localisation for bNOS and the negative control sections showed no staining, (Figure 4.3).

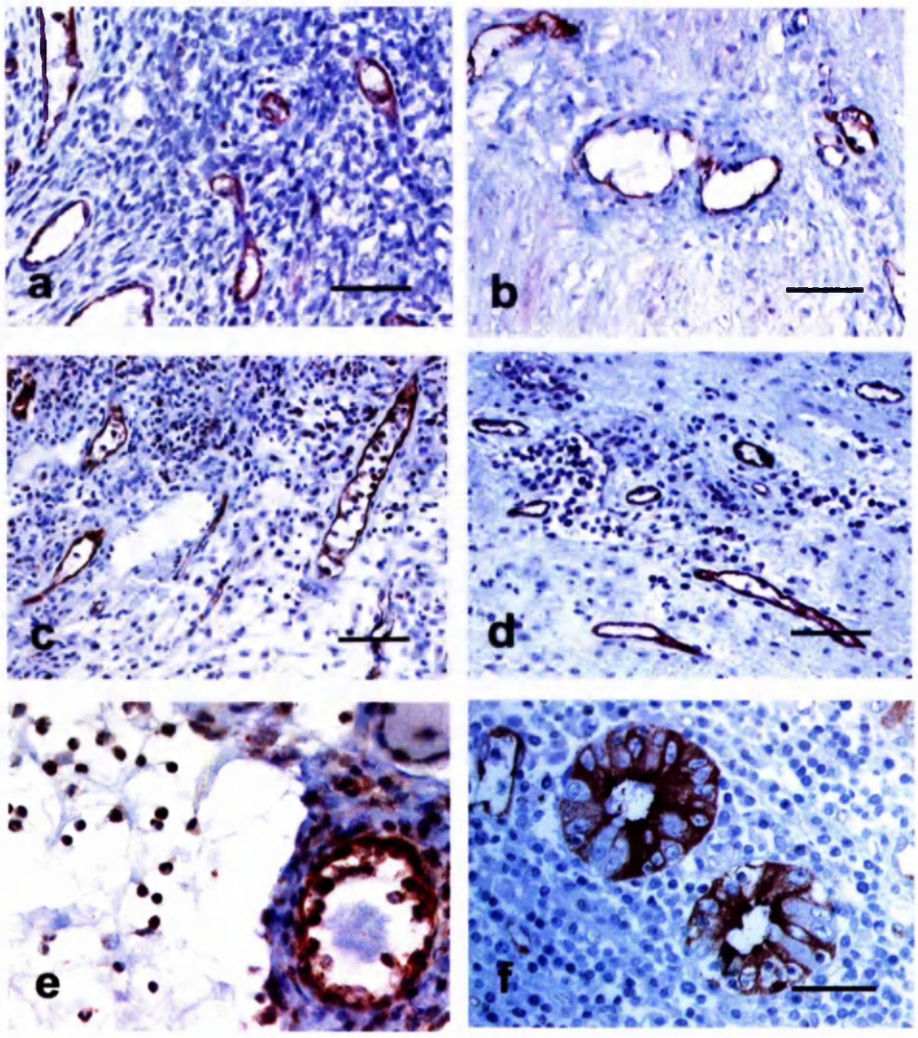


Figure 4.1. Immunolocalisation of iNOS in cervical biopsies collected from (**a**) non-pregnant women, (**b**) women in the first trimester of pregnancy, (**c**) pregnant women at term before the onset of labour, and (**d**) pregnant women at term during spontaneous labour. In each of the biopsies, iNOS localised to the vascular endothelium. In biopsies collected from pregnant women at term, iNOS was also identified within leukocytes (**e**). The positive control slides, small bowel from a patient with ulcerative colitis, showed appropriate localisation of iNOS (**f**). The negative control sections (see text) showed no staining. Scale bars (**a, b, c, d** and **f**) = 50μm. Figure (**e**) was obtained using a high powered (×100) oil immersion lens.

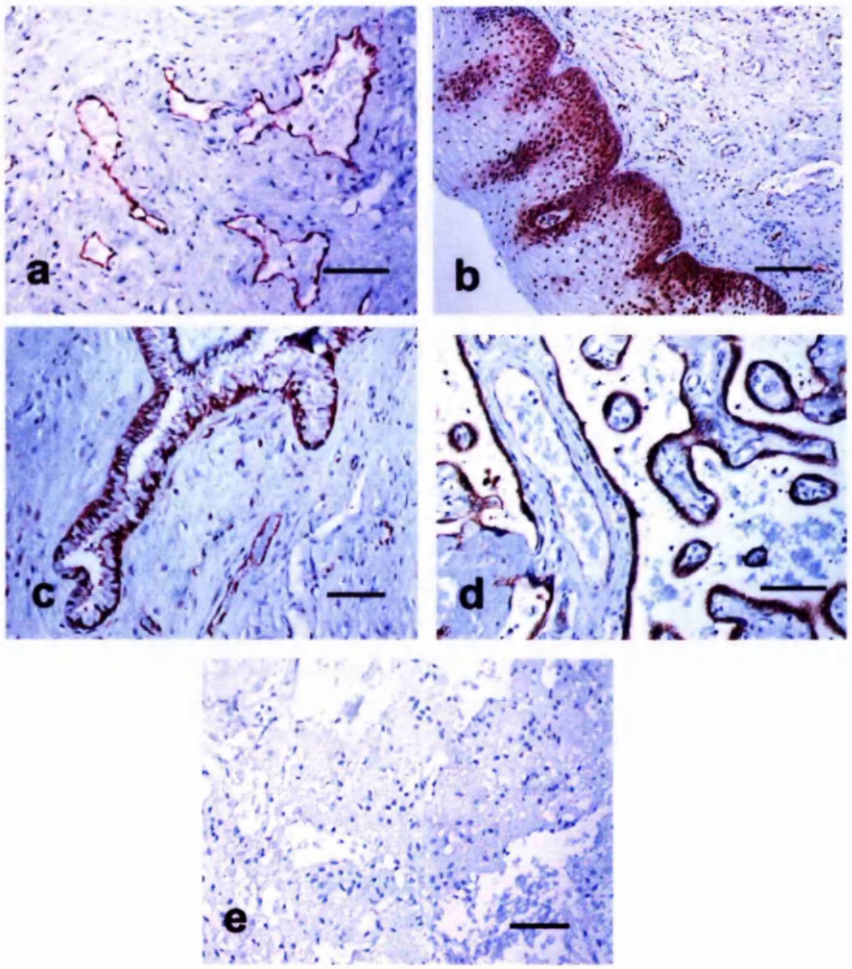


Figure 4.2. Localisation of eNOS in the human uterine cervix. In each of the biopsies, eNOS localised to (a) the vascular endothelium, (b) the parabasal cells of the surface epithelium and (c) the cervical glands. The positive control slides (d), term placenta, showed appropriate localisation of eNOS. The negative control slides (e, see text) showed no staining. Scale bars (a, c, d and e) = 50 μm, and (b) = 100 μm.

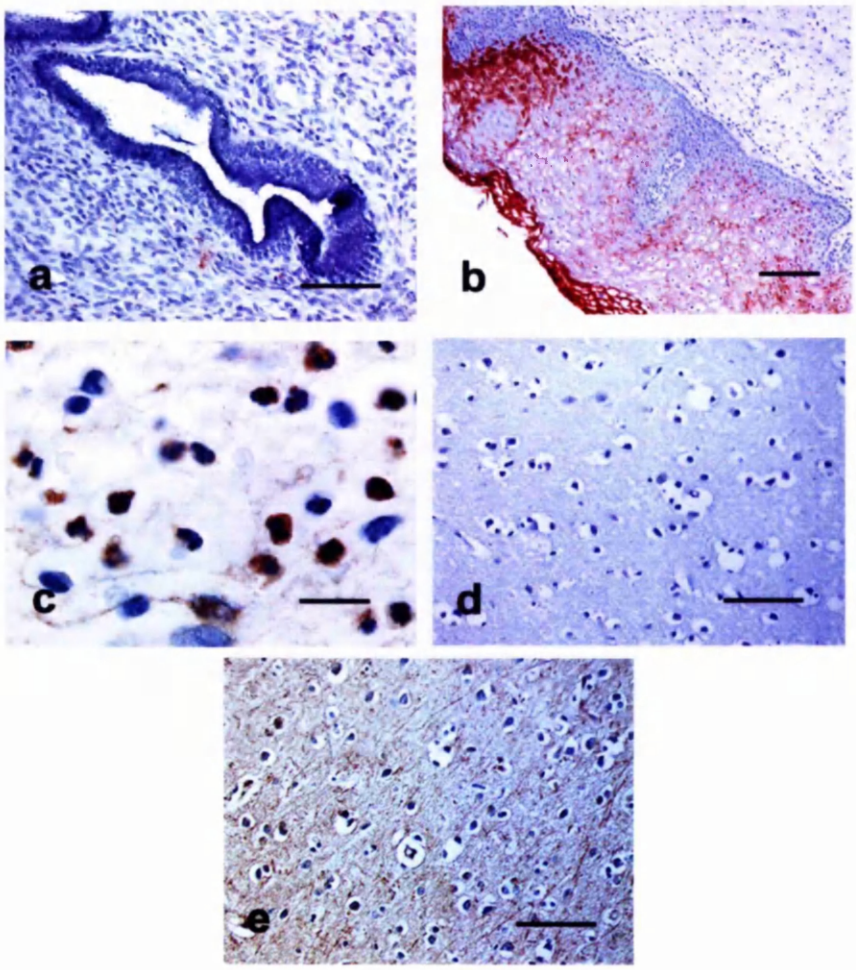


Figure 4.3. Immunolocalisation of bNOS in the human uterine cervix. bNOS protein was absent from the cervical glands (a) but was identified within the intermediate and superficial cells of the surface epithelium (b). Staining for bNOS was identified within leukocytes in biopsies collected from pregnant women at term (c). Sections of rat brain were employed as the negative controls and showed no staining (d) and the positive control slides (rat brain) showed appropriate staining for bNOS (e). Scale bars (a,d and e) = 50μm, (b) = 100 μm and (c) = 20 μm.

Western blotting analysis

Western blotting confirmed the presence of protein for each of the three NOS isoforms within cervical tissue biopsies.

iNOS

iNOS protein was detected at 130kDa. There was a 2.7 fold greater expression of iNOS in the first trimester compared with cervix obtained from non-pregnant subjects ($p<0.005$). There was a further 1.6 fold increase at term prior to the onset of labour ($p<0.01$). iNOS protein expression did not change following the onset of spontaneous labour ($p=0.27$), (Figure 4.4).

eNOS

No significant changes were observed in eNOS expression when comparing the non-pregnant and first trimester groups. However, there was an increase in eNOS expression in the cervix of those samples obtained from term subjects both not in labour and in labour when compared to non pregnant subjects ($p=0.002$ and $p=0.0016$ respectively), (Figure 4.6).

bNOS

In the cervix, bNOS protein was represented by positive bands at 160 kDa. bNOS protein increased in the first trimester of pregnancy compared to the non-pregnant state ($p<0.005$). There were no further significant changes in bNOS expression at term or with the onset of labour, (Figure 4.5).

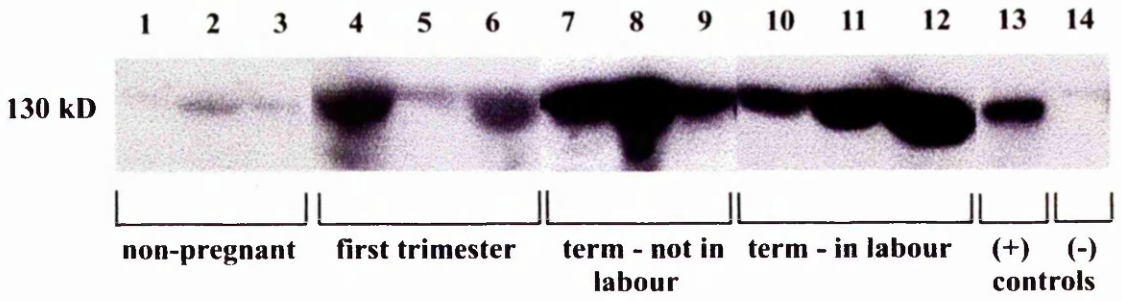


Figure 4.4. Western blot analysis of cervical samples collected from non-pregnant and pregnant women using an antibody directed against iNOS, (lanes 1-3, non-pregnant; lanes 4-6, pregnant first trimester; lanes 7-9, term pregnant not in labour and lanes 10-12, term pregnant in spontaneous labour). The putative iNOS band is seen at 130 kDa. The positive and negative controls are shown in lanes 13 and 14 respectively, (see text). The figure shows representative bands from 3 out of the 8 women in each group. Densitometric analysis showed a significantly greater expression of iNOS in the first trimester cervix compared with pregnant subjects. There was a further increase by term with no change following the onset of labour.

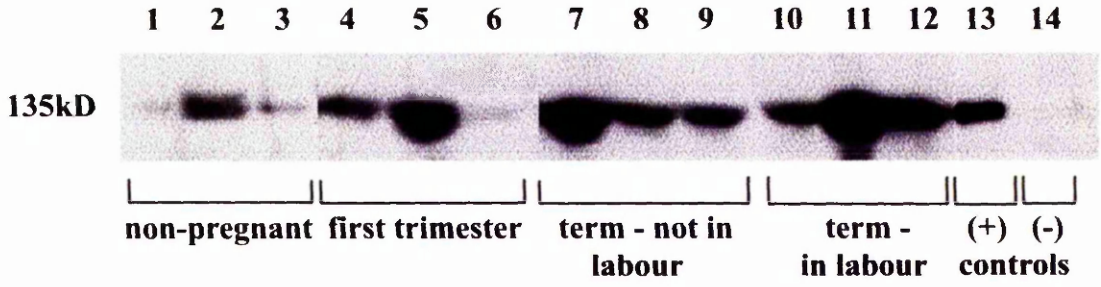


Figure 4.5 Western blot analysis of cervical samples collected from non-pregnant and pregnant women using an antibody directed against eNOS, (lanes 1-3, non-pregnant; lanes 4-6, pregnant first trimester; lanes 7-9, term pregnant not in labour and lanes 10-12, term pregnant in spontaneous labour). The putative eNOS band is seen at 135 kDa. The positive and negative controls are shown in lanes 13 and 14 respectively, (see text). The figure shows representative bands from 3 out of the 8 women in each group. Densitometric analysis showed a significantly greater expression of eNOS in cervical tissue samples collected at term compared with those collected from non-pregnant women, (see text).

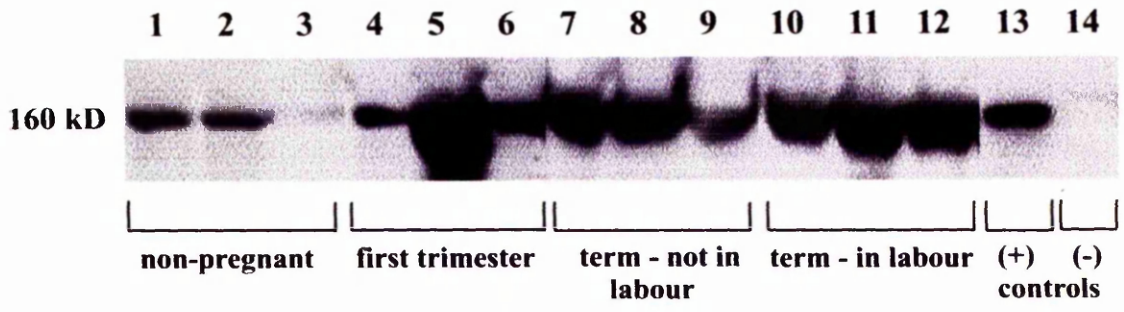


Figure 4.6 Western blot analysis of cervical samples collected from non-pregnant women using an antibody directed against bNOS, (lanes 1-3, non-pregnant; lanes 4-6, pregnant first trimester; lanes 7-9, term pregnant not in labour and lanes 10-12, term pregnant in spontaneous labour). The putative bNOS band is seen at 160 kDa. The positive and negative controls are shown in lanes 13 and 14 respectively, (see text). The figure shows representative bands from 3 out of the 8 women in each group. Densitometric analysis showed a significantly greater expression of bNOS in the first trimester compared with non-pregnant subjects. There were no further changes in bNOS expression by term or following the onset of labour, (see text).

Discussion

This is the first study to examine the localisation and expression of the three NOS isoforms in the human uterine cervix in non-pregnant subjects and in pregnant women in the first trimester and at term, both before and after the onset of labour. We have shown that the three NOS isoforms are present in the human cervix in the non-pregnant and pregnant states. Expression of each of the NOS isoforms is increased in pregnancy compared to the non-pregnant state. iNOS expression is further increased in the third trimester of pregnancy. None of the isoforms of NOS increases following the onset of labour. These results suggest that an increase in endogenous cervical NOS enzyme activity in the third trimester may be important in physiological cervical ripening in human pregnancy. The timing of these events would seem appropriate, given that the changes in the cervix during pregnancy are thought to occur at term prior to the onset of labour as part of a 'conditioning phase' before uterine activity can commence (Calder, 1994; Chwalisz and Garfield, 1997). At this time the cervix becomes soft and compliant secondary to alterations in its water content and constitutive connective tissue matrix components and the uterus undergoes changes which allow it to contract in a synchronous manner (Calder, 1994; Ekman, et al., 1991; Garfield, et al., 1998; Uldbjerg, et al., 1985).

Previous studies in animals have provided evidence that NO may have a physiological role in cervical ripening. The NO generating system has been identified in the rat cervix where it may also be involved in regulating cervical extensibility (Shi, et al., 2000). Increased expression of iNOS and bNOS has been demonstrated using western blotting in the labouring cervix at term and iNOS expression is increased following onapristone induced preterm labour (Buhimschi, et al., 1996). Using RT-PCR, Ali *et al.*, have demonstrated an increase in iNOS mRNA in the cervix of labouring rats when compared to controls with

only minor changes in the constitutive NOS isoforms during gestation (Ali, et al., 1997). In both human and animal studies, nitric oxide donors have been shown to ripen the cervix when artificially applied to this tissue (Chwalisz, et al., 1997; Qing, et al., 1996) (Thomson, et al., 1997; Thomson, et al., 1998). Furthermore, in rats the ripening process can be inhibited using L-NAME, a nitric oxide synthase inhibitor (Buhimschi, et al., 1996). Our data therefore support the hypothesis that NO is a pharmacological and physiological regulator of cervical ripening in the human.

Recent studies have described NOS expression within the human uterine cervix in samples obtained from non-pregnant women and in women following vaginal delivery at term (Tschugguel, et al., 1999). The authors found an increase in calcium independent NOS activity (i.e. the inducible NOS activity) within the pregnant cervix following delivery using a commercially available arginine to citrulline conversion assay. However there was no corresponding change in iNOS mRNA using RT-PCR. The authors suggested that the discrepancy between the RT-PCR and the enzyme activity was attributable to the fact that the iNOS mRNA may have been replaced by the more stable protein within the cervix thus accounting for these findings. A possible concern about this study is the effect of confounding variables such as pregnancy, labour and delivery of the baby on NOS expression. Our study has examined the expression of protein within this tissue during pregnancy and our results are in keeping with the above authors' findings. We have demonstrated an increase in iNOS protein at term in the cervix prior to the onset of labour. However, we have clarified that the increase in NOS activity occurs prior to the onset of labour and is not merely attributable to factors associated with cervical dilatation or vaginal delivery of the fetus.

In contrast to studies performed on the rat (Ali, et al., 1997; Buhimschi, et al., 1996), our studies in the human cervix have demonstrated an increase in expression of iNOS towards the end of gestation with no further increase with the onset of labour at term. Similarly, eNOS expression is increased at term in pregnancy compared to the non-pregnant state. We failed to demonstrate an increase in bNOS expression at term although this isoform was increased in the first trimester of pregnancy. This would suggest that in the human NO is involved in the gradual changes in cervical extensibility and not in the acute process of cervical dilatation, which occurs with spontaneous labour, as has been demonstrated in the rat model. This supports the hypothesis that NO is involved in cervical ripening.

Using immunohistochemistry, we have demonstrated iNOS localisation in the cervical vascular endothelium. This is in keeping with previous reports where iNOS has been identified in endothelial cells (Cheung, et al., 1999; Purcell, et al., 2000). We also demonstrated iNOS immunostaining in a proportion of cells within the tissue collected from labouring women. Using antibody directed against CD45, the common leukocyte antigen, these cells were identified as leukocytes infiltrating the tissue. This data is also in keeping with the findings of Marx *et al.*, who have recently reported similar findings in the pregnant rat cervix (Marx, et al., 2000). Tschugguel *et al.*, (Tschugguel, et al., 1999) also reported iNOS immunostaining in epithelial cells and in stromal spindle cells in cryosections of the human cervix. In comparison, our studies were carried out on paraffin embedded sections of cervical tissue as this method of tissue fixation provides better morphological specimens for analysis. This however may account for the observed differences in tissue localisation. The specificities of each of the NOS antibodies had been confirmed for the purpose of our studies using western blotting analysis as previously discussed.

We demonstrated the presence of bNOS protein within the cervix of non-pregnant and pregnant women using both immunohistochemistry and western blotting. Staining for bNOS was identified within the surface epithelium in both non-pregnant and pregnant subjects. bNOS was also localised to a proportion of leukocytes invading the cervix in labouring samples. This is in contrast to the findings of Tschugguel *et al* (Tschugguel, et al., 1999) who did not demonstrate either bNOS mRNA expression using RT-PCR or the presence of bNOS protein using immunohistochemistry. However our results are in keeping with studies recently performed in humans reporting the presence of bNOS mRNA and protein in the cervix of non-pregnant and pregnant subjects using RT-PCR and Western blotting respectively (Bao, et al., 2000). Further, localisation of bNOS in cervical epithelium has also been confirmed previously in studies on rat uterus (Schmidt, et al., 1992).

eNOS was confined to the vascular endothelium and the glandular and surface epithelium. Previous studies in rats have also shown eNOS immunoreactivity in endothelial and epithelial cells in non-pregnant animals (Chatterjee, et al., 1996). Our findings support those of Tschugguel *et al* (Tschugguel, et al., 1999) who have also reported the localisation of endothelial NOS to the vasculature in non-pregnant and pregnant human cervix. Tschugguel *et al* failed to demonstrate eNOS immunostaining in glandular epithelia. The discrepancy in these results again may be accounted for by the differences in the tissue specimens studied (cryosections vs paraffin sections).

It is now acknowledged that cervical ripening is an inflammatory process involving leukocytic infiltration and release of inflammatory mediators such as IL-1, IL-8 and TNF-α (Barclay, et al., 1993; Chwalisz, et al., 1994; Liggins, 1981). It is therefore not surprising that the inflammatory mediator NO should be involved in these events. The

exact role that NO plays in the ripening process is unclear. However, it is interesting to speculate as to the mechanism whereby NO is involved. During ripening the collagen content of the tissue decreases secondary to the action of degradative enzymes known as matrix metalloproteinases (MMPs) (Junquiera, et al., 1980; Osmers, et al., 1995). The composition of the constituent proteoglycans is also altered (Uldbjerg, et al., 1983; Von Maillot, et al., 1979). NO is capable of stimulating MMP activity and suppressing proteoglycan synthesis in other tissues (Murrell, et al., 1995; Trachtman, et al., 1996 Hauselmann, et al., 1994; Hauselmann, et al., 1998; Sasaki, et al., 1998) and hence may function in a similar manner in the cervix. In chapter 5 we investigate the potential interaction between NO and MMP-2 and -9 in the human cervix during cervical ripening (Ledingham, et al., 1999). NO may also contribute to the ripening process via induction of apoptosis (Brune, et al., 1998; Nicotera, et al., 1997), another mechanism which may be important in this physiological process (Leppert, 1992). The effects of NO may also be mediated via cross-talk with cyclo-oxygenase and an increase in prostaglandin synthesis which in turn would stimulate other inflammatory mediators such as IL-8 and secretory leukocyte protease inhibitor (Denison, et al., 1999; Ledingham, et al., 1999). This is further investigated and discussed in chapter 6.

In conclusion, we have shown that all three NOS isoforms are present in the human uterine cervix in non-pregnant and pregnant individuals. Each of the isoforms of NOS is upregulated during pregnancy and a further increase in the expression of iNOS is detected prior to the onset of labour at term. This supports a role for NO in the remodelling of the cervix that constitutes the ripening process. We speculate that, in the future it may be possible to influence cervical ripening when it occurs prematurely or is delayed, using agents directed against the NO generating system.

Chapter 5

The effects of nitric oxide donors on the secretion of matrix metalloproteinases -2 and -9 and their inhibitors (TIMPs) in the human uterine cervix.

Introduction

At the end of pregnancy, during the ripening process, the cervix changes its biochemical composition and structure to permit delivery. Ripening has been compared to an inflammatory reaction (Liggins, 1981) in which the tissue develops a marked leukocytic cell infiltrate (Junquiera, et al., 1980; Owiny, et al., 1995) and the collagen fibres, which comprise the bulk of the extracellular matrix (ECM) of the cervix, become disorganised. Recent research has proposed that these structural alterations may be due to changes in the water content of the ECM, alterations in the proteoglycan content and/or changes in activity of collagenases (Leppert, 1995)

Collagenases, now termed matrix metalloproteinases (MMPs) are a family of at least 17 different types of zinc-dependent enzymes (Hulboy, et al., 1997) which are also capable of degrading other ECM components such as proteoglycans, fibronectins and laminin present in interstitial matrix and basement membranes. The MMPs are divided into different subgroups depending on their domain structures and their substrate specificities. These groups comprise the fibrillar collagenases (MMPs -1, -8 and -13), gelatinases/type IV collagenases (MMPs -2 and -9), stromelysins (MMPs -3, -7, -10 and -11) and membrane-type MMPs (MMPs -14, -15, -16 and -17). MMPs are secreted as proenzymes and are modulated by activating proteases (Woessner, 1994) and four endogenous tissue inhibitors of metalloproteinases (TIMPs) which form a 1:1 complex with the MMPs (Herron, et al.,

1986).

Collagenolytic activity has been shown to increase in the cervix during late pregnancy and cervical dilatation in both human and animal studies (Rajabi, et al., 1991; Rajabi and Singh, 1995; Uldbjerg, et al., 1983). Increased levels of proMMP-2 and proMMP-9 have also been demonstrated in the cervix of rabbits in late pregnancy (Imada, et al., 1997). Neutrophils and macrophages, both of which invade the cervix during pregnancy and ripening, are known sources of leukocyte collagenase (MMP-8) and fibroblast collagenase (MMP-1), respectively (Osmers, et al., 1992) (Maeda, et al., 1998). However the exact MMPs and TIMPs, which are present in the human cervix in pregnancy, and the mechanism by which their activity is controlled in cervical ripening are not fully understood.

In previous chapters of this thesis we demonstrated that nitric oxide was involved in human cervical ripening and that cervical ripening can be effected using nitric oxide donors. The mechanism whereby NO is involved in the ripening process and how it interacts with other mediators of cervical ripening remains unclear, although NO has been shown to increase the activity of the gelatinases MMP-2 and -9 in other tissues (Murrell *et al.*, 1995, Trachtman *et al.*, 1995, Sasaki *et al.*, 1998, Tamura *et al.*, 1996).

We hypothesised that the process of cervical ripening mediated by NO involves activation of MMPs -2 and -9, which facilitate breakdown of type IV collagen in the blood vessel endothelium. This would allow neutrophil and macrophage influx into the tissue with the release of other agents involved in cervical ripening e.g. other MMPs and proinflammatory cytokines such as interleukin-1 (IL-1), IL-8 and prostaglandins. The aims of this study were to examine whether MMPs -2 and -9 are released by the cervix and if NO donors,

agents known to cause cervical ripening, regulate these MMPs.

Materials and methods

The studies were approved by the local research ethics committees and written informed consent obtained from each woman prior to surgery.

Study Patients

(i) Non-pregnant

Cervical biopsies were obtained from healthy, non-pregnant, premenopausal women (n=15) with regular menstrual cycles undergoing a hysterectomy for non-malignant conditions. Preliminary studies demonstrated that stage of the menstrual cycle did not affect the results. A longitudinal section of the anterior lip of the cervix was taken using a scalpel within 10 min of removal of the uterus. Biopsies were placed immediately in RPMI 1640 culture medium at 4°C for transport to the laboratory.

(ii) Pregnant

20 healthy women in the first trimester of pregnancy (6-12 weeks gestation) undergoing suction termination of pregnancy were recruited into the study. Women were randomised into 2 groups (n=10 in each group) and treatment given as follows:

(i) 40 mg isosorbide mononitrate (IMN) Tablet (Schwarz Pharma Ltd., East Sussex, Chesham, Bucks, England), a nitric oxide donor, per vaginam 2-3 h prior to surgery.

(ii) a control group given no treatment.

Cervical biopsies were taken from each woman from the anterior lip of the cervix using a 6 mm biopsy needle (Stiefel Laboratories, Wooburn Green, Bucks, UK) under general anaesthetic after evacuation of the uterus. Tissue was immediately transferred into

Dulbecco's medium (Sigma, Poole, UK) at 4°C prior to transport to the laboratory.

Tissue Culture

The culture media used for these experiments was that used as standard for tissue culture in the respective laboratory sites.

(i) Cervical biopsies from non-pregnant women:

Biopsies, which were approximately 20 to 35 mg in weight, 15-20 mm in length and 2-3 mm in diameter were washed once in phosphate buffered saline (PBS), dissected into small pieces (1-2 mm^3, 2-4 mg) and cultured in 24-well plates (Costar, High Wycombe, UK) in culture medium (RPMI 1640 supplemented with 2 mM L-glutamine; 50 μg/ml streptomycin, Gibco, Paisley, UK; 20 μg/ml gentamicin and 50 IU/ml penicillin) for 24 h at 37°C in 95 % air and 5 % CO_2 under humid conditions. Tissue was treated with either (i) the NO donor spermine nonoate (50 μg/ml, Sigma) or (ii) culture medium only. A single biopsy from each patient was used for all studies. Biopsies were divided and treatments were run in quadruplicate. Supernatant was removed after 24 h and biopsies were weighed and paraffin embedded. Media were stored at - 20°C until analysis.

(ii) Cervical fibroblast cell preparation:

Cervical biopsies from non-pregnant women were washed twice in PBS and incubated in buffer (80 μg/ml gentamicin; 5 μg/ml amphotericin B (Sigma) in PBS) for 1 h at 23°C. Biopsies were digested with Dispase I (1 U/ml in PBS; Boehringer Mannheim, Lewes, UK) for 2 -3 hours at 37°C with gentle agitation then washed twice in PBS. Next, the ecto- and endo-cervical epithelium were sheared off in sheets by scraping with a scalpel and the underlying cervical stroma scraped and dissected into small pieces (1 mm^3). The stromal tissue specimens were placed into 75 mm^3 tissue culture flasks and cultured in complete medium (RPMI 1640 supplemented with 10% fetal calf serum; 2 mM L-glutamine; 50 μg/ml streptomycin, Gibco, Paisley, UK; 20 μg/ml gentamycin and 50

IU/ml penicillin) at 37°C in 95 % air and 5 % CO_2 under humid conditions. Cervical fibroblasts grew to confluence within 28 days and were used up to passage 6. For experimentation, confluent fibroblasts were plated out at 2 x 10^5 cells/ml, washed in PBS and cultured for 24 h in RPMI cultured for 24 hours in RPMI 1640 with treatments added in quadruplicate. Media were stored at -20°C until analysis. Viability was >95 % by trypan blue exclusion and >95 % of cells were positive for vimentin immunoreactivity.

(iii) Cervical biopsies from pregnant women:

Biopsies weighing around 12 mg, 3-4 mm diameter and 10-14 mm length were dissected into small pieces (1-2 mm^3, 2-4 mg) and cultured in a 24 well plate in Dulbecco's medium supplemented with streptomycin 100 μg/ml, penicillin 100 U/ml and Fungizone 100 U/ml in 5% CO_2 and 95% air for 24 h at 37°C. A single biopsy from each patient was used for all studies and divided for culture with experiments run in quadruplicate. Biopsies were weighed after treatment and paraffin embedded. Supernatant was stored in 250 μl aliquots at -20°C for subsequent analysis.

Gelatinase zymography

Gelatinase zymography was used to detect MMP-2 and MMP-9 activities as described previously (Rawdanowicz, et al., 1994; Riley, et al., 1999) with minor modifications. Gelatinase activity degrades the gelatin substrate and therefore appears as clear bands against a dark background of Coomassie blue staining. Lyophilised samples of culture medium (1ml lyophilised sample reconstituted with 50 μl 0.1% SDS, 7.5 μl per sample loaded onto gel) conditioned by cervical explants and fibroblasts were separated by SDS-polyacrylamide gel electrophoresis (PAGE; 7.5% gels; Minigel apparatus; BioRad, Hemel Hempstead, Herts) containing gelatin (1 mg/ml; Sigma) using non-reducing conditions. Gels were washed twice using 2.5% (v/v) Triton X-100 (Merk-BDH), then incubated in zymography digestion buffer (200 mM NaCl, 50 mM Tris, 5 mM $CaCl_2$, 1 mM $ZnCl_2$,

0.02% (v/v) Brij-35, pH 7.6; all Merck-BDH except Brij obtained from Sigma) for 18 h at 37°C. Gels were immersed in staining solution comprising 0.5% Coomassie blue R250 (Bio-Rad) in 30 % methanol/10 % glacial acetic acid in H_2O for 3 h at 23°C, then destained (staining solution with Coomassie blue omitted).

Reverse zymography

Reverse zymography was performed for detection of TIMPs using a commercial kit, as described previously (University Technologies Inc., Calgary, Canada; (Hampton, et al., 1995) with some minor modifications. Lyophilised cervical explant and fibroblast samples (1ml lyophilised sample reconstituted with 50 µl 0.1% SDS, 7.5 µl per sample loaded onto gel) were separated according to molecular weight by PAGE using 12% gels containing 1 mg/ml gelatin and an MMP preparation (from BHK-21 cells that constitutively express proMMP-2; University Technologies Inc.) The gels were washed (50 mM Tris, 5 mM $CaCl_2$, 2.5% (v/v) Triton X-100; for 2.5 h at 23°C), then incubated in reverse zymography digestion buffer (50 mM Tris, 5 mM $CaCl_2$) for 17 h at 37°C. The gel was counterstained (as for zymography) with staining buffer then destained to detect the presence of protein, predominantly the incorporated gelatin. The presence of TIMPs was determined by their discrete inhibition of MMP activity, which was visualised as a darker band on a lighter background. TIMPs were identified and characterised by comparison with molecular weight markers, with control standards of conditioned medium containing mouse TIMP-1, -2 and -3 expressed by transfected BHK cells (University Technologies Inc.) and human recombinant TIMP-2 (Calbiochem, Nottingham).

Western blot analysis for MMPs -2 and -9 and TIMPs -1 and -4

Cervical explant and cervical fibroblast culture media samples were dialysed (retention size >12 kDa) against water, lyophilised and reconstituted in 0.1 % SDS. Samples (20 µl) were prepared in equal volumes of sample application buffer, separated by PAGE and

transferred to a nitrocellulose membrane, pore size 0.45 μm (Bio-Rad) by wet blotting (100 V for 1 h). Gels were stained with 0.5 % Coomassie Blue to check protein transfer. Membranes were blocked with 5 % BSA in 0.05 % v/v Tween Tris Buffered Saline (TTBS) prior to antibody application. The following primary antibodies were used: MMP-2 and MMP-9 (mouse monoclonal, Insight Biotechnology, Wembley, Middlesex) at 1:1000 dilution; TIMP-1 and TIMP-4 (Affinity purified rabbit polyclonal, Insight Biotechnology) at 1:1000 dilution. These were detected using sequentially a biotinylated horse anti-mouse or goat anti-rabbit second antibody in TTBS (1:200 dilution) as appropriate and an avidin-biotinylated peroxidase complex according to the manufacturer's instructions (Vector Labs, Peterborough, UK). 3,3'-diaminobenzidine with nickel chloride enhancement was used as chromagen.

Immunocytochemistry for MMP-2 and -9 and TIMPs -1 and -4

Tissue was fixed at the time of collection in 10 % neutral buffered formalin. Paraffin embedded sections of cervix were cut to 5 μm thickness, dewaxed, rehydrated and endogenous peroxidase activity blocked in 2 % H_2O_2 (Sigma) in H_2O for 30 min at 23°C. Sections of fetal membranes obtained at elective caesarean section at term were used as positive controls. Slides were washed in 0.01 M PBS for 10 min and blocked in diluted normal horse serum or goat serum respectively (Vectastain: Vector Laboratories) for at least 30 min. Excess blocking solution was removed and slides were incubated with primary antibody (antibodies used as per Western blotting protocol) for 18 h at 4°C under humid conditions. Primary antibodies were detected using horse anti-mouse and goat anti-rabbit biotinylated secondary antibodies (Vectastain) incubated for 1 h at 23°C. Avidin-biotin complex (Vectastain) was added according to the manufacturer's instructions. Specific immunoreactivity was identified by the application of the chromagen 3,3'-diaminobenzidine (Vectastain) that produces a brown colour. Sections were counterstained with haematoxylin prior to mounting.

Data analysis

Transmission densitometry (G-700 Densitometer; BioRad) was used to quantify MMP and TIMP activity in the zymograms. Parallel background readings of equal area were obtained in order to calculate relative intensities from the zymogram gels and calculated using dedicated software (Molecular Analyst, BioRad). Densitometric readings were only compared between samples run in parallel under precisely the same conditions (i.e. same electrophoresis run, same buffers, stains and incubation periods) and were not used for comparisons between the pregnant and non-pregnant groups. Statistical analysis was performed using Student's 't'-test with $p<0.05$ regarded as significant.

Results

Production of MMP-2 and -9 by cervix

(i) Non-pregnant subjects

Zymography showed gelatinase activities corresponding to MMP-2 and MMP-9 at 72 and 92 kDa molecular weight, respectively (Fig. 5.1). Cervical tissue explants released predominantly MMP-2 (72 kDa latent pro-form, 66 kDa active form) with lesser amounts of MMP-9 activity (92 kDa latent pro-form, 86 kDa active form; Fig 5.1a). Cervical fibroblasts released only MMP-2 (Fig. 5.1b). Western blotting confirmed the presence of MMP-2 protein in non-pregnant cervical explants and in cervical fibroblasts (Fig. 5.2a). MMP-9 protein was not detected in either tissue by Western blotting (Fig. 5.2b). Localisation using immunohistochemistry showed that MMP-2 was present predominantly in the stromal connective tissue with minimal staining in blood vessels or cervical smooth muscle (Fig. 5.3a). MMP-9 was localised weakly in some perivascular cells and connective tissue stroma.

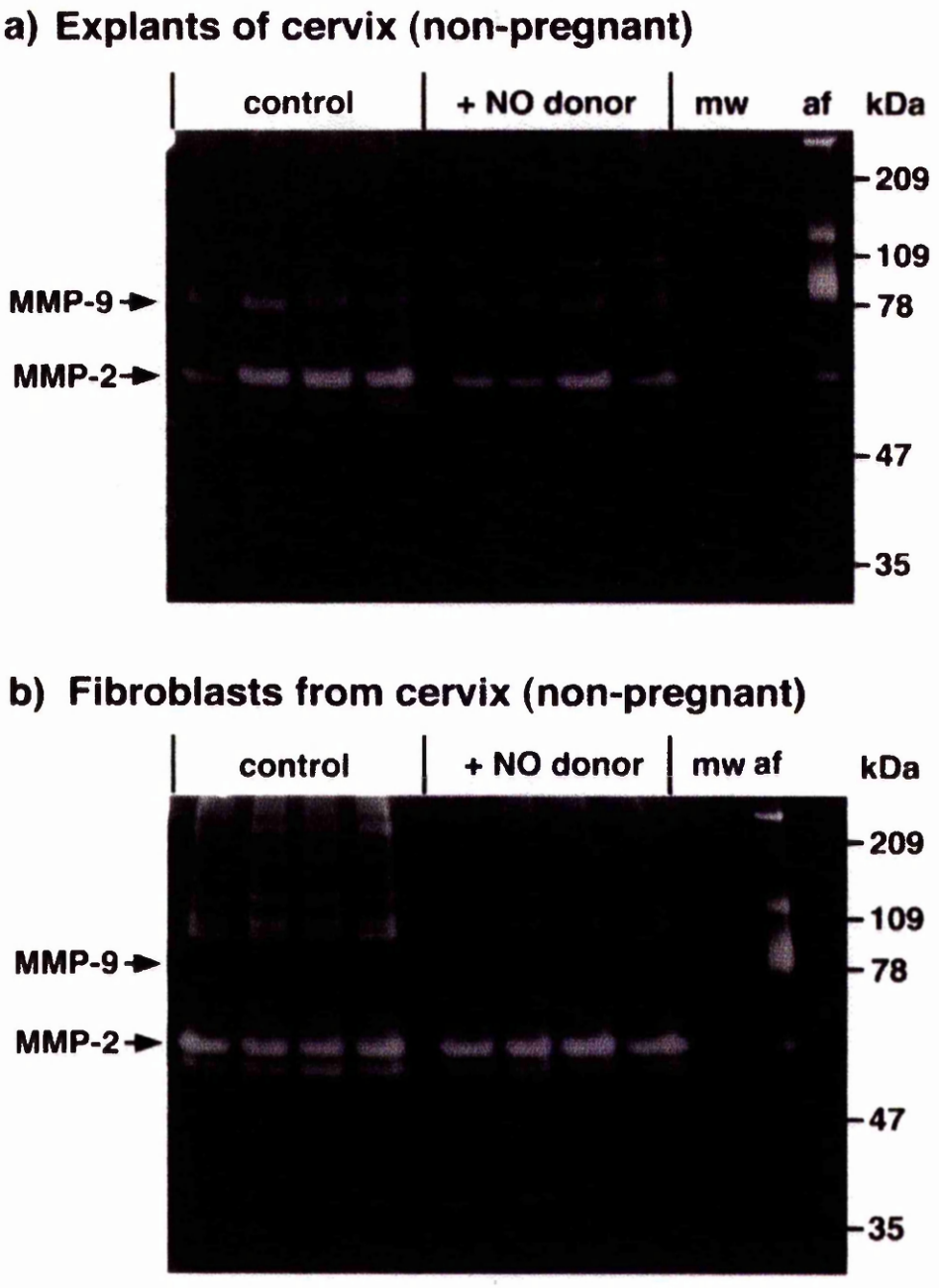


Figure 5.1. Zymography showing gelatinase activity (visualised as light bands) in culture media from (**a**) non-pregnant cervical tissue explants and (**b**) cervical fibroblasts. Matrix metalloproteinase (MMP)-2 activity is observed predominantly at 72kDa (latent pro-form) and 66 kDa (active form) in both non-pregnant explants and fibroblasts (**a,b**). MMP-9 activity is shown at 92kDa (latent pro-form) (**a**). MMP-9 activity was not detected in cultured fibroblasts (**b**). Both explants and fibroblasts were either untreated (control) or treated with NO donor (+ NO donor). mw, standard molecular weight markers (BioRad) as marked (kDa): af, amniotic fluid used as positive control.

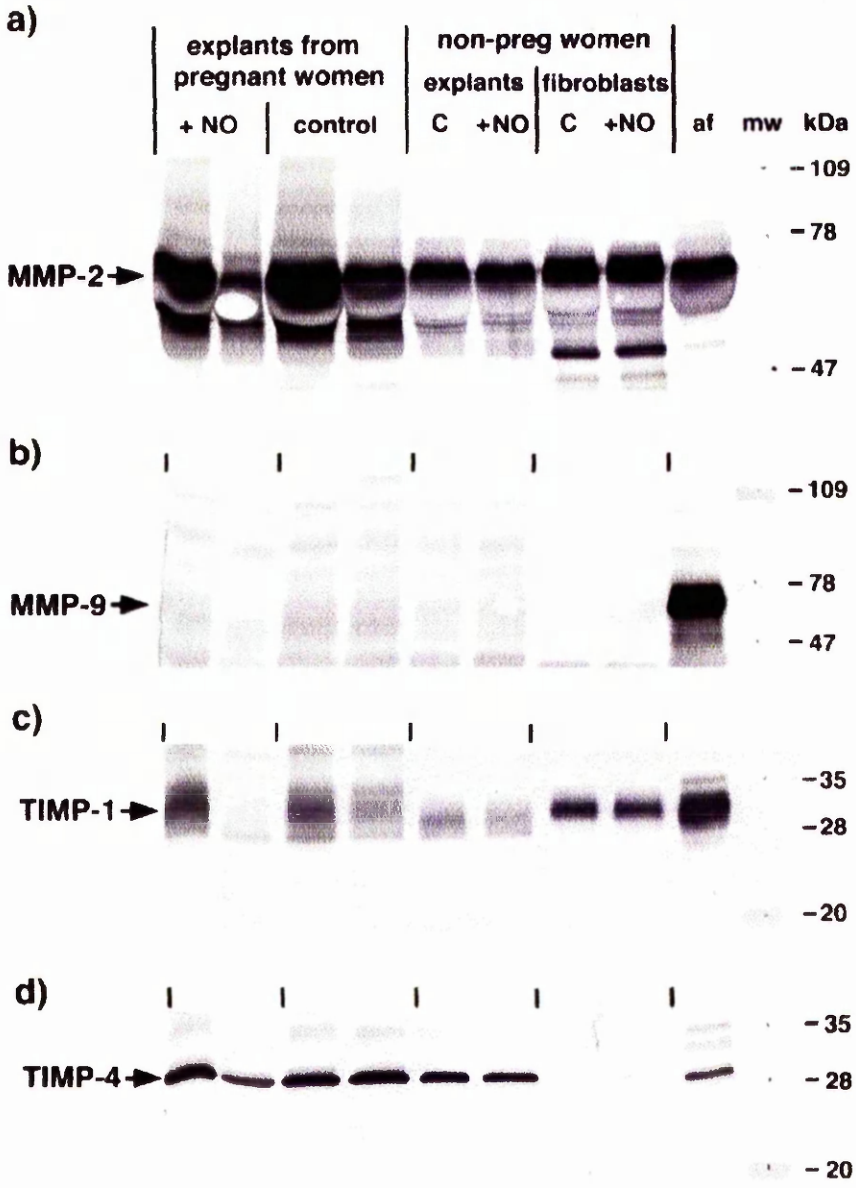


Figure 5.2. Western blot analysis on matched samples from pregnant and non-pregnant patients using antibodies to (**a**) MMP-2; (**b**) MMP-9; (**c**) TIMP-1 and (**d**) TIMP-4. MMP-2 is shown at 72 kDa (latent pro-form) and 66kDa (active form). MMP-9 is shown at 92kDa (latent pro-form) and 86kDa (active form). MMP-9 activity was not detected. TIMP-1 and TIMP-4 are shown at 27-30kDa. Samples of culture supernatant from pregnant women treated with NO donors (*in vivo*) and untreated (control) are shown. Samples from cervical explants and fibroblasts from non-pregnant women include those treated with NO donor *in vitro* (+NO) and controls (**c**). af, amniotic fluid used as a positive control; mw, molecular weight markers.

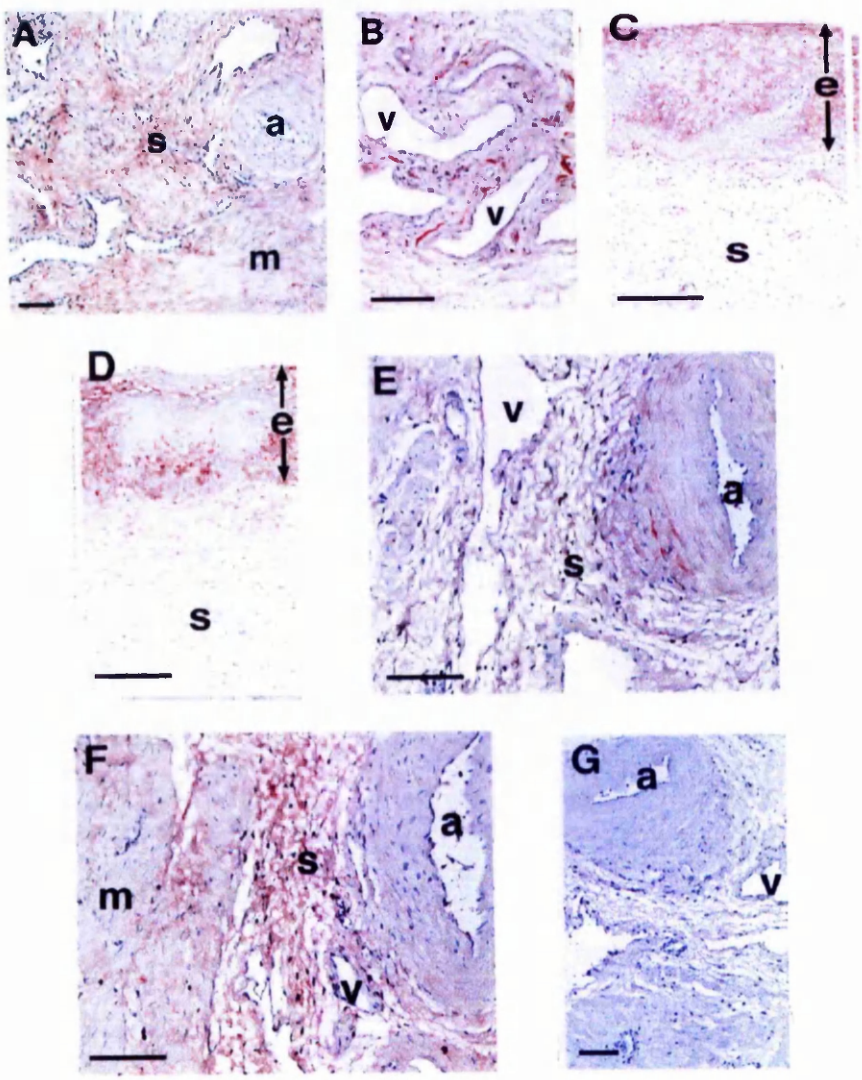


Figure 5.3. Immunohistochemical localisation in representative sections of cervical tissue of (**A**) MMP-2 (non-pregnant), (**B**) MMP-9 (non-pregnant), (**C**) MMP-2 (pregnant), (**D**) MMP-9 (pregnant), (**E**) TIMP-1 (non-pregnant), (**F**) TIMP-4 (non-pregnant), (**G**) negative (non-pregnant) control. In non-pregnant samples, MMP-2 was present mainly in stromal connective tissue with lesser amounts of staining in blood vessels and smooth muscle (**A**). MMP-9 was weakly present in perivascular cells and connective tissue stroma (**B**). TIMP-1 was localised in blood vessels and connective tissue stroma (**E**), while TIMP-4 was identified predominantly in cervical connective tissue stroma (**F**). In pregnant tissue, MMP-2 and –9 were localised in connective tissue stroma, surface epithelium and blood vessels (**C,D**). The localisation of TIMP-1 and –4 were similar to that of the non-pregnant tissue (data not shown). Key: **a**, artery; **v**, vein; **s**, stroma; **e**, epithelium; **m**, muscle. All scale bars are 100μm.

(ii) Early pregnant subjects

Zymography demonstrated that the explants released MMP-2 but not MMP-9 (Fig. 5.4). Expression of MMP-2 protein was confirmed by Western blotting (Fig. 5.2a). Immunohistochemistry localised MMP-2 and MMP-9 to the connective tissue stroma, surface epithelium and blood vessels.

(iii) Effect of NO donors

Treatment with NO donors *in vitro* or *in vivo* had no effect on release of MMP-2 and MMP-9, as characterised by zymography (Fig. 5.1), Western blot (Fig. 5.2) or immunohistochemistry (data not shown).

Production of TIMPs by cervix

(i) Non-pregnant subjects

Reverse zymography detected TIMP secretion by non-pregnant cervical tissue and fibroblasts (Fig. 5.5). A band representing the 27-30 kDa TIMPs (which may include TIMP-1, -4 and the glycosylated form of TIMP-3) can be seen in both explant and fibroblast groups. Western blotting was performed to further delineate the components of the 27-30 kDa band obtained by reverse zymography (Fig. 5.2). This was unlikely to consist of TIMP-3 since no discrete banding pattern had been obtained on zymography for the unglycosylated 28 kDa form. Analysis for TIMPs -1 and -4 therefore was performed by Western blotting which confirmed the presence of both proteins in the conditioned medium from cervical tissue explants of cervical biopsies from non-pregnant women (Fig. 5.2). Cervical fibroblasts produced TIMP-1 but not TIMP-4 (Fig. 5.2c and d). TIMP-1 was localised to blood vessels and connective tissue stroma (Fig. 5.3e). TIMP-4 was present predominately in the cervical connective tissue stromal cells (Fig 5.3f).

(ii) Early pregnant subjects

Reverse zymography showed a similar pattern of secretion of TIMPs by the cervical tissue explants of women in early pregnancy as that described for the non-pregnant group (Fig.

5.6). Cervical tissue released mainly TIMPs in the 27-30 kDa range. Smaller amounts of 21 kDa TIMP-2 were detected. Western blotting confirmed that the banding pattern in the 27-30 kDa range on zymography corresponded to both TIMP -1 and -4 protein (Fig. 5.2). The localisation of TIMP-1 and TIMP-4 were similar to the non-pregnant (data not shown).

(iii) Effect of a NO donor

In vitro and *in vivo* treatment with a NO donor in biopsies from non-pregnant and pregnant women had no effect on TIMP activity as confirmed by reverse zymography (Fig.5.5a and b), Western blot (Fig 5.2c and d) or immunohistochemistry (Fig. 5.3c and d).

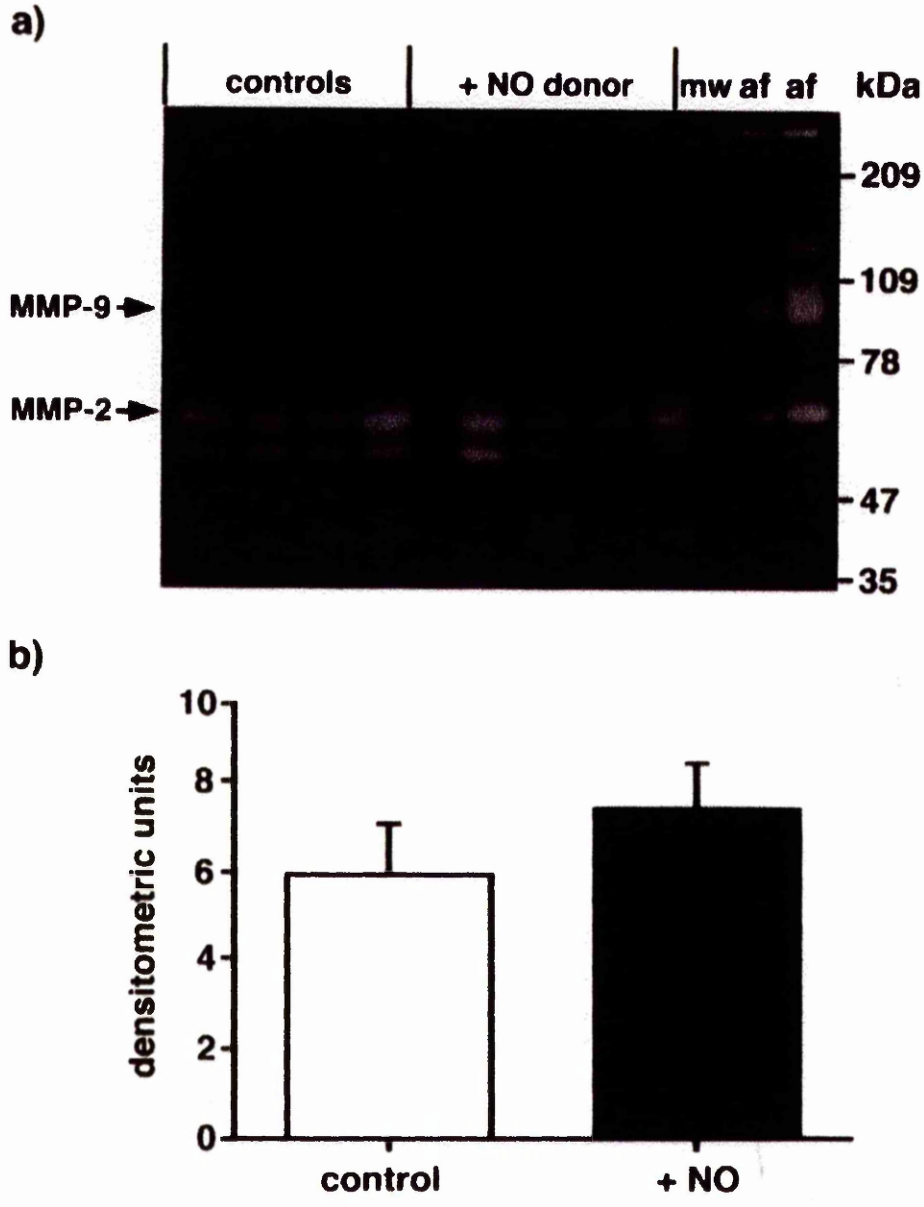


Figure 5.4. (**a**) Zymography showing gelatinase activity in supernatant from culture of matched samples of pregnant cervical tissue explants untreated (control) and treated *in vivo* with NO donor (+ NO). Matrix metalloproteinase (MMP)-2 activity is observed predominantly at 72kDa (latent pro-form) and 66 kDa (active form). MMP-9 activity is shown predominantly 92 kDa (latent pro-form) and at 86 kDa (active form). mw-molecular weight markers as marked (BioRad); af-amniotic fluid used as positive control.

(**b**) MMP-2 activity in pregnant cervical tissues in control and samples treated *in vivo* with NO donor, assessed using densitometry. There was no significant difference in MMP-2 activity between the groups. Values shown are mean + SEM.

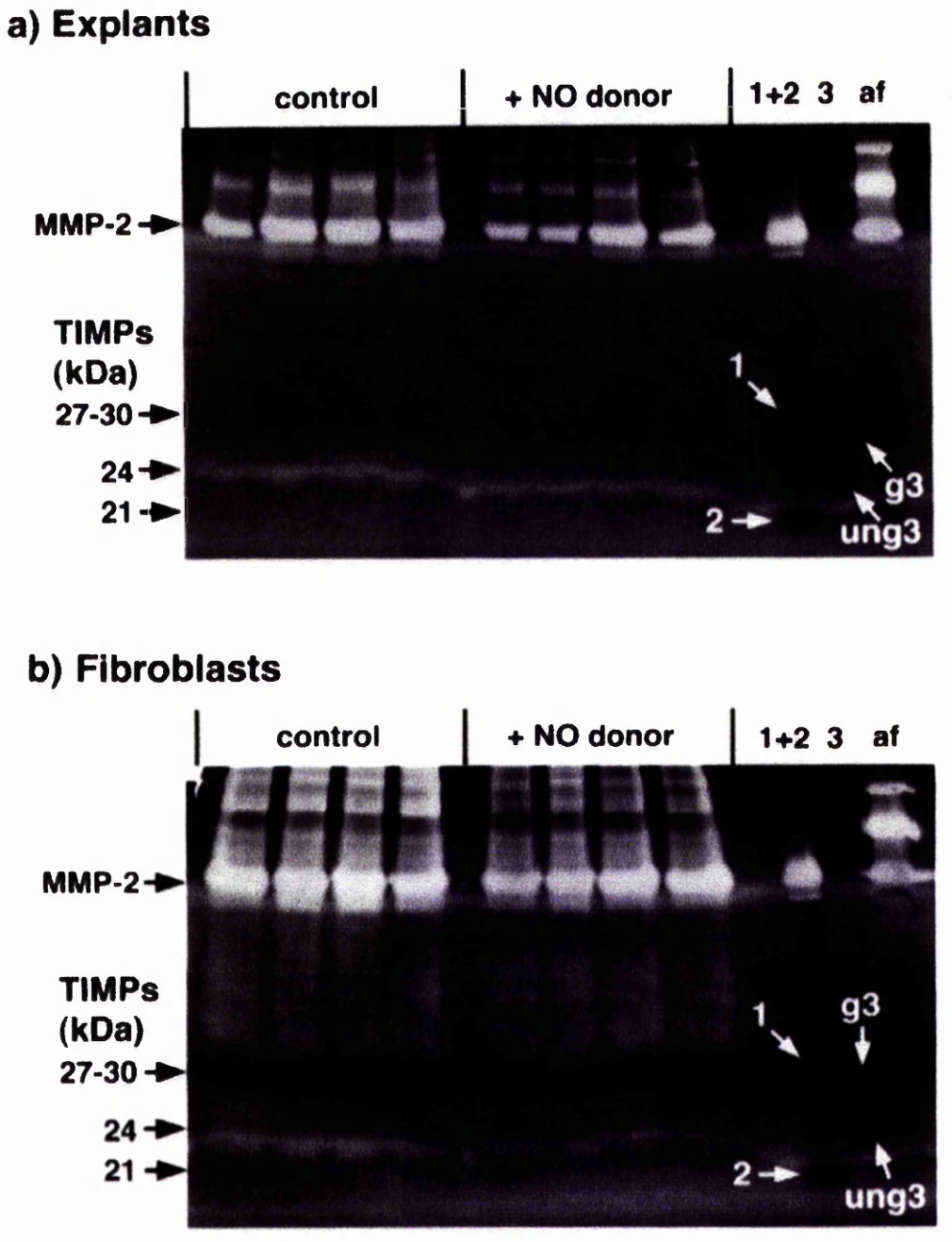


Figure 5.5. Reverse zymography to detect TIMP (visualised as dark bands) in supernatant from matched samples of (a) cervical explants from non-pregnant tissue, and (b) cervical fibroblasts either untreated (control) or NO donor treated (+ NO). Standards of TIMP 1 and 2 (1 + 2; separately identified on the gel by arrows) and of TIMP 3 (3; identified on the gel in the 27-30 kDa glycosylated form [g3] and 24 kDa unglycosylated form [ung3]). af = amniotic fluid used as a control.

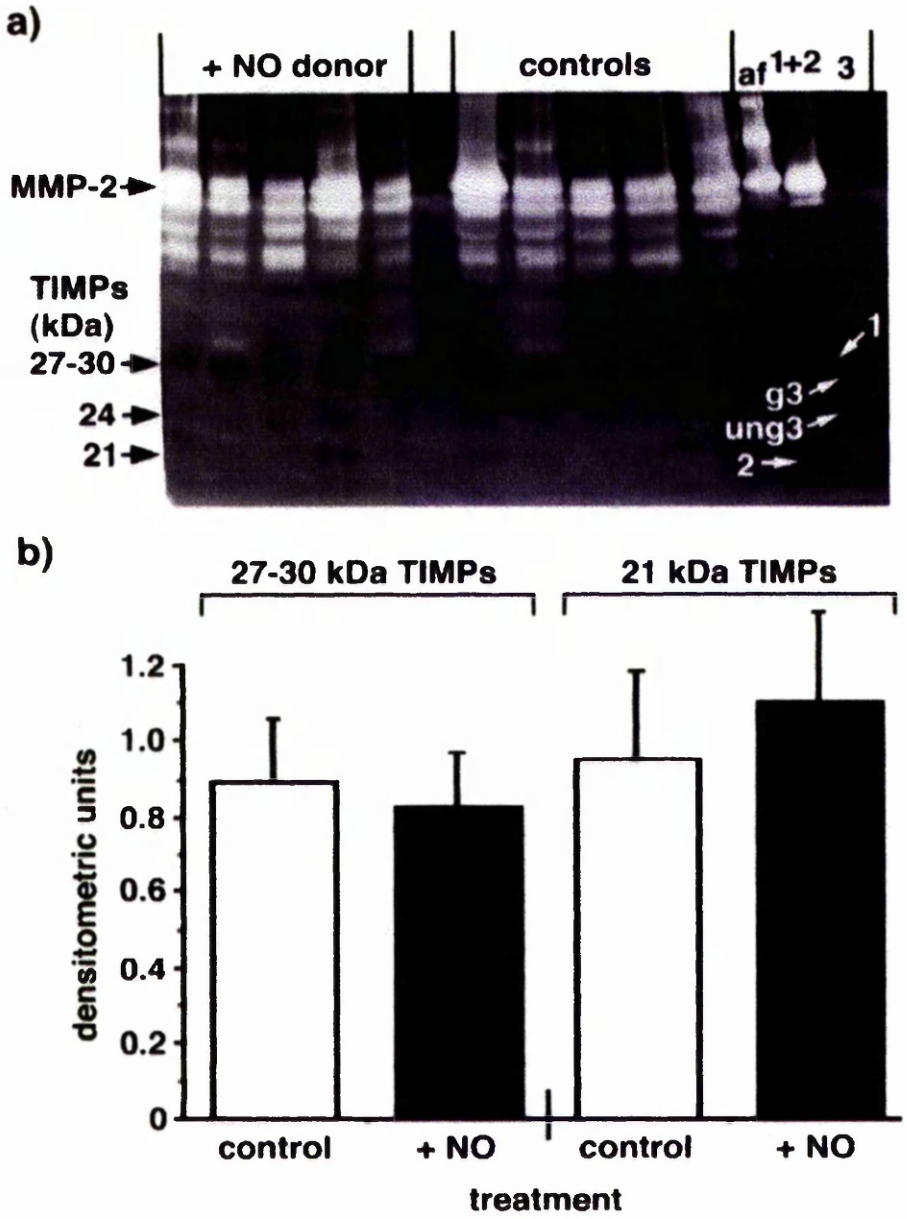


Figure 5.6 (a) Reverse zymography to detect TIMPS in culture supernatant from culture of pregnant cervical tissue explants either untreated (control) or treated with NO donor (+NO donor). Standards of TIMPS 1 and 2 (1+2; separately identified on the gel by arrows) and of TIMP 3 (3; identified on the gel in the 27-30 kDa glycosylated form [g3] and 24 kDa unglycosylated form [ung3]). af = amniotic fluid, used as a control.

(b) TIMP activity (mean and standard error of the mean) in control and NO treated pregnant cervical tissues assessed using densitometry. The TIMPs of mw 27-30 kDa represent TIMPs 1 and 4, the 21 kDa TIMP is TIMP-2. There was no significant difference in TIMPs when NO treated and control tissues were compared.

Discussion

We have demonstrated that MMPs -2 and -9 and TIMPs -1, - 2 and -4 are present in both the pregnant and non-pregnant human uterine cervix. These MMPs and their endogenous inhibitors may play a role in the normal turnover of the ECM type IV collagen, elastin and fibronectin in the cervix. Our results are supported by that of Agarwal *et al.*, (Agarwal, et al., 1994) who have previously demonstrated the presence of MMPs -2 and -9 in cultured non-pregnant endocervical cells by zymography and Western blotting. MMP-9 activity has also been shown to be present in the lower uterine segment, considered by some to represent the uterine cervix, and to increase along with MMP-8 activity during labour (Osmers, et al., 1995).

These studies did not reveal any differences in secretion of MMP-2 and TIMPs -1, -2 and -4 in the cervix from pregnant and non-pregnant women. The activity of MMP-9 present was lower in the cervix of the pregnant compared with the non-pregnant subjects. This decline in MMP-9 may be related to the increase in circulating progesterone levels during pregnancy. Physiological concentrations of progesterone suppress IL-1 and PMA mediated production of proMMP-9 at the transcriptional level in rabbit cervical fibroblasts (Imada, et al., 1997) and a suppressive effect of progesterone on MMP-9 secretion has also been shown in the endometrium (Marbaix, et al., 1992; Rodgers, et al., 1994). The reduction in activity of MMP-9 during early pregnancy in the human cervix may be a protective effect mediated by progesterone, to prevent premature cervical ripening (Salamonsen, 1996). Indeed, inhibition of progesterone stimulates cervical ripening as shown by studies using anti-progestogens (Leppert, 1995; Rechberger, et al., 1996).

MMP -9 was localised in the cervix of pregnant women by immunocytochemistry although

its secretion was not detected by zymography. This may be explained by the fact that samples of supernatant used for zymography contained very small quantities of MMP-9 which were below the detection limits of this method and which were therefore substantially less than the levels of MMP-9 present in the non-pregnant state.

We did not detect MMP-9 secretion by cervical fibroblasts in culture. This suggests that these cells are not the source of MMP-9 in the non-pregnant human cervix. These data are supported by studies in rabbits where cervical fibroblasts in cell culture did not produce the gelatin degrading enzyme corresponding to proMMP-9 (Imada, et al., 1997). In normal cycling human endometrium MMP-9 protein has been localised to eosinophils, neutrophils and monocyte-macrophages by immunohistochemistry (Jesiorska, et al., 1996). It is probable, therefore, that bone marrow derived cells and not fibroblasts are the source of MMP-9 located in the non-pregnant cervix. In this study we used zymography as a semi-quantitative measure of the amount of MMP and TIMP activity in our samples. We did not attempt to quantitate the protein content of the samples used for this process although tissue samples were all of approximately similar weights (2-4 mg). An alternative explanation for the lack of MMP-9 secretion seen in the cervical fibroblast group may therefore be that the amount of protein present in the fibroblast preparations was considerably less than that in the equivalent whole tissue culture sample.

We used the effects of the potent vasodilator NO on the cervix as a model for cervical ripening in our study. NO stimulates cervical ripening in both humans and animals (Ali, et al., 1997; Buhimschi, et al., 1996; Chwalisz, et al., 1996; Chwalisz, et al., 1997; Thomson, et al., 1997) and inhibits human cervical contractile activity (Ekerhovd, et al., 1998). Previous studies using the NO donor IMN in the first trimester of pregnancy demonstrated a reduction in the cumulative force required to dilate the cervix prior to suction termination

of pregnancy (Thomson, et al., 1997). This is an established objective method of measuring cervical ripening (El Refaey, et al., 1994; Norman, et al., 1991; Henshaw and Templeton, 1991). Since we had used the same NO donor, IMN, under the same experimental conditions as in our previous clinical studies, we were confident that the tissue obtained from pregnant women in these studies represented that from a ripened cervix.

It has been postulated that cervical ripening may be mediated by the NO and prostaglandin pathways acting in concert (Chwalisz, 1988), although the mechanism of NO induced cervical ripening remains unclear. NO has powerful vasodilatory properties as well as the ability to function as an immune regulator and inflammatory mediator (Moncada, 1997). Cervical ripening involves macrophage and neutrophil invasion of the cervix with release of inflammatory mediators and activation of MMPs capable of degrading the extracellular matrix. The activity of MMPs can be modulated by NO and its reactive metabolites via interactions with zinc and calcium residues (Drapier and Bouton, 1996). Thus, it would seem reasonable that cervical ripening mediated by NO would involve activation of MMPs. However the NO donors used in our study, isosorbide mononitrate and spermine nonoate, had no effect on activity or protein expression of MMPs-2 or 9 or TIMPs -1, -2 and -4 in the human uterine cervix when given *in vitro* to non-pregnant subjects or when given *in vivo* to pregnant subjects. Our findings may reflect the effects of using different NO donors compared to those investigated previously. Alternatively, other MMPs may be stimulated by NO during physiological cervical ripening in humans. The collagenases MMP-1 and MMP-8 may be activated by NO (Murrell, et al., 1995) and MMP-8 activity has been shown to be increased in the lower uterine segment during labour and delivery (Osmers, et al., 1995; Osmers, et al., 1995). Activation of proMMP-1 requires the

secretion of MMP-3 (Ito, et al., 1988) and cervical change may therefore also be dependent upon the presence of this MMP.

In summary therefore, the data presented in this chapter has shown that MMP-2 and -9 and TIMPs -1, -2 and -4 are active in the human uterine cervix in the non-pregnant and early pregnant state. Cervical ripening mediated by NO does not affect the expression of these mediators. It should be emphasised that these studies have been done using an *in vitro* system and may therefore not take account of the effects of other endogenous mediators acting via a cascade mechanism. Remodelling of the cervix during pregnancy is likely to involve MMPs regulated by a complex interaction between progesterone, cytokines, prostaglandins and NO. The interaction between NO, prostaglandins and various inflammatory cytokines in the process of cervical ripening is evaluated in the next chapter of this thesis.

Chapter 6

The effect of nitric oxide donors on prostaglandin and cytokine expression in the human cervix during the first trimester of pregnancy

Introduction

A wide variety of mediators have been implicated in the control of cervical ripening, including prostaglandins and various inflammatory cytokines. Through observation of the effects of various antiprogestins in the cervix, it is clear that progesterone is also fundamentally involved in the hormonal regulation of these events (Chwalisz, 1994; Chwalisz and Garfield, 1994). There is evidence that various cytokines are also involved. IL-8, a C-X-C chemokine, has been shown *in vivo* (Sennstrom, et al., 1997) and *in vitro* (Barclay, et al., 1993) to be produced in the cervix and to be capable of causing ripening when artificially applied to the cervix. IL-1 can induce cervical ripening in animal models (El Maradny, et al., 1995) and its mechanism of action may involve the co-induction of IL-8 (Uchiyama, et al., 1992). Other cytokines, such as TNFα (Chwalisz, et al., 1994) may act in concert with IL-6 to facilitate neutrophil chemotaxis, IL-1 gene expression and endothelial adhesion molecule upregulation (Rees, 1992) during this process.

Prostaglandins (PGs) were previously thought to be the final common mediators of cervical ripening. Prostaglandin synthesis is controlled by the enzyme cyclooxygenase (COX) which converts arachadonic acid to the prostaglandins (PG), prostacyclin (PGI_2) and thromboxane A_2 (TXA_2). COX-1 is the constitutive form of the enzyme while COX-2 can be induced by a number of other mediators including proinflammatory cytokines and growth factors (DeWitt, 1991). PGE_2 and $PGF_{2\alpha}$ have both been used to artificially

mediate cervical ripening in the first trimester of pregnancy and at term (Calder, 1990; Neilson, et al., 1983). However other agents must also be fundamental to this process since the ripening action of antiprogestins in the cervix cannot be blocked by the use of indomethacin (Radestad and Bygdeman, 1992) or the specific cyclooxygenase (COX)-2 inhibitor, flosulide (Shi, et al., 1996). Candidate agents for cervical ripening include inflammatory cytokines and NO.

The mechanism of action of NO in the inflammatory cervical ripening process remains unknown. NO has been shown to stimulate prostaglandin production via induction of COX-2 (Salvemini, et al., 1993; Sautebin, et al., 1994) and also cytokine release (Brady, et al., 1998; Cuthbertson, et al., 1998) possibly through activation of the transcription factor Nuclear Factor Kappa B (NF-κ B) (Nathan, 1992; Umansky, et al., 1988).

The purpose of this study therefore was to test the hypothesis that NO mediates cervical ripening as part of an inflammatory reaction and that it does so via induction of a variety of inflammatory cytokines and prostaglandins. We also attempted to compare the effects of NO on the production of cytokines and prostaglandins with that of other known mediators of cervical ripening.

Materials and methods

All studies were approved by the local research ethics committees and written informed consent obtained from each woman prior to surgery.

Subjects

(i) Pregnant women

Healthy women in the first trimester of pregnancy (7-12 weeks gestation, age17-41 years, mean age 28, n=20) undergoing suction termination of pregnancy were recruited to the study. Women were randomised into 2 groups and treated with either:

(i) 40 mg isosorbide mononitrate (IMN) Tablet (Schwarz Pharma Ltd., East Sussex, Chesham, Bucks, England), an NO donor, per vaginam 2-3 h prior to surgery (n=10)

(ii) no treatment (controls, n=10).

Biopsies were taken from the anterior lip of the cervix using a 6 mm biopsy needle (Stiefel Laboratories, Wooburn Green, Bucks, UK) under general anaesthetic after evacuation of the uterus. Tissue was immediately transferred into Dulbecco's medium for transport. All reagents were from Sigma, Poole, UK unless otherwise stated.

(ii) Non-pregnant women

Non-pregnant healthy pre menopausal women undergoing hysterectomy for benign disease (ages 34-49, mean age 41, n=10) were recruited to the study. A longitudinal section of the anterior lip of the cervix was taken using a scalpel following removal of the uterus. Biopsies were placed immediately in Dulbecco's medium for transport to the laboratory.

Tissue Culture

(i) Cervical biopsies from pregnant women:

Biopsies (12 mg weight, 3-4 mm diameter and 10-14 mm length) were dissected into 14-15 small pieces (1-2 mm^3) and cultured in a 24 well plate in 1.5 mls Dulbecco's medium supplemented with streptomycin 100 µg/ml, penicillin 100 U/ml and fungizone 100 U/ml in 5% CO_2 and 95% air for 24 h at 37°C. Biopsies were weighed after treatment and tissue was either snap frozen in liquid nitrogen and stored at –80°C, or formalin fixed and paraffin embedded. Culture media were divided in two portions and either frozen in 250 µl

aliquots at –20°C or treated with methyloximating solution (0.1M methoxylamine hydrochloride in 10% alcohol diluted in 1M sodium acetate, pH 5.6) prior to freezing.

(ii) Cervical biopsies from non-pregnant women:

Biopsies (20 to 35 mg weight, 15-20 mm length and 2-3 mm diameter) were dissected into small pieces (1-2 mm^3) and cultured in 24-well plates (Costar, High Wycombe, UK) in Dulbecco's medium as previously described. Explants were treated with one of the following: (i) Medroxyprogesterone acetate 10^{-6} M, (ii) Medroxyprogesterone acetate (MPA) 10^{-6} M with Mifepristone 175ng/ml (Roussel Uclaf, Cedex, France) PGE_1 1.0 μg/ml, (iii) Lipopolysaccharide (LPS) 1.0 μg/ml with interferon γ (IFN γ) 60 U/ml, (iv) the nitric oxide donor S-nitroso–N-acetyl-D, L-penicillamine (SNAP) at 100 μM or (v) SNAP at 200 μM. Experiments were run in triplicate, cultured and stored as previously described.

ELISA assays

IL-1β assay

96 well plates (Costar, High Wycombe, UK) were passively coated overnight at 4°C with 4 μg/ml IL-1β capture antibody (R&D Systems, Abingdon, Oxon, UK; diluted in phosphate buffered saline (PBS), pH 7.2). Plates were washed after incubation in cold water, coating solution added (Polyvinyl pyrollidone 2%, BSA 5 mg/ml, preservatives [(1mM 2-methylisothiazolone and 1mM bromonitrodioxane) Boehringer Mannheim UK Ltd., Lewes, East Sussex, UK; 0.1%, (EDTA 5mM, Tris 50mM)] at 100 μl /well for 30 mins, plates were then rewashed, air dried and stored at 4 °C. Plates were washed once in cold water prior to adding standards (diluted in ELISA buffer [10mM TRIS pH 7.2, preservatives, BSA 2mg/ml, 300 μl 0.5% Phenol Red solution/l, Na Cl 9 g/l, EDTA 2mM, Tween-20 0.05% to final pH 7.2]) and added at 100 μl/well with 250 pg/well as top standard. Samples were added (undiluted - 100 μl / well) and incubated overnight at 4°C.

After washing x4 in wash buffer (0.05% Tween-20, 9g/l NaCl, 100 mM tris, pH 7-7.5) detection antibody (25 ng/ml) was added (100 μl /well) and plates were incubated on an orbital shaker (1.5 hrs at 23°C) then washed x4 as before. Streptavidin peroxidase (Boerhinger Mannheim) was then added at 0.2U/ml and plates were incubated at room temperature for 30 mins. Plates were washed again and 100μl tetramethyl benzidine (TMB) substrate added to each well. Plates were left for 20 mins before quenching with 50μl 2N sulphuric acid and were read at 450 nm within 30 mins of quenching.

IL-8 assay

IL-8 ELISA was performed as previously described (Denison, et al., 1999) using paired capture and biotinylated labelled detection antibodies. Capture antibody was used at 4µg/ml with 100 μl /well and detection antibody at 30ng/ml (both R & D Systems). Standards were donated from Toray Industries Inc, Tokyo, Japan with 500pg/well as top standard. Streptavidin peroxidase was added to each well at 0.2U/ml and antibody binding was detected using TMB as substrate. Detection limit of the assay was 15pg/ml. The intra- and inter-assay coefficients were 9.1% and 11% respectively.

IL-6 assay

A similar protocol was followed for the detection of IL-6 with the use of capture and biotinylated secondary antibodies. Capture antibody was used at 4µg/ml and detection antibody at 50 ng/ml. Recombinant standards (R&D Systems) and samples were added to wells with 250 pg/ml as top standard. Plates were read and detected as before.

MCP-1 assay

MCP-1 ELISA was as previously described (Denison, et al., 1997). Capture antibody (gifted from Toray) was used at 4 μg/ml and peroxidase coupled detection antibody added at 60 μl/well. Top standard was 500 pg/well. Plates were read and detected as before. The intra- and inter-assay coefficients were 6.3% and 8.6% respectively. Detection limit of the assay was 7.5 pg/ml.

IL-10 assay

IL-10 assay was performed as previously described (Denison, et al., 1999). Capture antibody (Pharmingen, Sandiego, USA) was used at 200 ng/ml and detection antibody at 125ng/ml. Recombinant standards (Pharmingen, Sandiego, USA) were added with 500 pg/ml as top standard. Poly-peroxidase (CLB Laboratories, Amsterdam, Holland) was used at 1 ng/ml in ELISA buffer and plates read and detected as before. The intra- and inter-assay coefficients were 6.4% and 10.1% respectively. Detection limit was 15 pg/ml.

IL-15 assay

Anti-human IL-15 capture antibody (R&D Systems) was used diluted in 0.1M $NaHCO_3$ pH 8.4 and incubated overnight at 4 °C. Capture antibody was removed, plates were blocked (10% FCS in PBS at 200 μl /well at 37 °C for 2 hrs) washed (x2 in PBS/Tween) and standards [diluted in 10% FCS in PBS with 1.5pg/ml as top standard (Gifted by Dr A Gracie, Dept of Medicine, Glasgow Royal Infirmary)] and samples added (100 μl /well). Plates were incubated (37°C for 2 hrs) washed x4 as before and detection antibody added [(R&D Systems); diluted at 200 ng/ml; 100 μl /well and incubated at 37°C for 2 hrs]. Plates were washed x6 and extravidin-peroxidase (SAPU 1/1000) diluted in 10% FCS in PBS added at 100 μl /well. Plates were detected and read as described previously.

TNF-α

Paired capture (4 μg/ml) and detection antibodies [(100 ng/ml); both R&D Systems)] were used to detect bound standards and samples. Standards (R&D Systems) were added with 5000pg/well as top standard.

PGE2 Assay

PGE2 assay was performed as previously described (Denison, et al., 1999). The intra- and inter-assay coefficients were 7.8% and 15 % respectively and the ED50 was 195 pg/ml.

PGEM assay

A similar protocol was used to detect PGEM. Peroxidase conjugated PGEM was added at 1 in 50,000 diluted in ELISA buffer and anti-sera at 1.0 $x10^5$ in assay buffer. Standard range of the assay was 1280pg/ml to 2.5 pg/ml. Methyloximating solution was present in all standards and samples at a final concentration of 12.5%. ED50 was 220 pg/ml.

6–OXO-$PGF_2\alpha$

6–OXO-$PGF_2\alpha$ was detected using a similar protocol. Peroxidase conjugate was added at 1 in 2.0 x 10^5 and antisera added at 1 in 10, 000. The standard range of the assay was 10240 pg/ml to 5 pg/ml. 25% methyloximating solution was present in all samples and standards.

Thromboxane B_2

Assay was performed using the same protocol. Peroxidase conjugate was used at 1 in 1.25 x 10^5 and antisera at 1 in 25, 000. Standard range of the assay was 327.7 ng/ml to 0.04 ng/ml. Methyloximating solution was present in all standards and samples at a final concentration of 12.5%.

$PGF_2\alpha$

Peroxidase conjugated $PGF_2\alpha$ was added at 1 in 1.0 x 10^6 and antisera at 1 in 20,000. Standard range of the assay was 5120 to 10 pg/ml. ED50 was 220 pg/ml.

PGFM

Peroxidase conjugated PGFM was added at a concentration of 1 in 40, 000 and antiserum at 1 in 50,000 diluted in ELISA buffer. The standard range of the assay was 327.7 ng/ml to 0.04 ng/ml.

RNA extraction

Total RNA was isolated from cervical tissue explants using an adaptation of the method of Slater *et al.,* (Slater, *et al.*, 1995). Briefly, 1ml trizol (Gibco Life Technologies, Paisley, UK) was added to tissue samples and incubated overnight. RNA was isopropyl alcohol-chloroform (BDH, Glasgow, UK) precipitated and the supernatant removed. The pelleted RNA was washed in 75% ethanol and resuspended in DEPC-treated water. The RNA yield was determined spectrophotometrically at 260/280nm.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Reverse transcription was used to identify expression of COX-1 and COX-2 in cervical tissue explants by an adaptation of the method of Slater *et al.,* (Slater, et al., 1995). 3 μg total RNA was reverse transcribed into cDNA using Superscript II reverse transcriptase (Gibco Life Technologies) in 20μl of reaction buffer [(10xPCR buffer, 25μM $MgCl_2$, 0.1M DTT (Gibco Life Technologies), 10 mM dNTP and 50ng/ml random hexamers (Boehringer Mannheim)]. 5 μl cDNA was used for PCR amplification with either COX-1, COX-2 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (Gibco). Primer sequences were: COX-1 (sense) 5'- TGCCCAGCTCCTGGCCCGCCGCTT-3', COX-1

(antisense) 5'-CCATGGCCCAAGGCCTTG-3' (Slater, *et al.*, 1998); COX-2 (sense) 5'-TTCAAATGAGATTGTGGGAAAATTGCT-3', COX-2 (antisense) 5'-CCACCCATGGCAAATTCCATGGCA-3' (Iniguez, et al., 1998); GAPDH (sense) 5'-CCACCCATGGCAAATTCCATGGCA-3',5'-TCTAGACGGCAGGTCAGGTCCACC-3' (Slater, et al., 1998). PCR was performed in a 50 µl volume of reaction buffer containing 10xPCR buffer, 25mM $MgCl_2$, 2mM dNTPs, 1.3 μl primer 1, 1.0 μl primer 2, 5% dimethyl sulphoxide (DMSO) and 0.1μl taq polymerase (Gibco Life Technologies). The reaction was amplified by 35 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. Products were run on agarose gels and bands visualised using ethidium bromide.

Immunohistochemistry

Immunohistochemistry protocols for the detection of IL-1β, IL-6, IL-10, IL-15, MCP-1, TNFα, COX-1, COX-2, and prostaglandin dehydrogenase (PGDH) were established to determine the correct conditions for optimal staining (Table 6.1). PGDH is a nicotinamide adenine dinucleotide (NAD^+)-dependent 15-hydroxy-prostaglandin dehydrogenase responsible for prostaglandin metabolism.

Table 6.1.

Antigen	Clone	Pretreatment	Dilution	Supplier
COX-1	monoclonal	nil	1/750	Cayman Chemical
COX-2	AA 585-604	nil	1/750	Santa Cruz
PGDH	monoclonal	nil	1/1500	Cayman Chemical
IL-1β	rhIL-1β	microwave	1/50	R&D Systems
IL-6	rhIL-6	microwave	1/150	R&D Systems
IL-8	natural IL-8, rhIL-8	microwave	1/25	R&D Systems
IL-10	rhIL-10	microwave	1/350	R&D Systems
IL-15	monoclonal	microwave	1/150	R&D Systems
TNFα	rhTNFα	microwave	1/150	R&D Systems
MCP-1	77AA MCP-1	NBF	1/400	Gifted

COX-2, IL-1β, IL-6, IL-8, IL-10 and TNFα.

Sections 5μm thick were cut from paraffin embedded cervical samples and mounted on silane coated slides, heated to 60°C for 35 min, deparaffinised in xylene and rehydrated in graded alcohol series. Sections were placed in 0.5% hydrogen peroxide in methanol to block endogenous peroxide activity. If required, sections were pre-treated to retrieve the antigen by microwaving at full power for 5 mins in 0.01M citrate buffer pH 6.0. Sections were washed in PBS (PBS +0.1% saponin for microwaved sections) then blocked in 20%

rabbit serum with 20% human serum for 30 mins at room temperature. Slides were then incubated overnight at 4°C with the primary antibody diluted in 2% normal rabbit serum with 5% normal human serum. Sections were washed in PBS (+/- 0.1% saponin) before incubation with biotinylated anti-goat (Vector Laboratories) diluted 1:200 in 2% normal rabbit serum with 5% normal human serum added. The sections were washed as before in PBS (+/- 0.1% saponin) then incubated for 30 mins with avidin/ biotin horseradish peroxidase reagent (Vector Laboratories, Peterborough, UK) in PBS before final washing. The antigens were localised by incubating slides for 10 mins with 1mg/ml diaminobenzidene tetrahydrochloride (DAB), 0.02% H_2O_2 in 50mM Tris HCl, pH 7.6 and appeared as a brown end product. Sections were then counterstained with Harris haematoxylin.

Negative controls included sections incubated without the primary antibody. Kidney and endometium (Jones, et al., 1997) were used as positive controls for COX-2 and tonsillar tissue was used as a positive control for IL-1β, IL-6, IL-8, IL-10 and TNFα. To assess the specificity of the staining for COX-2 and TNFα representative slides were included where the primary antibody was preabsorbed with the appropriate peptide (COX-2 blocking peptide from Santa Cruz Biotechnology, sc-1745P; recombinant human TNFα from R&D Systems, 210-TA-010) as per the method of Van Noorden (Van Noorden, 1993).

IL-15, COX-1 and PGDH

Paraffin embedded sections were prepared as before and pretreated in order to retrieve the antigen if necessary (Table 6.1). Sections were then preincubated in 20% goat serum and 20% human serum for 30 mins at room temperature. They were then incubated with the appropriate monoclonal antibody diluted in 2% normal goat serum in PBS (+/- saponin) with 5% human serum added and left overnight at 4°C. Primary antibody was omitted from the negative control slides. Sections were then washed in PBS (+/- saponin) and incubated

with biotinylated goat anti-mouse (Dako) diluted 1/200 in 2% normal goat serum in PBS (+/- saponin) with 5% normal human serum added for 30 mins at room temperature in a humidified box. Sections were washed and incubated again with streptavidin peroxidase (Dako, Cambridge, UK) diluted 1/400 in PBS (+/- saponin) before washing and final treatment with DAB as before.

MCP-1

MCP-1 was localised in frozen tissue sections as described previously (Jones, et al., 1997). Tonsillar tissue was used as positive control and negative control slides were set up with either no primary antibody or non-immune rabbit IgG.

Analysis

Statistical analysis of IL-1β, IL-6, IL-8, IL-10, IL-15, MCP-1, TNFα, $PGF_{2\alpha}$, PGE_2, PGFM, PGEM, 6-OXO-$PGF_{2\alpha}$ and TXB_2 levels in culture media was performed using analysis of variance (Statview SE + Graphics v.1.04, Abacus Inc; Berkley, CA, USA). Significance was determined using Scheffe's F-test as a post-hoc test.

Results are expressed as mean level pg/ml $\pm$ s.e.m with $p<0.05$ taken to indicate significance.

Results

Effect of NO donors on pregnant first trimester human cervix:

(i) IL-1β, IL-6, IL-8, IL-10, IL-15, MCP-1, TNFα release:

Tissue explants of first trimester cervix released IL-6, IL-8, IL-10, IL-15, MCP-1 and TNFα (Figure 6.1). IL-1β was not released. *In vivo* treatment with the NO donor isosorbide mononitrate did not significantly alter the release of these cytokines from the first trimester cervix in culture.

(ii) Immunohistochemistry for IL-1β, IL-6, IL-8, IL-10, IL-15, MCP-1, TNFα:

Immunohistochemistry localised staining for IL-1β to the epithelium, glands and blood vessel endothelium (Figure. 6.2a). IL-6 was present in the epithelium and in perivascular structures (Figure 6.2b). Staining for IL-8 was confined to the epithelium and blood vessel endothelium (Figure 6.2c). IL-10 stained strongly in the epithelium and weakly in the blood vessels (Figure 6.2d). IL-15 stained strongly in the epithelium and blood vessels and more weakly in the cervical connective tissue stroma (Figure 6.2e). Staining for TNFα was localised to the surface epithelium with a small amount of perivascular staining also being present (Figure 6.2f). MCP-1 staining was present strongly in perivascular structures and in the surface epithelium (Figure 6.2g).

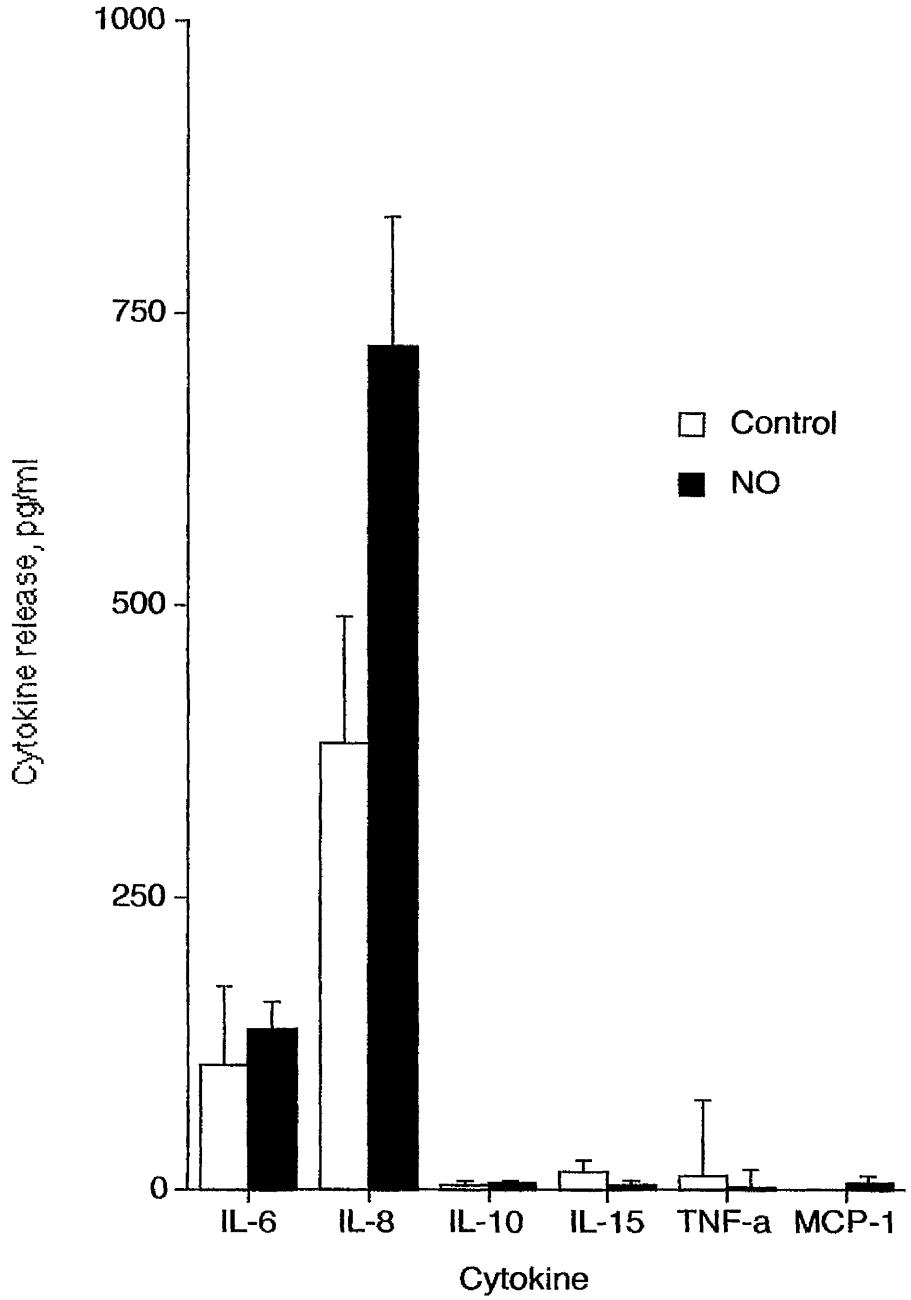


Figure 6.1 The effect of *in-vivo* treatment with the nitric oxide (NO) donor isosorbide mononitrate (IMN) on cytokine levels in cervical tissue in the first trimester of pregnancy. Concentrations of interleukin (IL)-6, IL-8, IL-10, IL-15, monocyte chemoattactant protein (MCP)-1, tumour necrosis factor-α (TNF)-α in supernatant from cervical explants of 10 control patients and 10 patients treated with the NO donor IMN 40mg *in vivo* were measured by enzyme linked immunosorbent assays. The increase in IL-8 concentration in the NO donor treated group was not statistically significant.

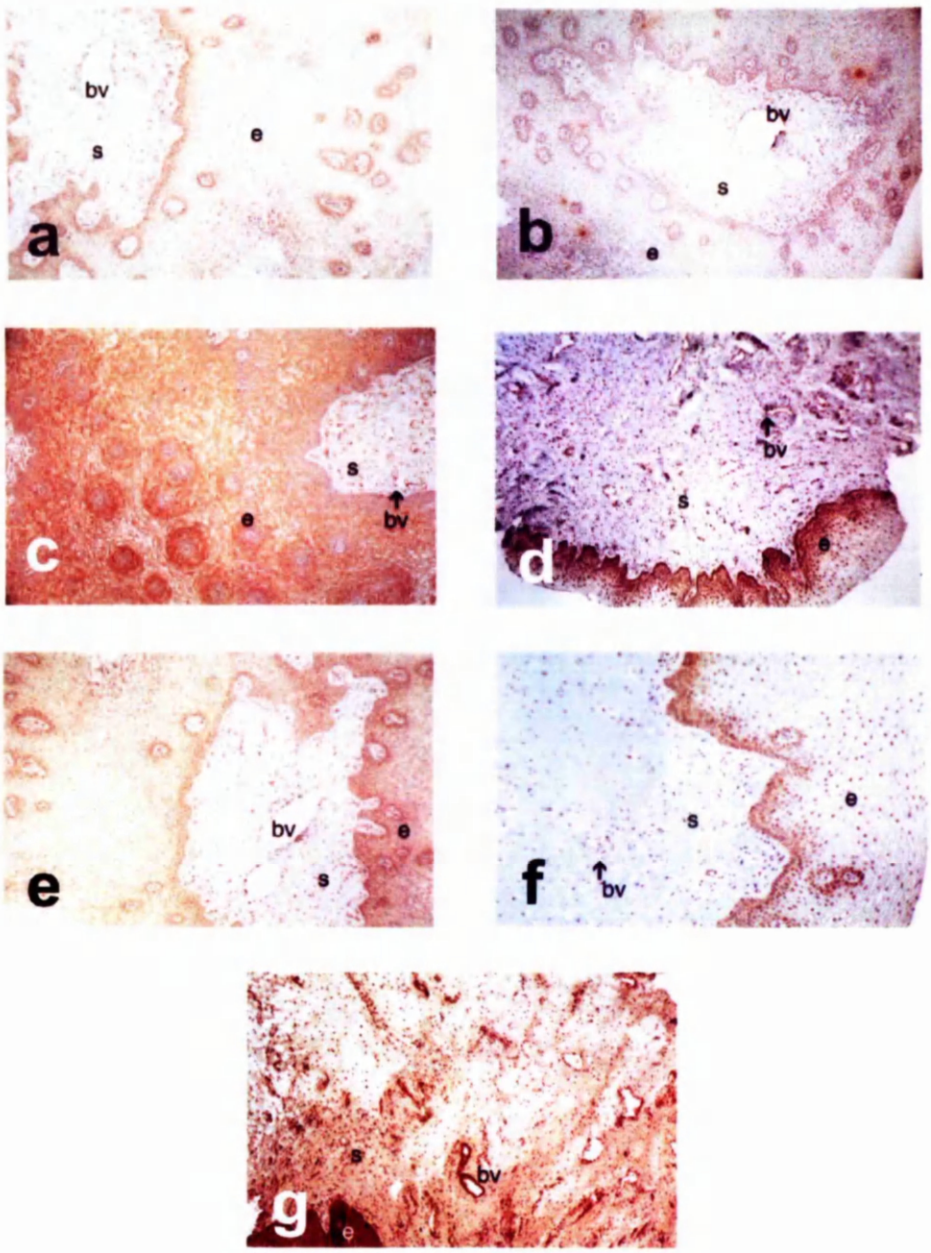


Figure 6.2 Immunostaining of interleukin (IL)-1 (**a**), IL-8 (**b**), IL-15 (**c**), monocyte chemoattractant protein-1 (MCP-1) (**d**), IL-6 (**e**), IL-10 (**f**), tumour necrosis factor (TNF)-α (**g**) in cervical tissue biopsy specimens from early pregnant subjects. bv = blood vessels, e = epithelium, s = stroma. Original magnification: (**a, e, f**) ×94, (**b,g**) ×48, (**c**) ×60, (**d**) ×75

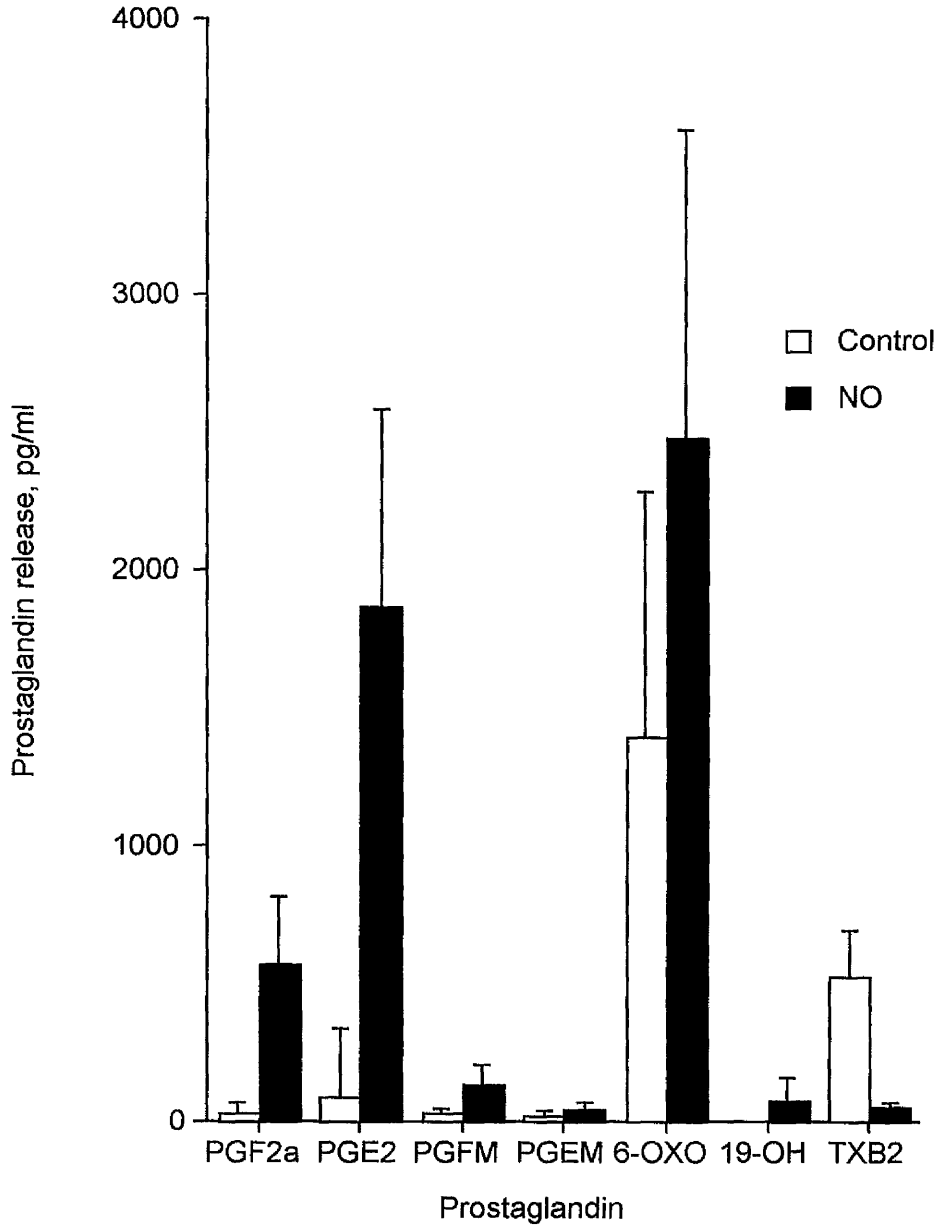


Figure 6.3 The effect of *in-vivo* administration of the nitric oxide (NO) donor isosorbide mononitrate (IMN) on prostaglandins in the cervix in the first trimester of pregnancy. Concentrations of prostaglandin (PG) $F_2\alpha$, PGE_2, PGFM, PGEM, 6-OXO-$PGF_2\alpha$, and thromboxane (TX)B_2 were measured in supernatant from cultured cervical explants by enzyme linked immunosorbent assays in 10 control patients and 10 patients treated with the NO donor IMN 40 mg *in vivo*. Treatment with IMN stimulated $PGF_2\alpha$ release ($P < 0.05$) and inhibited TXB_2 release ($P < 0.01$). Values are expressed as pg/ml ± SEM.

(iii) $PGF_{2\alpha}$, PGE_2, PGFM, PGEM, 6-OXO-$PGF_{2\alpha}$ and TXB_2 release:

Cervical explants from first trimester cervix released $PGF_{2\alpha}$, PGE_2, PGFM, PGEM, 6-OXO-$PGF_{2\alpha}$ and TXB_2 (Figure 6.3). Treatment with the NO donor IMN *in vivo* stimulated $PGF_{2\alpha}$ release ($P<0.05$) and inhibited TXB2 release ($p<0.01$). There was no significant effect of the NO donor isosorbide mononitrate on the levels of PGE_2, PGFM, PGEM and 6-OXO-$PGF_{2\alpha}$.

(iv) Immunohistochemistry for COX-1, COX-2 and PGDH:

Immunohistohemistry was performed to localise the enzymes COX-1, COX-2 and prostaglandin dehydrogenase (PGDH) to the cervical tissue in both NO treated and control patients (Figure 6.4).

Staining for COX-1, COX-2 and PGDH was present in both NO treated and control subjects. COX-1 was localised strongly to the superficial layers of the surface glandular epithelium and weakly to the connective tissue stroma (Figure 6.4a). COX-2 staining was also strong in the glandular epithelium and perivascularly with weaker staining in the stroma (Figure 6.4b). Staining for PGDH showed a similar pattern (Figure 6.4c).

(v) RT-PCR for COX-1 and COX-2:

RT-PCR was performed to identify the presence of mRNA for COX-1 and COX-2 in the cervix. The primer pairs yielded amplified products of the expected sizes: 304 bp for COX-1, 305 bp for COX-2 and 598 bp for GAPDH. Gel electrophoresis for COX-1 and COX-2 is shown (Figure 6.5; Figure 6.6). There was no contamination by amplified cDNA as assessed by appropriate negative controls. COX-1was present in the pregnant cervix. Treatment with NO donors *in vivo* had no apparent effect on COX-1 expression. COX-2 was not present in cervical tissue samples obtained from pregnant women in the first

trimester (n=2) but was expressed in two of three samples obtained after treatment with the NO donor isosorbide mononitrate.

Effect of NO donors in vitro on non-pregnant human cervix

(i) IL-6, IL-8, IL-10, IL-15, MCP-1, TNFα release:

Non pregnant cervical explants released IL-6, IL-8, IL-10, IL-15, MCP-1, TNFα as assessed by ELISA (Figure 6.7). The production of these cytokines was not affected by treatment with either the nitric oxide donor SNAP at concentrations of 100μM or 200μM, by bacterial LPS and IFN γ in combination, by PGE_1, by MPA or by mifepristone + MPA (data not shown).

(ii) $PGF_{2\alpha}$, PGE_2, PGFM, PGEM, 6-OXO-$PGF_{2\alpha}$ and TXB_2 release:

In contrast to the *in vivo* pregnant group, non-pregnant cervical explants treated with the NO donor SNAP *in vitro* did not show any significant change in the release of PGF2α or TXB2 (Figure 6.8). RT-PCR for COX-1 and COX-2 confirmed the presence of mRNA in non-pregnant control cervical tissue and tissue treated with the NO donor SNAP (Figure 6.5, Figure 6.6).

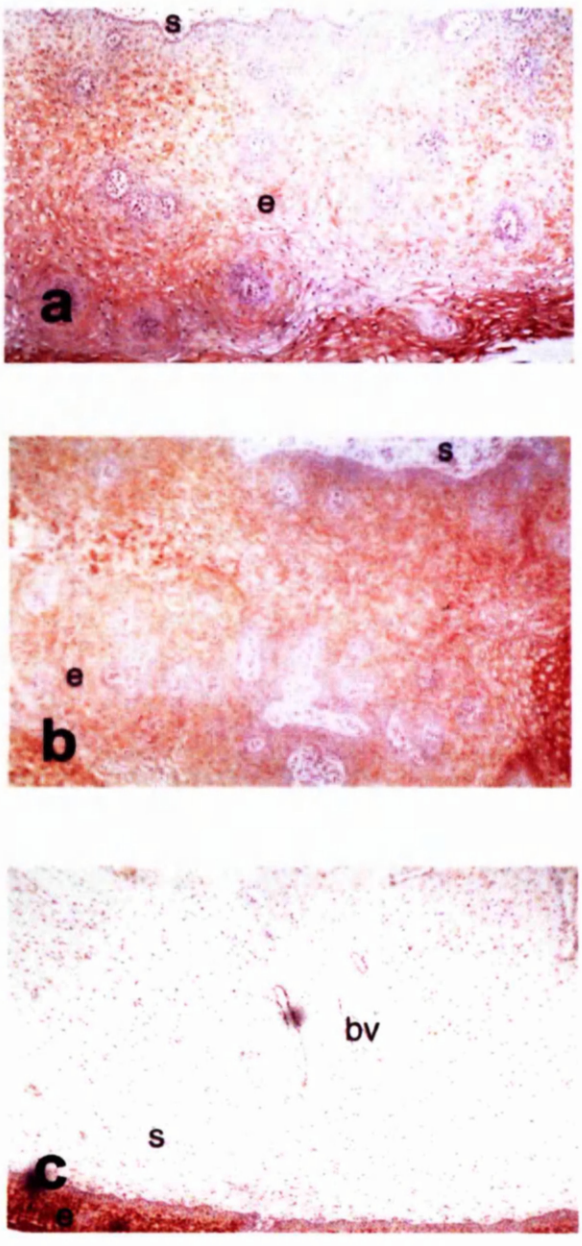


Figure 6.4. Immunostaining of cyclooxygenase (COX)-1 (**a**), COX-2 (**b**) and prostaglandin dehydrogenase (**c**) in cervical tissue biopsies from early pregnant subjects. bv = blood vessels; e = epithelium; s = stroma. Original magnification: (**a**) ×94; (**b**) ×83; (**c**) ×65.

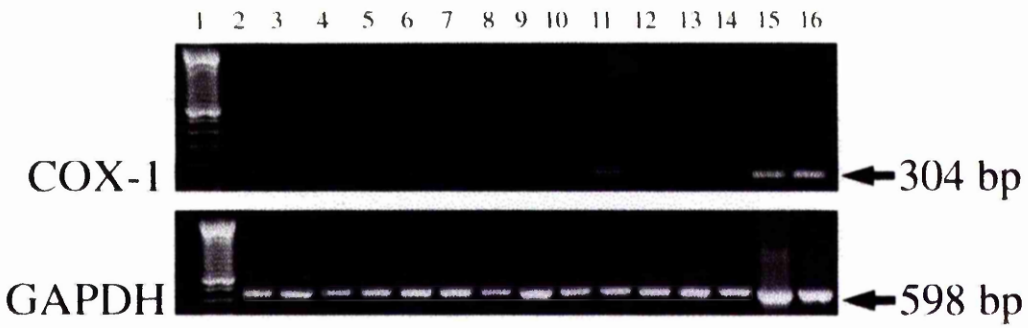


Figure 6.5 Reverse transcription-polymerase chain reaction showing COX-1 expression in cervical tissue biopsies from non-pregnant [± the NO donor S-nitrosos-N-acetyl-D,L-penicillamine (SNAP) in vitro] and pregnant subjects [± the NO donor isosorbide mononitrate (IMN) in vivo]. Lane 1: molecular weight markers.Lane 2: positive control. Lanes 3-6: non-pregnant control tissue. Lanes 7 and 8: pregnant control tissue. Lanes 9-11: pregnant tissue treated with IMN in vivo. Lanes 12-14:non-pregnant tissue treated with SNAP in vitro. Lanes 15 and 16: positive controls. Control lanes for GAPDH are also shown. COX-1 mRNA is expressed in all tissue samples.

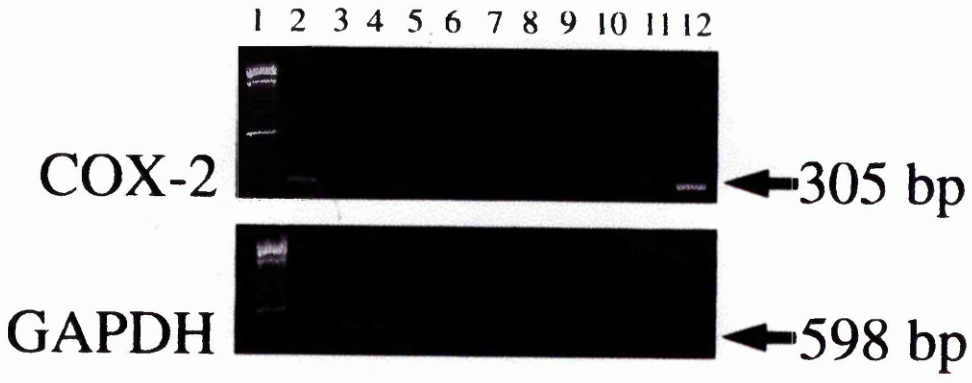


Figure 6.6 Reverse transcription-polymerase chain reaction showing COX-2 expression in cervical tissue biopsies from non-pregnant [± the NO donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP) in vitro] and pregnant subjects [± the NO donor isosorbide mononitrate (IMN) in vivo]. Lane 1: molecular weight markers. Lane 2-4 non-pregnant control tissue. Lanes 5 and 6: pregnant control tissue. Lanes 7-9: pregnant tissue treated with IMN in vivo. Lanes 10 and 11:non-pregnant tissue treated with SNAP in vitro. Lane 12: positive controls. Control lanes for GAPDH are also shown. COX-2 mRNA was present in non-pregnant tissue ± the NO donor SNAP given in vitro. In pregnant tissues COX-2 mRNA was not detected in the two control samples shown (Lanes 5 and 6). COX-2 mRNA was detected in two of the three samples from pregnant women treated with the NO donor IMN in vivo (Lanes 7 and 8).

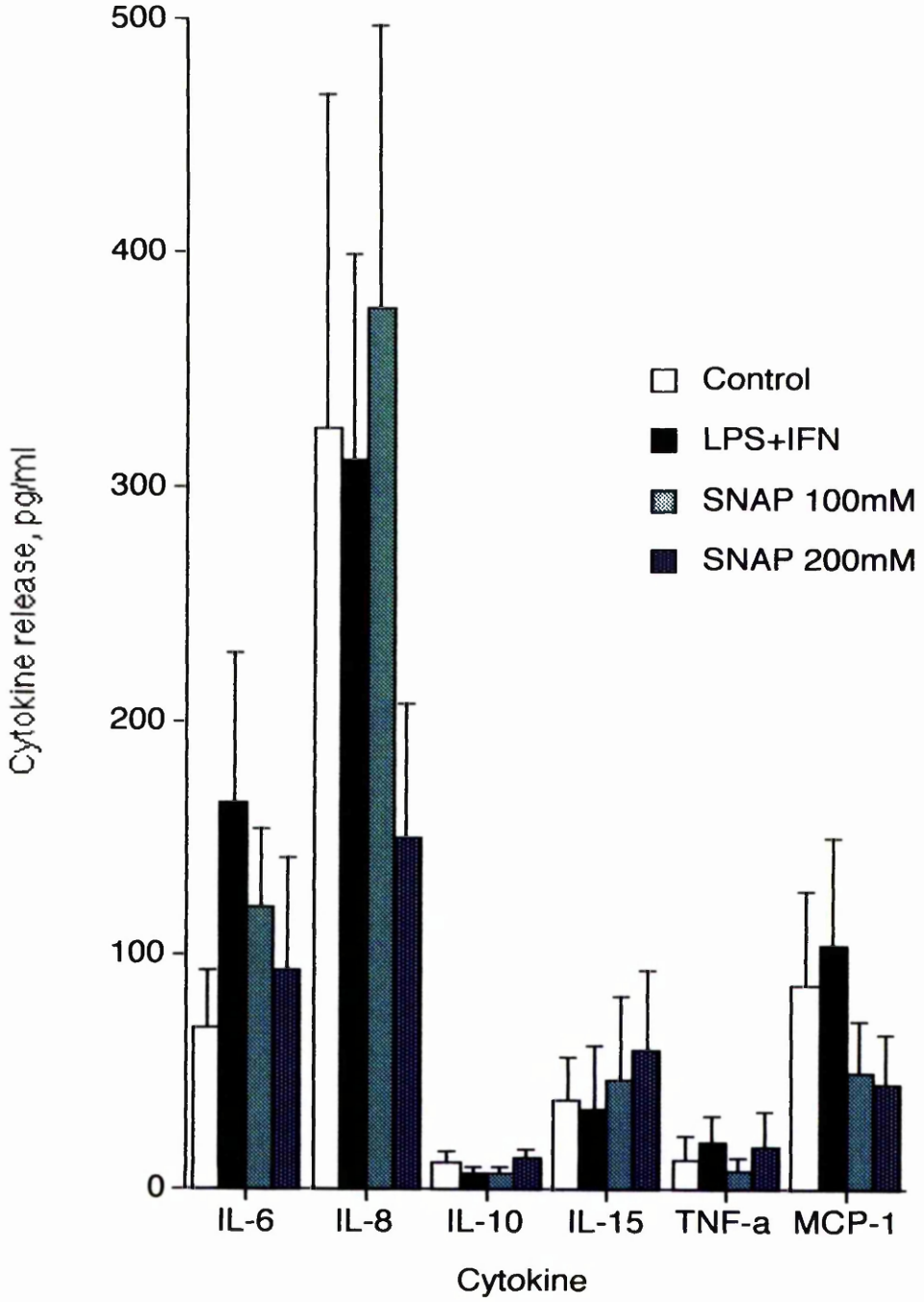


Figure 6.7. The effect of *in vitro* administration of the nitric oxide donor SNAP on cytokine production in the non-pregnant cervix. IL-6, IL-8, IL-10, IL-15, TNF and MCP-1 levels were measured by enzyme linked immunosorbance assay in supernatant from cultured cervical tissue explants treated *in vitro* for 24 h with lipopolysaccharide and interferon γ (LPS+IFNγ); SNAP at 100μM; or SNAP at 200μM. Tissue treated with culture medium only acted as controls. Values are expressed as pg/ml ± s.e.m.

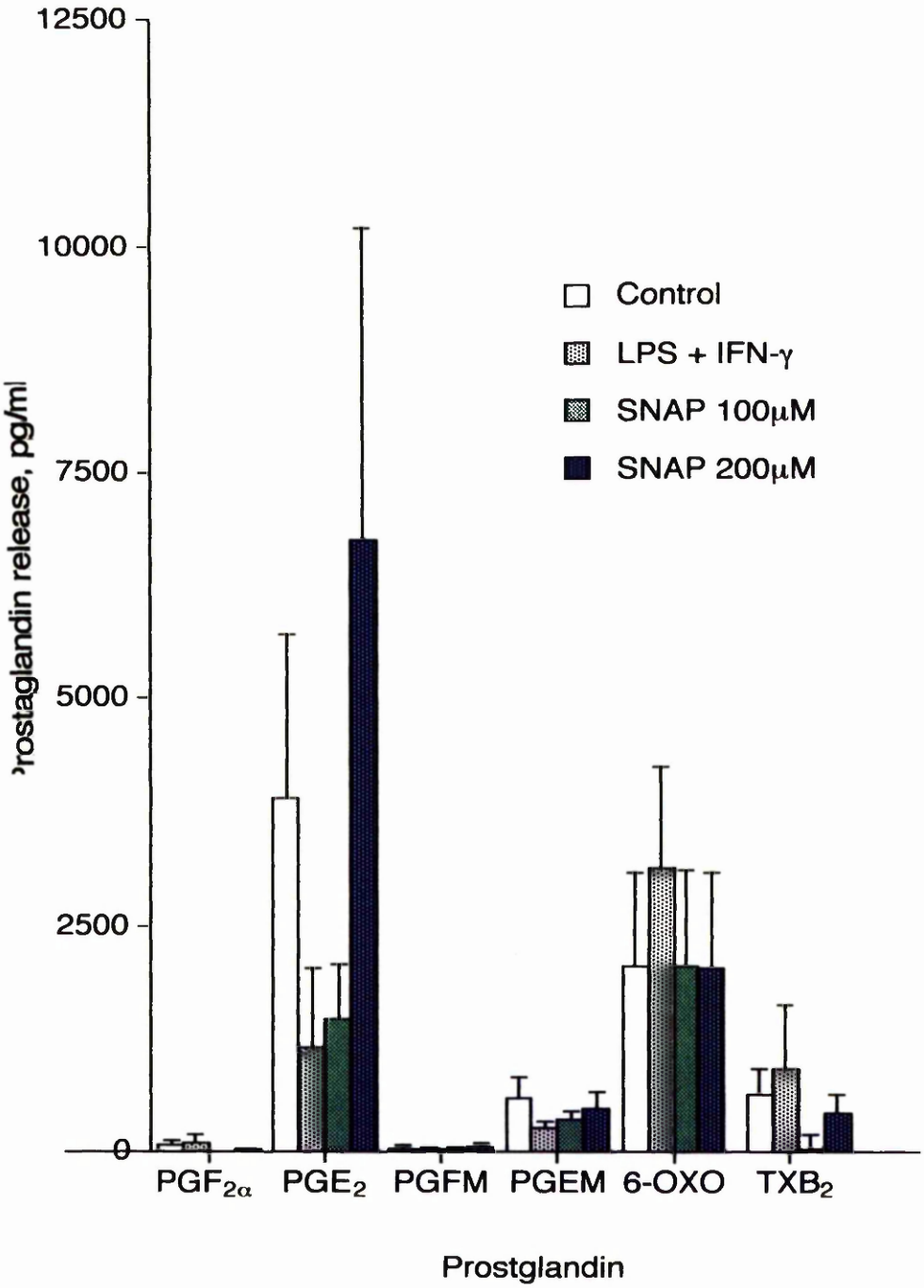


Figure 6.8. The effect of *in vitro* administration of the nitric oxide donor SNAP on prostaglandin production in non-pregnant cervical explants in culture. Prostaglandin production was measured in tissue culture supernatant after 24 hour's culture by enzyme linked immunosorbance assay. Explants were either untreated (controls) or stimulated with lipopolysaccharide and interferon γ (LPS+IFNγ). SNAP at 100μM or SNAP at 200μM. Values are expressed as pg/ml ± s.e.m.

Discussion

The data presented here shows that the *in vivo* administration of the NO donor, IMN, in the first trimester of pregnancy stimulates increased cervical production of PGF2α. Therefore previously reported effects of IMN in inducing cervical ripening seem to be in part mediated through the production of PGF2α. Our findings are in agreement with previously published reports where NO has been shown to activate PGF2α in human microglial cells (Janabi, et al., 1996).

Cervical ripening in pregnancy is known to involve increased production of the prostanoids PGE2 PGF2α and PGI2 within the cervix (Ellwood, et al., 1980). Although PGE2 is considered to be the most important of these (Calder and Greer, 1992), PGF2α may also be fundamentally involved. Animal studies have shown that the histological changes, which occur in the cervix after the administration of PGF2α are comparable with the changes observed in control animals undergoing spontaneous labour. Studies in humans have also shown that PGF2α can be used to artificially induce cervical ripening in both the first trimester of pregnancy prior to suction termination (Arias, 1984; Rath, et al., 1982) and at term (MacLennan, et al., 1994; MacLennan and Green, 1979). PGE2 and PGF2α have similar effects on cervical ripening when used in equipotent doses (MacKenzie and Embrey, 1979; Keirse, 1993) but PGE_2 remains the most commonly used agent for this purpose due to the reduced incidence of side effects encountered using a clinically effective dose.

We postulated that any increase in PGF2α in the cervix might be mediated *via* either an increase in COX activity or expression. The NO and COX systems have often been shown

to be present in concert in inflammatory conditions (Salvemini, 1997) and NO may activate cyclooxygenase through a cGMP independent mechanism (Salvemini, et al., 1993; Uno, et al., 1997). Immunohistochemistry localised COX-1 to the superficial epithelium and to the connective tissue stroma while COX-2 was localised in the cervix in a similar pattern. RT-PCR on first trimester cervical tissue did not attempt to be quantitative and showed the presence of mRNA for COX-1 in both NO treated patients and controls but only showed the presence of mRNA for COX-2 in two of the samples from the NO treated group. The difference in the control and NO treated groups should be interpreted with caution because of the small number of patients studied. NO may directly interact with COX-2 to cause an increase in $PGF2\alpha$ but whether this is achieved by an increase in enzyme activity via free radical stimulation of COX-2 or an increase in enzyme production requires further study.

In vitro, SNAP appeared to have no effect on prostaglandin production. We hypothesise that this may be due to lack of paracrine interaction in the *in vitro* situation. During cervical ripening, NO may act as an inflammatory mediator causing vasodilatation, changes in vascular permeability and activation of cytokines and other proinflammatory mediators. Although the role of NO in the process of lymphocyte trafficking is unclear, it has been suggested that high levels of NO produced in response to iNOS upregulation during acute inflammation contribute to leukocyte and platelet adhesion to the vascular endothelium (Clancy, et al., 1998). NO is also involved in lymphocyte signalling through enhanced activation of a tyrosine kinase p56 (Clancy, et al., 1998). Thus the lack of active tissue perfusion and hence the inability for such complex interactions to take place within the *in vitro* tissue culture system may explain the lack of effect witnessed in the group treated with SNAP *in vitro*. Alternatively, the difference between the groups could be related to the fact that the *in vivo* studies were carried out on pregnant cervix and may

therefore reflect changes which may occur in the maternal immune response during pregnancy designed to prevent fetal allograft rejection (Wegmann, et al., 1993).

Other previously published reports however show that NO donors *in vitro* are capable of stimulating prostaglandins in non-pregnant cervix (Denison, et al., 1999). This may reflect the different NO donors used in these studies compared to those that we employed. Under different *in vitro* experimental conditions it has also been shown that NO can either have no effect on prostaglandin release (Curtis, et al., 1996; Tsai, et al., 1994) or can actually inhibit prostaglandin production at high concentrations (Swierkosz, et al., 1995). The discrepancies between our own and other reported studies may reflect differences in cell types, alterations in the active state of the cells examined and differences in the amount of iNOS and COX-2 present as well as variation in the type and doses of the NO donors used.

Our studies have also demonstrated that IMN administered to the first trimester cervix causes a decrease in thromboxane B2 release. Thromboxane B2 is the metabolic break down product of the arachadonic acid derivative thromboxane A2 which plays a crucial role in platelet functioning. Following platelet activation, the release of TXA2 causes vasoconstriction and stimulates platelet aggregation. Organic nitrates such as IMN are known to reduce platelet adhesion and aggregation as well as causing vasodilatation (Parker and Parker, 1998) and endogenous NO has similar effects (Radomski, et al., 1987; Salvemini, et al., 1990). Our studies suggest that the effect of NO in inhibiting platelet aggregation may be in part mediated by a decrease in thromboxane synthesis. Alternatively the decrease in thromboxane B2 after treatment with NO may reflect substrate shift the arachadonic acid pathway being preferentially driven to increase production of PGF2α.
Our studies failed to show any significant effect of *in vivo* or *in vitro* administration of NO donors on cytokine production within the cervix. *In vivo* administration of IMN to the

pregnant cervix resulted in an increase in IL-8 release, which was not statistically significant. Using other NO donors, NO has been shown previously to stimulate IL-8 production in both the cervix (Denison, et al., 1997) and in peripheral blood monocytes (Cuthbertson, et al., 1998). However, this relationship seems to vary with the NO donor used as Cuthbertson *et al.* (Cuthbertson, et al., 1998) also showed that 3-morpholinosydnonimine (SIN), a combined NO and superoxide donor, was capable of decreasing IL-8 release from blood monocytes. Our results may be attributable to the specific effects of the NO donors used or to the small sample size studied.

In summary, this study has shown that the effects of the nitric oxide donor IMN when used to effect cervical ripening in pregnancy may be in part mediated by an increase in $PGF2\alpha$. TXB2 release is inhibited during this process. We suggest that the paracrine interaction of NO with other inflammatory mediators and growth factors is important in this process since the same effect was not seen in the *in vitro* situation. Further studies are required to examine how NO interacts with cytokines and prostaglandin metabolites in the process of endogenous cervical ripening at term during pregnancy and how interactions occur with NO and $PGF2\alpha$ at a molecular level.

Chapter 7

Changes in cell adhesion molecule expression in the human uterine cervix and myometrium during pregnancy and parturition

Introduction

In chapter 4 of this thesis we showed that at least part of the increase in NO production within the cervix during ripening is attributable to increased expression in invading leukocytes. Accumulating evidence suggests that parturition in the human represents an inflammatory process involving a wide variety of mediators, with leukocytes now recognised to play a crucial role (Bokstrom, et al., 1997; Junquiera, et al., 1980; Liggins, 1981; Owiny, et al., 1995; Thomson, et al., 1999). The placenta, fetal membranes and decidua develop a leukocytic infiltrate and leukocytes, predominantly neutrophils and macrophages, infiltrate the cervix during ripening (Bokstrom, et al., 1997; Junquiera, et al., 1980; Liggins, 1981; Owiny, et al., 1995) and the myometrium during spontaneous labour (Thomson, et al., 1999;Halgunset, et al., 1994; Rosenberg, et al., 1996).

The factors controlling leukocyte infiltration into the uterus and cervix during parturition are incompletely understood. However, in other tissues leukocyte infiltration from the vasculature is controlled by the expression of a number of cell adhesion molecules present on both the leukocyte cell surface and the vascular endothelium (Frenette and Wagner, 1996; Zimmerman, et al., 1997). During inflammation, increased expression of these cell adhesion molecules controls the aggregation and extravastation of leukocytes. Cell adhesion molecules of the selectin, integrin and immunoglobulin families are involved in this complex process which involves leukocyte slowing, rolling, activation and migration into tissues (Bevilacqua, 1993; Imhof and Dunon, 1995; Ley, et al., 1998; Springer, 1990;

Tedder, 1995). Recent evidence suggests that cell adhesion molecules have a variety of other biochemical and physiological functions including the regulation of cellular differentiation, gene transcription, angiogenesis, apoptosis and cell signalling (Bischoff, 1997; Buckley, et al., 1998; Cavenagh, et al., 1998; Freemont, 1998; Gumbiner, 1996; Zimmerman, et al., 1997).

Clearly an improved understanding of the mechanisms involved in controlling the inflammatory events in both the cervix and myometrium is crucial to our understanding of the control of parturition. Previous immunohistochemical studies have described changes in cell adhesion molecule expression in the vascular endothelium of human lower segment myometrium during parturition (Thomson, et al., 1999; Winkler, et al., 1998). More recently, Winkler et al., (Winkler, et al., 2000) have reported a significant increase in intercellular adhesion molecule-1 (ICAM-1) protein levels in lower segment myometrium following the onset of labour, determined using enzyme-linked immunosorbent assay. Whilst cell adhesion molecules have been reported in non-pregnant human cervix, (Coleman and Stanley, 1994; Johansson, et al., 1999), their expression in the cervix during pregnancy and parturition has not been determined.

We hypothesised that cell adhesion molecules are fundamental to the control of leukocyte infiltration in the human cervix and myometrium, during late pregnancy and the onset of labour. Further, we hypothesised that changes occurring in the cervix are fundamentally different from those occurring in the myometrium, reflecting the functional and structural differences between these tissues (Bokstrom, et al., 1997; Calder, 1994; Danforth, 1947; Thomson, et al., 1999). The aims of this study were therefore (i) to examine the expression of ICAM-1, vascular cell adhesion molecule (VCAM), platelet-endothelial cell adhesion

molecule (PECAM) and E-selectin in human uterine cervix during pregnancy and parturition using both qualitative (immunohistochemistry) and quantitative (Northern blotting) methods; (ii) to determine whether expression of these adhesion molecules in cervix and myometrium is influenced by pregnancy, (by analysing samples from non-pregnant and pregnant women); and (iii) to identify whether cells within myometrium and cervix, other than the vascular endothelium, express adhesion molecules during pregnancy and labour.

Materials and Methods

Subjects

Non pregnant women undergoing hysterectomy and pregnant women undergoing Caesarean section were recruited to the study. The study was approved by the local research ethics committee and written informed consent was obtained from each woman prior to the onset of surgery. Biopsies of cervical tissue and of myometrium were obtained from three groups of women:

(i) Non pregnant women with regular menstrual cycles undergoing hysterectomy for benign disease (n=8)

(ii) Pregnant women at term ($\geq$ 37 weeks gestation) undergoing elective Caesarean section prior to the onset of labour (n=8)

(iii) Pregnant women at term undergoing emergency Caesarean section during spontaneous labour (cervical dilatation >4cm and <9cm) (n=8)

Cervical biopsies in group (i) were obtained from the anterior cervical lip immediately following hysterectomy. In group (ii) biopsies were obtained from the lower part of the cervix *per vaginam* prior to Caesarean section. Cervical biopsies in group (iii) were obtained after the delivery of the infant, the cervix being approached via the uterine

incision. Myometrial biopsies in group (i) were obtained from the anterior wall of the lower uterine body following removal of the uterus at the time of hysterectomy. In groups (ii) and (iii), the biopsy of myometrium was obtained from the upper margin of the lower uterine incision at Caesarean section. In all myometrial biopsies, myometrium was separated from surrounding structures i.e. endometrium and decidua, by sharp dissection.

The cervical and myometrial samples were divided; half was snap frozen in liquid nitrogen and stored at -70°C for use in Northern blotting and half was fixed in 10% neutral buffered formalin and paraffin embedded for immunohistochemistry.

Characterisation of biopsies

Immunohistochemistry using anti-myosin smooth muscle antibody (Table 7.1) was performed to confirm tissue origin by quantifying the smooth muscle content of paraffin embedded sections of cervical and myometrial biopsies (see Immunohistochemistry methods). The presence of leukocytes in the tissue in each of the three groups was examined using immunohistochemistry with an antibody directed against CD 45, the common leukocyte antigen (Table 7.1).

Table 7.1 Primary antibodies used in immunohistochemistry

Antibody	Clone	Section type (pretreatment)	Dilution	Source
E-Selectin	polyclonal	paraffin (microwave)	1:500	R+D (Abingdon,UK)
ICAM 1	polyclonal	paraffin (microwave)	1:1000	R+D (Abingdon,UK)
PECAM	clone JC/70A	paraffin (microwave)	1:500	Dako (Cambridge,UK)
CD 45	2B11+PD7/26	paraffin (microwave)	1:100	Dako (Cambridge,UK)
VCAM	polyclonal	paraffin (no pretreatment)	1:1000	R+D (Abingdon,UK)
Myosin	hSM-V	paraffin (microwave)	1:250	Sigma (Poole,UK)

Northern analysis of myometrium

Total RNA was extracted from the myometrial samples (n=5 in each group) using the RNAzol^{TM} B method according to the manufacturers instructions (Biogenesis, Bournemouth, UK). The isolated RNA was redissolved in diethylpyrocarbonate (DEPC)-treated distilled water and quantified by UV spectrophotometry. The integrity of the RNA was confirmed as being free of protein and DNA by having a ratio of >1.8 (optical density at 260nm/280nm) and by the presence of intact 18S and 28S bands on the agarose gels. RNA sample loading buffer (Sigma, UK) was added to 10μg of total RNA and separated on 1.2% agarose gels containing 6% formaldehyde and 20mmol/L MOPS (0.2 mol/L 3-[N-Morpholino] propane-sulphonic acid; 0.05 mol/L Na acetate pH 7.0; 0.01 mol/L

Na_2EDTA). Gels were electrophoresed at 60 volts for 2.5 hours. RNA was transferred overnight onto Hybond-N nylon membranes (Amersham, Buckinghamshire, UK) in 20 x SSC (3mol/L NaCl; 0.3 mol/L $Na_3Citrate$, pH 7.0) and fixed to the membrane by ultra violet irradiation. Membranes were prehybridised for a minimum of 4 hours in 12ml of the prehybridisation buffer: 5 x SSC; 5 x Denhardt's solution (50 x Denhardt's is 1% ficoll (Pharmacia Biotech, Hertfordshire, U.K.), 1% polyvinylpyrolidone and 1% BSA [factor V]); 0.5% sodium dodecyl sulphate (SDS); 10% dextran sulphate and 100μg/ml boiled salmon sperm DNA.

Nylon filters were hybridised in the same buffer overnight with the appropriate ^{32}P labelled (Oligolabelling kit, Pharmacia Biotech) cDNA added to the prehybridisation buffer. The ICAM-1 (1.8Kb insert), VCAM (1.9Kb) and PECAM (2.5Kb) cDNA were a gift from Dr. Simmons, Oxford, UK. The E-Selectin (1.2Kb) cDNA was a gift from Dr. M. Bevilacqua, California, USA. The cDNA probe for the house-keeping gene human glyceraldehyde-3-phosphate dehydrogenase (GAPDH: 1.1Kb) was purchased from Clontech Laboratories UK Ltd (Basingstoke, UK). Nylon filters were washed to a final stringency of 0.5 x SSC, 0.1% SDS at 65°C and autoradiography was carried out with Fuji X-ray film at -70°C for 3 days. The intensity of the bands on the autoradiographs for each of the cell adhesion molecules was compared with GAPDH and the ratio determined.

Human umbilical vein endothelial cells (HUVECs) were isolated by collagenase digestion. Once confluent, the cells were incubated in medium containing IL-1β (10 IU/ml) for 4 hours and used as a positive control for the expression of ICAM-1, VCAM, PECAM and E-Selectin. Total RNA was extracted as above, and loaded at 5 μg/lane.

Northern analysis of cervix

Total RNA was extracted from the cervical samples (n=6 in each group) using the Trizol™ method according to the manufacturers instructions (Life Technologies, Paisley, UK). The RNA was quantified by UV spectrophotometry and the integrity of the RNA confirmed as before. RNA sample loading buffer was added to 10μg of total RNA and separated on 1.2% agarose gels as for myometrial samples. Gels were electrophoresed at 60 volts for 2.5 hours. RNA was transferred overnight onto Hybond-N nylon membranes in 20 x SSC and fixed to the membrane by ultra violet irradiation. Membranes were prehybridised for 1-2 hours, at 42°C, in 12ml of Ultrahyb™ (AMS Biotechnology, Oxon, UK) and then hybridised with the appropriate ^{32}P labelled (Oligolabelling kit, Pharmacia Biotech, Hertfordshire, U.K.) cDNA added to the prehybridisation buffer. Nylon filters were washed in 0.5 x SSC, 0.1% SDS at 65°C and if necessary a further wash was carried out in 0.1 x SSC, 0.1% SDS at 65°C. Autoradiography was carried out with Fuji X-ray film at -70°C for between 2 and 7 days.

Human umbilical vein endothelial cells (HUVECs) were used as a positive control for the expression of each cell adhesion molecule as before.

Immunohistochemistry

Immunohistochemistry was performed on paraffin embedded cervical and myometrial biopsies (n=8 in each group) using antibodies against ICAM-1, VCAM, PECAM, E-selectin, smooth muscle myosin and CD45 as detailed in Table 7.1.

Biopsies of cervix and myometrium were fixed in 10% neutral buffered formalin (BDH, Poole, UK) on collection and embedded in paraffin. 5μm thickness paraffin sections were cut and collected on silane coated slides. Sections were heated to 60°C for 30 mins, dewaxed in xylene and rehydrated through graded alcohols and endogenous peroxide

activity was blocked with 0.5% hydrogen peroxide in methanol. Sections were washed in Phosphate Buffered Saline (PBS) and antigen was retrieved by microwaving in a pressure cooker (Lakeland Plastics Ltd, Cumbria, UK) if required (Table 7.1). Tissue sections were then blocked in either 20% normal horse (SAPU, Carluke, UK) /human serum (PECAM, Myosin and CD45) or 20% rabbit /human serum (ICAM 1, E-Selectin) for 30 mins at room temperature and incubated for 16 hr at 4°C with the primary antibody (Table 7.1) diluted in 2% normal rabbit serum /2% normal horse serum. Sections were washed in PBS then incubated for 30 mins with biotinylated rabbit anti-goat (Vector Laboratories) or with biotinylated horse anti-mouse (Vector Laboratories) diluted 1:200 in 2% normal rabbit serum or 2% normal horse with 5% normal human serum added. Sections were washed in PBS, then incubated with avidin DH/ biotinylated horseradish peroxidase H reagent (Vector Laboratories, UK) in PBS for 30 mins before final washing. Antigen was localised using 1mg/ml diaminobenzidene tetrahydrochloride (Sigma, UK), 0.02% H_2O_2 in 50mM Tris-Cl, pH 7.6 and appeared as a brown end product. Sections were then counterstained with Harris haematoxylin. Negative controls included slides incubated without the primary antibody and sections incubated with a mouse monoclonal antibody against IgG_1 Aspergillus niger glucose oxidase (DAKO Ltd), an enzyme that is neither present nor inducible in mammalian tissues. Placenta was used as a positive control for ICAM-1 and PECAM. Arthritic joint was positive control for VCAM (Littler, et al., 1997) and kidney was used for E-selectin (Patey, et al., 1994).

Data analysis

Northern blotting

The intensity of the bands on the autoradiographs for each of the cell adhesion molecules undergoing Northern analysis was compared with GAPDH and the ratio determined using

the Bio-Rad Multi-Analyst™/PC Version 1.1. Statistical analysis of band intensity for the Northern analysis was performed using Kruskal-Wallis with Mann Whitney-U as a post hoc test. $P<0.05$ was considered to be significant.

Immunohistochemistry

Each section was analysed by 2 observers who were blind to the source of the biopsy and the localisation of staining was recorded for each antibody used. Staining for CD45 was used to co-localise leukocytes in the tissues.

Results

Characterisation of biopsies

Staining for anti-smooth muscle myosin was used to determine the muscle content of each biopsy. Myometrial tissue from non-pregnant and pregnant non-labouring subjects had an abundant muscle content and the bundles of smooth muscle were compacted. The staining pattern became less compacted in the labouring myometrial samples, consistent with the disruption of this tissue during labour (Thomson, et al., 1999). In comparison, the smooth muscle content of cervical tissue biopsies was sparse as determined by immunolocalisation of anti-smooth muscle myosin antibody. There was no change in smooth muscle distribution in this tissue during pregnancy and labour (Figure 7.1).

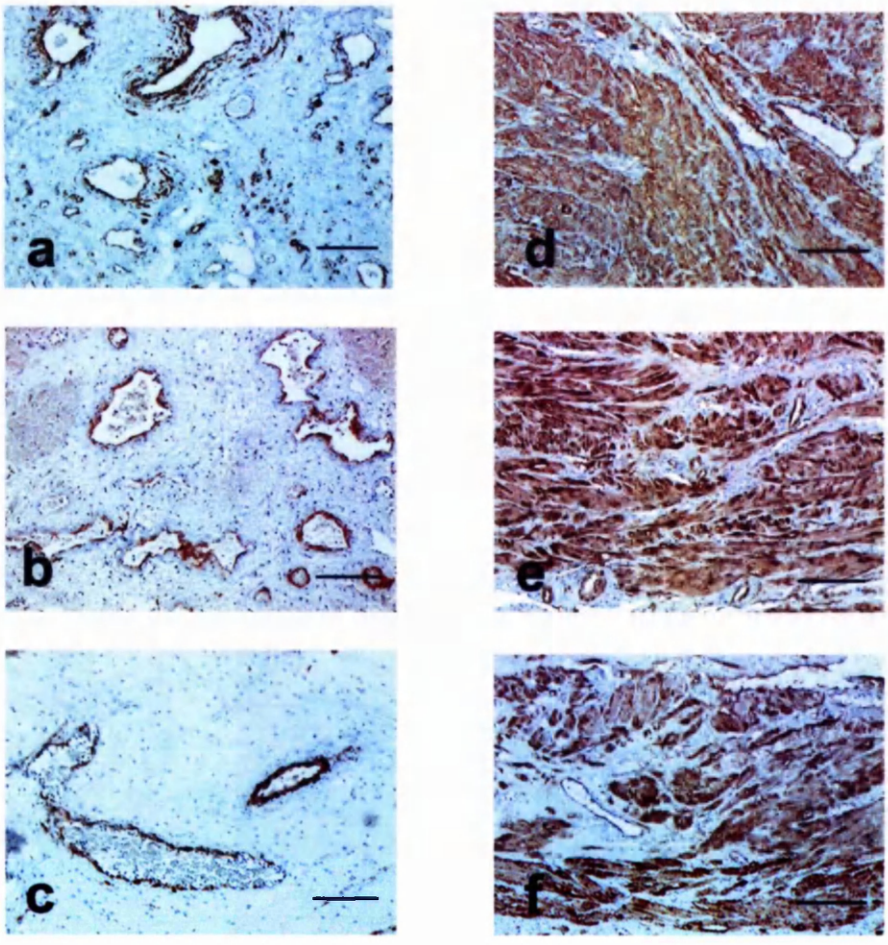


Figure 7.1. Immunolocalisation of smooth muscle myosin in human cervix and myometrium. The smooth muscle content was sparse in cervical biopsies collected from each group of women, (**a**) non-pregnant, (**b**) pregnant before the onset of labour and (**c**) pregnant during spontaneous labour. In contrast, myometrial tissue from (**d**) non-pregnant women, (**e**) pregnant, non-labouring women and (**f**) pregnant labouring women, had an abundant muscle content. The negative controls (see text) exhibited no reactivity. Scale bars = 50 μm.

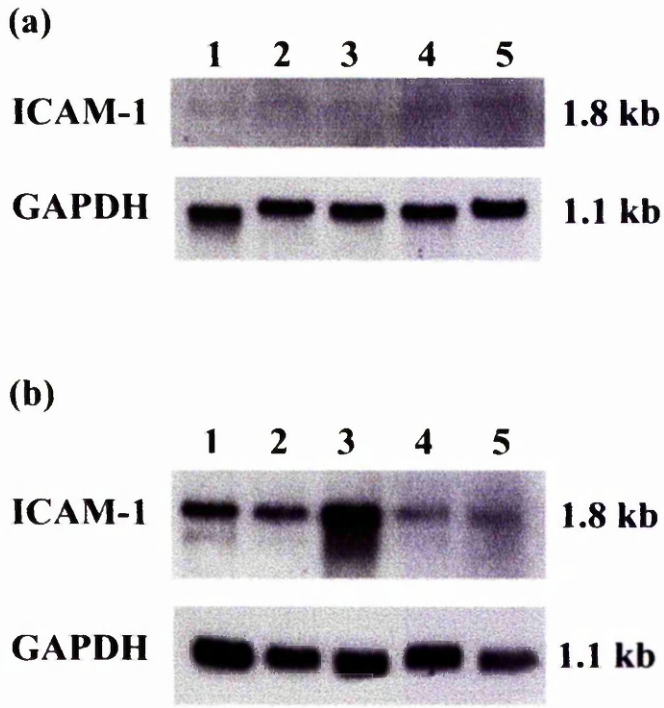


Figure 7.2. Northern blot hybridization of total RNA (10 μg/lane) from human myometrial samples (n=5) collected (**a**) before labour and (**b**) during labour. Intercellular adhesion molecule (ICAM)-1 hybridization was compared with the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

ICAM-1 expression and localisation

Northern blotting confirmed the presence of mRNA for ICAM-1 in myometrial and cervical samples. There was no significant change in the expression of ICAM-1 between the pregnant and non-pregnant groups. ICAM-1 was upregulated in labour in both myometrium (Figure 7.2) and cervix ($p<0.01$), (Figure 7.3). Immunohistochemistry localised ICAM-1 strongly to the vascular endothelium in both tissues. In pregnant and labouring samples, ICAM-1 was present in leukocytes that had infiltrated the tissues, as confirmed by CD45 staining, (Figure 7.4). Substantially greater numbers of these cells were visualised in the labouring samples from both tissues. Staining was absent in the negative control slides.

VCAM expression and localisation

VCAM mRNA was expressed in cervical and myometrial tissues from non-pregnant and pregnant subjects. Expression was increased in the cervix during pregnancy ($p<0.01$) with no further increase with the onset of labour (Figure 7.5). The expression of VCAM in the myometrium was not altered during pregnancy. VCAM was localised weakly to the vascular endothelium in both the cervix and myometrium from all groups of women (Figure 7.6). Significant staining was also seen in some pregnant labouring samples of cervix and myometrium in the cellular infiltrate, identified as leukocytes by CD45 staining. There was no staining observed in the negative control slides.

PECAM expression and localisation

PECAM mRNA was present in the cervix and myometrium from all three groups of women. Expression of PECAM mRNA was significantly elevated in pregnant myometrium ($p<0.01$) with no further change with the onset of labour (Figure 7.7). There was no corresponding change in cervical tissue. Immunohistochemistry localised PECAM strongly to the vascular endothelium in both tissues and also to leukocytes (Figure 7.8).

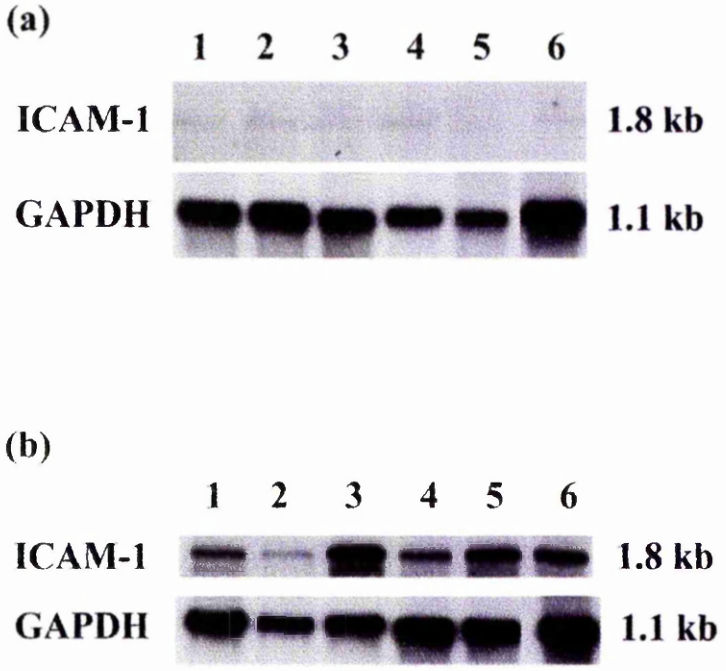


Figure 7.3. Northern blot hybridization of total RNA (10 μg/lane) from human cervical samples (n=6) collected (a) before labour and (b) during labour. Intercellular adhesion molecule (ICAM)-1 hybridization was compared with the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

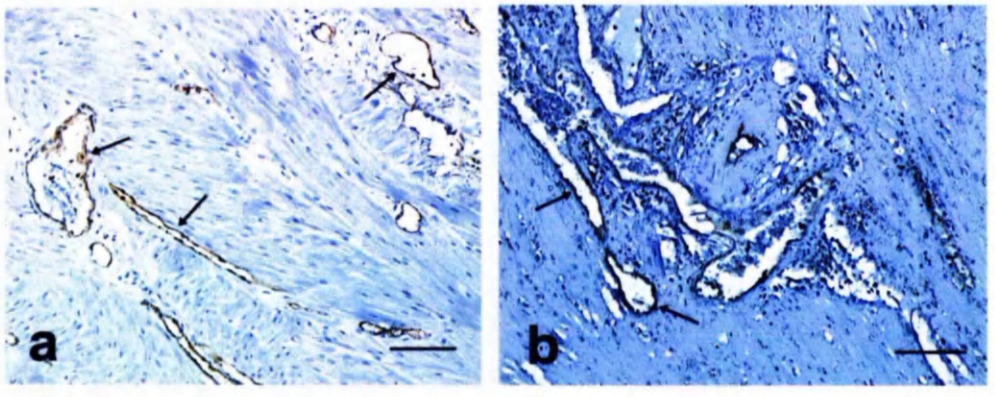


Figure 7.4. Immunolocalisation of intercellular adhesion molecule (ICAM)-1 in human myometrium (**a**) before and (**b**) during the onset of spontaneous labour at term. In each of the biopsies, ICAM-1 localised to the vascular endothelium (arrows). Additionally, ICAM-1 was identified within leukocytes, which were abundant in tissues collected during labour. The negative controls (see text) exhibited no reactivity. Scale bars = 50 μm.

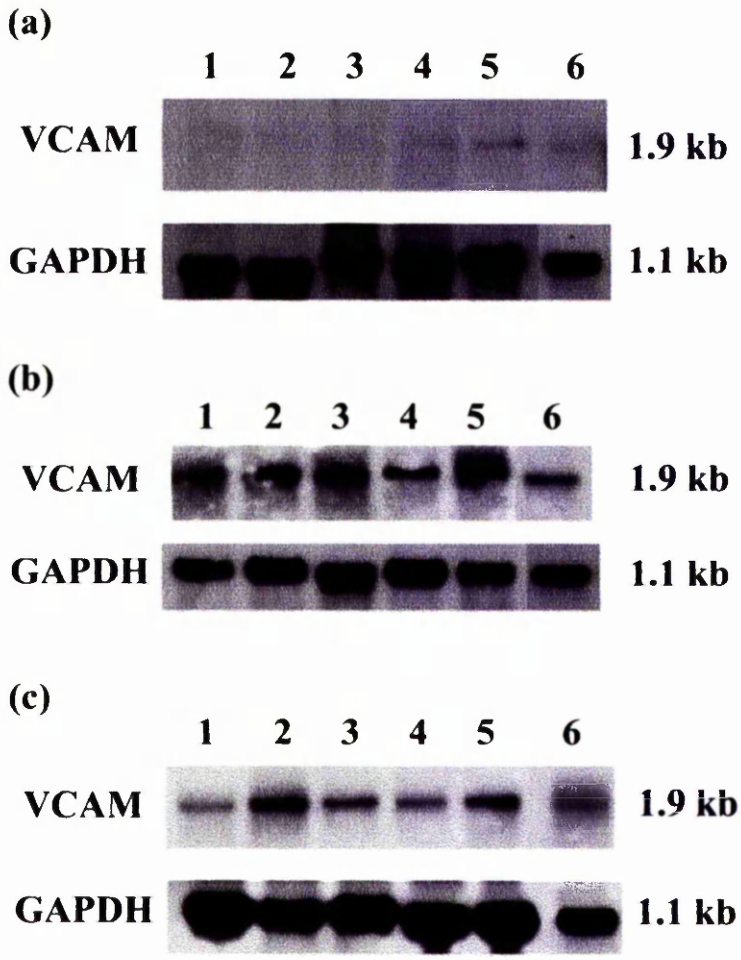


Figure 7.5. Northern blot hybridization of total RNA (10 μg/lane) from human cervical samples (n=6) collected from (**a**) non-pregnant women, (**b**) pregnant women before labour and (**c**) pregnant women during labour. Vascular cell adhesion molecule (VCAM) hybridization was compared with the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The expression of VCAM mRNA was upregulated in the cervix during pregnancy.

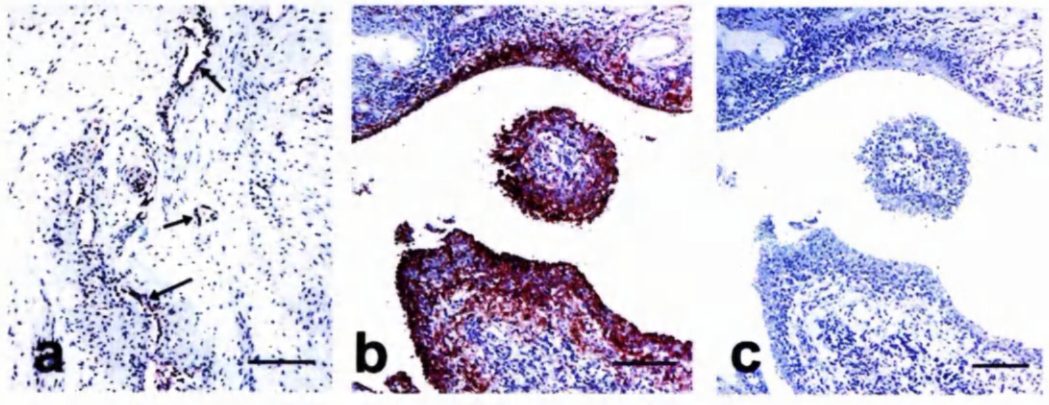


Figure 7.6. Expression of vascular cell adhesion molecule (VCAM) in (**a**) cervix collected before the onset of labour at term. VCAM localised strongly to the vascular endothelium (arrows). VCAM was strongly expressed within (**b**) arthritc joint (positive control), whilst the negative controls (**c**) exhibited no reactivity. Scale bars = 50 μm.

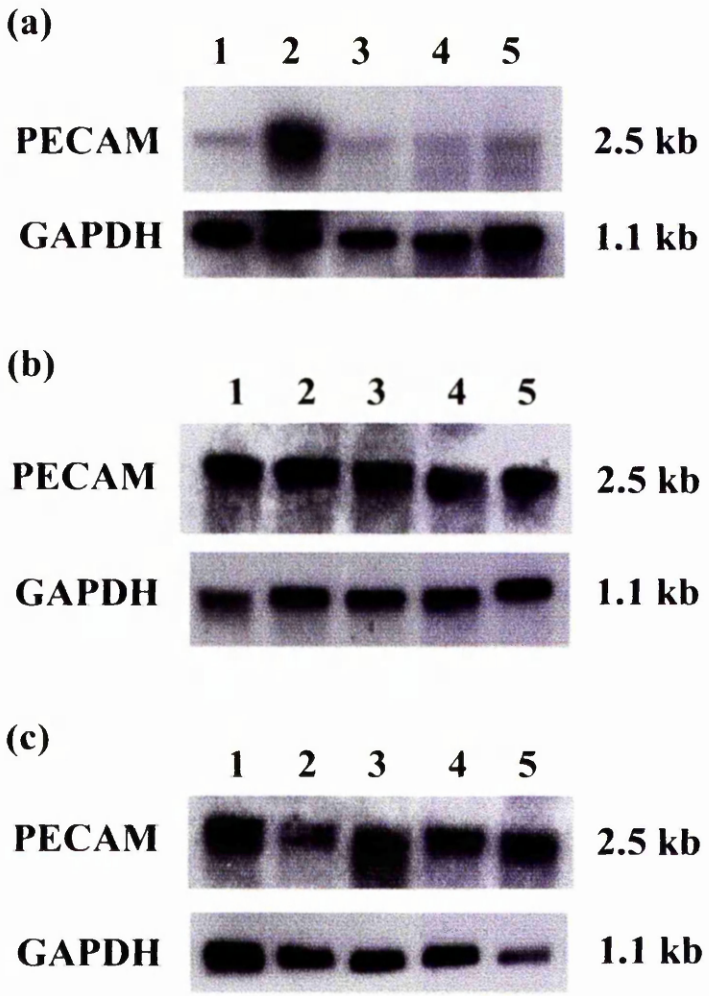


Figure 7.7. Northern blot hybridization of total RNA (10 μg/lane) from human myometrial samples (n=5) collected from (a) non-pregnant women, (b) pregnant women before labour and (c) pregnant women during labour. Platelet-endothelial cell adhesion molecule (PECAM) hybridization was compared with the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The expression of PECAM mRNA was upregulated in myometrium during pregnancy.

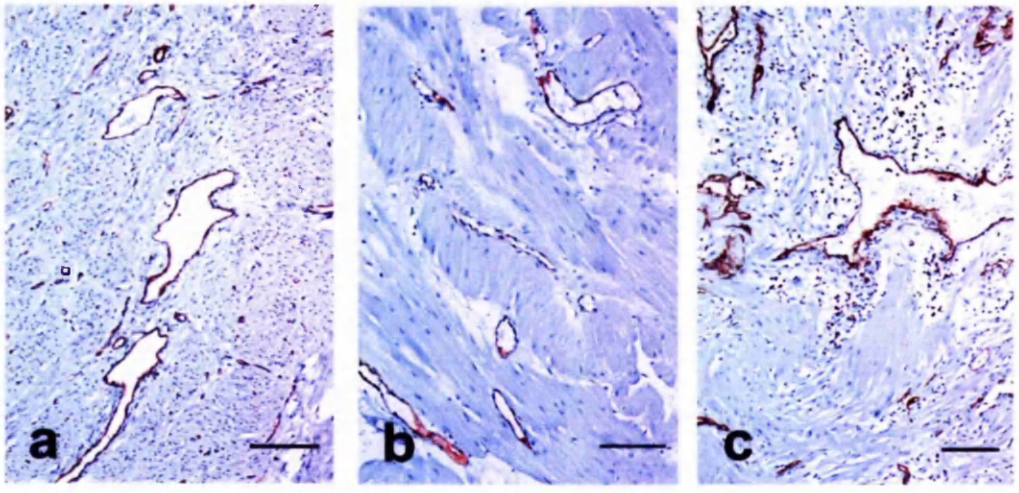


Figure 7.8. Immunolocalisation of platelet-endothelial cell adhesion molecule (PECAM) in human myometrium, (a) non-pregnant, (b) pregnant before labour and (c) pregnant during labour. PECAM antigen was identified on the vascular endothelium in all tissues and also in infiltrating leukocytes. The negative controls (see text) exhibited no reactivity. Scale bars = 50 μm.

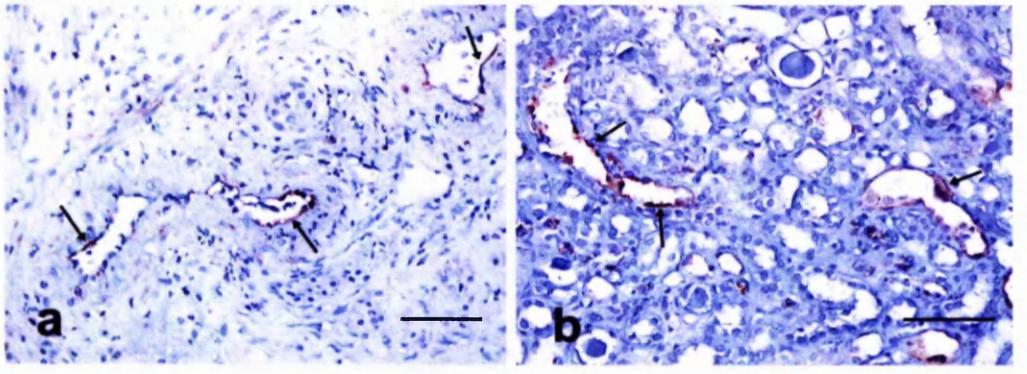


Figure 7.9. Expression of E-selectin on the vascular endothelium of (a) cervix collected during spontaneous labour (arrows). E-selectin was not identified in non-pregnant cervix nor in cervix collected before the onset of labour. E-selectin was expressed within kidney (b) which was employed as the positive control (arrows; see text). The negative controls (see text) exhibited no reactivity. Scale bars = 50 μm.

E-selectin expression and localisation

There were no significant changes in E-selectin mRNA expression comparing the three groups in either myometrium or cervix, (Kruskal Wallis test, p=0.065 and p=0.07, respectively). Using immunohistochemistry, E-selectin was not expressed in the vascular endothelium in either the cervix or myometrium in non-pregnant biopsies or biopsies obtained before the onset of labour. However, E-selectin was present in the vascular endothelium in four of the eight biopsies from the cervix and four of the eight myometrial biopsies obtained during labour (Figure 7.9)

Discussion

This study describes the parallel changes in expression of ICAM, VCAM, PECAM and E-selectin in cervix and myometrium during pregnancy and labour. Our findings are in agreement with previously published reports describing the expression of these adhesion molecules in pregnant and non-pregnant tissues (Coleman and Stanley, 1994; Johansson, et al., 1999; Thomson, et al., 1999; Winkler, et al., 1998; Winkler, et al., 2000). ICAM, VCAM and E-selectin have been demonstrated in non-pregnant cervix and myometrium (Coleman and Stanley, 1994; Thomson, et al., 1999) and in lower segment myometrium obtained during pregnancy before and after the onset of labour (Thomson, et al., 1999; Winkler, et al., 1998; Winkler, et al., 2000). Our results have demonstrated an increase in expression of ICAM-1 in the cervix and myometrium during labour. VCAM expression is increased during pregnancy in the uterine cervix while PECAM expression is increased in the lower segment myometrium at this time. These findings support the hypothesis that pregnancy and labour represent an inflammatory process with cell adhesion molecules playing an important role.

In view of the technical difficulties involved in obtaining cervical biopsies for research purposes, it has been suggested that the lower uterine segment can be taken as representative of the cervix (Rajabi, et al., 1988; Winkler et al., 1998; Rechberger and Woessner, 1993). However, it has long been recognised that the cervix and myometrium are different during pregnancy and labour, both structurally and functionally (Calder, 1994; Danforth, 1947). In the cervix at term, a significantly greater number of neutrophils and macrophages are present compared to the first trimester, with no increase noted following the onset of labour (Bokstrom, et al., 1997). In contrast, in the myometrium neutrophils and macrophages are sparse in lower segment myometrium before, and abundant during labour (Thomson, et al., 1999; Bokstrom, et al., 1997). This is consistent with cervical ripening being an inflammatory process involving leukocytic infiltration of the tissue in preparation for the onset of labour (Junquiera, et al., 1980; Liggins, 1981; Owiny, et al., 1995).

In this study, we have characterised the structure of each of the myometrial and cervical biopsies by determining its smooth muscle content, and have confirmed that lower segment myometrium is composed of substantially more smooth muscle during pregnancy and labour in comparison to the cervix. During pregnancy the smooth muscle composition of the myometrium was dispersed while that of the cervix remained constant. Our results have also shown that changes in cell adhesion molecule expression in cervix and lower segment myometrium are not identical. ICAM-1 mRNA expression increases in both cervix and myometrium during labour. However, the expression of VCAM mRNA is increased in the cervix during pregnancy, while PECAM mRNA expression is increased in the myometrium at this time. Thus we have provided further evidence that lower segment myometrium cannot be considered as being representative of the cervix for the purpose of research in this field.

The expression of cell adhesion molecules on the vascular endothelium of lower uterine segment myometrium has been described in previous studies (Thomson, et al., 1999; Winkler, et al., 2000; Winkler, et al., 1998). In the present study we have investigated whether other cell types within uterus and cervix can express adhesion molecules. We have found that infiltrating leukocytes in the labouring cervix and myometrium expressed ICAM-1, VCAM and PECAM. In light of these immunohistochemical findings, we conclude that the increase in ICAM-1 mRNA expression within the lower segment myometrium and cervix with the onset of labour seems to be attributable, at least in part, to the leukocytic infiltration occuring in these tissues with the onset of labour. Leukocytic influx is a characteristic feature in the cervix and myometrium prior to and at the onset of labour. Leukocyte expressed ICAM-1 may be involved in modulating cell signalling pathways, activation of cytokines and metalloproteinases, and apoptosis, events which take place in the cervix and myometrium around the time of the onset of parturition

We have demonstrated an increase in VCAM expression in the cervix during labour at term. VCAM is principally expressed on endothelial cells, but is also found on macrophages, dendritic cells and bone marrow fibroblasts and plays a significant role in the migration of leukocytes that express VLA-4, that is lymphocytes, monocytes eosinophils and basophils. VCAM is not expressed on endothelial cells in the resting state but, like ICAM-1, is upregulated by inflammatory mediators such as IL-1β, IL-4, TNF α and IL-13. Prostaglandins may also regulate VCAM expression as *in vitro* experiments have shown that it can be induced by phospholipase A 2, the enzyme responsible for catalysing the release of arachadonic acid from cell membrane phospholipids (Yokote, et al., 1993). Prostaglandins, IL-1β and TNF α are also recognised mediators of cervical ripening (Chwalisz, et al., 1994; Greer, 1992; Winkler and Rath, 1996), hence the increased expression of VCAM in the cervix during pregnancy may reflect one of the

mechanisms of action of these agents. Increased VCAM transcription in the cervix during pregnancy would promote leukocyte recruitment and cervical ripening. The exact timing of the increase in VCAM transcription is unclear from the present study.

PECAM is constitutively found on platelets, leukocytes and endothelial cells and mediates leukocyte-endothelial and platelet-endothelial interactions. It is essential for the transendothelial migration of leukocytes through intracellular junctions of vascular endothelial cells. Its transcription is not affected by cytokine release. PECAM mRNA was seen to increase in the myometrium during pregnancy with no further increase with the onset of labour. PECAM is a marker of endothelial cell differentiation, hence the increase in PECAM may refect increased turnover

E-selectin is expressed by cytokine activated endothelial cells and mediates neutrophil, monocyte and lymphocyte recruitment (Bevilacqua, 1993; Ley, et al., 1998). In this study, we found E-selectin was localised to leukocytes in 4 out of 8 cervical biopsies obtained after the onset of labour and to 4 out of 8 myometrial biopsies. Quantitative assessment using Northern analysis however failed to demonstrate any difference in E-selectin expression in either tissue during pregnancy or labour. These results may reflect the small sample size studied and further investigations are required to determine whether E-selectin is involved in leukocyte recruitment during pregnancy.

Recent research has suggested that cell adhesion molecules may be potential targets for therapeutic intervention (Murray, et al., 1999). Antibodies directed against cell adhesion molecules may have a major functional role in the treatment of such inflammatory conditions as rheumatoid arthritis (Kavanaugh, et al., 1994), allergic airways disease and asthma (Abraham, et al., 1994), inflammatory bowel disease (Podolsky, et al., 1993) stroke

(Connolly, et al., 1996; McCarron, et al., 1994) and ischaemia reperfusion injury (Dragun and Haller, 1999). The mechanisms controlling the onset of human parturition remain unclear. However, our studies have demonstrated that the processes of cervical ripening and the initiation of labour constitute an inflammatory response with increased cell adhesion molecule expression in both the cervix and the myometrium. Improved understanding of the mechanisms involved in these events will ultimately lead to new therapeutic strategies for the treatment of preterm labour, which remains a leading cause of neonatal morbidity and mortality despite improvements in neonatal intensive care facilities. In the future, antibodies directed against cell adhesion molecules in the cervix and myometrium may be of benefit in reducing the morbidity and mortality associated with preterm labour.

Chapter 8

Conclusions

Cervical ripening in humans is clearly an inflammatory reaction involving a variety of mediators. The purpose of this thesis was to investigate the role of the inflammatory mediator nitric oxide in endogenous and artificially produced cervical ripening in human pregnancy.

Prostaglandin analogues are the most commonly used agents for the purpose of cervical ripening prior to suction termination of pregnancy. Nitric oxide donors have recently been shown in animal and human studies to ripen the cervix in the first trimester of pregnancy. A randomised controlled trial comparing the nitric oxide donor IMN with the prostaglandin analogue gemeprost, presented in chapter 2 of this thesis, demonstrate that IMN has fewer side effects than the prostaglandin analogue gemeprost when administered per vaginam in the first trimester of pregnancy. The nitric oxide donor is associated with a lower incidence of abdominal pain and pre-operative vaginal bleeding and a higher proportion of patients remaining asymptomatic. However, more women receiving the nitric oxide donor experienced a headache. Although the cervical ripening effects of IMN (40 and 80 mg) are less than those of gemeprost (1 mg), results from the concurrent observational study indicate that these agents are clinically sufficient for ripening the cervix at this gestation.

The results of the randomised controlled trial presented in chapter 3 demonstrate that the prostaglandin analogue misoprostol and the nitric oxide donor isosorbide mononitrate are effective for the purpose of cervical ripening in the first trimester of pregnancy. Combination of these two agents does not improve the cervical ripening effect. Side

effects are more common when these two agents are used in combination. These findings may be attributable to the mechanism of action of NO donors in the process of cervical ripening, as NO donors increase the production of PGF2α, as discussed in chapter 6. Combination therapy with these two agents may therefore enhance prostaglandin mediated side effects rather than exploit the smooth muscle relaxant properties of NO donors. Misoprostol at a dose of 400μg appears to be the agent of choice for preoperative cervical ripening prior to suction termination in the first trimester. However NO donors may be useful for this purpose in those individuals for whom prostaglandin therapy is contra-indicated.

The data presented in chapter 4 of this thesis demonstrates that cervical expression of each of the NOS isoforms is up-regulated during pregnancy. iNOS and eNOS protein expression is greater in the third trimester compared to the non-pregnant state. The increased expression of NOS isoforms, particularly iNOS, in the cervix in late pregnancy at the time when cervical ripening is occuring support the hypothesis that the inflammatory mediator NO is involved in the remodelling of the cervix that constitutes the ripening process.

The mechanism of action of nitric oxide in the process of cervical ripening remains obscure. Possible interactions are with prostaglandins and other inflammatory agents, MMPs, proteoglycans and via stimulation of apoptosis. The data presented in chapter 5 investigate the interaction between NO donors and MMPs. In artificially mediated cervical ripening, the NO donors IMN and spermine nonoate have no effect on the expression of MMPs-2 or 9 or TIMPs-1, -2 or –4 in the human cervix when these agents are administered *in vitro* to non-pregnant subjects or when given *in vivo* to pregnant subjects. These data suggest that other MMPs must be involved in the interaction with NO in cervical ripening.

In chapter 6 we investigated the potential interaction between nitric oxide donors, cytokines and prostaglandins in the cervix. The nitric oxide donor IMN stimulates production of PGF2α *in vivo* in the pregnant human uterine cervix. Administration of the nitric oxide donor SNAP *in vitro* to non-pregnant cervix does not produce this effect. Administration of these NO donors *in vivo* in the pregnant cervix or *in vitro* in the non-pregnant cervix has no effect on the cytokines IL-1, IL-6, IL-8, IL-10, IL-15, TNFα, or MCP-1. The mechanism of nitric oxide induced cervical ripening during pregnancy may be mediated in part via an increase in prostaglandin synthesis. Further studies are needed to examine whether there is an interaction between NO and prostaglandins / inflammatory mediators in spontaneous cervical ripening.

In human pregnancy at term leukocytes infiltrate the cervix and myometrium. These cells appear to be the source of nitric oxide involved in ripening the cervix, as demonstrated in the studies presented in chapter 4. The mechanisms that control the process of leukocyte recruitment in the cervix are incompletely understood although in other tissues cell adhesion molecules are intimately involved. In chapter 7 we demonstrate that there are changes in the expression of cell adhsion molecule expression mRNA in the cervix and myometrium during pregnancy. ICAM-1 expression and VCAM expression are altered in the cervix in the third trimester of pregnancy. Immunohistochemistry has confirmed that at laest part of the incresed expression of these cell adhesion molecules is attributable to the infiltrating leukocytes themselves. In the future, therapeutic strategies for the treatment of premature labour may be aimed at preventing cell adhesion molecule expression and activity in the cervix and myometrium.

In summary, the changes occuring within the cervix prior to the onset of labour appear to be dependent upon a functioning NO-cGMP system. Nitric oxide expression is increased in the cervix in the third trimester concurrently with cervical ripening. Therapeutic advances

in the treatment of preterm and post-dates pregnancy may be possible through manipulation of the NO generating system.

Chapter 9

Further Research

The work presented in this thesis provides further insight into the mechanisms involved in human cervical ripening. However there are clearly further avenues for future research based upon these findings. The role of the NO generating system in preterm labour remains to be established. It would be both interesting and beneficial to investigate whether similar changes as those that occur at term are initiated in the preterm cervix. This in turn may provide future therapies for the treatment of this condition.

We have shown that NO is upregulated in the cervix at term and that, at least in the first trimester, that the cervix can be artificially ripened using the nitric oxide donors glyceryl trinitrate and isosorbide mononitrate. Since NO inhibits myometrial contractility, cervical ripening at term would be theoretically possible using NO donors, with the added advantage of failing to stimulate uterine activity during the ripening process. Preliminary data would suggest that NO donors are a safe and effective alternative to prostaglandins in this clinical setting (Chanrachakul et al., 2000), but further work is required to confirm these findings.

The exact mechanisms by which NO ripens the cervix remains unclear. MMP-2 and MMP-9 appear not to be the candidate MMPs involved and further studies should address the role of other MMPs, including MMP-8 (neutrophil collagenase) and MMP-1. Cervical ripening is characterised by an alteration in the glycosaminoglycan composition of the tissue. NO has the potential to suppress glycosaminoglycan synthesis (Trachtmann et al., 1996), and the interaction between NO this connective tissue composite therefore also merits further investigation. An increase in apoptosis has been observed in rat and human cervix, particularly in fibroblasts and smooth muscle cells (Allaire et al., 2000), and the

involvement of NO remains to be elucidated as NO has been shown to stimulate this process in other tissues (Leppert, et al., 1994)

The control of NO expression within the cervix also remains obscure. Of particular interest for future studies is the potential role of the transcription factor NF Kappa B. A number of proinflammatory cytokines, cell adhesion molecules and reactive oxygen species appear to be under the transcriptional control of the NF Kappa B family members, and they are therefore thought to play a central role in inflammatory responses in a number of tissues. Data on whether NF Kappa B is present in the human cervix are sparse. However, nuclear extracts from cervical cell carcinoma lines contain dimers of the p65 and p50 subunits of NF Kappa B (Altenberg et al., 1999). Further research will examine the role of NF Kappa B in cervical ripening and the potential control of NO and COX-2 expression. Future therapies directed at the treatment of preterm labour may well involve inhibition of NF Kappa B, either by gene transfer of the inhibitor of NF Kappa B (I Kappa B) or by the use of I Kappa B kinases, or drugs directed at inhibiting the degradation of I Kappa B (Barnes, 1999).

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Published Papers.

The following papers have been published based on the text of this thesis:

Original Articles.

1. Thomson AJ, Lunan CB, Ledingham MA, Howat RCL, Cameron IT, Greer IA, Norman JE. (1998) A randomised trial of nitric oxide donors for cervical ripening: more acceptable than prostaglandins? The Lancet, 352, 1093-1096.

2. Ledingham MA, Thomson AJ, Lunan CB, Greer IA, Norman JE (2000) A comparison of isosorbide mononitrate, misoprostol and combined therapy for first trimester surgical termination of pregnancy; a randomised controlled trial. Br J Obstet Gynaecol, 108, 276-280.

3. Ledingham MA, Thomson AJ, Young A, Macara LM, Greer IA, Norman JE (2000) Changes in the expression of nitric oxide synthase in the human uterine cervix during pregnancy and parturition. Mol Hum Reprod, 6, 1041-1048.

4. Ledingham, M., Denison, F. C., Kelly, R., et al. (1999) Nitric oxide donors stimulate PGF-2 alpha and inhibit thromboxane B2 production in the human cervix during the first trimester of pregnancy. Mol Hum Reprod, 5, 973-982

5. Ledingham, M. A., Denison, F. C., Riley, S. C., et al. (1999) Matrix metalloproteinases -2 and -9 and their inhibitors are produced by the human uterine cervix but their activity is not stimulated by nitric oxide. Hum Reprod, 14, 2089-2096

6. Ledingham, M.A., Thomson, A.T., Jordan, F., et al. (2000) Changes in cell adhesion molecule expression in the cervix and myometrium during pregnancy and parturition. Obstet Gynecol, 97, 235-242.

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