Molecular Human Reproduction, Vol.16, No.12 pp. 907–915, 2010
Advanced Access publication on July 20, 2010 doi:10.1093/molehr/gaq063

MHR Basic science of reproductive medicine

NEW RESEARCH HORIZON *Review*

Nitric oxide synthases and tubal ectopic pregnancies induced by *Chlamydia* infection: basic and clinical insights

Ruijin Shao[1,*], Sean X. Zhang[2], Birgitta Weijdegård[1], Shien Zou[3], Emil Egecioglu[1], Anders Norström[4], Mats Brännström[4], and Håkan Billig[1]

[1]Department of Physiology/Endocrinology, Institute of Neuroscience and Physiology, The Sahlgrenska Academy at University of Gothenburg, Gothenburg, Sweden [2]Public Health Laboratories, Ontario Agency for Health Protection and Promotion, Department of Lab Medicine and Pathobiology, University of Toronto, Toronto, Canada [3]Obstetrics and Gynaecology Hospital of Fudan University, Shanghai, China [4]Department of Obstetrics and Gynecology, The Sahlgrenska Academy at University of Gothenburg, Gothenburg, Sweden

*Correspondence address. Tel: +46-31-7863408; Fax: +46-31-7863512; E-mail: ruijin.shao@fysiologi.gu.se

Submitted on May 12, 2010; resubmitted on June 22, 2010; accepted on July 11, 2010

ABSTRACT: Human ectopic pregnancy (EP) remains a common cause of pregnancy-related first trimester death. Nitric oxide (NO) is synthesized from L-arginine by three NO synthases (NOS) in different tissues, including the Fallopian tube. Studies of knockout mouse models have improved our understanding of the function of NOS isoforms in reproduction, but their roles and specific mechanisms in infection-induced tubal dysfunction have not been fully elucidated. Here, we provide an overview of the expression, regulation and possible function of NOS isoforms in the Fallopian tube, highlighting the effects of infection-induced changes in the tubal cellular microenvironment (imbalance of NO production) on tubal dysfunction and the potential involvement of NOS isoforms in tubal EP after *Chlamydia trachomatis* genital infection. The non-equivalent regulation of tubal NOS isoforms during the menstrual cycle suggests that endogenous ovarian steroid hormones regulate NOS in an isoform-specific manner. The current literature suggests that infection with *C. trachomatis* induces an inflammatory response that eventually leads to tubal epithelial destruction and functional impairment, caused by a high NO output mediated by inducible NOS (iNOS). Therefore, tissue-specific therapeutic approaches to suppress iNOS expression may help to prevent ectopic implantation in patients with prior *C. trachomatis* infection of the Fallopian tube.

Key words: nitric oxide / nitric oxide synthase isoforms / *Chlamydia trachomatis* / tubal ectopic pregnancy

Introduction

Ectopic implantation in the first trimester of pregnancy is a common cause of human maternal morbidity and mortality (Corpa, 2006). It accounts for 1.5–2% of all pregnancies in the western world (Barnhart, 2009), and ~97% of ectopic pregnancies (EPs) are in the Fallopian tube (Mukul and Teal, 2007). Tubal EP is a growing problem in developing countries (Farquhar, 2005; Barnhart, 2009). The mammalian Fallopian tube is a dynamic, steroid-responsive tissue (Jansen, 1984) composed of heterogeneous cell types: ciliated and secretory epithelial cells as well as smooth muscle cells, all of which appear to be specialized to perform different functions (Shao *et al.*, 2007a, b).

Tubal abnormalities and dysfunction (e.g. altered abnormal ciliary activity or contractility) are thought to lead to tubal EP, but the precise aetiology of its initiation and development is unknown (Shao, 2010). A major risk factor is previous pelvic inflammatory disease, which is quite common in women with tubal EP (Brunham and Rey-Ladino, 2005). According to the World Health Organization (WHO) (http://www.who.int/mediacentre/factsheets/fs110/en/index.html), 10–40% of women with untreated *Chlamydia trachomatis* infection worldwide develop symptomatic pelvic inflammatory disease associated with complications. Women infected with *C. trachomatis* in the genital tract are highly susceptible to tubal EP (Chow *et al.*, 1990). An untreated *C. trachomatis* infection is likely to injure tissue, cause permanent scarring and obstruct the Fallopian tubes at multiple sites (Paavonen and Lehtinen, 1996; Farquhar, 2005) as a consequence of the host inflammatory immune response (Entrican *et al.*, 2004), ultimately inhibiting or disrupting gamete transport. However, the

pathological mechanisms that induce tubal EP are unknown. Since 70–90% of women with *C. trachomatis* infection do not have symptoms (Peipert, 2003), the WHO has recommended screening strategies to decrease the *C. trachomatis* spread and thereby indirectly decrease the tubal EP rate. In many industrialized countries and China, the prevalence of *C. trachomatis* infection is 2–5% of the population (Fenton *et al.*, 2001; Parish *et al.*, 2003; Miller *et al.*, 2004).

How *C. trachomatis* infection induces Fallopian tube damage is poorly understood at the molecular level, and a better understanding of the biological basis of infection-induced tubal EP is needed to aid its prevention and treatment. Nitric oxide (NO) is an endogenous short-lived signalling molecule involved not only in multiple physiological processes but also in diseases in different organs/tissues (Moncada and Higgs, 1991; Moncada *et al.*, 1991), including the Fallopian tube (Rosselli *et al.*, 1998). The role of NO in regulating gene expression, enzyme activity, and transcription factor activation has been extensively demonstrated in several experimental models other than Fallopian tube (Moncada and Higgs, 1991; Moncada *et al.*, 1991). NO is a double-edged sword. At low levels, it alters intracellular Ca^{2+} levels and activates uterine smooth muscle relaxation (Telfer *et al.*, 1995). But at high levels, it causes infection-induced immune reaction and inflammation-induced tissue lesions (Moncada and Higgs, 1991; Moncada *et al.*, 1991; Rosselli *et al.*, 1998).

The purpose of this review is to highlight the potential role of nitric oxide synthases (NOS) in Fallopian tube physiology and to discuss how *C. trachomatis* infection changes the tubal microenvironment by regulating NO production by different NOS isoforms, leading to tubal EP. The current review also suggests future research directions.

NO signalling and NOS isoforms in the Fallopian tube

The diverse biological actions of NO as an intra- and inter-cellular messenger are mediated by binding to metal-containing centres of enzymes and by stimulation of the soluble NO-sensitive form of guanylate cyclase (sGC). This event leads to increases in cyclic guanosine monophosphate (cGMP) levels and activation of cGMP-dependent protein kinase (PKG) or cGMP-independent intracellular effects in target cells (Moncada *et al.*, 1991). In the clinic, treatment of vagina with sildenafil citrate, which inhibits cGMP breakdown, increases endometrial thickness (Jerzak *et al.*, 2008), which may increase the chance of successful pregnancy at spontaneous conception or IVF in recurrent miscarriage patients. As far as caution on cardiovascular effects of sildenafil in humans is concerned, there is a need for studies to evaluate toxicities associated with sildenafil treatment, in particular the human risk assessment, before clinical use of sildenafil in women with EP.

The precise role of the NO-cGMP signalling pathway in the Fallopian tube is unclear. Rat Fallopian tube cells express a number of NO-cGMP signalling components, such as sGCα1, sGCβ1, PKG1α and PKG1α (Zhan *et al.*, 2003). The mammalian Fallopian tube is a site of endogenous production of NO (Rosselli *et al.*, 1998), which is quickly metabolized to its stable end product, nitrate (Wennmalm *et al.*, 1993). One clinical study showed a significant increase in circulating nitrite/nitrate levels after the luteinizing hormone surge in the menstrual cycle (Ekerhovd *et al.*, 2001). NO signalling is largely regulated at the level of NO biosynthesis, and the primary source of NO production *in vivo* is oxidation of L-arginine by NOS enzymes (Alderton *et al.*, 2001).

Three distinct tissue-localized NOS isoforms with predicted molecular weights of 130–160 kDa have been identified: calcium-independent inducible NOS (iNOS), calcium/calmodulin-dependent neuronal NOS (nNOS) and endothelial NOS (eNOS) (Moncada and Higgs, 1991; Moncada *et al.*, 1991; Rosselli *et al.*, 1998). NOS isoforms are encoded by three distinct genes, exhibit 50–60% amino acid sequence similarity, and fulfil different functions (Nathan and Xie, 1994; Alderton *et al.*, 2001). nNOS and eNOS are constitutively expressed. iNOS is expressed in response to stimuli (e.g. immune and inflammatory responses) and increased iNOS expression leads to massive NO production that has both cytotoxic and cytoprotective effects in a wide range of tissues and cells (Mashimo and Goyal, 1999). Moreover, activation of nNOS or eNOS results in transient NO release within seconds or minutes, whereas iNOS generates cellular production of NO that continues for hours or even days (Moncada and Higgs, 1991; Moncada *et al.*, 1991).

Studies of the roles of NOS isoforms in NO production and activity in knockout mouse models indicate that NO activity is maintained at appropriate levels by a potent NOS network of mechanisms (Tranguch and Huet-Hudson, 2003). All nucleated mammalian cells possess at least one of the three conserved NOS isoforms; however, their expressions vary in different tissues and cells *in vivo* (Bryan *et al.*, 2009) and may reflect their biological roles. The three NOS isoforms are expressed differentially in different cell types of the Fallopian tube in humans (Rosselli *et al.*, 1996; Ekerhovd *et al.*, 1997; Tschugguel *et al.*, 1998; Ekerhovd *et al.*, 1999; Machado-Oliveira *et al.*, 2008; Al-Azemi *et al.*, 2010; Refaat *et al.*, 2009), rats (Bryant *et al.*, 1995; Chatterjee *et al.*, 1996; Zhan *et al.*, 2003; Kilic *et al.*, 2008), cattle (Majewski *et al.*, 1995; Rosselli *et al.*, 1996; Lapointe *et al.*, 2006; Ulbrich *et al.*, 2006) and pigs (Majewski *et al.*, 1995; Andronowska *et al.*, 1999; Gawronska *et al.*, 2000) (Table I). However, only tubal epithelial cells express all NOS isoforms, suggesting that this cell type is a potentially important determinant of the effects of NO in the Fallopian tube (Table I). Tubal iNOS and eNOS are the predominant isoforms in both humans and animals. As discussed below, NOS appears to have physiologically important, isoform-specific functions in the Fallopian tube.

Regulation of NOS isoforms in the Fallopian tube

Although gene expression of NOS isoforms depends on various factors, hormonal regulation is quite important in the Fallopian tube. Expression of NOS isoforms and nicotinamide adenine dinucleotide phosphate-diaphorase (a histochemical, non-specific marker for all NOS isoform activities) varies across the normal reproductive cycle in human, rat, cattle and porcine Fallopian tubes (Bryant *et al.*, 1995; Chatterjee *et al.*, 1996; Gawronska *et al.*, 2000; Lapointe *et al.*, 2006; Ulbrich *et al.*, 2006; Al-Azemi *et al.*, 2010). These results raise the possibility that changes in NOS-derived NO production during fluctuating physiological conditions may be involved in regulation of important Fallopian tube functions, such as ciliary activity or contractility.

Table I Summary of reported cell type-specific differences of NOS isoforms in the Fallopian tube.

Species	nNOS	iNOS	eNOS	References
Human	Epithelial cells (*ampulla*)	Epithelial cells[a] Smooth muscle cells (*AIJ, isthmus*) Endothelial cells (*AIJ, isthmus*)	Epithelial cells[a] Endothelial cells (*AIJ, isthmus*)	Al-Azemi *et al.* (2010) Ekerhovd *et al.* (1997, 1999) Machado-Oliveira *et al.* (2008) Refaat *et al.* (2009) Rosselli *et al.* (1996) Tschugguel *et al.* (1998)
Rat	Epithelial cells	Epithelial cells	Epithelial cells[a] Smooth muscle cells Endothelial cells	Bryant *et al.* (1995) Chatterjee *et al.* (1996) Kilic *et al.* (2008) Zhan *et al.* (2003)
Bovine	Epithelial cells[a] Smooth muscle cells[a]	Epithelial cells[a] Smooth muscle cells[a]	Epithelial cells[a] Smooth muscle cells[a] Endothelial cell	Lapointe *et al.* (2006) Majewski *et al.* (1995) Rosselli *et al.* (1996) Ulbrich *et al.* (2006)
Porcine	Nerve fibre (*isthmus*)	Epithelial cells (*ampulla, isthmus*) Endothelial cells (*IF, ampulla, isthmus*)	Epithelial cells (*ampulla, isthmus*) Endothelial cells (*ampulla, isthmus*)	Andronowska *et al.* (1999) Gawronska *et al.* (2000) Majewski *et al.* (1995)

AIJ, ampullary isthmic junction; IF, infundibulum; nNOS, neuronal NOS; iNOS, inducible NOS; eNOS, endothelial NOS.
[a]Demonstrated in the ampulla, AIJ and isthmus.

Given the complex interactions between fluctuating and declining levels of the major ovarian-derived steroid hormones (17β-estradiol and progesterone) during the reproductive cycle, local expression of NOS isoforms may be regulated by 17β-estradiol and/or progesterone. Indeed, NOS expression and activity in the Fallopian tube are related to the ratio of 17β-estradiol and progesterone (Rosselli *et al.*, 1998). Several clinical and experimental observations suggest a regulatory role for estrogens in the expression of NOS isoforms and endogenous NO production. First, during follicular development, increases in estrogen levels are associated with circulating nitrate levels in reproductive-age women (Rosselli *et al.*, 1994a; Ekerhovd *et al.*, 2001), and circulating estrogen levels correlate with circulating/follicular nitrite/nitrate levels during ovarian stimulation in IVF treatment (Rosselli *et al.*, 1994a; Anteby *et al.*, 1996). Second, in postmenopausal women, administration of 17β-estradiol significantly increases circulating nitrite/nitrate levels (Ramsay *et al.*, 1995; Cicinelli *et al.*, 1997). Third, in non-pregnant guinea pigs, the experimental manipulation of endogenous estrogen levels induces expression of nNOS and eNOS mRNA and NOS activity in the heart, kidney and skeletal muscle (Weiner *et al.*, 1994). Fourth, treatment with 17β-estradiol selectively up-regulates iNOS mRNA and protein expression in the bovine Fallopian tube during the periovulatory period (Lapointe *et al.*, 2006).

Both genomic and non-genomic effects of estrogen may contribute to the regulation of NOS isoform expression, but the role of tubal estrogen receptors in this regulation is poorly characterized. On the other hand, although the effect of pregnancy on NOS isoform expression does not support progesterone-mediated induction of nNOS and eNOS expression (Weiner *et al.*, 1994), progesterone can up-regulate tissue-dependent iNOS mRNA and protein expression in pregnant rodents *in vivo* (Maul *et al.*, 2003). Moreover, acute *in vitro* treatment of bovine oviduct epithelial cells with progesterone rapidly increases iNOS and eNOS mRNA expression (Ulbrich *et al.*, 2006). Thus, multiple steroid-related mechanisms may contribute to the overall regulation of NOS isoform expression and NO production in the Fallopian tube *in vivo*.

Probing the biological functions of NOS isoforms

In mammals, NOS activity is highly controlled because its product is a potent regulator of many physiological processes (Moncada and Higgs, 1991; Moncada *et al.*, 1991; Rosselli *et al.*, 1998). Tubal transport of the embryo is a complex and highly regulated process, which spans several days. One line of evidence suggests that NO helps regulate tubal contractility and relaxation under physiological conditions (Rosselli *et al.*, 1996, 1998). *In vitro* treatment with two NO donors (nitroglycerin and spermine NONOate) and 8-bromo cGMP decreased the contractility of the human Fallopian tube (Ekerhovd *et al.*, 1999). In line with these findings, the opposite effect was observed in human and bovine Fallopian tubes treated with *N*-nitro-*L*-arginine methyl ester (L-NAME), an inhibitor of NO synthesis (Rosselli *et al.*, 1994b; Ekerhovd *et al.*, 1997). Moreover, local administration of spermine NONOate abolished the effects of L-NAME, reducing the transport speed of ovulated oocyte-cumulus complexes in rat Fallopian tubes (Perez Martinez *et al.*, 2000).

Use of pharmacological blockers of NO synthesis does not distinguish the competitive inhibitory properties of the three NOS isoforms or the diversity of specific NOS-derived NO production that

may result in cell-specific responses in the Fallopian tube under physiological conditions. Interestingly, the transiently increased iNOS expression (Shao *et al.*, unpublished data) corroborates reported increases in circulating NO production (Ekerhovd *et al.*, 2001) and frequency of contraction (Ekerhovd *et al.*, 1999). Taken together, these results imply that iNOS, rather than nNOS and eNOS, is the major contributing NOS for local NO production, which might be needed to suppress tubal transport in the specific period. Since Fallopian tubal transport is under precise temporal control by specific hormonal and signalling pathways (Jansen, 1984), research is needed to determine whether tubal transport requires iNOS, and if steroid hormonal regulation of tubal transport are independent of NOS-derived NO network.

Key insights into the physiological significance of each NOS isoform have come from gene knockout studies in mice. For example, central but not ovarian nNOS activity is crucial for ovulation in nNOS-deficient female mice (Gyurko *et al.*, 2002), whereas studies of iNOS-deficient female mice have shown that iNOS contributes to the maintenance of decidual cellular integrity for implantation and embryo survival (Burnett *et al.*, 2002). In contrast, eNOS-deficient female mice have a reduced ovulatory capacity, a prolonged oestrous cycle, impaired early embryonic viability and decreases in implantation during pregnancy (Tempfer *et al.*, 2000; Hefler and Gregg, 2001; Hefler *et al.*, 2001; Pallares *et al.*, 2008). However, typically the deletions remove simultaneously more than one NOS gene, hindering the establishment of genotype-phenotype correlations. In fact, nNOS-iNOS-, nNOS-eNOS- and iNOS-eNOS-double homozygous mutant female mice show insufficient alterations of oestrous cyclicity and fertility (Tranguch and Huet-Hudson, 2003), suggesting that, when one NOS isoform is deleted, compensatory responses of other NOS isoforms maintain overall NOS activity in female reproductive events *in vivo*.

However, it is not clear how deletion of NOS isoforms, individually or in combination, affects Fallopian tube function. Furthermore, isoform-specific NOS-deficient mouse models show diverse immune responses (Liu and Huang, 2008). For example, nNOS-deficient mice are resistant to neuronal inflammatory injury, while eNOS-deficient mice show increased susceptibility to neuronal and vascular inflammatory injury. More interestingly, iNOS-deficient mice show decreased neuronal inflammatory injury and increased susceptibility to bacterial and viral pathogens (MacMicking *et al.*, 1995; Wei *et al.*, 1995). These provocative findings shed light on the mechanisms that regulate the dynamic expression of different NOS isoforms under pathophysiological conditions.

C. trachomatis infection induces an inflammatory microenvironment in the Fallopian tube

Chlamydia are unique obligate intracellular pathogens that can exist in two forms: the elementary body (EB) and the reticulate body (RB). EBs, the transmissible form of the organism capable of extracellular survival, attach to susceptible host cells to initiate infection. After entering the host cell, the EB induces endocytosis into a host-derived, membrane-bound vacuole designated as an inclusion. Inside the inclusion, the EB rapidly differentiates into the non-infective, intracellular and replicative RB (Muschiol *et al.*, 2006). *C. trachomatis* has a unique biphasic developmental cycle, alternating between metabolically inert EBs and metabolically active RBs (Moulder, 1991). RBs divide exponentially by binary fission before condensing back into EBs, which are released after lysis of infected cells, allowing for further propagation of the infection (Moulder, 1991).

Chlamydia shares morphological and structural properties of gram-negative bacteria (Bush and Everett, 2001) and is presented in two ways: cellular lysis and/or reminiscent exocytosis in host cells (Hybiske and Stephens, 2007). Human *Chlamydia* genital infection causes a wide range of pathologies. In women undergoing microtuboplasty for surgical correction of damaged Fallopian tubes, tubal biopsies contain *Chlamydia* (Henry-Suchet *et al.*, 1981; Shepard and Jones, 1989), and women with tubal factor infertility often have *C. trachomatis* DNA or its antigen in the Fallopian tubes (Campbell *et al.*, 1993). In human Fallopian tube organ culture, *C. trachomatis* replicates within both ciliated and non-ciliated epithelial cells (Cooper *et al.*, 1990). Epithelial cells, but not muscle cells, in the Fallopian tube are the primary targets for *Chlamydia* infection (Henry-Suchet *et al.*, 1996; Rasmussen *et al.*, 1997; Igietseme *et al.*, 1998) and may serve as the first line of defence against such infection by regulating both innate and adaptive immune responses (Wira *et al.*, 2005a, b). The infected epithelial cells trigger local innate immune responses for the recognition of invading pathogens through pattern recognition receptors such as Toll-like receptors (Hart *et al.*, 2009). A polymorphonuclear leukocyte response is initiated; these cells produce interleukin (IL) 8 and other proinflammatory cytokines (Rasmussen *et al.*, 1997) and stimulate the initial neutrophilic response, which is followed by tissue infiltration of macrophages, lymphocyte, plasma cells and eosinophils (Kuo, 1988).

The important feature of innate immunity is cytokine production, and regulation of inflammation-induced cytokine expression in the Fallopian tube has been demonstrated by several groups. Hess *et al.* (2009) demonstrated that epithelial specific-IL-1β expression in the Fallopian tubes is higher in women with EP than in those with a normal menstrual cycle; however, the number of women with EP who had a *Chlamydia* infection was not determined. Hvid *et al.* (2007) reported that *C. trachomatis* infection induces epithelial IL-1β and IL-1 receptor Type I synthesis, which could be blocked by treatment with IL-1 receptor antagonist in human Fallopian tubes. This group, as well as Prantner *et al.* (2009), reported that either inhibition of IL-1β signalling or deletion of IL-1β can prevent pathology in human Fallopian tubes after infection with *C. trachomatis in vitro* or in mouse Fallopian tubes after infection with *Chlamydia muridarum*, a murine strain of *Chlamydia in vivo*. Finally, potential sites for IL-1β synthesis and secretion are present in macrophages in mouse Fallopian tube (Prantner *et al.*, 2009). Infections favour macrophage recruitment and stimulate cytokine synthesis and secretion (La Verda and Byrne, 1994).

Another proinflammatory cytokine, tumour necrosis factor-α (TNF-α), an inflammatory central modulator, is also increased in response to *C. trachomatis* infection in human Fallopian tubes *in vivo* and *in vitro* (Toth *et al.*, 1992; Ault *et al.*, 1996). Moreover, TNF-α has an apoptotic role in mouse Fallopian tubes infected with *C. trachomatis in vivo* (Perfettini *et al.*, 2000). Thus, epithelial cells

and macrophages appear to co-operate to regulate IL-1 and TNF-α expression and activation, which may be pathogenic in Fallopian tubes infected with *Chlamydia*.

The other major proinflammatory cytokines and chemokines, including IL-2, IL-6, IL-8, IL-10, interferon gamma (IFN-γ) and granulocyte/macrophage-colony stimulating factor (GM-CSF), are likely involved in the propagation and progression of inflammation in the Fallopian tubes after *Chlamydia* infection (Rasmussen *et al.*, 1997; Van Voorhis *et al.*, 1997; Entrican *et al.*, 2004; Strandell *et al.*, 2004; Hvid *et al.*, 2007). Analysis of patients according to prior exposure to genital *C. trachomatis* infection has revealed that the TNFα 308A allele and the IL-10 1082A allele are risk factors for Fallopian tube damage in infected women (Ohman *et al.*, 2009), further illustrating the possibility that cytokines and chemokines mediate tubal damage induced by *C. trachomatis* infection.

Inflammation is a physiological process used by the organism in response to infection, tissue damage or cellular irritants (Stutz *et al.*, 2009). Inflammation progresses through different stages. Under normal circumstance the dynamics of acute inflammation are tightly regulated and self-limited. However, the onset of an unfavourable chronic inflammation by abnormal persistence of a stimulus may induce tissue damage and impair tissue function. The mechanisms responsible for Fallopian tube occlusion are not fully understood, but presumably they involve a combination of chronic inflammation and scarring induced by infections.

Inappropriate regulation of iNOS-derived NO production facilitates tubal EP

Little is known about the molecular pathogenesis of tubal EP, and in particular, infection-induced factors (Shao, 2010). Epithelial and smooth muscle cells are key players in tubal transport (Jansen, 1984; Shao, 2010). Indeed, it has been suggested that initial alterations in tubal cell mobility, which result in abnormal ciliary activity, epithelial secretion and contractility, may be responsible for EP. Epithelial cells and macrophages are the targets of *C. trachomatis* infection in the Fallopian tube (Henry-Suchet *et al.*, 1996; Rasmussen *et al.*, 1997; Igietseme *et al.*, 1998), highlighting the major cellular sites for the disease. This link has been strengthened by studies demonstrating that *C. trachomatis* infection directly induces loss of microvilli and cilia in epithelial cells, tubal oedema and extensive disruption of the mucosal surface in human Fallopian tubes *in vitro* (Cooper *et al.*, 1990) and in mice *in vivo* (Tuffrey *et al.*, 1990). Notably, in mice with *C. muridarum* infection, the transport of ovulated oocyte-cumulus complexes is inhibited by loss of spontaneous contractile activity and up-regulation of iNOS protein expression in Fallopian tubes *in vivo* (Dixon *et al.*, 2009).

iNOS is important because its expression and activity are enhanced in inflammatory immune processes associated with tissue damage (Nathan and Xie, 1994; Mashimo and Goyal, 1999). Although the mechanisms linking *C. trachomatis* infection, iNOS and tubal tissue/cell destruction are not completely understood, there is evidence that chronic, intense inflammation contributes to tissue remodelling and scarring in the Fallopian tube (Stephens, 2003; Entrican *et al.*, 2004), increasing the risk for tubal EP. *C. trachomatis* infection stimulates the expression of a number of proinflammatory cytokines that reflect the induction of inflammatory processes, thereby resulting in Fallopian tube pathology and female infertility (Rasmussen *et al.*, 1997; Stephens, 2003; Entrican *et al.*, 2004). It is tempting to hypothesize that inflammation is a mechanism that links *C. trachomatis* infection to tubal EP.

Cytokines produced in the Fallopian tube in response to *Chlamydia* infection may regulate iNOS and subsequently generate NO. In fact, NO is considered to be part of the innate immune response because it is a bactericidal agent that is lethal to intracellular pathogens such as *C. trachomatis*. NO production is a feature of innate immune cells, such as macrophages and dendritic cells, but NO is also produced in epithelial cells (Bogdan, 2001). NO production in response to *C. trachomatis* infection in the Fallopian tube can be both protective and pathogenic. NO can participate in microbe-triggered immune responses within the Fallopian tube; however, their pathological expression may lead to tubal damage and EP (Refaat *et al.*, 2009). The ability of *C. trachomatis* to target both immune and non-immune cell responses may explain its tendency to establish chronic, latent infection and cause tissue damage (Stephens, 2003).

In macrophages and other cell types, iNOS expression is transcriptionally induced by stimulation with cytokines or exposure to microbial products (Xie *et al.*, 1992; Nathan and Xie, 1994). Since both epithelial and smooth muscle cells in the human Fallopian tube express iNOS (Table I), it is assumed that the tubal cells targeted for cytokine action by NO production can be the cytokine-producing cell itself (e.g. epithelial cells) or, through direct release, neighbouring cells such as macrophages. iNOS-synthesized NO is equally important in human immune regulation and disease (Nathan and Xie, 1994) but in large amounts is thought to disrupt cellular signalling cascades, resulting in anti-inflammatory or immunosuppressive effects. Interestingly, iNOS-deficient mice show greater inflammation, more dissemination of *C. trachomatis* infection (Igietseme *et al.*, 1998; Ramsey *et al.*, 2001) and impaired clearance (Perry *et al.*, 1998), demonstrating that iNOS may contribute to infection-induced immunopathology, rather than the host immunity against *Chlamydia*.

It has been suggested that NO is important for implantation and early pregnancy and activation of inflammatory response in mice (Rosselli *et al.*, 1998; Purcell *et al.*, 1999). If optimally balanced, NOS-derived NO accumulation is crucial for implantation in rodents (Barroso *et al.*, 1998; Ota *et al.*, 1999; Purcell *et al.*, 1999; Novaro *et al.*, 2002). In the Fallopian tube, iNOS is expressed in multiple cells, where it regulates NO production through autocrine or paracrine effects. iNOS participates in the growth associated with the decidual response in the rat uterus (Spencer *et al.*, 1998). The effects of NO are highly dependent on the local microenvironment, tissue/cell type and redox state (Rosselli *et al.*, 1998). Although the cause of tubal EP seems to be multifactorial, NO and iNOS are likely to contribute to tubal EP because tubal iNOS mRNA and protein expression is increased in the ampullary region, a site of fertilization, of women with tubal EP (Al-Azemi *et al.*, 2010).

The reason why high levels of NO favour permanent tubal damage, resulting in tubal EP may be that large and sustained fluxes of NO can induce cytotoxicity and transcriptional disturbance in various tissues/cells (Moncada and Higgs, 1991; Moncada *et al.*, 1991). Thus, it is most likely that cytokine/chemokine-triggered iNOS-derived NO production in women infected with *Chlamydia* causes Fallopian tube scarring and blocks tubal transport by inhibiting ciliary beats and smooth muscle contractions, ultimately resulting in EP (Fig. 1). If this

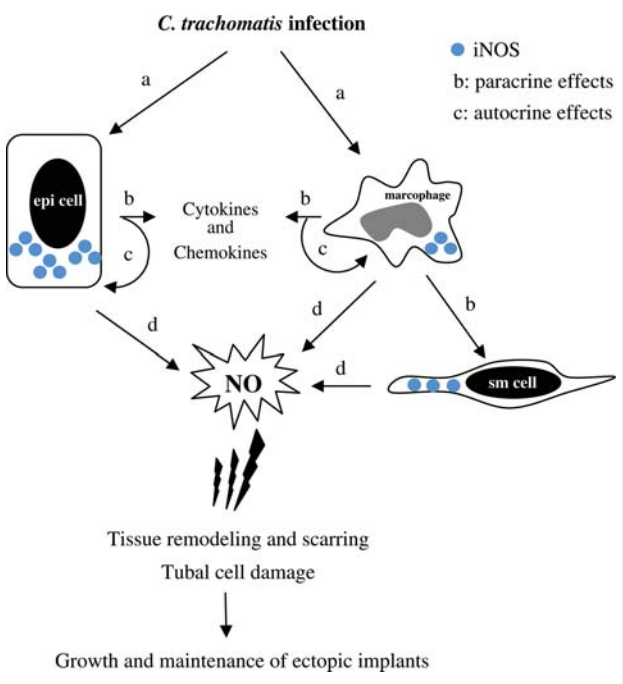

Figure 1 A simplified view of inflammation-induced iNOS-derived NO production that accounts for tubal EP in humans infected with *C. trachomatis*. Fallopian tubes infected with *C. trachomatis* (a) results in the synthesis of cytokines and chemokines in epithelial cells (epi) and macrophages (b). As secretion, in turn, autocrine and paracrine up-regulation of iNOS expression (b, c) induces an inappropriate NO production (d). The excessive iNOS-derived NO production promotes tissue remodelling and scarring, leading to tubal cell damage and destruction that may contribute to the development of infection-induced tubal EP. Pharmacological tools to modulate this process might be of therapeutic relevance. sm cell, smooth muscle cell.

is true, it may provide a therapeutic target for suppression of tubal inflammation by manipulation of cytokine and chemokine production to inhibit the NO pathways.

Further studies are warranted to examine the molecular interaction between pathophysiological inflammatory responses and iNOS-derived NO regulation after acute and chronic infection with *Chlamydia*. For example, evaluating cytokine and chemokine production and secretion under physiological conditions (e.g. normal uterine implantation) or between *Chlamydia* infection and the onset of tubal damage in iNOS-deficient mice *in vivo* and Fallopian tubal tissue culture *in vitro* will shed light on the significance of NO signalling in the development of tubal EP induced by *Chlamydia* infection. Even though NO protects against infection (Nathan and Xie, 1994; Bogdan, 2001), iNOS-derived NO overproduction might have deleterious consequences for the implanted embryo if there is re-infection and persistence of *Chlamydia*. Although no data provide specific insight into how implantation occurs in damaged Fallopian tubes after *Chlamydia* infection, the tubal microenvironment may facilitate the survival of the implanted embryo (Mastroianni, 1999; Lyons *et al.*, 2006). In view of the process by which *C. trachomatis* infection induces tubal EP is being probably more complicated than a simple step regulation, there must be research priority to discern how *C. trachomatis* infection promotes tubal scarring and/or fibrosis so that appropriate prevention and treatment strategies may be adopted.

Concluding remarks and perspective

Tubal EP induced by *C. trachomatis* infection is still a clinical problem and the diagnosis remains a challenge. As the complexity of Fallopian tubal transport under physiological conditions is being revealed (Jansen, 1984; Shao, 2010), so is the complexity of the mechanisms responsible for NOS regulation and inflammation-related EP after *Chlamydia* infection. To our knowledge, obligatory participation of the different NOS isoforms in the Fallopian tube in a physiological context has not been defined.

In this brief review, we have attempted to present NOS isoforms, in particular iNOS, as a component of the NO signalling pathway that is invoked when tubal cells undergo chronic inflammatory processes. Moreover, cytokines and chemokines produced and released in response to *Chlamydia* infection may cause progressive damage and functional decline of the Fallopian tube, resulting in tubal EP. Although estrogen can exhibit both anti-inflammatory and immunomodulatory properties in a tissue/cell-specific manner (Straub, 2007), there is little evidence for the importance of steroid hormone interactions with cytokines after *Chlamydia* infection. However, in contrast to progesterone (Tuffrey *et al.*, 1990), high levels of endogenous estrogen or treatment with 17β-estradiol increases resistance to *C. trachomatis* infection in mice (Brunham and Rey-Ladino, 2005) and the host estrogen receptor complex influence *C. trachomatis* attachment in human endometrial epithelial cells (Davis *et al.*, 2002). Because estrogen acts through activation of estrogen receptors in the Fallopian tube (Shao *et al.*, 2007a, b), targeted deletion of estrogen receptor α/β lowers iNOS expression in female mouse vascular smooth muscle cells (Liang *et al.*, 2003). Since estrogen up-regulates iNOS expression in the Fallopian tube, studies of the molecular mechanisms by which estrogen modulates cell-specific pro-inflammatory cytokine secretion and NO production are increasing our understanding the role of estrogen in Fallopian tubal pathology after *Chlamydia* infection. Such knowledge might facilitate the development of therapeutic approaches to mitigate tubal dysfunction.

Funding

This review was supported by the Swedish Medical Research Council (Grant 5859), the Sahlgrenska Academy Research Council, the Mary von Sydow Foundation and Göteborgs Läkaresällskap. Funding to pay the Open Access Charge was provided by the Swedish Research Council.

References

Al-Azemi M, Refaat B, Amer S, Ola B, Chapman N, Ledger W. The expression of inducible nitric oxide synthase in the human fallopian tube during the menstrual cycle and in ectopic pregnancy. *Fertil Steril* 2010;**94**:833–840.

Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. *Biochem J* 2001;**357**:593–615.

Andronowska A, Doboszynska T, Janiszewska L. Localization of NADPH-diaphorase and NOS isoforms in the oviduct of the pig. *Folia Histochem Cytobiol* 1999;**37**:93–94.

Anteby EY, Hurwitz A, Korach O, Revel A, Simon A, Finci-Yeheskel Z, Mayer M, Laufer N. Human follicular nitric oxide pathway: relationship to follicular size, oestradiol concentrations and ovarian blood flow. *Hum Reprod* 1996;**11**:1947–1951.

Ault KA, Tawfik OW, Smith-King MM, Gunter J, Terranova PF. Tumor necrosis factor-alpha response to infection with *Chlamydia trachomatis* in human fallopian tube organ culture. *Am J Obstet Gynecol* 1996; **175**:1242–1245.

Barnhart KT. Clinical practice. Ectopic pregnancy. *N Engl J Med* 2009; **361**:379–387.

Barroso RP, Osuamkpe C, Nagamani M, Yallampalli C. Nitric oxide inhibits development of embryos and implantation in mice. *Mol Hum Reprod* 1998;**4**:503–507.

Bogdan C. Nitric oxide and the immune response. *Nat Immunol* 2001; **2**:907–916.

Brunham RC, Rey-Ladino J. Immunology of *Chlamydia* infection: implications for a *Chlamydia trachomatis* vaccine. *Nat Rev Immunol* 2005;**5**:149–161.

Bryan NS, Bian K, Murad F. Discovery of the nitric oxide signaling pathway and targets for drug development. *Front Biosci* 2009;**14**:1–18.

Bryant CE, Tomlinson A, Mitchell JA, Thiemermann C, Willoughby DA. Nitric oxide synthase in the rat fallopian tube is regulated during the oestrous cycle. *J Endocrinol* 1995;**146**:149–157.

Burnett TG, Tash JS, Hunt JS. Investigation of the role of nitric oxide synthase 2 in pregnancy using mutant mice. *Reproduction* 2002; **124**:49–57.

Bush RM, Everett KD. Molecular evolution of the Chlamydiaceae. *Int J Syst Evol Microbiol* 2001;**51**:203–220.

Campbell LA, Patton DL, Moore DE, Cappuccio AL, Mueller BA, Wang SP. Detection of *Chlamydia trachomatis* deoxyribonucleic acid in women with tubal infertility. *Fertil Steril* 1993;**59**:45–50.

Chatterjee S, Gangula PR, Dong YL, Yallampalli C. Immunocytochemical localization of nitric oxide synthase-III in reproductive organs of female rats during the oestrous cycle. *Histochem J* 1996;**28**:715–723.

Chow JM, Yonekura ML, Richwald GA, Greenland S, Sweet RL, Schachter J. The association between *Chlamydia trachomatis* and ectopic pregnancy. A matched-pair, case-control study. *JAMA* 1990;**263**:3164–3167.

Cicinelli E, Ignarro LJ, Lograno M, Matteo G, Falco N, Schonauer LM. Acute effects of transdermal estradiol administration on plasma levels of nitric oxide in postmenopausal women. *Fertil Steril* 1997;**67**:63–66.

Cooper MD, Rapp J, Jeffery-Wiseman C, Barnes RC, Stephens DS. *Chlamydia trachomatis* infection of human fallopian tube organ cultures. *J Gen Microbiol* 1990;**136**:1109–1115.

Corpa JM. Ectopic pregnancy in animals and humans. *Reproduction* 2006; **131**:631–640.

Davis CH, Raulston JE, Wyrick PB. Protein disulfide isomerase, a component of the estrogen receptor complex, is associated with *Chlamydia trachomatis* serovar E attached to human endometrial epithelial cells. *Infect Immun* 2002;**70**:3418–3418.

Dixon RE, Hwang SJ, Hennig GW, Ramsey KH, Schripsema JH, Sanders KM, Ward SM. *Chlamydia* infection causes loss of pacemaker cells and inhibits oocyte transport in the mouse oviduct. *Biol Reprod* 2009;**80**:665–673.

Ekerhovd E, Brännström M, Alexandersson M, Norström A. Evidence for nitric oxide mediation of contractile activity in isolated strips of the human Fallopian tube. *Hum Reprod* 1997;**12**:301–305.

Ekerhovd E, Brännström M, Weijdegard B, Norström A. Localization of nitric oxide synthase and effects of nitric oxide donors on the human Fallopian tube. *Mol Hum Reprod* 1999;**5**:1040–1047.

Ekerhovd E, Enskog A, Caidahl K, Klintland N, Nilsson L, Brännström M, Norström A. Plasma concentrations of nitrate during the menstrual cycle, ovarian stimulation and ovarian hyperstimulation syndrome. *Hum Reprod* 2001;**16**:1334–1339.

Entrican G, Wattegedera S, Rocchi M, Fleming DC, Kelly RW, Wathne G, Magdalenic V, Howie SE. Induction of inflammatory host immune responses by organisms belonging to the genera *Chlamydia*/ Chlamydophila. *Vet Immunol Immunopathol* 2004;**100**:179–186.

Farquhar CM. Ectopic pregnancy. *Lancet* 2005;**366**:583–591.

Fenton KA, Korovessis C, Johnson AM, McCadden A, McManus S, Wellings K, Mercer CH, Carder C, Copas AJ, Nanchahal K *et al.* Sexual behaviour in Britain: reported sexually transmitted infections and prevalent genital *Chlamydia trachomatis* infection. *Lancet* 2001; **358**:1851–1854.

Gawronska B, Bodek G, Ziecik AJ. Distribution of NADPH-diaphorase and nitric oxide synthase (NOS) in different regions of porcine oviduct during the estrous cycle. *J Histochem Cytochem* 2000; **48**:867–875.

Gyurko R, Leupen S, Huang PL. Deletion of exon 6 of the neuronal nitric oxide synthase gene in mice results in hypogonadism and infertility. *Endocrinology* 2002;**143**:2767–2774.

Hart KM, Murphy AJ, Barrett KT, Wira CR, Guyre PM, Pioli PA. Functional expression of pattern recognition receptors in tissues of the human female reproductive tract. *J Reprod Immunol* 2009;**80**:33–40.

Hefler LA, Gregg AR. Influence of the angiotensinogen gene on the ovulatory capacity of mice. *Fertil Steril* 2001;**75**:1206–1211.

Hefler LA, Tempfer CB, Moreno RM, O'Brien WE, Gregg AR. Endothelial-derived nitric oxide and angiotensinogen: blood pressure and metabolism during mouse pregnancy. *Am J Physiol Regul Integr Comp Physiol* 2001;**280**:R174–R182.

Henry-Suchet J, Catalan F, Loffredo V, Sanson MJ, Debache C, Pigeau F, Coppin R. *Chlamydia trachomatis* associated with chronic inflammation in abdominal specimens from women selected for tuboplasty. *Fertil Steril* 1981;**36**:599–605.

Henry-Suchet J, Askienazy-Elbhar M, Orfila J. Clinical consequences of immune response to CT upper genital tract infection in women. *Infect Dis Obstet Gynecol* 1996;**4**:171–175.

Hess AP, Baston-Buest DM, Schanz A, Hirchenhain J, Bielfeld P, Kruessel JS. Interleukin-1 system in the human fallopian tube-No spatial but a temporal regulation of mRNA and protein expression. *Mol Cell Endocrinol* 2009;**303**:7–12.

Hvid M, Baczynska A, Deleuran B, Fedder J, Knudsen HJ, Christiansen G, Birkelund S. Interleukin-1 is the initiator of Fallopian tube destruction during *Chlamydia trachomatis* infection. *Cell Microbiol* 2007;**9**:2795–2803.

Hybiske K, Stephens RS. Mechanisms of *Chlamydia trachomatis* entry into nonphagocytic cells. *Infect Immun* 2007;**75**:3925–3934.

Igietseme JU, Perry LL, Ananaba GA, Uriri IM, Ojior OO, Kumar SN, Caldwell HD. *Chlamydia*l infection in inducible nitric oxide synthase knockout mice. *Infect Immun* 1998;**66**:1282–1286.

Jansen RP. Endocrine response in the fallopian tube. *Endocr Rev* 1984; **5**:525–551.

Jerzak M, Kniotek M, Mrozek J, Gorski A, Baranowski W. Sildenafil citrate decreased natural killer cell activity and enhanced chance of successful pregnancy in women with a history of recurrent miscarriage. *Fertil Steril* 2008;**90**:1848–1853.

Kilic S, Tasdemir N, Lortlar N, Yuksel B, Budak G, Batioglu S. Vascular endothelial growth factor (VEGF) and inducible nitric oxide synthase (iNOS) immunoreactivities in rat ovaries and uterine tubes after tubal ligation: a controlled immunohistochemical study. *Eur J Contracept Reprod Health Care* 2008;**13**:431–437.

Kuo C. Host response. In Barron A (ed). *Microbiology of Chlamydia*. Boca Raton, FL: CRC Press, 1988, 193–208.

La Verda D, Byrne GI. Interactions between macrophages and chlamydiae. *Immunol Ser* 1994;**60**:381–399.

Lapointe J, Roy M, St-Pierre I, Kimmins S, Gauvreau D, MacLaren LA, Bilodeau JF. Hormonal and spatial regulation of nitric oxide synthases (NOS) (neuronal NOS, inducible NOS, and endothelial NOS) in the oviducts. *Endocrinology* 2006;**147**:5600–5610.

Liang M, Ekblad E, Lydrup ML, Nilsson BO. Combined lack of estrogen receptors alpha and beta affects vascular iNOS protein expression. *Cell Tissue Res* 2003;**313**:63–70.

Liu VW, Huang PL. Cardiovascular roles of nitric oxide: a review of insights from nitric oxide synthase gene disrupted mice. *Cardiovasc Res* 2008; **77**:19–29.

Lyons RA, Saridogan E, Djahanbakhch O. The reproductive significance of human Fallopian tube cilia. *Hum Reprod Update* 2006;**12**:363–372.

Machado-Oliveira G, Lefievre L, Ford C, Herrero MB, Barratt C, Connolly TJ, Nash K, Morales-Garcia A, Kirkman-Brown J, Publicover S. Mobilisation of Ca^{2+} stores and flagellar regulation in human sperm by S-nitrosylation: a role for NO synthesised in the female reproductive tract. *Development* 2008;**135**:3677–3686.

MacMicking JD, Nathan C, Hom G, Chartrain N, Fletcher DS, Trumbauer M, Stevens K, Xie QW, Sokol K, Hutchinson N *et al*. Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell* 1995;**81**:641–650.

Majewski M, Sienkiewicz W, Kaleczyc J, Mayer B, Czaja K, Lakomy M. The distribution and co-localization of immunoreactivity to nitric oxide synthase, vasoactive intestinal polypeptide and substance P within nerve fibres supplying bovine and porcine female genital organs. *Cell Tissue Res* 1995;**281**:445–464.

Mashimo H, Goyal RK. Lessons from genetically engineered animal models. IV. Nitric oxide synthase gene knockout mice. *Am J Physiol* 1999;**277**:G745–G750.

Mastroianni L Jr. The fallopian tube and reproductive health. *J Pediatr Adolesc Gynecol* 1999;**12**:121–126.

Maul H, Longo M, Saade GR, Garfield RE. Nitric oxide and its role during pregnancy: from ovulation to delivery. *Curr Pharm Des* 2003;**9**: 359–380.

Miller WC, Ford CA, Morris M, Handcock MS, Schmitz JL, Hobbs MM, Cohen MS, Harris KM, Udry JR. Prevalence of chlamydial and gonococcal infections among young adults in the United States. *JAMA* 2004;**291**:2229–2236.

Moncada S, Higgs EA. Endogenous nitric oxide: physiology, pathology and clinical relevance. *Eur J Clin Invest* 1991;**21**:361–374.

Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 1991;**43**:109–142.

Moulder JW. Interaction of chlamydiae and host cells *in vitro*. *Microbiol Rev* 1991;**55**:143–190.

Mukul LV, Teal SB. Current management of ectopic pregnancy. *Obstet Gynecol Clin North Am* 2007;**34**:403–419.

Muschiol S, Bailey L, Gylfe A, Sundin C, Hultenby K, Bergstrom S, Elofsson M, Wolf-Watz H, Normark S, Henriques-Normark B. A small-molecule inhibitor of type III secretion inhibits different stages of the infectious cycle of *Chlamydia trachomatis*. *Proc Natl Acad Sci USA* 2006;**103**:14566–14571.

Nathan C, Xie QW. Nitric oxide synthases: roles, tolls, and controls. *Cell* 1994;**78**:915–918.

Novaro V, Pustovrh C, Colman-Lerner A, Radisky D, Lo Nostro F, Paz D, Jawerbaum A, Gonzalez E. Nitric oxide induces gelatinase A (matrix metalloproteinase 2) during rat embryo implantation. *Fertil Steril* 2002; **78**:1278–1287.

Ohman H, Tiitinen A, Halttunen M, Lehtinen M, Paavonen J, Surcel HM. Cytokine polymorphisms and severity of tubal damage in women with *Chlamydia*-associated infertility. *J Infect Dis* 2009;**199**:1353–1359.

Ota H, Igarashi S, Oyama N, Suzuki Y, Tanaka T. Optimal levels of nitric oxide are crucial for implantation in mice. *Reprod Fertil Dev* 1999; **11**:183–188.

Paavonen J, Lehtinen M. Chlamydial pelvic inflammatory disease. *Hum Reprod Update* 1996;**2**:519–529.

Pallares P, Garcia-Fernandez RA, Criado LM, Letelier CA, Esteban D, Fernandez-Toro JM, Flores JM, Gonzalez-Bulnes A. Disruption of the endothelial nitric oxide synthase gene affects ovulation, fertilization and early embryo survival in a knockout mouse model. *Reproduction* 2008;**136**:573–579.

Parish WL, Laumann EO, Cohen MS, Pan S, Zheng H, Hoffman I, Wang T, Ng KH. Population-based study of chlamydial infection in China: a hidden epidemic. *JAMA* 2003;**289**:1265–1273.

Peipert JF. Clinical practice. Genital chlamydial infections. *N Engl J Med* 2003;**349**:2424–2430.

Perez Martinez S, Viggiano M, Franchi AM, Herrero MB, Ortiz ME, Gimeno MF, Villalon M. Effect of nitric oxide synthase inhibitors on ovum transport and oviductal smooth muscle activity in the rat oviduct. *J Reprod Fertil* 2000;**118**:111–117.

Perfettini JL, Darville T, Gachelin G, Souque P, Huerre M, Dautry-Varsat A, Ojcius DM. Effect of *Chlamydia trachomatis* infection and subsequent tumor necrosis factor alpha secretion on apoptosis in the murine genital tract. *Infect Immun* 2000;**68**:2237–2244.

Perry LL, Feilzer K, Caldwell HD. Neither interleukin-6 nor inducible nitric oxide synthase is required for clearance of *Chlamydia trachomatis* from the murine genital tract epithelium. *Infect Immun* 1998;**66**:1265–1269.

Prantner D, Darville T, Sikes JD, Andrews CW Jr, Brade H, Rank RG, Nagarajan UM. Critical role for interleukin-1beta (IL-1beta) during *Chlamydia* muridarum genital infection and bacterial replication-independent secretion of IL-1beta in mouse macrophages. *Infect Immun* 2009;**77**:5334–5346.

Purcell TL, Given R, Chwalisz K, Garfield RE. Nitric oxide synthase distribution during implantation in the mouse. *Mol Hum Reprod* 1999;**5**:467–475.

Ramsay B, Johnson MR, Leone AM, Steer PJ. The effect of exogenous oestrogen on nitric oxide production in women: a placebo controlled crossover study. *Br J Obstet Gynaecol* 1995;**102**:417–419.

Ramsey KH, Sigar IM, Rana SV, Gupta J, Holland SM, Byrne GI. Role for inducible nitric oxide synthase in protection from chronic *Chlamydia trachomatis* urogenital disease in mice and its regulation by oxygen free radicals. *Infect Immun* 2001;**69**:7374–7379.

Rasmussen SJ, Eckmann L, Quayle AJ, Shen L, Zhang YX, Anderson DJ, Fierer J, Stephens RS, Kagnoff MF. Secretion of proinflammatory cytokines by epithelial cells in response to *Chlamydia* infection suggests a central role for epithelial cells in chlamydial pathogenesis. *J Clin Invest* 1997;**99**:77–87.

Refaat B, Al-Azemi M, Geary I, Eley A, Ledger W. Role of activins and inducible nitric oxide in the pathogenesis of ectopic pregnancy in patients with or without *Chlamydia trachomatis* infection. *Clin Vaccine Immunol* 2009;**16**:1493–1503.

Rosselli M, Imthurm B, Macas E, Keller PJ, Dubey RK. Circulating nitrite/ nitrate levels increase with follicular development: indirect evidence for estradiol mediated NO release. *Biochem Biophys Res Commun* 1994a; **202**:1543–1552.

Rosselli M, Imthurn B, Macas E, Keller PJ, Dubey RK. Endogenous nitric oxide modulates endothelin-1 induced contraction of bovine oviduct. *Biochem Biophys Res Commun* 1994b;**201**:143–148.

Rosselli M, Dubey RK, Rosselli MA, Macas E, Fink D, Lauper U, Keller PJ, Imthurn B. Identification of nitric oxide synthase in human and bovine oviduct. *Mol Hum Reprod* 1996;**2**:607–612.

Rosselli M, Keller PJ, Dubey RK. Role of nitric oxide in the biology, physiology and pathophysiology of reproduction. *Hum Reprod Update* 1998;**4**:3–24.

Shao R. Understanding the mechanisms of human tubal ectopic pregnancies: new evidence from knockout mouse models. *Hum Reprod* 2010;**25**:584–587.

Shao R, Egecioglu E, Weijdegard B, Kopchick JJ, Fernandez-Rodriguez J, Andersson N, Billig H. Dynamic regulation of estrogen receptor-alpha isoform expression in the mouse fallopian tube: mechanistic insight into estrogen-dependent production and secretion of insulin-like growth factors. *Am J Physiol Endocrinol Metab* 2007a;**293**:E1430–E1442.

Shao R, Weijdegard B, Fernandez-Rodriguez J, Egecioglu E, Zhu C, Andersson N, Thurin-Kjellberg A, Bergh C, Billig H. Ciliated epithelial-specific and regional-specific expression and regulation of the estrogen receptor-beta2 in the fallopian tubes of immature rats: a possible mechanism for estrogen-mediated transport process in vivo. *Am J Physiol Endocrinol Metab* 2007b;**293**:E147–E158.

Shepard MK, Jones RB. Recovery of *Chlamydia trachomatis* from endometrial and fallopian tube biopsies in women with infertility of tubal origin. *Fertil Steril* 1989;**52**:232–238.

Spencer F, Chi L, Zhu MX. Antiproliferative effects of inducible nitric oxide synthase inhibition on decidualization in pseudopregnant rats. *Proc Soc Exp Biol Med* 1998;**218**:45–50.

Stephens RS. The cellular paradigm of chlamydial pathogenesis. *Trends Microbiol* 2003;**11**:44–51.

Strandell A, Thorburn J, Wallin A. The presence of cytokines and growther factors in hydrosalpingeal fluid. *J Assist Reprod Genet* 2004;**21**:241–247.

Straub RH. The complex role of estrogens in inflammation. *Endocr Rev* 2007;**28**:521–574.

Stutz A, Golenbock DT, Latz E. Inflammasomes: too big to miss. *J Clin Invest* 2009;**119**:3502–3511.

Telfer JF, Lyall F, Norman JE, Cameron IT. Identification of nitric oxide synthase in human uterus. *Hum Reprod* 1995;**10**:19–23.

Tempfer C, Moreno RM, O'Brien WE, Gregg AR. Genetic contributions of the endothelial nitric oxide synthase gene to ovulation and menopause in a mouse model. *Fertil Steril* 2000;**73**:1025–1031.

Toth M, Jeremias J, Ledger WJ, Witkin SS. In vivo tumor necrosis factor production in women with salpingitis. *Surg Gynecol Obstet* 1992; **174**:359–362.

Tranguch S, Huet-Hudson Y. Decreased viability of nitric oxide synthase double knockout mice. *Mol Reprod Dev* 2003;**65**:175–179.

Tschugguel W, Schneeberger C, Unfried G, Czerwenka K, Weninger W, Mildner M, Bishop JR, Huber JC. Induction of inducible nitric oxide synthase expression in human secretory endometrium. *Hum Reprod* 1998;**13**:436–444.

Tuffrey M, Alexander F, Inman C, Ward ME. Correlation of infertility with altered tubal morphology and function in mice with salpingitis induced by a human genital-tract isolate of *Chlamydia trachomatis*. *J Reprod Fertil* 1990;**88**:295–305.

Ulbrich SE, Rehfeld S, Bauersachs S, Wolf E, Rottmayer R, Hiendleder S, Vermehren M, Sinowatz F, Meyer HH, Einspanier R. Region-specific expression of nitric oxide synthases in the bovine oviduct during the oestrous cycle and *in vitro*. *J Endocrinol* 2006;**188**:205–213.

Van Voorhis WC, Barrett LK, Sweeney YT, Kuo CC, Patton DL. Repeated *Chlamydia trachomatis* infection of Macaca nemestrina fallopian tubes produces a Th1-like cytokine response associated with fibrosis and scarring. *Infect Immun* 1997;**65**:2175–2182.

Wei XQ, Charles IG, Smith A, Ure J, Feng GJ, Huang FP, Xu D, Muller W, Moncada S, Liew FY. Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature* 1995;**375**:408–411.

Weiner CP, Lizasoain I, Baylis SA, Knowles RG, Charles IG, Moncada S. Induction of calcium-dependent nitric oxide synthases by sex hormones. *Proc Natl Acad Sci USA* 1994;**91**:5212–5216.

Wennmalm A, Benthin G, Edlund A, Jungersten L, Kieler-Jensen N, Lundin S, Westfelt UN, Petersson AS, Waagstein F. Metabolism and excretion of nitric oxide in humans. An experimental and clinical study. *Circ Res* 1993;**73**:1121–1127.

Wira CR, Fahey JV, Sentman CL, Pioli PA, Shen L. Innate and adaptive immunity in female genital tract: cellular responses and interactions. *Immunol Rev* 2005a;**206**:306–335.

Wira CR, Grant-Tschudy KS, Crane-Godreau MA. Epithelial cells in the female reproductive tract: a central role as sentinels of immune protection. *Am J Reprod Immunol* 2005b;**53**:65–76.

Xie QW, Cho HJ, Calaycay J, Mumford RA, Swiderek KM, Lee TD, Ding A, Troso T, Nathan C. Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science* 1992; **256**:225–228.

Zhan X, Li D, Johns RA. Expression of endothelial nitric oxide synthase in ciliated epithelia of rats. *J Histochem Cytochem* 2003;**51**:81–87.