Department of Obstetrics and Gynecology

Helsinki University Central Hospital

University of Helsinki, Finland

NITRIC OXIDE IN HUMAN UTERINE CERVIX:

ROLE IN CERVICAL RIPENING

Mervi Väisänen-Tommiska

Academic Dissertation

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Supervised by

Professor Olavi Ylikorkala, M.D., Ph.D.
Department of Obstetrics and Gynecology
Helsinki University Central Hospital

Tomi Mikkola, M.D., Ph.D.
Department of Obstetrics and Gynecology
Helsinki University Central Hospital

Reviewed by

Eeva Ekholm, M.D., Ph.D.
Department of Obstetrics and Gynecology
Turku University Hospital

Hannu Kankaanranta, M.D., Ph.D.
The Immunopharmacology Research Group
Medical School
University of Tampere

Official Opponent

Professor Seppo Heinonen, M.D., Ph.D.
Department of Obstetrics and Gynecology
Kuopio University Hospital

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

I. M. Väisänen-Tommiska, M. Nuutila, K. Aittomäki, V. Hiilesmaa and O. Ylikorkala. Nitric oxide metabolites in cervical fluid during pregnancy: Further evidence for the role of cervical nitric oxide in cervical ripening. Am J Obstet Gynecol 2003; 188:779-785.

II. M. Väisänen-Tommiska, T.S. Mikkola and O. Ylikorkala. Increased release of cervical nitric oxide in spontaneous abortion before clinical symptoms: A possible mechanism for preabortal cervical ripening. J Clin Endocrinol Metab 2004; 89:5622-5626.

III. M. Väisänen-Tommiska, M. Nuutila and O. Ylikorkala. Cervical nitric oxide release in women postterm. Obstet & Gynecol 2004; 103:657-662.

IV. M. Väisänen-Tommiska, T.S. Mikkola, O. Ylikorkala. Misoprostol induces cervical nitric oxide release in pregnant, but not in nonpregnant women. Am J Obstet Gynecol 2005; 193:790-796.

V. M. Väisänen-Tommiska, R. Butzow, O. Ylikorkala, T.S. Mikkola. Mifepristone-induced nitric oxide release and expression of nitric oxide synthases in human cervix at early pregnancy. Submitted.

In addition, some unpublished data are included.

ABBREVIATIONS

ANOVA	analysis of variance
cGMP	cyclic guanosine 3´,5´-monophosphate
CI	confidence interval
COX	cyclooxygenase
CV	coefficient of variation
DNA	deoxyribonucleic acid
ECM	extracellular matrix
eNOS	endothelial nitric oxide synthase
ER	estrogen receptor
FAD	flavin adenine dinucleotide
fFN	fetal fibronectin
GTN	glyceryl trinitrate
GTP	guanosine triphosphate
HbNO	nitrosylhemoglobin
hCG	human chorionic gonadotropin
IL	interleukin
IMN	isosorbide mononitrate
iNOS	inducible nitric oxide synthase
LPS	lipopolysaccharide
MCP-1	monocyte chemoattractant protein 1
MMP	matrix metalloprotease
NADPH	reduced nicotinamide adenine dinucleotide phosphate
nd	not detectable
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
Nox	nitric oxide metabolites, nitrate and nitrite
NS	not significant
PAF	platelet-activating factor
PG	prostaglandin
PR	progesterone receptor
RANTES	regulated upon activation, normal T cell expressed and secreted
SD	standard deviation
SE	standard error
SLPI	secretory leukocyte protease inhibitor
SNP	sodium nitroprusside
TNFα	tumor necrosis factor α

ABSTRACT

The human uterine cervix is capable of producing nitric oxide (NO), a free radical gas with an ultra-short half-life. We studied cervical NO release by measuring the levels of NO metabolites (Nox) in cervical fluid in 664 nonpregnant and pregnant women. In addition, the expression of inducible and endothelial NO synthases was studied in cervical tissue.

Cervical fluid Nox was more often detectable and higher in concentration in the follicular phase (93%, median 18.6 μmol/l) than in the luteal phase (46%, median < 3.8 μmol/l). Cervical fluid Nox was more often detectable and higher in concentration in cases of blighted ovum (87%, median 25.6 μmol/l) and in missed abortion (90%, median 59.4 μmol/l) than in normal early pregnancy (55 to 68%, median 4.3 to 11.4 μmol/l); Nox levels in women with tubal pregnancy were not elevated. The lower the circulating progesterone level, the higher the cervical NO release in nonviable pregnancy. Cervical NO release was reduced in postterm pregnancy. Postterm women with low cervical NO failed more often to progress in labor and had longer duration of labor than postterm women with high NO release.

The riper the cervix, the higher was the cervical NO release. Parous women had higher cervical fluid Nox than nulliparous women. Cervical NO release was induced by spontaneous uterine contractions (3.5-fold), and by cervical manipulation (6.6-fold).

The prostaglandin (PG) E1 analogue misoprostol administrated vaginally induced in three hours a 5.2-fold elevation in cervical NO in early pregnancy and an 18.2-fold elevation in late pregnancy, but had no effect in nonpregnant women. The antiprogestin mifepristone induced in three hours a 17.2-fold elevation in cervical NO in early viable pregnancy.

The expression of both iNOS and eNOS was detected by immunohistochemistry and Western blotting in the cervical cells: both of them in the vascular endothelium, iNOS in pericytes and fibroblasts, and eNOS in the parabasal cells of the surface epithelium and the cervical glandular epithelial cells. The expression of iNOS was stimulated by mifepristone and, additionally, the presence of iNOS was seen in the cervical glands.

Cervical NO release became stimulated during both physiological and pharmacologically induced cervical ripening in pregnant women. Increased preabortal cervical nitric oxide release in women with nonviable pregnancy may contribute to onset of clinical abortion. Reduced cervical NO release may contribute to postterm pregnancy. Prostaglandin-induced cervical NO release suggests a joint action of NO and PGs in cervical ripening. Mifepristone-induced release of NO and elevated expression of iNOS implies that mifepristone may initiate cervical ripening by the NO pathway.

INTRODUCTION

The discovery of an endothelium-derived relaxing factor (Furchgott and Zawadzki 1980), and its later identification as nitric oxide (NO) (Ignarro *et al* 1987) have to be considered as among the most exciting discoveries in medicine in the 1980s. Therefore, it was no surprise that NO was nominated Science's "molecule of the year" in 1992, and its discovery was rewarded with the Nobel Prize in 1998. Nitric oxide is a small uncharged gas molecule that is a highly reactive free radical with an extremely short half-life of approximately four seconds. It has been shown to be a major paracrine mediator of numerous biological processes, including smooth muscle relaxation, host defense and inflammation (Ignarro *et al.* 1987, Moncada and Higgs 1993, Alderton *et al.* 2001, Korhonen *et al.* 2005). In fact, NO is involved in almost all areas of biology and medicine.

The uterine cervix has a pivotal role in the physiology of gestation and parturition; it has to be firm enough to retain the conceptus throughout pregnancy and, on the other hand, have the ability to soften before and during labor to enable the birth of the infant. Cervical ripening is actively controlled and shows features similar to those in inflammation in rearrangement of the cervical collagen fibers (Denison *et al.* 1999, Sennström *et al.* 2000). Cervical ripening is thus associated with changes in local cytokines, prostaglandins, and metalloproteases, as well as in other bioregulators that play roles in inflammation and in collagen metabolism (Denison *et al.* 1999, Sennström *et al.* 2000). These factors also take part in the regulation of NO synthesis and release (Maul *et al.* 2003a), and indeed, animal data have suggested that NO is a factor in cervical ripening (Chwalisz and Garfield 1997, Chwalisz *et al.* 1997, Chwalisz and Garfield 1998, Chwalisz *et al.* 1999). Therefore, the possibility existed that cervical NO also plays a role in ripening of the human uterine cervix. The present study was designed to clarify this question.

REVIEW OF THE LITERATURE

1. NITRIC OXIDE

Nitric oxide is a gaseous, colorless, highly reactive short-lived molecule which regulates various physiological and pathophysiological conditions in the body (Änggård 1994, Aktan 2004). It is formed in almost all cell types. Despite its extremely short half-life *in vivo* of approximately four seconds, it penetrates the surrounding tissues and activates a variety of cellular signaling pathways (Henry *et al.* 1993). It is soluble both in water and lipids (Henry *et al.* 1993).

1.1 Synthesis

Nitric oxide is formed from L-arginine (Palmer *et al.* 1988) through nitric oxide synthases (NOS) (Palmer and Moncada 1989), a group of enzymes that structurally resemble cytochrome P-450 reductase (Marletta 1994). The biosynthesis of NO takes place from L-arginine and molecular oxygen, utilizing nicotinamide adenine dinucleotide phosphate as an electron donor and heme, tetrahydrobiopterin, calmodulin, and flavin adenine mono- and dinucleotides as cofactors through a reaction that consumes five electrons (Figure 1). The overall reaction consists of a two-step oxidative conversion of L-arginine to NO and L-citrulline via N^{w}-hydroxy-L-arginine as an intermediate, with monooxygenase I and monooxygenase II, in each step a mixed-function oxidation taking place (Aktan 2004) (Figure 1).

Three NOS isoenzymes have been characterized as neuronal NOS (type I, nNOS, also called bNOS), inducible NOS (type II or iNOS), and endothelial NOS (type III or eNOS) (Pollock *et al.* 1991, Xie *et al.* 1992, Nakane *et al.* 1993). Their syntheses are regulated by genes located in chromosomes 12, 17 and 7, respectively (Mayer and Hemmens 1997). The amino acid identity between different human NOS isoforms is approximately 55% (Michel and Feron 1997). Both nNOS and eNOS are expressed constitutively, and their activity is calcium/calmodulin-dependent, whereas the expression of iNOS is induced by bacterial lipopolysaccharides (LPS) and cytokines, independently of calcium (Knowles and Moncada 1994) (Table 1).

Figure 1: The synthesis of nitric oxide (NO) from L-arginine. Nitric oxide is synthesized by the conversion of L-arginine to L-citrulline. During this reaction, NADPH (1.5 molecules) is used as an electron donor and hydroxyl-arginine is generated as an intermediate.

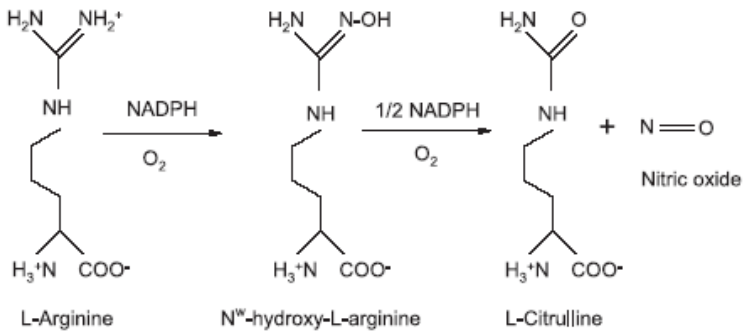


Table 1: Comparison of the constitutive and inducible nitric oxide synthases.

	Nitric oxide synthase		
	Neuronal	***Inducible***	***Endothelial***
Molecular mass (kDa)	160	130	140
Examples of			
• cellular sources	neurons	macrophages smooth muscle cells	endothelial cells
• target organs	nerves	microbes	vascular smooth muscle
Expression	constitutive	inducible	constitutive
Activity regulation	Ca-calmodulin	transcriptional increased	Ca-calmodulin
Amount of NO release	10^{-12} (pmol)	10^{-9} (nmol)	10^{-12} (pmol)
Activators and inducers	sex hormones cytokines stress physical exercise	inflammatory mediators cytokines kinases LPS PG	acetylcholine bradykinin sex hormones mechanical pressure physical exercise

LPS, lipopolysaccharide; PG, prostaglandin

Endothelial NOS is mainly expressed in endothelial cells (Pollock *et al.* 1991) and platelets (Radomski *et al.* 1990), while nNOS is expressed in the cerebellum and skeletal muscle (Nakane *et al.* 1993). Inducible NOS was first cloned from activated mouse macrophages (Xie *et al.* 1992), and thereafter it has been demonstrated in various human cells including macrophages (Moilanen *et al.* 1997, Aktan 2004) (Table 1).

1.2 As a mediator

Nitric oxide serves as a highly diffusible first messenger that can affect cells both directly and indirectly. The direct effects are mediated by the NO molecule itself, while the indirect ones are mediated by reactive nitrogen produced by the interaction of NO with oxygen or superoxide radicals (O_2-). At the low concentrations of NO produced through eNOS and nNOS, the direct effects prevail, while at higher concentrations of NO, produced through iNOS, the indirect effects dominate (Murad 1999, Davis *et al.* 2001) (Table 1).

The formation of cyclic guanosine 3',5'-monophosphate (cGMP) accounts for many of the direct physiological effects of NO (Ignarro *et al.* 1999, Murad 1999). Nitric oxide may also interact with nonheme iron-containing and zinc-containing proteins, or form S-nitrosothiols by nitrosylation (Davis *et al.* 2001, Hogg 2002).

The indirect effects of NO include oxidation, nitrosation and nitration (Davis *et al.* 2001). Cytokine-induced NO production mediates cytotoxicity in the target cells of macrophages (Farrell and Blake 1996). In a reaction with O_2 (auto-oxidation) NO forms dinitrogen trioxide (N_2O_3), which can mediate DNA deamination and nitrosylation. By reacting with superoxide NO produces peroxynitrite (ONOO-), which is a toxic nitrating agent and a powerful oxidant, modifying proteins, lipids, tyrosine and nucleic acids (Davis *et al.* 2001).

1.3 Assessment

The detection of endogenous NO in biological systems is challenging because of its very short half-life. Nitric oxide was first quantified by means of chemiluminescence assay, since its interaction with ozone produces light (Palmer *et al.* 1987). *In vitro*, NO-specific microelectrodes have been used for the detection of NO (Tsukahara *et al.* 1993). Furthermore, a rapid-response chemi-luminescence analyzer has been used for the detection of NO (Lee *et al.* 2000). Measurement of the conversion of radio-labeled arginine to citrulline can be used to measure NO production, as well as the formation of its second messenger cGMP (Ogden and Moore 1995). Unfortunately, this method may give false negative results because hemoglobin catches NO before its reaction with guanylate cyclase. The production of NO can also be detected by positive NADPH diaphorase activity (Rosselli *et al.* 1996, Ekerhövd *et al.* 1997).

The assessment of NO *in vivo* is even more difficult. Endothelial vasomotor function, reflecting NO release *in vivo*, can be measured by forearm venous occlusion plethysmography and by pulse-wave analysis. In plethysmography endothelium-dependent vasodilatation is measured as the increase in blood flow in response to intra-arterial administration of drugs such as acetylcholine that increase NO production (Benjamin *et al.* 1995). In pulse-wave analysis the shape of the arterial pressure waveform reflecting arterial stiffness is measured (Wilkinson *et al.* 2002). The expression of NOSs responsible for NO production has been assessed in various tissues by Western blotting and immunohistochemistry (Tschugguel *et al.* 1999, Alderton *et al.* 2001, Kakui *et al.* 2004, Aktan 2004, Törnblom *et al.* 2005), and the results

have been related to NO release. However, the collection of tissue biopsy samples *in vivo* can be considered unethical and it certainly causes trauma, which may artificially modify the release of NO.

Nitric oxide is rapidly converted to stable NO metabolites, nitrate and nitrite (Nox) which can be assayed both *in vitro* and *in vivo* by means of the Griess reaction in physiological fluids, e.g. plasma, urine, peritoneal and follicular fluid (Green *et al.* 1982, Orpana *et al.* 1996, Dong *et al.* 2001, Osborn *et al.* 2002). Vaginal fluid has also been assayed for Nox (Nakatsuka *et al.* 2000). Griess reagent forms azo dye with nitrite, which can be spectrophotometrically measured (Green *et al.* 1982). Nitrate in the sample must be reduced to nitrite before the assay (Green *et al.* 1982), and plasma samples need to be deproteinized (Moshage *et al.* 1995). Food rich in nitrate (such as red meat, many vegetables, teas, malt beverages and wine) elevate plasma nitrate levels. Therefore, oral intake of Nox-rich food should be restricted for 48 h before taking samples for plasma Nox assay (Jungersten *et al.* 1996).

No method for measurement of Nox in cervical fluid existed when the present study was designed.

1.4 General effects

Nitric oxide is an important intra- and intercellular signaling molecule involved in the regulation of diverse physiological and pathophysiological mechanisms in cardiovascular, nervous and immunological systems (Moncada and Higgs 1993, Alderton *et al.* 2001, Aktan 2004, Korhonen *et al.* 2005). It relaxes vascular smooth muscles, inhibits platelet aggregation, stimulates angiogenesis, reduces blood pressure and transmits neuronal signals. It also activates macrophages to synthesize large amounts of microorganism-destroying NO, mainly through iNOS (Ignarro *et al.* 1987, Moncada and Higgs 1993, Alderton *et al.* 2001, Aktan 2004). On the other hand, it can act as a cytotoxic agent in inflammatory disorders (Moncada and Higgs 1993, Alderton *et al.* 2001, Aktan 2004, Korhonen *et al.* 2005). Nitric oxide may also play a role in asthma (Korhonen *et al.* 2005) and interestingly, patients with asthmatic symptoms but normal lung function have also been shown to have increased alveolar and bronchial NO concentrations (Lehtimäki *et al* 2005). In summary, nitric oxide is involved on a very large scale in human physiology.

1.5 In reproduction

Nitric oxide takes part in various functions of female and male reproduction (Rosselli *et al.* 1998) (Figure 2). In the reproductive system NO was first recognized to have a role in male penile erection (Ignarro *et al.* 1990), and nowadays it is known to play a key role in the physiology of erection as well as in sperm motility (Lewis *et al.* 1996, Maul *et al.* 2003a).

In females, circulating NO is increased during follicle development and decreased immediately after ovulation (Agarwal *et al.* 2005). In rats, iNOS-inhibition results in a 50% reduction of ovulation, an effect completely reversed by NO (Maul *et al,* 2003a). Endothelial NOS knock-out mice exhibit reduced hCG-induced ovulation (Maul *et al.* 2003a). Data regarding the length of the cycle are controversial: whereas Drazen *et al.* (1999) found mice with eNOS deletions to exhibit shorter estrous cycles, Jablonka-Shariff *et al.* (1999) observed longer estrous cycles in eNOS knock-out mice compared with controls. In this regard iNOS may be without effect, because the lack of iNOS did not alter the cycle length (Jablonka-Shariff *et al.* 1999). Neuronal NOS knock-out mice also seem not to be different from wild-type mice regarding ovulation or cycle length, suggesting nNOS not to be of

importance in this process (Jablonka-Shariff and Olson 1998).

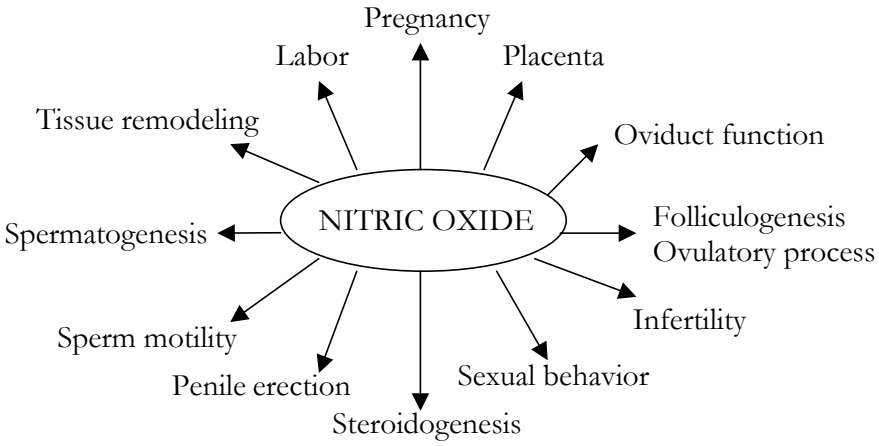
Both the constitutive and the inducible NOSs are present in human tubal cells (Rosselli *et al.* 1996, Ekerhövd *et al.* 1997, Agarwal *et al.* 2005). Nitric oxide relaxes smooth muscles (Agarwal *et al.* 2005). Deficiency of NO may lead to tubal motility dysfunction, resulting in retention of the ovum, delayed sperm transport and infertility (Agarwal *et al.* 2005). Furthermore, increased NO levels in the Fallopian tubes are cytotoxic to invading microbes (Rosselli *et al.* 1995, Rosselli 1997). Thus, NO may protect against ascending pelvic infection. High tubal NO release may also be toxic to spermatozoa (Rosselli *et al.* 1995, Rosselli 1997).

Nitric oxide regulates endometrial functions such as endometrial receptivity, implantation and menstruation (Shi *et al.* 2003, Sun *et al.* 2003, Mörlin and Hammarström 2005). It mediates spiral arterial changes in decidualization (Maul *et al.* 2003a), and promotes embryo implantation (Zhang *et al.* 2005). In guinea pigs and baboons NO may account for the vasodilatation during the initial stages of trophoblast migration (Maul *et al.* 2003a).

In addition to the above, NO production is essential for maintaining pregnancy. In early preimplantation embryonic development NO regulates mitotic division (Tranguch *et al.* 2003). Placental perfusion is also controlled in part by NO (Maul *et al.* 2003a). Fetal membranes are rich in NO, and oxytocin stimulates NO release in fetal membranes at term (Ticconi *et al.* 2004).

Endothelial dysfunction is important in the pathophysiology of preeclampsia (Ranta *et al.* 1999, Jokimaa *et al.* 2000, Maul *et al.* 2003a). Endothelial function changes before the clinical development of preeclampsia (Khan *et al.* 2005). Recent studies have revealed NOS gene polymorphisms in women at risk of preeclampsia (Akbar *et al.* 2005, Biondi *et al.* 2005, GOPEC Consortium 2005, Schiessl *et al.* 2005), which implies a primary role of NO deficiency in this condition.

Figure 2: Nitric oxide controls reproduction.

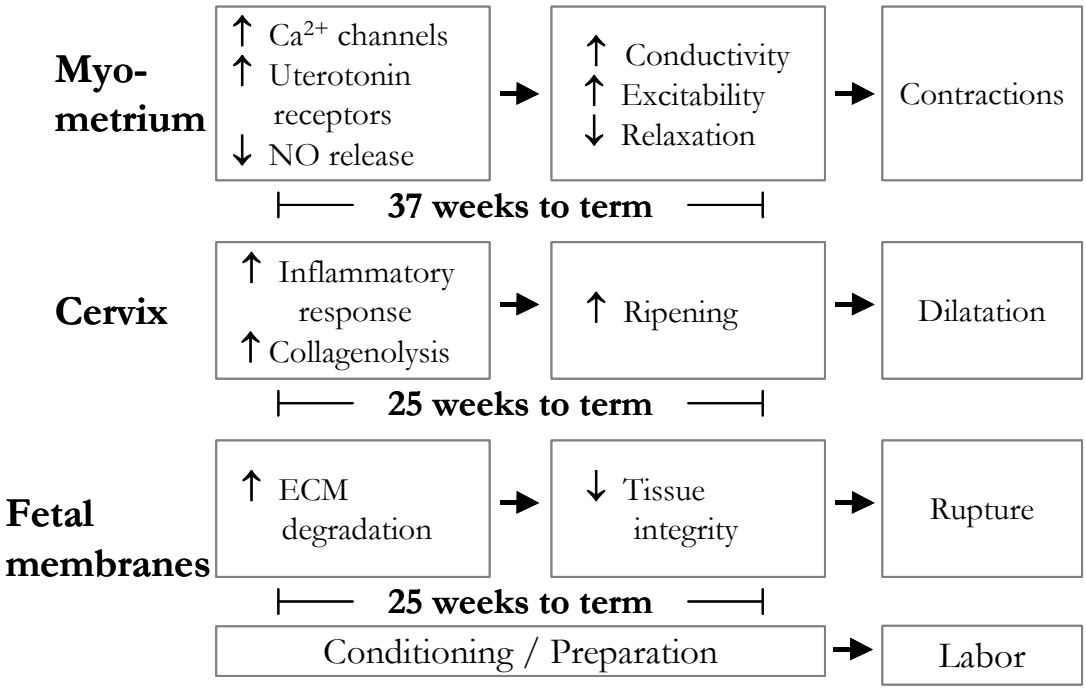


In myometrial tissue all three NOS isoforms have been found in various species, including humans (Buhimschi *et al.* 1996, Ali *et al.* 1997, Ekerhövd *et al.* 1999). Nitric oxide inhibits uterine contractility during pregnancy (Maul *et al.* 2003a, Maul *et al.* 2003b) via activation of the soluble guanylate cyclase-cGMP pathway, but NO-induced relaxation is independent of cGMP (Buxton *et al.* 2001, Hoffmann *et al.* 2003, Tichenor *et al.* 2003). The decreased production of NO, as well as the decreased sensitivity to NO close to term, may promote the initiation of labor (Hertelendy and Zakar 2004, Okawa *et al.* 2004a, Okawa *et al.* 2004b) (Figure 3). Various NO donors inhibit myometrial contractility in nonpregnant women and pregnant laboring and non-laboring women (Norman *et al.* 1997, Ekerhövd *et al.* 1999, Longo *et al.* 2003), probably by mimicking the action of NO. Furthermore, transdermal NO donors decrease the uterine pulsatility index and resistance index (Cacciatore *et al.* 1998). In fact, NO produced by the trophoblast and placenta plays a significant role in maintaining uterine quiescence and blood flow (Cacciatore *et al.* 1998, Al-Hijji *et al.* 2003).

In conclusion, NO appears to be a key element in reproduction and pregnancy.

Figure 3: Changes in the myometrium, cervix and fetal membranes during pregnancy. In the myometrium the preparation phase involves changes in transduction mechanisms, in the synthesis of calcium ion channels and receptors for uterotonins. At the same time, downregulation of the myometrial nitric oxide (NO) system leads to withdrawal of uterine relaxation.

ECM: extracellular matrix.



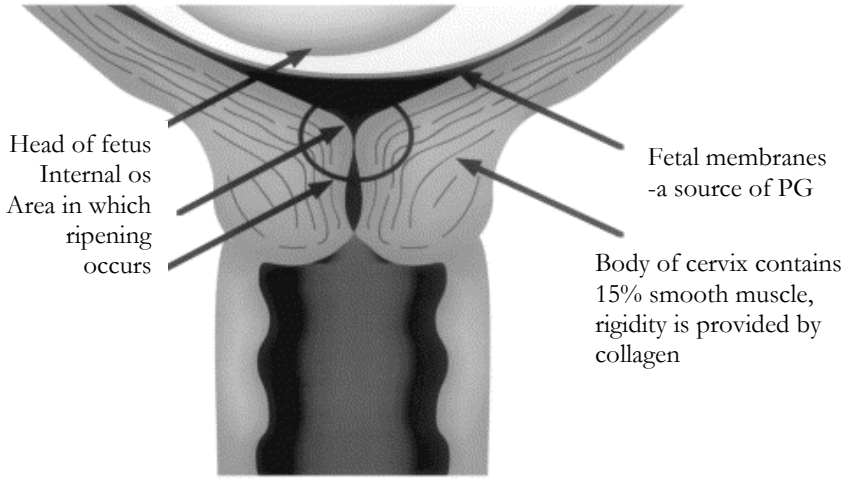
2. CERVICAL RIPENING

The human cervix consists of smooth muscle cells (10–15%) and connective tissue (85–90%) (Danforth 1983) (Figure 4). The columnar epithelium lining of the endocervical canal contains large branched glands (Danforth 1983). The underlying stroma consists predominantly of extracellular connective tissue, mainly type I and III collagen bundles (Leppert 1995, Kelly 2002). In addition, type IV collagen is present in smooth muscle cells and blood vessel walls (Minamoto *et al.* 1987). Collagen bundles provide a rigidity that can be removed rapidly by collagenases; the source and control of collagenases are not yet fully understood (Kelly 2002).

The matrix consists of water, glycosaminoglycans and proteoglycans as well as dermatane sulfate, hyaluronic acid and heparin sulfate (Leppert 1995). Elastic fibers with functional elastin are located between the bundles of collagen fibers in a thin band under the epithelium. The ratio of elastin to collagen is highest in the area of the internal os (Leppert 1995).

The cervix undergoes changes in two phases: ripening, which involves collagen realignment, and dilation (Figure 3). Cervical ripening is an integral part of the conditioning phase of parturition, and it occurs independently of uterine contractions (Leppert 1995, Chwalisz and Garfield 1998) (Figure 3). Cervical ripening resembles an inflammatory reaction, which involves a complex cascade of degradative enzymes accompanied by rearrangement of extracellular matrix (ECM) proteins and glycoproteins (Leppert 1995, Leppert 1998, Maul *et al.* 2003a, Sennström *et al.* 2003). The physiologic changes occurring in gestation involve hyperplasia and hypertrophy of cervical fibroblasts and smooth muscle cells, along with increasing hydration of the tissue (Leppert 1995).

Figure 4. The internal os of the cervix, where ripening starts, lies in close proximity to the fetal membranes. The rigidity of the cervix is largely due to collagens; and thus collagenases soften it.



PG: prostaglandin

2.1 Control

Cervical ripening is the result of digestion of collagen within the cervix and this is followed by an increase in water content (Leppert 1995). As the cervix effaces, the upper part (the internal os) opens and becomes indistinguishable from the lower segment of the myometrium (Kelly 2002). Thus, at the internal os of the cervix the ripening is maximal (Figure 4). In cervical dilatation during parturition, catabolic enzymes lead to collagen degradation and changes in collagen architecture, and also to degradation of other structural matrix proteins (Kelly 2002). Increased production of tumor necrosis factor α (TNF α) and interleukin (IL)-1β induces a rise in the expression of endothelial adhesion molecules, and neutrophils extravasating into the cervical stroma (Winkler and Rath 1999). Rising concentrations of hyaluronic acid have been considered as potent inducers of IL-1 β and TNF- α (Winkler and Rath 1999).

Parturition is associated with an increase in IL-1β and IL-6 mRNA expression in the cervix, IL-6 and IL-8 mRNA expression in the chorio-decidua and IL-1β and IL-8 mRNA expression in the amnion (Osman *et al.* 2003, Sennström *et al.* 2000). Interleukin-8 is localized in stromal cells, macrophages and granulocytes of the human cervix (Osman *et al.* 2003, Sakamoto *et al.* 2004). Levels of cervical IL-8 correlate with the release of collagenases, which then regulate the remodeling of cervical ECM (Garcia-Velasco and Arici 1999). Cervical IL-8 levels increase at term vaginal delivery (Osman *et al.* 2003, Sennström *et al.* 1997) and correlate with cervical opening and with cervical matrix metalloprotease-8 (MMP-8) content (Osmers *et al.* 1995a, Osmers *et al.* 1995b). Recently, no correlation was found between IL-8 and cervical ripening, but IL-8 was involved in cervical dilatation (Sakamoto *et al.* 2004). Nevertheless, interleukin-8 has been be utilized pharmacologically to ripen animal cervix (Kelly *et al.* 1992, Chwalisz *et al.* 1994).

The increase in IL synthesis stimulates PG and leukotriene production, causing dilatation of cervical vessels and further promoting the extravasation of leukocytes (Winkler and Rath 1999). The presence of activated and degranulated polymorphonuclear granulocytes is accompanied by degradation of the ECM (Leppert 1995). The proteases released after degranulation of neutrophils encounter an already destabilized collagenous fiber network (Winkler and Rath 1999). Since the action of proteases may lead to severe tissue damage, this is strictly limited in time and is controlled by increasing concentrations of tissue inhibitors of protease (Winkler and Rath 1999).

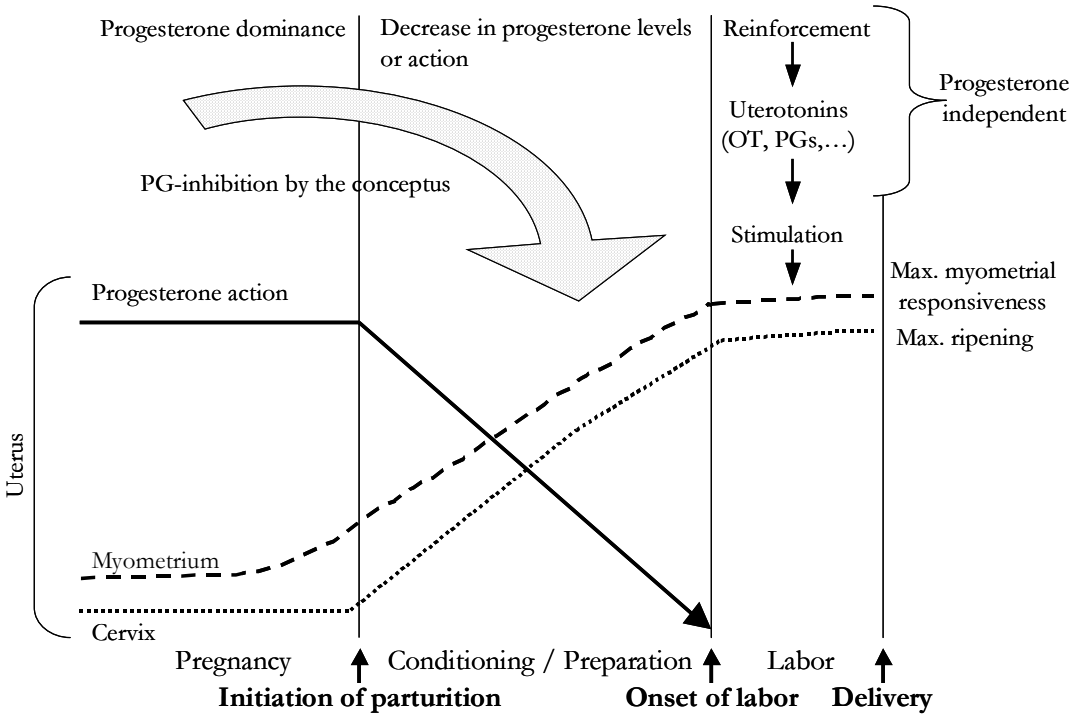
Matrix metalloprotease-8 seems to correlate most closely to cervical ripening and it is localized primarily in the stromal tissue (Sennström *et al.* 2003, Aronsson *et al.* 2005). Matrix metalloproteases -1 and -3 may also be involved (Sennström *et al.* 2003), although their inhibitors resulted in no change in cervical ripening induced by misoprostol (Aronsson *et al.* 2005).

Progesterone seems to be involved in the control of cervical ripening (Figure 5), and all antiprogestins studied so far are effective agents in inducing cervical ripening in all species investigated to date, including humans (Garfield *et al.* 1998, Neilson 2004). However, the mechanism of progesterone action has remained poorly understood in women. Serum progesterone levels decrease in miscarriages (Schindler 2005a), but not before term parturition (Schindler 2005a). Nevertheless, treatment with an antiprogestin is successful for labor induction at term (Neilson 2004). The human progesterone receptor (PR) exists

in two isoforms (PR-A and PR-B), mediating different biological responses. Functional progesterone withdrawal may take place in many ways (Figure 5), i.e. a change in PR receptor affinity, PR concentration, or a post-receptor effect may occur in the myometrium and/or cervix. In fact, there is preliminary data supporting the hypothesis that progesterone withdrawal may take place in human myometrium via changes in the expression of PR coactivators (Condon *et al.* 2003) or via differential expression of PR isoforms (Madsen *et al.* 2004). Interestingly, a recent study showed a change in PR isoforms in cervical biopsies of women before and after term labor (Stjernholm-Vladic *et al.* 2004b) supporting the idea that progesterone withdrawal occurs at the receptor level in the cervix at parturition.

Cervical ripening involves a wide range of inflammatory mediators, including PGs and IL-8 (Leppert 1995, Kelly 2002) (Figure 5). Uterotonins, like oxytocin and endothelin-1, are progesterone-independent. One such mediator is secretory leukocyte protease inhibitor (SLPI), which is present in cervical mucus (Denison *et al.* 1999). It is a potent inhibitor of neutrophil function (Sallenave *et al.* 1997) and thus opposes the action of IL-8, perhaps also in cervical ripening. In addition, platelet-activating factor (PAF), which is a proinflammatory cytokine, accelerates collagenolysis via induction of monocyte chemoattractant protein 1 (MCP 1) and regulated upon activation, normal T cell expressed and secreted (RANTES) during cervical ripening (Sugano *et al.* 2001). Finally, a number of neuropeptides, such as substance P, capsaicin, neurokinin A, calcitonin-gene-related peptide, and secretoneurin, belong to the substances that may contribute to cervical ripening (Collins *et al.* 2002).

Figure 5. Effects of progesterone on the myometrium and cervix during pregnancy and parturition. OT: oxytocin; PG: prostaglandin.



Although an impressive number of molecules have been identified as factors involved in cervical ripening, the question remains of how they work together to enable the process, and whether NO may have a role in this phenomenon.

2.2 Assessment

Cervical status can predict the success of induction and duration of labor (Jackson and Regan 1997). Cervical assessment has progressed from qualitative (soft or firm, ripe or unripe) to quantitative, numerically based classifications that provide more information. However, no objective method for assessing cervical ripeness exists (Fuentes and Williams 1995, Jackson and Regan 1997).

Bishop scoring is based on five factors easily evaluated during pelvic examination: cervical dilation (cm), effacement (%) or length (cm), station, consistency (firm, medium, soft) and position (posterior, middle, anterior) (Bishop 1964). The Bishop score is still widely used although it is poorly reproducible and suffers from large inter- and intra-observer variation (Fuentes and Williams 1995, Laube 1997).

Fetal fibronectin (fFN) is a high-molecular-weight glycoprotein produced by the trophoblast and other fetal tissues which functions in the maintenance of the chorionic-decidual ECM interface (Feinberg *et al.* 1991). It is also present in human cervicovaginal fluid (Sennström *et al.* 1998). In the first half of pregnancy, fFN normally occurs in cervicovaginal fluid (Feinberg *et al.* 1991), but not after the 20th week of gestation (Sibille *et al*.1986). A high level of fFN in the cervical fluid may predict preterm birth (Ascarelli and Morrison 1997, Goepfert *et al.* 2000). In fact, a cervicovaginal fFN value of ≥ 50 ng/mL has been used to define women at risk of preterm labor (Goepfert *et al*. 2000). The assessment of fFN is confounded by its presence in amniotic fluid and in sperm (Lockwood *et al.* 1991, Aumuller and Riva 1992). In addition, the measurement of fFN gives false results when the sample is bloody (Ascarelli and Morrison 1997). Therefore, the usefulness of fFN is still limited in prediction of the risk of preterm birth.

Insulin-like growth factor-binding protein-1 (IGFBP-1) is synthesized and secreted by the fetal and adult liver, and it is a major product of maternal decidualized endometrium (Rutanen *et al.* 1986, Julkunen *et al.* 1988). Different phosphoisoforms of IGFBP-1 can be identified in cervical fluid by use of monoclonal antibodies (Rutanen 2000). The detection of amniotic fluid originating nonphosphorylated and less phosphorylated isoforms of IGFBP-1 in cervical samples is diagnostic of the rupture of fetal membranes (Rutanen *et al.* 1993, Rutanen *et al.* 1996). In women with intact fetal membranes, detection of the highly phosphorylated isoform may reflect cervical ripening at term (Nuutila *et al.* 1999) and predict the risk of preterm birth (Kekki *et al.* 2001).

The markers mentioned above are clinically used, but have limitations. Therefore, there is a strong need for a more reliable biological marker which could be used in clinics for the assessment of cervical ripening or for the prediction of the risk of preterm birth.

2.3 Induction

Labor induction is routinely used in modern obstetrics. In Finland the rate of induced labor rose from 13% of live births in 1993 to 17% in 2003 (Finnish Birth Register 2005). The optimal drug for inducing labor should be efficient but not cause uterine hyperactivity or have other side effects.

Misoprostol

Misoprostol, a PG E1 analogue, is routinely used for cervical ripening before

termination of early pregnancy and for induction of labor (Song 2000, Goldberg *et al.* 2001, Goldberg *et al.* 2003, Alfirevic 2004, Lin *et al.* 2005). Misoprostol, administered either orally or intravaginally, shortens induction-to-delivery intervals, and lowers the oxytocin doses needed during labor (Song 2000, Goldberg *et al.* 2001, Goldberg *et al.* 2003, Alfirevic 2004,). In addition, the use of misoprostol for labor induction has reduced the rate of cesarean section (CS) when compared with previously used PGs (such as dinoprost) (Sanchez-Ramos and Kaunitz 2000). Although misoprostol can be administered orally, rectally or buccally, the vaginal route of administration is favored in clinical routine owing to its superior clinical efficacy, and lack of gastrointestinal side effects (Goldberg *et al.* 2001, Goldberg *et al.* 2003). This may be the result of more stable plasma levels of the drug after vaginal application compared with oral administration (Zieman *et al.* 1997, Danielsson *et al.* 1999, Tang *et al.* 2002). After vaginal application, misoprostol reaches its peak plasma level in 80 minutes (Zieman *et al.* 1997), but these levels show great inter-individual variation (Danielsson *et al.* 1999, Tang *et al.* 2002).

Vaginal administration of misoprostol carries a risk of uterine hyperstimulation in 5–30% of women (Hofmeyr and Gulmezoglu 2004, Ramsey *et al.* 2005). Therefore, in many countries misoprostol is not used in women with previous CS because it may cause rupture of the uterine scar (Dodd and Crowther 2004).

The sensitivity of the cervix to misoprostol becomes enhanced during pregnancy, and, therefore, smaller doses of misoprostol (around 25–50 µg per 4 hours) are needed in late pregnancy than in early pregnancy (around 400–800 µg per 4 hours) (Goldberg *et al.* 2001, Goldberg *et al.* 2003). The sensitivity is further enhanced in early nonviable pregnancy (Barnhart *et al.* 2004). The cause of this phenomenon is unknown.

Misoprostol may stimulate MMP activity, either directly or indirectly. It directly increases the activity of MMP-1 (Yoshida *et al.* 2002), MMP-8 and -9 (Shankavaram *et al.* 1998, Aronsson *et al.* 2005). The indirect effect of misoprostol on MMPs could be mediated via vasodilatation and influx of leukocytes rich in MMPs and cytokines into the cervix (Denison *et al.* 1999, Ledingham *et al.* 1999a, Denison *et al.* 2000).

Although PGs and NO may act in concert in many physiological events, the effect of misoprostol on cervical NO release has not been studied.

Mifepristone

Mifepristone is a PR antagonist used in termination of early or mid-pregnancy and in inducing labor in late pregnancy (Neilson 2004). In nonpregnant women it fails to ripen the cervix (Ben-Chetrit *et al.* 2004). Beside its antiprogestin effect, it also has anti-glucocorticoid and estrogen-related properties (Olive 2002).

Following oral ingestion, mifepristone is rapidly absorbed and the time to peak plasma levels is approximately 1–2 h (Heikinheimo *et al.* 1986). It may have side effects, such as nausea and vomiting (Neilson 2004).

Mifepristone induces uterine contractions and bleeding by blocking PRs (Olive 2002) and by inducing cyclooxygenase (COX) activity (Hapangama *et al*. 2002), whereas it is less clear how it brings about cervical ripening. Mifepristone administration causes an influx of leukocytes, specifically neutrophils and monocytes, and an increase in MMPs -1, -8 and -9 in human cervix (Denison *et al.* 2000). In rat cervix the antiprogestin onapristone markedly suppressed both

cellular proliferation and apoptotic cell death (Leppert 1998).

Onapristone has been reported to cause a 2.5-fold increase of iNOS mRNA in rat cervix (Ali *et al.* 1997). The effect of mifepristone on human cervical NO release is unknown.

2.4 Nitric oxide

All three isoforms of NOS (nNOS, iNOS and eNOS) are present in the human uterine cervix (Tschugguel *et al.* 1999, Ledingham *et al.* 2000, Bao *et al.* 2001): Neuronal NOS is localized in the stroma and in epithelial cells (Bao *et al.* 2001), iNOS in the epithelial cells and stromal spindle cells (Tschugguel *et al.* 1999), and eNOS in vascular endothelium (Tschugguel *et al.* 1999).

In human studies, inducible NOS has been reported to become stimulated in the cervix during vaginal delivery (Tschugguel *et al.* 1999, Ledingham *et al.* 2000), although not in all studies (Thomson *et al.* 1997). In addition, data are not uniform as regards the changes in expression of cervical nNOS and eNOS during term vaginal delivery: in some studies no change was seen (Tschugguel *et al.* 1999, Ledingham *et al.* 2000), but in the others, cervical nNOS expression became stimulated (Bao *et al.* 2001).

In women the concentration of Nox in vaginal secretions has been reported to be elevated before preterm birth (Nakatsuka *et al.* 2000). Although the source of this Nox is not known, both infiltrating inflammatory cells and cells in the uterine cervix may be responsible. Because NO can activate MMPs (Yoshida *et al.* 2001, Biondi *et al.* 2005) and induce apoptotic cell death (Brune *et al.* 1998, Leppert 1998), overproduction of NO may be involved in cervical ripening, fragility of membranes, and subsequent premature delivery.

In animal studies, NO induced cervical ripening (Chwalisz *et al.* 1997) and cervical NO release was elevated during labor (Buhimschi *et al.* 1996). Nitric oxide shares with TNF-α a unique ability to initiate and to block apoptosis, depending on multiple variables that are being elucidated (Brune *et al.* 1998). Therefore, NO is both an antiapoptotic and an apoptotic substance, which may arrest cellular turnover and allow reorganization of the collagen (Chwalisz *et al.* 1997, Leppert *et al.* 2000). Nitric oxide may act in concert with PGE2 by inducing local vasodilatation and by increasing vascular permeability and leukocyte infiltration (Ekerhövd *et al.* 2002). In addition, NO may directly regulate the activity or the production of MMPs (Maul *et al.* 2003a), although Ledingham *et al.* (1999b) demonstrated that the secretion of MMP-2 and MMP-9 in cervical fibroblasts was not regulated by exogenous NO. If NO modulates MMPs, the action of NO both in the uterus and cervix may be mediated partly by MMPs.

In summary, myometrial NO may contribute to uterine quiescence during pregnancy. In contrast, animal data imply that cervical NO is downregulated during pregnancy but becomes upregulated when the time of labor approaches. However, NO regulation in the uterus and cervix is not yet fully understood.

Nitric oxide donors

In animals, NO donors have been found to ripen the cervix (Chwalisz and Garfield 1997, Shi *et al.* 2000). In women, NO donors such as isosorbide mononitrate (IMN) (Thomson *et al.* 1998, Nicoll *et al.* 2001, Ekerhövd *et al.* 2003, Li *et al.* 2003a, Li *et al.* 2003b, Eppel *et al.* 2005), sodium nitroprusside (Facchinetti *et al.* 2000, Chan *et al.* 2005), and glyceryl trinitrate (Thomson *et al.* 1998, Chanrachakul *et al.* 2000, Sharma *et al.* 2005), administered intravaginally or intracervically, ripen the cervix during

pregnancy (Table 2). In general, NO donors appear to be less effective than PGs, at least in viable pregnancies, but in nonviable early pregnancy IMN was more effective than misoprostol (Arteaga-Troncoso *et al.* 2005) (Table 2). Sodium nitroprusside ripens the cervix even in nonpregnant women (Piccinini *et al.* 2003).

Nitric oxide donors are safe and have no major side effects on the fetus or mother (Cacciatore *et al.* 1998, Bates *et al.* 2003, Ekerhövd *et al.* 2003, Kahler *et al.* 2004). When compared with misoprostol, NO donors were less effective (Ledingham *et al.* 2001), but did not cause uterine hyperstimulation (Nicoll *et al.* 2001, Maul *et al.* 2003b). Thus, NO donors hold promise in cervical ripening in women, although additional data are needed before they can be routinely used in clinics.

Table 2. Randomized controlled trials on nitric oxide donors in cervical ripening in pregnant women.

Reference	No. of women	Drug & dose	Compared with	Trim	Exposure time (hrs)	Comparison of efficacy
Thomson *et al.* 1997	48	IMN 40mg	Placebo	I	3	IMN>Placebo
		GTN 0.5mg	Placebo		3	GTN=Placebo
Thomson *et al.* 1998	66	IMN 40mg	Gemeprost 1mg	I	3	IMN<Gemeprost
		IMN 80mg	Gemeprost 1mg		3	IMN<Gemeprost
Facchinetti *et al.* 2000	36	SNP 5mg	Placebo	I	6	SNP>Placebo
		SNP 10mg	Placebo		3	SNP>Placebo
Ledingham *et al.* 2001	65	IMN 40mg	Misoprostol 0.4mg	I	2–3	IMN<Misoprostol
Li *et al.* 2003a	126	IMN 40mg	Placebo or Misoprostol 0.4mg	I	4–6	IMN=Placebo IMN<Misoprostol
Chan *et al.* 2005	200	SNP 10mg + Placebo	Misoprostol 0.4mg + Placebo	I	3	SNP<Misoprostol
Arteaga-Troncoso *et al.* 2005	60	IMN 80mg	Misoprostol 0.4mg	I	12 max 4 doses	IMN>Misoprostol
Li *et al.* 2003b	100	IMN 40mg	Placebo	II	12 after 1 dose Miso	IMN=Placebo
Eppel *et al.* 2005	72	IMN 40mg + Gemeprost 1mg	Placebo + Gemeprost 1mg	II	48 max 3 doses/d	IMN>Placebo
Chanrachakul *et al.* 2000	110	GTN 0.5mg	Dinoprost 3mg	III	6	GTN<Dinoprost CS rate 35 vs. 35%
Nicoll *et al.* 2001	36	IMN 20mg	Vaginal exam.	III	6	IMN=vag. exam. CS rate 46 vs. 33%
		IMN 40mg	Vaginal exam.		6	IMN=vag. exam. CS rate 18 vs. 33%
Ekerhövd *et al.* 2003	60	IMN 40mg	Placebo	III	4	IMN>Placebo Elective CS all
Sharma *et al.* 2005	65	GTN 0.5mg	Misoprostol 0.05mg	III	6	GTN<Misoprostol CS rate 43 vs. 48%
		GTN 0.5mg	Dinoprost 3mg		6	GTN<Dinoprost CS rate 43 vs. 52%

IMN: isosorbide mononitrate; GTN: glyceryl trinitrate; SNP: sodium nitroprusside; CS: cesarean section; Comparison of efficacy: which drug caused more cervical ripening; trim: pregnancy trimester

AIMS OF THE STUDY

The present study was undertaken to evaluate cervical NO release in human cervical ripening. For this we developed a novel method of Nox assessment in cervical fluid.

The specific aims were to study cervical NO release

1. in normal pregnancy
2. in early nonviable pregnancy
3. in postterm pregnancy
4. in response to the PG analogue misoprostol given vaginally
5. in response to the antiprogestin mifepristone given orally

SUBJECTS AND METHODS

1. SUBJECTS

Altogether, 664 women (638 pregnant and 26 nonpregnant) were studied during the years 2000–2004 (Table 3). The Ethics Committee of the Department of Obstetrics and Gynecology approved the study protocols, and the subjects gave informed consent prior to participation.

Table 3. Characteristics of study subjects, and design (mean, *n*, %, range)

	Study				
	I	II	III	IV	V
Number of women	117	239	208	72	28
Age (yrs)	30.9 (18–45)	29.2 (18–46)	31.5 (18–44)	32.3 (20–52)	30.5 (20–42)
Nulliparous	62 (53)	126 (53)	76 (37)	30 (42)	0 (0)
Nonpregnant	11 (9)	-	-	15 (21)	-
Pregnant	106 (91)	239 (100)	208 (100)	57 (79)	28 (100)
Gestational age (weeks)					
Early	8.8 (6–11) (*n*=19)	8.6 (5–16)	-	8.8 (7–12) (*n*=26)	9.0 (7–12)
Late	39.7 (37–42) (*n*=87)	-	40.7(36–42)	40.4 (37–42) (*n*=31)	-
Comparison	early vs. late non- vs. laboring	nonviable vs. viable	postterm vs. term	early vs. late	Nox and NOSs
Intervention	cervical palpation amniotomy NO donor	-	-	misoprostol	mifepristone

2. SAMPLES

2.1 Cervical fluid samples

Cervical fluid samples were collected by the introduction of a Dacron polyester swab into the cervix under visual control. The swab, kept in the cervical canal precisely 20 seconds, was then flushed in 1.5 mL of physiological saline solution for 2 minutes. The saline solution was stored frozen (-21 °C) until it was analyzed. Macroscopically bloody cervical fluid samples were discarded. To assess the volume of cervical fluid that had been soaked up by the Dacron swabs, we weighed eleven swabs before and after sample collection; the weight increase (0.080 ± 0.006 g [mean ± SD]) represented the volume of cervical fluid obtained (0.080 ± 0.006 mL). By multiplying the Nox levels in saline solution by the dilution factor (1.5 mL/0.08 mL = 18.8), we obtained the Nox concentrations in the cervical fluid that had been soaked up by the Dacron swabs. This dilution factor was used for each Nox value in studies I–V.

2.2 Cervical biopsies

Cervical tissue specimens were taken under general anesthesia using Shumaker punch biopsy forceps (Stifle Lab., Wooburn Green, Bucks, UK). In nonpregnant women, this was done before the introduction of a Sairges instrument into the cervix in association with laparoscopic tubal sterilization. In the women with first trimester pregnancy, the biopsies were taken before Hegar dilators were introduced into the cervix. Then, pregnancy was terminated by means of vacuum suction. Two cervical specimens were taken: one was fixed in formalin and embedded in paraffin for immunohistochemistry and the other was snap-frozen in liquid nitrogen and stored at -80 °C for subsequent Western blotting.

2.3 Blood samples

A blood sample was collected at the time of cervical sampling from 156 women: plasma EDTA samples in 46 women in Study I (8 nonpregnant, 15 with viable early pregnancy, and 23 in late pregnancy) and serum samples in 110 women in Study II (80 with viable early pregnancy and 30 with nonviable early pregnancy).

Plasma EDTA samples in Study I were used for the assessment of Nox. Since some food products (for example red meat and some vegetables) are known to lead to an increase in plasma concentration of nitrate (Jungersten *et al.* 1996), these women had kept to a low-Nox diet for 24 hours before sampling, and blood was taken after a 12-hour fast.

Serum samples in Study II were used for assay of human chorionic gonadotropin (hCG) and progesterone.

3. MEASUREMENT OF NITRIC OXIDE

Cervical fluid samples (500 µL) were treated undiluted straight from supernatant (first centrifugation: 2200 x g, for 10 min +4 °C). Other samples were diluted as follows: plasma 1:4 and amniotic fluid 1:4 with aqua, and semen 1:3 with physiological saline. Nox concentrations were measured spectrophotometrically. This was done after nitrate reduction by incubating 125 µL of the sample with 5 µL (10 U/mL) nitrate reductase (Boehringer Mannheim), 5 µL (20 mM) NADPH (Boehringer Mannheim), 5 µL (1 mM) FAD (Boehringer Mannheim), and 50 µL PBS for 15 minutes. The remaining NADPH, which interferes with the chemical detection of nitrite, was removed by incubation with 1.25 µL (3.75 mM) lactate dehydrogenase (Boehringer Mannheim), and 100 µL (15 mM) pyruvate (Sigma Chemical Co., St. Louis, MO) for 10

minutes. Thereafter, protein in the sample was precipitated by adding zinc sulfate (Merck, Darmstadt, Germany) (1.67 M in H_2O; 5%), mixing and centrifuging the sample at 2200 x g for 10 minutes. Total nitrite was then measured by adding Griess reagent to the supernatant. This was prepared by mixing equal volumes of 10% p-aminobenzenesulfoamide (Sigma) in 25% phosphoric acid (Riedel-de Haen AG, Seelze, Germany) and 1% N-(1-naphthyl)ethylenediamine dihydrochloride (Sigma) immediately before use (Green *et al.* 1982, Orpana *et al.* 1996). The Griess reaction was performed in duplicate, and absorbance was read at 546 nm against sodium nitrate (Merck) standards (0, 1.25, 2.5, 5, 12.5, 25, and 50 μmol/L) prepared in water and processed in the same way as the samples. An individual blank was prepared for every sample, and the absorbance obtained from the blank was subtracted from that of the sample. The detection limits of the assay were 3.8 μmol/L (cervical fluid) and 1 μmol/L (plasma, amniotic fluid and semen). The intra- and interassay coefficients of variation for cervical fluid Nox were 1.6 and 2.4% and for plasma Nox 3.2 and 9.6%, respectively.

4. EXPRESSION AND LOCALIZATION OF NITRIC OXIDE SYNTHASES

4.1 Immunohistochemistry

Paraffin sections (5 μm) were used and a standard immunohistochemical technique (HRP-linked antibody conjugates method) was carried out to visualize eNOS and iNOS. After the tissues had been dewaxed and rehydrated, an antigen retrieval procedure was performed. The sections were pre-treated by heating in a microwave oven at 700 W in 0.01 M citric acid buffer (pH 6.0). Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide. Power Vision +^{TM} poly-HRP IHC Detection Kits were used, and a Lab Vision Autostainer (Lab Vision Corp., Fremont, CA, USA). The polyclonal antibodies used for the detection of iNOS and eNOS (Neo Marker, Fremont, CA, USA) (Table 4) were diluted to a concentration of 20 μg/mL (1:50) and incubated for 60 minutes at room temperature. Positive controls for iNOS and eNOS were sections of umbilical cord, and negative controls included slides incubated without primary antibody. After the Lab Vision Autostainer procedure, counterstaining was carried out for ten seconds with Mayer´s hemalum solution (Merck 1.09249). The slides were then manually mounted.

Table 4. Endothelial and inducible nitric oxide synthase antibodies for immunohistochemistry

Antibody	Type	Immunogen	Dilution	Pretreatment	Source
eNOS (RB-1711-P1)	rabbit polyclonal	Peptide from C-terminal of human eNOS	1:50	microwave	Neo Marker
iNOS (RB-1605-P1)	rabbit polyclonal	Peptide from C-terminal of mouse macrophage iNOS	1:50	microwave	Neo Marker

Three observers blind to the identity of the slides performed all the assessments. Staining was evaluated semi-quantitatively using the following system: (0) no staining, (1) weak, (2) moderate, and (3) strong staining.

4.2 Western blotting

Total protein was extracted from the cervical tissue biopsy specimens using the TriPure Isolation Reagent method according to the manufacturer's instructions (Roche Applied Science, IN, USA). Protein was quantified using the Bio-Rad protein assay method (Bio-Rad Laboratories, CA, USA) and spectrophotometry at 750 nm. Samples containing 25 µg protein were prepared with application buffer, separated by means of Novex® 3–8% tris-acetate gel electrophoresis (NuPage™) and transferred to a PVDF (polyvinylidene fluoride) membrane (pore size 450 µm) (Immobilon-P, Millipore Corp., Bedford, MA, USA) by wet blotting (30 V for 2 h). The membranes were blocked in 3% bovine albumin (Sigma Chemical Co., St Louis, MO, USA) in 0.05% v/v Tween–Tris-buffered saline (TBS-T) for at least 1 h prior to antibody application. The antibodies and concentrations were: iNOS (iNOS/NOS Type II, BD Transduction Laboratories, Pharmingen, USA) at 1:2000, and eNOS (eNOS/NOS Type III, BD Transduction Laboratories, Pharmingen, USA) at 1:2000. Lysates of IFNγ/LPS-treated mouse macrophages (Transduction Laboratories), and human endothelial cells (Transduction Laboratories) were used as controls for iNOS and eNOS, respectively. Immunoreactivity was visualized using peroxidase-conjugated secondary antibody against the appropriate species and stained with 3,3´-diaminobenzidine tetrahydrochloride (DAB, Fluka Chemie GmbH, Germany). Stained molecular weight markers (Bio-Rad and Fermentas) were transferred to the PVDF membrane and used to identify and characterize the molecular weights of the NOS isoforms examined.

5. OTHER MEASUREMENTS

Serum concentrations of hCG were measured by solid phase, two-site fluoroimmunometric assay (Auto-DELFIA® Wallac, Turku, Finland) and those of progesterone by coated tube radioimmunoassay (Spectria, Orion Diagnostica, Espoo, Finland), using routine laboratory methods. The intra- and interassay coefficients of variation of the assays were 3.3% and 3.7% for hCG and 2.7% and 3.2% for progesterone, respectively.

6. STATISTICAL ANALYSES

Categorial data were analyzed by means of the Chi-square test and Fisher's exact probability test (Studies I–V), by linear regression (Study II), by the Armitage test for trend (Studies I–II), and by repeated measures ANOVA (Studies IV–V). Medians with their 95% confidence intervals were used to describe Nox levels. Because the Nox values were not normally distributed they were analyzed by means of non-parametric tests, such as the Mann-Whitney *U*, the Kruskal-Wallis one-way ANOVA, and rank correlation tests. All tests were two-sided and processed by using NCSS 2000 software (NCSS Inc., Kaysville, Utah). Values of $p < 0.05$ were considered statistically significant.

The data on hCG and progesterone were analyzed as absolute values, and also on a relative scale, when hCG and progesterone concentrations in the nonviable pregnancies were expressed as percentages of the mean levels of these hormones at the same gestational point in the control group (Study II). To better describe treatment-induced changes in cervical fluid Nox levels, we also present the Nox data as percentages of pretreatment values (Studies IV-V).

RESULTS

The main data are presented here; details are shown in the original publications.

1. METHODOLOGICAL ASPECTS

1.1 Nitric oxide metabolites in cervical fluid (Study I)

Because this assessment was novel, we studied the possible effects of various confounders (Table 5) and assessed the levels of possibly interfering sources of Nox (Table 5) in Study I. No correlation (r = 0.14, p = 0.41) was observed between plasma and cervical fluid Nox. Palpation of the cervix caused an increase in cervical fluid Nox, and rupture of fetal membranes was accompanied by a decrease (40%) in Nox concentrations (Table 5). The cervical application of a NO donor was followed by an increase in cervical NO release and the baseline Nox concentration was reached in 18 minutes. Hence, women with ruptured membranes were always excluded from our studies, and no manual palpation of the cervix was allowed for three hours before sample collection.

The assay was reproducible as regards cervical fluid; when two parallel samples were collected simultaneously they showed a mean inter-sample difference of 11% (n = 16). The detection limit of cervical fluid Nox concentration was 3.8 µmol/L.

The cervical fluid range of Nox concentration (from undetectable to 2068 µmol/L) differed from the ranges in plasma, amniotic fluid and semen (Table 5).

Table 5. The possible confounders of cervical nitric oxide (NO) release. GTN: glyceryl trinitrate, Nitro® Orion, Espoo, Finland

Confounder	*n*	Effect/ ×-fold of initial	*p*	Nox range (µmol/L)
Cervical palpation	11	6.6	0.007	
Amniotomy	7	0.6	0.3	
Amniotic fluid	11			14.5–102.1
Plasma	46			2.0–48.3
Blood in sample	19	0.6	0.07	
Administration of the intracervical NO donor GTN 0.5 mg	3	5–300	0.02	
Semen	10			5.6–9.4

1.2 Expression and localization of nitric oxide synthases in the cervix (Study V)

Because we measured NO as its metabolites we wanted to confirm that the enzymes responsible for NO release are present in cervical cells.

Immunohistochemistry

iNOS

Inducible NOS was detected in the vascular endothelium, pericytes and fibroblasts in women with early viable pregnancy. The ratio of iNOS expression in the endothelium vs. that in the pericytes was low. Cervical iNOS staining was considered weak (grade 1) in 5 of the 6 women and moderate (grade 2) in one woman.

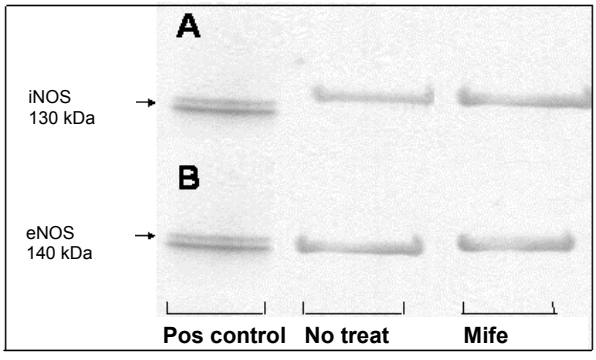
eNOS

Endothelial NOS was present in the vascular endothelium, the parabasal cells of the surface epithelium and the cervical glandular epithelial cells in early pregnancy. The endothelium/pericyte ratio in staining was high as regards eNOS expression. Cervical eNOS staining was considered weak (grade 1) in 2, moderate (grade 2) in 2, and strong (grade 3) in 2 of the 6 women.

Western blotting

Western blotting confirmed the presence of protein for both of iNOS (130 kDa) and eNOS (140 kDA) isoforms in the cervix (Figure 6).

Figure 6. Examples of the detection of inducible nitric oxide synthase (iNOS) (panel A) and endothelial nitric oxide synthase (eNOS) (panel B) by Western blotting in one woman with early viable pregnancy and no treatment (No treat), and in one woman pretreated with mifepristone (Mife).

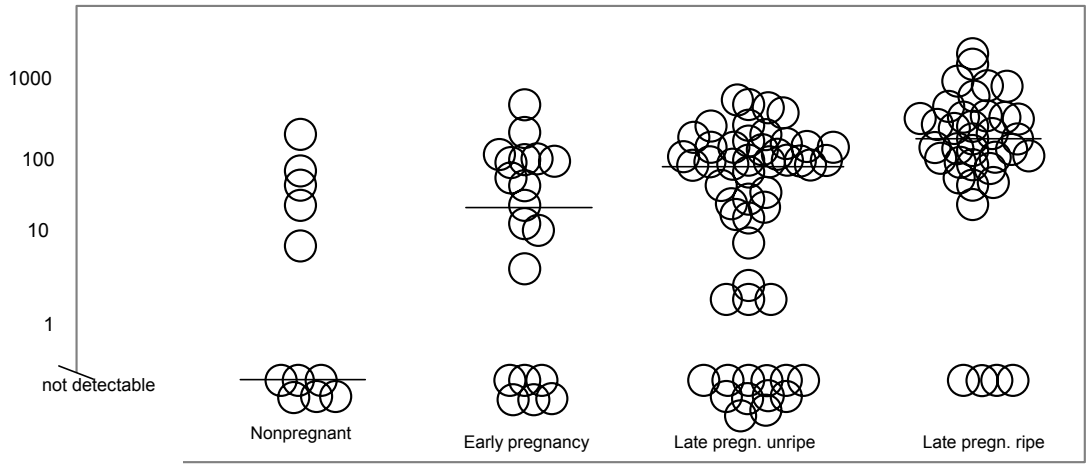


2. NITRIC OXIDE IN NORMAL PREGNANCY (Study I)

Cervical fluid Nox was detectable in 46% of the nonpregnant women, in 68% of the women in early pregnancy, and in 82% of the women in late pregnancy (Figure 7).

The cervical fluid Nox concentration in term pregnancy with a ripe cervix but without uterine activity was higher (p < 0.0001) than that in term pregnancy with an unripe cervix (Figure 7). Cervical NO release was higher (p = 0.008) in parous than in nulliparous women, and it was related to the Bishop score (r = 0.39; p = 0.01).

Figure 7: Cervical fluid Nox concentrations and group-specific medians in nonpregnant and pregnant (pregn.) women (μmol/L).



3. NITRIC OXIDE IN EARLY NONVIABLE PREGNANCY (Study II)

Women with missed abortion or blighted ovum more often had detectable and higher cervical fluid Nox levels than did women with early viable pregnancy (Figure 8). In addition, the Nox concentration in the missed abortion group was significantly higher than that in the blighted ovum group (Figure 8). In contrast, tubal pregnancy did not induce cervical NO release (Figure 8). The duration of amenorrhea was not a determinant as regards cervical fluid Nox, but women with a history of previous miscarriage had higher (p = 0.02) Nox levels (n = 21, median 73.9 μmol/L, 95% CI 52.2–95.1) than women without such a history (n = 71, median 20.0 μmol/L, 95% CI 12.6–46.4).

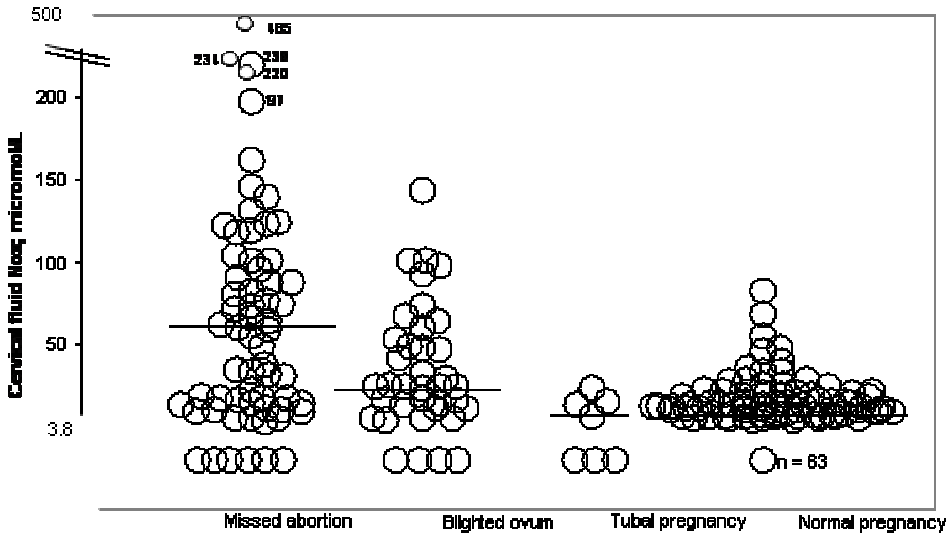
Cervical fluid Nox concentrations were inversely related to serum progesterone levels, but bore no relationship to serum hCG levels.

The likelihood of experiencing incomplete abortion following mifepristone-misoprostol or expectant management in the missed abortion or blighted ovum group was higher in women with median or lower cervical fluid Nox concentrations than in those with Nox levels above the group-specific median (Table 6).

Table 6. Rate of complete or incomplete abortion following a mifepristone-misoprostol regimen or expectant management in women with nonviable early pregnancies with regard to the group-specific median cervical fluid nitric oxide metabolite (Nox) level (*n*; %).

Variable	Cervical fluid Nox		*p*
	≤ median	>median	
Nonviable pregnancy	25	25	
• Complete	18 (72%)	24 (96%)	0.12
• Incomplete	7 (28%)	1 (4%)	0.04

Figure 8: Levels of cervical fluid nitric oxide metabolites (Nox) in women with missed abortion (*n* = 56), blighted ovum (*n* = 36), tubal pregnancy (*n* = 7) or normal intrauterine pregnancy (*n* = 140). The medians are shown by lines.



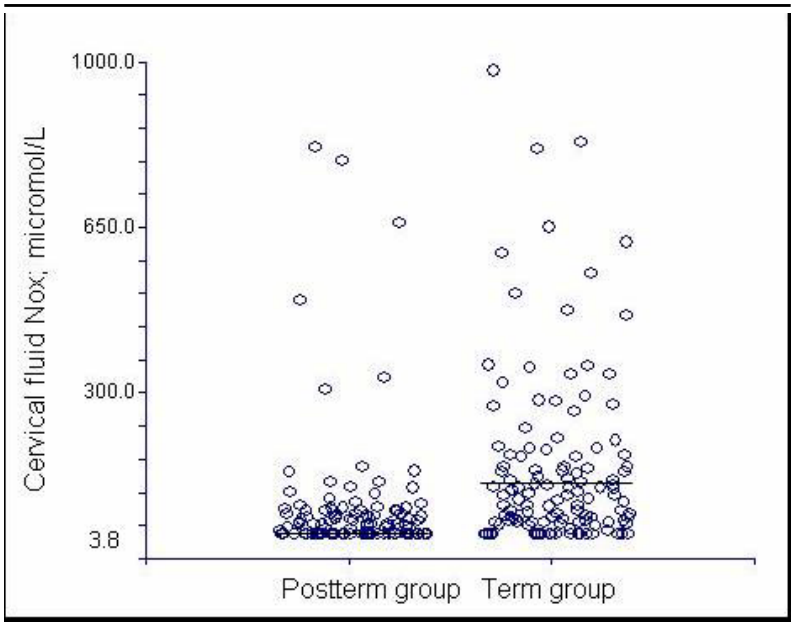
4. NITRIC OXIDE IN POSTTERM PREGNANCY (Study III)

Cervical fluid Nox levels were less often detectable and 4.5 times lower in women going postterm than in those delivering at term (Figure 9). Cervical fluid Nox levels were significantly and similarly related to Bishop score. However, women with postterm pregnancy exhibited a lower median cervical fluid Nox concentration against one Bishop score; this ratio was 7.8 in the postterm group compared with 17.7 in the term group.

The cervical fluid Nox concentration was inversely related to the time elapsed from sample collection to spontaneous initiation of labor and to the duration of delivery in women delivering postterm, but not in women delivering at term.

In women with postterm pregnancy, a cervical fluid Nox level below the median concentration (low Nox) was associated with a less ripe cervix, lower inducibility of labor and a longer duration of labor than in women with Nox above the median level (high Nox). Women with failed progression of labor were 8.1 times more likely to belong to the low Nox group than to the high Nox group.

Figure 9: Cervical fluid nitric oxide metabolite (Nox) concentrations in women going postterm and in women delivering spontaneously at term (μmol/L). Medians are shown by lines. The detection limit of the assay was 3.8 μmol/L.



5. EFFECT OF MISOPROSTOL (Study IV)

Cervical NO release was induced by vaginally administered misoprostol in pregnant, but not in nonpregnant women (Figure 10). Moreover, relatively similar stimulation in early and in late pregnancy could be accomplished with very different doses: the dose in late pregnancy was only 6% of the misoprostol dose administered in early pregnancy. This effect of misoprostol was specific, since placebo had no effect on cervical NO release either in early or in late pregnancy (Figure 10).

After misoprostol administration in the nonpregnant group, the cervix was softened in 29% of nulliparous women and in 38% of parous women. In the early pregnancy group this happened in 50% of nulliparous and in 71% of parous women. Use of placebo was associated with a softened cervix in 33% of parous women in early pregnancy. The baseline levels of Nox, and their responses, showed no differences between women with softened or tight cervixes. However, in the early pregnancy group, elevation of Nox levels (median or more) tended to occur more often (p = 0.09) in women with a softened cervix (70%) than in those with a tight cervix (29%) (Figure 11).

In the late pregnancy group, elevation of Nox levels following misoprostol was not related to changes in Bishop score (p = 0.33), but the median cervical fluid Nox concentration per one Bishop score rose 4-fold (97.7 vs. 24.3; p = 0.04) after misoprostol.

Figure 10: Cervical fluid nitric oxide metabolite (Nox) levels in percentages of initial values (mean ± SE) at 1, 2 and 3 hours after vaginal administration of misoprostol in nonpregnant women (-□-), in women in early pregnancy (-Δ-), in women in late pregnancy (-○-), and after placebo in women in early or late pregnancy (-◊-). The dose of misoprostol was 400 μg in nonpregnant subjects and in the early pregnancy group, and 25 μg in the late pregnancy group.
☆ = $p < 0.01$, ☆ = $p < 0.001$ in comparison with baseline.

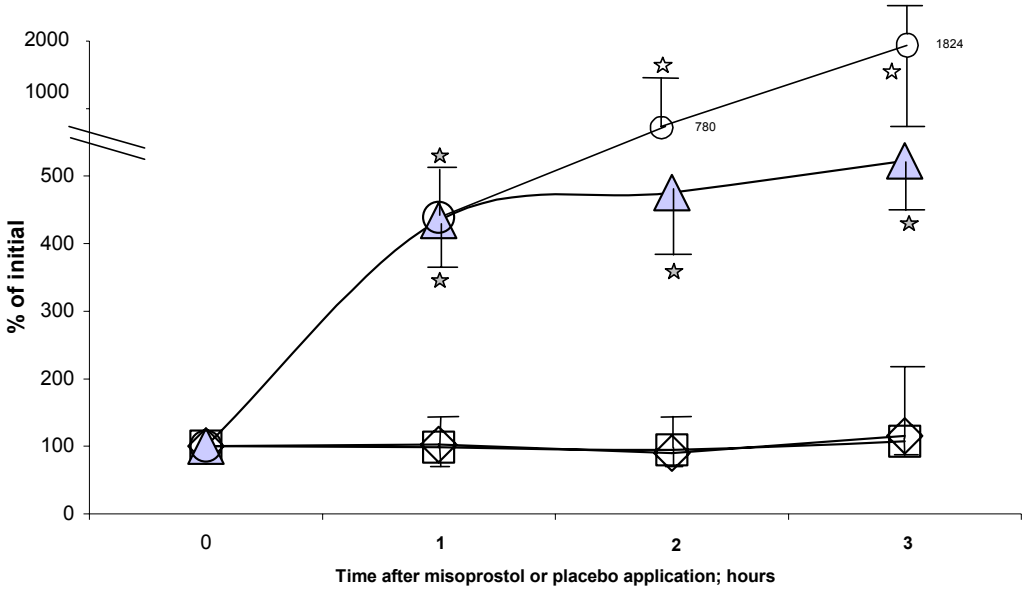
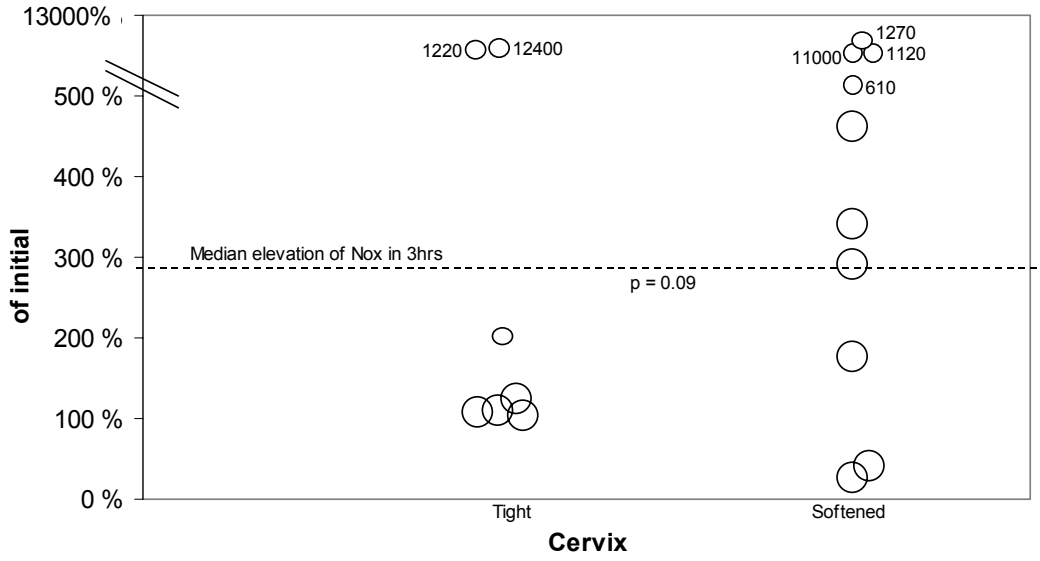


Figure 11: Cervical nitric oxide metabolite (Nox) elevations in 3 hours after vaginal misoprostol observed during Hegar dilation in 17 women with early pregnancy. Softened cervix = Hegar dilator of size 7 introduced into the cervix without force. Tight cervix = Hegar 7 introduction needing force.



6. EFFECT OF MIFEPRISTONE (Study V)

The administration of mifepristone in early viable pregnancy was followed by significant elevation in cervical fluid Nox concentrations (7.4- to 17.2-fold rises) in 1 to 3 hours (Figure 12).

Immunohistochemistry

iNOS

All women except one treated with mifepristone showed strong (grade 3) cervical iNOS staining, as compared with none in the control group (83% vs. 0%; $p = 0.002$). Additionally, iNOS was detected in the cervical glands.

eNOS

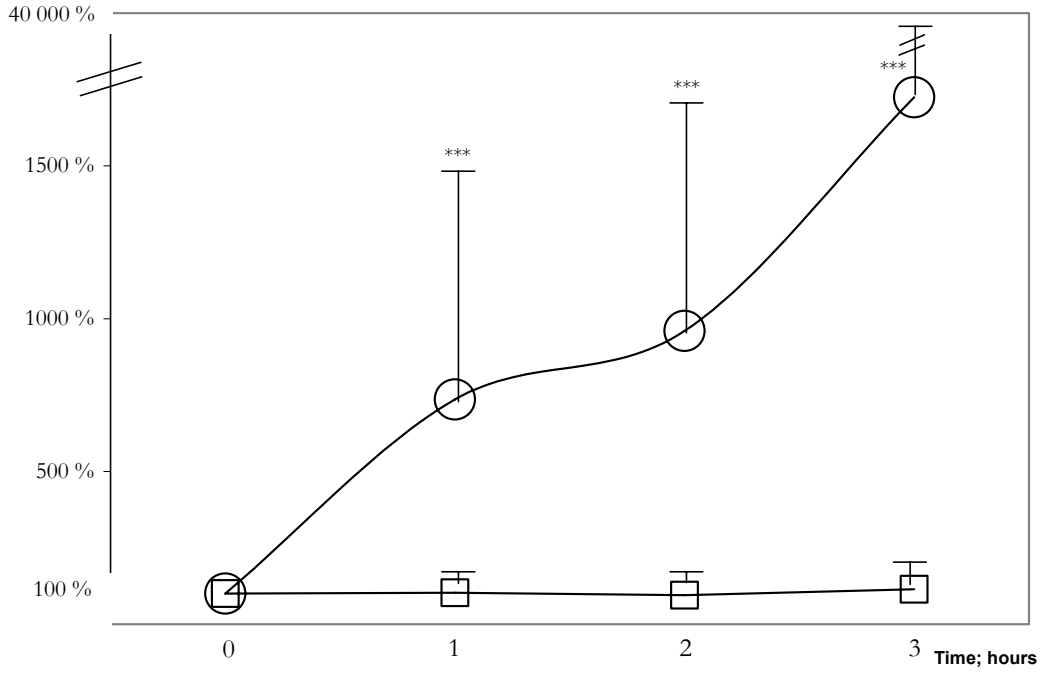
The localization and the pattern of immunostaining of eNOS in mifepristone-treated women did not differ from that in the controls.

Western blotting

Western blotting confirmed the presence of protein for both of iNOS (130 kDa) and eNOS (140 kDA) isoforms in the cervix (Figure 6).

Figure 12: Levels of nitric oxide metabolites (Nox) in cervical fluid (percentages of initial values; mean ± SD) in women in early pregnancy (-○-) at 1, 2 and 3 hours after administration of mifepristone (200 mg), and placebo (-□-).

*** = $p < 0.001$ in comparison with baseline. The 1-hour response differed significantly (p = 0.02) from the 3-hour one in the mifepristone group, and placebo had no effect.



DISCUSSION

Since the discovery that endothelium-derived relaxing factor was NO (Ignarro *et al.* 1987, Palmer *et al.* 1987), a large body of research has revealed that NO acts as a mediator in a variety of physiological and pathophysiological conditions (Moncada and Higgs 1993, Davis *et al.* 2001, Korhonen *et al.* 2005). Because impaired endothelium-derived vasodilatation is related to coronary heart disease and atherogenesis (Hambrecht *et al.* 2000), NO is described as the primary endothelium-derived anti-atherosclerotic factor (Sumi *et al.* 2001). In addition to its vasoregulatory properties, NO exerts inhibitory effects on leukocyte adhesion and platelet aggregation (Änggård 1994, Mayer and Hemmens 1997, Ignarro *et al.* 1999, Aktan 2004). Furthermore, NO is also involved in inflammation, host defense, as well as in reproduction (Maul *et al.* 2003a, Aktan 2004, Korhonen *et al.* 2005).

Feasible assay

Although NO has been implicated most strongly with vascular physiology, a plausible possibility existed (Chwalisz and Garfield 1997, Chwalisz *et al.* 1999, Maul *et al.* 2003a) that it could also be involved in human cervical ripening. Therefore, we developed a method for the assessment of NO metabolites (Nox) in cervical fluid, a method that employs spectrophotometry and Griess reagent (Green *et al.* 1982). This method was reproducible and no correlation was observed between plasma and cervical fluid Nox concentrations. Cervical fluid Nox levels were not affected by dietary intake in Study I. Although we instructed our subjects in Study I to follow a Nox-free diet before sampling, we were unable to confirm if dietary restrictions were followed at home. Furthermore, the Nox concentration in sperm was only 1 to 10% of that in cervical fluid, suggesting that sperm, possibly present in cervical fluid, is not a major confounder as regards assessment of cervical fluid Nox, nor does it account for high values assayed. In contrast, cervical palpation accelerated cervical NO release, as it is known to trigger cervical ripening through a number of local bioregulators (McColgin *et al.* 1993), and therefore no palpation was allowed during a three-hour period prior to sample collection. Rupture of the membranes decreased cervical fluid Nox values by 40%. This may be due to the flushing effect of amniotic fluid, since Nox concentrations in amniotic fluid were only 10% of the level of cervical Nox. Therefore, all women with ruptured membranes were excluded. The presence of blood in cervical fluid resulted in a reduction of Nox levels, because hemoglobin binds to NO and forms a complex of nitrosylhemoglobin (HbNO) (Kankaanranta *et al.* 1996). That is why we carefully excluded all women whose cervical fluid samples were visibly bloody. Cervicovaginal infections, accompanied by a release of cytokines and PGs, may stimulate NO production (Fang *et al.* 1999, 2001, Maul *et al.* 2003a) and therefore we excluded women with any signs of cervicovaginal infection. Finally, to confirm the synthesis of NO in cervical cells, we assessed the concomitant expression of iNOS and eNOS by immunohistochemistry and Western blotting in early pregnancy, and related them to Nox levels. Both these methods carry some uncertainties, since the used antibodies may cross-react with enzymes other than NO synthases, and therefore, for further confirmation of the presence of iNOS, the polymerase chain reaction (PCR) method should be employed. Nevertheless, on the whole, we are confident that assay of cervical fluid Nox is a feasible and reliable method for assessing cervical NO release.

Stimulatory effect of pregnancy

Our data show that cervical NO release increases during human pregnancy. These results are in line with those from animal studies (Buhimschi *et al.* 1996, Ali *et al.* 1997, Chwalisz and Garfield 1998, Garfield *et al.* 1998, Maul *et al.* 2003a) and some observations on humans, obtained by methods different from ours (Tschugguel *et al.* 1999, Ledingham *et al.* 2000, Bao *et al.* 2001). The correlation between cervical NO release and Bishop scores, and the finding that parous women have higher cervical fluid Nox concentrations than nulliparous women are novel, to the best of our knowledge, and these data fit well with the hypothesis that local NO has a role in cervical ripening (Biondi *et al.* 2005). A recent study showed that the iNOS gene was downregulated in the cervixes of women after term vaginal labor (Huber *et al.* 2005). This may imply that during active labor iNOS-derived cervical NO release is no longer needed, because the primary task of cervical iNOS is in cervical ripening.

Nonviable early pregnancy

Women with failed early intrauterine pregnancy showed elevated cervical NO release. We do not believe that increased cervical NO, although a very reactive molecule, is a primary cause of miscarriage. Our view is supported by the reduced placental expression of iNOS at the fetomaternal interface in women with spontaneous abortion when compared with that in women with early viable pregnancy (Marinoni *et al.* 2004). Our findings could have the following explanations. First, elevated release of NO in the dying fetus, decidua, and/or fetal membranes has been found in LPS-induced abortions in animal experiments (Haddad *et al.* 1995, Ogando *et al.* 2003b), but no such human data exist so far. However, it appears plausible that remnants of the conceptus could have released NO excessively, which could have leaked, as either NO or Nox, into the cervical canal. This hypothesis is supported by our findings: cervical fluid Nox levels were elevated only in intrauterine miscarriages, not in tubal ones, and missed abortion, with potentially more abundant remnants of conception, was characterized by higher cervical fluid Nox levels than cases of blighted ovum. Second, spontaneous abortion is often associated with a local inflammatory reaction in the cervix, and this may result in the stimulation of NO release through PGs or cytokines (Sennström *et al.* 2000, Maul *et al.* 2003a). In addition, many other hormones, such as inhibins (Reis *et al.* 2000, Muttukrishna *et al.* 2002, Lahiri *et al.* 2003), may be involved in spontaneous abortion and may have secondarily stimulated cervical NO release in our subjects. Third, increased cervical NO release may be a specific phenomenon in abortion, perhaps triggered by a fall in the level of progesterone either locally or in the serum (Figure 13).

Postterm pregnancy

Cervical NO release was deficient in postterm pregnancy. We do not know if cervical NO deficiency is a primary phenomenon, and thus a true contributing factor to postterm pregnancy, or whether it is a reflection of relative insufficiency of PGs, cytokines, MMPs, or some other agents which may be primarily involved in cervical ripening (Sennström *et al.* 1997, Sennström *et al.* 2000, Yoshida *et al.* 2001, Kelly 2002, Stygar *et al.* 2002, Yoshida *et al.* 2002, Osman *et al.* 2003, Sennström *et al.* 2003, Stjernholm-Vladic *et al.* 2004a) and which may stimulate NO release. A high progesterone level seems to downregulate the synthesis and release of cervical NO. As many as 20–30% of parous women repeatedly carry postterm (Olesen *et al*. 2003). This characteristic seems to be genetically

determined (Laursen *et al.* 2004), and therefore we speculate that "postterm genes" are functionally linked to the genes regulating NO synthases. Such women might benefit from the administration of a vaginal NO donor when induction of labor is needed.

Relationships to prostaglandins

It is well established that NO and PGs operate jointly in many cells (Dong *et al.* 1999, Ledingham *et al.* 1999a, Boiti *et al.* 2003, Hausman *et al.* 2003, Aktan 2004, Gookin *et al.* 2004, Timoshenko *et al.* 2004). Nitric oxide may either stimulate or inhibit the release of COX-2, and likewise, PGs may have a stimulatory or inhibitory effect on iNOS, depending on the cell type and/or the presence of cofactors (Goharkhay *et al.* 2002, Maul *et al.* 2003a, Ogando *et al.* 2003a, Timoshenko *et al.* 2004). Our data show that misoprostol as a PG analogue induces NO release in the uterine cervix of pregnant women, and furthermore, the response of cervical NO release to PG becomes enhanced from early to late pregnancy. Thus, PG analogues such as misoprostol can perhaps initiate a chain reaction in the cervix of pregnant women; the initial NO stimulation caused by misoprostol is followed by endogenous release of PGs triggered by NO. As a result, NO, PG and COX pathways may have a joint action in human cervical ripening, as schematically shown in Figure 13.

Reducing effect of progesterone

The results of numerous animal experiments support the view of progesterone having opposing effects on NO release in the endomyometrium and cervix; it upregulates NO release in the former, but downregulates it in the latter (Buhimschi *et al.* 1996, Ali *et al.* 1997, Chwalisz and Garfield 1998, Garfield *et al.* 1998, Maul *et al.* 2003a). Our data imply a link between progesterone and cervical NO (Figure 13). First, the lower the progesterone level the higher the detection rate of cervical fluid Nox in nonpregnant women. In fact, 93% of women in the follicular phase, versus 46% of women in the luteal phase showed detectable cervical fluid Nox. Circulating Nox levels are also higher during the follicular phase and at the time of ovulation than in the luteal phase (Ekerhövd *et al.* 2001). Second, cervical NO release was inversely related to circulating progesterone concentrations in early nonviable pregnancy in our study. Women with threatened abortion or preterm birth have considerably lower levels of circulating progesterone than women with normal pregnancy (Gruber and Huber 2005). Progesterone insufficiency could well have stimulated cervical NO release in nonviable intrauterine pregnancy, which may be needed for ripening of the cervix during the course of miscarriage. Third, cervical NO responded to the antiprogestin mifepristone with a 17-fold increase in cervical fluid Nox and with increased expression of iNOS in early viable pregnancy. Furthermore, mifepristone induced the appearance of iNOS in cervical glands, which is a novel finding.

The mechanisms behind mifepristone-induced NO release are not known, but a PR-mediated pathway may be involved (Stjernholm-Vladic *et al.* 2004b). Local progesterone withdrawal in the cervix brought about by mifepristone may lead specifically to the stimulation of iNOS. Alternatively, the anti-glucocorticoid properties of mifepristone, blocking glucocorticoid receptors, may stimulate iNOS (Alderton *et al.* 2001). This would fit well with data showing that the levels of glucocorticoid receptor decrease in the human cervix during labor (Stjernholm-Vladic *et al.* 2004a). Furthermore, it is possible that mifepristone triggers an influx of inflammatory cells, such as macrophages, neutrophils and

monocytes, which are rich in iNOS. Moreover, mifepristone upregulates various MMPs and/or the secretion of cytokines in cervical cells (Denison *et al.* 2000, Maul *et al.* 2003a, Stjernholm-Vladic *et al.* 2004a). These mediators may either induce or inhibit iNOS depending on the availability of various cofactors (Denison *et al.* 2000, Alderton *et al.* 2001). Furthermore, NO may act in concert with the COX pathway, especially with COX-II (Brune *et al.* 1998, Hapangama *et al.* 2002, Hausman *et al.* 2003, Maul *et al.* 2003a) (Figure 13). Nitric oxide in turn may soften the cervix by remodeling the ECM (Maul *et al.* 2003a) (Figure 13), where cytokine-induced NOS is centrally involved (Tschugguel *et al.* 1999, Maul *et al.* 2003a), or by inducing apoptosis of cervical cells (Brune *et al.* 1998, Maul *et al.* 2003a). Taken as a whole, our data and those of others (Ali *et al.* 1997, Chwalisz and Garfield 1997, Chwalisz *et al.* 1999, Maul *et al.* 2003a) can be seen as good evidence that NO in the cervix, and progesterone, are functionally related in pregnancy (Figure 13). In fact, administration of progesterone is used in treatment of threatened abortion or preterm birth (Brancazio *et al.* 2003, da Fonseca *et al.* 2003, Greene 2003, Di Renzo *et al.* 2005, Schindler 2005b).

Possible clinical applications

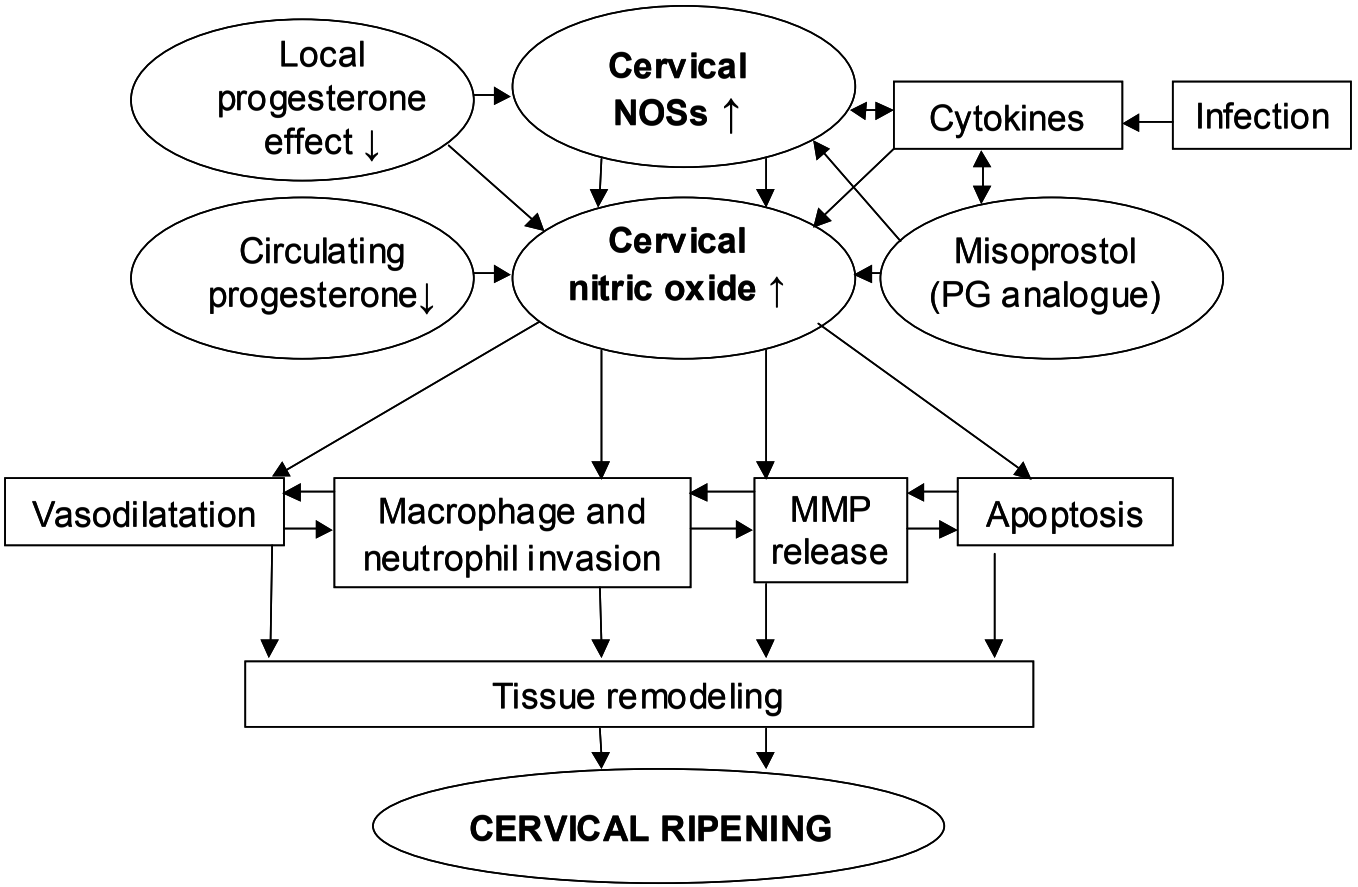
Cervical fluid Nox may perhaps be used as a marker of cervical ripeness in clinics. Clinicians could benefit from its assessment both in early and late pregnancy because this test may indicate the readiness of the cervical canal for misoprostol or mifepristone priming. As regards early pregnancy, women with nonviable early pregnancy and “high” cervical fluid Nox could perhaps be treated expectantly, because these women might belong to the 50% of such women who will bleed and abort spontaneously within 2 weeks (Condous *et al.* 2003). If the test result is “low”, priming of the cervix with PGs or mifepristone can be considered. In addition, our data allow us to speculate that women with an unripe cervix may benefit from treatment with a NO donor. These questions should be studied in clinical trials. Likewise, as deficient cervical NO release was related to failed progression of labor, the cervical fluid Nox level may perhaps be used to predict the likelihood of successful induction of labor in postterm pregnancy. In early as well as in postterm pregnancy, NO donors may hold a promise, but this question has not been studied so far.

No reliable biochemical marker for the identification of women at risk of preterm birth exists. Fetal fibronectin and IGFBP-1 have been extensively studied as marker candidates, but they have poor predictive values and there are major confounders in sample collection (Ascarelli and Morrison 1997, Goepfert *et al.* 2000, Kekki *et al.* 2001). Transvaginal ultrasonography is also insufficient in predicting preterm birth (Honest *et al.* 2003, Iams 2003). Although we did not study women with preterm birth, our data suggest that cervical fluid Nox might prove to be a feasible marker for this condition. An epidemiological study in this connection would need a huge number of women; the risk of spontaneous preterm birth being 2–3% (Perinatal statistics in Nordic countries 2004, Boggess 2005). Therefore, we should first study women who report preterm contractions in order to see if this test can differentiate between those women who carry to term and those who deliver preterm. Some preliminary data suggest that this test could be useful in such women (Facchinetti *et al.* 2005). Moreover, it could be worthwhile studying if cervical fluid Nox levels are related to fFN or IGFPB-I levels, which are both of some value in prediction of preterm birth (Ascarelli and Morrison 1997, Goepfert *et al.* 2000, Kekki *et al.* 2001). A possible

correlation between cervical fluid Nox and the concentrations of these substances may pave the way to more extensive clinical studies on the clinical usefulness of the cervical fluid Nox test in the prediction of preterm birth.

In summary, our data support the concept that the NO pathway, alone or jointly with PGs and progesterone (Figure 13), is involved in human cervical ripening.

Figure 13. A schematic model of the possible role of cervical nitric oxide in human cervical ripening, as evidenced and supported by our data (ellipses) and by literature (boxes). NOSs: nitric oxide synthases PG: prostaglandin MMP: matrix metalloprotease



CONCLUSIONS

On the basis of the present work, the following conclusions can be drawn:

1. Cervical NO release as analyzed by cervical fluid Nox levels is related to cervical ripening during pregnancy.
2. Cervical NO release increases in early nonviable intrauterine pregnancy, and "low" NO release predicts incomplete abortion after medical or expectant management.
3. Cervical NO release is deficient in postterm pregnancy and this deficiency may contribute to failed progression of labor in postterm women.
4. Cervical NO release is upregulated by misoprostol in pregnant, but not in nonpregnant women. The sensitivity of NO release towards misoprostol is enhanced from early to late pregnancy.
5. Cervical NO release is downregulated by progesterone both in pregnant and nonpregnant women. Moreover, antiprogestin stimulates cervical NO release in early viable pregnancy.
6. Nitric oxide is suggested to have a role in human cervical ripening.

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Mervi Väisänen-Tommiska

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