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Clinical, biochemical and morphological aspects of cervical ripening in the first trimester

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Anja 8 år

To my family

Abstract

Background: The uterine cervix has the ability to be transformed from being a rigid organ in non-pregnant women to become a loose structure that dilates and allows passage of the fetus at parturition. This process, cervical ripening, has been described similar to an inflammatory reaction of the extracellular matrix involving activation of inflammatory cytokines, matrix metalloproteinases and breakdown of the collagen framework. Cervical ripening can be induced prior to surgical termination of pregnancy by agents such as prostaglandins (PGs) and nitric oxide (NO). It appears reasonable that cervical ripening takes place as a spontaneous event in women with miscarriage before expulsion of gestational products.

Aims: The aims of the thesis were to investigate clinical, morphological and biochemical aspects of cervical ripening in the first trimester, both when induced by PGs or NO donors and when spontaneously occurring in women with symptomatic and silent miscarriage.

Methods: Nulliparous women admitted for surgical termination of pregnancies in the first trimester were randomized to cervical priming with either gemeprost or misoprostol (Study I), and to misoprostol or isosorbide mononitrate (IMN) (Study II). In Study I and II, the efficacy of cervical priming was measured by tonometry. Side effects were also estimated. In Study III cervical biopsies from women treated with misoprostol or IMN were analyzed using electron microscopy (EM). Inflammatory parameters were analyzed by ELISA (IL-8) and immunohistochemistry (IHC) (MMP-1, MMP-9). In Study IV biopsies were obtained from nulliparous women suffering symptomatic or silent miscarriage and from women undergoing surgical termination of pregnancy. Morphology was studied by EM and inflammatory parameters by ELISA (IL-8) and IHC (IL-8, MMP-1, MMP-8, MMP-9).

Results: There was no difference in baseline cervical dilation and cumulative force to dilate the cervix to 10mm in women treated with misoprostol compared to gemeprost. Cervical resistance was higher in women treated with IMN compared to women treated with misoprostol. Abdominal pain and vaginal bleeding were frequent following cervical priming with misoprostol, while headache was common following IMN. In cervical specimens from women treated with misoprostol the collagen framework was disorganized and fibroblasts and mast cells appeared activated. Similar ultrastructural changes were observed in specimens from women treated with IMN, though less pronounced. Cervical tissue levels of IL-8 were higher in women treated with misoprostol compared to IMN and controls. Immunohistochemical staining for MMP-1 and MMP-9 was of higher intensity in women treated with IMN compared to misoprostol. In cervical tissue from women with miscarriage the organization of the collagen framework was deranged. Fibroblasts were reactive and mast cells were frequently observed and demonstrated secretory activity. Tissue levels of IL-8 were increased in women with miscarriage. Immuno-

positivity of MMP-1 and MMP-8 did not differ between women with miscarriage and control women. MMP-9 was lower in women with symptomatic miscarriage compared to women with silent miscarriage and controls.
Conclusions: Misoprostol is as effective as gemeprost for cervical priming in the first trimester. Misoprostol induces a more pronounced cervical ripening than IMN, but both treatments are associated with side effects when the treatment interval exceeds 4 hours. Both misoprostol and IMN induces a tissue response consistent with an inflammatory reaction. In women suffering either symptomatic or silent miscarriage an inflammatory response takes place, indicating an ongoing ripening process. Therefore, inadequate cervical remodelling does not seem to be the reason why some miscarriages remain silent.

Key words: cervical ripening, misoprostol, nitric oxide, IL-8, MMP-1, MMP-8, MMP-9, miscarriage, electron microscopy

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Contents

List of publications

This thesis is based on the following articles :

I. Gemeprost versus misoprostol for cervical priming before first-trimester abortion: a randomized controlled trial.
Ekerhovd E, Radulovic N, Norström A.
Obstet Gynecol. 2003;101:722-5.

II. Outpatient cervical ripening before first-trimester surgical abortion: a comparison between misoprostol and isosorbide mononitrate.
Radulovic N, Norström A, Ekerhovd E.
Acta Obstet Gynecol Scand. 2007;86:344-8.

III. Cervical priming in the first trimester: morphological and biochemical effects of misoprostol and isosorbide mononitrate.
Vukas Radulovic N, Ekerhovd E, Abrahamsson G, Norström A.
Acta Obstet Gynecol Scand. 2009;88:43-51

IV. Cervical tissue changes in women with miscarriage: a morphological and biochemical investigation.
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Abbreviations

CD45	leukocyte common antigen
COX-1	cyclooxygenase-1
COX-2	cyclooxygenase-2
ECM	extracellular matrix
EM	electron microscopy
ELISA	enzyme-linked immunosorbent assay
FACITs	fibril-associated collagens with interrupted triple helices
GAG	glycosaminoglycan
IHC	immunohistochemistry
IF-γ	interferon gamma
IL-1	interleukin-1
IL-6	interleukin-6
IL-8	interleukin-8
IMN	isosorbide mononitrate
MMP	matrix metalloproteinase
MPA	misoprostol acid
N	Newton
NADPH	nicotinamide adenine dinucleotide phosphate-oxidase
NFκB	nuclear factor kappa B
NO	nitric oxide
NOS	nitric oxide synthase
PG	prostaglandin
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
RCOG	Royal College of Obstetricians and Gynaecologists
TNFα	tumor necrosis factor alpha
TIMPs	tissue inhibitors of metalloproteinases

Background

Introduction

Cervical ripening is a physiological and crucial event in pregnancy and parturition. It is part of an ongoing process of tissue remodelling. At term softening of the cervix is a prerequisite for uncomplicated vaginal delivery. Remodelling of cervix prior to onset of labour has been described to be similar to an inflammatory process. Thus, it includes activation of inflammatory agents and influx of leucocytes, which via complex pathways leads to rearrangement and breakdown of the collagen framework and tissue oedema. Several lines of evidence have shown that the mechanisms involved in cervical ripening at term are comparable to those that occur following pharmacologically or mechanically induced cervical softening prior to elective surgical abortion in the first trimester or spontaneous cervical softening in early miscarriages. The aims of this thesis were to provide further clinical, biochemical and morphological insight into the process of cervical ripening in women having cervical priming with either prostaglandin (PG) or nitric oxide (NO) prior to elective surgical abortion in the first trimester as well as in women having miscarriages.

Anatomy and histology

The nonpregnant human uterus is pear-shaped. It is located between the bladder and the rectum. The uterus of an adult nulliparous woman is 6-8 cm long and weighs in average 50-70 g, while in multiparous women the uterus is 9-10 cm long and weighs approximately 80 g. However, there are considerable individual differences in length and weight of the uterus (Danforth 1947). The uterus consists of two major parts, the corpus and the cervix. These parts are connected by a minor segment, the isthmus. The cervix is of a cylindrical shape. It protrudes into the vagina with its portio vaginalis. The upper part of the cervix is named portio supravaginalis. The uterine corpus is mainly composed of smooth muscle (70%), while the cervix contains mostly connective tissue and only 10-15% smooth muscle.

The endocervical canal connects the uterine corpus cavity with the vagina from the internal os to the external cervical os. It is coated with single layer columnar epithelium that at the external os continues as squamous epithelium covering the portio vaginalis and vagina. The endocervical mucosa contains highly branched glands that open into the endocervical canal (Cunningham 2005). The underlying connective tissue stroma, the extracellular connective matrix (ECM), consists predominantly of collagen fibers and a minor portion of elastic fibers. Fibroblasts are the dominating cells within the cervical stroma. Among other connective cells, mast cells appear relatively frequently. Smooth muscle cells are distributed in bundles with an increasing proportion towards the internal os. The interfibrillar, intercellular ground substance constitutes mainly of proteoglycan complexes, which regulate the organization of the fibrillar network (Leppert, Keller et al. 1983; Uldbjerg, Malmström et al. 1983).

Both blood vessels and nerves are encountered in the cervix. The vascular supply of the uterus derives from a. uterina and a. ovarica. Venous sinuses in the uterine wall

empty into v. uterina and further into v. iliaca interna. Sympathetic nerves arise from the aortic plexus just below the promontory of the sacrum, enter the pelvis via plexus iliacus internus and join the uterovaginal plexus of Frankenhäuser, giving branches to supply the uterus, bladder and upper part of vagina. The pelvic nerve, that also joins the plexus of Frankenhäuser, represents the parasympatic nervous system. It derives from the second, third and fourth sacral nerves. Painful stimuli of myometrial contractions are transmitted in the 11th and 12th thoracic nerves, while sensory stimuli from the cervix and upper part of the birth canal are transmitted via the 2nd, 3rd and 4th sacral nerves.

Physiology of the uterus in pregnancy

Early views of uterine function at parturition were emphasized on the role of the expulsive forces, giving the cervix a passive role. Following several decades of research it became clear that the cervix is a dynamic structure, having an important role during late gestation and at parturition. During pregnancy the uterus is transformed into a thin-walled muscular organ with a volume capacity that is 500 to 1000 times greater than in the non-pregnant state (Cunningham 2005). It accommodates the fetus, placenta and amniotic sac keeping the myometrium in a relatively quiescent state, the cervix being closed, rigid and resisting tension throughout the most part of gestation (Schwalm and Dubrauszky 1966). Remodelling of the uterine tissue, both within the corpus and the cervix, occurs throughout pregnancy where both smooth muscle and connective tissue constituents increase and undergo biochemical and physiological alterations (Hjelm, Barchan et al. 2002). In late stages of pregnancy, *i.e.* from about the 32nd week of gestation, softening of the cervix gradually starts as an ongoing process right up to parturition and proceeds in a reparative involution immediately *post partum*. In cases where the ripening starts early in pregnancy it might lead to preterm birth (Iams, Goldenberg et al. 1996). On the other hand, if cervical ripening fails, it might contribute to cervical dystocia and dysfunctional labor (Ekman, Malmström et al. 1986; Kjaergaard, Olsen et al. 2008).

Extracellular matrix (ECM)

The extracellular matrix is vital to sustain the structural integrity of all tissues. It is not a passive part of the tissue. On the contrary, it has an effect on cell shape, cell adhesion, cell migration, cell growth and differentiation as well as cell death (Madri and Basson 1992; Lin and Bissell 1993; Friedl and Brocker 2000). Synthesis, accumulation and catabolism of the ECM are involved in wound healing, the initiation and progression of numerous diseases as well as in physiological processes. Each type of anatomical structure has its own specific biochemical composition of the ECM, adapted to specific functional demands. For example, it reinforces the arrangement of collagen fibres in ligaments, adapts to transparency in the cornea and attains capacity to be calcified in bone.

The major components of the ECM in the cervix include collagen and elastic fibers and non-fibrillar components, mainly proteoglycans, fibronectin and laminin. Collagen fibers form the basis for the tensile strength of the tissue. Elastic fibers, composed of elastin, contribute to the viscoelastic properties of the tissue. Proteoglycans

are responsible for the organization of the fibrillar components and tissue hydration (Leppert 1995). Fibronectin, by binding to integrins on the cell surface, influences cell anchoring and may further affect tissue integrity by binding to collagens and proteoglycan complexes.
Fibroblasts, the predominant cell type in the cervix, are responsible for producing ECM proteins, *i.e.* collagen, proteoglycans and fibronectin as well as enzymes involved in the turnover of these proteins.

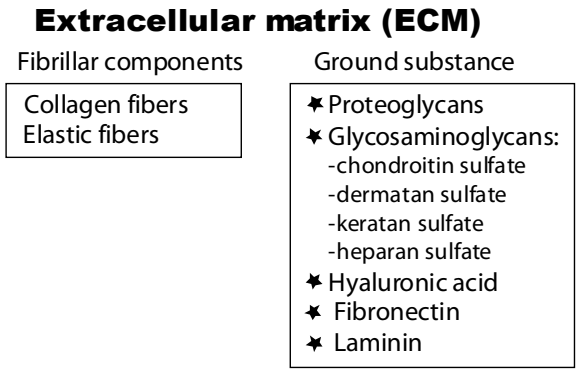

Figure 1. Main components of the extracellular matrix.

Collagens

Collagens are a family of ECM proteins that play a dominant role in maintaining the structure of various tissues. In vertebrates there are at least 27 types of collagen and they are numbered with roman numerals in the order of their discovery. All collagen molecules consist of three polypeptide chains, coiled to each other into a helix and wound around a common axis to form a triple helical structure, giving a final structure of a rope like rod (Myllyharju and Kivirikko 2004). Amino acids, important for the packing and the stability of the triple helix, are glycine, proline and 4-hydroxyproline. The collagens can form different supramolecular assemblies such as fibrils (collagen type I, II, III, V, XI, XXIV, XXVII), basement membrane collagen (collagen type IV), beaded filaments (collagen type VI), hexagonal networks (collagen type VIII, X), anchoring fibrils (collagen type VII), transmembrane collagen (collagen type XIII) and fibril-associated collagens with interrupted triple helices (FACITs) found on the surfaces of collagen fibrils.
Collagen I is the most abundant type of fibril forming collagen and is the major collagen of bone, tendons, ligaments, skin and cornea. The collagen typ I triple helix is usually formed as a heterotrimer and provides tensile strength in the tissue. Collagen type III is widely distributed in tissues that contain collagen I, with the exception of bone. It is localized in elastic tissues and is part of reticular fibres in the interstitial tissue of lungs, spleen, liver, dermis and vessels (Prockop and Kivirikko 1995; Gelse, Poschl et al. 2003). In wound healing and embryo development the collagen type III is abundant.

The predominant cervical collagen is type I with a smaller content of type III (Kleissl, van der Rest et al. 1978). Collagen IV is mainly located in the basal lamina at the border between epithelial cells and adjacent stroma. During pregnancy, the total amount of collagen decreases by approximately 30%, which, concomitantly with increasing tissue hydration, leads to decreased collagen concentrations at term (Uldbjerg, Ekman et al. 1983; Minamoto, Arai et al. 1987; Timpl and Brown 1996). The biochemical and biophysiological properties of cervical collagen, secondarily to changes in the proteoglycans, undergo major changes during pregnancy leading to increased collagen solubility (Ito, Kitamura et al. 1979). During cervical ripening collagen turnover increases and there is a disruption of the tightly aligned collagen fibrils. The net loss in collagen fiber alignment and decrease in fiber length lead to loss of tensile strength, thereby contributing to the ripening process (Leppert 1995).

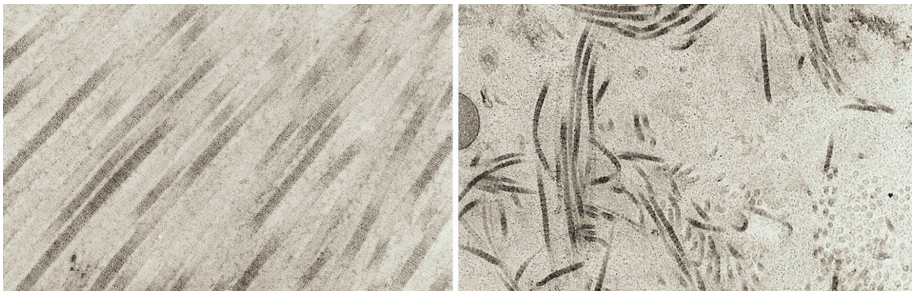

Figure 2. Electron microscopy of cervical tissue. Intact collagen x 52500 (left); disorganized collagen network x10250 (right).

Matrix proteoglycans

Proteoglycans are core proteins with a single core protein and large numbers of unbranched glycopolysaccharide side chains, termed glycosaminoglycans (GAGs). Matrix proteoglycans are divided into three main families, *i.e.* the hyalectans (proteoglycans interacting with hyaluronan and lectins), the small leucine-rich repeat proteoglycans (SLRPs) and basement membrane proteoglycans (Iozzo 1998). There are four major GAGs found in covalent association with proteoglycans, chondroitin sulfate, (chondroitin-4-sulfate and chondroitin-6-sulfate), dermatan sulfate, keratan sulfate and heparan sulfate. The GAGs constitute up to 90% of the mass of proteoglycans. Hyaluronic acid is a space-filling GAG. It is not covalently associated in the proteoglycan aggregate, though one end of the core protein interacts in a highly specific way with hyaluronic acid (Naftolin 1980; Iozzo 1998). Hyaluronic acid is essential for the organization of the proteoglycans in the ECM and tissue hydration. The composition and turnover of GAGs in the cervix appear to be a dynamic process during pregnancy and labour. Glycosaminoglycan concentrations in cervical tissue increase during pregnancy and peak at the onset of labour with a marked decrease during parturition (Osmers, Rath et al. 1993). However, during labor there is a relative increase in heparan sulfate proteoglycans as well as hyaluronic acid (Gran-

ström, Ekman et al. 1989; Ruscheinsky, De la Motte et al. 2008). Dermatan sulfate proteoglycans are suggested to regulate cervical tissue stability (Toole and Lowther 1968). A decrease of dermatan sulfate proteoglycans during cervical ripening implies a reduction of bridges between dermatan sulfate and collagen, which, together with increasing hyaluronic levels and subsequent tissue hydration, contributes to loss of cervical tissue strength.

Cervical ripening

In 1895, softening of the lower uterine segment was described as an early event during pregnancy, known as the Hegar's sign. Being characteristic and evident on physical examination, it was customarily used for diagnosis of pregnancy. However, the most overt changes in the cervix during pregnancy occur in late gestation and at parturition. The clinically evident change of cervical consistency is an expression of connective tissue remodelling and occurs in four stages: softening, ripening, dilation, and repair (Word, Li et al. 2007). The softening stage of cervical remodelling is a slow process, involving the turnover and rearrangement of the ECM leading to increased tissue compliance. During softening of the cervix the collagen fibers reorganize with an alignment in the direction of the mechanical stress (Leppert 1992). The reduction of dermatan sulfate bridges to collagen fibrils underlies this rearrangement (Uldbjerg, Ekman et al. 1983; Rajabi, Dean et al. 1988; Osmers, Rath et al. 1993). Elastic fibers, muscle fibers and fibroblasts are likewise joined in parallel alignment giving this structural arrangement a polarized strength. Towards term, synthesis of hyaluronic acid is stimulated and a double increase is observed at parturition (Osmers, Rath et al. 1993). Hyaluronic acid attracts water, giving increased tissue hydration, which contributes to the softening of the cervix (Leppert 1995). The softening of the cervix overlaps with the ripening phase. During the ripening phase, which is preceded by a shift in sex steroid levels with a relative decline of progesterone (Romero, Scoccia et al. 1988; Challis, Matthews et al. 2000), there is a further increase in tissue compliance and an increased influx of inflammatory cells in the cervical stroma. The onset of uterine contractions initiates the rapid phase, dilation of the cervix. The tissue water contents further increase and contribute to increased cervical distensibility (Ruscheinsky, De la Motte et al. 2008). The last phase, *post partum* repair, starts right after the expulsion of the fetus and is consistent with repair and return to the dense and ordered connective tissue of the non-pregnant cervix (cervical involution). During the repair there is a further increased expression of genes encoding matrix metalloproteinases (MMPs), ECM proteins, *i.e.* proteoglycans and proteins needed for the reestablishment of mature collagen (Timmons and Mahendroo 2007). It is reasonable to assume that softening and dilation of the cervix in women having early miscarriages or in cases where cervical ripening is induced in the first trimester, are associated with similar biochemical and biomechanical events as those during labor.

Inflammation

In 1981 cervical ripening was described to be similar to an inflammatory process involving tissue oedema and invasion of neutrophils (Liggins 1981). This hypothesis has been supported by many studies demonstrating influx of leucocytes into the cervical stroma at term pregnancy (Junqueira, Zugaib et al. 1980; Chwalisz 1994; Bokström, Brännström et al. 1997; Kelly 2002).

When an acute inflammation occurs, local mast cells and macrophages are activated. They subsequently secrete inflammatory agents, which induce vasodilatation, decreased blood flow, increased capillary permeability and tissue oedema. This process promotes adhesion molecules on the leucocyte cell surface to attach to the endothelium and penetrate the vessel wall (diapedesis), being attracted by chemokines (Mölne 2007).

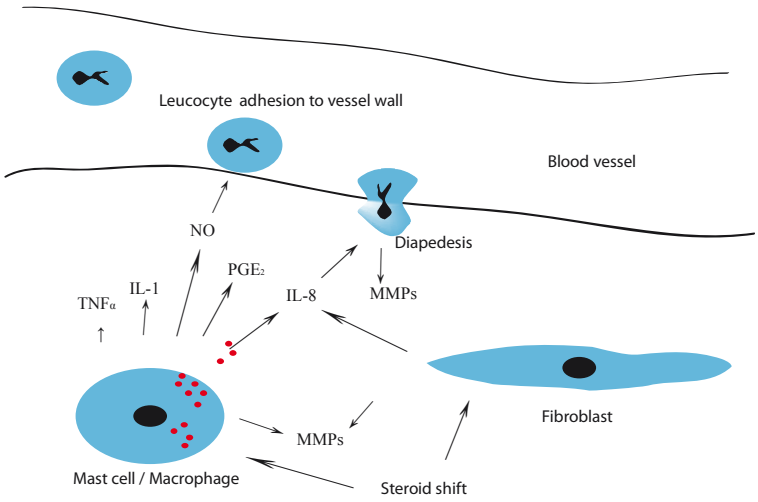

Figure 3. Inflammatory events associated with cervical ripening. Activation of macrophages, mast cells and fibroblasts in the cervical stroma with the subsequent attraction of leucocytes.

As mentioned, the inflammatory process during cervical ripening is believed to be regulated by a shift in the levels of sex steroids (Chwalisz and Garfield 1997). This mechanism triggers stromal cells to produce inflammatory mediators, which accumulate to a substantial amount and subsequently direct the ripening process. Thus, the mediators influence endothelial reactions, attract leukocytes into the tissue stroma (chemotaxis) and control intracellular responses to inflammation throughout the ripening process. The secretion of collagenolytic enzymes by neutrophil and eosinophil granulocytes as well as histamine by mast cells and basophil granulocytes is an early event in the inflammatory response. These agents are stored in granules coated with a membrane in the cell plasma and are released within seconds by degranulation. Subsequent mediators to arise at the inflammatory sight are free

radicals (NO, O_2^-, H_2O_2) being produced within a couple of minutes after activation of NADPH-oxidase and NO synthetase (NOS). Other agents accompaning the inflammation are synthesized from fatty acids of the cell membrane, *i.e.* PGs, leukotrienes, tromboxanes and platelet activating factor. Nitric oxide and PGs relax smooth muscle cells thereby causing further vasodilatation. Chemotactic (IL-8, IL-6) and proinflammatory cytokines (TNFα, IL-1), mainly produced by macrophages and monocytes, act as messengers between cells and attract inflammatory cells. They are synthesized after intracellular transcription and released a couple of hours after the start of the inflammatory process.

Chemokines

In 1992 the term chemokines, an abbreviation for chemotactic cytokines, was accepted for a subgroup of cytokines. Chemokines are sectretory proteins, mainly produced by leucocytes and act via heptahelical G-protein coupled receptors which are typical for leucocyte attractants (Baggiolini 2001). Interleukin-8, considered to play a key role during cervical ripening (Sennström, Brauner et al. 1997), is selectively chemotactic for neutrophils and is thought to be the primary regulatory molecule of inflammation. The attractant action on neutrophils is enhanced by PGE_2 (Colditz 1990) as well as other cytokines like IL-1 and TNFα (Baggiolini, Loetscher et al. 1995). Interleukin-8 may also be synthesized by monocytes as well as fibroblasts, which, in addition, are potent sources of PGE_2 and NO (Kelly 2002).

Prostaglandins

A major breakthrough in PG research came in the late 1950s when Sune Bergström purified the first PGs and determined their structure, which rendered the Nobel Prize in Physiology or Medicine 1982. Virtually every cell in the body is capable to produce prostanoids due to the ubiquity of arachidonic acid. Prostaglandins are 20-carbon cyclopentane carboxylic acids. The levels of PGs in tissues depend on the presence and activity of various enzymes (phospholipases, cyclooxygenases (COX-1, COX-2)), catalyzing the biosynthesis of PGs. Most data point to that the levels of the constitutive isoform, COX-1, do not change circumstantially during pregnancy and labor (Hertelendy and Zakar 2004; Bullarbo, Norström et al. 2007). The inducible isoform, COX-2, is typically undetectable under normal physiological conditions but can be expressed at high levels after specific stimulation, *i.e.* by proinflammatory cytokines, especially IL-1 and NF-kB and growth factors (Belt, Baldassare et al. 1999; Hertelendy and Zakar 2004).

Prostaglandins are naturally occurring mainly as PGE_1, PGE_2 and $PGF_{2\alpha}$. Prostanoids of the PGE_2-series have the greatest biological activity. Prostaglandins interfere with a variety of physiological processes within the reproductive tract. $PGF_{2\alpha}$ is a potent stimulator of myometrial activity whereas PGE_2 inhibits cervical smooth contractions (Bryman, Sahni et al. 1984; Wiqvist, Bryman et al. 1985). Furthermore, especially PGE_2, due to the more than 10 times higher cervical receptor density for PGE_2 than for $PGF_{2\alpha}$ (Wakeling and Wyngarden 1974), has been demonstrated clinically and experimentally to play a fundamental role during cervical ripening. Natural PGs are poorly absorbed after oral administration and are rapidly metabolized

(Bygdeman 2003). In addition, they are chemically unstable at room temperature giving a short shelf life. The use of natural prostanoids has been shown to be associated with obvious side effects. Nevertheless, the clinical applicability of natural PGs in clinical practice is well documented. Prostaglandin $F_{2\alpha}$ has been successfully used to initiate or reinforce myometrial contractions in second trimester abortions as well as at labor (Wiqvist, Bygdeman et al. 1972; Kelly, Kavanagh et al. 2003). Further, PGE_2 applied intracervically in a viscous gel, induces cervical softening and dilation before induction of labor as well as preoperatively prior to vacuum aspiration in the first trimester (Ulmsten, Wingerup et al. 1979; Wingerup, Ulmsten et al. 1979). The development of potent PG analogues gave the benefit of PGs protected from rapid degradation, thereby preserving biological activity after oral, vaginal or rectal administration (Collins, Pappo et al. 1985). Therefore, during recent years various PGs have preferentially used to induce cervical ripening in the first trimester when uterotonic side effects can be neglected.

Gemeprost, a PGE_1 analogue, (16,16-dimethyl-trans-Δ2 –PGE_1 methyl ester) registered as Cervagem® 1mg, is administered *per vaginam*. The peak plasma level is seen 2-3 hours after administration and suitable intervals for administration are 6 hours. It is approved for cervical priming prior to vacuum aspiration and medical termination of pregnancy in the first and second trimester. Gemeprost, like natural PGE_2, requires refrigeration and is chemically unstable at room temperature. Due to these factors and being relatively costly, the clinical use of gemeprost is declining.

Misoprostol, a PGE_1 analogue, ((11α, 13E)-11,16-dihydroxy-16-methyl-9-oxoprost-13-en-1-oic acid methyl ester) is the current drug of choice to soften the cervix in early pregnancy. In addition to induce cervical ripening misoprostol increases uterine tonus. These effects are applied for cervical ripening in connection with surgical abortion, as an abortifacient for medical abortion, in the management of postpartum hemorrhage and during recent years for induction of labor (Tang, Gemzell-Danielsson et al. 2007). Misoprostol is manufactured for oral administration and was initially approved for the prevention and treatment of gastric ulcer. It is stable at room temperature, cheap, widely available and can be used for oral, vaginal, rectal and sublingual administration. Consequently, in countries with scarce resources misoprostol has become a life-saving drug and is approved for obstetrical and gynecological use in many countries word-wide (www.misoprostol.org). The clinically active metabolite of misoprostol detectable in plasma, misoprostol acid (MPA), is produced in the liver after de-esterification of misoprostol. Misoprostol is eliminated in 80% via the urine (Karim 1987), and does not affect the cytochome P-450 enzyme system in the liver substantially (Watkinson and Akbar 1987). Side effects of misoprostol are diarrhea, abdominal pain, vaginal bleeding, nausea, vomiting, fever and chills.

Irrespective of the route of administration, misoprostol is extensively absorbed. After administration *per os*, misoprostol levels peak in plasma at 30 min and decline rapidly after 120 min, whereas administration *per vaginam* gives plasma concentrations that increase gradually with a peak after 80 min and decline slowly with still more than half of the peak concentration after 240 min. Systemic bioavailability after vaginal administration compared to oral administration is three times higher after

six hours (Zieman, Fong et al. 1997). A slow release oral formulation of misoprostol has been developed giving lower peak levels and prolonged elevation of plasma concentrations compared to conventional oral tablets (Fiala, Aronsson et al. 2005).
Generally, the main advantage of vaginal drug delivery over conventional drug delivery *per os* is the ability to by-pass first pass metabolism, since blood, leaving the vagina, enters the peripheral circulation via a venous plexus, which primarily empties into the internal iliac veins. However, considerable variability in the extent of absorption of vaginally administered drugs is observed due to the condition of the vaginal epithelium (Hussain and Ahsan 2005).
As an alternative to vaginal and oral routes to administer misoprostol, a sublingual route has been applied yielding faster MPA increase and higher systemic bioavailability than oral and vaginal administration (Tang, Schweer et al. 2002). A buccal route, placing the misoprostol tablet between the cheek and the teeth, has similar time to peak concentration in plasma as the vaginal route but the bioavailability is four times higher after vaginal administration (Schaff, DiCenzo et al. 2005).
Rectal administration of misoprostol is mainly used for postpartum hemorrhage. It shows lower peak levels and less adverse effects than oral administration. The absorption curve is similar to that of vaginal route, but the area under the curve is three times higher after vaginal administration (Khan and El-Refaey 2003; Meckstroth, Whitaker et al. 2006).
Regardless the route of administration, precaution has to be taken in cases with postoperative scarring of the uterine wall, due to the risk of uterine hypertonus and rupture, which may be rather associated with labor induction than in first trimester abortions (Plaut, Schwartz et al. 1999; Kim, Han et al. 2005).

Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a family of enzymes (endopeptidases) that degrade ECM proteins. They are at least 25 proteolytic, structurally related, zinc-dependent enzymes divided into four classes: collagenases, gelatinases, stromeolysins and membrane type proteinases (Curry and Osteen 2003). Collagenases (MMP-1, MMP-8, MMP-13) and gelatinases (MMP-2, MMP-9) mainly degrade collagen while stromeolysins (MMP-3, MMP-7, MMP-10, MMP-11) mainly degrade proteoglycans and fibronectin (Mölne 2007).
Collagenases are the only MMPs that can efficiently cleave the fibrillar collagens (I, II and III) at their triple-helical domains (Lovejoy, Welch et al. 1999), making the collagen molecules thermally unstable so that they unwind to form gelatine, after which they can be degraded further by other MMPs such as gelatinases MMP-2 and MMP-9.
MMP-1 degrades collagen type III more efficiently than type I and II. MMP-8 degrades collagen type I more efficiently than II and III (Netzel-Arnett, Fields et al. 1991). Neutrophils are the major source of MMP-8, which is stored in specific subsets of granules in the cytoplasm (Kelly 2002). MMP-8 has also been found in other cells such as arthritic chondrocytes and gingival fibroblasts (Cole, Chubinskaya et al. 1996; Tervahartiala, Pirila et al. 2000).
The MMPs are released as proenzymes, a latent zymogene form, from different cell

types such as fibroblasts, macrophages, smooth muscle cells and endothelial cells and are activated extracellularly by plasmin, oxygen radicals and already activated MMPs. Most of the MMPs have optimal enzymatic activity around neutral pH (Birkedal-Hansen, Moore et al. 1993). During normal morphogenesis and tissue remodelling, MMP activity is precisely controlled by regulation at the levels of gene transcription, zymogen activation and neutralization of the active enzymes by the endogenous tissue inhibitors of MMPs (TIMPs) (Somerville, Oblander et al. 2003). The transcription of MMP is influenced by hormones (progesterone, estrogen, glucocorticoids) and cytokines (IL-1, IL-8, TNF-α) (Hulboy, Rudolph et al. 1997; Garcia-Velasco and Arici 1999). Thus, increased concentrations of IL-8 in the lower uterine segment at term were associated with increased levels of MMP-8 and MMP-9 (Osmers, Blaser et al. 1995).

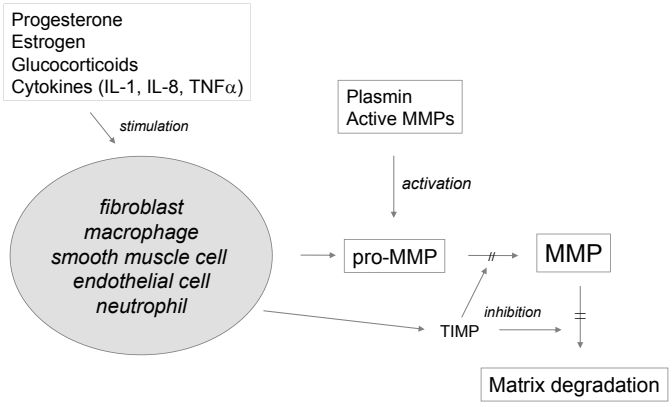

Figure 4. MMP regulation of ECM degradation

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Nitric oxide

Nitric oxide (NO) is a simple free biologically active radical gas, soluble in both water and lipid and therefore freely diffusible in cell environment (Henry, Lepoivre et al. 1993). Nitric oxide is synthesized from the amino acid L-arginine, has a short half-life (approximately 4 seconds) and it is not stored *in vivo* (Palmer, Rees et al. 1988). The synthesis of NO is regulated by NO synthases (NOS) of which three isoforms are identified: inducible NOS (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS). Nitric oxide synthases require calcium / calmodulin for activation (Busse and Mulsch 1990a). Nitric oxide is rapidly converted to the final metabolites nitrate and nitrite. This reaction is catalyzed by transition metals including iron. The half-life of NO and the ratio of nitrate and nitrite in aqueous solutions, depend upon surrounding conditions *i.e.* presence of oxygen-derived radicals, pO_2, pH and concentrations of transition metals and thiols (Kelm 1999).

Nitric oxide is a mediator in many biological processes such as inflammation, immune response, smooth muscle relaxation, vascular homeostasis and neurotransmission. In the female reproductive tract NO is involved in ovulation, tubal transport, implantation, pregnancy maintenance, labor and delivery (Ekerhovd, Brännström et al. 1997; Ekerhovd, Weidegård et al. 1999; Maul, Longo et al. 2003).

In inflammation iNOS can be induced by cytokines (IL-1, IL-2, IL-12), TNF-α, interferon gamma (IF-γ) and endotoxins, where NO is synthesized with a delay of 6-8 hours after stimulation (Busse and Mulsch 1990b; Beck, Eberhardt et al. 1999). Newly synthesized NO, in turn, can stimulate the formation of cytokines, such as IL-8 in mast cells in the endometrial stroma during implantation (Okada, Asahina et al. 2001). Nitric oxide can regulate COX activity, both constitutive COX-1 and inducible COX-2 and thereby affect the production of PGs under normal and inflammatory conditions (Salvemini, Seibert et al. 1994; Salvemini 1997; Bullarbo, Norström et al. 2007).

To take benefits of the biological effects of NO clinically, one has to utilize NO donors which, when metabolized, release NO. Thus, the nitric oxide donors glyceryl trinitrate (nitroglycerine), sodium nitroprusside and isosorbide mononitrate (IMN) are used for treatment of angina pectoris, acute myocardial infarction and congestive heart failure (Abrams 1987).

In the pregnant uterus NO donors have been used as uterine relaxants (Yallampalli, Garfield et al. 1993; Ekerhovd, Weidegård et al. 1999; Caponas 2001; Leszczynska-Gorzelak, Laskowska et al. 2001; Bullarbo, Tjugum et al. 2005). Nitric oxide donors also promote cervical smooth muscle relaxation in early pregnancy as well as at term (Ekerhovd, Brännström et al. 1998; Ekerhovd, Brännström et al. 2000) and have been applied clinically as cervical ripening agents (see below).

Isosorbide mononitrate is manufactured for oral administration as a vasoactive agent, causing vasodilatation. IMN is a potent drug, being rapidly absorbed and almost 100% bioavailable, as there is no significant first pass metabolism. Peak levels in plasma occur within one hour and the half-life is 5 hours. Isosorbide mononitrate is excreted via the urine (Abshagen 1992). In gynecological practice IMN is preferentially administered *per vaginam,* when peak serum levels are half of the levels after oral administration but remain constant at least for six hours (Bates, Nicoll et al. 2003). It is suggested that IMN given *per vaginam*, by a first uterine pass effect leads to higher concentrations in the uterus than in the serum thereby maximizing the desired effect (Bulletti, de Ziegler et al. 1997).

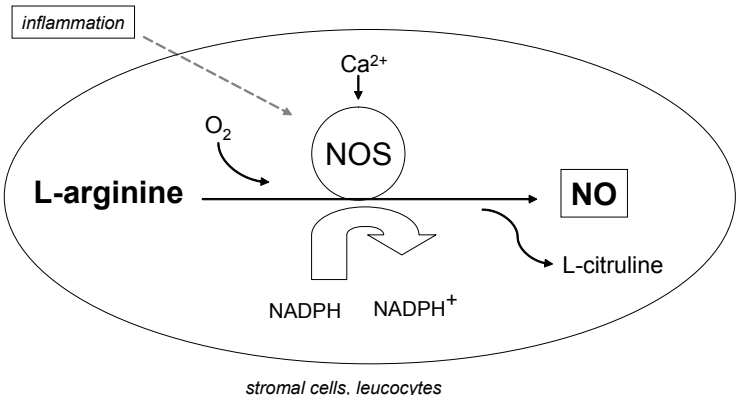

Figure 5. The synthesis of NO.

Induction of cervical ripening

In the first and second trimester of pregnancy induction of cervical ripening is applied for cervical priming before surgical abortions, medical abortions as well as in the management of miscarriages. In the third trimester of pregnancy cervical priming is undertaken as a part of induction of labor.

Cervical priming prior to surgical evacuation is a standard procedure in many clinics. Cervical dilatation before vacuum aspiration is a critical step of the procedure since forceful and difficult dilatations can lead to cervical laceration, incomplete evacuation, hemorrhage and uterine perforation (Hulka and Higgins 1961; Moberg 1976). Using cervical priming prior to vacuum aspiration the dilatation procedure becomes easier and the blood loss and operating time are reduced (Ngai, Tang et al. 1995). According to RCOG guideline from 2004 cervical priming is beneficial prior to surgical abortion and should be routinely used in women below 18 years of age and in women beyond the 10th gestational week (RCOG 2004).

Over the years, different regimens for cervical ripening in the first trimester have been applied such as cervical tents, PGs, antiprogestins and NO donors.

Tents, being osmotic dilators, act upon the cervix by swelling after insertion in the cervical canal, thereby dilating the cervix. Laminaria tents, prepared from sea weed (Laminaria Japonica, Laminaria digitata) need a longer priming time interval compared with synthetic tents such as Lamicel and Dilapan (Darney 1986). Tents require early admission and cannot be self-administered.

As mentioned, natural PGE_2, prepared in a viscous gel for cervical and vaginal administration, is successfully used for cervical ripening before vacuum aspiration as well as induction of labor. During the last two decades the PG analogues misoprostol and gemeprost have been the most commonly used cervical priming agents before surgical abortion. Gemeprost (1mg), administered vaginally 3-5 hours prior to vacuum aspiration, has been shown to dilate the cervix effectively (Ho, Liang et al. 1983; el-Refaey, Calder et al. 1994). However, gemeprost is expensive, needs refrigeration and has a short shelf-life. Misoprostol is nowadays more widely used than gemeprost since it is more easily handled, comparably cheap and stable at room temperature. As mentioned, misoprostol can be administered both orally, vaginally, sublingually and buccally for cervical priming prior to surgical termination of pregnancy in the first trimester. The oral and vaginal routes have demonstrated significant increase in cervical dilation compared with placebo (Bugalho, Bique et al. 1994; el-Refaey, Calder et al. 1994; Ngai, Tang et al. 1995; Ficicioglu, Tasdemir et al. 1996; Bokström, Atterfelt et al. 1998). The efficacy of the oral route compared to the vaginal route seems to be similar (Ashok, Hamoda et al. 2003; Cakir, Dilbaz et al. 2005) whereas the vaginal route is associated with less inter-individual variability regarding clinical effect as well as side-effects (Lawrie, Penney et al. 1996). Various doses and time intervals for vaginally administered misoprostol have been investigated demonstrating the optimal dose and time interval being 400μg for three hours (Singh, Fong et al. 1998; Singh, Fong et al. 1999). This regimen can be used for oral administration as well (Ashok, Hamoda et al. 2003).

The sublingual route has shown the same efficacy as the oral route, though being associated with more side effects (Aronsson, Hellström et al. 2004; Vimala, Mit-

tal et al. 2004). In one study, comparing cervical priming in the second trimester abortions, buccal misoprostol was as effective as laminaria tents (Todd, Soler et al. 2002).
Antiprogestins like mifepristone require a long period to exert their ripening action in combination with misoprostol for medical abortion (WHO 1990). Mifepristone has been shown to be equally effective as gemeprost (2-4 hours) for cervical priming when given 36 hours prior to evacuation and even more effective with a priming time interval of 48 hours compared to misoprostol for 2-4 hours (Henshaw and Templeton 1991; Ashok, Flett et al. 2000). (see below)
Nitric oxide donors (IMN, sodium nitroprusside, glyceryl trinitrate) have been shown, both in animal and human studies, to induce ripening of the cervix in the first trimester as well as at term (Chwalisz, Shao-Qing et al. 1997; Thomson, Lunan et al. 1997; Facchinetti, Piccinini et al. 2000; Ekerhovd, Bullarbo et al. 2003). The route of administration is either *per vaginam*, in the posterior fornix (tablets) or intracervically (gels).

Medical abortion

Ripening of the cervix is obtained in regimens applied for medical (pharmacological) abortions. The introduction of mifepristone, having high affinity for progesterone receptors, thereby blocking the action of endogenous progesterone and making the myometrium more sensitized to PGs, gave the starting point for pharmacological termination of pregnancy (Lahteenmaki, Heikinheimo et al. 1987; Swahn and Bygdeman 1988). Mifepristone, orally administered before surgical abortion, has a ripening effect on the cervix (Rådestad, Christensen et al. 1988; Bokström, Norström et al. 1995). Thus, mifepristone alone has been applied for cervical ripening but is more commonly used in various combinations of PGs for medical abortion. The most widely used combination is mifepristone (200 mg) followed 48 hours later by misoprostol (800μg) (Kulier, Gulmezoglu et al. 2004).

Miscarriage

Generally, miscarriage is defined as pregnancy loss before the fetus reaches a viable gestational age. The exact incidence of miscarriages is hard to determine. Sensitive immunological tests for hCG make early diagnosis of pregnancy possible and it is evident that many pregnancies are short-lived and diagnosed as "biochemical" (Miller, Williamson et al. 1980). The use of high-resolution transvaginal ultrasound is today the most important tool in the diagnosis of early pregnancy (Schwimer and Lebovic 1984; Wikland, Enk et al. 1985). In clinical practice, it is reasonable to consider that miscarriages account for about 12 % of registered pregnancies (Blohm, Fridén et al. 2008). The great majority occurs early, before the 12 weeks of gestational age and fewer than 5 % after identification of fetal heart activity (Brigham, Conlon et al. 1999). Loss in the second trimester constitutes less than 4% of all pregnancies (Ugwumadu, Manyonda et al. 2003).
Numerical chromosomal anomalies, *i.e.* most commonly trisomy 13, 16, 18, 21 and 22, have been shown to be frequently associated with miscarriages (Goddijn and Leschot 2000). Chromosomal abnormalities are linked to maternal age, which *per*

se implies an increased risk of miscarriage (Nybo Andersen, Wohlfahrt et al. 2000). Other documented risk factors of miscarriage are uterine anomalies, submucous fibroids (George, Granath et al. 2006), intrauterine adhesions (Ashermans syndrome) and maternal infections, especially rubella (Edlich, Winters et al. 2005).

In order to bring better clarity in the diagnostic nomenclature for the different types of miscarriages, changes in terminology were recommended by the Royal College of Obstetricians and Gynaecologists (RCOG) in 1997. It was recommended that the term spontaneous abortion should be replaced by miscarriage, the terms blighted ovum or missed abortion by early embryonic or fetal demise and the term incomplete abortion should be replaced by incomplete miscarriage. The nomenclature has more recently been updated and revised (Farquharson, Jauniaux et al. 2005). In the present thesis women with miscarriages were divided into two subgroup: symptomatic, *i.e.* women having bleeding and/or pain without fetal heart activity when examined by ultrasound and asymptomatic *i.e.* women with no symptoms of miscarriage where ultrasound revealed a gestational sac without a fetal pole or a gestational sac with a fetal pole but with no heart beats (Bernard and Cooperberg 1985; Gemzell-Danielsson, Ho et al. 2007).

Surgical evacuation of the uterus used to be the standard management of miscarriage. It is a quick procedure with a very high success rate. During recent years, expectant management and medical management using PG analogues have become increasingly established. Expectant management has been shown to have similar outcome as vacuum aspiration regarding delivery of gestational products and complications in women having symptomatic, incomplete miscarriages (Nielsen and Hahlin 1995). Thus, expectant management has become accepted as a method of choice (Luise, Jermy et al. 2002; Blohm, Fridén et al. 2003), though a 100% success rate is unpredictable. Medical management may be a better alternative to some women. Several studies have been performed to investigate the optimal regimens of PG analogues with or without mifepristone. The use of mifepristone in addition to misoprostol does not seem to improve the success rate of neither symptomatic nor silent miscarriages (Nielsen, Hahlin et al. 1997; Nielsen, Hahlin et al. 1999; Grönlund, Grönlund et al. 2002).

Aims of the studies

- To evaluate if 400μg of misoprostol is as effective as 1mg of gemeprost for cervical priming in the first trimester prior to surgical termination of pregnancy.

- To compare efficacy and side effects of IMN with misoprostol for cervical priming in the first trimester.

- To study morphology of cervical biopsies obtained from women following cervical priming with IMN or misoprostol.

- To study the inflammatory parameters IL-8, MMP-1 and MMP-9 in cervical biopsies obtained from women treated with IMN or misoprostol.

- To study morphology of cervical biopsies from women with symptomatic as well as silent miscarriage.

- To study the inflammatory parameters IL-8, MMP-1, MMP-8 and MMP-9 in cervical biopsies obtained from women with symptomatic or silent miscarriage.

MATERIAL AND METHODS

Study population

All studies were approved by the human ethics committee of Gothenburg University. A total number of 275 women gave their informed consent to participate. The women were between 15-36 years of age, nulliparous, healthy and had not undergone any type of cervical surgery.

Table 1. Study population included in study I-IV

	Total number of subjects	Number of subjects included in subsequent studies
Study I	90	
Study II	148	24 (Study III)
Study III	32	
Study IV	29	

Study I

90 healthy nulliparous women between 9-12 weeks of gestation, requesting surgical termination of pregnancy with a viable singelton pregnancy confirmed by transvaginal ultrasonography were recruited.

Exclusion criteria were symptoms or signs of threatening miscarriage and any kind of serious disease.

Study II

Included were 148 nulliparous women scheduled for surgical termination of pregnancy, having a viable singleton pregnancy and gestational age less than 12 weeks as assessed by transvaginal ultrasonography. 76 primigravid women were included for the assessment of cervical ripening and side effects and 72 nulliparous women were included for assessment of side effects only. Exclusion criteria were previous cervical surgery, ongoing vaginal bleeding, uterine related pain and known allergy to IMN or misoprostol.

Study III

Cervical biopsies were obtained from 24 healthy primigravid women who had also been included in study II. The tissue specimens were used for morphological as well as biochemical analyses. Cervical biopsies from 8 primigravid women, scheduled for surgical termination of pregnancy in the first trimester who had not received any preoperative cervical priming, served as controls.

Study IV

Women who were diagnosed with either a symptomatic (n=7) or a silent (n=11) miscarriage in the first trimester (< 12 weeks of gestational age) and women with a viable singleton pregnancy (n=11), requesting termination of first trimester pregnancy (con-

trols), were included. A miscarriage with symptoms such as ongoing vaginal bleeding, low abdominal pain and no fetal heart activity registered by vaginal ultrasound, was defined as a symptomatic miscarriage. A miscarriage diagnosed unexpectedly without any symptoms and without fetal heart activity, registered by ultrasound, was defined as a silent miscarriage. In control women a normal viable pregnancy was registered. All women underwent surgical abortion without any pretreatment and cervical biopsies for morphological and biochemical analyses were obtained prior to dilatation of the cervix.

Tonometry

A cervical tonometer was used in study I and II, for the assessment of the cervical ripening, measuring the baseline cervical dilation and the force needed to dilate the cervical canal (Fisher, Anthony et al. 1981). The procedure involves the use of cervical dilators from 3 mm to 10 mm that are locked on to a handle connected to a tonometer. The instrument measures the force in Newton (N) when the dilator is inserted into the cervical canal. Baseline cervical dilation was defined as the first dilator to produce a peak force more than 5 N. Peak force was measured for each dilator as the force needed to enter the internal os. The cumulative force was the sum of peak forces for each dilator, needed to dilate the cervix up to 10 mm (Study I) or up to 9 mm (Study II).

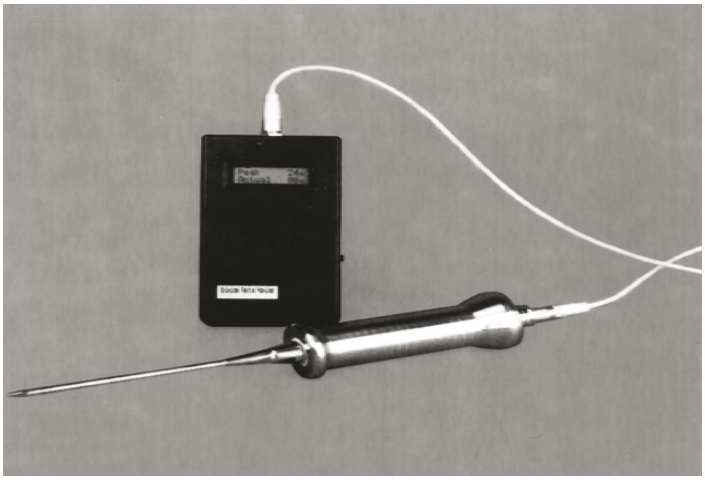

Figure 6. A cervical tonometer.

Questionnaire

For evaluation of side effects in study I and II questionnaires were used. In study II side effects were assessed by a visual analogue scale (VAS). The women completed the questionnaires in the morning following cervical priming and shortly before entering the operating theatre. The questionnaire concerned the following symptoms: abdominal pain, nausea, vomiting, headache, hot flushes, vaginal bleeding and palpitations. Each symptom was evaluated separately and was graded from 0 (no symptoms) to 10 (maximal intensity) at analysis.

Cervical biopsy procedure

Cervical biopsies, approximately 18 x 1.5mm in size, for morphological and biochemical analyses were obtained from the central portion of the anterior lip with the use of a True-Cut® biopsy needle (Allegiance, Healthcare Corporation, McGaw Park, IL, USA). The biopsies were obtained under general anesthesia before cervical dilatation. For electron microscopy (EM) the cervical biopsies were immediately fixed in 0.1M Na-Cacodylate-buffered glutaraldehyde.
For analysis of IL-8 and MMPs, the specimens were snap frozen in liquid nitrogen and stored at -70°C until analysis.

Electron microscopy

Electron microscopy was used for morphological analysis of cervical tissue in study III and IV. The fixed specimens were washed in 0.1M Na-Cacodylate-buffer containing 4 % sucrose, postfixed with 1% osmium tetroxide, dehydrated in graded series of ethanol and propylene oxide and embedded in epoxy resin (Agar 100 Resin®, Agar Scientific Ltd., Stansted, Essex, England). Appropriate areas for analysis were selected from semithin (1µm) sections stained with toluidine blue. Ultrathin sections (60-90 nm) were then prepared, retrieved on 150 mesh Formvar-coated copper grids, contrasted with uranyl acetate/lead citrate and examined using a Philips CM 10 electron microscope.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was used for quantification of IL-8 in study III and IV. Without thawing, tissue specimens were grounded on dry ice and the tissue powder was suspended in Tris-HCl buffer (0.04M, pH 8.0) containing 0.005M $CaCl_2 \cdot 2H_2O$, 0.02M benzamidine, 0.01M phenylmethane sulfonyl fluoride, 0.05% Briz-35 TM and 0.02% NaN_3 (10:1). The samples were sonicated, centrifuged (15 minutes at 10700 rpm, +4°C) and the supernatants were collected for Quantikine® Human CXCL8/IL-8 Immunoassay (R&D Systems, Minneapolis, MN, USA), according to the manufacturers protocol.

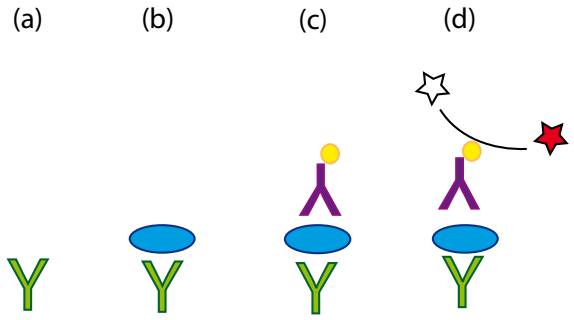

Figure 7. Principle of the quantitative sandwich enzyme immunoassay (ELISA)

This assay employs the quantitative sandwich enzyme immunoassay technique. Microplates pre-coated with monoclonal antibody specific for IL-8 were supplied by the manufacturer (Fig. 7a). Standards and samples were pipetted into the wells and any IL-8 present was bound by the immobilized antibody (Fig. 7b). After washing of any unbound substances, an enzyme-linked polyclonal antibody specific for IL-8 was added to the wells (Fig. 7c). Following washing to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of IL-8 bound in the initial step (Fig. 7d). The color development was stopped and the intensity of the color was measured. The average of the duplicate readings for each standard, control and sample were counted and subtracted from the average zero standard optical density. A standard curve was created by using a four parameter logistic (4-PL) curve fit. Levels of IL-8 were expressed as pg/mg protein. Protein concentrations were measured using the Micro BCA protein assay kit® (Pierce, Rockford IL, USA).

Immunohistochemistry

Immunohistochemistry was used for detection and semiquantitative analysis of MMP-1, MMP-8, MMP-9 and IL-8 (Study III and Study IV).

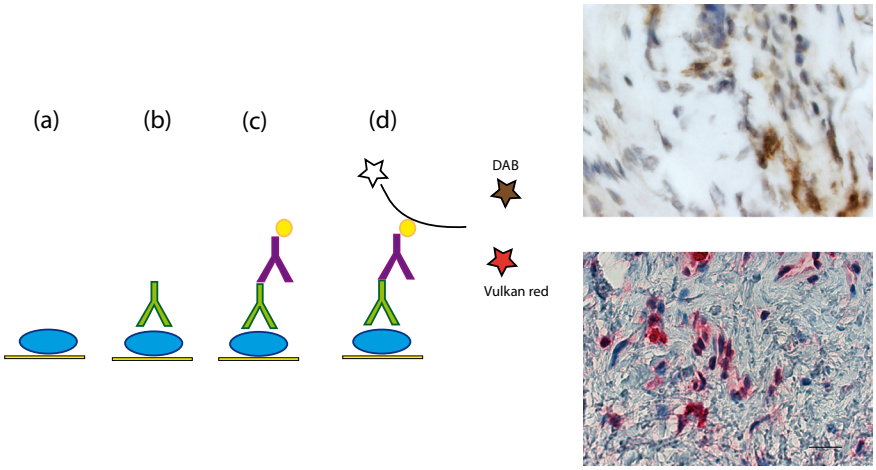

Figure 8. Principle of the immunohistochemical assay. ABC method detected with DAB substrate; polymer-based MACH3 system detected with Vulcan Red.

In Study III immunhistochemical staining was done using the Avidin-Biotin Complex (ABC) method on frozen sections. Avidin, a large glycoprotein, can be labelled with peroxidase and has a very high affinity for biotin. Biotin, a low molecular weight vitamin, can be conjugated to a variety of biological molecules such as antibodies. The technique involves three layers. The first layer is unlabeled primary antibody. The second layer is biotinylated secondary antibody. The third layer is a complex of avidin-biotin peroxidase. The peroxidase is then developed by the DAB substrate to produce colorimetric end products.

The middle portion of snap-frozen specimens from five women in each group was cut out to yield appropriate areas for comparison. The specimens were mounted in O.C.T.™ compound (Sakura Finetek, Zouterwoude, The Netherlands). Six mm sections were prepared in a Leica CM3050 cryostat, transferred to glasses (Fig. 8a) and rinsed in phosphate buffered saline (PBS). They were then treated with 0.3% (v/v) hydrogen peroxide in methanol for 30 minutes and exposed to normal horse serum (Vector Laboratories, Inc., Burlingame, CA, USA) for 20 minutes at room temperature to eliminate unspecific binding. Thereafter, they were incubated with the primary monoclonal antibody (Fig. 8b) against either MMP-1 or MMP-9 (Calbiochem, EMD Biosciences, Inc/Merck KGaA, Darmstadt, Germany), diluted 1:100 in 1% bovine serum albumin/PBS at 48°C overnight. The sections were rinsed three times in 0.05% Triton in PBS and incubated with biothinylated horse anti-mouse immunoglobulin G (Vector Laboratories) as secondary antibody for 30 minutes, followed by the addition of avidin-biotin peroxidase complex (Vector Laboratories) for 60 minutes (Fig. 8c). To visualize immunoreactivity, the specimens were exposed to 0.05% 3'3' diaminobenzidine-tetrahydrochloride (Sigma Chemical Co., St.Louis, MO, USA) in PBS containing 0.3% hydrogen peroxide for seven minutes (Fig. 8d). Two sections were prepared from each biopsy specimen. Microscopy and photography were performed by means of a Nikon EFD-3 microscope connected to a Nikon Coolpix 990 or a Nikon Digital Sight DS-U1 Camera. A positive reaction was demonstrated by a brown reaction product. Negative controls were treated with PBS without the monoclonal antibody. Sections of placental tissue were used as positive control. Analysis was performed blindly at two occasions by two independent observers whose findings displayed a high degree of comparability. The specimens were evaluated at x20 magnification and immuno-positivity was scored on a 0-3 graded scale (0=absent, 1=weak, 2=moderate, 3=strong), taking into regard the intensity of staining and number of stained cells. The mean scoring value from 20 arbitrarily chosen areas of each specimen was calculated to form the basis for statistical analysis.

Polymer-based alkaline phosphatase or horseradish peroxidase detection systems are widely accepted as the most sensitive in the field of immunohistochemistry. Secondary antibodies conjugate with a dextran polymer backbone containing a large number of enzyme molecules. An advantage is that the system is biotin free and therefore does not display the non-specific staining associated with biotin-binding proteins or endogenous biotin. This technique was applied in Study IV were frozen cervical specimens were postfixed in buffered 4% formalin for 12 hours and embedded in paraffin. Tissue sections (5µm) were dewaxed in Tissue Clear® (Histolab Products AB, Gothenburg, Sweden) and rehydrated in graded ethanol (Fig. 8a). For antigen retrieval sections were heated in Antigen Unmasking Solution® (Vector Laboratories, Inc.Burlingame, CA, USA) for 20 min at 121°C in an autoclave. The slides were rinsed in distilled water and blocked with Background sniper® (Biocare Medical, Concord, CA, USA) for 10 min at room temperature to eliminate unspecific binding. Further, the slides were rinsed in 1xTBS (Tris buffered saline), pH 7.6, and, after application of the primary antibody, incubated overnight in a humid chamber

at +4°C (Fig. 8b). The antibodies used were: MMP-1, rabbit polyclonal antibody (Neomarkers for Lab Vision, Termo Scientific, Freemont, CA, USA 1:100); MMP-8 mouse monoclonal antibody (Abcam, Cambridge, UK 1:10); MMP-9 mouse monoclonal antibody (Abcam, Cambridge, UK 1:100); IL-8 rabbit polyclonal antibody (Abcam, Cambridge, UK 1:200). The slides were rinsed in TBS and incubated with the secondary antibody (Fig. 8c) according to manufacturers protocol of MACH 3 Alk Phos Polymer Kit® (Biocare Medical, Concord, CA, USA). The slides were stained with Vulcan Fast Red® (Vulcan Fast Red Chromogen Kit, Biocare Medical, Concord, CA, USA) for 10 min to visualize immunoreactivity (Fig. 8d), counterstained with hematoxylin, dehydrated and mounted with Pertex® (Histolab Products AB, Gothenburg, Sweden). Microscopy and photography were performed by means of a Nikon EFD-3 microscope connected to a Nikon Digital Sight DS-U1 Camera. A positive reaction was demonstrated by a red reaction product. Negative controls were incubated with IgG negative control serum (Biocare Medical, Concord, CA, USA) from mouse or rabbit depending on the origin of the antibody. Sections of placental tissue were used as positive control. For assessment of positive staining, two sections from each specimen of five women in each group were evaluated and scored as described above.

Statistics

In study I, the Fisher nonparametric permutation test was used for comparison of the cumulative force.

In study II the non-parametric Mann-Whitney U-test for comparison of the cumulative force and the frequency of side effects. Fisher's exact test was used for comparison of the intensity of side effects.

In study III and IV the non-parametric Mann-Whitney U-test was used for comparing semiquantitative immunohistochemical data of IL-8, MMP-1, MMP-8 and MMP-9 as well as quantitative data of IL-8 (ELISA).

A p-value < 0.05 was considered significant.

Methodological considerations

The study population of nulliparous healthy women was chosen to minimize any effect on the cervix due to previous vaginal delivery or cervical surgery. Further, the women did not take any medication and they did not suffer from cervical or vaginal infections. None of them had undergone previous dilation of the cervix. Cervical biopsies were obtained before cervical dilatation to avoid possible effects on the cervix by the surgical procedure.

One and the same investigator carried out the operative procedures using dilators connected to a tonometer. Once familiar with this instrument, the dilatation procedure is accomplished as easily as by using conventional Hegar dilators. Cervical tonometry is an objective method which overcomes the surgeon's subjective impression.

In study I and II, questionnaires were used to register side effects. Prior to be included into the study, the women were informed about the study and side effects. The awareness of possible side effects might have had an impact on the way they answered the questionnaire.

Cervical tissue samples were stored at -70°C to keep proteins such as MMPs and IL-8 intact. Still, in spite of these precautionary measures some deterioration during storage cannot be totally excluded.

The middle portion of the approximately 18mm long cervical tissue strips was selected for EM. Semithin sections were prepared to yield appropriately comparable areas of analysis before ultrathin sectioning. In spite of these measures, a slight variation in tissue composition especially with regard to smooth muscle components could be observed in single cases.

All tissue samples analyzed by ELISA, were managed within one single assay thereby reducing the methodological error. The sensitivity (detection limit) of the test was 3.5 pg/mL, implying that registered IL-8 values were kept within the standard curve.

In immunohistochemical experiments, negative controls were incubated with IgG negative control serum from mouse or rabbit depending on the origin of the antibody in study IV. This step was, however, omitted in study III, where negative controls where incubated with buffer solution. Therefore, unspecific binding of the antibody might have occurred in Study III. On the other hand, the results of complementary experiments using IgG negative control serum gave similar results (unpublished data).
Both ELISA and immunohistochemistry are based upon the use of antibodies. Monoclonal antibodies are generated by one single clone of antibody producing cells, while polyclonal antibodies are generated by different clones of antibody producing cells with the possibility of higher unspecific binding.

Results and comments

Study I.

Gemeprost versus misoprostol for cervical ripening before first-trimester abortion

90 women were enrolled into a double-blind randomized study. The women were randomized to vaginal treatment with either 1mg gemeprost (n=45) or 400μg misoprostol (n=45) for 3 to 4 hours prior to surgical termination of pregnancy in the first trimester. There were no significant differences between the groups regarding age, gestational age and priming to operation interval.

A cervicometer was used for registration of cervical resistance as a measure of cervical ripening. Both baseline cervical dilation and cumulative peak force to dilate the cervix to 10 mm were registered. No significant difference was found between the two groups with regard to baseline cervical dilation and cumulative peak force. The peak force registered for the 8, 9 and 10mm dilators did not show any significant difference between the groups.

Table 2. Results of cervical tonometry (mean±SD, (range)). N= Newton. NS, not significant.

	Gemeprost	Misoprostol	p
Baseline cervical dilation (mm)	7.4±1.5 (4-10)	7.6±1.6 (4-10)	NS
Cumulative peak force to 10mm (N)	45±12.3 (22-72)	44.2±13.2 (18.0-74.0)	NS

Comments:

The results of preoperative vaginal treatment for cervical ripening showed that vaginal administration of 400μg misoprostol is as effective as 1mg gemeprost as assessed by cervical tonometry. Side effects such as low abdominal pain were of low intensity and did not differ between the groups. Bearing in mind that misoprostol is easy to administer, inexpensive, and stable at room temperature with a long shelf-life makes this medication convenient and attractive as a cervical ripening agent in the first trimester of pregnancy.

Study II.

Outpatient cervical ripening before first-trimester surgical abortion: a comparison between misoprostol and isosorbide mononitrate

In this study vaginally administered IMN (40mg) was compared with vaginal misoprostol (200μg) overnight (9-13 hours) as treatment for cervical ripening. A total number of 148 women were enrolled into a double-blind randomized study. Seventy six women were included for assessment of both cervical ripening and side effects, while 72 additional women were included for assessment of side effects only. Sixteen women were excluded from analysis (decision to continue pregnancy (n=3); miscarriage (n=2); treatment interval > 13 hours (n=11)). Thus, a total number of 30

women treated with IMN and 30 women treated with misoprostol were analysed for cervical ripening, while 61 women treated with IMN and 59 women treated with misoprostol were analysed with regard to side effects only.
There was no significant difference in mean age, gestational age, treatment interval and intraoperative blood loss.
Cervical ripening was assessed by measuring baseline cervical dilation and cumulative peak force up to 9mm. In the misoprostol group there was a significantly higher median baseline cervical dilation and a significantly lower median cumulative peak force compared to the IMN group.

Table 3. Results of cervical tonometry (median (range)). N = Newton

	Isosorbide mononitrate	Misoprostol	p-value
Baseline cervical dilation (mm)	6(3-9)	9(3-9)	<0.001
Cumulative peak force to 9mm (N)	73(26-138)	15(10-110)	<0.009

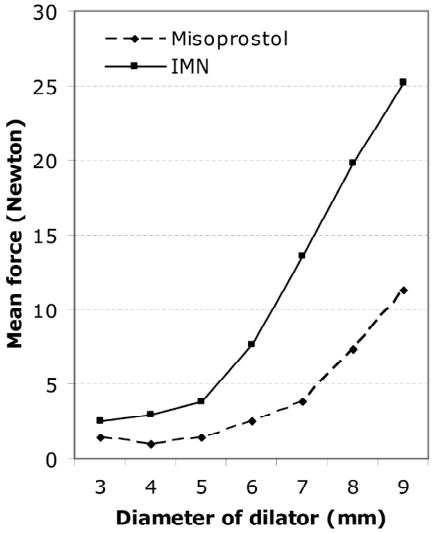

Figure 9. Force required for cervical dilatation in women treated with misoprostol and IMN.

Both treatments were associated with a high frequency of side effects. Headache was common and significantly increased after treatment with IMN compared to misoprostol. Hot flushes were common but not significantly different between the groups. The frequency of abdominal pain and intraoperative bleeding as well as the intensity of abdominal pain was significantly higher in the misoprostol group compared to the IMN group.

Comments:
The results demonstrate that 200μg misoprostol induced a more pronounced cervical ripening than 40mg IMN when self-administered vaginally overnight. On the basis of the present results misopristol appears superior to IMN for cervical ripening in the first trimester. Both treatments were associated with a high frequency of side effects. Misoprostol caused vaginal bleeding, abdominal pain and nausea more frequently, while IMN gave more headache and a tendency towards more hot flushes. The registered side effects may be due to the stimulatory effects of PGs on uterine and gastrointestinal smooth muscle and the vasodilatory effect of NO. The long treatment period (up to 13 hours) appears inconvenient due to the high frequency of adverse effects by both regimens.

Study III.
Cervical priming in the first trimester: morphological and biochemical effects of misoprostol and isosorbide mononitrate

In this study cervical ultrastructure and inflammatory parameters (MMP-1, MMP-9 and IL-8), known to be involved in cervical ripening, were investigated following vaginal treatment with either IMN 40mg or misoprostol 200μg overnight. Cervical biopsies were obtained from 24 women who also were included in study II (12 women in each group). In addition, 8 nulliparous women, requesting surgical termination of pregnancy in the first trimester, were included. These women did not receive any cervical priming and served as controls.
The median cervical resistance at dilatation was significantly lower in women treated with misoprostol (22.0 N; range 8-34 N) compared to women treated with IMN (71.5 N; range 45-97 N) and controls (76.0 N; range 69-97 N).

Electron microscopy
The ultrastructure of cervical specimens following treatment with misoprostol showed a disorganized collagen framework with pronounced splitting of the collagen fibers (Fig.10a). In fibroblasts, the nuclear chromatin was dispersedly distributed with condensations under the nucleolemma and the cytoplasm contained proliferative, dilated granular endoplasmatic reticulum (gER) and frequent dense bodies (lysosomes) (Fig.10b). The number of encountered mast cells appeared increased and the endothelial surface facing the vessel lumen exhibited frequent buddings and pinocytotic vesicles.
The ultrastructure of specimens from women in the IMN group was similar as in specimens obtained from women treated with misoprostol although the reactive phenomena were less pronounced in the IMN group. The collagen framework generally exhibited a lower degree of disintegration and fibroblasts appeared less reactive as judged from the more densely packed nuclear chromatin (Fig.10c). However, in areas where collagen fibers were highly disorganized and disrupted, *i.e.* exhibiting signs of collagen degradation, fibroblasts appeared activated with an enriched gER (Fig.10d).
In specimens obtained from women who had no presurgical treatment the cervical ultrastructure was clearly different from the two other groups. The collagen frame-

work appeared intact (Fig.10e) with a rather regular distribution of fibroblasts, with less dilated gER and more condensed chromatin. In specimens from women in the control group a small number of mast cells was observed. The mast cells were mainly located in the vicinity of scarce areas with disorganized collagen fibers (Fig.10f).

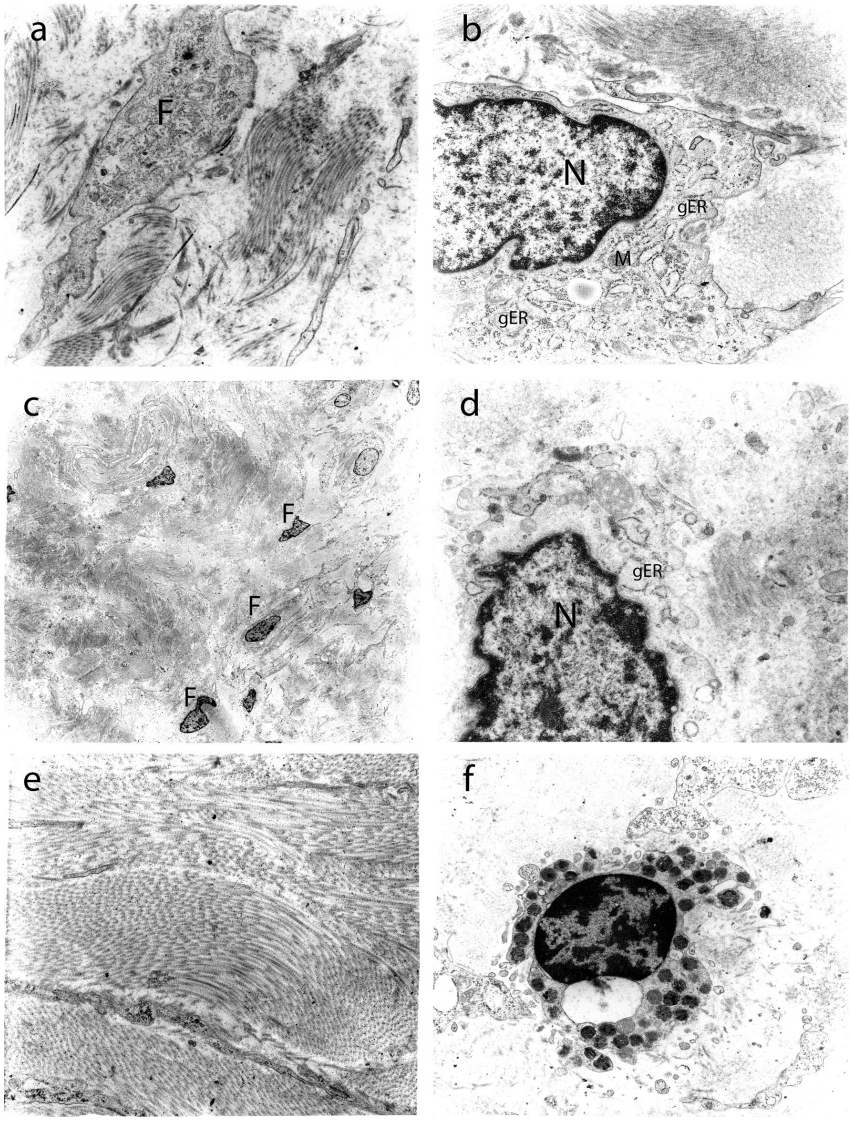

Figure 10. Electron micrographs of cervical tissue obtained from women following treatment with misoprostol :(a x10250; b x14500); Isosorbide mononitrat: (c x1800; d x14500) and women with no treatment (e x14500; f x10250). F=fibroblast, N=nucleus, M=mitochondria, gER=granular endoplasmatic reticulum

Matrix metalloproteinases

The expression of MMP-1 and MMP-9 was scored by the number of positively stained cells and the intensity of staining. The scoring was higher for both MMPs in specimens obtained from treated women compared to specimens from women in the control group. The scoring for MMP-9 in IMN-treated women was higher than in women treated with misoprostol (Table 4, Fig. 11).

*Table 4. Immunohistochemical scoring for MMP-1 and MMP-9. Median (range). *=(p<0.05).*

	Misoprostol (n=5)	Isosorbide mononitrate (n=5)	Control (n=5)
MMP-1	2.0(1.5-3.0)*	3.0(1.5-3.0)*	1.5(1.0-2.0)
MMP-9	2.5(1.0-2.5)	3.0(2.5-3.0)*	2.0(1.0-2.0)

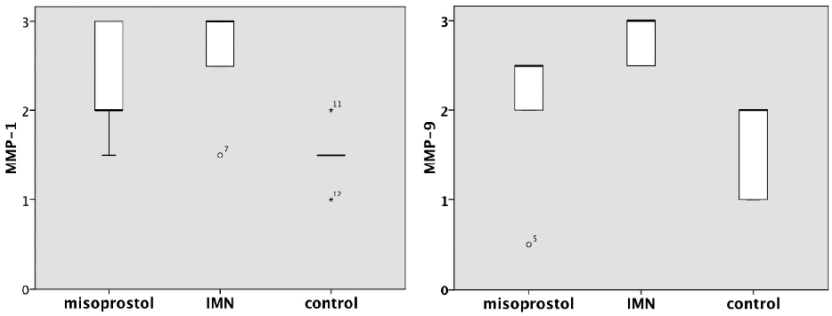

Figure 11. Boxplots illustrating immunohistochemical scoring for MMP-1 (left) and MMP-9 (right).

Positively stained cells for MMP-1 were distributed as single cells or as clusters of cells in the interstitial connective tissue and as aggregates of intensely stained cells in the vicinity of stromal capillaries (Fig.12).

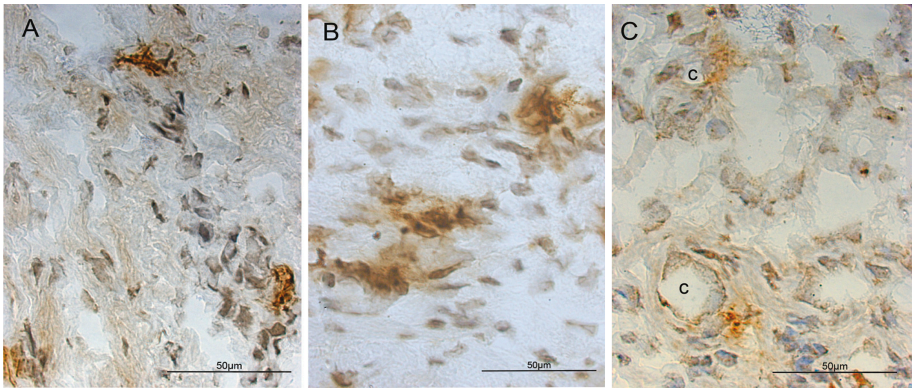

Figure 12. Immunohistochemical staining for MMP-1. A=misoprostol, B=isosorbide mononitrate, C=no treatment. Bar 50µm

Positive immunostaining of MMP-9 was observed in the endothelium in the vessel wall of veins, arteries as well as capillaries. In the IMN group it was evident that an increased sprouting of stained capillaries contributed to the high score. In addition, positively stained single cells or clusters of cells in the interstitial tissue were more frequent in the IMN group. In women who had no treatment the number of positively stained interstitial cells was low and staining was mainly observed in endothelial cells (Fig.13).

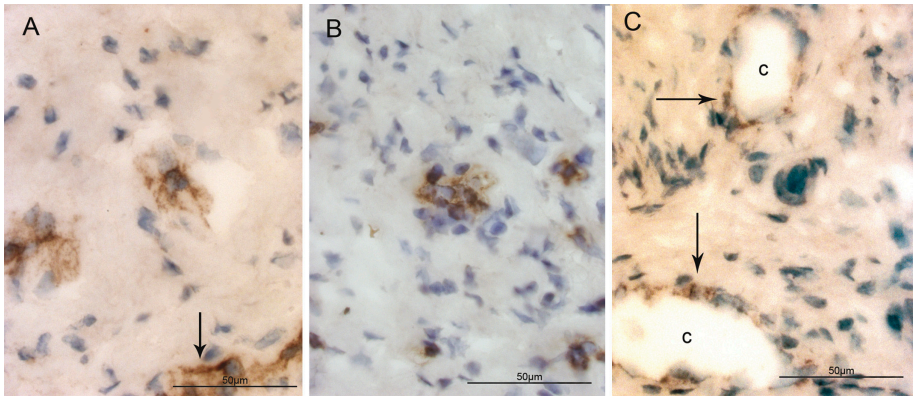

Figure 13. Immunohistochemical staining for MMP-9. Arrows indicate stained endothelial cells. A=misoprostol, B=isosorbide mononitrate, C=no treatment. Bar 50µm

IL-8

Cervical levels of IL-8, measured by means of ELISA, were significantly increased ($p < 0.05$) following treatment with misoprostol compared to IMN and controls (Table 5, Fig. 14).

*Table 5. IL-8 (pg/mg protein) presented as median (IQR). *=p<0.05*

	Misoprostol (n=10)	Isosorbide mononitrate (n=10)	Control (n=6)
IL-8	8.8(4.0-55.3)*	2.7(1.6-4.9)	2.4(1.9-3.8)

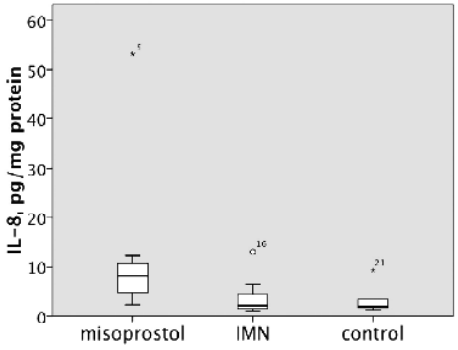

Figure 14. Boxplot illustrating IL-8 levels in cervical tissue specimens.

Comments:
Ultrastructural changes of the cervical tissue were obvious after treatment with misopristol as well as IMN compared to controls. The tissue exhibited an inflammatory-like reaction demonstrating collagenolysis with disorganization of the ECM, splitting of collagen fibres, tissue oedema, activation of fibroblasts and endothelial cells as well as increased number of mast cells. This tissue reaction was most prominent in women treated with misoprostol. Tissue from women in the IMN group demonstrated typically sprouting of capillaries. Activation of mast cells with degranulation and secretion of granules in the stroma appeared specific. Interestingly, in cervical tissue from women who had no treatment scarce reactive areas with disintegrated collagen fibres and reactive mast cells were seen. These findings most likely indicate cervical tissue plasticity and constantly ongoing physiological remodelling of the cervical collagen framework.
The inflammatory tissue reaction was further documented by biochemical and histochemical data of cervical tissue specimens from both treatment groups, demonstrating expressions of an inflammatory reaction with significantly increased levels of IL-8 in the misoprostol group and intensive staining for MMP-1 and MMP-9 in the misoprostol as well as the IMN group compared to controls. Overall, the present data illustrate that the clinically estimated cervical ripening (tonometry) is similar to an inflammatory reaction following treatment with both misoprostol or IMN.

Study IV.
Cervical tissue changes in women with miscarriage: a morphological and biochemical investigation.

In this study cervical ultrastructure and inflammatory parameters (MMP-1, MMP-8, MMP-9 and IL-8) were compared in women with symptomatic and silent miscarriage. Demographics of included women are given in Table 6.

Table 6. Age and gestational age of women included. Data are presented as mean (range).

	Symptomatic miscarriage n=7	Silent miscarriage n=11	Control group n=11
Age, mean years (range)	30 (23-36)	31 (25-36)	22 (19-29)
Gestational age, mean days (range)	64 (42-84)	64 (49-77)	58 (23-36)

Electron microscopy
The ultrastructure of cervical specimens from women with symptomatic miscarriage typically displayed a disorganized collagen framework with splitting and disruption of the collagen fibers with frequently observed granular material in the extracellular matrix. The fibroblasts appeared activated with dispersed nuclear chromatin and proliferative gER. The fibroblast cytoplasmic projections were elongated, containing

granular material (Fig 15a). Mast cells were frequently observed exhibiting cytoplasmic elongations and cytolemmal buddings (Fig 15b).
The ultrastructure of specimens from women with silent miscarriage displayed even more pronounced disruption of the collagen framework compared to specimens from women with symptomatic miscarriage with scarce areas of intact collagen bundles (Fig 15c). In the distorted collagen framework, granules, probably of mast cell origin, were frequently observed. Reactive mast cells were often observed and demonstrated cytoplasmic buddings, secretory granules at various stages of disruption and secreted granules in the neighbouring stroma (Fig 15c). The fibroblasts appeared activated and fibroblast cytoplasma was markedly elongated with folded and distorted projections containing granular material (Fig 15d).
In specimens from women in the control group the collagen framework appeared predominantly well organized (Fig 15e), though scarce minor areas appeared disorganized. Fibroblasts appeared less activated and mast cells were less frequently observed compared to specimens from women with miscarriage (Fig 15f).

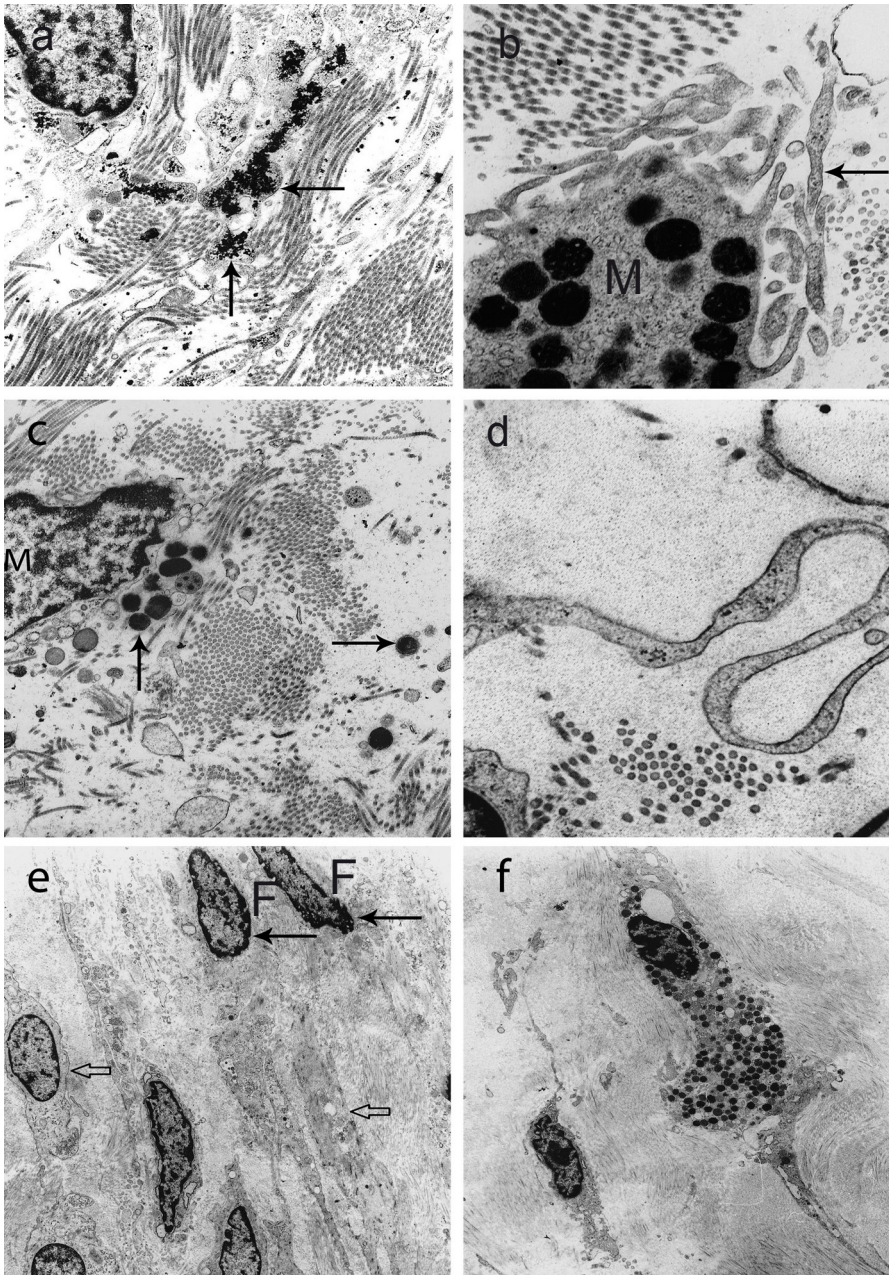

Figure 15. Electron micrographs of cervical tissue obtained from women with symptomatic miscarriage(a,b): a. granular material in cytoplasmic projection (x14500), b. arrow indicates cytoplasmic budding (x41000); silent miscarriage (c,d): c. arrows indicate mast cell granula (x14500), d. cytoplasmic projection (x41000); control women (e,f): e. intact collagen network, regularly distributed fibroblasts. arrow indicates densely packed chromatin. smooth muscle cells (open arrow) (x14500), f. intact collagen surrounding a mast cell (x6500). M=mast cell. F= fibroblast.

IL-8

Cervical tissue levels of IL-8, measured by ELISA and semiquantitatively estimated by immunohistochemical analysis on the basis of number of immunopositive cells, were significantly increased in women with symptomatic miscarriage and in women with silent miscarriage compared to women in the control group. There was no significant difference between women with symptomatic miscarriage and silent miscarriage (Table 7, Fig 16). No significant difference was registered between the groups with respect to staining intensity.

Table 7. IL-8 (pg/mg protein) presented as median (IQR).

	Misoprostol (n=10)	Isosorbide mononitrate (n=10)	Control (n=6)
IL-8	8.8(4.0-55.3)*	2.7(1.6-4.9)	2.4(1.9-3.8)

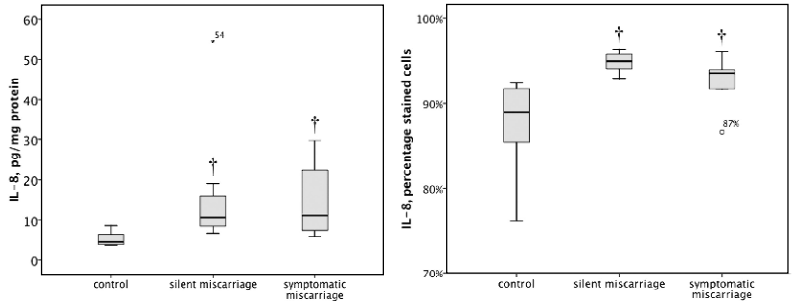

Figure 16. IL-8 assessed by ELISA (pg/mg protein) in the left boxplot; IL-8 assessed by immunohistochemistry (%) positively stained cells (right boxplot). †= significantly higher compared to controls.

Positive staining for IL-8 was observed in single cells and clusters of cells within the stroma, in smooth muscle cells of stromal muscle bundles, in smooth muscle cells in vessel walls and in endothelial cells (Fig. 17). Enrichment of immunopositive cells was observed in the vicinity of capillaries (Fig. 17). In specimens where glandular crypts were present, accumulations of IL-8 positive cells were noticed in the subepithelial stroma.

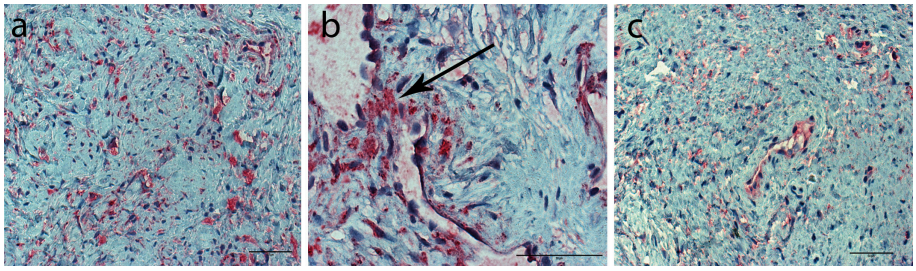

Figure 17. Immunohistochemical localization of IL-8 in symptomatic miscarriage (a), silent miscarriage (b) and controls (c) Arrow indicates perivascular staining. Bar 50μm

Matrix metalloproteinases

In general, light microscopy revealed that the connective tissue in specimens of women in the control group appeared more compact in comparison with specimens from women with symptomatic and silent miscarriage.

Semiquantitative analysis of immunopositive staining for MMP-1 as well as for MMP-8 showed a tendency for higher staining intensity in specimens from women with miscarriage compared to controls, but there was no significant difference between the groups. Immunopositive staining for MMP-9 was significantly lower in specimens from women with symptomatic miscarriage compared to specimens from women with silent miscarriage and controls.

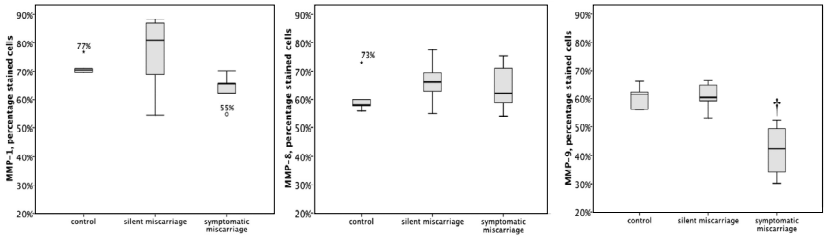

Figure 18. Frequency of immunohistchemical staining for MMP-1 (left), MMP-8 (middle), MMP-9 (right). †=significantly lower compared to silent miscarriage and controls.

Immunopositive staining for all MMPs was observed in single cells within the stroma and smooth muscle cells organized in bundles and in smooth muscle cells in vessel walls. Especially intensively staining was observed in endothelial cells. Stained cells within the capillary wall or in its immediate vicinity, possibly indicating diapedesis, were most evident for MMP-9 and most frequently observed in specimens from women with silent miscarriage.

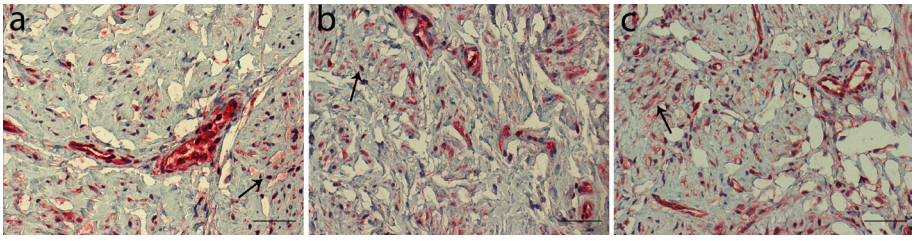

Figure 19. Immunohistochemical localization of MMP-1 in controls (a), symptomatic miscarriage (b), silent miscarriage (c). Arrows indicates smooth muscle. Bar 50μm

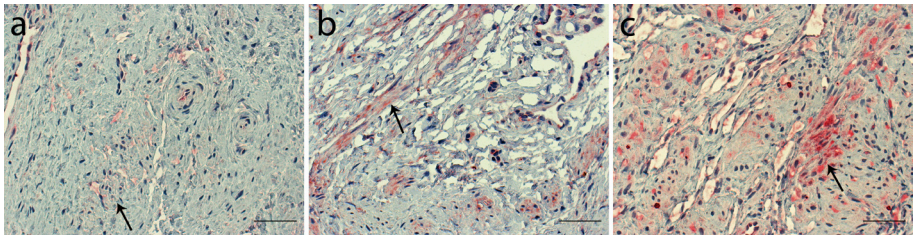

Figure 20. Immunohistochemical localization of MMP-8 in controls (a), symptomatic miscarriage (b), silent miscarriage (c). Arrows indicates smooth muscle. Bar 50μm

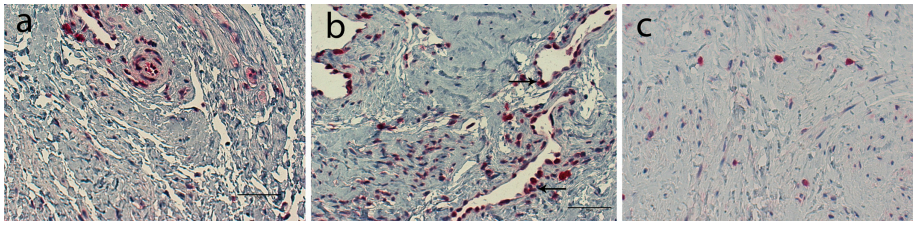

Figure 21. Immunohistochemical localization of MMP-9 in symptomatic miscarriage (a), silent miscarriage (b), controls (c). Arrows indicates immunopositive cells in the vicinity of vessels. Bar 50μm

Comments:

Only primigravid women were included to avoid bias due to previous gestations. The age of women in the control group happened to be less than that of women suffering miscarriage. However, it seems reasonable that parity more than age could influence the observations in this study.

Cervical tissue of women having symptomatic as well as silent miscarriage demonstrated ultrastructural changes with disorganized collagen framework, activated fibroblasts exhibiting dispersed chromatin, cytoplasmic elongations, proliferative gER and accumulation of granular material. A consistent observation in areas where collagen fibres were disrupted and disorganized was the enrichment and activation of mast cells. The overt mast cell reactivity points to a key role of these cells in the inflammatory reaction associated with cervical ripening.

The significantly higher tissue levels of IL-8 and the tendency to increased staining for MMP-1 and MMP-8, as observed in women with miscarriage, further support the inflammatory process associated with cervical tissue remodelling. Unexpectedly, the staining for MMP-9 was significantly lower in women with symptomatic miscarriage compared to women with silent miscarriage and controls. This could indicate a difference in the time-course of the inflammatory response in symptomatic compared to silent miscarriage. MMP-9 is especially expressed in the initial phase of an inflammatory reaction. Therefore, it can not be excluded that MMP-9 in cervical tissue from women with symptomatic miscarriage might have decreased substantially at the time point of tissue sampling.

To summarize, the present study demonstrates that an inflammatory process occurs in the cervix in both women with symptomatic and silent miscarriage. The inflam-

matory responses appear to be similar in the two groups of women. Therefore, an inadequate inflammatory response does not seem to be the reason why some miscarriages remain silent.

DISCUSSION

Surgical termination of pregnancy with a gestational age < 7 weeks may be performed without mechanical dilatation of the cervical canal. The so called Karman technique for evacuation can be successfully performed with a low risk of complications (Karman and Potts 1972; Atterfelt 1977). Beyond 8 weeks of gestation dilatation of the cervix is a prerequisite for successful vacuum aspiration (Herczeg 1990). Several studies have shown that rapid mechanical dilatation *per se* may be harmful (Hulka and Higgins 1961; Molin 1993). Currently, pharmacological regimens are increasingly being used to induce abortion in early pregnancy, making mechanical dilatation unnecessary. Thus, 20 years ago Swahn and Bygdeman presented a pioneering study combining antiprogestins with PGs in the management of medical abortion up to the 9th gestational week (Swahn and Bygdeman 1989). Still, surgical evacuation remains an alternative procedure, which may be preferred by some women. At present, surgical termination of pregnancy is the procedure of choice from the 10th to 12th gestational week. Irrespective of previous parity or history of previous gestations treatment to induce presurgical softening of the cervix is in common use in order to reduce the risk of mechanical trauma and other complications, such as infections, and to facilitate the surgical procedure. Presurgical priming of the uterine cervix is strongly recommended especially in young women (RCOG 2004). The resistance of the cervical tissue to mechanical dilatation can be divided into three phases according to Atienza *et al* (Atienza 1980). (1) An initial low resistance phase, reflecting the elastic properties of the cervical tissue. (2) A phase of firm resistance where the metal dilator must be held under pressure, the so called visco-elastic creep. (3) A phase of sudden decreasing resistance, reflecting tear of the tissue. At the point where the cervical visco-elasticity is exceeded (approximately 9mm) small cervical tears may appear (Hulka and Higgins 1961; Molin 1993). If the cervix is being dilated > 12mm the fibrous matrix may be irreversibly destroyed (Johnson 1989). After preoperative cervical priming with either the PG analogue gemeprost or synthetic Dilapan tents the yielding point to dilatation is moved from 9 to 11mm, a dilatation sufficient for vacuum aspiration up to 12 completed gestational weeks (Robinson 1991).

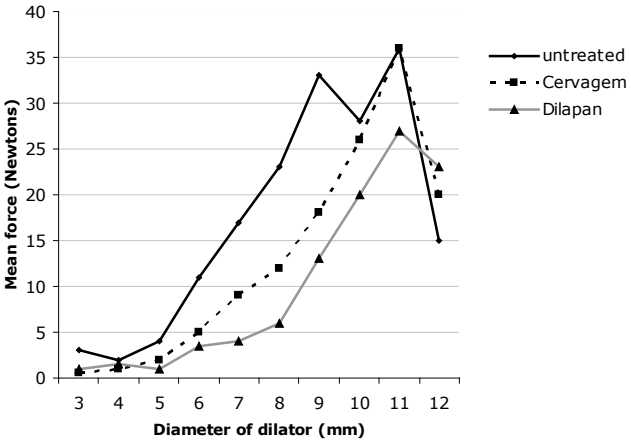

Figure 22. Mean force required to dilate the uterine cervix with Hegar dilators (3-12 mm) in untreated women and women treated with Cervagem or Dilapan.

In the present thesis the PG analogues misoprostol and gemeprost and the NO donor IMN were used for cervical priming in the first trimester. Misoprostol was shown to be as effective as gemeprost based upon baseline cervical dilation and the mechanical force to dilate the cervix up to 10mm. This observation confirms the results of earlier studies comparing misoprostol and gemeprost (el-Refaey, Calder et al. 1994; Platz-Christensen, Nielsen et al. 1995; Henry and Haukkamaa 1999). For medical termination of pregnancies in the first and second trimester PGs have been used in combination with mifepristone, where misoprostol was found to be equally effective as gemeprost (Tang and Ho 2002; Svendsen, Rorbye et al. 2005).

Prostaglandins have an important role in cervical ripening and their action appears to be mainly on the ECM. Biochemical studies on cervical tissue obtained from women in the first trimester have shown that a lowering of hydroxyproline levels takes place after intracervical treatment with $PGF_{2\alpha}$, indicating a decrease of cervical collagen content (Rath, Theobald et al. 1982). An influence of PGs on cervical collagen metabolism was further documented by an increased radio-labelling with tritiated proline, a precursor for collagen-specific hydroxyproline, in cervical tissue obtained from women in the first trimester after treatment with PGE_2, gemeprost or misoprostol (Norström 1982; el-Refaey, Calder et al. 1994). Moreover, both PGE_2 and $PGF_{2\alpha}$ were shown to stimulate collagenolytic activity in isolated cervical fibroblasts (Goshowaki, Ito et al. 1988). Our EM observations of a pronounced splitting and disorganization of the collagen fibers after treatment with misoprostol further support the effect of PGs on the ECM.

Pro-collagens are synthesized by fibroblasts and secreted to the ECM where they are transformed into tropocollagen, which further via cross-linking forms mature collagen fibrils. Fibroblasts, when specifically stimulated, may also have the capacity to produce collagenases, *i.e.* contributing to breakdown of their collagen products. Thus, the activation of fibroblasts as seen in cervical tissue from women treated with

misoprostol demonstrates their central role in remodelling of the collagen network. The main collagens of the uterine cervix are of type I and III. Both collagen type I and collagen type III are degraded by collagenases, *i.e.* MMP-1 and MMP-8, and further, after degradation, by gelatinases, *i.e.* MMP-9. Vaginal treatment with misoprostol was shown to increase cervical tissue levels of MMP-8 and MMP-9 (Aronsson, Ulfgren et al. 2005). In this thesis a tendency for increased intensity of immunohistochemical staining for both MMP-1 and MMP-9 following priming with misoprostol was noticed.

Cervical ripening, as an inflammatory process, is associated with a migration of inflammatory cells into the cervical tissue (Junqueira, Zugaib et al. 1980). The role of PGs in the initiation of the inflammatory process was demonstrated by a higher staining of CD45 positive leukocytes following treatment with misoprostol (Greer, Millar et al. 1992; Aronsson, Ulfgren et al. 2005). The influx of leukocytes is directed by the chemoattractant action of IL-8, produced in stationary macrophages and monocytes. The formation of IL-8 might be additionally enhanced by administered PG (Denison, Calder et al. 1999). Migrated, activated neutrophils may become the main source of IL-8 production. Increased cervical tissue levels of IL-8, as demonstrated in this thesis following treatment with misoprostol, is in accordance with this concept. Most data concerning physiological and biochemical events during cervical ripening have been elucidated at term pregnancy. Thus, an increase of IL-8 as well as an increased expression of IL-8 mRNA were documented in the cervix and in the lower uterine segment in term pregnant women (Osmers, Blaser et al. 1995; Sennström, Brauner et al. 1997; Sennström, Ekman et al. 2000; Osman, Young et al. 2003; Malmström, Sennström et al. 2007). A different inflammatory response was observed in post-term women with failed induction of labor after treatment with PGs, compared to women with successful induction. Accordingly, failed induction was associated with a reduced number of CD45 positive cells indicating an impaired influx of leucocytes as well as decreased immunostaining of IL-8 and MMP-9 (Sahlin, Stjernholm-Vladic et al. 2008).

In the present thesis vaginal misoprostol (200µg) was shown to be more effective than the NO donor IMN (40mg) to induce cervical dilatation prior to surgical termination of early pregnancy. In other studies, the ripening effect of vaginally administered NO donors was demonstrated in guinea-pigs (Chwalisz, Shao-Qing et al. 1997) as well as in humans, both in early pregnancy and at term (Thomson, Lunan et al. 1997; Facchinetti, Piccinini et al. 2000; Bullarbo, Orrskog et al. 2007). However, a similar difference in ripening effect as in our study was found when 40mg IMN was compared to misoprostol with a priming interval of 4-6 hours (Li, Chan et al. 2003). The poor clinical response of IMN in that study as well as in our investigation and the discrepancy in effects compared to other investigations are hard to explain. It is unlikely that the dosage of IMN has any major impact since a higher dose of IMN (80mg) gave the same ripening effect as 40mg IMN (Thomson, Lunan et al. 1998). The prolonged treatment interval (overnight) does not explain the minimal effect of the NO donor. The treatment interval was chosen since the serum levels of vaginal levels of IMN proceed to increase at least up to 6 hours after vaginal administration (Bates, Nicoll et al. 2003). Previously, it was shown that IMN

increases the expression of COX-2 as well as stimulates the synthesis of both PGE_2 and $PGF_{2\alpha}$ in the cervix in the first trimester (Ekerhovd, Weijdegård et al. 2002). A concomitant PG formation could theoretically reinforce a priming effect of NO. An effect of NO on PG synthesis was also demonstrated in other studies. Cervical explants from women treated with IMN produced increased amount of $PGF_{2\alpha}$ *in vitro* (Ledingham, Denison et al. 1999a). Likewise, IMN increased the expression of COX-2 in the cervix at term (Bullarbo, Norström et al. 2007). Nitric oxide in turn may stimulate the synthesis of PGs (Salvemini 1997). Thus, NO may act in concert with PGE_2 as part of the inflammatory process during cervical ripening. As both PGs and NO donors have shown a ripening effect on the cervix a combination IMN and misoprostol was applied for cervical priming. However, the combination of NO and PG did not result in an enhanced efficiency on cervical ripening (Ledingham, Thomson et al. 2001).

No considerable clinical effect of vaginal IMN on cervical dilation was noticed in our study. However, it seems evident that the drug initiates an inflammatory reaction in the cervix. Consequently, we observed an apparent alteration of the cervical ultrastructure following vaginal application of IMN. These changes included derangement of collagen fibers, tissue oedema and activation of fibroblasts and mast cells. In addition, the expressions of the MMP-1 and the MMP-9 were increased. In cultured fibroblasts or explants of cervical tissue NO donors increased MMP-1 production (Yoshida, Sagawa et al. 2001) but did not affect the expression of MMP-2 and MMP-9 (Ledingham, Denison et al. 1999b). A role of NO in cervical function and its involvement in physiological cervical ripening was documented by the identification of an endogenous NOS system in the cervix (Ekerhovd, Brännström et al. 1998; Ekerhovd, Brännström et al. 2000) and the demonstration of elevated expression of NOS at term (Tschugguel, Schneeberger et al. 1999; Ledingham, Thomson et al. 2000).

In our study cervical levels of IL-8, a key mediator of cervical ripening, were not affected by NO treatment. However, an *in vitro* study on cultured cervical fibroblasts demonstrated elevated IL-8 after both PGE_2 and NO stimulation (Denison, Calder et al. 1999).

Adverse effects of the priming agents were registered in both clinical studies and systematically analysed in Study II. Side effects known to be common after treatment with PGs (Honkanen, Piaggio et al. 2004; Svendsen, Rorbye et al. 2005) were reported by women treated with misoprostol and gemeprost. The most frequent side effects of the PG analogues appear to be related to PG action on smooth muscle contractile activity, *i.e.* abdominal pain, vaginal bleeding and nausea. Women treated with IMN experienced side effects related to vasodilation, *i.e.* headache and hot flushes. According to the VAS scale, all side effects registered were of moderate to mild intensity. As compared to our results, previous studies have registered less frequent side effects following treatment with NO donors in the first trimester (Thomson, Lunan et al. 1998; Li, Chan et al. 2003), although a high frequency of headache was noticed when IMN was administered at term (Bullarbo, Orrskog et al. 2007).

When miscarriage occurs, it is likely that the same pathways are activated as at parturition, including softening and dilation of the cervix and establishment of uterine

contractions to expel gestational products. This is seen in women having symptomatic miscarriage with vaginal bleeding and abdominal pain. Women with silent miscarriage do not experience any symptoms. For termination of pregnancy an induction of an abortive process might be considered. In women with silent miscarriage the cervix appears well preserved without obvious signs of maturation. Knowledge of biochemical and biophysical mechanisms which regulate cervical ripening have been obtained in studies of spontaneous cervical ripening at term and in the first trimester of pregnancy where various regimens have been in use to induce cervical softening. Few investigations concerning cervical tissue events in relation to miscarriage, as in study IV, have been carried out. As expected, cervical tissue from women with symptomatic miscarriage exhibited reactions consistent with cervical ripening. On the other hand, it was unexpected that the same phenomenon also was observed in cervical tissue specimens from women with silent miscarriage.

Electron microscopy of symptomatic and silent miscarriage demonstrated a deranged collagen framework compared to controls. In silent miscarriage the areas with intact collagen bundles were even more scarce than in symptomatic miscarriage. In addition, fibroblasts and mast cells appeared activated with cytoplasmic elongations, often containing granular material. These findings indicate an ongoing cervical remodelling, though not being clinically overt. Mast cells were more frequently observed in specimens from silent and symptomatic miscarriage compared to controls. Generally, mast cells are engaged in inflammatory processes. The secretory granules contain a variety of inflammatory mediators, *i.e.* histamine, cytokines, proteases and proteoglycans (heparin). On activation, the secretory granules are released and the inflammatory agents are being synthesized *de novo*. An enrichment of activated mast cells appears clearly associated with cervical ripening and was, as mentioned, noticed as a specific reaction in women treated with both misoprostol and IMN. A potential role of mast cells in cervical ripening has been indicated in previous studies. An increase in the number of mast cells was seen following cervical priming with Lamicel and mifepristone in the first trimester (Nicolaides, Welch et al. 1983; Rådestad, Thyberg et al. 1993). Moreover, increased numbers of decidual mast cells were demonstrated in first trimester miscarriage. It was suggested that mast cells play a role in the onset of miscarriage due to their production of cytokines (Marx, Arck et al. 1999). It was also proposed that local corticotropin-releasing hormone may activate endometrial mast cells to release high levels of tryptase and IL-8, seen in gestational products of recurrent miscarriage (Madhappan, Kempuraj et al. 2003). Mast cell degranulation might stimulate normal angiogenesis in pregnancy (Varayoud, Ramos et al. 2004). They also play a key role in neutrophil recruitment and may contribute to degradation of the ECM by releasing MMPs (Chen, Ning et al. 2001).

In our semiquantitative estimation of the expression of MMPs assessed by immunohistochemistry there was a tendency to higher immunopositive staining of MMP-1 and MMP-8 in silent miscarriage and a significant decrease of MMP-9 in symptomatic miscarriage. This finding could indicate a different response in silent versus symptomatic miscarriage. In an inflammatory process various MMPs are stepwise and transiently activated and MMP-9 is expressed with highest levels in the initial

phase (24-48 hours) of the inflammatory reaction (Tarlton, Vickery et al. 1997). Therefore, one could speculate that the expression of MMP-9 in women with symptomatic miscarriage might have decreased substantially at the time point of tissue sampling. MMP-9, a gelatinase, degrades especially collagen IV. Its high affinity to collagen IV, even when inactive, yields by the juxtaposition a pool of the enzyme, which is rapidly available for any remodelling (Olson, Toth et al. 1998). Nevertheless, the importance of MMPs in cervical remodelling is well documented and was recently evaluated by modern techniques, demonstrating that the expression mRNA for MMP-1 and MMP-9 as well as protein levels of MMP-8 and MMP-9 are high during cervical ripening at term (Dubicke, Åkerud et al. 2008).

Interleukin-8 was significantly higher in cervical specimens from women with both symptomatic and silent miscarriage. Interleukin-8 is considered to be an important mediator of cervical ripening since it is detected in increased amounts in cervical tissue at term, in women having non-infected preterm delivery, after induced cervical ripening in the first trimester as well as in women with miscarriage (Sennström, Brauner et al. 1997; Törnblom, Klimaviciute et al. 2005). Our IL-8 data support that a ripening process is established in the cervix of women with both symptomatic and silent miscarriage. Since circulating levels of IL-8 are not elevated in spite of elevated IL-8 levels in cervical tissue, the ripening process stands out as a local inflammatory reaction (Koumantaki, Matalliotakis et al. 2001; Paradisi, Porcu et al. 2003; Whitcomb, Schisterman et al. 2007).

The reason why some miscarriages remain silent remains unclear. The present study shows that, also in first trimester silent miscarriages, the cervix undergoes ripening as based on biochemical, immunohistochemical and EM parameters. Obviously, persistent myometrial quiescence may underlie the failed "spontaneous" abortion. Myometrial contractility is inhibited by progesterone and a physiological relative lowering of progesterone towards term may both initiate myometrial contractions (Csapo, Bernard et al. 1980) and cervical ripening (Chwalisz and Garfield 1997).

Nitric oxide, which is a myometrial relaxant, might be another central mediator of uterine quiescence. Moreover, progesterone may play a physiological role not only in the uterine body but also in the uterine cervix since receptors for progesterone are present in the cervix (Sanborn, Held et al. 1976). In animals it was found that a fall in progesterone levels inhibits the release of NO in the uterine body but promotes NO production in the cervix (Chwalisz and Garfield 1997). In accordance with these observations, it was demonstrated that low serum levels of progesterone in women with non-viable first trimester pregnancy was associated with higher levels of NO metabolites in the cervical secretion. Furthermore, women with low levels of NO metabolites failed more often to abort completely after mifepristone-misoprostol or expectancy management (Väisänen-Tommiska, Nuutila et al. 2003). It is also noteworthy that women with silent miscarriage respond less successfully to combined treatment with mifepristone and misoprostol in attempts to eliminate gestational products (Nielsen, Hahlin et al. 1997). Therefore, it is tempting to speculate that mechanisms related to local levels of progesterone may regulate the initiation of the abortive process. This hypothesis awaits to be elucidated in future investigations.

CONCLUSION

Misoprostol (400μg) is as effective as gemeprost (1mg) for cervical priming in the first trimester prior to surgical termination of pregnancy.

Misoprostol (200μg) induces a more pronounced cervical ripening than IMN (40mg) in the first trimester prior to surgical termination of pregnancy. Both regimens are associated with a high frequency of side effects when administered 9-13 hours before surgery.

Both misoprostol and IMN induce ultrastructural changes in the cervix consistent with collagenolysis. These changes are less pronounced following cervical priming with IMN compared to misoprostol.

Cervical tissue levels of IL-8 are elevated in women following cervical priming with misoprostol in the first trimester. There is an increased expression of MMP-1 and MMP-9 following cervical priming with IMN.

In the cervix of women with both symptomatic and silent miscarriage in first trimester an inflammatory-like process occurs as ascribed in cervical ripening at term:
-The ultrastructure demonstrates derangement of collagen fibrils.
-Cervical tissue levels of IL-8 are elevated.

Tissue expressions of MMP-1 and MMP-8 were not significantly altered in women with miscarriage, whereas the expression of MMP-9 was decreased in women suffering from symptomatic miscarriage compared to women with silent miscarriage.

SAMMANFATTNING

Introduktion: Livmoderhalsen har förmågan att förändras från att vara ett organ med kompakt vävnad hos icke gravida kvinnor till att bli mjuk mot slutet av en graviditet och vidgas vid förlossning och på så sätt möjliggöra passage för ett foster. Denna uppmjukningsprocess benämns "livmoderhalsmognad" och beskrives som en inflammatorisk reaktion med aktivering av inflammatoriska cytokiner (ex. IL-8), matrix metalloproteinaser (MMP) och nedbrytning av den kollagena bindväven. Uppmjukning av livmoderhalsen kan induceras före kirurgiska aborter av farmaka såsom prostaglandiner (PG) och kväveoxid (NO). Det är logiskt att tro att en utmognad av livmoderhalsen inträffar hos kvinnor i samband med missfall på likartat sätt som vid förlossning.

Målsättning: Målsättningen med denna avhandling var att undersöka kliniska, morfologiska och biokemiska förhållanden vid uppmjukningen av livmoderhalsen hos kvinnor i tidig graviditet (första trimestern) inför kirurgisk abort både efter förbehandling med PG samt NO och i samband med spontant missfall med eller utan kliniska symptom.

Metod: Kvinnor som ej genomgått en vaginal förlossning och som söker för kirurgiskt avbrytande av graviditet i första trimestern randomiserades till förbehandling i Studie I med antingen PG-analogerna misoprostol eller gemeprost och i Studie II med misoprostol eller NO-donatorn isosorbide mononitrate (IMN). I Studie I och II mättes livmoderhalsmognaden med tonometri, illustrerande livmoderhalsens eftergivlighet. Biverkningar bedömdes med hjälp av en enkät. I studie III togs vävnadsprov från livmoderhalsen från kvinnor förbehandlade med misoprostol eller IMN och analyserades med elektronmikroskopi (EM). Inflammatoriska parametrar analyserades med ELISA (IL-8) och immunohistokemi (IHC) (MMP-1, MMP-9). I Studie IV erhölls vävnadsprov från kvinnor med missfall i första trimestern (symptomatiska och asymptomatiska) och från kvinnor i samband med kirurgiskt avbrytande av tidig graviditet. Vävnadens morfologi studerades med EM och inflammatoriska parametrar med ELISA (IL-8) samt IHC (MMP-1, MMP-8, MMP-9).

Resultat: Det var ingen skillnad i hur mycket livmoderhalsen var vidgad initialt eller i den samlade kraft som behövdes för att vidga livmoderhalskanalen till 10mm vid jämförande av förbehandling med misoprostol och gemeprost. Motståndet att vidga livmoderhalsen var högre hos kvinnor, förbehandlade med IMN, jämfört med kvinnor, förbehandlade med misoprostol. Buksmärta och vaginala blödningar var vanligare hos kvinnor förbehandlade med misoprostol, medan huvudvärk var vanligare hos kvinnor förbehandlade med IMN. I vävnadsprover från kvinnor, som erhållit förbehandling med misoprostol, var det kollagena nätverket delvis upplöst och bindvävsceller såsom fibroblaster och mastceller visade reaktiva tecken. På ett likartat sätt, men om än mindre uttalat, observerades ultrastrukturella förändringar i vävnadsprover från kvinnor förbehandlade med IMN. Vävnadsmängden av IL-8 var högre i kvinnor förbehandlade med misoprostol jämfört med IMN och kvin-

nor utan förbehandling. Med IHC visades att uttrycket av MMP-1 och MMP-9 i vävnaden var högre hos kvinnor förbehandlade med IMN jämfört med misoprostol eller ingen behandling. I livmoderhalsvävnaden från kvinnor med missfall observerades en uppluckring av den kollagena bindväven med reaktiva fibroblaster samt ofta förekommande mast celler med sekretorisk aktivitet. Vävnadsmängden av IL-8 var förhöjd hos kvinnor med missfall. Uttrycket av MMP-1 och MMP-8, värderat med IHC, skiljde sig inte för kvinnor med missfall jämför med kvinnor i kontrollgruppen. Däremot var MMP-9 lägre hos kvinnor med symptomatiska missfall.

Sammanfattning: Misoprostol är lika effektiv som gemeprost för utmognad av livmoderhalsen i första trimestern. Misoprostol ger en mer uttalad livmoderhalsmognad jämfört med IMN, men båda behandlingarna är associerade med biverkningar. Trots att den kliniskt tydliga vidgningen av livmoderhalsen efter behandling med IMN ej är jämförbar med misoprostol, gav förbehandling med IMN en ”inflammatorisk ”vävnadsreaktion, talande för en initierad utmognadsprocess. I livmoderhalsen hos kvinnor med både symptomatiskt och asymptomatiskt missfall ses en ”inflammatorisk ”vävnadsreaktion som tyder på en pågående uppmjukning av livmoderhalsen. Därför verkar inte en utebliven utmognad av livmoderhalsen vara orsaken till att en del missfall förblir asymptomatiska.

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