

Comparative Study of Nerve Fiber Density between Adenomyosis Patients with Moderate to Severe Pain and Mild Pain

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Background: Since some retrospective studies have given inconsistent findings about innervation in adenomyosis, its role in the pain mechanism is still inconclusive.

Objective: Define the nerve fiber density in adenomyotic tissue as it correlated to pain symptoms.

Material and Method: A cross-sectional study was performed in twenty-five uterine samples from reproductive age women with adenomyosis who underwent either laparotomy or laparoscopic surgery. The nerve fiber density from hysterectomized specimens as measured by immunohistochemistry staining for Protein gene product (PGP) 9.5 and Neurofilament (NF) were compared with the level of pain in the patients as defined by a visual analogue scale and a verbal rating scale.

Results: Nerve fibers as detected by PGP9.5 and NF staining in the myometrium were significantly increased in the group of women with adenomyosis experiencing moderate and severe pain as compared to the group experiencing less pain (4 (0, 7) vs. 1.55 (0, 7)/mm², p-value <0.001, and 6 (3, 10) vs. 0 (0, 4)/mm², p-value <0.001 respectively). At both phases of the menstrual cycle, the densities of nerve fibers stained with PGP9.5 and NF showed no significant difference.

Conclusion: These results suggested that the increased of nerve fibers shown in the more severe pain group might play a role in the pathogenesis or symptoms of adenomyosis.

Keywords: Adenomyosis, Nerve fibers, Pain, Immunohistochemistry

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Adenomyosis is a common gynecologic disorder with poorly understood pathogenesis⁽¹⁾. It is generally described as “the benign invasion of endometrium into the myometrium, producing a diffusely enlarged uterus which microscopically exhibits ectopic non-neoplastic, endometrial glands and stroma surround by the hypertrophic and hyperplastic myometrium”⁽²⁾. The frequency of adenomyosis in hysterectomy specimens reported in the literature varies from 5 to 70%^(2,3).

Clinically, symptoms of adenomyosis include dysmenorrhea, heavy menstrual bleeding, and subfertility. Treatment has been a challenge, and hysterectomy is still the treatment of choice for adenomyosis⁽¹⁾. Surprisingly, although the disease is

hormone-sensitive, progestogenic agents are still not a very effective means of treatment. Gonadotropin-releasing hormone (GnRH) agonist use is an effective treatment but its use is restricted due to its adverse effects⁽¹⁾. In addition, the symptoms often recurrent after discontinuation of the therapy⁽⁴⁾. Adenomyosis is being found incidentally in younger infertile women or those who present with dysmenorrhea and/or menorrhagia. Given that the diagnosis is being made more frequently in younger women, new therapeutic options are being investigated to eliminate the need for hysterectomy⁽⁵⁾.

Adenomyosis and endometriosis share some similarities in definition, including their estrogen-dependence, symptomatology, treatment modality, and their many documented molecular aberrations^(6,7). Some investigators suggest that certain common mechanisms may be involved in the pathogenesis of both diseases⁽⁸⁾.

One common symptom of both diseases that places a burden on the quality of life is pain, but the

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exact cause of the pain is still unknown. Increasing evidence demonstrates that the endometrium is innervated by small nerve fibers in women with endometriosis but not in women without endometriosis⁽⁹⁻¹¹⁾. Therefore, it is suggested that innervation of the endometrium is unique to women with endometriosis, and may be used as a diagnostic tool for this disease⁽⁹⁾. Adenomyosis and endometriosis are believed to be under the control of the same steroid hormones^(12,13), share remarkable similarities^(6,7,14) and often occur concurrently⁽¹⁴⁾. One of the publications investigated the expression of nerve growth factor (NGF) in the ectopic endometrium in adenomyosis patients and explored the relationship between NGF expression and innervation or pain scales. They also had found that the NGF level and the density of PGP9.5 positive nerve fibers were higher in adenomyosis pain group than painless group⁽¹⁵⁾. The nerve fibers could not be detected in adenomyotic lesions, and were nonexistent at the endometrial-myometrial interface of the adenomyotic uterus⁽¹⁶⁾. On the contrary, some investigators demonstrated the presence of nerve fibers in the hysterectomized specimen in women with pain symptoms whether the women had endometriosis, adenomyosis, fibroids, or endometriosis with adenomyosis, but not in women without pain, suggested the role of nerve fibers in the functional layer of the endometrium being played in pain generation^(17,18). Since these retrospective studies have given inconsistent findings about innervation in adenomyosis, its role in the pain mechanism is still inconclusive. The authors conducted the present study to define the nerve fiber density in adenomyotic tissue in correlation with pain symptoms, as this may be one important part of pain mechanism.

Material and Method

Thirty-two healthy reproductive age women with adenomyosis and having indications for hysterectomy were consecutively recruited in the study. The present study was conducted between August 2012 and March 2013, and was approved by the Ramathibodi Hospital Ethics Committee on Human Research. Informed consents were obtained from all participants. The medical histories and investigations were reviewed and patients were excluded for other suspected concurrent diseases or for hormonal used within three months. In addition, women who had pain symptoms due to conditions other than adenomyosis were excluded. The women in the present study were operated on either by laparoscopy or laparotomy.

Dysmenorrhea and pelvic pain were evaluated before the day of surgery using a 10-cm Visual Analog Scale (VAS). The authors classified the patients into two groups depending on their pain levels and using VAS cut off point level 0-4 as mild pain and 5 to 10 as moderate to severe pain. The specimens were collected immediately after the uterus was removed from the body. The hysterectomized specimens were sampled in full thickness fashion from uterine isthmus and uterine corpus based on gross appearance of the adenomyosis on the cut-surface. Samples were immediately fixed in 10% neutral buffered formalin for approximately 18 to 24 hours, processed, and embedded in paraffin wax according to the standard protocol. Three-micrometer-thick sections were cut and routinely stained with hematoxylin and eosin. To evaluate the nerve bundles, the tissue sections were mounted on positively charged slides and baked overnight at 60°C prior to immunostaining. The primary antibodies included Protein gene product (PGP) 9.5 (Agilent Technology, Santa Clara, CA, USA; dilution 1:1,000) and Neurofilament (NF) (Agilent Technology, Santa Clara, CA, USA; dilution 1:200). Immunostaining was performed on the Ventana Benchmark auto-immunostainer (Tucson, AZ, USA) using the ultraVIEW Universal DAB (diaminobenzidine) Detection Kit (Ventana DAB Universal kit; Ventana-Bio Tek Solutions; Tucson, AZ, USA). Normal skin was used as positive control since it reliably contains myelinated and unmyelinated nerve fibers. Phosphate buffered saline was used as a negative control.

Both PGP9.5- and NF-stained slides were examined at low power (10x objective) for areas of increased numbers of neural bundles (hot spots). The hot spots were photographed using a 40x objective on a Nikon Eclipse E600W microscope equipped with a digital Nikon camera and DXM1200F software (Nikon Instrument Inc., Tokyo, Japan). Four fields were selected per slide providing a total area of 1 mm². The neural bundles of the studied cases at the uterine isthmus and uterine corpus were counted in each of the four fields, and the combined results were the count for neural densities/mm². The nerve fiber density was counted (with blinded patient data) by both the author and an experienced consultant pathologist. The discordant results were re-evaluated to reach a consensus.

Statistical analysis was performed by using STATA version 12 (Statacorp LP, TX, USA). Sample size was calculated by the comparison two independent means between two groups with power 0.80 and mean

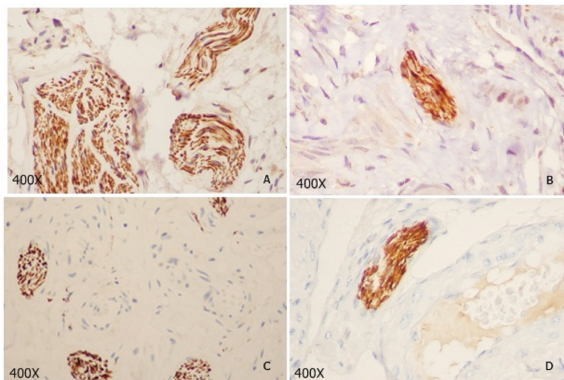


Fig. 1 Show PGP9.5 stain; moderate to severe pain (A) and mild pain (B) NF stain; moderate to severe pain (C) and mild pain (D).

(SD) in control group of 1.275 (1.069). Continuous data were presented as the mean with standard deviation (SD) for normal distribution and median with interquartile range (IQR) for non-normal distribution. Categorical data were presented as numerical with percentage. Student's t-test and quartile regression were used to assess the difference of continuous data between two independent groups. Chi-square test and Fisher's exact test were used to assess the difference of categorical data between two independent groups. A *p*-value less than 0.05 was considered statistically significant.

Results

Seven patients who had other coexisting lesions were excluded. The study was performed on the remaining twenty-five patients. The average patient age was 43.44 years old. The indications for hysterectomy included pain and hypermenorrhea (32% with pain, 28% with hypermenorrhea, and 40% who complained of both). The average VAS for pain was 5.024 (range 0-10).

The patients were divided into two groups, using the visual analogue scale (VAS) cut off point adopted from cancer pain grading⁽¹⁹⁾ and pelvic pain level study⁽²⁰⁾. A VAS rating of 5 or more was categorized as the moderate to severe pain group.

The baseline characteristics were presented in Table 1. Mean age was comparable in both groups (44.55±3.3 vs. 42.57±6.9, *p*-value 0.397). There were no statistical differences in BMI, marital status, or menstrual phases. As in Table 2, the mean and median of the nerve fiber density were statistically significant. It was greater in the moderate to severe pain group when compared with the mild pain group as determined

by PGP9.5 (6.07±1.98 vs. 4.45±0.69, *p*-value 0.011 for isthmus sampling, 4 (4, 5) vs. 1.55 (0, 2), *p*-value <0.001 for lesion sampling), and by NF (7.36±2.50 vs. 5.09±1.92, *p*-value 0.021 for isthmus sampling, 6 (5, 8) vs. 0 (0, 0), *p*-value 0.001 for lesion sampling).

There were no statistical differences in comparison between menstrual phase on the day of surgery, and nerve fiber density in all areas as shown in Table 3.

Discussion

Based on the literatures, the role of nerve fiber density is still controversial. It has been reported that the innervation of the endometrium is uniquely present in women with endometriosis⁽¹¹⁾. PGP9.5-immunoreactive small nerve fibers appeared in the functional layer of the endometrium in women with endometriosis, which may be considered as a diagnostic marker for this disease⁽¹¹⁾. However, some studies suggest that these nerve fibers could also be found in women with painful uterine fibroids and adenomyosis, but not in women with painless uterine fibroids or adenomyosis⁽¹⁸⁾. These studies were conducted in retrospective fashion; the inconsistent results might due to recall bias. Furthermore, it was impossible to evaluate entire specimens from block specimens.

To investigate the types of nerve fibers in the endometrium and myometrium in women with endometriosis, Tokushige et al had conducted a study using many specific immunohistochemical neural markers. They included polyclonal rabbit

Table 1. Demographic data between moderate to severe pain group and mild pain group

Variables	VAS <5 n = 11	VAS ≥5 n = 14	<i>p</i> -value
Age (years)	44.55±3.3	42.57±6.9	0.397 ^a
BMI	23.83±2.6	21.98±3.5	0.140 ^a
Marital Status (%)			0.423 ^b
Single	2 (18.2%)	2 (14.3%)	
Married	7 (63.6%)	12 (85.7%)	
Divorced	1 (9.1%)	0	
Widowed	1 (9.1%)	0	
Menstrual phase (%)			0.607 ^b
Proliferative phase	6 (54.5%)	8 (57.1%)	
Secretory phase	5 (45.5%)	6 (42.9%)	

VAS = visual analog scale; BMI = body mass index
Data presented as mean ± SD, number in cohort (%)

^a Student's t-test

^b Fisher's exact test

Table 2. PGP9.5 and NF-immunoreactive nerve fiber density in the isthmus area and adenomyotic lesion area (per mm²)

Nerve fiber protein	Location	VAS <5 (n = 11)	VAS ≥5 (n = 14)	p-value
PGP9.5	Isthmus area, mean ± SD	4.45±0.69	6.07±1.98	0.011 ^a
	Lesion area, median (IQR)	1.55 (0, 2)	4 (4, 5)	<0.001 ^b
NF	Isthmus area, mean ± SD	5.09±1.92	7.36±2.50	0.021 ^a
	Lesion area, median (IQR)	0 (0, 0)	6 (5, 8)	<0.001 ^b

VAS = visual analog scale; NF = neurofilament protein; PGP9.5 = protein gene product 9.5

Data presented as mean ± SD

^a Student's t-test which compare mean between two independent groups

^b Quantile regression analysis which compare Median between two independent groups

Table 3. Comparison of visual analog scale and nerve fiber density between proliferative and secretory phase specimens

	Location	Proliferative phase (n = 14)	Secretory phase (n = 11)	p-value
VAS, median (IQR)		5.9 (0, 7.8)	7.5 (1.8, 7.9)	0.455 ^b
PGP9.5	Isthmus area, mean ± SD	5.64±1.65	5.00±1.84	0.367 ^a
	Lesion area, median (IQR)	4 (0, 5)	4 (2, 4)	1.000 ^b
NF	Isthmus area, mean ± SD	6.71±2.92	5.91±1.87	0.435 ^a
	Lesion area, median (IQR)	3 (0, 5)	6 (0, 8)	0.157 ^b

VAS = visual analog scale; NF = neurofilament protein; PGP9.5 = protein gene product 9.5

^a Student t test which compare mean between two independent groups

^b Quantile regression analysis which compare Median between two independent groups

anti-protein gene product 9.5 (PGP9.5), monoclonal mouse antihuman neurofilament (NF), polyclonal rabbit anti-SP, rabbit anti-CGRP, polyclonal rabbit anti-vesicular acetylcholine transporter (VACHT), monoclonal antityrosine hydroxylase (TH), polyclonal rabbit anti-vasoactive intestinal polypeptide (VIP), and polyclonal rabbit anti-neuropeptide Y (NPY). They found that the nerve fibers that stained with PGP9.5 and NF in the endometrium and myometrium were significantly increased in women with endometriosis compared to women without endometriosis⁽¹⁰⁾. Therefore, we decided to use PGP9.5 and NF immunohistochemistry staining as the nerve fiber detection method.

The authors separated the patients into two groups depending on their pain levels using VAS cut off point level of 5 or more as significant pain (moderate to severe pain)^(19,20). The present study results showed that patients with high preoperative pain scores displayed higher densities of nerve fibers in the uterine isthmus and within and/or around adenomyotic lesions than patients with lower preoperative pain scores, which was consistent with the previous study^(17,18). It also supported the hypothesis that these nerve fibers might play a role as part of the pain mechanism more than being a pathognomonic sign of disease. In adenomyosis, it could help to explain the poor correlation between the extent of the disease and the

intensity of the pain symptoms, as illustrated by some cases having huge infiltrative uterus, which presented with hypermenorrhea but with no pain, as opposed to some cases in which a small lesion appeared to be the cause of pelvic pain.

In the present study, the authors also attempted to compare nerve fiber densities in different phases of menstruation. The result showed the nerve fiber density was not statistically significant different between proliferative and secretory phase, according to both PGP9.5 and NF staining. These findings might explain why some patients have painful symptoms such as chronic pelvic pain unrelated to the menstrual cycle.

To determine the exact factor related to nerve fiber proliferation, more studies are needed. Nerve growth factor (NGF) is most interesting. The polypeptide belongs to the neurotrophin family and binds two classes of transmembrane receptors: high-affinity receptor tyrosine kinase receptor-A (TrkA) and low-affinity receptor NGF receptor p75 (NGFR p75). NGF and its receptors play an important role in mediating neuropathological and non-neuropathological pain. Studies in a mouse model demonstrated a gradually increasing level of Nerve Growth Factor beta (NGF-beta) and its receptors in the uterus as the disease becomes more severe which suggests an effect of NGF-beta on the pathogenic mechanisms of adenomyosis⁽²¹⁾. In correlation with

nerve fiber density, one study showed NGF expression in peritoneal fluid from women with endometriosis by using in vitro neuronal growth assay⁽²²⁾, and demonstrated that NGF is the important factor that mediated nerve growth and innervation in peritoneal endometriosis. These relationships may provide explanations for pain symptoms. However, further studies are needed to define other possible factors involve nerve fiber proliferation and may be of possible benefit in adenomyosis treatment.

Compared to previous retrospective studies that investigated nerve fiber density and innervation of the adenomyotic uterus^(16,18), the authors conducted the present study in a prospective fashion, so that it offered evaluation of the severity of pain accurately without confounding factors such as recall bias. The specimens could be selected and sampled from the entire uterus. This being better than those sampled from a paraffin block because of its limited specimen volume and long storage time. The effect of long-term storage on immunohistochemistry reactions had not been proved.

Some of these cases were excluded due to concurrent pathologic findings precluding subgroup analysis and the ability to show a correlation between levels of pain and nerve fiber density.

The result of our study presented the correlation between the increase in nerve fibers density and pain level, but more studies are needed to explain the cause and the role of this change at the molecular level.

Conclusion

There was significant correlation between nerve fiber density and pain in adenomyosis. This indicated that nerve fiber density could play an important role in the pain mechanism of this disease. In addition, it may also explain why the extent of lesion may not always correlate with the severity of pain symptoms. However, the exact mechanisms or factors inducing nerve proliferation and the role of future treatment associated with nerve proliferation will require further study.

What is already known on this topic?

The most frequently presenting symptom of adenomyosis is pelvic pain, but the exact cause of the pain is still unknown. Adenomyosis and endometriosis are believed to be under the same pathophysiology. The nerve fibers cannot be detected in adenomyotic lesions, on the contrary, some

investigators demonstrated the presence of nerve fibers in the hysterectomized specimen in women with pain symptoms whether the women had endometriosis, adenomyosis, fibroids, or endometriosis with adenomyosis, but not in women without pain, suggesting a role of nerve fibers in the functional layer of the endometrium being played in pain generation. Since these retrospective studies have given inconsistent findings about innervation in adenomyosis, its role in the pain mechanism is still inconclusive, that is why we conducted a study to define the nerve fiber density in adenomyotic tissue in correlation with pain symptoms, as this is may be one important part of pain mechanism.

What this study adds?

According to the previous study, we cannot conclude the correlation between nerve fiber density and pain mechanism in adenomyosis. The present study shown significant correlation between nerve fiber density and pain in adenomyosis. This indicated that nerve fiber density could play an important role in the pain mechanism of this disease. In addition, it may explain why the extent of lesion may not always correlate with the severity of pain symptoms.

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Potential conflicts of interest

None.

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การศึกษาเปรียบเทียบความหนาแน่นของใยประสาทในรอยโรคแอดดีโนไมโอซิสที่มีและไม่มีภาวะปวด

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ภูมิหลัง: หลายการศึกษาแบบย้อนหลังพบว่ายังไม่ชัดเจนเกี่ยวกับเส้นประสาทที่มาเลี้ยงเนื้ออกแอดดีโนไมโอซิสกับอาการปวด

วัตถุประสงค์: เพื่อเปรียบเทียบระหว่างความหนาแน่นของใยประสาทในรอยโรคแอดดีโนไมโอซิสกับระดับความรุนแรงของความปวด
วัสดุและวิธีการ: การศึกษาภาคตัดขวาง ภาควิชาสูติศาสตร์รีเวชวิทยา คณะแพทยศาสตร์ โรงพยาบาลรามธิบดี ผู้ป่วยสตรีวัยเจริญพันธุ์ 25 ราย ที่เข้ารับการตัดมดลูก เนื่องจากมีอาการปวดหรือเลือดออกผิดปกติที่มีสาเหตุจากโรคแอดดีโนไมโอซิส ผู้ป่วยทุกรายได้รับการประเมินระดับอาการปวดก่อนการผ่าตัดร่วมกับตรวจวินิจฉัยชิ้นเนื้อทางพยาธิวิทยา เพื่อยืนยันการวินิจฉัยโรคแอดดีโนไมโอซิส และย้อมเทคนิคทางอิมมูโนฮิสโตเคมี *protein gene product (PGP) 9.5* และ *neurofilament (NF)* เพื่อตรวจนับจำนวนใยประสาท โดยตัวชี้วัดคือความหนาแน่นของใยประสาทจากชิ้นเนื้อมดลูกที่ได้จากการผ่าตัดโดยการย้อม *protein gene product (PGP) 9.5* และ *neurofilament (NF)* ด้วยเทคนิคทางอิมมูโนฮิสโตเคมี และระดับความรุนแรงของความปวดโดยประเมินจาก *visual analogue scale*

ผลการศึกษา: พบว่าจำนวนใยประสาทที่ตรวจโดยการย้อม *PGP9.5* และ *NF* ในส่วนกล้ามเนื้อมดลูกมีความหนาแน่นมากขึ้นในชิ้นเนื้อมดลูกที่ได้จากกลุ่มผู้ป่วยแอดดีโนไมโอซิสที่มีระดับความปวดปานกลางจนถึงรุนแรง เมื่อเปรียบเทียบกับกลุ่มผู้ป่วยที่มีระดับความปวดน้อย (4 (0, 7) กับ 1.55 (0, 7)/ตารางมิลลิเมตร, $p < 0.001$, และ 6 (3, 10) กับ 0 (0, 4)/ตารางมิลลิเมตร, $p < 0.001$ ตามลำดับ) ไม่พบความแตกต่างอย่างมีนัยสำคัญของความหนาแน่นของใยประสาทระหว่างช่วงต่างๆ ของรอบประจำเดือน

สรุป: การศึกษาแสดงให้เห็นว่ามีการเพิ่มขึ้นของความหนาแน่นของใยประสาทในกลุ่มที่มีอาการปวดระดับปานกลางจนถึงรุนแรง ซึ่งอาจมีบทบาทเกี่ยวข้องกับของการเกิดโรคหรืออาการของโรคแอดดีโนไมโอซิส
