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Review

Gas transmitters in female reproductive system

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Abstract: Nitric oxide, carbon monoxide and hydrogen sulfide synthesized endogenously in living organisms produce an array of disparate biological effects, so as to be considered as gas transmitters. These three gaseous molecules play important roles in many physiological and pathological processes in the bodies, such as the regulation of vascular tone and inflammatory responses as well as reproductive function. This review mainly focuses on the distribution and biological functions of these three gas transmitters in female reproductive tissues.

Key words: nitric oxide; carbon monoxide; hydrogen sulfide; female; reproduction

气体信号分子在女性生殖系统中的作用

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摘 要：生物体内合成的内源性气体分子：NO，CO以及H_2S，具有多种生物学功能因而被称为气体信号分子。这三种气体信号分子在许多生理与病理过程中发挥重要作用，如调节血管紧张性、炎症反应、生殖功能等。本文主要对这三种气体信号分子在女性和雌性动物生殖系统中的分布和生物学功能进行综述。

关键词：一氧化氮；一氧化碳；硫化氢；生殖
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1 Introduction

With the fame of "Molecule of The Year" nominated by *Science* in 1992 and the *Viagra* becoming one of the global best-selling drugs, nitric oxide (NO), and the subsequent resurgence of carbon monoxide (CO) and, more recently, hydrogen sulfide (H_2S) have evoked a marked escalation in interest of researchers in various areas of life sciences in the last decade. NO, CO and H_2S have many common features. They all can be produced endogenously in the body and are involved in various physiological functions. In this review, we focus on these gaseous transmitters in female reproductive system during pregnancy.

2 NO

NO is derived from *L*-arginine by the action of nitric oxide synthase (NOS). This enzyme exists in three main forms: neuronal NOS (nNOS, brain NOS or type I NOS); inducible NOS (iNOS or type II NOS) and endothelial NOS (eNOS or type III)[1,2]. Both eNOS and

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nNOS are constitutive enzymes (termed as cNOS). They have been shown to be controlled by Ca^{2+} and calmodulin and generate small amounts of NO. In contrast, iNOS is a Ca^{2+}-independent enzyme, which is expressed by macrophages and other tissues in response to inflammatory mediators and liberates large, uncontrolled amounts of NO when it is stimulated. NO is produced by almost all of the human genital duct and intrauterine tissues and involved in various functions such as modulation of vascular tone and hormone secretion in placenta, platelet activation during menstruation and suppression of myometrial contractility [3–7].

2.1 NO in fallopian tube

Both eNOS and iNOS were found in human fallopian tube. Positive immunostaining for eNOS was found in the luminal epithelial cells and in the endothelial cells of blood vessels. The expression of iNOS was identified in tubal smooth muscle cells, epithelial cells as well as the walls of the blood vessels [8]. More recently, NO production by human fallopian tube during the menstrual cycle is found to be region-specific and cyclic [9]. Positive immunoreactivity of iNOS was found in both the ampulla and the isthmus. The intensity of the iNOS immunoreaction in the epithelial cells decreased toward the isthmic region, showing a gradient of expression along the fallopian tube. The expression of iNOS was weak in the menstrual phase, increased in the follicular phase, and was strong in the luteal phase. The iNOS gene expression showed similar periodic pattern as its protein distribution.

The fallopian tube is a delicate organ, which is responsible for transport of the embryo into the uterine cavity. Transportation within the tube is achieved by a complex interaction among muscle contractions, ciliary activity, and the flow of tubal secretions [10]. *L*-arginine methyl ester (*L*-NAME, an inhibitor of NO synthesis), enhances tubal contractility, while *L*-arginine, the substrate for NOS, caused relaxation of the strips, suggesting a potent relaxing effect of NO on human tubal smooth muscle [8, 11].

2.2 NO in corpora uteri

NOS isoforms have also been found in human corpora uteri. All three NOS isoforms are expressed in endometrium[4, 12–14]. The activity of NOS is increased in epithelial cells and blood vessels during the secretory phase of the menstrual cycle[15]. A number of studies have demonstrated that eNOS is expressed in the glandular and epithelial endometrium and endometrial microvasculature, whereas iNOS is present in various immunocompetent endometrial cells, decidualized stromal cells, as well as epithelial cells[13, 16–20]. It has been found that expression of eNOS mRNA is increased in glandular epithelial cells in the late secretary phase, whereas iNOS mRNA is expressed in the glandular epithelium during menstruation. The eNOS may be the predominant isoform of NOS in the non-pregnant human endometrium and is increased in secretory endometrium compared with that in proliferative endometrium [13]. In peri-menstruation and during early pregnancy, iNOS turns into the predominant NOS isoform in endometrium[17]. The activity of iNOS showed a six-fold increase in menstruation compared with that in proliferative or secretory phases[17]. The immunoreactivity of iNOS in decidual cells is increased in early pregnancy, suggesting that decidualization may generally be accompanied by the up-regulation of iNOS [18].

The endometrium is receptive to blastocyst implantation only in a specific period, which is termed as "implantation window". Under the influence of progesterone, the endometrial stroma undergoes a dramatic differentiation into the deciduas in the early pregnancy. NO is believed to support and maintain the decidualization process and plays a crucial role in implantation. It improves endometrial vascularity and receptivity, thereby promoting pregnancy rate[21]. Both of iNOS and eNOS are identified in the connective tissue surrounding spiral arterioles in the baboon endometrium during implantation [22]. All three NOS isoforms are found in the mouse implantation site, with iNOS and eNOS predominance[23]. During the peri-implantation phase, *L*-NAME inhibited implantation in mice and rats[24], whereas oral supplementation of *L*-arginine, a NO donor, improved endometrial receptivity and pregnancy rate in poor responder *in-vitro* fertilization patients[21].

In myometrium, the expression pattern of three types of NOS differs with that in endometrium. The expression of iNOS is undetectable whilst eNOS and nNOS expression level is very low in non-pregnant myometrium during secretory phase. In the proliferative phase, eNOS expression is up-regulated compared with that in secretory phase[13]. During pregnancy, the expression of both constitutive and inducible NOS is greatly increased in human myometrium with gestational length, which contributes to retention of pregnancy[26–28]. Toward term, iNOS and eNOS expression is greatly declined, concurrently with the increase in oxytocin

receptor and prostaglandin F2α receptor[26,27,29].

In vitro studies show that *L*-arginine causes a rapid and substantial relaxation of spontaneous activity in the uterine strips of pregnant women[30]. These relaxation effects were reversed by *L*-NAME. Sodium nitroprusside (SNP), a NO donor, completely abolishes spontaneous contractions. Methylene blue, an inhibitor of guanylate cyclase, can block *L*-arginine-induced relaxation. NO appears to be at least as effective as the commonly used tocolytics today. Moreover, NO application for inhibition of preterm labor is safe, and it is characterized by only minor side effects such as headache[31].

2.3 NO in cervix

All three NOS isoforms are found in the cervix[32–34], suggesting an important role of NO in the cervix. Interestingly, contrast to the reduced expression of NOS in the uterus during term and preterm labor as mentioned above, NOS is up-regulated in the cervix during labor, and even higher in preterm labor[33, 34]. The nNOS is not expressed in myometrium, but intensively expressed in cervix. It was localized to the stroma, the glandular epithelium and the basal membrane of the squamous epithelium [34, 35]. In the myometrium, the NO system may contribute to the maintenance of uterine quiescence during pregnancy. In contrast, in the cervix, it may contribute to the activation of the inflammation cascade and the remodelling of the extracellular matrix, thereby contributing to the onset of labour[34–36]. Interestingly, the expression of NOS and NO activity are up-regulated in cervix at parturition, concomitant to a complete disappearance of NO activity in the myometrium[34–36]. In first trimester pregnant women undergoing surgical termination of pregnancy, clinically assessed ripening could be achieved with locally applied NO donors[37]. However, the mechanism responsible for the differential regulation of the NO system in the uterus and cervix remains yet to be established.

2.4 NO in placenta

The placenta may be an important source of NO during pregnancy. Human villous trophoblasts express eNOS. The expression of eNOS in placenta is increased with gestation. Abnormal elevated eNOS is associated with pathological pregnancies including fetal retardation and diabetes, but not with pregnant hypertension. The expression of eNOS in syncytiotrophoblasts is found in all stages of pregnancy, but in cytotrophoblasts, it is found at the first trimest and absent at term. The staining of eNOS is also found in endothelium surrounding the vascular tree[38]. NO in placenta maintains the low basal tone of uterine and placental vessels by attenuating the action of vasoconstrictors[39] and preventing platelet aggregation in intervillous space[40]. NO has also been shown to up-regulate MMP-2 and MMP-9, which are required for trophoblast invasion during embryo implantation[25,41]. The process of trophoblast-derived arterial wall destruction starts at 8 weeks of gestation[42]. At the same time, NO produced by trophoblast cells causes the relaxation of the vascular walls at the implantation site. Thus, NO is required to promote the cytotrophoblast endovascular invasion, which is an essential feature of normal placentation[43]. Dash *et al*.[44] have shown that NO protects cultured extravillous trophoblast cells from apoptosis through a mechanism involving the activation of soluble guanylyl cyclase (sGC). During pregnancy, there is a physiological vascular adaptation that consists of increased blood volume, increased cardiac output, and decreased vascular resistance. Such changes are accompanied by an increase in endogenous NO. Lack of NO during gestation is associated with the development of pregnancy-induced hypertension and preeclampsia (PE)[5]. It has been suggested that a derangement in the NO system occurs in women who are suffering from PE[45]. The placental eNOS is not different between normotensive pregnancy and PE, whereas the staining of nitrotyrosine, a marker of peroxynitrite, is stronger in preeclamptic villi compared with that in normal placenta[46]. Sandrim *et al*.[47] indicated that eNOS polymorphisms affect endogenous NO formation in normal pregnancy, but not in PE, and that the 'C Glu b' haplotype may protect against the development of PE by increasing endogenous NO formation. The modulating effect of NO on the angiogenic balance might be important during PE[48,49]. NO production in primary human trophoblasts increased placental growth factor and vascular endothelial growth factor (VEGF) and decreased soluble fms-like tyrosine kinase receptor 1 (sFlt1) mRNA expression resulting in an enhanced proangiogenic environment *in vitro*. In addition, lack of eNOS aggravates the sFlt1-induced PE-phenotype in mice[48].

2.5 Regulation of NO in female reproductive tissues

The expression of NOS in endometrium and myometrium is regulated by various hormones and cytokines. The expression levels of eNOS in the postmenopausal endometrium and myometrium were significantly lower than those in premenopausal compartments. Estrogen

replacement therapy can restore endometrial and myometrial eNOS expression to premenopausal levels. Khorram *et al*.'s study showed that estrogen regulates myometrial eNOS, whereas progesterone or a combination of estrogen and progesterone regulates endometrial eNOS[13]. Mifepristone, a progesterone receptor antagonist, decreases eNOS expression in the endometrial glandular epithelium but does not affect eNOS in vascular endothelium in endometrium, suggesting a role of epithelial eNOS in human endometrial receptivity[50]. Dong *et al*. [51] demonstrated that progesterone increases iNOS expression, whereas mifespristone can induce preterm labor by decreasing iNOS expression in myometrium. 17β-estradiol can act on estrogen receptor (ER) α and ERβ to stimulate iNOS gene expression in the human myometrium [52]. Mifepristone administration induces NO release and increases expression of iNOS in cervix during early pregnancy[53].

Endometrial proinflammatory cytokines are up-regulated during implantation. In the endometrium and deciduas, the leukocytes may in fact represent the most important source of NO during menstruation, implantation and early pregnancy. Various cytokines secreted from endometrial cells, immune cells, or macrophages stimulate eNOS and thereby release NO[19, 54]. These abnormal immune responses might eventually stimulate macrophages and/or endometrial cells to persistently produce a large amount of NO and inhibit implantation[55]. The iNOS can be induced by IL-1, IL-2, IL-12, tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), or lipopolysaccharide (LPS) [2]. IL-1 and TNF-α downregulate eNOS expression, whilst adenyl-cyclase activators increase eNOS expression[56]. Macrophage migration inhibitory factor, a multifunctional proinflammatory cytokine, can reduce iNOS expression and NO release by fetal membranes[57]. Prostaglandins (PGs) inhibit cytokine-induced NO production in uterus, suggesting that increased PG production at term may down-regulate uterine NO production, thereby facilitating labor [58].

Clifton *et al*. [59] reported that placental corticotrophin-releasing hormone (CRH) causes a vasodilatory response via a NO-/cGMP-dependent pathway in human placenta. In human myometrial cells, CRH acts on CRH receptor type 1 to stimulate expression of eNOS and nNOS but not iNOS isoforms[60]. The activity of eNOS can be regulated by eNOS-traffic inducer (NOSTRIN) which is abundant in highly vascularized tissues such as the placenta[61]. NOSTRIN is part of a protein network that controls the activity, trafficking and targeting of eNOS, and functions by facilitating the intracellular trafficking of eNOS between different intracellular compartments, and by targeting eNOS at structures that contain dynamin, e.g. endocytic vesicles and at actin filaments[62].

3 CO

Cells are known to produce CO before NO is discovered. Endogenous CO is formed by a specific haem oxygenase (HO) enzyme, which exists as three isoforms, i.e. HO-1, HO-2 and HO-3. Similar with NOS, HO can also be subclassified into constitutive (HO-2 and HO-3) and inducible enzymes (HO-1). The increased synthesis of the HO-1 protein occurs as a general response to stress in biological systems. The response appears to occur ubiquitously among most tissues. HO-2 is constitutively expressed in many mammalian cells. HO-3 may be derived from retrotransposition of the HO-2 gene since the HO-3 gene does not contain introns [63].

HO-1 and HO-2 are found to be expressed in theca cells, granulosa cells of follicles, and luteal cells of mature rat ovaries. HO-2 was also expressed in the ovarian stroma [64]. Hemin injection *in vivo* increased androstenedione and estradiol but not progesterone production. Inhibition of HO activity causes a decrease in progesterone and androstenedione but not 17β-estradiol secretion, suggesting that endogenous CO stimulates the production of ovarian steroids [64].

HO activity has been detected in human umbilical cord [65] and human placenta [66–69]. It has also been demonstrated that placenta is capable of producing CO through HO [66, 67]. HO-2 expression is strong in syncytiotrophoblast, which is reduced toward term. HO-2 is also identified in endothelia of blood vessels, which is increased toward term[69]. In PE and fetal growth restriction (FGR), immunohistochemical analysis showed HO-2 expression in endothelial cells was down-regulated whereas its expression in villous trophoblast did not differ among normal pregnancy, PE and FGR[70]. The reduced expression of HO-2 in endothelia in PE and FGR may attribute to reduced placental blood flow in these abnormal pregnancies. HO-1 is very low or undetectable in placenta [69, 70]. CO can reduce placental perfusion pressure, suggesting that CO can induce vasodilatation in placenta[71]. It seems that CO-induced vasodilatation is through sGC signalling pathway[71]. CO is also found to modulate placental CRH secretion [72].

Both HO-1 and HO-2 are expressed in human uterus [73]. The expression of HO-1 and HO-2 as well as CO production is up-regulated during pregnancy and reaches peak toward the end of pregnancy. CO can inhibit both the spontaneous and oxytocin-induced contractions of myometrial strips *in vitro* [73, 74]. It has also been found that CO level is lower in pregnant woman with premature uterine contractions [75]. However, some studies showed that HO-1 and HO-2 were not up-regulated during pregnancy in myometrium [76]. Animal studies showed that uterine HO activity and HO-1 expression are up-regulated in late gestation in rats [77].

4 H_2S

H_2S is a colourless, flammable, water-soluble gas with the characteristic smell of rotten eggs. Like CO, H_2S received attention primarily as a toxic gas and as an environmental hazard for many decades. Increasing body of evidence suggests that it is also produced in mammals including humans, and is now considered as the third endogenous gaseous signaling transmitter in mammalian tissues in addition to NO and CO[78]. H_2S is synthesized endogenously in various mammalian tissues by two pyridoxal-5'-phosphate-dependent enzymes responsible for metabolizing *L*-cysteine: cystathionine β-synthase (CBS, EC 4.2.1.22) and cystathionine γ-lyase (CSE, EC 4.4.1.1). The substrate of CBS and CSE, *L*-cysteine, can be derived from alimentary sources or can be liberated from endogenous proteins. It can also be synthesized endogenously from *L*-methionine through the trans-sulphuration pathway, with homocysteine being an intermediate in the process[78, 79]. In some tissues, CBS and CSE are both needed for generation of H_2S, whereas in others one enzyme suffices. The tissue specific expression and molecular regulation of CBS and CSE in various systems have been characterized and elegantly reviewed by multiple groups[80–82].

The expression of H_2S generating enzymes and the endogenous production of H_2S have been identified in female reproductive systems in various mammalian species[83–85]. It is indicated that CSE and CBS play different roles in female reproduction. CSE-knockout mice are fertile and give birth normally[86]. By contrast, female offspring of CBS-knockout mice have reduced fertility whereas male offspring are fertile, suggesting that CBS is essential for female reproductive function[87].

CBS is ubiquitously distributed in the ovary with the strongest expression in follicular cells at all stages, but not expressed in the oocytes [88]. CBS-knockout female mice have a decreased number of developed follicles, a shortened and irregular estrus cycle, and a decreased time of estrus and diestrus period compared with wild-type females [89]. Dramatic decreases in uterine mass and the percentage of surviving fetuses were found in CBS-knockout females. Fertility was fully restored when CBS-deficient ovaries were transplanted to wide-type or heterozygous recipients, suggesting that uterine failure, but not ovarian dysfunction, might account for infertility of CBS-deficient females[89]. Since the metabolism of homocysteine is closely regulated by CBS, hyperhomocysteinemia in the uterine environment associated with CBS-knockout mice would be the cause for the dysfunctional uterus. However, the study of Liang *et al*. indicated that CBS in granulose cells plays an important role in oocyte maturation by showing that knockdown of CBS expression in granulose cells results in inhibition of oocyte maturation[89].

CBS and CSE have also been detected in female reproductive tract and gestational tissues, including uterus, vagina, placenta and fetal membranes. A number of studies including ours have shown that both CBS and CSE are expressed in human and rat uterus, fetal membranes and placenta[83–85,90]. The mRNA and protein expressions of CBS as well as H_2S production rate were down-regulated in human laboring myometrium compared with those in nonlaboring myometrium[84]. A number of studies have shown the relaxatory effects of H_2S on smooth muscle of female reproductive tract. Sidhu *et al*. [91] demonstrated both H_2S donor and precursor inhibit the spontaneous contractility of pregnant rat uterine strips. Srilatha *et al.* [92] found that NaHS significantly relaxes rabbit vaginal and cavernosa smooth muscle strips. NaHS and *L*-cysteine both inhibit spontaneous contractility of human myometrium, with a decrease in amplitude[84, 93], suggesting H_2S is a potentially tocolytic agent. *L*-cysteine, at higher concentration, increases the frequency of spontaneous contractions and induces tonic contraction, suggesting that endogenous H_2S may have dual effects on the contractility of human myomentrium. These effects of *L*-cysteine were blocked by the inhibitors of CBS and CSE, as well as glibenclamide, an inhibitor of ATP-sensitive potassium (K_{ATP}) channels[84].

It was found that low oxygen conditions significantly increase H_2S production in human placenta homoge-

nates [83]. As placental hypoxia has been considered to be one of the risk factors for PE, it would be interesting to know whether H_2S is involved in the pathogenesis of PE. Numerous studies have reported the potent vasodilatory effects of H_2S in various isolated vascular preparations including aortic arteries, mesenteric arteries, pulmonary arteries, internal mammary arteries, saphenous veins and coronary arteries [94]. We recently found that NaHS caused relaxation of human umbilical arteries and veins *in vitro*[90] and both CBS and CSE expression were dramatically lower in PE placentas compared with those in normal placentas[90,95], suggesting the impairment of vasodilation in PE is due to the decrease of endogenous H_2S production. In addition, a number of studies have shown that CBS and CSE are localized in the syncytiotrophoblasts in placenta and the endothelium in the fetal vessels from the chorionic- and stem-villi and smooth muscle of human umbilical arteries and veins [85,90,96,97]. NaHS and *L*-cysteine can both increase the expression of angiotensin-converting enzyme (ACE) and VEGF in human placental trophoblasts, which indicates that H_2S participates in angiogenesis in placenta and may contribute to initiation or prevention of PE[95]. In a very recent research, CBS expression was shown in Hofbauer cells[96], the macrophages in placenta, which might be another important H_2S-produced cells contributing to placental vasculogenesis.

5 Prospective

As mentioned, increasing body of evidence implicates that three gas transmitters (NO, CO and H_2S) play an important role in the regulation of ovary functions and maintenance of pregnancy, and aberrant functionality of gasotransmitters is associated with development of various gynaecological and obstetrical disorders such as PE and premature delivery. Recently, some studies suggest that factors being able to increase functionality or availability of gasotransmitters may be beneficial as therapeutic agents in PE[49]. For instance, *L*-arginine did not lower blood pressure in women with pre-existent hypertension, but supplementation resulted in less need for additional antihypertensive medication[98]. The combination of *L*-arginine and antioxidant vitamins (C, E) prolonged the latency to develop PE in a high-risk population[99]. H_2S-based therapies are emerging in the field of cardiovascular diseases, and H_2S-releasing compounds are developed for clinical use. Recently, Wang *et al*. [85] showed that PE symptoms and alterations in angiogenic factors in mice induced by CSE inhibition by *DL*-propargylglycine were restored by a slow releasing H_2S compound (GYY4137). However, the biological actions of these molecules in female reproductive system remain largely unknown, particularly the molecular mechanisms responsible for gasotransmitters are unclear. Further investigating the physiological functions of these molecules and defining their molecular mechanism would help develop new therapeutic strategies for the gynaecological and obstetrical diseases.

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