Immunohistochemical Expression of Inducible Nitric Oxide Synthase and Distribution of Secretory Cells in Human Fallopian Tube

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Abstract

Background: The human fallopian tube provides an environment that enhances and supports fertilization and early embryonic development. Inducible nitric oxide synthase (iNOS) has been suggested that it participates in the regulation of tubal function. Additionally, secretions of secretory cells contain nutrients for spermatozoa, oocytes, and zygotes. The secretions also promote capacitation of the sperm.

Aim of the Work: To examine expression of iNOS and distribution of the secretory cells in the ampulla and isthmus of human fallopian tube during various stages of the reproductive and the postmenopausal periods.

Material and Methods: Fallopian tubes were obtained from 36 women at proliferative, secretory and postmenopausal periods. Samples were collected from the isthmus and the ampulla. Slides were stained with Haematoxyline & Eosin and immunohistochemical demonstration of iNOS. The following parameters were done; counting of the secretory cells and the area percent of expression of iNOS in the isthmus and the ampulla.

Results: During the proliferative phase, the mucosa was relatively thick, and covered by crowded columnar epithelial cells; the cells were of 2 types ciliated and non ciliated (secretory). Ciliated cells were more obvious in the ampulla than the isthmus. While secretory cells appeared more frequent in the isthmus as compared to the ampulla especially during the secretory phase. This was confirmed by counting of the secretory cells and the area percent of expression of iNOS in the isthmus and the ampulla.

Conclusions: The results of this study revealed difference in iNOS expression between the isthmus and the ampulla at the different phases which suggest a different role of NO in the different phases.

Key Words: Human Fallopian tube – iNOS – Secretory cells.
is generated from L-arginine by the group of enzymes called nitric oxide synthase (NOS) [8].

At least three different isoforms of this enzyme exist, i.e. constitutively expressed endothelial NOS (eNOS), neural NOS (nNOS) and an inducible form (iNOS). During the last decade nitric oxide (NO) has been ascribed an essential role as a regulator of various physiological processes. Among these, it has been shown that NO mediates smooth muscle relaxation of vascular [9], gastrointestinal [10], airway [11] and cavernous [12] tissues by increasing intracellular concentrations of cyclic guanosinemonophosphate (cGMP) [13]. It has also been shown that NO may be involved in maintaining uterine quiescence during pregnancy [14].

Recently, NOS has been identified in human, bovine [15] fallopian tubes. It has also been suggested that NO participates in the regulation of tubal function [8]. Tubal abnormalities and dysfunction (e.g. altered abnormal ciliary activity or contractility) are thought to lead to tubal ectopic pregnancy [18].

Thus the present study was carried out to examine expression of iNOS and distribution of secretory cells ampulla and isthmus of human fallopian tube during various stages of the reproductive and postmenopausal periods.

Material and Methods
I- Fallopian tube specimen collections:
A total of 36 fallopian tubes were obtained from women undergoing gynaecological operations such as total abdominal hysterectomy or salpingo-oophorectomy for benign conditions. The structural normality of the fallopian tubes was confirmed by later histologic examination. All specimens were collected from Almadinah Maternity and Child Hospital (MMCH) Saudi Arabia, between Nov. 2012 – March 2013. Approval for the study and informed consent was obtained from the patients according to the guidelines of the Ethics Standards Protocol. The 36 specimens were classified according to menstrual cycle and histological examination into three groups (each 12); proliferative, secretory and postmenopausal. Histological dating of the endometrium was performed as described by Palter et al., [19] Menopause was defined as the spontaneous cessation of menses for 12 months or more. None of the participants had received any hormonal medication within the 3 months before surgery.

Immediately after salpingectomy, oviductal samples were collected from isthmus and ampulla. Isthmic tubal segments were obtained 2cm from the fimbriated segment. Tubes with gross pathology were discarded from this study.

II- Tissue processing:
Specimens from fallopian tube in the three groups were taken and immediately placed in 10% buffered formalin and processed to prepare paraffin blocks, then cut into 5 µm thick sections and stained with Hematoxylin and Eosin [20]. Histological and morphometric study of distribution of secretory cells were done at the Research Unit, Faculty of Medicine, Taibah University, Al Madina Al Munawarrah, KSA.

III- Immunocytochemical staining:
For the immunohistochemical demonstration of iNOS, the specimens were dehydrated and embedded in paraffin wax. Serial sections (5 µm) were cut on a Leitz microtome and collected on gelatin/chrom alum-coated slides. To expose antigenic sites, dewaxed sections were heated four times to 95ºC in a 600W microwave oven in citrate buffer for 5min. Endogenous peroxidase activity was then eliminated by incubation with 0.5% (v/v) H2O2 solution in absolute methanol for 15min. at 20ºC. Non-specific protein binding was eliminated by incubation with 10% normal goat serum in PBS for 1h at 20ºC. Sections were then incubated with polyclonal rabbit antibody against iNOS (Upstate, Lake Placid, NY, USA). It was used at a dilution of 1:200. Incubation was performed at 18h at 4ºC in a humidified chamber. This was followed by incubating the sections with biotinylated anti-rabbit IgG 1:400 (Amersham-Pharmacia) for 1h. The sections were then incubated with ABC reagent from a commercial kit (Vector Laboratories, Burlingame, CA, USA). The bound antibody complex was made visible by reaction with 0.05% 3,3-DAB and 0.0006% H2O2 in 0.1g PBS. Sections were viewed unstained or counterstained in Mayer’s haematoxylin, dehydrated, cleared and mounted. Controls were performed by either replacing primary antibody with buffer or non-immune serum, or incubating with DAB reagent alone to exclude the possibility of non-suppressed endogenous peroxidase activity. Lack of detectable staining in the controls demonstrated the specificity of the reactions [18]. Immunohistochemical staining was done at Faculty of Medicine, Mansoura University, Egypt.

IV- Morphometric study:
Counting of secretory cells and immunohistochemical evaluations were carried out using the Image Analyzer (Digital camera CH-9435 DFC 290 coupled to photomicroscope; Leica Qwin standard, Wetzlar, Germany) at the Research Unit, Faculty of Medicine, Taibah University, Al Madina
Histological results:

Haematoxylin and Eosin stained sections of normal human fallopian tubes during the proliferative phase revealed that the mucosa was relatively thick, and covered by crowded columnar epithelial cells; the cells were of 2 types ciliated with prominent cilia and non-ciliated (secretory). Ciliated cells and mucosal folds were obvious in the ampulla while it was less frequent in the isthmus. The ciliated cell has a columnar shape and contains oval or round nucleus, located perpendicular or parallel to the long axis of the cell. The secretory cell is narrow columnar cell with approximately the same height as the ciliated cell. The nucleus is ovoid and perpendicular to the long axis of the cell. The chromatin was denser than that seen in the ciliated cell. Secretory cells appeared more frequent in the isthmus when compared to the ampulla. The stroma consisted of connective tissue fibers and blood vessels (Figs. 1,2).

In the secretory phase, the epithelial cells were less crowded with some darkly stained nuclei (pyknotic). Pulging of the secretory cells and prominent stromal blood vessels were observed (Figs. 3,4). While in postmenopausal phase, the tubal mucosa was atrophied and widened less vascular stroma can be seen (Figs. 5,6).

Immunohistochemical results:

Sections of the ampulla and the isthmus stained immunohistochemically with iNOS showed moderate immunopositive epithelial cells and immunopositive cells in the lamina muscularis and wall of blood vessels in the proliferative phase (Figs. 7,8).

While in the secretory phase, the reaction was more intense in the isthmus (Fig. 10) when compared to the ampulla (Fig. 9).

In the postmenopausal phase, the reaction was mild in the ampulla (Fig. 11) and moderate in the isthmus (Fig. 12).

Statistical analysis:

The statistical analysis of the morphometric measurements in the ampullary and the isthmic mucosa among the studied groups showed a significant increase in the number of the secretory cells in the isthmic epithelium during the secretory phase when compared with the ampullary epithelium as shown in the graph below. The area % of iNOS expression showed that the expression of iNOS was more in the isthmus when compared with the ampulla in different phases. However, the difference was significant only in the proliferative phase and highly significant in the secretory and the postmenopausal phases.

In the ampulla, the expression of iNOS decreased from the proliferative to the postmenopausal phases. While in the isthmus, the expression of iNOS increased from the proliferative to the secretory then decreased again in the postmenopausal phase as shown in the table below.

| Table (1): Mean values of area % of iNOS (±SE) in different studied groups. |
|-----------------------------|-------------------------------|-----------------------------|-----------------------------|
| Menstrual phase     | Ampulla (N=18) | Isthmus (N=18) | p-value |
| Proliferative       | 21.90±0.23       | 23.03±0.19       | 0.001* |
| Secretory          | 10.26±0.20       | 32.45±0.25       | 0.000** |
| Postmenopausal      | 8.49±0.19        | 20.39±0.19       | 0.000** |

SE : Standard error. ** : Significant p<0.001. * : Significant p<0.05. N : Number.
Figs. (1,2): Photomicrographs of sections from the ampulla (1) and the isthmus (2) of the human uterine tube during the proliferative phase showing crowded epithelial cells (E) with prominent cilia (arrowheads) and secretory cells (arrows) with underlying stroma (S). (Hx. & E.; X400).

Figs. (3,4): Photomicrographs of sections from the ampulla (3) and the isthmus (4) of the human uterine tube during the secretory phase showing less crowded epithelial cells with short cilia (red arrows), with pyknotic darkly stained nuclei (arrowheads), and prominent stromal blood vessels (v). Surface domes and pulging of secretory cells were observed (black arrows). (Hx. & E.; X400).

Figs. (5,6): Photomicrographs of sections from the ampulla (5) and the isthmus (6) of the human uterine tube during the postmenopausal phase showing atrophied epithelial layer (arrows) with widened less vascular stroma (S). Ciliated and secretory cells (arrow heads) appeared darkly stained. (Hx. & E.; X400).
Figs. (7,8): Photomicrographs of sections from the ampulla (7) and the isthmus (8) of the human uterine tube in proliferative phase showing moderate immunopositive epithelial cells (thick black arrows) and immunopositive cells in the lamina muscularis (thin black arrow) and wall of blood vessels (red arrow). (Anti-iNOS, X400).

Fig. (9): A photomicrograph of sections from the ampulla of the human uterine tube in secretory phase showing mild immunopositive reaction in epithelial cells (thick black arrows) and in the lamina muscularis (thin black arrow) and wall of blood vessels (red arrow). (Anti-iNOS, X400).

Fig. (10): A photomicrograph of sections from the isthmus of human uterine tube in secretory phase showing intense immunopositive reaction in epithelial cells (thick black arrows) and in the lamina muscularis (thin black arrow) and wall of blood vessels (red arrow). (Anti-iNOS, X400).

Fig. (11): A photomicrograph of sections from the ampulla of the human uterine tube in postmenopausal phase showing mild immunopositive reaction in epithelial cells (thick black arrow) and in the lamina muscularis (thin black arrow) and wall of blood vessels (red arrow). (Anti-iNOS, X400).

Fig. (12): A photomicrograph of sections from the isthmus of the human uterine tube in postmenopausal phase showing moderate immunopositive reaction in epithelial cells (thick black arrows) and in the lamina muscularis (thin black arrow) and wall of blood vessels (red arrow). (Anti-iNOS, X400).
Discussion

Gamete transport, sperm capacitation, final oocyte maturation, fertilization, and early embryonic development occur in direct contact with the mucosal epithelium of the oviduct. Hence, the functional state of the uterine tubes plays a major role in fertility and reproduction and the histopathological cytology may provide new information in this field [21].

The ampullary and the isthmic region of the uterine tube has been selected in this study as the ampulla showed prominent cyclic changes than other regions, indicating the sensitive response of this region to female hormonal levels; in addition fertilization usually takes place in the ampulla [22]. In the isthmic region, the smooth muscle cells constitute major tissue components. The muscle function of the isthmic segment is a sphincter-like contractile activity may be of special importance [23], thus, functional disorders of tubal contractile activity, i.e., prolonged spasms of the isthmic region, may lead to the retention of the fertilized ovum, thereby causing infertility or tubal pregnancy [24]. The secretory cells produce a nutrient rich fluid that bathes the sperm and egg and provides the environment in which the gametes can find each other [25].

Light microscopic examination of the ampullary and the isthmic mucosa of uterine tubes during the proliferative phase showed that the mucosa is covered by crowded columnar epithelial cells (ciliated and secretory). In secretory phase, the epithelial cells were less crowded with some darkly stained nuclei. These findings are in agreement with Lyons et al., [26], who reported that the human uterine tube has the capacity to undergo dynamic endocrine-induced changes during the menstrual cycle, including cell growth and regeneration, providing the unique environment required for the maintenance of male and female gamete viability, fertilization, and early embryonic development as well as transport to the uterus.

Atrophied mucosa was observed in postmenopausal phase in the current study, which is in agreement with previous investigators, who observed a postmenopausal gradual atrophy of all cell types of human oviductal epithelium with a significant decrease in the epithelial height, the percentage of ciliated cells, and in the secretory function [27].

In the current work, the ciliated cells with prominent cilia in the proliferative phase were obvious in the ampulla when compared to the isthmus. While the secretory cells showed significant increase in the isthmus during the secretory phase. In women, the ciliogenesis in the follicular phase occurs under the influence of estrogen, whereas progesterone leads to deciliation [21]. The presence of a normal ciliary activity during the follicular phase and at ovulation is a very important factor regulating gamete transport rate and the reproductive process [28]. Ekerhovd et al., [13] reported that, the secretory cells are more numerous in the the isthmus, while ciliated cells dominate in the ampulla.

This study investigated iNOS expression in the ampulla and the isthmus at different phases. iNOS expression was more in the isthmus as compared to the ampulla. The difference was high significant in the secretory phase as compared to the proliferative phase. iNOS expression was observed in epithelial cells, muscle layer and wall of blood vessels. These results are in agreement with Ekerhovd et al., [13] who observed positive staining for iNOS in smooth muscle, epithelium, vascular endothelium, and connective tissues of human oviduct. In contrast, Gawronska et al., [8] reported that, iNOS immunoreactivity was confined to the endothelium of the lymphatics and some blood vessels. They explained that, these differences may result from species differences or may be attributable to low antigenicity of the primary antibody, which could not detect low concentration of iNOS in porcine oviduct. Because progesterone can be locally concentrated in the blood, reaching the oviduct immediately after ovulation, it is possible that NO (apart from its own direct influence on the oviduct) activates cyclooxygenase enzymes to increase the production of PGE2. The increase of NADPH-d activity in myosalpinx exclusively at the postovulatory stage of the estrous cycle can be connected with relaxation of the oviduct for passage of the embryos to the uterus after fertilization [13].

The increase in NOS activity in myosalpinx of the isthmus up to Day 4 of the estrous cycle can inhibit motility of this part of oviduct, facilitating passage of embryos into the uterus after fertilization [8].

Using L-NAME (N-nitro-L-arginine methyl ester), a well-known inhibitor of NOS, Perez et al., [29] found evidence of increased tubal motility that resulted in accelerated ovum transport into the uterus. Moreover, oestradiol treatment caused increasing contraction frequency of the smooth muscle of the isthmus [18]. This could be mediated by oestrogen receptor, which is more abundant in the isthmus [30]. The endogenous local downregulation of iNOS in the isthmus could support similar effects. Therefore,
our hypothesis is that the downregulation of iNOS at oestrus in the isthmus leads to an increase of oviduct motility by circular smooth muscle activity. Furthermore, through the ability of iNOS to produce cytotoxic levels of NO [31], the downregulation of iNOS in the isthmus at oestrus could be an implicit protective mechanism for advancing sperm and the developing embryo. Al-Azem et al., [32] reported that, the cyclicity in iNOS expression by the tube suggests its involvement in fertilization and early embryonic development. Pathologic generation of nitric oxide through increase iNOS production may decrease tubal ciliary beats and smooth muscle contractions and thus affect embryo transport, which may consequently result in ectopic pregnancy. Moreover, Refaat et al., [33] reported that, the expression of iNOS was statistically significantly increased in the tubes bearing an ectopic pregnancy (EP).

Conclusion:

There is a difference in iNOS expression between the isthmus and the ampulla at the different phases which suggest a different role of NO in the ampulla and the isthmus regions. Also, the distribution of the secretory cells at the isthmus was more as compared to the ampulla which is important for fertilized ovum nutrition. The present findings underline the physiological influence of both iNOS and the secretory cells in supporting a successful fertilization by regulating the oviduct environment.

References

19- PALTER S.F., MULAYIM N., SENTURK L. and ARICI A.: Interleukin-8 in the human fallopian tube. Journal of
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