

From the DEPARTMENT OF WOMEN'S AND CHILDREN'S HEALTH
Karolinska Institutet, Stockholm, Sweden

STUDIES ON FEMALE GENITAL TRACT INFECTIONS AND THE ROLE OF NITRIC OXIDE IN DIAGNOSIS

Angelos Sioutas



**Karolinska
Institutet**

Stockholm 2010

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Published by Karolinska Institutet.

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ISBN 978-91-7409-896-9

Printed by
 **REPROPRINT AB**
Stockholm 2010
www.reproprint.se

Gårdsvägen 4, 169 70 Solna

“Nature does nothing uselessly”
Aristotle

To Evangelia

ABSTRACT

Female genital tract infections are among the most common conditions causing individuals to seek medical care, and are associated with a high gynaecologic and reproductive morbidity. Thus, prompt and precise diagnosis of genital tract infections is essential to institute effective therapy and prevent sequelae. It is therefore desirable to develop an easily performed, objective test that can be useful to the practicing physician. Local measurement of nitric oxide (NO) gas in hollow organs has been used to detect and monitor inflammatory processes, but as of today NO has not been studied in the female genital tract.

The aim of the present thesis was to investigate whether it is possible to measure accurate NO formation in the female genital tract. We also sought to explore the role of NO as a diagnostic marker for inflammation in the female genital tract. Finally we wanted to evaluate the effect of bacterial vaginosis (BV) on the pharmacokinetics (plasma concentration C_{max}, T_{max} and bioavailability measured as the AUC₂₄₀) of vaginally administered misoprostol in the first trimester of pregnancy.

NO has a very short half-life in biological tissues, but it is more stable in the gaseous phase, which makes it possible to measure this free radical in luminal structures. We have developed a new method for measurement of luminal NO formation involving the insertion of a silicon catheter in the vagina or the uterine cavity. The balloon is filled with air, which is incubated in the vagina or the uterine cavity for sampling of NO from the vagina or the uterus respectively.

We observed an almost 100-fold increase in intrauterine concentration of NO in patients diagnosed with pelvic inflammatory disease compared to those diagnosed with appendicitis or healthy controls. Uterine NO levels were uniformly low in healthy women throughout the menstrual cycle. Intrauterine NO levels did not rise after manipulation in the uterine cavity, and after an incubation time of just two minutes, NO levels were significantly increased in patients with diagnosed PID compared to control subjects. Moreover, in patients with symptoms of vaginitis, NO concentration was almost 100-fold increased compared to healthy controls. Vaginal NO levels were uniformly low among healthy women, both of reproductive age and in menopause.

Finally, we observed that the bioavailability of misoprostol was reduced in patients with BV, but there was no significant difference in the pharmacokinetics of vaginally administered misoprostol between the patients with BV and healthy control subjects.

In conclusion, BV did not affect the pharmacokinetics of vaginally administered misoprostol in early pregnancy. Furthermore, we describe a simple, reliable, rapid, safe and well tolerated method for measuring NO in the female genital tract. We also suggest that NO could be further evaluated as a biomarker of inflammatory disease in the female genital tract.

LIST OF PUBLICATIONS

- I. Sioutas A, Ehrén I, Lundberg JO, Wiklund NP, Gemzell-Danielsson K. "Intrauterine Nitric Oxide in Pelvic Inflammatory Disease" *Fertil Steril.* 2008 Apr;89(4):948-52.
- II. Sioutas A, Ehrén I, Gemzell-Danielsson K. "Measurement of nitric oxide in the vagina." *Acta Obstet Gynecol Scand.* 2008;87(10):1055-9.
- III. Sioutas A, Gemzell-Danielsson K, Lundberg JO, Ehrén I. "Measurement of luminal nitric oxide in the uterine cavity using a silicon balloon catheter" *Submitted*
- IV. Sioutas A, Sandström A, Fiala C, Watzer B, Schweer H, Gemzell-Danielsson K. "Effect of bacterial vaginosis on the pharmacokinetics of vaginally administered misoprostol in early pregnancy" *Submitted*

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LIST OF ABBREVIATIONS

AUC	Area under the curve
BH ₄	Tetrahydrobiopterin
BV	Bacterial vaginosis
CaM	Calmoduline
CDC	Centers for Disease Control and Prevention
DNA	Deoxyribonucleic acid
EDRF	Endothelium-derived relaxing factor
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GBS	Group B streptococci
GC	Neisseria gonorrhoeae
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HSV	Herpes simplex virus
LH	Luteinizing hormone
LPS	Lipopolysaccharides
MPA	Misoprostol acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NO ₂ ⁻	Nitrite
NO ₃ ⁻	Nitrate
NOS	Nitric oxide synthase
NSAID	Nonsteroidal anti-inflammatory drug
PID	Pelvic inflammatory disease
PPB	Parts per billion
PPM	Parts per million
STI	Sexually transmitted infection
VAS	Visual analogue scale
WHO	World Health Organization

1 INTRODUCTION

1.1 ANATOMY OF THE FEMALE GENITAL TRACT

The female genital system encompasses a variety of organs and epithelial surfaces.

The external genital organs include the mons pubis, labia majora, labia minora, Bartholin's glands, and clitoris. The area containing these organs is called the vulva. The internal genital organs form a pathway (the genital tract). This pathway consists of the vagina, the uterus, the fallopian tubes (oviducts) and the ovaries (1).

1.1.1 Vagina

The vagina is a narrow, muscular but elastic organ about 10 cm long in an adult woman. It is the terminal portion of the female genital tract and its cavity is continuous proximally with that of the uterus at the external os of the cervix. It connects the external genital organs to the uterus.

The vagina is lined with a mucous membrane, kept moist by fluids oozing from cells on its surface and by secretions from glands in the cervix (the lower part of the uterus).

1.1.2 Uterus

The uterus is a thick-walled, muscular organ having a narrow cavity which is continuous with the peritoneal cavity through the uterine tubes and with the perineum through the vagina. It is about the size and shape of a pear, with the narrow portion directed inferiorly.

The cervix is the lower part of the uterus, which protrudes into the upper end of the vagina. The channel through the cervix is lined with glands that secrete mucus. The uterus is lined with the endometrium. The stratum functionale of the endometrium sloughs off during menstruation. The deeper stratum basale provides the foundation for rebuilding the stratum functionale.

1.1.3 Fallopian tubes

The two fallopian tubes or oviducts, which are about 5 to 7 cm long, extend from the upper edges of the uterus toward the ovaries. The tubes do not directly connect with the

ovaries. Instead, the end of each tube flares into a funnel shaped infundibulum with fingerlike extensions called fimbriae.

The fallopian tubes are lined with tiny hair like projections (cilia). The cilia and the muscles in the tube's wall propel an oocyte downward through the tube to the uterus.

1.1.4 Ovaries

The ovaries are usually pearl-coloured, oblong, and measure approximately 3 cm x 1.5 cm x 1.5 cm. The ovaries aren't attached to the fallopian tubes but to the outer layer of the uterus via the ovarian ligaments. The ovaries are uncovered in the peritoneal cavity. Thus, the ovary is the only organ in the human body which is totally invaginated into the peritoneum, making it the only interperitoneal organ.

1.2 VAGINAL INFECTION

1.2.1 Vaginitis

Vaginitis is a term used to describe infectious diseases and other inflammatory conditions affecting the vaginal mucosa and sometimes secondarily involving the vulva (2).

These conditions can result from an infection caused by bacteria, usually *Gardnerella vaginalis* and *Mycoplasma hominis* in combination with various anaerobes (3, 4). Other less commonly encountered bacteria are *Chlamydia trachomatis*, *Escherichia coli*, *Neisseria gonorrhoeae*, streptococci and staphylococci (5). Protozoa (*Trichomonas vaginalis*) cause 1/3 of all cases, while *Candida* is a frequent cause in pregnant women and diabetics, and occasionally oral contraceptives increase susceptibility (6). Another cause is viral infections such as the human papillomavirus (HPV) and herpes simplex (7).

Noninfectious vaginitis is often associated with atrophic changes, but can also be caused by radiation of the pelvis, dystrophies or tumors of the genital tract, as well as by allergic reaction or irritation from vaginal sprays, douches, coital lubricants or spermicidal products (7). The skin around the vagina can also be sensitive to perfumed soaps, detergents, and fabric softeners.

1.2.2 Epidemiological aspects of infectious vaginitis

The true prevalence and causes of vaginitis are indeterminable, in part because the condition is so often self-diagnosed and self-treated. In addition, vaginitis is frequently asymptomatic or has more than one cause. Experts assume that up to 90 percent of vaginitis cases are secondary to vulvovaginal candidiasis, bacterial vaginosis, and trichomoniasis and available figures are mostly derived from studies which involved symptomatic women presenting in primary care; the prevalence of vaginal candidiasis ranged from 17% to 39%; bacterial vaginosis, 22% to 50%; and trichomoniasis, 4% to 35% (8). The incidence of trichomoniasis is decreasing in most industrialized countries (9).

Vaginal candidiasis is a common infective cause of pathological vaginal discharge that affects about 75% of women at some time during their life, with 40-50% having two or more episodes (6). *Candida albicans* is the causative agent in approximately 80% of those infected. It is estimated that as many as 50% of asymptomatic women have candidal organisms as part of their endogenous vaginal flora (6, 8).

Despite the fact that 35% to 75% of cases of bacterial vaginosis are asymptomatic, this condition is still one of the most common diagnoses in women attending genitourinary clinics. Depending on the clinical setting the prevalence of BV varies from 5-26% in pregnant women to 24-37% in those attending sexually transmitted infection (STI) clinics (10).

1.2.3 Clinical picture of infectious vaginitis

The most common symptom of vaginitis is vaginal discharge that is different from the normal secretions, accompanied by pruritus, erythema, and sometimes burning, pain, or mild bleeding, with or without vulvar irritation (11). Discomfort during urination or dyspareunia may also occur.

Although symptoms vary among particular types of vaginitis, there is much overlap. Trichomonal vaginitis is marked by a profuse, malodorous, yellow-green discharge and the patients may have dysuria, dyspareunia, erythema and severe itching.

Candida vaginitis is suggested by moderate to severe vaginal and sometimes vulvar pruritus with or without burning. Dyspareunia, redness, edema and possibly excoriation are common as is a thick, white, cottage cheese-like vaginal discharge that tends to cling to the vaginal walls. Acute candida infection has several known predisposing

factors including antibiotic and high estrogen dose oral contraceptive usage, hormone replacement therapy, pregnancy, and poorly controlled diabetes mellitus (9, 12). Recurrent candida infection can be multifactorial in etiology, but is usually defined as idiopathic with no known predisposing factors. However, susceptibility to recurrent candida vaginitis had been postulated to result from an adaptive immune dysfunction or deficiency (12, 13).

Various diagnostic methods are available to identify the etiology of an abnormal vaginal discharge (7, 11). Laboratory testing fails to identify the cause of vaginitis in a minority of women. The cause of vaginal symptoms usually can be determined by pH and microscopic examination of fresh samples of the discharge.

1.2.4 Bacterial vaginosis

Non-specific vaginitis or BV was first reported in 1955 by Gardner and Dukes, who described the unique clinical signs and symptoms and the distinctive nature of the vaginal discharge associated with it. They also described a “new” causative organism, which has variously been named *Haemophilus vaginalis* (14), *Corynebacterium vaginalis* (15), and subsequently renamed and reclassified as the only member of a new genus, *Gardnerella* (16).

BV is vaginitis due to a complex alteration of vaginal flora in which normal hydrogen peroxide (H_2O_2) producing *Lactobacillus* species decrease and anaerobic pathogens (e.g., *Prevotella* sp., *Peptostreptococcus* sp. and *Mobiluncus* sp.), *Gardnerella vaginalis*, and *Mycoplasma hominis* overgrow (3, 4, 17, 18). The cause of the microbial alteration is not fully understood. BV is associated with having multiple sex partners, a new sex partner, douching, and lack of vaginal lactobacilli (19, 20); whether BV results from acquisition of a sexually transmitted pathogen is unclear. Women who have never been sexually active are rarely affected.

BV is the most prevalent cause of vaginal discharge or malodor; BV tends to produce a white, gray, or yellowish turbid discharge with a foul or ‘fishy’ odor that becomes stronger when the discharge becomes alkaline (e.g., after coitus or washing with soap) (21). Vulvar pruritus or irritation may be present, but redness or edema is not usually marked.

BV, once considered inconsequential, appears to be associated with pelvic inflammatory disease (PID), infectious complications after abortion or gynecological invasive procedures, and increased risk of HIV transmission and acquisition (22, 23).

BV during pregnancy is associated with significant reproductive morbidity, including premature rupture of membranes, preterm delivery, intraamniotic infection, and postpartum endometritis (10, 24-26).

1.2.4.1 Diagnostic Considerations of BV

BV can be diagnosed by the use of the clinical criteria or Gram stain (21, 27). The clinical criteria, often called Amsel's criteria, require three of the following symptoms or signs:

- a thin and homogeneous appearing discharge that smoothly coats the vaginal walls (no reference to colour or amount of discharge is made as these factors are difficult to assess in a standard manner);
- presence of clue cells on microscopic examination;
- pH of vaginal fluid >4.5; and
- a fishy amine odor of vaginal discharge before or after addition of 10% KOH (i.e., the sniff test).

1.2.5 Recommendations for treatment

Specific causes of discharge require specific therapy. *Candida* infection is treated with short-course topical formulations (i.e., single dose and regimens of 1–3 days) (28). The topically applied azole drugs (i.e., miconazole or clotrimazole) result in relief of symptoms and negative cultures in 80%–90% of patients who complete therapy. Short duration oral therapy (i.e., flukonazol or itrakonazol) is often used in recurrent disease. In women with recurrent candida infection, antifungal oral or topical therapy is highly effective for individual symptomatic attacks with little evidence for drug resistance but does not prevent recurrence (29). Intravaginal preparations of azoles are available over-the-counter in many countries.

Trichomonas is treated with oral metronidazole or tinidazole. Topically applied antimicrobials (e.g., metronidazole gel) are unlikely to achieve therapeutic levels in the urethra or perivaginal glands; therefore, use of topical formulations is not recommended (28). Ideally, the sexual partner should also be treated.

Current BV treatment includes both intravaginal and oral formulations of the antibiotics clindamycin and metronidazole (30). As recommended treatments for BV are reported to have similar initial clinical cure rates, the choice of treatment should also consider such factors as patient's preference of the route of administration, frequency of

administration, and side effect profile. Treatment of male sex partners has not been beneficial in preventing the recurrence of BV. Several studies have evaluated the clinical and microbiologic efficacy of using lactobacilli to restore normal flora and treat BV. However, no currently available probiotic therapy was determined to be better than placebo (30-32).

1.3 PELVIC INFECTION

Pelvic infection is defined as infection of the upper reproductive tract organs, adjacent parametrium and overlying pelvic peritoneum (33). The term ‘pelvic inflammatory disease’ (PID) is used to describe the clinical condition representing infection and inflammation of all or some of the pelvic organs. Although it is often used synonymously with salpingitis, the inflammation may be present at any point along a continuum that includes endometritis, salpingitis, salpingo-oophoritis, and peritonitis. The vast majority of PID cases occur when microorganisms ascend from the lower genital tract to the mucosa of the endometrium, the fallopian tubes and/or pelvic peritoneal cavity. The list of causative organisms, which have been implicated as etiologic agents of PID, is long but includes *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Mycoplasma* spp., *Escherichia coli*, *Streptococcus* spp., and other aerobic and anaerobic bacteria associated with bacterial vaginosis (e.g. *Gardnerella vaginalis*, *Bacteroides*, *Mobiluncus* and *Peptostreptococcus* spp.) (34-37).

1.3.1 Epidemiological aspects

PID is the most common serious complication of sexually transmitted diseases. Both symptomatic and asymptomatic infections can lead to the development of serious complications and sequelae, as chronic pelvic pain, infertility, ectopic pregnancy and pelvic adhesions (38, 39). The risk of ectopic pregnancy increases seven- to ten-fold after an episode of PID, and 40% to 50% of ectopic pregnancies can be attributed to previous pelvic inflammatory disease (40). According to a Scandinavian study, up to 60% of women who have laparoscopy verified salpingitis become infertile depending on the number of episodes of PID, the woman’s age and the severity of tubal inflammation (41). It is estimated that lack of inflammatory signs occurs 50% of the time and according to WHO up to 70% of women with gonococcal and/or chlamydial infections may experience no symptoms at all (42). In addition, the true prevalence and

incidence of PID is indeterminable due to the fact that the symptoms in PID range from subclinical (asymptomatic) to severe, and thus segregate the disease into ‘silent’ PID and PID (38, 43, 44). Moreover, the diagnostic criteria used can have a major impact on the prevalence and incidence of PID. In a study of incarcerated adolescents the new diagnostic criteria of PID , recommended by the Centers for Disease Control and Prevention (CDC) in 2004, doubled the prevalence and more than tripled the incidence of the disease (45).

It is rare for women not having menstrual periods to acquire PID. On the other hand, women at risk for acquiring any sexually transmitted infection are at high risk for developing PID. Risk factors include multiple or new sexual partners, previous episode of PID and presence of BV, younger age, low socioeconomic status, inconsistent condom use, commercial sex work, drug use, or a partner with STI (46, 47).

1.3.2 Investigations and diagnosis

As mentioned previously many episodes of PID go unrecognized. Although some cases are asymptomatic, others are not diagnosed because the patient or the practicing physician fails to recognize the implications of mild or non-specific symptoms and signs such as lower abdominal pain, abnormal uterine bleeding, dyspareunia, and vaginal discharge. A classic dilemma can be to differentiate between PID and other conditions (e.g. acute appendicitis, endometriosis, adnexal torsion, ruptured ovarian cyst, ectopic pregnancy, gastrointestinal disorders and pelvic adhesions). Because of the difficulty of diagnosis and the potential for damage to the reproductive health of women, even by apparently mild or subclinical PID, a syndromic approach to diagnosis of PID is recommended (33, 48-50). According to CDC 2006 empiric therapy is indicated before laboratory test results are available if the patient belongs to the high risk population for STIs and if the woman is experiencing pelvic or lower abdominal pain, if no cause for the illness other than PID can be identified, and if one or more of the minimum criteria are present on pelvic examination (Table 1) (33). The requirement that all three minimum criteria be present before the initiation of empiric therapy could result in insufficient sensitivity for the diagnosis of PID. The presence of signs of lower genital tract inflammation, in addition to one of the three minimum criteria, increases the specificity of diagnosis (Table 1). All patients who have suspected or confirmed PID should be tested for *N. gonorrhoeae* and *C. trachomatis* and a pregnancy test should be done. The pregnancy test is done to exclude ectopic pregnancy. Testing for

chlamydia is strongly recommended because of the increased utility and availability of highly sensitive and specific testing methods and because a specific diagnosis might enhance partner notification and improve compliance with treatment, especially in the exposed partner. Although common causes of lower abdominal pain are unlikely to be impaired by initiating empiric antimicrobial therapy for PID, elaborate diagnostic evaluation is necessary because incorrect diagnosis and management might cause unnecessary morbidity.

Table 1. Diagnostic criteria for acute PID

Lower abdominal pain
One or more of the following minimum criteria are present on pelvic examination
<ul style="list-style-type: none">• Cervical motion tenderness• Uterine tenderness• Adnexal tenderness
Additional criteria that enhance the specificity of the minimum criteria
<ul style="list-style-type: none">• Elevated oral temperature ($>38.3^{\circ}\text{C}$)• Abnormal cervical or vaginal discharge• Elevated WBC count on saline microscopy of vaginal secretions• Elevated erythrocyte sedimentation rate and/or C-reactive protein• Laboratory documentation of cervical infection with <i>N. gonorrhoeae</i> or <i>C. trachomatis</i>• Endometrial biopsy with histopathologic evidence of endometritis• Tubo-ovarian abscess on sonography or magnetic resonance imaging techniques• Visual evidence of PID in laparoscopy

1.3.3 Treatment considerations

Antibiotics therapy with broad spectrum coverage of likely pathogens (inclusive *N. gonorrhoeae* and *C. trachomatis*) should be initiated as soon as the presumptive diagnosis has been made. The goals of treatment of PID are 1) microbiologic cure of infection, 2) improvement of signs and symptoms, 3) a decrease in potential complications (e.g., infertility or chronic pain) and 4) prevention of transmission to others.

Many randomized trials have demonstrated the efficacy of both parenteral and oral regimens. Recommended regimens for treatment of PID according to CDC: “Sexually transmitted diseases treatment guidelines, 2006” are shown in Table 2.

Although general trends in the treatment of PID over the past decade have been towards the use of oral therapy on an outpatient basis, hospitalization should be considered when severe pain suggests other diagnoses (e.g., adnexal torsion, appendicitis, or abscess) or when patients are febrile or might be noncompliant with an antimicrobial regimen.

Finally, effective clinical management of patients with PID and subsequently diagnosed STIs requires treatment of the patients' recent sex partners to prevent reinfection and curtail further transmission (42). Patients should be instructed to refer their sex partners for evaluation and treatment.

Table 2. Regimens for treatment of PID*.

Treatment	Regimen A	Regimen B	Alternative Regimens
Parenteral	<p>Cefotetan 2 g IV every 12 hours <i>or</i> Cefoxitin 2 g IV every 6 h <i>plus</i> Doxycycline 100 mg orally or IV every 12 h</p>	<p>Clindamycin 900 mg IV every 8 hours <i>plus</i> Gentamicin loading dose IV or IM (2 mg/kg of body weight), followed by a maintenance dose (1.5 mg/kg) every 8 hours. Single daily dosing may be substituted.</p>	<p>Levofloxacin 500 mg IV once daily <i>with or without</i> Metronidazole 500 mg IV every 8 hours OR Ofloxacin 400 mg IV every 12 hours <i>with or without</i> Metronidazole 500 mg IV every 8 hours OR Ampicillin/Sulbactam 3 g IV every 6 hours <i>plus</i> Doxycycline 100 mg orally or IV every 12 hours</p>
Oral	<p>Levofloxacin 500 mg orally once daily for 14 d <i>or</i> Ofloxacin 400 mg orally twice daily for 14 days <i>with or without</i> Metronidazole 500 mg orally twice a day for 14 days</p>	<p>Ceftriaxone 250 mg IM in a single dose <i>plus</i> Doxycycline 100 mg orally twice a day for 14 days <i>with or without</i> Metronidazole 500 mg orally twice a day for 14 days OR Cefoxitin 2 g IM in a single dose and Probencid, 1 g orally administered concurrently in a single dose <i>plus</i> Doxycycline 100 mg orally twice a day for 14 days <i>with or without</i> Metronidazole 500 mg orally twice a day for 14 days OR Other parenteral third-generation cephalosporin (e.g., ceftizoxime or cefotaxime) <i>plus</i> Doxycycline 100 mg orally twice a day for 14 days <i>with or without</i> Metronidazole 500 mg orally twice a day for 14 days</p>	<p>Amoxicillin/clavulanic acid and doxycycline</p>

* Available at www.cdc.gov/std/treatment

1.4 MISOPROSTOL

1.4.1 Background

In the 1930s, during artificial insemination to treat sterility in women, it was observed that there was a contraction of the uterus after injection of semen into the uterine cavity. Human semen was discovered to contain an acid and lipid soluble substance that is a powerful vasodilator and can stimulate smooth-muscle activity (51). Prostaglandins received their name because at that time they were thought to originate from the prostate gland. Furthermore, it has been discovered that prostaglandins are produced in almost all tissues of the body, and are ubiquitous messenger molecules with a variety of actions (52). They are fatty acids and have been classified into groups from prostaglandin A to prostaglandin I according to structural variations in the five-carbon ring at one end of the molecule. There is further subdivision based on the number of double-bonds in the two side chains. These small variations in their chemical structures are believed to be responsible for the immense diversity of effects they have in a wide range of body functions such as the contraction and relaxation of smooth muscle, the dilation and constriction of blood vessels, control of blood pressure, and modulation of inflammation (53). Due to their potency and varying effects, prostaglandins have been of significant interest over the last several decades. Their highly unusual biochemical properties have attracted the notice of pharmaceutical manufacturers, and many are now commercially available.

1.4.2 Pharmacology of Misoprostol

Misoprostol ($C_{22}H_{38}O_5$; M:W.=382.5; Formal Chemical Name (IUPAC): methyl 7-((1R,2R)-3-hydroxy-2-((S,E)-4-hydroxy-4-methyloct-1-enyl)-5-oxocyclopentyl)heptanoate) is a synthetic prostaglandin E1 analogue developed in 1973 by Searle (Figure 1). Since 1985 it is marketed by G.D. Searle & Company (now incorporated into Pfizer, NY, USA) under the trade name Cytotec®, but generic versions are available as well.

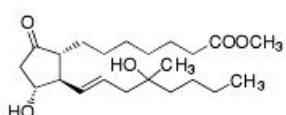


Figure 1. Chemical structure of misoprostol.

It is a drug that is approved in most countries for the treatment and prevention of stomach ulcers. The uterotonic and cervical softening effects of misoprostol were considered as side effects rather than therapeutic effects. However, it is because of these effects that misoprostol is so widely used in obstetric and gynecological practice today. In a few countries, misoprostol is also specifically approved for obstetric and gynecologic uses, including labor induction, incomplete abortion management, and, in conjunction with mifepristone, early pregnancy termination. More recently 25 μ g tablets for vaginal use have been approved in Brazil and Egypt and application for European Marketing Authorisation is planned by among others a pharmaceutical group based in the United Kingdom (54). However, Misoprostol is included on WHO's essential medicine list on three indications (medical abortion following mifepristone, labor induction and incomplete abortion) and in a large number of countries, misoprostol is commonly prescribed off-label to cause labor induction, prevention and treatment of post partum haemorrhage and termination of pregnancy (54).

Misoprostol is an inexpensive drug, stable at ambient temperatures, easy to transport, easy to administer, and does not require refrigeration, even in hot climates.

Early studies focused on the pharmacokinetic profile after oral administration of a single dose of misoprostol. Misoprostol is extensively absorbed through mucosal surfaces and undergoes rapid de-esterification by the liver to form the free misoprostol acid (MPA), which is responsible for its clinical activity. The plasma MPA level increases rapidly and peaks at about 30 minutes declines rapidly by 120 minutes and remains low thereafter (55-59).

However, due to its clinical effectiveness in the field of reproductive health, misoprostol has been extensively studied in obstetric and gynaecological applications and different routes of administration have been evaluated (56-66). Pharmacokinetic studies on oral, vaginal, sublingual, buccal and rectal application have shown that the shortest time to peak concentration, the highest peak concentration and the greatest bioavailability is after sublingual administration (57, 58, 60). The area under the curve (AUC) of sublingual misoprostol is 4 times that of buccal administration (67). Another study comparing MPA levels for different routes of administration have shown that the serum MPA levels are lower after buccal application compared with vaginal administration (57). In the same study the AUC₃₀₀ of MPA serum levels after rectal administration is only 1/3 that of vaginal administration. Moreover, in contrast to the oral route, the AUC is higher after vaginal administration. When misoprostol is given

vaginally, the plasma concentration increases gradually, reaching its maximum level after 70-80 minutes before slowly declining with detectable MPA levels still present after 6 hours. In fact, at the end of 4 hours the serum level of MPA after vaginal application is higher than those of the sublingual and oral routes (60).

It was found in clinical studies that vaginal administration was more effective than oral administration in medical abortion (63, 64). This was explained later by the greater bioavailability of vaginal misoprostol compared to oral misoprostol. However, it has been shown that the coefficient of variation of the AUC after vaginal administration is greater than that after oral administration, indicating that the vaginal absorption of misoprostol is inconsistent (58, 59). In clinical practice, particulate remnants of tablets are sometimes seen many hours after vaginal administration, meaning that the dissolution and absorption are variable and incomplete (68). These reports of undissolved tablets in some patients have raised concern that this may be due to the variation in the vaginal environment between women. Thus, variation in the amount and pH of the vaginal discharge or in the amount of bleeding during medical abortion may affect the dissolution and absorption of misoprostol. Numerous attempts have been made to improve the absorption of vaginal misoprostol, and moistening the tablets with an aqueous solution (e.g. water or acetic acid) is a common praxis in many clinical settings (69-72). However, this has been shown not to improve the bioavailability of the drug (58, 73-75).

Studies on the effect of misoprostol on uterine contractility have shown that a sustained MPA level, rather than a high serum level, is required for the development of regular uterine contractions (76, 77). These regular uterine contractions are sustained for a longer period after vaginal application than after oral or sublingual administration, with decreased activity occurring first after 4 hours (76). Thus, the development of regular contractions and the cervical priming effect of misoprostol in the pregnant state may explain the clinical efficacy of different routes of administration (61, 63, 64, 78).

Recommended doses of misoprostol (200-800 µg/day) do not have clinically significant adverse haematological, endocrine, biochemical, immunological, ophthalmic, respiratory, platelet or cardiovascular effects (79). Gastrointestinal symptoms (e.g. diarrhoea, vomiting) are the major adverse reactions that have been reported consistently with misoprostol, but are usually mild and self-limiting, and usually resolve once misoprostol is withdrawn. Fever and chills were reported in studies using misoprostol for medical abortion, especially in cases of oral administration.

Although mutagenicity studies of misoprostol have been negative and the drug has not been shown to be embryotoxic, fetotoxic or teratogenic, a wide range of congenital defects is possible depending on the time of exposure to misoprostol. These fetal malformations may be due to a disturbed blood supply to the developing embryo during misoprostol-induced uterine contractions (80, 81).

1.5 NITRIC OXIDE

1.5.1 History

The biological effects of nitric oxide (NO) were observed for more than a century ago. In 1867, Lauder Brunton, a distinguished British physician, discovered the ability of organic nitrates to relieve anginal pain (53). Following Dr Brunton's discovery, William Murrell experimented with the use of nitroglycerin (a substance which was discovered by Nobel to be an excellent explosive) to alleviate angina pectoris and reduce blood pressure. Nitroglycerin was soon adopted into widespread use, but it took a century until NO was recognized. In early 1980's, when NO was considered to be an atmospheric pollutant, Furchtgott and co-workers showed that endothelial cells produced a mediator which caused relaxation of blood vessels (82). The unknown mediator was initially termed endothelium-derived relaxing factor (EDRF). This simple and important discovery stimulated a scientific gold-rush by many groups to be the first to identify the unknown mediator. Attempts to identify it were, however, initially frustrated by its very short lifetime of only a few seconds in plasma, and it was not until 1987 that the surprising answer was obtained that the unknown mediator is NO. It was shown by independent research groups, including Moncada, Ignarro and Murad, that EDRF was equivalent to NO (83-85). Since then it has been shown that NO is a biologically active substance involved in many processes and diseases throughout the body. Nitric oxide was named "Molecule of the Year" in 1992 by the journal *Science* (86), and the Nobel Prize in Physiology or Medicine in 1998 was awarded to Furchtgott, Murad, and Ignarro for the discovery of the signalling properties of nitric oxide.

1.5.2 Chemistry and Synthesis

Nitric oxide (common name) or nitrogen monoxide (systematic name) is a chemical compound with chemical formula NO and molecular weight 30D. NO is a small highly diffusible colorless gas and a ubiquitous bioactive molecule. The NO molecule is a free radical, which is relevant to understanding its high reactivity. At ambient temperatures in the absence of a catalyst the oxidation of low concentrations of NO is very slow, e.g. at 1000 parts per billion (ppb) in air containing 20% oxygen, 24 hours are required for 25% oxidation (87). In vivo NO is highly reactive having an elimination half life in the range of seconds due to presence of scavenger proteins and by forming complexes with transition metals, e.g. the iron in oxyhemoglobin to form NO_3^- and methemoglobin (88).

NO can be formed by the uncatalyzed endothermic reaction of molecular nitrogen (N_2) and oxygen (O_2), which requires high temperatures ($>1000^\circ\text{C}$). In the human body most of the NO is produced from the terminal guanidino group of the amino acid L-arginine via an oxidation reaction catalyzed by a family of enzymes known as NO synthases (NOS) with equimolar production of the amino acid L-citrulline (fig.2). This enzymatic reaction requires nicotinamide adenine dinucleotide phosphate (NADPH) as co-substrate and heme, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), calmodulin (CaM) and tetrahydrobiopterin (BH₄) as co-factors (89). At present, three different isoforms of the enzyme have been identified in human, both calcium-dependent constitutive (eNOS, nNOS) and calcium-independent inducible (iNOS) (90-95). The two constitutively expressed forms of NOS are named after the tissue in which their cDNA was first cloned; neural NOS (nNOS, NOS I) (96), and endothelial NOS (eNOS, NOS III) (97). However, these isoforms have later been found in a variety of tissues. The third isoform (iNOS, NOS II) was first identified in macrophages and it differs from the other two isoforms in that it is calcium-independent, requires de novo protein synthesis which is controlled at the transcriptional level, and produces larger amounts of NO for extended periods of time (98). The expression of iNOS is stimulated by bacterial products such as lipopolysaccharides (LPS) and pro-inflammatory cytokines (e.g. tumour necrosis factor- α , interleukin-1 and interferon γ) (99-101).

There is also a nonenzymatic formation of NO in vivo first described in 1994 by two independent groups (102, 103). Chemical synthesis of NO is accomplished after

concentration in the saliva of dietary NO_3^- (e.g. from green leafy vegetables) and reduction to NO_2^- by tongue surface anaerobic bacteria, and subsequently in the acidic conditions of the stomach NO_2^- is converted to nitrous acid (HNO_2), which rapidly decomposes into nitrogen dioxide (HNO_2), NO, and water when in solution (104). In addition to the stomach, nonenzymatic pathway for NO production has also been shown in the heart and on the skin (105, 106).

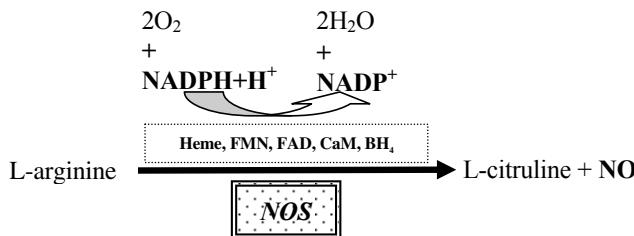


Figure 2. Enzymatic formation of NO

1.5.3 NO measurement technique

The most common way of measuring NO concentration is by using a simple chemiluminescent reaction involving ozone: A sample containing gaseous NO is mixed with a large quantity of ozone. The nitric oxide reacts with the ozone to produce oxygen and nitrogen dioxide with an electron in an excited state (NO_2^\bullet). This NO_2^\bullet changes back to ground state while emitting light (chemiluminescence), which can be measured with a photodetector that proportionally converts the intensity of luminescence into an electric signal for display. The amount of light produced is proportional to the amount of nitric oxide in the sample ($\text{NO} + \text{O}_3 \rightarrow \text{NO}_2 + \text{O}_2 + \text{hv}$).

Other techniques that have been used include electroanalysis (amperometric approach), where NO reacts with an electrode to induce a current or voltage change, and a group of fluorescent dye indicators (e.g. 4,5-diaminofluorescein) for intracellular measurements (107). Another practical method is spin trapping of nitric oxide with iron-dithiocarbamate complexes and subsequent detection of the mono-nitrosyl-iron complex with Electron Paramagnetic Resonance (EPR) (108, 109).

1.5.4 NO and inflammation

Since 1980s intensive research on NO have shown that this diatomic free radical plays a major role in a variety of mammalian biological processes including blood pressure homeostasis, transmission of signals by the nervous system, platelet aggregation, and non-specific immune responses to infection, host defense, and cytotoxicity. The role of NO in host defense and immune responses was started to be understood at the second half of the 1980s, when Stuehr and Marletta reported that LPS stimulated mouse macrophages produce nitrite and nitrate from an unknown precursor molecule (110). Hibbs and co-workers were able to identify the L-arginine-dependent biochemical pathway synthesizing L-citrulline and nitrite in macrophage mediated cytotoxicity (111). Since then, numerous reports on the effects of NO in inflammatory conditions have been published. Today, NO is clearly regarded as an important mediator in host defense mechanisms and an effective antimicrobial agent (112). Increased iNOS activity and NO formation have been described in many inflammatory processes, such as asthma, colitis, cystitis, prostatitis and rheumatoid arthritis (113-118). Although the role of NO in inflammation is not yet completely understood, it has been suggested to be very useful as a non-invasive, diagnostic and monitoring marker of inflammatory disorders (e.g. asthma, cystitis) (119-122). In addition, a dramatic up-regulation of iNOS activity and the production of high levels of NO appear to limit replication of bacteria, protozoa, and viruses, at the risk of potential inflammatory damage to host cells and tissues (112). Thus, the high levels of NO that are produced from increased iNOS expression in response to inflammatory stimuli, may also contribute to the morbidity of infection by acting as vasodilator, myocardial depressant and cytotoxic mediator (123, 124). A large part of the cytotoxicity commonly attributed to NO is actually mediated indirectly by reactive nitrogen species produced by the interaction of NO with superoxide anion or with oxygen. NO reacts very quickly with superoxide anion (O_2^-), resulting in the formation of peroxynitrite ($ONOO^-$) (125). Peroxynitrite is a nitrating agent and a powerful oxidant that is able to damage proteins, lipids and DNA.

In summary, while it is clear NO plays an important role in innate immunity and inflammation, the pathologic processes are complex and there remain considerable gaps in our knowledge regarding the exact role of NO *in vivo*, particularly in humans.

1.5.5 NO in the female genital tract

In the female reproductive organs, immunohistochemical and molecular studies of NOS expression have shown that NO is abundantly expressed in endometrial, myometrial, tubal, and ovarian tissue (126, 127). eNOS has been detected in endometrial vascular endothelium and glandular epithelium (128). In the human myometrium NO is thought to play a key role in uterine relaxation during pregnancy (129). Recent data suggests that in the human uterine cervix NO is synthesized via eNOS and iNOS and that this endogenous NO system is involved in cervical ripening (130, 131). It is suggested that the cervical NO release may be controlled, at least in part, by circulating progesterone, but other factors such as cytokines, prostaglandins and metalloproteases take part also in the regulation of NO synthesis and release (132). Thus, NO is implicated as an important regulatory agent in various female reproductive processes such as ovulation, implantation, pregnancy maintenance, labor, and delivery (128, 133). However, it is important to mention that because the detection of NO radicals in biological tissues is particularly difficult due to the short lifetime and concentration of these radicals in tissues, NO was measured indirectly by testing for NOS in various tissues or by testing fluid samples (e.g. cervical fluid) for nitrite and nitrate levels.

2 AIMS OF THE STUDY

The aims of this thesis were:

- ❖ To evaluate whether it is possible to measure accurate NO concentration in air introduced into a catheter balloon into the uterus, and to determine the optimal time of incubation.
- ❖ To examine whether NO level rises after manipulation in the uterine cavity both in patients with pelvic inflammatory disease (PID) and in healthy women.
- ❖ To explore intrauterine NO as a biomarker in the diagnosis of PID.
- ❖ To evaluate whether it is possible to detect and measure the amount of NO produced in the vagina of healthy women both of reproductive and in post menopausal age as well as in patients with vaginitis.
- ❖ To investigate whether NO formation in the vagina is increased in patients with vaginitis.
- ❖ To evaluate the pharmacokinetics (plasma concentration Cmax, Tmax and bioavailability measured as the AUC₂₄₀) of vaginally administered misoprostol in the first trimester of pregnancy in healthy women and in women with diagnosed BV.

3 MATERIAL AND METHODS

For a detailed account of materials and methods the reader is referred to the individual papers.

3.1 STUDY SUBJECTS

All studies were conducted at the Department of Obstetrics and Gynaecology, Karolinska University Hospital. The studies were approved by the local ethics committee, and all women gave their informed consent prior to participation.

In *paper I* 20 premenopausal, non pregnant women from 18 to 48 years of age with lower abdominal pain and 9 healthy women (aged 26-45 years) with regular menstrual cycles were included.

In *paper II* 18 non pregnant women from 19 to 65 years of age with symptoms of vaginitis, eight healthy women (aged 21-43 years) in reproductive age with regular menstrual cycles and nine healthy post-menopausal women (aged 53-79 years) were enrolled in the study.

In *paper III* we studied 6 non pregnant women from 22 to 50 years of age with PID and 10 healthy women (aged 23-38 years) with regular menstrual cycles.

In *paper IV* A total of 20 women with good general health from 18 to 40 years of age with a viable intrauterine pregnancy of up to 63 days of gestation, who had requested medical termination of pregnancy, were recruited to the study. A gynecological examination was performed on admission and 10 women with diagnosed BV were allocated to the BV-group, and 10 healthy women with no symptoms or signs of vaginal infection were allocated to the control group.

In all studies exclusionary criteria were use of an intrauterine device, presence of vaginal bleeding, and inability to understand the information provided. In papers I-III pregnancy was an exclusion criteria while abnormal pregnancy was an exclusion criteria in study IV.

All women underwent vaginal examination and specimen collection for microbiological examination according to clinical routine. In all women a thorough

assessment was performed, including speculum examination, pH testing with Macherey-Nagel pH-Fix indicator sticks pH 3.6 – 6.1 (D-5160 Düren, Germany), wet mount and potassium hydroxide (KOH) preparations of vaginal fluid.

In *study I* we measured NO levels in air incubated for five minutes in a catheter balloon in the uterine cavity. All patients were assessed for PID according to the diagnostic criteria formulated by CDC 2006 (33). There were 14 patients with PID and 6 patients with appendicitis. In all the healthy volunteers daily urine samples were collected for measurement of the luteinizing hormone (LH) peak, and measurement of NO was performed at different stages of the menstrual cycle.

In *study III* we measured NO levels in air incubated for 2, 5 and 10 minutes in a catheter balloon in the uterine cavity in 6 patients with PID and in 6 healthy women. In addition, we measured NO concentration in air incubated for 10, 5 and 2 minutes in a catheter balloon in the uterine cavity in another control group with 6 healthy volunteers. In all the healthy volunteers an endometrial biopsy was obtained after the measurement of NO. Visual analogue scale (VAS) pain scores were used to evaluate any pain experienced during measurement of NO.

In *study II* all the women were assessed for infectious vaginitis according to the following criteria: (I) vaginal and/or cervical swab culture positive for GC, GBS, HSV or Chlamydia; (II) positive wet smear for motile trichomonas, hyphae or yeast form fungus; or (III) BV according to Amsel criteria (9), as follows: homogenous vaginal discharge, vaginal pH >4.5, positive whiff test and presence of clue cells. Measurements of NO levels in air incubated for five minutes in a catheter balloon in the vagina were performed directly after the gynecological examination.

In *study IV* the gestational length according to last menstruation period was confirmed with the help of gynaecological examination and vaginal ultrasound. BV was diagnosed using the Amsel criteria: thin homogenous vaginal discharge, vaginal pH >4.5, positive sniff test and presence of clue cells on microscopy. All the women received the standard treatment for medical abortion; they were administered 200 mg mifepristone followed 24-48 hours later by a single dose of 800 µg misoprostol vaginally. Blood samples were taken before (0h) and 0.5, 1, 2, 3 and 4 hours after misoprostol administration. All patients diagnosed with BV at the pre abortion screening for infection were also given treatment with metronidazol orally. All treatment was handed out to the participants in accordance with routine by a research nurse. Prophylactic analgesics (in the form of NSAIDs and paracetamol 500mg + dihydrocodeine 10mg)

were given and any additional pain intervention or medication, type and dose were noted. Side-effects to misoprostol (e.g. pain, shivering, fever, nausea, vomiting or diarrhoea) were noted. All women were scheduled to return for a follow-up visit two to three weeks after the treatment.

3.2 ANALYSIS OF INTRAUTERINE NO (PAPER I AND III)

For measurement of NO concentration a chemiluminescence NO analyzer (CLD 77 AM, Eco Physics, Dürnten, Switzerland) was used. The detection limit for NO was 1 parts per billion (ppb) and the analyzer was calibrated at known concentration of NO in N₂, using an electromagnetic flow controller. The chemiluminescence assay is highly specific for NO and there is no interference from other nitrogen oxides (134). At a NO level of 10 parts per million (ppm) in air, containing 20% oxygen, at 20°C there is an oxidation rate of 25% after 2.3 hours (87).

For NO measurements, an 8 French silicon catheter was used. The ability of NO to diffuse into the catheter balloon of an 8F silicon catheter was tested by placing the catheters with the balloon filled with 2.5mL of room air in a canister with NO at known concentrations for different time periods. The recovery rate for the silicon catheter placed for 2, 5 and 10 minutes in a canister with NO at known concentration was 30%, 40% and 43% respectively.

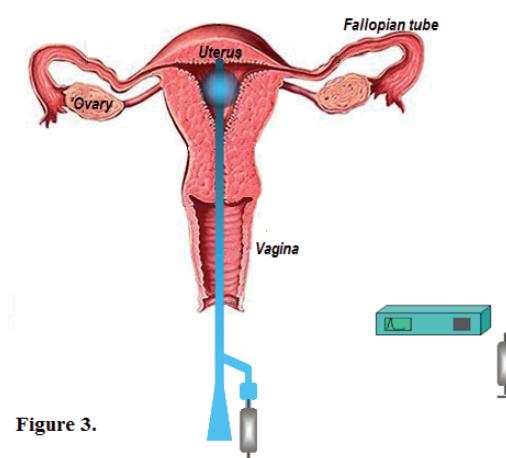


Figure 3.

The catheter balloon was easily inserted in the uterine cavity and room air was introduced into the balloon (2.5 mL) and incubated for 5 minutes (*paper I*) and 2, 5 and 10 minutes (*paper III*). After incubation, the air was aspirated into a syringe and within a few minutes injected into a chemiluminescence NO analyzer (CLD 77 AM, Eco Physics,

Dürnten, Switzerland) and peak levels of NO were registered (fig. 3). The NO level in air from the examination room was also measured and subtracted from the intrauterine NO level. The registered peak values of NO were corrected for the 40% recovery in *study I* and 30%, 40% and 43% recovery in *study III*.

3.3 ANALYSIS OF VAGINAL NO (PAPER II)

For NO measurements, a 14 French silicon catheter was used. The catheter balloon was positioned in the vagina and room air was introduced into the balloon (25 mL) (Fig.4).

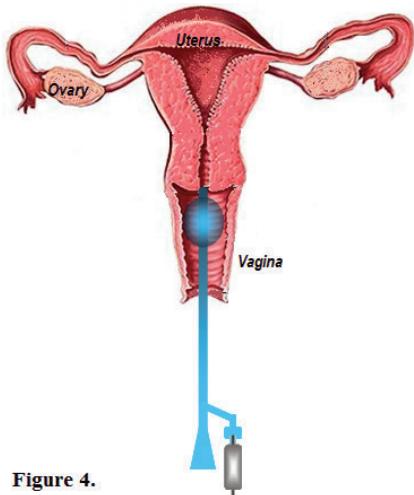


Figure 4.

After 5 minutes incubation, the air was aspirated into a syringe and within a few minutes injected into a chemiluminescence NO analyzer (CLD 77 AM, Eco Physics, Dürnten, Switzerland), for measurement of the NO level. The NO level in air from the examination room was also measured and subtracted from the NO level measured in the vagina. The nitric oxide concentration was measured as parts per billion (ppb) and the detection limit for NO was one ppb. The recovery rate for the silicon

catheter placed for five minutes in a canister with NO at known concentration was 80%. The registered peak values of NO were corrected for the 80% recovery.

3.4 PHARMACOKINETICS AND ANALYSIS OF MISOPROSTOL ACID (PAPER IV)

To evaluate the pharmacokinetics of vaginal misoprostol up to 4 hours after administration, we measured the serum levels of the active metabolite, misoprostol acid (MPA). Blood samples taken before (0h) and 0.5, 1, 2, 3 and 4 hours after misoprostol administration were immediately stored in a refrigerator for half an hour and centrifuged in a cooled centrifuge. The serum was then withdrawn and stored at -20°C. The samples were analyzed at the Department of Paediatrics, Philipps University Marburg. MPA was measured in serum samples using a liquid chromatography (LC)-MS/MS isotope dilution assay (60, 135).

3.5 STATISTICS

Data were analyzed using SigmaStat for Windows version 2.03, SigmaStat for Windows version 3.0.1a, and SigmaPlot for Windows Version 11.0. The test results were analyzed using standard statistical methods, including the Fisher's Exact Test, Mann-Whitney U test or the t-test when appropriate. The Bonferroni correction was used for multiple comparisons.

4 RESULTS

4.1 MEASUREMENT OF NO LEVELS IN THE UTERINE CAVITY (PAPER I & III)

The NO concentration in the uterine cavity in all the patients with a diagnosis of PID was found to be significantly higher compared with the controls. The procedure was well tolerated with no adverse events or complications.

In *study I*, intrauterine NO levels measured in healthy women were low, and NO levels showed no significant variation during the menstrual cycle (Fig. 5). There was no significant difference in the NO levels between healthy women and patients with diagnosed appendicitis. Conversely, there was a clear difference in NO levels between patients diagnosed with PID and those diagnosed with appendicitis ($P<0.001$). The difference in the NO levels between patients diagnosed with PID and healthy women was also significant ($P<0.001$) (Fig.6).

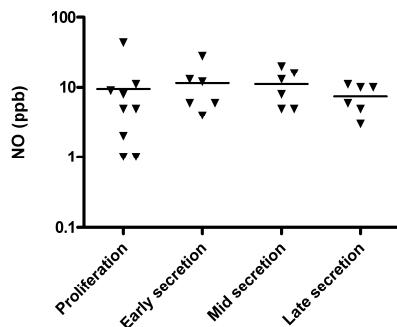


Figure 5: NO concentration measured as parts per billion (ppb) in air incubated in a catheter balloon in the uterine cavity of 9 healthy women. The measurement of NO was performed at different times during the menstrual cycle. Note logarithmic scale on Y-axis.

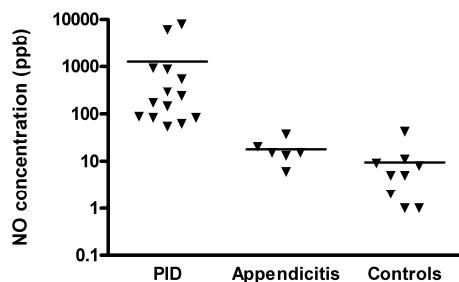


Figure 6: NO concentration measured as parts per billion (ppb) in air incubated in a catheter balloon in the uterine cavity of 20 patients with low abdominal pain (14 patients with PID and 6 patients with appendicitis) and in the uterine cavity of 9 healthy women (measurement of NO was performed at different times during the menstrual cycle and the mean was calculated). Note logarithmic scale on Y-axis.

In *study III*, the difference in NO concentration in the uterine cavity between patients with PID and controls was visible after just 2 minutes of incubation ($P=0,026$). In both control groups, maximal levels of NO were registered when the air had been incubated for the first time (for either two or 10 minutes). Subsequent incubation for five and ten minutes or for five and two minutes, respectively, did not result in any further increase in NO concentration. Within the groups there was no significant difference between NO levels in air incubated for 2, 5 and 10 minutes or for 10, 5 and 2 minutes. There were 16 women with median endometrial thickness 9 mm (range 5-14) who reported having mild pain during measurement of NO in the uterine cavity. One woman with endometrial thickness 2 mm reported moderate pain, and one woman with endometrial thickness 5 mm reported severe pain. Analgesics were not required during measurement of NO, neither in patients nor in healthy women.

4.2 MEASUREMENT OF NO LEVELS IN THE VAGINA (PAPER II)

All 18 patients recruited into study II had symptoms associated with vaginal infection, including abnormal vaginal discharge, odor and pruritus. A majority of the women had more than one symptom; odor and pruritus rarely being noted without an abnormal discharge.

Vaginal NO levels in all patients were found to be elevated compared with the healthy controls. Vaginal NO levels measured in healthy women, regardless of menopausal status, were low (Fig. 7). The NO levels in women at reproductive age, as well as in postmenopausal women, showed no significant variation (Fig. 7).

Conversely, there was a clear difference in NO levels between patients diagnosed with vaginitis and healthy controls ($p<0.001$).

The procedure was well tolerated with no complications. Analgesics were not required during measurement of vaginal NO, neither in patients nor in healthy women.

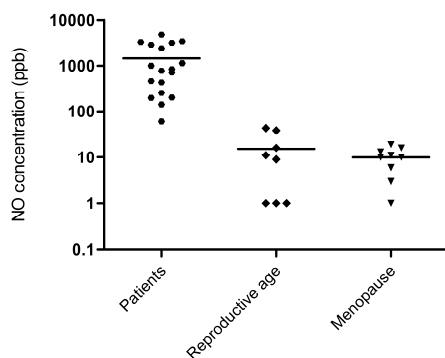


Figure 7: Nitric oxide concentration measured as parts per billion (ppb) in air incubated in a catheter balloon in the vagina of 18 patients with diagnosed vaginitis and in the vagina of 17 healthy women (eight women in reproductive age and nine in menopause). Note logarithmic scale on Y-axis.

4.3 EFFEKT OF BV ON THE PHARMACOKINETICS UP TO 4 HOURS OF VAGINALLY ADMINISTERED MISOPROSTOL DURING EARLY PREGNANCY (PAPER IV).

In study IV, we determined a number of pharmacokinetic parameters, namely the peak serum concentration of MPA (Cmax), time to the peak concentration (Tmax) and the AUC up to 240 minutes after administration of misoprostol vaginaly. The individual variability of these pharmacokinetic parameters was denoted by the coefficient of variation. The mean serum concentrations of MPA after administration of 800 µg vaginal misoprostol are shown in Fig.8.

Although the Cmax of MPA was higher in the control group, it did not differ significantly between the two groups. The Tmax of MPA was slightly shorter in the women with BV, but there was no significant difference between the two groups (Fig.8; Table 3).

The AUC₂₄₀ was larger in the control group compared to the BV group, but the difference was not statistically significant. The cumulative AUC for the two groups is shown in figure 9.

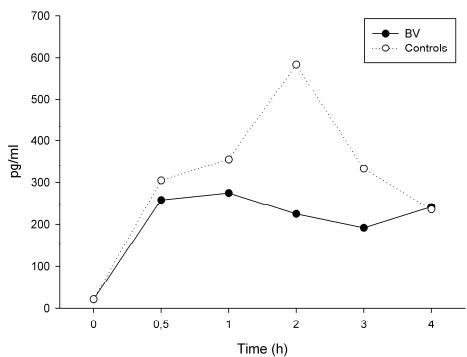


Figure 8: Mean plasma concentrations of misoprostol acid over time.

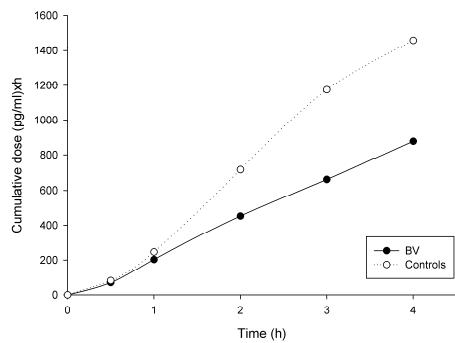


Figure 9: Cumulative dose of misoprostol (pg/ml) by time Curves showing the result of vaginal administration in women with BV and healthy controls.

Table 3. Pharmacokinetic parameters of 800 μ g misoprostol vaginally

	BV (n=10)	Controls (n=10)	P-value ^a
Cmax of MPA (pg/ml)			
Mean \pm SD	342.5 \pm 140	630.7 \pm 490.5	0.186 NS
CV	40.88	77.76	
Tmax (hours)			
Mean \pm SD	2.15 \pm 1.6	2.5 \pm 0.7	0.370 NS
CV	74.39	28.28	
AUC₂₄₀ (pg h/ml)			
Mean \pm SD	878.065 \pm 324.968	1458.695 \pm 847.245	0.140 NS
CV	37.01	58.08	

^a Mann-Whitney Rank Sum Test; CV, coefficient of variation (%); NS, no significant

5 GENERAL DISCUSSION

Female genital tract infections cause major medical, social, and economic problems worldwide. An infection of the genital tract is a possible cause of obstetrics complications and gynecological disease, and some of the affected women face a lifetime of gynaecologic and reproductive morbidity. Although these infections generally respond to treatment, delayed or missed diagnosis and pharmacologic resistance may occur. Thus, rapid and precise diagnosis of genital tract infections is essential to institute effective therapy and prevent sequelae. The CDC has formulated diagnostic criteria which are focused on syndromic diagnosis and the exclusion of competing diagnoses. Unfortunately all signs and symptoms are not always present, and even when present they often are uncertain and lack sensitivity.

5.1 NO AS MARKER FOR INFLAMMATION AND LUMINAL NO MEASUREMENT.

NO is synthesized by a family of NOS. iNOS is normally not present in resting cells but is usually up regulated during inflammation and it produces large amounts of NO in response to inflammatory signals (100, 136, 137). NO has previously been shown to be a marker for the detection of inflammatory disorders in various organs such as the lungs, intestines and urinary bladder (113, 122, 138, 139). It has been argued that NO is a non-specific marker of inflammation and that NO contributes to non-specific host defence against bacterial, viral and fungal infections. However, it is still unknown whether elevated NO levels in the genital tract in patients with genital infection is beneficial or harmful for the tissue. NO production could occur in response to bacterial endotoxins, known to induce NOS expression, and high NO levels in the vagina or uterus may be beneficial in host defence reactions due to its bacteriostatic properties (112, 140). However, increased levels of NO or subsequent reactive products can have cytotoxic actions against host cells (141).

Investigators have shown that an endogenous nitric oxide system is present in the uterine cervix at term (130), and Väistö-Tommiska et al noticed that cervical fluid nitric oxide metabolite levels rise after cervical manipulation, and that vaginal misoprostol stimulates cervical nitric oxide release in pregnancy, suggesting a joint action of nitric oxide and prostaglandins in cervical ripening (131, 142). Furthermore,

there have been attempts to evaluate the local immune response and the role of NO produced in the vagina in women with vaginal infections (143, 144). Unfortunately until now NO in the female genital organs was only measured indirectly by testing tissue specimens for NOS or by testing vaginal fluid samples for nitrite and nitrate levels. A weakness of this method is that analysis of the metabolites of the NO in the vaginal fluid may reflect other conditions such as dietary discrepancies, making it a less reliable technique. Moreover, the biological role of the anion nitrite has undergone a significant transformation in recent years. For many decades nitrite was considered an inert by-product of NO metabolism and has traditionally been viewed as a diagnostic marker of NO formation in biological systems. More recently, this view has changed and nitrite is recognized as a major storage form of NO in blood and tissues that can readily be reduced to NO under certain conditions and initiate cytoprotective signaling during pathological states (145).

On the other hand, the chemiluminescence's analysis of gaseous NO is a direct method which measures the actual local NO production. It has been shown that the direct measurement of NO concentration in the gaseous phase is a simple and reliable method of detecting inflammation in various organs (113, 117, 138). Thus, using luminal measurement of NO it is possible to detect a local formation of NO and the NO concentration measured reflects the amount of NO produced in that specific organ. In *paper I, II* and *III* we demonstrated for the first time how NO levels can easily and directly be measured in the vagina and the uterine cavity. NO gas was detectable in all study subjects, and in women with a clinical diagnosis of infectious vaginitis or PID the NO levels measured in the vagina or the uterus respectively were significantly higher compared to healthy controls. In addition, in an unpublished pilot study we measured luminal NO levels in the vagina of six women at term pregnancy who were admitted at the Department of Obstetrics and Gynecology, Karolinska University Hospital for labor induction. According to this study findings there was no significant difference in the NO levels measured in the vagina prior to labor induction compared to NO levels measured 45 minutes after administration of 2 mg Dinoprostone vaginally and cervical manipulation. Similar results were seen in *study III*, when we measured intrauterine NO levels for different periods of time. In *study III* we did not see any rise of intrauterine NO levels at one minute after manipulation. It is important to note that we measured luminal NO and not its oxidation products. The luminal NO concentration reflects NO produced in the vagina or the uterine cavity respectively, since the biological half life of NO is only a few seconds.

The results of our studies suggest that measurement of vaginal or intrauterine NO level is a feasible, safe and well tolerated procedure and may be used to detect inflammation of the female genital tract. It is tempting to speculate that determination of vaginal or intrauterine NO could be a way to strengthen the clinical diagnosis of infectious diseases in the female genital tract, but it is important to note that our studies are clearly too small to be used as an evidence base for the formulation of diagnostic criteria.

5.2 BACTERIAL VAGINOSIS AND PHARMACOKINETICS OF MISOPROSTOL

Although BV is a very common cause of symptomatic and asymptomatic vaginal infection and it has been associated with a high incidence of obstetric and gynaecologic complications and an increased risk for post abortion endometritis, so far there is no data on the effect of BV on the pharmacokinetics of misoprostol in early pregnancy. While vaginally administered misoprostol is clinically effective, wide variability in observed efficacy between patients is occasionally noted (58). In many circumstances, the sources of such variations have not been well characterized. Recent investigations have suggested that vaginal pH may be one such factor that may account for the variability observed clinically when misoprostol is administered by the vaginal route (69, 146). On the other hand, Karim et al, who evaluated the effect of antacids on gastric absorption of orally administered misoprostol, demonstrated that antacids (ie, alkalinization of gastric secretions) resulted in no significant changes in absorption of misoprostol, but the bioavailability was reduced by about 16% (147). Thus, it could be expected that MPA concentrations after vaginal administration of misoprostol would be lower in women with diagnosed BV than in healthy controls. In *study IV* we have demonstrated that BV and subsequently vaginal pH appears to have no significant effect on the bioavailability, measured as AUC₂₄₀, or the peak serum level of MPA. Thus, the effects of vaginal acidity on the efficacy of the vaginally administered misoprostol extend beyond affecting the pharmacokinetics of the drug. It will be of interest to study whether the pharmacokinetic data of MPA correlates with the clinical efficacy and the side effects of misoprostol, but this will require larger groups of patients.

6 CONCLUSIONS

- ❖ We have developed a novel method for measuring NO in the female genital tract using a silicon catheter. This method is simple, minimally invasive, reliable, rapid, safe and well tolerated, which makes it useful in clinical practice.
- ❖ PID is associated with increased intrauterine NO levels compared to healthy women and patients with acute appendicitis. The levels of NO are increased in women diagnosed with PID already after an incubation time of two minutes.
- ❖ The levels of NO are increased in patients with vaginitis, but are uniformly low among healthy women, both in reproductive age and in menopause.
- ❖ Studies in PID as well as vaginitis open the possibility of using NO as a biomarker of inflammatory disease in the female genital tract.
- ❖ Although BV increases the risk of a post abortion endometritis it does not affect the pharmacokinetics of vaginally administered misoprostol.

7 ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to everyone who through their kindness and generosity have helped me through all these years to make this thesis possible. In particular I would like to thank the following persons:

Professor *Kristina Gemzell-Danielsson*, my main supervisor, for introducing me to the fascinating world of research and sharing your experiences from both science and life, for believing in me, for encouragement in every step, for your never-ending enthusiasm and optimism, for being helpful in every situation, for all your care. Your kindness and support helped me not to give up and go on.

Associate Professor *Ingrid Ehrén*, my co-supervisor for nice research talks over lunches and always being interested, friendly and encouraging, for your great experience in clinical practice and wonderful ideas in clinical research. You are always busy but never too busy for giving advice or answering an e-mail.

Professor *Peter Wiklund*, co-author, for your support with my first manuscript and for your positive and helpful attitude.

Professor *Jon Lundberg*, co-author, for sharing the knowledge in the field of nitric oxide and for your kind support and interest in my study.

Professor *Bernhard Wutzer*, and Dr *Horst Schweer*, Department of Pediatrics, Philipps University Marburg, co-authors, for interesting collaboration. I am very grateful to your help in laboratory analysis of misoprostol, and for your quick responses in spite of lack of time.

My co-authors, *Anna Sandström* and *Christian Fiala* for joyful collaborations.

Professor *Bo von Schoultz*, former head of the division of Obstetrics and Gynaecology at the Department of Women's and Children's Health, for encouraging a stimulating research environment in the department.

Professor *Gunvor Ekman-Ordeberg* for your kind support, encouragement, hospitality and for interesting discussions.

Marianne van Rooijen, the head of the Dept. of Obstetrics and Gynaecology at Karolinska University Hospital, for creating a stimulating and pleasant working atmosphere.

Associate Professor *Lennart Nordström*, and Associate Professor *Oddvar Bakos* the former heads of the Dept. of Obstetrics and Gynaecology at Karolinska University Hospital for your support.

Associate Professor *Lena Marions* for introducing me to Professor Kristina Gemzell-Danielsson, and for always being friendly.

Research nurse at the WHO centre *Margareta Hellborg* and *Lena Elfors-Söderlund*, and midwife *Eva Broberg* for your interest and friendship, your excellent work, perfect administration of protocols and highly professional care of the patients.

Birgitta Byström and *Eva Andersson*, in FHR, for all your kindness and assistance.

Dr *Lalitkumar PGL, Aurelija Dubicke, Chun-Xia Meng, Johanna Sundqvist and Sujata Lalitkumar*, my fellow doctoral students, research colleagues and co-authors in the research group, for fruitful meetings, discussions, and collaboration.

Monica Johansson, Eva Sterner, Catharina Zätterström, Majken Ullmark, Eva-Lisa Nordlund, Marianne Racke, Inga-May Andersson, staff of SESAM and C21 at the Department of Obstetrics and Gynaecology, Karolinska University Hospital, for the warm and professional care of patients.

Birgitta Mörlin and *Gunnel Lindell*, who supervised my clinical work, and know how much I have always appreciated your support, help and kindness.

Barbro Larsson, Kjell Schedvins and Brita Jonsson for all your kindness and encouragement in my clinical work, for all good advice on my surgical career and creating an incredible working atmosphere.

Associate Professor *Masoumeh Rezapour*, the head of our clinic at the Dept. of Obstetrics and Gynaecology at the Karolinska University Hospital, for creating a pleasant working atmosphere.

Lisskulla Sylvén and *Måns Edlund* for your kind support.

Olof Stephansson and *Henrik Falconer*, my office colleagues and friends, for fruitful discussions and practical support throughout my thesis work.

Ingrid Sääv, my colleague and friend, for your kindness and your hospitality.

All other colleagues in our clinic for support and interest in clinical research, and for always being friendly.

All the nurses and midwives at the Department of Obstetrics and Gynaecology, Karolinska University Hospital, for always being happy and friendly.

Clary Georgantzi and *Apostolos Tsolakis*, my best man, for all the happy hours during weekends and skiing travels.

Gerasimos Tzortzatos, my office colleague and best friend, for everything we have experienced together from our first moments in Sweden.

The Greek “gang”: *Ioannis* and *Katerina Rigopoulos*, *Georgios Vavilis*, *Fotis* and *Alkistis Papadopoulos*, *Dimitris* and *Christina Sarikostas*, *Kyriakos Konstantinidis*, *Kostas Arvanitis*, *Evangelos Argiriou*, *Evangelos Chandanos*, *Konstantinos* and *Katerina Michos*, *Georgios Charonis*, *Georgios Kapodidis* and *Georgios Garefalakis*, for all the time we have spent together during these years, for parties, exciting discussions and all other activities we did together.

My dear parents, *Spyridon* and *Lambrini*, for your endless love and support and guidance through life. Thank you for always being around, for all sacrifices and for making my life so easy. My brother *Georgios*, my sister *Paraskevi*, my sister in law *Natalia*, my brother in law *Christos*, my mother in law *Eleni*, my nephews *Spyridon-Aetios* and *Nikolaos-Christianos* for your love.

Finally I would like to thank *Evangelia*, my life partner and the woman in my life, for your endless love, support, and understanding, for cheering me up and reminding me of what life is really about. Our wonderful son, for the great serenity and happiness you bring to my life.

The studies were supported by grants from the Swedish Research Council (grant no 0855), ALF (Karolinska Institutet–Stockholm County Council, Agreement on Medical Research and Training) funding, and the Karolinska Institute Research Funds.

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