ANTIBACTERIAL EFFECTS OF NITRITE IN URINE

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To Anne, Emma, Adam and Fabian
”Hela bollen måste ligga stilla”

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Antibacterial effects of nitrite in urine

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Abstract

Urinary tract infections (UTI’s) are among the most common conditions causing individuals to seek medical care. In addition, catheter-associated infections account for most of the hospital-acquired UTI’s and is also a major source of resistant nosocomial pathogens. The majority of bacterial strains causing UTI have a nitrate-reducing capacity and since the 1920s, when the urinary nitrite test was first developed, this dipstick assay has been an important component of modern UTI diagnosis. In this thesis we have expanded the role of nitrite beyond diagnosis of infection and explored its role as an antimicrobial agent. From earlier studies it is known that acidification of nitrite results in the formation of nitric oxide (NO) and other reactive nitrogen oxides, which are toxic to a variety of microorganisms.

The aim of the present thesis was to investigate NO formation and possible antibacterial effects of acidified nitrite-containing urine. We also sought to determine the antibacterial effect of a two-step procedure in which bacteria were first fed with nitrate to form nitrite, followed later by acidification of the urine. Finally we wanted to evaluate in vitro a novel concept for intravesical delivery of antibacterial NO via the retention balloon of a urinary catheter. NO formation was measured in headspace gas using a chemiluminescence-technique, while antibacterial effects were evaluated by turbidity measurements and by viable counts.

We show that large amounts of the antimicrobial gas NO are generated in mildly acidified nitrite-containing human urine and this NO formation is greatly enhanced by the addition of ascorbic acid. Furthermore, we show that mildly acidified nitrite-containing human urine has potent antimicrobial activity against three of the most common urinary pathogens and this inhibitory effect is further increased by ascorbic acid. We also found, that Escherichia coli self destructs when it is first allowed to generate nitrite and then transferred to mildly acidified urine. Translated into clinical terms, this could imply that ingestion of nitrate followed some hours later by acidification of urine could be a new approach for treatment of UTI. Finally, potent antibacterial effects were also observed in infected urine when the nitrite-derived nitrogen oxides were delivered via the retention balloon of a urinary catheter.

In conclusion, we describe potent antibacterial effects of acidified nitrite-containing urine and also suggest a novel attractive alternative to prevent catheter-associated urinary tract infections.

Key words: nitrite, nitrate, nitric oxide, urinary tract infection, catheter-associated urinary tract infection, antimicrobial, Escherichia coli.

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LIST OF PUBLICATIONS

This thesis is based on the following papers which are referred to in the text by their Roman numerals (I-IV):


ABBREVIATIONS

ABU  asymptomatic bacteriuria
BCG  bacillus calmette-Guérin
CAUTI  catheter-associated urinary tract infection
CE  capillary electrophoresis
CFU  colony-forming units
cGMP  cyclic guanosine monophosphate
cNOS  constitutive nitric oxide synthase
E. coli  Escherichia coli
EDRF  endothelium-derived relaxing factor
eNOS  endothelial nitric oxide synthase
HNO2  nitrous acid
H2O2  hydrogen peroxide
HPLC  high-performance liquid chromatography
IL-8  interleukin 8
iNOS  inducible nitric oxide synthase
L-NAME  N⁰-nitro-L-arginine-methylester
L-NMMA  N⁰-monomethyl-L-arginine
LPS  lipopolysaccharides
MBC  minimum bactericidal concentration
MIC  minimum inhibitory concentration
mRNA  messenger ribonucleic acid
NADPH  nicotinamide adenine dinucleotide phosphate
NO2⁻  nitrite
NO3⁻  nitrate
NO2  nitrogen dioxide
N2O3  di-nitrogen trioxide
N2O4  di-nitrogen tetroxide
nNOS  neuronal nitric oxide synthase
NO  nitric oxide
NOS  nitric oxide synthase
O₂⁻  superoxide
OH  hydroxyl radical
ONOO⁻  peroxynitrite
PDE-5  phosphodiesterase type-5
PMNL  polymorphonuclear leukocytes
PHOX  phagocyte oxidase
ppm  parts per million
ppb  parts per billion
pO2  partial pressure of oxygen
RNI  reactive nitrogen intermediates
ROS  reactive oxygen intermediates
RSNO  S-nitrosothiols
sGC  soluble guanylyl cyclase
TMP  trimethoprim
SMX  sulfamethoxazole
SOD  superoxide dismutase
UTI  urinary tract infection
INTRODUCTION

Urinary tract infections

Urinary tract infection (UTI) is defined as an inflammatory response of the urothelium to bacterial invasion [1]. Acute uncomplicated UTI with an estimated incidence of 150 million yearly on a global basis is among the most common conditions causing individuals to seek medical care [2]. At the age of about thirty, 50% of all women report having had a UTI. In contrast, only 20% of men in their seventies have experienced a UTI [3, 4]. Approximately 25% of women who have had an episode of acute cystitis develop recurrent UTI [5]. UTI affects up to 10% of the childhood population and is an important cause of morbidity [6]. It is a heterogeneous disease which can be sub-divided into a variety of clinical conditions ranging from the asymptomatic presence of bacteria in the urine to severe, sometimes life-threatening, infections of the kidney with septicaemia [7].

Aetiology

Microorganisms reach the urinary tract by the ways of the ascending route and via the haematogenous or lymphatic routes. The ascending route via urethra from periurethral colonization is the most common pathway [8]. This explains the greater frequency of UTI in women since the female urethra is short and situated in close proximity to the vaginal vestibule and rectum. It also explains the increased risk of infection following bladder catheterization or instrumentation. Other risk factors, apart from gender and the use of catheters/surgical instruments, are vaginal intercourse, spermicide use and underlying conditions affecting the urinary tract such as diabetes and pregnancy. Obstruction of urinary flow (e.g. because of prostate enlargement) and diminished ability to empty the bladder (e.g. because of spinal cord injury, decreased bladder contractility or neuropathy) also increase the risk of UTI [4]. Haematogenous infections of the urinary tract can be caused by Staphylococcus aureus, Candida species or Mycobacterium tuberculosis. These infections are uncommon in normal adults, but can occasionally occur in immunocompromised patients and in neonates [9, 10]. UTI via the lymphatic route is probably not of importance, but may occur in unusual circumstances such as a severe bowel infection or retroperitoneal abscess [10, 11]. Escherichia coli (E. coli) is by far the most common cause of UTI, accounting for 80-85 % of community-acquired infections and 50% of hospital-acquired infections. Other gram-negative enterobacteriaceae including Proteus, Klebsiella and gram-positive Enterococcus faecalis
and *Staphylococcus saprophyticus* are responsible for the remainder of most community-acquired infections [12, 13]. In hospital-acquired UTI *E. coli* is still the leading cause of UTI, but other bacteria such as *Pseudomonas aeruginosa*, *Klebsiella species* and *Proteus mirabilis* are seen more frequent as causative agents [14].

**Bacterial virulence**

Uropathogenic bacteria are selected from the faecal flora by presence of virulence factors that enable them to adhere to and colonize the perineum and urethra and migrate to the urinary tract [15, 16]. These virulence factors include adhesins, siderophores, toxins, polysaccharide coating, proteases and invasins. However, no single virulence factor is common to all clinical UTI isolates and none of these factors is absolutely necessary for development of UTI pathogenesis [17]. The more compromised the natural defense mechanism (e.g. obstruction, bladder catheterization, diabetes with poor blood glucose control), the fewer virulence factors are needed for any bacterial strain to induce infection. This is supported by the observation that bacteria isolated from patients with a complicated UTI frequently fail to express virulence factors [17-19]. There has also been suggested that if a bacterial strain had a doubling time of about 50 minutes or less in urine, it could maintain itself in the normal bladder without adhering [20].

**Host defense mechanisms**

Flow of urine through the urinary tract and voiding are effective natural bladder defenses against infection [8, 21]. In addition, low pH, urea and high urine osmolality can be inhibitory to bacterial growth. Both concentration of urea and urine osmolality are decreased by urine dilution, which has led some authors to suggest that diuresis actually could favour bacterial proliferation [22-24]. The asymptomatic bacteriuria (ABU) strains do not activate a host response because these strains stop expressing adherence factors once they are established in the urine thereby avoiding the antibacterial defense [25]. In contrast, the uropathogenic strains, continue to adhere and activate the uroepithelial cells to release cytokines that recruit polymorphonuclear leukocytes (PMNL) to the site of infection. During their passage across the mucosa, the PMNL kill the bacteria [26]. The cytokine interleukin-8 (IL-8) seems to be the main driving force for the PMNL to cross the urinary tract epithelium and the emergence of leukocytes in the urine, known as *pyuria*, is a classical sign of UTI [25]. The attachment of a uropathogenic strain may result in exfoliation of host bladder epithelial cells as part of an innate host defense system [27]. Another host defense strategy is to produce soluble receptor
molecules that compete with bacterial adhesins and block bacterial attachments [28]. The vaginal flora, dominated by *Lactobacillus* species, is an additional important host defense system against UTI. Particularly the *Lactobacillus* species that produce hydrogen peroxide (H$_2$O$_2$) seems to have a protective effect [29]. *Lactobacilli* have also been shown to generate an acidic vaginal pH, which appear to be of importance for lowering the rate of UTI [30]. Unfortunately, use of antibiotics and intravaginal antimicrobials including antimycotics and spermicides reduces the vaginal flora and increases susceptibility to UTI [31].

**Diagnosis**

Urine cultures are still the golden standard to diagnose UTI as it has been for decades, although the number of bacteria considered relevant for diagnosis has been changed since the classical “Kass criteria” of $10^5$ bacteria/ml were described in 1956 [32]. Stamm and co-workers showed in 1982 that a count of $\geq 10^5$/ml identified only about 50% of dysuric women with bacteriuria, whereas a count of $\geq 10^2$/ml had a predictive value of 0.88 for diagnosing a probable urinary tract infection [33]. The diagnostic criteria for UTI in acute uncomplicated cystitis in women is $\geq 10^3$ colony-forming units (CFU) of uropathogen/ml of mid-stream sample of urine according to EAU guidelines [2, 34]. Since the 1920s, when the urinary nitrite test was first developed, this dipstick assay has been an important component of modern UTI diagnosis [35, 36]. Most dipstick assays will indicate positive nitrite (change of colour) when urinary nitrite levels exceed 7-25 µM, as a result of bacterial nitrate-reducing capacity [37, 38]. Today the common urine dipstick test in use detects nitrite and leukocyte esterase. These tests are rapid and cheap alternatives, but show less accuracy compared with a quantitative culture [39]. Another clear disadvantage with the dipstick assays is that they do not give information about the exact species that caused the UTI or the antimicrobial susceptibility profiles. The majority of bacterial strains causing UTI have a nitrate-reducing capacity, like for example *E. coli, Pseudomonas aeruginosa and Proteus mirabilis*. *Staphylococcus saprophyticus* can also express nitrate-reductase, but normally very low levels [40]. However, streptococci, including strains of *Enterococcus faecalis*, do not have nitrate reductase enzymes and lack the capacity to reduce urinary nitrate to nitrite [41, 42]. Recently, a meta-analysis demonstrated that sensitivities of the combination of both nitrites and leukocyte-esterase vary between 68 and 88% in different patient groups. The sensitivity was also analysed in different patient settings and found to be highest in studies carried out in family medicine (90%). The sensitivity of the urine dipstick test for nitrite alone was 45-60% with higher level of specificity (85-98%) [43]. Reasons for false negative results using
dipstick test for nitrite i.e., negative nitrite assay in urine with more than $10^5$ nitrate-reducing organisms/ml could be urinary pH below 6.0, urobilinogen, lack of dietary nitrate or intake of ascorbic acid [37]. Also high frequency of micturition might be a reason for false negative results, since an incubation at $37^\circ$ C of four to six hours is preferable for generation of detectable nitrite in infected urine samples [41].

**Treatment and Antimicrobial resistance.**

In treatment of uncomplicated cystitis, a short course of antibiotics are highly effective and also desirable because of the improved compliance that they promote, their low cost and the low frequency of adverse reactions. According to American guidelines, trimethoprim-sulfamethoxazole (TMP-SMX) for three days is considered the current standard therapy [44]. TMP alone and other fluoro-quinolones (ofloxacin, norfloxacin, ciprofloxacin, and fleroxacin) are equivalent to TMP-SMX [44]. Pivmecillinam and nitrofurantoin are alternative oral drugs, especially in situations in which fluoroquinolones are not indicated [45, 46]. In Sweden, clinical practice guidelines have been developed by the STRAMA-group, which recommends pivmecillinam or nitrofurantoin or TMP for seven days as a standard treatment of uncomplicated cystitis in women [47]. However, there are other Swedish studies supporting short-term treatment for 3-5 days of uncomplicated lower urinary tract infections in women [48, 49]. A growing problem of worldwide concern is the increasing resistance of pathogens to conventional antibiotics [50]. In UTI much of the increase in bacterial resistance is in acute uncomplicated cystitis with increasing TMP-SMX and β-lactam resistance. Of more concern, however, are the emerging issues of fluoroquinolone resistance and multidrug-resistance among community-acquired urinary isolate. Important strategies to help slow the progression of resistance is a judicious use of antibiotics and to develop novel methods for the prevention of UTI [51].

**Prophylaxis and Acidification of urine**

Urinary acidification has long been used as an aid in the treatment and prevention of urinary tract infections. Various agents have been used in attempts to lower urinary pH, these include mandelic acid, gluconic acid, ammonium chloride, methionine and ascorbic acid (vitamin C) [52-55]. Urinary acidification with vitamin C has traditionally been used as a household remedy, although evidence to support its efficacy has largely been anecdotal and any antibacterial action still remains unclear [56]. Effective acidification of urine has been poor and antibacterial effects variable [57-59]. Methenamine hippurate, a weak base that slowly
hydrolyzes in acidic urine to ammonia and the nonspecific antibacterial formaldehyde, are often used for the prevention of urinary tract infection. However, there is not enough evidence to conclusively support the use of methenamine hippurate for UTI prophylaxis [60]. Today most prophylaxis of UTI consists of antimicrobial therapy. The increasing prevalence of _E. coli_ isolates that are resistant to antimicrobial agents, however, has stimulated interest in novel, non-antibiotic methods for preventing UTI. Natural compounds like cranberry products have been more popular and used as a household remedy for treating and preventing UTI [61]. It has been postulated that acidification of urine is an important means by which cranberry products might protect against UTI. However, effective acidification of urine through cranberry ingestion has not been proved [62]. The mechanism of action is likely to be a tannin (proanthocyanidin) that block adherence of P-fimbriated _E. coli_ [63]. Although there are some clinical trials that have shown positive effects with cranberry ingestions in preventing UTI a Cochrane review in 2001 concludes that there is no compelling evidence to recommend cranberries for prevention of UTI [64].

**Catheter-associated urinary tract infection and Biofilms**

Catheter-associated urinary tract infection (CAUTI) accounts for most of the hospital-acquired UTI [65]. In medical intensive care units in the US as much as 95% of urinary tract infections were associated with urinary catheters [66]. CAUTI is also a major source of resistant nosocomial pathogens [67]. The incidence of bacteriuria in catheterized patients varies between 3% and 10% per day [68]. A biofilm is a collection of microbial organisms on a surface that is surrounded by an extra cellular matrix composed primarily of poly-saccharide materials. Urinary catheters can develop biofilm in two ways. The extra luminal route, by direct inoculation at time of catheter insertion or by migration in the mucous sheath surrounding the catheter, is the most common way (about 70%) [69]. Adherent bacteria aggregate, multiply and form a biofilm within a few hours [70]. Microorganism may also enter the catheter from the internal lumen of the catheter, but this intra luminal route presupposes a contamination of the closed drainage system [71]. The biofilm provides a protective environment for the microorganisms and make them less susceptible to antimicrobial agents. The reason for this poor effect of antimicrobial agents is that the bacteria in the biofilm grow much slower than bacteria growing freely within the urine and also that the extra cellular matrix prevent penetration of antimicrobial agents into the biofilm [72]. Another problem with biofilm production is that freely growing bacteria found in cultures obtained from catheterized patients may not reflect bacterial population growing
within the biofilm [73]. Guidelines recommend aseptic technique during catheter insertion and stress the importance of using a closed urinary drainage system to minimise the development of CAUTI [74]. Other approaches in preventing these infections include systemic antibiotics and anti-infective catheters [70]. Silver alloy catheters have been used and the result of a meta-analysis indicates that they likely prevent bacteriuria and despite a higher cost also seem economically efficient when used in patients receiving indwelling catheterization for 2 to 10 days [75, 76]. Impregnation with salicylic acid and even electrification have been showed to reduce bacterial adhesion to urethral catheters [77, 78]. Additional methods are suprapubic catheters and clean intermittent catheterization. Suprapubic catheters are mainly used in patients requiring long-term catheterization and several trials have indicated that patients have lower risk of bacteriuria and higher rate of satisfaction compared with those using indwelling catheters [79]. Clean intermittent catheterization is associated with a decreased risk for UTI compared with long-term indwelling catheters [80]. Despite these preventive efforts CAUTIs are very common and result in substantially increased health care costs and leads to substantial morbidity and mortality with data suggesting an almost threefold increase in mortality even when comorbid conditions and other factors are accounted for [81, 82].

**Nitric Oxide**

*The history of nitric oxide.*

In 1980, Furchgott and co-workers showed that acetylcholine-induced relaxation of blood vessels was dependent on the endothelium and as a consequence the endothelium-derived relaxing factor (EDRF) was discovered [83]. Murad and co-workers had three years earlier shown that nitroglycerine and nitroprusside cause relaxation of smooth muscle cells via activation of the enzyme soluble guanylyl cyclas (sGC) which increase cyclic guanosine monophosphate (cGMP) [84]. Murad and co-workers then found that Nitric Oxide (NO) was the mediator of this cGC activation and, in 1983, that EDRF induced relaxation in smooth muscle cells via increased cGMP [85, 86]. Eventually in 1987, Ignarro and Moncada with co-workers independently showed that NO is identical to EDRF [87, 88]. With these Nobel Prize rewarded discoveries (Furchgott, Ignarro and Murad in 1998), NO went from being regarded merely an atmospheric pollutant to a highly significant biological molecule. The discovery of NO led to an explosion of research regarding the biological significance of this small diatomic molecule with, until today, more than 65 000 publications on the topic.
Enzymatic formation of NO

Mammalian cells synthesize NO enzymatically from the amino acid L-arginine and molecular oxygen by nitric oxide synthase (NOS) [89] (fig 1). This enzymatic reaction is nicotineamide adenine dinucleotide phosphate (NADPH)-dependent and results in formation of equimolar amounts of L-citrulline [90]. NOS also needs the cofactors heme, flavin adenine dinucleotide, flavin mononucleotide and tetrahydrobiopterin [91]. At present, three main isoforms of the enzyme have been identified, out of which two are expressed constitutively and one isoform is inducible. The isoforms of the enzymes were named after the tissue in which they were initially found [92]. The two constitutively expressed (cNOS) are neuronal NOS (nNOS) and endothelial NOS (eNOS) and activation of these enzymes requires calmodulin, which in turn is controlled by the intracellular concentration of Ca$^{2+}$ [93, 94]. Within seconds from stimulation with agonists such acetylcholine or bradykinin, cNOS produce femtomolar to picomolar concentration of NO. The third isoenzyme, iNOS, is on the other hand independent of free Ca$^{2+}$ and its expression can be induced by bacterial products such as lipopolysaccharide and proinflammatory cytokines (e.g. tumour necrosis factor-α, interleukin-1β and interferon-γ) a process involving transcription factors (e.g. nuclear factor-κB) and thereby increasing iNOS mRNA [95, 96]. The time that is required for iNOS mRNA and protein to be synthesized results in a delay of several hours between cell activation and NO synthesis. Once iNOS is expressed, which can happen in a wide variety of human cells, it can produce nanomolar concentration of NO and this production is sustained over a prolonged period of time.

Nonenzymatic formation of NO

Chemical synthesis of NO in vivo was first described 1994 when Benjamin and co-workers and Lundberg and co-workers independently showed NO production in the stomach, which relies on the secretion of nitrate in saliva and bacterial conversion to nitrite on the tongue with reduction to NO by stomach acid [97, 98]. The dorsal surface of the tongue harbours a specialized flora of symbiotic nitrate-reducing facultative anaerobic bacteria [99]. Nonenzymatic formation occurs in the stomach at low pH, when the nitrite ion (NO$_2^-$) is converted to nitrous acid (HNO$_2$), which subsequently decomposes to various nitrogen oxides including NO in the parts per million (ppm) range [97, 98] (fig 1).
Apart from pH, this nonenzymatic formation depends on pO$_2$, proximity to heme containing proteins, redox state and thiol concentration [100]. Reducing agents, like ascorbic acid, will further enhance this reaction. Ascorbic acid and ascorbate ion will generate NO over a wide pH range in aqueous solution by rapid reduction of nitrous acid (HNO$_2$) and formation of dehydroascorbic acid [101] (fig 1). In addition to the stomach, *in vivo* non-enzymatic formation of NO has also been shown in the oral cavity, the heart, and on the skin [99, 102-104]. The enzyme nitrate reductase is not found in humans, but a number of commensal bacteria inhabiting mucosal surfaces have multiple nitrate reductases and can catalyse the reduction of nitrate to nitrite especially during oxygen limitation. There are now increasing data suggesting that these nitrate-reducing commensals have a true symbiotic role in mammals [105, 106]. Other enzymes in humans, that can reduce nitrite to NO are mitochondrial enzymes, xantineoxidase and cytochrome P450 [107-110]. In addition, deoxy-hemoglobin has recently been shown to reduce nitrite to NO and thereby causing vasodilatation in the human circulation [111].

**Nitrate in medicine**

During the 19$^{th}$ century inorganic nitrate was a very common component in many galenic preparations and was widely used in treatment of different disorders (eg. oedemas, pain and inflammation) [112]. In the early decade of the 20$^{th}$ century it was shown that inorganic nitrate could cause methaemoglobinaemia in infants and its therapeutic use clearly fell into
disgrace [113]. Organic nitrates, like glyceryl trinitrate and isorbiddinitrate on the other hand, have been used therapeutically for over 100 years and are still in use in the treatment and prophylaxis of angina pectoris [114]. Since around 1950 when inorganic nitrate was suspected to be associated with development of gastric cancer its bad reputation grew even worse [115]. The metabolism of nitrate and nitrite can result in N-nitrosoamines which are carcinogenic in cell cultures and animal models [116]. However, despite numerous studies, there is still no clear evidence in humans for a link between inorganic nitrate intake and gastric cancer and most studies show no relationship between a high intake of nitrate and gastric cancer in humans [117-120]. More recent studies even indicate the opposite, that in humans the bacterial metabolism of nitrate to nitrite and the subsequent formation of biologically active nitrogen oxides could in fact be beneficial [105, 121].

**Metabolism of nitrate/nitrite**

Green vegetables such as lettuce and spinach, root vegetables such as beetroot and drinking water are the main sources of exogenous nitrate intake [122]. Exogenous intake of nitrite is much smaller, but nitrite can be found in some food as a ingredient in red meat curing giving cured meat and hot dogs their red colour (nitrosylated myoglobin) and protecting it from oxidation and spoiling [123]. Nitrite is particularly effective against the pathogen *Clostridium Botulinum* [124]. About 90% of ingested nitrite comes from nitrate in saliva and less than 10% comes from food [125]. The main source of endogenous nitrate in mammals is the L-arginine-NO-pathway, which in fasting humans constitutes the major source of nitrate/nitrite in plasma [126]. After ingestion, nitrate is absorbed from the stomach and proximal small intestine into plasma, where it mixes with endogenously synthesized nitrate [127]. It is then concentrated, by a factor of ten, from the plasma into the saliva [128]. By this entero-salivary circulation of nitrate, about 25% of plasma nitrate is actively taken up by the salivary glands and secreted with saliva [129]. About 1/5 of this recycled nitrate (approximately 5% of the total ingested nitrate) is converted to nitrite by oral cavity organisms [130]. Studies with isotope labelled administrated nitrate have shown that only 60% is recovered in the urine. Consequently, the exact fate of all the nitrate in the body is still unresolved [131].

**Chemistry of NO, nitrate and nitrite**

The gas NO, with a molecule weight of only 30 Dalton, is certainly one of the smallest biological mediators. Due to it small size, uncharged character and lipid solubility it diffuses easily and rapidly over biological membranes. NO is also a free radical in that it has an
unpaired electron and is therefore extraordinary labile, with a half-life of only about 1-5 seconds in most biological systems [132]. NO exhibits a low level of solubility in water (approximately 9 times more lipid soluble than water soluble) and when inserted into a physiological buffer, the major part of the gas immediately appears in the headspace [133]. Despite being a radical the reactivity of NO under physiologically circumstances is quite limited to either a reaction with other radicals, with oxygen or with transition metals [134]. The in vivo reaction of NO and superoxide (\(O_2^-\)), that forms peroxynitrite (ONO\(O^-\)), is an example of two relatively unreactive free radicals that will produce a much more reactive species (fig 2). Peroxynitrite is a potentially cytotoxic substance that can be protonated to peroxynitrous acid (OONO\(H\)), which in turn dissociates to oxidizing hydroxyl radical (OH) and nitrogen dioxide radical (NO\(_2\)) within seconds at physiological pH [135] (fig 2). However, eukaryotic cells contain large amounts of superoxide dismutas (SOD), an enzyme that keep concentration of \(O_2^-\) remarkably low [136]. High concentrations of gaseous NO can react with oxygen to form NO\(_2\), a poisonous gas called “brown gas” (fig 2). However, the reaction is of second order with respect to NO which means that it will be slower when NO concentrations are low for example in vivo (normally 100-300 nM and no more than 1-3 \(\mu\)M during inflammation). Much more common in biological fluids, with oxygen present, is the rapid oxidation of NO to nitrite (NO\(_2^-\)) or nitrate (NO\(_3^-\)) [137]. One of the most important examples of a reaction with metals is when NO interacts with the heme group in the enzyme sGC and thereby increases cGMP, which in turn mediates many of the biological actions of NO [138]. Another example of reaction with metals occurs in the blood, where NO reacts with Hb(Fe\(^{2+}\))O\(_2\) to form NO\(_3^-\) and methaemoglobin. This is suggested to be an important pathway for NO elimination [139] (fig 2). NO can also react with thiol groups allowing the formation of S-nitrosothiols (RSNOs) that may represent a physiologically important source of NO [140]. Acidification of nitrite will generate nitrous acid (HNO\(_2\)), which will spontaneously yield di-nitrogen trioxide (N\(_2\)O\(_3\)), NO and nitrogen dioxide (NO\(_2\)) [100]. The in vivo chemistry of these reactive nitrogen intermediates (RNIs), many of which have biological effects, is very complex and still not fully characterized [141, 142]. Until recently, the above mentioned oxidation of NO to the inert and stable end products nitrite and nitrate was generally accepted as a mechanism for inactivation of NO. However, several recent studies have shown that different pathways exist to recycle nitrite back into bioactive NO in blood and tissue [97, 98, 102, 108, 110, 111, 143].
Fig 2. NO reaction with oxygen, superoxide and oxyhaemoglobin. Nitrate reduction by bacterial nitrate reductase (NR).

**Beneficial effects of NO**

NO is a unique messenger molecule. In the autonomic nervous system NO functions as a major non-adrenergic non-cholinergic neurotransmitter. Moreover, NO has a particularly important role in relaxation of smooth muscle in the gastrointestinal and urogenital tracts and NO is responsible for the smooth muscle relaxation of corpus cavernosus leading to penile erection. These important smooth muscle relaxations are mediated by the reaction of NO with sGC leading to increased cGMP [83, 144, 145]. Sildenafil (Viagra®), a drug that inhibits phosphodiesterase type-5 (PDE-5) and thereby inhibit the degradation of cGMP leading to a prolonged effect of cGMP and better erection, has revolutionised the treatment of erectile dysfunction [146]. In the urinary tract of animal models, NOS activity has been found in the urothelium, smooth muscle, striated muscle, nerves and blood vessels [147]. In addition, NO is an important mediator in dilation of bladder neck, urethra and urethral sphincter during the micturation reflex [148]. NO is also a potent vasodilator and inhibitor of platelet aggregation and adhesion [149-151]. Inhibitors of NO synthases have been very useful to explore the biological activities of NO. Two L-arginine analogues, N⁶-monomethyl-L-arginine (L-
NMMA) and NG-nitro-L-arginine-methylester (L-NAME), that lack selectivity for the NOS isoforms, have been shown to increase blood pressure in normotensive humans, confirming that NO is important in the regulation of vascular tone [152, 153]. There are also several NO-donors currently being used in various types of experimental studies [154]. Some of these different NO-donors appear to be of interest for their potential therapeutic applications, for example the diazeniumdiolates (formerly NONOates), that have been used in studies regarding inhibiting restenosis after angioplasty, preparing thromboresistant medical devices, reversing vasospasm, and relieving pulmonary hypertension [155]. Another very exciting group of NO-donors, are the NO-non-steroidal anti-inflammatory (NO-NSAIDs) drugs, which are characterized by a reduction in gastrointestinal side effects [156, 157]. Since NO is also thought to play a role in detrusor instability, a NO-releasing and prostaglandin synthesis-inhibitory-drug has been used in patients with neurogenic bladder instability with promising results [157].

**Beneficial effects of acidified nitrite.**

Dietary nitrate has an important role in protection against ingested pathogens. After a high nitrate containing meal, like a portion of lettuce, the levels of nitrite and nitrate in saliva increase. When this saliva enters the acidic environment in the stomach, it results in an increased NO concentration from basal levels of 20 parts per million (ppm) to > 400 ppm. The acid environment alone is not sufficient for killing of pathogenic bacteria in the stomach. However, after mixture of salivary nitrite in gastric juice, most pathogenic bacteria are killed within a hour in vitro [105, 127]. Dietary nitrate and the above described acidified-nitrite-derived NO are also of great importance as a gastroprotective agent. Bjorne and co-workers have shown in a rat in vivo model that mucosal blood flow and mucus secretion increased after luminal application of nitrite-rich saliva, whereas saliva from a fasting individual had no effect [158]. Acidified nitrite has antimicrobial effect on periodontal bacteria, which might explain some of the protective effects of normal saliva against dental caries [159]. Commensal skin bacteria have been shown to produce NO by the reduction of sweat nitrate to nitrite and its subsequent conversion to NO by the acidic environment [103]. In vitro studies have shown that acidified nitrite is microbiocidal to common cutaneous pathogens [160]. As described earlier, acidification of nitrite results in production of a complex mixture of nitrogen oxides including nitrous acid (HNO₂), di-nitrogen trioxide (N₂O₃), nitrogen dioxide (NO₂) and NO. All these nitrogen oxides may act as nitrosating agents and can rapidly react with reduced thiols to form nitrosothiols. Nitrosothiols as well as many other of these nitrogen
oxides can kill microbes and therefore the exact mechanism responsible for the above described in vivo antibacterial effects of acidified nitrite is not fully understood and needs to be further investigated.

**NO in inflammation and host-defense**

In 1983, it was shown that that *E. coli* lipopolysaccharides (LPS) stimulates urinary nitrate excretion and it was proposed that this mammalian nitrate biosynthesis was the result of oxidation of reduced nitrogen compounds [161]. In 1985, Marletta and Stuehr reported that LPS-stimulated peritoneal macrophages from mice, synthesize nitrite and nitrate from an unknown precursor molecule [162]. Three years later the same group showed that NO, was produced and that L-arginine, was the precursor [162, 163]. Independently in 1987, Hibbs and co-workers, showed that the anti-tumour effect of macrophages were abolished when arginine was removed from the medium [164]. In addition to macrophages, cytokine-induced high-output NO synthesis, has been found in most somatic cells involved in cell-mediated immune reactions [165]. Today, NO is clearly regarded as a central component of innate immunity and an effective antimicrobial agent [166]. On the other hand, the large amounts of NO resulting from increased iNOS expression, may also contribute to the morbidity of infection by acting as vasodilator, myocardial depressant and cytotoxic mediator [167]. Not all of the host–derived NO is synthesized by NOS. As described earlier, dietary nitrate can be reduced by oral bacteria to nitrite and then in the acidic environment in the stomach further reduced to NO and other RNIs may also be formed. The first line of host defense is the innate immune response, where phagocytic cells are very important components. In addition to the iNOS pathway that generate NO radicals, phagocytic cells use another important antimicrobial system, the NADPH phagocyte oxidase (PHOX) pathway responsible for superoxide (O$_2^-$) generation. Superoxide (O$_2^-$) is like hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (OH) intermediate reduction products of oxygen en route to water and they are commonly called “reactive oxygen species” (ROS) [168]. The microbial targets of ROS and RNI are mainly thiols, metal centres and DNA. RNI mainly inhibit respiration and interfere with DNA replication, whereas ROS work mainly through direct DNA damage. The antimicrobial actions of RNI are more complex than those of ROS and multiple cellular targets are almost certainly involved, although the exact mechanism for the antimicrobial effects are still not known [167, 169, 170]. ROS/ RNI and phagocytic cells represent nonspecificity in the immune system and are effective killers of microbes and essential for clearing invading microbes from an infected area. Phagocytes are quick to sacrifice themselves and surrounding
host tissue to prevent microbial metastasis, thus resulting in further tissues damage [169]. Increased NO production in vivo has been described in many chronic inflammatory processes, such as for example cystitis [171], asthma [172], colitis [173] and rheumatoid arthritis [174]. NO has been suggested to be very useful as a non-invasive marker of several inflammatory diseases, although the role of NO in chronic inflammatory processes remains to be elucidated [175]. However, there are now studies questioning whether the sustained iNOS expression seen in many chronic inflammatory diseases, for example psoriasis, is actively producing significant amounts of NO in vivo and recent evidence, in fact, suggest that lack of NO formation in this disease is of importance in the pathophysiological process [176].

**NO and inflammation in the lower urinary tract.**

In studies of the mouse urinary tract, as discussed earlier, it has been shown that the uropathogenic strains adhere to the uroepithelial cells and activate them to release cytokines. The cytokines recruit polymorphonuclear leukocytes (PMNL) that clear the urinary tract from the infection [177]. In humans, increased iNOS activity has been detected during UTI from neutrophils in the urine and the iNOS activity persist for a period of time after initiation of antibiotic treatment [178]. Luminal NO in the urinary bladder is increased in patients with cystitis of different aetiology [179]. Cytokines, as well as bacillus calmette-Guérin (BCG), have been shown to increase iNOS activity in vitro in human uroepithelial cells [180, 181]. However, studies with UTI in iNOS-deficient mice have shown that there were no differences in bacterial clearance or persistence between mice with intact iNOS capacity compared with the deficient genotype [182]. It has been shown that human bladder smooth muscle cells respond to LPS and inflammatory cytokines with increased iNOS activity and collagen type III expression. The authors suggest that iNOS may be a critical mediator of the bladder wall fibrotic response to chronic UTI [183]. In ifosfamide induced hemorrhagic cystitis in mice, NO produced by iNOS, is suggested to be responsible for urothelial damage and inflammation [184]. In conclusion, the exact role of NO in human UTI is not clear, but taking the well-known antimicrobial properties into account it is very likely that NO in some way enhance local host defense in the bladder.
**Bacterial defense against RNIs.**

Since protection against ROI have been studied for over 100 years, detoxification-enzymes like microbial catalase, SOD and peroxides that convert ROS to less toxic species have been well documented [185]. In contrast, until about seventeen years ago, RNI production in mammals was not thought to exist, because RNI was regarded to be too toxic [169]. Knowledge about resistance to RNI is therefore a relatively new concept. However, some pathways for microbial defense against RNI have now been identified and include scavenging of RNIs, suppressed RNI production, repair of nitrosative damage and enzymatically detoxification of RNIs. The most studied bacterial enzymatic detoxification defense to NO and other RNIs are in *E* coli, where nitrite reductases and NO reductases, as well as a flavohaemoglobin, have been characterized [105, 186]. Studies in *E* coli have also demonstrated specific antioxidant regulons. One example is a family of approximately 12 genes (even including the SOD gene) called the soxRS regulon, which may protect bacteria against NO [187].
AIMS

The general aim of this thesis was to investigate antibacterial effects of nitrite in urine.

The specific aims were:

- To investigate the changes in NO formation in relation to different concentrations of nitrite and different pH-levels with or without ascorbic acid in normal and infected urine.

- To study antibacterial effects of acidified nitrite and ascorbic acid against three common urinary pathogens at different urinary pH levels.

- To evaluate the minimal bacteriostatic and the minimal bactericidal concentrations of acidified nitrite and ascorbic acid and to compare the effects with conventional antibiotics.

- To evaluate the antibacterial effect of a new two-step procedure in which bacteria are first fed with nitrate, followed by acidification of the urine.

- To evaluate the in vitro antibacterial effect of a novel concept for delivery of NO via a catheter retention balloon into the surrounding urine.
MATERIAL AND METHODS

Study subjects

In paper I urine was collected from 8 patients (41-70 years old) with bacteriuria as confirmed by urinary cultures. Urine was also collected from 5 healthy subjects before and after ingestion of vitamin C (2g/day) for 2 days.

In paper III 8 healthy subjects mean age 37 years (range 25-47) fasted overnight. Basal urinary samples were collected and then the subjects ingested sodium nitrate (10 mg/kg) dissolved in 150 mL water. Urinary samples were collected at 1, 2 and 3 hours.

Chemical analysis of NO, nitrite and nitrate

Analysis of NO in gaseous phase by chemiluminescence (paper I-II and IV)

In this thesis we have used two chemiluminescence NO analysers when measuring gaseous NO. In paper I and II CLD 700, Eco Physics, Dürnten, Switzerland was used and in paper IV, Aerocrine AB, Stockholm, Sweden was used. Measurement of gaseous NO with chemiluminescence, is based on the reaction of NO with an excess of ozone (O\textsubscript{3}) to produce nitrogen dioxide (NO\textsubscript{2}), which is partly excited (NO\textsubscript{2}\textsuperscript{*}). When NO\textsubscript{2}\textsuperscript{*} returns to its ground state the excess energy is released as a photon (hv) and light (luminescence) in the 640-3000 nm wavelength range is emitted, which is measured by a sensitive photomultiplier tube. The intensity of this luminescence is proportionally converted into an electrical signal and displayed as NO levels with a response time of less than 0.7 s. The chemiluminiscence assay is extremely sensitive for NO and has a detection limit of 1 part per billion (ppb) and a linear response for concentrations between 1-100 000 ppb. The assay also shows a high specificity for NO without interference from other nitrogen oxides [188]. When \geq 50 ml of NO-containing gas (0.5-20 ppm) was injected into the NO analyser the recovery of the NO signal was 100%. In this thesis we made use of the well documented properties of NO, as a gas with poor solubility in water and extremely high diffusibility. Thus, when NO is injected into a physiological buffer, \geq 80% immediately appears in the gas space (head space) [133]. NO has also been shown to diffuse through the inflatable retention balloon of a urinary catheter [133, 179, 189]. In paper I and II, urinary samples (10 ml) were incubated at 37°C in closed syringes with a headspace of 50 ml. After 30 min, the headspace gas was removed and immediately injected into the chemiluminescence analyser (CLD 700, Eco Physics, Dürnten, Switzerland). Ambient NO levels were below 5 ppb. In paper I the urinary samples consisted
of 10 ml infected urine or 10 ml control urine with or without addition of 100 µM sodium nitrite and NO release was measured at different pH levels. NO was also measured before and after ingestion of vitamin C (2g/day) for 2 days in 10 ml urinary samples with addition of 100 µM sodium nitrite at different urinary pH levels (pH 4-7). In paper II NO was measured in 10 ml urinary samples consisting of sodium nitrite (50-500 µM) with or without addition of vitamin C (10 mM) at different urinary pH levels (pH 4.5-6.0). Ambient NO levels were below 5 ppb in all experiments.

In paper IV An all-silicone catheter (Argyle®, Sherwood Medical, Tullamore, Ireland) was placed in a bladder model (50 ml flask) and the retention balloon was filled with 10 ml of saline containing ascorbic acid (10 mM) and sodium nitrite (5 mM) at pH 2.5. The flask was then closed and synthetic NO-free air was flushed via an inlet at a rate of 4 L/min. Headspace NO concentration was continuously measured from an outlet by a rapid-response chemiluminescence system (Aerocrine AB, Stockholm, Sweden) at room temperature (20°C). Ambient NO levels were below 5 ppb in all experiments.

Quantification of nitrite and nitrate by chemiluminescence

In paper III and paper IV nitrite and/or nitrate sample concentrations were determined by chemiluminescence after reductive cleavage and subsequent determination of the NO released into the gas phase. The samples were directly introduced via a gas-tight syringe into a reduction solution of a micro reaction purge vessel coupled with a condenser and heating jackets unit (Sievers, Boulder, CO, USA). The condenser jacket temperature was controlled by a continuous flow of cold water while the temperature of the heating jacket was controlled by a flow of warm water regulated by a constant-temperature circulating bath (MGW Lauda M3). Nitrogen gas, at flow of 192 mL/min, was used as the carrier gas of NO. The flow could be adjusted with a needle valve integrated with the purge vessel and the outlet of the gas stream was passed through a scrubbing bottle containing sodium hydroxide (1 M, 0°C) in order to trap traces of acid before transfer into the NO analyzer. A chemiluminescence NO analyser (Aerocrine AB, Stockholm, Sweden) was used to display the NO signals and collect the data, which were further manipulated with Origin for Windows, Version 7.0 (Microcal, Northampton, MA) and reported as area under the curve. Nitrite was determined according to Feelish et al [190]. The reducing mixture, consisting of 45 mmol/L potassium iodide (KI) and 10 mmol/L iodine (I₂) in glacial acetic acid, was kept at a constant temperature of 56 °C and continuously bubbled with nitrogen gas. The amount of nitrite in a given sample was quantified by simple subtraction of the peak areas of sample aliquots pretreated with
sulfanilamide from that of untreated aliquots (10% (v/v) of a 5% solution of sulfanilamide in 1N HCl is added to the biological sample (final concentration 29 mmol/L) and incubated for 15 min at room temperature. Under these conditions, nitrite reacts with sulfanilamide to form a stable diazonium ion that is not converted to NO. Nitrate was reduced to NO with a solution of Vanadium(III)chloride in hydrochloric acid 1N (saturated solution) at 95°C. Since Vanadium(III)/HCl will also convert nitrite to NO the amount of nitrate was quantified by subtraction of the nitrite concentration calculated before.

**Quantification of nitrite by capillary electrophoresis**

Capillary electrophoresis (CE) has been used in the analysis of several ions and micromolecules, because of its high efficiency of separation and short analysis time compared to other available techniques such as high-performance liquid chromatography (HPLC). In paper I CE was used to analyse urinary nitrite concentrations in 8 patients with bacteruria. After collecting midstream urine the samples were stored at -80°C until analysis. The CE system used was Hewlett Packard capillary electrophoresis system (HP 3D, Waldbrunn, Germany) with UV detection at 214 nm. The background electrolyte consisted of 25 mM sodium sulphate and 5% NICE-Pack OFM Anion-BT, dissolved in freshly brought Milli-Q water. Samples were injected hydrodynamically by 30 mbar for 60 seconds onto extended light path capillaries (104 cm effective length and 75 µm ID). The analysis was done at an applied negative voltage of 300 kV/m and the temperature of the capillaries was set to 40°C. Data were analyzed with a HP 3D CE Chem Station data system. Standard curves for nitrite was plotted. Before being used for the first time, capillars were conditioned with sodium hydroxide (1 and 0.1 M) and the background electrolyte for 5 min each while the temperature of the capillary was set to 60°C.

**Bacterial cultures and media**

The strains used in this thesis were *E. coli* U1106024 (paper IV) isolated from a patient with urinary tract infection and reference strains *E. coli* ATCC 25922 (paper I-IV), *P. aeruginosa* ATCC 27853 (paper II), *S. saprophyticus* SS2 (paper II), all obtained from the department of Clinical Microbiology, Uppsala, Sweden. In paper III *E. coli* RK 4353 and a RK 4353 mutant, lacking all three known nitrate reductase enzymes were used [191]. The RK 4353 strains were a generous gift from Dr. JA. Cole, Birmingham, UK. Before each experiment
bacteria were grown aerobically in Mueller-Hinton broth for 6 hours at 37° C, resulting in 2-5 x 10^8 colony-forming units (CFU)/mL.

All growth experiments in this thesis were carried out in pooled urine. Midstream urine was collected from healthy subjects (paper I: 5 subjects, paper II: 8 subjects, paper III: 10 subjects, paper IV: 4 subjects) pooled, divided into batches (50 ml) and immediately frozen (-20 C) until use.

**Analysis of bacterial growth**

*Turbidity measurements*

The basic concept for turbidity measurements is that bacteria scatter light in proportion to their number. The turbidity or optical density of a suspension of cells is directly related to the cell mass or cell number, after construction and calibration of a standard curve. The method is efficient, reproducible and has been widely used as a rapid screening method for large number of antimicrobial agents [192, 193]. In paper I-II the bacterial strains were grown in Mueller-Hinton broth for 6 hours at 37° C, resulting in 2-5 x 10^8 colony-forming units (CFU)/mL. The strains were diluted to a bacterial density of 10^6 CFU/mL in the pooled urine. After 2 hours of incubation at 37° C in a closed tube (in pooled urine with various amounts of nitrite, with or without addition of vitamin C and at various urinary pH-levels), 40 µL of the bacterial suspension was transferred to micro well plates containing 360 µL sterile filtered control urine (pH 6). After this dilution (1/10) and transfer to the recovery medium (filtered control urine, pH 6), bacterial growth was measured continuously for 10 hours (paper I) and 20 hours (paper II) by vertical photometry (optical density) at a wavelength of 540 nm in a computerized incubator for bacteria (Bioscreen C, Labsystems, Helsinki, Finland). In paper III the bacterial culture was diluted to a bacterial density of 2-5 x 10^6 CFU/mL in flat-bottom micro well plates (96 wells of 300 µL) containing the pooled urine and various amounts of nitrite, with or without addition of vitamin C and at various urinary pH-levels. Note that in paper III, there is no use of a recovery medium. Bacterial growth was measured continuously for 20 hours at 37° C by vertical photometry (optical density) at a wavelength of 540 nm in a computerized incubator (Molecular Devices Spectra Max 340 Sunnyvale, CA). Minimum inhibitory concentration (MIC) was defined as the lowest concentration at which no visible growth had taken place after 20 hours. MICs of nitrite and ascorbic acid were determined. We also determined MIC values for nitrite in combination with a fixed concentration of ascorbic
acid (10 mM). MIC was also determined for nitrofurantoin and trimethoprim using the same urine.

**Viable counts measurements**

Viable count involve spreading (plating out) a sample of a culture on a nutritient agar surface. The sample is diluted in a non-toxic diluent (e.g. phosphate-buffered saline) before plating. Each viable unit grows and forms a colony. Each colony that can be counted is called a CFU and the number of CFU’s is related to the viable number of bacteria in the sample. An excellent correlation has been shown by Dominguez and co-workers between antibacterial effects by turbidity (Bioscreen C) and viable counts methods for ciprofloxacin and standard strains of *E. coli* and *P. aeruginosa* [194].

In **paper III** we used the viable count method for determination of bactericidal activity of various amounts of nitrite, with or without addition of vitamin C and at various urinary pH-levels in the pooled urine. In **paper IV** we used the viable count method for determination of bactericidal activity of a novel *in vitro* concept of delivery of NO via a catheter retention balloon into the surrounding urine. An all-silicone catheter (Argyle, Sherwood Medical, Tullamore, Ireland) was placed in the flask and the retention balloon was filled with 10 ml of saline containing ascorbic acid (10 mM) and sodium nitrite (5 mM). The acidity of the solution was adjusted to pH 2.5 using hydrochloric acid (3 M). Ascorbic acid and nitrite were prepared and mixed immediately before administration. Ascorbic acid solution alone (pH 2.5) was used in control catheters. After filling the retention balloons the catheter was gently pulled outwards and fixed at the neck whereby the flask-opening was sealed off. Then the flasks were turned up-side-down and incubated at 37 degrees for 24 h.

After 20 hours (**paper III**) or 24 hours (**paper IV**) of bacterial growth/incubation (at 37° C, 10 μL urine was diluted in series with PBS (pH 7.3) and transferred to agar plates. In separate experiments (**paper IV**) we also tested shorter exposure times of 5, 30, 60 and 120 min. The agar plates were incubated for 24 hours and then a viable count was performed (counting CFU/ml). MBC (Minimum Bactericidal Concentrations) was defined as the concentration where at least 99.9% (a reduction of > 3 log CFU/ml) of the original inoculate was killed.

In **paper IV** we also compared the effects of nitrite with another NO-releasing compound DETA NONOate, which is considered to be a rather pure NO donor. In these experiments the sodium nitrite was replaced with DETA NONOate (0.05, 0.5 or 5 mM) and all other test parameters were identical. DETA NONOate was prepared immediately before the start of the experiment. We then used the viable count method for determination of bactericidal activity
as described above. To exclude any antibacterial effect of the parent compound (a polyamine), the same experiment was also performed with DETA NONOate solution that had been prepared and stirred for 72 h in open air to release all NO (complete NO release was confirmed with chemiluminescence showing that the NO signal at 24 h was <0.1 % of the initial peak level).

**Statistics**

Experimental data are expressed as mean ± S.E.M (paper I-II) and as mean ± S.D. (paper III–IV). Statistical significance was tested according to Mann-Whitney U test (paper I). For calculation of statistical difference between repeated measurements analysis of variance (ANOVA) was used (paper II).
RESULTS AND COMMENTS

NO formation from nitrite in urine (paper I-II, IV)

Infected urine may contain considerable amounts of nitrite as a result of bacterial nitrate reductase activity. We sought to determine whether NO was generated from acidified nitrite-containing urine. We found that at a basal urinary pH (5.5-6.5), NO formation from both control urine and infected urine was low. In contrast, large amounts of NO were generated from infected urine (containing 8 to 400µM nitrite) when the urine was acidified to pH 4.5-5.0 and from acidified non-infected urine if nitrite (100 µM) was added. Urinary NO formation was strongly pH dependent in the presence of nitrite and was markedly enhanced by addition of ascorbic acid. We also found that increasing concentrations of nitrite (0-500 µM) caused a dose-dependent enhancement of NO formation that again was greatly enhanced by the addition of ascorbic acid (Fig 3 and 4).

![Fig 3](image_url). Urinary NO formation (ppb) in urine from different concentrations of sodium nitrite (0-500 µM) with the addition of ascorbic acid (AA, 10mM) or from AA alone, at varying urinary pH values (4.5-6.0).
Fig 4. NO formation in urine from nitrite (500 µM) at different urinary pH values (4.5-6.0) with and without the addition of ascorbic acid (AA, 10mM).

We also studied whether a nitrite/ascorbic acid-containing solution when placed in a catheter retention balloon would generate NO that could be measured outside the balloons. Indeed, NO did traverse the silicone membrane and measured levels peaked initially at about10 ppm in the headspace gas. If the balloon was filled with nitrite alone (pH 7) or ascorbic acid alone (pH 2.5) no NO signal was noted (NO < 0.001 ppm). These results shows that a tiny uncharged gas such as NO can easily diffuse through the silicon membrane of the retention balloon of a silicone urinary catheter (Argyle®), which is in accordance with previous findings when a similar catheter was used as a sampling device [133, 179, 189].

**Effects of nitrite on bacterial growth**

*Effects of sodium nitrite (paper I-III)*

Urine is an excellent culture medium for bacteria that cause urinary tract infections [32]. Indeed, at a basal urinary pH and even in slightly acidic urine the bacterial growth was very good. In contrast, the growth of *E. coli*, *P. aeruginosa* and *S. saprophyticus* was dose-dependently inhibited by nitrite in acidified urine. The inhibitory effect of nitrite was greater at lower pH. Addition of ascorbic acid (10 mM) further enhanced the inhibition of bacterial growth by nitrite. Increasing concentrations of nitrite (0-500 µM) with a fixed concentration of ascorbic acid (10mM) caused a dose-dependent inhibition of *E.coli* growth which correlated to the NO formation observed (Fig 5). Note that by this method the bacteria are transferred to a recovery medium after 2 hours of nitrite exposure and thus we are studying post-antibiotic effects.
Fig 5. Growth of *E.coli*, after 2 hours of incubation at varying pH values (4.5-6.0) and nitrite concentrations (0-500 µM) with the addition of ascorbic acid (AA, 10 mM) and AA alone. Bacterial growth is expressed as the time needed for the bacteria to grow to A50 (A50 was defined as 50% of the maximal absorbency of the controls).

In the following experiments we studied the effect of continuous exposure to acidified nitrite. Again we found that the growth of *E.coli* ATCC 25922 was inhibited by sodium nitrite in acidified urine and that addition of ascorbic acid (10 mM) further enhanced the inhibition of bacterial growth by sodium nitrite (Fig 6). With the prolonged nitrite exposure the antibacterial effect was more pronounced which also allowed for calculations of MIC and MBC. The MICs of sodium nitrite alone was 160 µM at pH 5 and 1280 µM at pH 5.5. With the addition of ascorbic acid (10 mM) MICs for nitrite were reduced to as low as 20 µM at pH 5 and 160 µM at pH 5.5. Ascorbic acid itself showed poor antibacterial effects with MICs > 40 mM. Bactericidal effects (MBCs) of sodium nitrite + ascorbic acid for *E. coli* ATCC 25922, was 40 µM at pH 5.0 and 320 µM at pH 5.5.

Taken together, in paper I and II the antibacterial effects of acidified nitrite were a result of post antibiotic treatment, since the bacteria were only exposed for two hours before being transferred to recovery medium. In contrast, in paper III, when the bacteria were exposed to acidified nitrite and ascorbic acid during the entire experiments (20h), the effects were much more pronounced and in fact for urinary pH ≤ 5.5 the effects were bactericidal.
Having confirmed that exogenous nitrite had antibacterial effects in urine, we wanted to go on and study if nitrite generated by the bacteria themselves could be turned against them in the form of toxic RNIs. We first studied how much nitrate that would accumulate in urine after ingesting 10 mg/kg of body weight sodium nitrate (corresponds to the amounts found in about 300g of spinach). Basal nitrate levels in urine were about 0.7 mM in fasting individuals and these levels increased about 10-fold already one hour after ingestion of sodium nitrate. Now knowing the amount of nitrate that could easily be achieved in urine, we continued with studying how much nitrite the nitrate reducing bacteria, *E. coli* ATCC 25922, could form from this nitrate. For comparisons we used a *E. coli* mutant that lacked all nitrate reducing enzymes. When *E. coli* was incubated for 20 hours in basal urine (nitrate 0.7 mM) about 2 µM nitrite was generated. When the basal urine had been supplemented with 1 mM sodium nitrate the nitrite formation increased to 370 µM. Maximum formation of nitrite was seen after supplementation with 3 and 10 mM sodium nitrate (2470 and 2400 µM nitrite respectively). When the mutant lacking all three nitrate reductases was incubated for 20 hours with maximum substitution of sodium nitrate (10 mM) the formation of nitrite was negligible. Note that resulting nitrite concentrations after transferring the cultures to the acidic urine in the second part of the experiment were 1/10 of the values above, in other words 37, 247 and 240µM nitrite respectively.
Fig 7 Growth of two strains of *E. coli*. A control strain and a mutant lacking nitrate reductase. The bacteria were first grown at pH 7 with sodium nitrate (10 mM). After 20 hours the cultures were transferred to acidified urine (pH 5.0) and bacterial survival was monitored by viable counts.

Having shown that *E. coli* ATCC 25922 was able to produce significant amounts of nitrite from urinary nitrate, we then continued with studying whether acidification in a second step would result in antibacterial effects. We first incubated *E. coli* in urine at pH 7.0 with 0-10 mM sodium nitrate added, which resulted in accumulation of nitrite in the medium. Subsequent transferring of the nitrite-rich culture to acidic urine resulted in a marked decrease in viable counts. This effect was dose-dependent with more effective killing rates at higher pre-incubation levels of sodium nitrate. At the highest level of nitrate (10 mM), a final reduction of more than 3 logs CFU/ml (bactericidal effect) was observed after transfer to acidic medium. If bacteria were pre-incubated in basal urine without addition of sodium nitrate, no inhibition was noted when the culture was transferred to the acidic urine. This indicates that a normal diet in a patient treated with urinary acidification is probably insufficient to excrete enough nitrate in the urine to achieve antibacterial effects.

In experiments with the mutant lacking nitrate reductases and a control strain both strains grew similarly well at pH 7.0 with 10 mM sodium nitrate added. When transferring the cultures to acidic urine containing ascorbic acid the control strain was effectively killed while the mutant retained full viability (fig 7). Taken together, in paper III we have shown that *E. coli*
coli is effectively killed when it is first allowed to generate nitrite in nitrate rich urine at neutral pH and then transferred to mildly acidified urine. In contrast, a mutant lacking a nitrate reducing capacity, retained full viability by this procedure.

**Delivery of antibacterial nitrogen oxides via a catheter retention balloon (paper IV)**

A model of a catheterized urinary bladder was used to study a novel approach for intravesical delivery of an antibacterial agent. Bacteria were grown in urine outside of the retention balloon thereby simulating a catheterized bladder with infection. When the catheter balloon was filled with ascorbic acid without nitrite (pH 2.5), the bacterial counts of the reference strain had increased from $2.5 \times 10^6$ to $2.7 \times 10^8$ CFU/ml and the clinical isolate strain had increased from $9.0 \times 10^5$ to $2.0 \times 10^8$ CFU/ml after 24 h. In contrast, when using test catheters with ascorbic acid and nitrite (pH 2.5) both tested strains were effectively killed (fig 8).

To further try to pinpoint the compound responsible for antibacterial effects of acidified nitrite we also studied the effects of a pure NO donor. A 24 h exposure to the NO-donor DETA NONOate was completely bacteriostatic at 5 mM while 0.5 and 0.05 mM had no effect on bacterial growth. Thus, in paper IV we describe a novel concept, which allows for intravesical delivery of antibacterial gases into the urinary bladder model via the catheter retention balloon, resulting in bacteriostatic and bactericidal effects in the urine.

![Fig 8. Effects of test (5 mM nitrite + 10 mM ascorbic acid) and control (ascorbic acid alone) catheters on the growth of two strains of E coli in a model of urinary tract infection (pH 6.1).](image-url)
GENERAL DISCUSSION

Acidified nitrite and antibacterial effects in urine

Nitrite has been used in the meat curing process since the 1920s because it produces a desirable bright pink colour and also enhances flavour. In addition, in 1934 it was shown by Tanner and Evans that the growth of the anaerobic bacteria of the genus Clostridium was inhibited by this process [195]. Since 1941, when Tarr showed that bacteria were susceptible to low concentration of nitrite provided the meat was sufficiently acid, the antimicrobial effects of acidified nitrite have been used to prevent from botulism [196]. Later it was shown that addition of ascorbic acid enhanced the inhibition of Clostridium botulinum by nitrite and that antibacterial effects of nitrite in meat involves generation of NO [197, 198]. However, not until 1994 when Benjamin/Lundberg and co-workers demonstrated NO generation in the stomach from acidified nitrite, it was appreciated that this chemical reaction was of importance even in vivo [97, 98].

In this thesis we have shown (paper I) that large amounts of the antimicrobial gas NO is formed in mildly acidified nitrite-containing human urine and this NO formation is greatly enhanced by the addition of ascorbic acid. Furthermore, we have shown (paper I and II) that mildly acidified nitrite-containing human urine has potent antimicrobial activity against three of the most common urinary pathogens and this inhibitory effect is further increased by ascorbic acid. We have also shown (paper III), that E. coli is effectively killed when it is first allowed to generate nitrite and then transferred to mildly acidified urine. Finally we have suggested (in paper IV) a new approach to prevent CAUTI by intravesical delivery of antibacterial compounds generated from nitrite and ascorbic acid via the catheter retention balloon.

What is the mechanism of action?

It is obvious from this thesis that acidified nitrite together with ascorbic acid shows antimicrobial effects in urine. However, the exact chemical nature of the toxic nitrite-derived compound is not known and neither is the exact mechanism by which bacterial killing occurs. Dykhuizen and co-workers, showed bactericidal effects of acidified nitrite in the stomach against several common gut pathogens. They claimed that the antimicrobial effect is due to an additive contribution of reactive intermediates of nitrogen [199]. The chemistry of acidified nitrite is very complex and a variety of reactive nitrogen intermediates (RNIs) and other nitrogen oxides are generated directly or after reactions with other compounds.
Fig 9. Possible microbial cellular targets of reactive nitrogen intermediates (RNI). Figure slightly modified from Fang (1997).

These include NO, N₂O₃, N₂O₄, NO⁺, HNO₂, NO₂, ONOO⁻ and S-nitrosothiols, many of which have antimicrobial activity. Other authors have shown that the sensitivity to different RNIs differs greatly between microbial pathogens. NO has been shown to be microbicidal to *Mycobacterium tuberculosis* and inhibition of NO production has been shown to induce reactivation of the disease, but it has been suggested that NO has low antimicrobial activity against *E. coli* [200, 201]. The question then is if the bactericidal effects of acidified nitrite observed in this thesis are caused by NO. The fact that ascorbic acid potentiates bactericidal effects of nitrite in urine suggests that the production of NO at some stage is important for the antibacterial effects observed. Thus, ascorbic acid greatly increases the production of NO from nitrite at the expense of most other nitrogen oxides mentioned above [100]. Another fact, speaking in favour of NO, is that charged nitrogen oxides eg. NO⁺ or NO₂⁻ would not pass the silicone membrane of the urinary catheter balloon, while NO being a small uncharged molecule does. Indeed we did detect large amounts of NO outside of the membrane and we
did observe bactericidal effects in the urine outside of the catheter balloon. Yet another observation favouring NO as a candidate of the nitrite effect is that the pure NO-donor DETA NONOate, had antibacterial effects in the same system. In addition, the antibacterial effect and the amount of NO produced from acidified nitrite when tested in a Mueller-Hinton medium instead of urine, were clearly decreased (data not shown). The exact mechanism whereby NO and other products from acidified nitrite inhibit bacteria is still not settled but multiple cellular targets are most certainly involved (Fig 9) including DNA, surface proteins and key enzymes in the respiratory chain [167]. The effects of RNIs on DNA could be interaction with DNA repair system as well as direct inactivation of ribonucleotide reductase by NO [202].

**Bacterial defense systems**

Some urinary pathogens e.g. Proteus species have the capacity of producing ammonia from urea thereby increasing urinary pH to levels where nitrite is not converted to toxic nitrogen oxides. Once the higher pH has been established acidification of urine is more difficult [203]. This seems to be an effective mechanism developed by some pathogenic bacteria to protect against acid/nitrite induced damage. *Helicobacter pylori* for example, has the unique ability to thrive within the human stomach and has a urea-specific channel that opens as the pH of the medium drops and cytoplasmatic urease is able to gain access to urea [204]. Obviously, as shown in this thesis, when pH falls to very low levels (4.5 and below) urinary bacterial growth is retarded even in the absence of nitrite indicating also direct effects of acidity itself. At the pH levels more commonly found in mildly acidified urine (5-5.5) however, the effect of pH itself was only minor and required the presence of nitrite/ascorbic acid for more pronounced antibacterial effects (paper II-III).

An other bacterial defense mechanism found in many bacteria, including *E. coli*, is the flavohaemoglobins, an ancient family of proteins that have been shown to effectively bind NO thereby protecting the bacteria from nitrosative stress [186, 205]. Studies in *E coli* have also demonstrated specific antioxidant regulons (*soxRS* regulon), which may protect bacteria against NO when induced [187]. Indeed, *E. coli* with deficient *soxRS* locus, was more sensitive to NO-dependent killing [206]. Another important defense alternative for *E coli*, especially under low oxygen tensions, is the cytochrom c nitrite reductase (NrfA), a bacterial enzyme that may further reduce NO to other less toxic nitrogen metabolites [207]. In addition, many denitrifying bacteria, including *Pseudomonas species*, possess NO reductase enzymes (NOR), which convert NO to nitrous oxide and ultimately to nitrogen [208]. There are data
showing that a mutation of NO reductase gene was lethal to *Pseudomonas stutzeri* during anaerobic growth in the presence of nitrate or nitrite [209]. The pH optimum for the nitrate reductase is high (about pH 8) and it probably operates less effectively at a low pH [210]. Obviously, it is of value for the bacteria not to reduce nitrate to nitrite under acidic conditions, as nitrite will automatically convert to toxic nitrogen oxides thereby leading to self-destruction. Other compounds that can help bacteria to detoxify NO or related compounds include glutathione, homocysteine and superoxide dismutase [167]. Taken together there are many different possible bacterial defense mechanisms against RNIs, but apparently they were all insufficient in our experiments. One can speculate that it is the rapid kinetics of NO release from acidified nitrite described in this thesis, that prevent the bacteria from up regulating these defensive pathways.

**Possible clinical usefulness**

*Treatment of UTI*

Acidification of urine e.g. with vitamin C has been used as a household remedy for urinary tract infections although the mechanism of action remains uncertain. We here speculate that some of these effects are related to generation of bactericidal nitrogen oxides from urinary nitrite. In this process Vitamin C has a dual action; it helps to acidify the urine and in addition its intrinsic reducing capacity greatly enhances nitrite reduction. The reported clinical effects of Vitamin C are however not very consistent and there may be several reasons for this. First, vitamin C itself does not seem to be a very effective urinary acidifier and sometimes it completely fails to reduce urinary pH [57-59]. In addition, in cases where acidification of urine might have been effective, the nitrite concentration in urine may have been to low. As shown here vitamin C itself had poor antibacterial effect and acidity itself did not have a major influence on bacterial growth in urine. However, with the further addition of nitrite bacteria were effectively killed in this medium. Therefore, to refine and improve the clinical effects of vitamin C we probably need to create conditions with sufficient concentrations of nitrite in urine in combination with a consistent and effective acidification of the urine. Is this then possible to achieve in vivo? The urinary ascorbic acid concentration (10mM) used in this thesis is physiologically achievable and in fact, similar to those obtained after ingestion of 1-2 gram of vitamin C daily [211]. Urinary pH normally varies between 5 and 7 and urinary pH levels as low as 4.6 have been reported after treatment with drugs used for urinary acidification [212]. Urinary nitrite in patients with nitrite-positive urinary tract infection.
varies between 10-700 μM [213]. Thus, in some cases these amounts would be sufficient provided that the urine is made acidic and contain enough ascorbic acid. In addition, we could supplement the diet with nitrate to further increase substrate supply to the bacteria in the urine as shown here. Already one hour after ingestion of 10 mg/kg sodium-nitrate (which corresponds to what is found in about 300g spinach), nitrate levels in urine increased to a concentration that is well above what the bacteria’s need to generate enough nitrite.

Now, knowing that sufficient amounts of nitrite, ascorbic acid and low urinary pH is physiologically achievable. How can we accomplish this in the clinical setting? For this concept to work we need first to obtain high nitrite levels in urine. Then, we probably need to lower the urinary pH quite rapidly in order to convert the nitrite into toxic RNI’s. Thus, a sequential two-steps procedure may be preferable. A medicinal product resulting in sufficient urinary nitrate levels would be useful to start with. The dose of nitrate ingested and the time interval between nitrate intake and acidification of the urine will probably be critical. In this thesis (paper III) we used a pre-incubation period of 20 h in nitrate-rich urine. With this long incubation time maximum amounts of nitrite are generated. For practical reasons a shorter incubation time will be necessary in a clinical setting which may result in less nitrite generated. A potential problem is therefore that nitrite levels could be too low to effectively kill the bacteria when the urine is acidified. There are several ways of acidifying the urine, but even if pH can be sufficiently lowered it will likely be important that this process is rather rapid so that bacteria will not have time to up regulate defense mechanisms. In fact, studies with ammonium chloride (50 mg/kg) showed a significant decrease in urinary pH (4.7-5.4) already about 4 hours after a single dose [214]. Various other agents for acidifying the urine have been used, these include mandelic acid, gluconic acid and methionine [52-55].

Another potential problem is that infected urine often has higher pH and therefore acidification is probably more difficult. On the other hand, considering the potent antibacterial effect of acidified nitrite, a decrease in urinary pH below a critical level during a limited period, could be sufficient to inhibit bacterial growth if nitrite levels are sufficiently high. In this thesis we used pooled urine from many different volunteers on a normal western diet. It is likely that the antimicrobial activity of acidified nitrite in urine will vary because of different composition of urine e.g. in relation to diet. Also, urinary osmolality may influence the antibacterial effects of nitrite in acidified urine. One great advantage with the sequential procedure described here compared to many traditional antibiotics is that the bacteria in the gastro-intestinal tract are probably much less, if at all affected. On the other hand, a disadvantage with the concept is that it will only work in bacteria with nitrate reductases.
Bacteria that do not reduce nitrate e.g. *Enterococcus faecalis* will not be sensitive to this treatment similarly to the E. coli nitrate reductase mutant used in paper III. Taken together, we have suggested a new concept for treatment of urinary tract infections caused by nitrate-reducing bacteria. This involves a two-step procedure where bacteria are first fed with nitrate followed some hours later by acidification of the urine. According to this theory the bacteria self-destruct by producing nitrite which is later turned against them in form of toxic RNI’s when urine is acidified. Clearly, controlled *in vivo* studies are needed to evaluate the clinical potential for this antimicrobial regime and to test the timing of the therapeutic interventions (nitrate intake, ascorbic acid intake and urinary acidification).

**Prevention of CAUTI**

CAUTI continues to be large problem in modern medical care and this condition is associated with significant higher costs, morbidity and even higher mortality. Immense amount of work has been done trying to prevent these infections, for example the development of silver alloy catheters, catheters with salicylic acid impregnation, immersion of catheters in antibiotic solution, closed drainage systems and even electrification of catheters [70, 77, 78]. Despite these efforts CAUTI continues to be a major problem in modern health care.

In this thesis (paper IV) we describe a new concept, which allows for direct intravesical delivery of antibacterial gases into the urinary bladder via the catheter retention balloon. It is tempting to speculate that this regime could be useful to treat and to prevent CAUTI’s. Is then this concept applicable in vivo? Again a key question is if the *in vitro* concentrations of nitrite, ascorbic acid and the pH used in this set up are effective *in vivo* also without causing troublesome side-effects. The concentrations of ascorbic acid (10 mM) used here were the same as described above. Natural acidification of salivary nitrite in the stomach, continuously exposes the gastric mucosa to similar amounts of nitrogen oxides. Furthermore, a nitrite concentration of about 3 mM, which is similar to what we used in this set up, is allowed as an ingredient in meat curing. Thus, these antibacterial compounds are expected to have fairly low acute toxicity to host cells in the concentrations used here. Whether a prolonged exposure of the bladder to these RNSs could favour formation of nitrosamines, a group of potentially carcinogenic chemicals, must indeed be further studied. However, the addition of ascorbic acid as one component in the retention balloon likely inhibits the total formation of nitrosamines [215]. The urinary pH in the bladder-model was not influenced by the acidic solution in the intact catheter balloon. Even if the balloon broke by an accident, the small amount (10ml) of solution with pH 2.5 would likely have low acute toxicity to the bladder.
mucosa since probably at least some amount of residual urine is present thereby diluting and buffering the acidic solution.

In paper IV we examined two different strains of bacteria. Although not studied here, it is likely that also other urinary tract pathogens are sensitive to this procedure. Thus, from the results presented in this thesis it is known that RNSs generated from acidified nitrite have anti-bacterial effects against several of the most common urinary pathogens including also *P. aeruginosa* and *S. saprophyticus*.

A potential advantage with this regime is that NO will likely diffuse easily and rapidly into the biofilm generated by the bacteria. This biofilm normally provides a protective environment for the microorganisms and thereby making them less susceptible to antimicrobial agents. Naturally, this assumption has to be tested. Many other questions remain to answer before we can consider using this method in the clinic. For example we need to determine the durability of the antibacterial effect as well as, the exact dosing of nitrite and ascorbic acid and the risk of bacterial resistance. Nevertheless, this simple concept of substituting the content of the retention balloon of a urinary catheter with an active diffusible drug instead of normal saline, is an attractive new alternative to the existing methods used to treat and prevent CAUTI’s. With this novel method one could easily administrate repeated individual doses of the active drug at chosen intervals without disturbing the integrity of the closed drainage system. Considering the fact that CAUTIs are still very common and result in substantially increased morbidity and health care costs, new approaches for their prevention are highly warranted.
CONCLUSIONS

• Large amounts of NO were formed in mildly acidified nitrite-containing infected human urine and in acidified control urine after addition of nitrite. This NO formation was strongly pH dependent and was greatly increased by the addition of ascorbic acid.

• The growth of *E. coli*, *P. aeruginosa* and *S. saprophyticus* was dose-dependently inhibited after two hours of incubation in mildly acidified nitrite-containing urine. This bacteriostatic effect was greater at lower pH and was markedly potentiated by ascorbic acid.

• Prolonged exposure of to physiological achievable concentrations of sodium nitrite plus ascorbic acid resulted in bactericidal effects. In comparison with conventional antibiotics tested in this same model nitrite seemed to be at least equally potent.

• A two-step procedure where *E. coli* was first fed with nitrate followed later by acidification of the urine, resulted in effective bacterial killing. This killing was dependent on the nitrite generated by the bacteria themselves.

• A novel concept for intravesical delivery of NO via a catheter retention balloon was tested *in vitro*. The urinary pathogen *E. coli* was effectively killed by this procedure.
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