Acta Histochemica 116 (2014) 871–877

Contents lists available at ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.de/acthis

Decreased nerve fibers in the oviduct isthmus of women with endometriosis

Libo Zhu, Qiongshi Huang, Xiufeng Huang, Jing Zhang, Hong Xu, Xinmei Zhang*

Zhejiang University School of Medicine, Hangzhou, Zhejiang 310006, PR China

ARTICLE INFO

Article history:
Received 9 December 2013
Received in revised form 18 February 2014
Accepted 19 February 2014

Keywords:
Endometriosis
Nerve fibers
Immunohistochemistry
Fallopian tubes

ABSTRACT

Oviduct tubal motility is thought to be controlled by hormones and nerves and has been associated with endometriosis. However, it is still not known whether the fallopian tubes in women with endometriosis demonstrate an abnormal distribution of nerve fibers. The objective of this study was to determine the distribution of nerve fibers in the oviduct isthmus in women with and without endometriosis. Histological sections of the oviduct isthmus tissues were obtained from women undergoing hysterectomy for endometriosis ($n = 24$) and other benign gynecologic diseases ($n = 24$). The tissues were immunohistochemically stained for protein gene product (PGP) 9.5, substance P (SP), neuropeptide Y (NPY), and vasoactive intestinal peptide (VIP) to reveal all nerve fibers, sensory nerve fibers and sympathetic and parasympathetic nerve fibers. Nerve fibers stained with PGP9.5, VIP and NPY in the oviduct isthmus were all significantly decreased in women with endometriosis as compared with women without endometriosis ($P < 0.05$). In women with endometriosis, reduced nerve fibers stained with PGP9.5 and SP in the serosal layer, NPY in the muscular and mucosal layers, and VIP in the mucosal layer of the oviduct isthmus were all associated with the severity of the disease ($P < 0.05$). These results suggest that decreased nerve fibers in the oviduct isthmus in women with endometriosis in comparison to women without may imply a role in the pathogenesis of endometriosis.

Introduction

Endometriosis is defined by the presence of functional endometrium outside the uterine cavity, resulting in dysmenorrhea, dyspareunia, pelvic pain, and infertility (Sun et al., 2012). Although the causes of endometriosis still remain unclear, a composite theory of retrograde menstruation with implantation of endometrial fragments in conjunction with peritoneal factors to stimulate cell growth is widely accepted (Zhang et al., 2004). Obviously, apart from the abnormalities of tubal anatomy, tubal dysmotility may also be associated with endometriosis (Zhang et al., 2012).

It has been suggested that the oviduct motility is controlled by hormones and nerves (Eddy and Pauerstein, 1980; Atoji et al., 2000; Steffl et al., 2006; Neamţu et al., 2009). The fallopian tube is innervated with cholinergic and adrenergic fibers, which are supplied by the hypogastric plexus containing myelinated and unmyelinated, pre- and postganglionic fibers (Marshall, 1970; Jankovic et al., 2004). In addition to the cholinergic and adrenergic innervation of the fallopian tube, several neuropeptides have also been detected in nerve fibers of the fallopian tube (Samuelson and Dalsgaard, 1985; Samuelson et al., 1985; Heinrich et al., 1987). These nerves and neuropeptides in the fallopian tube are considered to supply the vasculature, the smooth muscle and the surface epithelium (Marshall, 1970; Samuelson and Dalsgaard, 1985; Samuelson et al., 1985; Heinrich et al., 1987; Jankovic et al., 2004), and thus regulating the smooth muscle contractility and ciliary beat activity of the fallopian tube (Marshall, 1970; Samuelson and Dalsgaard, 1985; Samuelson et al., 1985; Heinrich et al., 1987; Jankovic et al., 2004; Shaw et al., 2010). Apparently, abnormal distribution of nerve fibers in the fallopian tube may cause dysfunctional tubal motility, which is associated with endometriosis.

Recently, Cajal-like interstitial cells, which are thought to be the oviduct pacemaker cells and to regulate neurotransmission, have been shown to be decreased in the fallopian tube in women with endometriosis as compared with women without endometriosis (Popescu et al., 2005; Yang et al., 2013). This dysfunctional tubal motility associated with nerves may imply a possible abnormal distribution of nerve fibers in the fallopian tube in women with endometriosis, which plays a role in the pathogenesis of the disease

* Corresponding author at: The Department of Gynecology, Women's Hospital, School of Medicine, Zhejiang University, 1 Xueshi Road, Hangzhou, Zhejiang 310006, PR China.
E-mail address: zxm20130729@163.com (X. Zhang).

http://dx.doi.org/10.1016/j.acthis.2014.02.005
0065-1281/

(Zhang et al., 2012). It has been shown that the specific sphincter-like function of the fallopian tube is located at the isthmus, which is more densely innervated when compared with other segments of the fallopian tube (Zhu et al., 2013). Therefore, in this study we aimed to determine the distribution of nerve fibers in the oviduct isthmus in women with endometriosis in comparison with women without endometriosis. The oviduct isthmus tissues were immunochemically stained with protein gene product (PGP) 9.5, substance P (SP), neuropeptide Y (NPY) and vasoactive intestinal peptide (VIP) to reveal all nerve fibers, sensory nerve fibers and sympathetic and parasympathetic nerve fibers so as to compare the differences between women with and without endometriosis.

Materials and methods

Tissue collection

Between December 2011 and January 2013, the oviduct isthmus tissues were obtained from 48 women undergoing laparoscopic hysterectomy for endometriosis (median age: 39.5 years; range: 31–47 years; $n = 24$) and for other benign gynecologic diseases (median age: 38.5 years; range: 29–45 years; $n = 24$). In women with endometriosis (case group), 13 women (54.2%) had pain symptoms, while in women without endometriosis (control group), only 4 women (16.7%) complained of pain. Endometriosis was graded according to the Revised American Fertility Society scoring (rAFS) system (I + II = 11, III + IV = 13) (American Society for Reproductive Medicine, 1997).

All study subjects were fertile women, and had bilateral tubal patency, which was confirmed by chromotubation under laparoscopy. The samples from both groups were obtained from the right oviduct isthmus to facilitate the comparison between groups. The specimens of the oviduct isthmus sampled from all study subjects underwent thorough histological analysis (Vang and Wheeler, 2010). None of the patients received sex-hormone therapy prior to surgery. The study was approved by the Human Ethics Committee of the Women's Hospital, School of Medicine, Zhejiang University. All subjects gave their informed consent to participate in the study.

Histology and immunohistochemistry

After surgical excision, all samples of the oviduct isthmus were fixed in 10% neutral buffered formalin for approximately 18–24 h, processed and embedded in paraffin wax according to a standard protocol. We obtained five 6 μm thick sections from each isthmus, with one section used for hematoxylin and eosin staining and four sections for immunohistochemical staining. We used PGP9.5, a highly specific pan-neuronal marker, to determine the presence of nerve fibers in the isthmus without differentiating them, and used SP, NPY and VIP to stain sensory nerve fibers and sympathetic and parasympathetic nerve fibers, respectively.

Immunohistochemical staining was performed as previously described by Kelm et al. (2008). Serial sections, 6 μm thick, were immunostained using polyclonal rabbit anti-PGP9.5 antibody (dilution 1:700, Z5116; Dako Cytomation, Glostrup, Denmark), polyclonal rabbit anti-SP antibody (1:400, ab14184; Abcam, Cambridge, MA, USA), polyclonal rabbit anti-NPY antibody (1:100, 12833-1-AP; Proteintech Group, Chicago, IL, USA), and polyclonal rabbit anti-VIP antibody (1:150, ab8556; Abcam, Cambridge, MA, USA) for 60 min at room temperature. The sections were washed in phosphate-buffered saline (PBS) and incubated with Envision-labeled polymer-alkaline phosphatase mouse/rabbit (EnVisiont/HRP/Mo, GK400105; EnVisiont/HRP/Rb, GK400305/15; Novocastra, Newcastle-upon-Tyne, UK) for 60 min. The antigen–antibody reaction was visualized using diaminobenzidine (DAB) as chromogen (GK346810; Novocastra, UK). After washing, the sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted with a mounting medium. Normal vulval skin was used as the positive control group. Negative controls were incubated with rabbit immunoglobulin fraction (X 0936; Dako, Glostrup, Denmark) instead of the primary antibody. An experienced gynecological pathologist, who was unaware of the sample background, evaluated the samples using histopathology and immunostaining of the oviduct isthmus (Vang and Wheeler, 2011). If no immunoactive nerve fibers appeared on the slides, we defined them as negative cases. Otherwise, we designated them as positive cases and counted the percentage (positive cases/total cases) as well as positive nerve fibers.

Quantification of nerve fiber density

We used a microvascular density quantification method previously described by Weidner (1995) with minor modification to count the number of nerve fibers identified by PGP9.5, SP, NYP, and VIP staining in the isthmus (Weidner, 1995; Zhang et al., 2010a,b). After immunostaining, the entire section was scanned at low power (100×) (Leica MZ16 microscope system, Leica Microsytems, Wetzlar, Germany) to identify "hot spots", which represent the areas of highest innervation. The individual nerve fibers were then counted at high magnification (400×) to obtain a nerve count in a defined area. The total number of nerve fibers was divided by the total number of hot spots on each section to obtain an average of nerve fibers per hot spot (each hot spot measuring 1 mm^2). The results were expressed as the mean (±SD) number of nerve fibers/mm^2 in each specimen from all isthmus samples. These hot spots were present in the serosal, muscular and mucosal layers of the oviduct isthmus wall. The average nerve count in five hot spots was calculated because no significant difference was found in the total number of hot spots between the study groups. Two independent observers who were blind to the sample background counted the number of PGP9.5, SP, NYP, and VIP-positive nerve fibers.

Statistical analysis

We used the Statistical Package for the Social Sciences Version 13.0 (SPSS, IBM, Chicago, IL, USA) to perform statistical analyses. The results were expressed as the mean (±SD) number of nerve fibers/mm^2 in each specimen from all isthmus sections, although the measured values of the variables were not normally distributed. The Mann–Whitney *U*-test was used to compare the differences in the nerve fiber density, age, parity, and abortion between groups, respectively. The χ^2 test was used to compare the differences in the percentage of immunoreactive nerve fibers between groups. Differences were considered significant at $P < 0.05$.

Results

No significant differences were found with regard to age at operation, parity or history of previous abortion between women with and without endometriosis ($P > 0.05$), although women with endometriosis had a higher percentage with pain symptoms when compared with women without ($P < 0.01$, Table 1). SP and VIP-immunoreactive nerve fibers in the oviduct isthmus could be detected in all study subjects (100%, 48/48). PGP9.5 and NPY-immunoreactive nerve fibers in the oviduct isthmus could be detected in all 24 women without endometriosis (100%, 24/24), but reduced to 95.8% (23/24) in women with endometriosis (Table 1). There were no significant differences with respect to the percentage of PGP9.5, SP, NPY or VIP-immunoreactive nerve fibers in the oviduct isthmus between women with and without endometriosis ($P > 0.05$, Table 1).