Nitric oxide in female reproductive system

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Nitric oxide (NO), synthesized from L-arginine and oxygen by a family of enzymes known as nitric oxide synthase (NOS), is an effective and intercellular signal transduction molecule, and is ubiquitously present in vertebrates. To date, there are three distinct isoforms of NOS: neural NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). Among them, eNOS and nNOS, also called constitutive isoforms (cNOS), require calcium for activity, and are expressed constitutively in the physiological condition. The third isoforms, iNOS, whose activity is not dependent on calcium, are produced only in response to some stimulus, including cytokines and immune stimulating factors, etc.[1].

In living organism, NO is known to play a crucial role in diverse physiological processes. As a potent vasodilator, NO also functions as a powerful endogenous relaxant factor in smooth muscle. It can activate guanylate cyclase (GC), resulting in increased levels of cGMP, which is involved in various physiological responses through cGMP signaling pathway[2]. Recent studies show that NO is involved in regulating blood flow, thrombus forming, neurotransmission, and macrophage cytotoxicity[3]. NO has been reported to be related to many reproductive processes, including sperm capacitation, ovulation, embryo implantation, and pregnancy maintenance[4—7]. Now, the studies on NO have become the focus and front in the field of biomedicine.

1 Nitric oxide and signal transduction

NO is an important activator of GC, which is responsible for transforming GTP to cGMP. cGMP has been shown to exist in many species. cGMP contributes to a number of physiological functions, such as vascular smooth muscle relaxation, polymorphonuclear leucocyte chemotaxis increasing, and neurotransmission, which occur through regulation by phosphodiesterase and the phosphorylation by protein kinase[8]. The second signal transduction system, composed of GC, cGMP and NO, is present in various types of tissues and cells, and represents a new intracellular and intercellular signal transduction system regulating cell functions.

2 Nitric oxide and female reproductive system

As a multifunctional molecule, NO is involved in signal transduction in diverse physiological systems. It has been reported that NO is present in reproductive organs and is implicated in several reproductive events, including the regulation of ovarian function, embryo implantation, parturition, etc.[5—7].

(ⅰ) Nitric oxide and ovary. In ovary, NO is an important ovarian function modulator, and essential for follicular development, ovulation and luteolysis[5,9,10].

The positive staining for eNOS is present in theca cells and granulosa cells of ovarian follicles. There is specific expression of eNOS within both the follicle and ovulated oocytes. The level of the circulating nitrite/nitrate, NO metabolites, increases with follicular development, and parallels with follicular size. These data support that NO participates in follicular development. Furthermore, the function of NO in ovary has been studied using eNOS knockout female mice, which shows dysfunction in development of follicles and oocytes, decreasing number of growing follicles, abnormal meiotic maturation at the later stage of meiosis, and raising oocyte death rate[9]. Taken together, eNOS-derived NO is a possible key regulator of oocyte meiotic maturation, and involved in development of follicles and oocytes[11].

According to Shukovski et al.[5], administration of NOS inhibitors suppressed the human chorionic-gonadotropin hormone (hCG)-induced ovulation in rats, and the NO donor reversed the anti-ovulation effect of NO inhibitor. The results showed that NO was probably related to the ovulatory process. Targeted gene-disruption experiments provide clues to the function of NO. eNOS gene knockout results in much fewer ovulated oocytes and newly formed corpus luteum than that in wild-type female mice[9]. Therefore, these results prove the important role of NO in ovulatory process in murine. In addition, iNOS is a primary contributor to the ovulatory process. It has also been demonstrated that NO, derived from iNOS, induces the rupture of the follicular wall and ovulation via increasing prostaglandin (PG) production[12].

During the late luteal phase, eNOS is the most abundant isoforms in human ovary. The NO donor significantly decreases the production of ovarian progesterone in late luteal phase without influencing that in mid- and luteal phases. It has been suggested that NO leads to a significant increase of prostaglandin E (PGE) during the late luteal phase, which has been identified as an important luteolysis factor. The evidence elucidates that NO participates in luteolysis in the human corpus luteum[10].

(ⅱ) Nitric oxide and menstruation/estrous cycle. A body of data reported that NOS was present in the endometrium in a variety of animals. In human endometrium, eNOS is expressed in glandular epithelium and luminal epithelium cells, and reaches a peak in the midsecretory phase[13]. iNOS protein is localized predominantly in glandular epithelium of uterus, and increases signifycantly during the secretory phase. A large number of cy-

tokines are increasingly secreted in endometrium during this phase, such as tumor necrosis factor (TNF), interleukin-1 (IL-1), transforming growth factor-beta1 (TGF-β1), etc. These cytokines have been proved to be strong inducers of iNOS, so the production of NO is the elevated during the secretory phase. Recent findings suggest that NO might be involved in the onset of menstruation and regulation of blood flow[2,14]. The NO-cGMP signal pathway mediates the contractility of myometrium and vascular smooth muscle, which is related with the cyclic shedding of the endometrial lining[15]. These results support that NO is essential for the process of menstruation, including the onset of menstruation, regulation of blood flow, and the cyclic shedding of the endometrial lining.

Studies in rats have demonstrated that iNOS is localized in glandular epithelium and myometrial smooth muscle cells during estrous cycle. The expression of iNOS increases during proestrus and estrus in rats. This fact suggests that NO is necessary to maintain normal physiological function of endometrium[16]. Moreover, NO contributes to regulating estrus cycle in mice. Abnormal estrus cycle is observed in eNOS-knockout female mice. It shows that the estrus cycle extended for about 6—7 d, while that of the control wild-type female mice was 4—5 d[9]. The above evidence shows that NO plays an important role in regulating normal estrus cycle in mice.

(iii) NO and blastocyst implantation. Blastocyst implantation begins at special time after ovulation. In this process, two events are essential for successful blastocyst implantation; i.e. establishment of an implantation "window" in the uterus, and the invasive ability of blastocyst. Numerous studies show that NO has critical effects on implantation[6,17,18].

To verify NO production during blastocyst implantation, Purcell et al.[17] analyzed the expression of iNOS and eNOS in mice uteri during peri-implantation using immunohistochemistry and Western blot analysis, and found that the productions of iNOS and eNOS were higher at the blastocyst implantation site than that in the adjacent tissue, the non-implantation site. These findings indicate that NO is required for successful blastocyst implantation.

Pharmacological manipulation of NO production has been proved to be a powerful means to study the mechanism of NO in blastocyst implantation. Unilateral intrauterine administration of the NOS inhibitor, N-nitro-L-arginine methyl ester (L-NAME), reduces the number of successfully implanted embryos by 50% in mature female pregnant mice, and also significantly impairs the endometrial receptivity and embryonic development. To ensure that the failure of implantation is a consequence of NO deficiency, L-NAME is administered in association with sodium nitroprusside (SNP), a donor of NO. The result shows that SNP can reverse the anti-implantation effect of L-NAME[6]. According to Ota et al.[18], the uteri exhibited retarded decidualization of stromal cells and defected function of predecidualized cells after treatment with L-NAME. The dysfunctional of decidua leads to inhibited implantation. The above evidence shows that NOS is expressed in decidua, and the expression and activity of NOS increase at the implantation site during peri-implantation period. NO is indispensable to blastocyst implantation.

Uterus undergoes important changes during the implantation period, including increased vascular permeability and angiogenesis. Morphological and anatomical findings demonstrate that the functional condition of female reproductive system is related to angiogenesis. However, the mechanism of angiogenesis is still not clear. As an endogenous vasodilator, NO is involved in vascular relaxation and angiogenesis. It might be deduced that NO plays a role in implantation by this mechanism.

In pregnant rats, iNOS is distributed within the decidua surrounding vessels and the ectoplacental cone, and eNOS is localized in the vessels of the primary decidual zone adjacent to embryo[17]. The expression pattern of NOS suggests that NO may play an important role in implantation by regulating the angiogenesis of implantation sites. A number of vasodilatory substances are produced under the effect of oestrogen, such as acetylcholine, histamine, and vascular endothelial growth factor[19], which are the inducer of endogenous NO[20]. The evidence shows that VEGF is associated with angiogenesis, and positively regulates NO production in endothelial cells. In addition, NO attributes to the increasing of microvascular permeability mediated by VEGF[21].

NO affects VEGF-regulated angiogenesis and endothelial function. In endothelial cells, VEGF mediates the upregulation of NOS expression by VEGF receptor, KDR. Further studies show that VEGF induces the production of eNOS through a tyrosine kinase-activated pathway, resulting in increased angiogenesis[22,23]. Moreover, *in vitro* research shows that the increased NOS expression enhances the generation of VEGF[24]. Angiogenesis in eNOS knockout mice is defected, which could not be reversed by the addition of VEGF[25]. This finding suggests that eNOS is a downstream angiogenic mediator for VEGF. Taken together, NO is involved in successful implantation and normal function of female reproductive system by regulating VEGF-induced angiogenesis.

(iv) No in pregnancy and parturition. Establishment and maintenance of pregnancy have been demonstrated to be regulated by the orchestrated action and the levels of estrogen and progesterone. Under the effects of estrogen, the endothelium undergoes characteristic and sensitive changes during early pregnancy, including the increased microvascular permeability and stromal edema, which are necessary for successful implantation. The production of NO is involved in the effect of estrogen on uterus[26], and the NO expression is regulated by estrogen

and progesterone during pregnancy[27].

NO mediates smooth muscle cell contractility and spontaneous contraction, as well as distension of the uterus during pregnancy[28]. NO is also implicated in peripheral vasodilation during pregnancy and in the control of blood flow in the fetoplacental circulation. Other studies demonstrate that NO is involved in maintaining myometrial quiescence during pregnancy[7]. Endogenous NO suppresses myometrial connexin 43 gap junction protein (Cx43) expression during rat pregnancy. Cx43 is an important factor in uterus preparing for parturition. It links to increasing of uterine wall stretch due to inhibition of relaxation uterus and is important in parturition. These studies show that NO regulates the pregnancy maintenance by suppressing the expression of genes necessary for parturition.

As a key event in the preparatory changes preceding the onset of labor, the ripening of the cervix is similar to an inflammatory reaction accompanied by the remodeling of the extracellular matrix. In this process, the organization of the cervix becomes soft, which is required for rapid and safe delivery. Detailed mechanisms for this process are still poorly understood. Several studies in human and other animal models provide evidence that the expression of NO plays a critical role in cervical ripening. All three known NOS isoforms exist in rat cervix during pregnancy. In rat, the level of cervical iNOS mRNA and NO is elevated during natural cervical ripening[7]. Tschugguel et al.[29] reported that the production and activity of iNOS increased in human postpartum cervix. NO is probably involved in the degradation of extracellular matrix during cervical ripening by enhancing the production of matrix metalloproteinases (MMPs)[30]. In addition, NO is responsible for apoptosis and inflammatory reactions that have recently been proved to be tightly associated with cervical ripening. Therefore, one could speculate that NO may control the process of ripening by mediating the inflammatory reaction and apoptosis[31]. Further studies are necessary to elucidating the mechanism of NO in the cervical ripening and pregnancy.

The effects of three NOS isoforms on pregnancy are significantly different in pregnancy. Farina et al.[32] studied the production of NO during early, mid- and late pregnancy and found that NO synthesis was present in uterus throughout pregnancy. iNOS is the main producer of NO. The expression patterns of three NOS isoforms are different from each other during pregnancy. nNOS is not present throughout pregnancy, while eNOS is expressed during pregnancy and significantly increases on day 13 of pregnancy, with the lowest expression on the day after labor. iNOS production enhances substantially during pregnancy, and decreases at term and after labor in rat uteri.

3 Prospects of NO in research and application

As a signal molecule, NO is appreciated since the effects of NO on blood vessel were initially recognized. Recently, numerous studies show that NO plays an important role in both angiogenesis and reproductive events[33], and the former is tightly associated with normal female reproductive function[34]. These data extend the research field of NO in the female reproductive system.

Beside VEGF, a number of cytokines correlate with NO in the process of angiogenesis, whereby forms complicated regulating network involving NO. The effects of NOS in angiogenesis are dependent on the activated Akt pathway[35], and Akt mediates the activation of eNOS by calcium-independent regulatory mechanism[36]. Other studies suggest that NO might be involved in angiogenesis by TGF-β and integrin[37,38]. Chiarugi et al.[39] reported that cyclooxygenase-2 (COX-2) upregulated the PGE, which induced the regulators of angiogenesis, including VEGF, basic fibroblast growth factor (bFGF), TGF-β, and iNOS. Wild-type p53 protein inhibits angiogenesis process by various ways, such as enhancing the production of anti-angiogenic factor, diminishing angiogenic factors, and inducing apoptosis. iNOS is implicated in angiogenesis and metastasis of human cancers. *In vivo* studies show that wild-type p53 inhibits tumor growth through down-regulation of iNOS expression[40].

The importance of NO as a mechanism for angiogenesis has been well established. The cytokines, such as VEGF and TGF-β, interact with NO in angiogenesis, and also have an important role in female reproductive function. The coordination of various cytokines is necessary to the normal reproductive function. To further understand the mechanism of NO in the reproductive system, it is important to study the correlation of various cytokines at molecular level, by which the mechanisms of NO will be better understood.

Administration of traditional contraceptive medicines results in failure of pregnancy due to interference with one or some of the links in reproductive processes. Since NO is essential for diverse reproductive processes, such as follicular development, ovulation, implantation, and pregnancy maintenance, blockade of any events will result in the suppression of ovulation and pregnancy. During peri-implantation, treatment of rats with NOS inhibitors decreases the pregnancy rate by 50%[15]. These findings provide the theoretical basis for future development of contraceptive ways by blocking the production of NO.

There are many enigmas in reproductive system. Research on NO in the reproductive system is still limited. Future investigation will focus on elucidating the correlation of NO and cytokines in view of the role of NO in angiogenesis. This may provide a new idea for understanding the mechanism of reproduction.

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