# Immunohistochemical localization of nerve fibers in the pseudocapsule of fibroids

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## Abstract

The pseudocapsule surrounding fibroids consists of compressed myometrium containing nerves and blood vessels that continue into adjacent myometrium. Oxytocin (OXT) is thought to affect wound healing after myomectomy. We determined the presence of OXT and protein gene product 9.5 (PGP9.5) immunoreactive nerve fibers in pseudocapsule compared to adjacent myometrium. Samples (N=106) of pseudocapsule and adjacent myometrium were collected from 57 women with uterine fibroids undergoing myomectomy, and stained with anti-OXT and PGP 9.5 antibodies to demonstrate the presence of nerve fibers. Nerve fibers in the pseudocapsule stained positively with OXT (89/106, 84.0%) and PGP 9.5 (94/106, 88.7%). The densities of nerve fibers staining with PGP 9.5 and OXT in the pseudocapsule were highest in the isthmus ($23.68\pm22.45$/mm$^2$ and $43.35\pm40.74$/mm$^2$, respectively). There were no significant differences in the density of nerve fibers, stained with either OXT or PGP 9.5, between the pseudocapsule, and adjacent normal myometrium regardless of the fibroid location in the uterus (P>0.05). These results suggest that the pseudocapsule should avoid to be damaged during the myomectomy procedure.

## Introduction

Fibroids are the most common tumors in women of reproductive age.[1] Their pseudocapsule is a neurovascular bundle or fibrovascular network that separates the fibroid from adjacent normal myometrium.[2] Many fibroids do not contain nerve fibers though a small proportion carry a similar distribution to normal myometrium.[3] The reasons for these differences are unclear, though they may reflect an injury to nerves in the pseudocapsule of adjacent myometrium that releases that area of myometrium from normal constraints of growth. Pseudocapsule has similar architecture to normal myometrium but also contains different nerve fibers and neuropeptides.[4-8] Characterizing these nerve fibers may assist in our understanding of the etiology of some patterns of fibroids.

Oxytocin (OXT) is a nine amino acid neuropeptide that is synthesized in the hypothalamic supraoptic and paraventricular nuclei, that enhances uterine contractions during labor, and, mediates milk release from the mammary glands during suckling.[9,10] OXT is released to the periphery via the pituitary gland, and within the brain via multiple pathways;[10] the central and peripheral actions of OXT are mediated through one oxytocin receptor (OXTR), which are the product of a single gene.[11-13] Recently, accumulating evidence shows that OXT is involved in a variety of biological processes, possessing anti-inflammatory, antioxidative stress and tumorigenic properties as well as mediating human social behavior.[14-19] Obviously, OXT is associated with wound healing and pregnancy-related complications after myomectomy. Protein gene product 9.5 (PGP9.5) is a highly specific pan-neuronal marker for both myelinated and unmyelinatednerve fibers, which stains not only autonomic nerve fibers but also sensory nerve fibers.[20-23] Therefore, in this study we determined the distribution of nerve fibers staining with OXT, and PGP9.5 in the pseudocapsule of uterine fibroids.

## Materials and Methods

### Patients and samples collection

Between May 2011 and February 2012, a total of 57 non-pregnant women (mean age: 33.6 years; range: 27-42 years) who required to undergo laparoscopic myomectomy at the Women's Hospital, Zhejiang University School of Medicine, China were recruited in this study. The indications for myomectomy included intractable menorrhagia not responding to conservative treatment, a large uterus associated with urinary or/and rectal symptoms, or uncontrolled growth as verified by repeated ultrasounds. 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.

All fibroids were diagnosed using standardized transvaginal ultrasound myoma mapping by an expert technician, and evaluated under laparoscopy during the surgical procedure (Figures 1 and 2). If the pseudocapsule of uterine fibroids could not be identified under ultrasonography or distinguished from adenomyosis, we further performed a magnetic resonance imaging (MRI) examination. The fibroids were intramural, corporal, fundal, cornual and isthmus and the ultrasound data were recorded for postsurgical evaluation. Prior to surgery, pregnancy was excluded by a serum - HCG test. Moreover, a history of gynecological tumors, post-treatment of gonadotropin-releasing hormone agonists (GnRHa) and previous uterine scar (including cesarean section) were also excluded. Furthermore, all the participants had no history of endometriosis, adenomyosis or pelvic inflammatory diseases, which was confirmed by laparoscopy. In addition, none of them received sex-hormone therapy prior to surgery.

All uterine fibroids were single or multiple, located in the fundus, the corpus, the cornua or the isthmus, respectively. The diameter of the fibroids was between 3 and 14 cm. In order to obtain adequate samples of the uterine fibroid pseudocapsule and the surrounding normal myometrium, we chose intramural and subserosal fibroids (n. 5 and 4 by Wamsteker classification) as the study subjects.[24,25] Pedunculated, cervical and intraligamentary fibroids were excluded directly under ultrasonographic examination or during the surgical procedure. All myomectomies were performed by laparoscopic surgery under general

**Figure 1. Serosal fibroid image shown under sonography. A) Transvaginal ultrasound shows a serosal fibroid located in the uterine isthmus. B) Ultrasound image shows the surrounding ring of the fibroid for the pseudocapsule (highlighted by white arrows). C) Doppler ultrasound shows the surrounding *ring of fire* and vessels for the pseudocapsule.**

anesthesia with endotracheal intubation, which depends on the dimensions of the fibroid. After surgery, fibroids were confirmed by histopathological examination. All surgical procedures were performed by experienced gynecologists (YMS, XFH and XMZ, three of the authors). The laparoscopic myomectomies were performed using a standardized method as previously described by Malvasi *et al*.[5] Briefly, incisions were made longitudinally using monopolar or bipolar diathermy until opening the relatively bloodless plane between the pseudocapsule and the fibroid. Once the surface of the fibroid was breached, the fibroid was hooked and extracted from its capsule by traction and pushing down the capsule (Figure 2). The pseudocapsule and the surrounding normal myometrial tissues were excised using scissors during the procedure. The samples were immediately sent to the laboratory in a dry-ice container for histological and immunofluorescent studies.

## Histology, immunohistochemistry and immunofluorescence

All samples of the pseudocapsule and the surrounding normal myometrium 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 two sections (cut at 5 μm) from each pseudocapsule or myometrium, such that one section was used for the hematoxylinand eosin staining, and the other for the immunohistochemical staining. For the indirect immunofluorescence analysis, the specimens were frozen in dry ice and sectioned in a cryostat (6 μm thickness). The hematoxylin and eosin staining and the immunohistochemical staining were performed as described previously.[26,27] Serial sections were cut at 5 μm, and immunostained using polyclonal rabbit anti-PGP9.5 antibody (dilution 1:500, Z5116; Dako Cytomation, Glostrup, Denmark) for 60 min at room temperature. The sections were washed in phosphate-buffered saline (PBS) and incu-
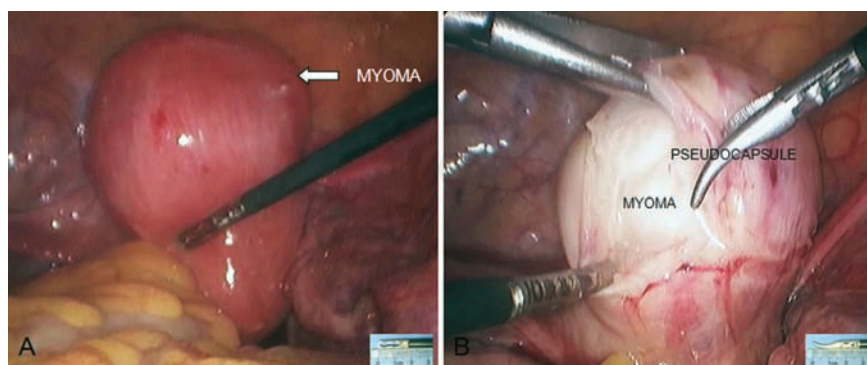


**Figure 2. A serosal uterine fibroid shown by laparoscopy during surgery. A) A serosal fibroid located at the uterine isthmus was shown under laparoscopy. B) A laparoscopic image showing the fibroid and the surrounding pseudocapsule.**

bated with Envision-labeled polymer-alkaline phosphatase mouse/rabbit (EnVision/HRP/Mo, GK400105; EnVision/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). After washing, the sections were counterstained with Mayer's hema-toxylin, dehydrated, and mounted with a mounting medium. Normal vulval skin was used as the positive control group. Negative controls were incubated with normal goat serum (X 0907; Dako) instead of the primary antibody. The indirect immunofluorescence detection of OXT was performed as described previously.[28] Briefly, after retrieving antigen for oxytocin, the sections were incubated with polyclonal rabbit anti-OXT antibody (dilution 1:100, AB911; Millpore, Germany) for 60 min at room temperature. The sections were washed in PBS and incubated with secondary fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit IgG antibody (R0156, Dako A/S, Denmark) for 30 min at 37°C in dark. After washing, the sections were observed under flu-

orescence microscopy (AX-70, Olympus, Tokyo, Japan). All the sections were finally evaluated by an experienced gynecological pathologist who was unaware of the sample background. 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 with minor modification to count the number of nerve fibers identified by using PGP9.5 staining in the pseudocapsule and the surrounding normal myometrium.[27,29] 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 mm²). The results were expressed as the mean (±SD) number of nerve fibers/mm² in each specimen from all the samples. 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. A single observer who was blind to the sample background counted the number of PGP9.5-positive nerve fibers.

The chromaticity-positive nerve cells showed green fluorescence under fluorescence microscopy. The density of oxytocin immunoreactive nerve fibers was calculated in 5 randomly selected and homogeneous areas under fluorescence microscopy (200×, Olympus, AX-70). The total number of nerve fibers was divided by the total number of areas on each section to obtain an average of nerve fibers per area (each area measuring 1 mm²). The results were expressed as the mean (±SD) number of nerve fibers/mm² in each specimen from all the samples.

### Statistical analysis

We used the Statistical Package for the Social Sciences version 13.0 (SPSS, Chicago, IL, USA) to perform statistical analyses. The results were expressed as the mean (±SD) number of nerve fibers/mm² in each specimen from all pseudocapsule and myometrium 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 between groups. The $\chi^2$ test was used to compare the differences in the percentage of immunoreactive nerve fibers between groups. The Spearman correlation was used to analyze the correlations between groups. Differences were considered significant at P<0.05.

## Results

This study included 15 women (26.3%) with single fibroids and 42 (73.7%) with multiple fibroids, which had a mean BMI of 23.8, a mean parity of 1.2, and a mean abortion of 1.8. Fifty-seven women had a total of 106 fibroids. Of the 106 fibroids, 27 fibroids (25.5%) were located in the posterior wall, 25 (23.6%) in the anterior wall, 15 (14.2%) in the lateral wall, 18 (17.0%) in the fundus, 9 (8.5%) in the cornua and 12 (11.3%) in the isthmus of the uterus, respectively (Tables 1 and 2). PGP9.5-immunoreactive nerve fibers were detected in the pseudocapsule in 89/106 fibroids (84.0%). The percentage of PGP9.5-immunoreactive nerve fibers in the fibroid pseudocapsule was different at different sites with the highest percentage in the isthmus (Table 1, Figure 3). In normal myometrium 95/106 (89.6%) stained positive with PGP9.5-immunoreactive nerve fibers. There were no significant differences in the percentage of PGP9.5-immunoreactive nerve fibers between fibroid pseudocapsule and surrounding normal myometrium either in the posterior, anterior, lateral, fundus, cornua or isthmus of the uterus (P>0.05, Table 1).

The density of PGP9.5-immunoreactive nerve fibers in fibroid pseudocapsule was also different at different sites in the uterus with the highest levels in the isthmus (23.68±22.45/mm², P<0.01, *versus* other sites), and the lowest levels in the fundus (3.08±3.43/mm², P<0.05, *versus* lateral or isthmus). In the surrounding normal myometrium, PGP9.5-immunoreactive nerve fibers showed similar patterns, as per quantity and quality, to fibroid pseudocapsule. No statistical significant differences with respect to the density of PGP9.5-immunoreactive nerve fibers between the fibroid pseudocapsule and the surrounding normal myometrium either in the posterior, anterior, lateral, fundus, cornua or isthmus of the uterus were found (P>0.05, Table 1). Oxytocin-immunoreactive nerve fibers in the pseudocapsule were detected in 94 /106 (88.7%) fibroids. The density of OXT-immunoreactive nerve fibers in the fibroid pseudocapsule was higher in the lateral wall,

**Table 1. Distribution of PGP9.5-immunoreactive nerve fibers in the fibroid pseudocapsule and the normal myometrium (Mean±SD/mm²).**

| Location | Number of uterine fibroids | PGP9.5-immunoreactive nerve fibers | | | |
| | | Percentage | | Density | |
| | | Pseudocapsule | Myometrium | Pseudocapsule | Myometrium |
|---|---|---|---|---|---|
| Anterior | 25 | 80.0% (20/25) | 84.0% (21/25) | 5.13±4.11 | 5.21±4.08 |
| Posterior | 27 | 77.8% (21/27) | 81.5% (22/27) | 4.76±5.51 | 4.84±5.75 |
| Lateral | 15 | 86.7% (13/15) | 86.7% (13/15) | 11.03±8.13 | 10.98±8.32 |
| Fundus | 18 | 88.9% (16/18) | 100% (18/18) | 3.08±3.43 | 3.23±3.51 |
| Cornua | 9 | 77.8% (7/9) | 100% (9/9) | 4.83±4.46 | 4.78±4.89 |
| Isthmus | 12 | 100% (12/12) | 100% (12/12) | 23.68±22.45 | 24.19±23.71 |

**Table 2. Distribution of oxytocin-immunoreactive nerve fibers in the fibroid pseudocapsule and the normal myometrium (Mean±SD/mm²).**

| Location | Number of uterine fibroids | Oxytocin-immunoreactive nerve fibers | | | |
| | | Percentage | | Density | |
| | | Pseudocapsule | Myometrium | Pseudocapsule | Myometrium |
|---|---|---|---|---|---|
| Anterior | 25 | 80.0% (20/25) | 92.0% (23/25) | 11.84±11.99 | 11.28±12.57 |
| Posterior | 27 | 88.9% (24/27) | 92.6% (25/27) | 15.13±9.97 | 15.41±10.14 |
| Lateral | 15 | 100% (15/15) | 100% (15/15) | 30.15±20.77 | 31.09±25.65 |
| Fundus | 18 | 77.8% (14/18) | 72.2 (13/18) | 10.08±9.97 | 9.96±10.52 |
| Cornua | 9 | 100% (9/9) | 100% (9/9) | 28.23±19.06 | 29.61±22.17 |
| Isthmus | 12 | 100% (12/12) | 100% (12/12) | 43.35±40.74 | 40.97±44.23 |

cornu and isthmus of the uterus, yet the differences at different sites of the uterus did not reach statistical significance (P>0.05, Table 2, Figure 4). Normal myometriun showed similar distribution of OXT-immunoreactive nerve fibers to fibroid pseudocapsule. No significant differences were detected in OXT-immunoreactive nerve fibers between the fibroid pseudocapsule and surrounding normal myometrium at different sites in the uterus (P>0.05, Table 2). The density of OXT-immunoreactive nerve fibers in the fibroid pseudocapsule was also different at different sites in the uterus with the lowest levels in the fundus ($10.08\pm9.97$ /mm$^2$, P<0.01, *versus* lateral, cornua or isthmus), and the highest in the isthmus ($43.35\pm40.74$/mm$^2$, P<0.01, *versus* other sites; Table 2). The normal myometrium exhibited the same distribution of OXT-immunoreactive nerve fibers as fibroid pseudocapsule. The density of OXT-immunoreactive nerve fibers were similar in the fibroid pseudocapsule and the normal myometrium either in the posterior, anterior, lateral, fundus, cornua or isthmus of the uterus (P>0.05, Table 2).

## Discussion

Nerve fibers staining with PGP9.5 are present in similar quantities in the pseudocapsule of uterine fibroids, and adjacent normal myometrium no matter where the fibroids are located in the uterus. Our results also showed that oxytocin-immunoreactive nerve fibers are also present in the pseudocapsule and adjacent normal myometrium, and both showed similar patterns of distribution. The densities of nerve fibers stained with PGP9.5 and oxytocin were both highest in the isthmus, and then in the lateral wall of the uterus. PGP9.5 is a pan-neu-



**Figure 3. PGP9.5-immunoreactive nerve fibers in the fibroid pseudocapsule (original magnification 400×). Nerve fibers stained with PGP9.5 exhibited small single varicosities as well as bead-like or trunk-like structures that seemed to be along the smooth muscle and fibrous tissue in close relation to the vessels. The density of PGP9.5-immunoreactive nerve fibers in the fibroid pseudocapsule was significantly higher in the posterior wall of the uterine isthmus (B) than that in the anterior wall of uterine body (A) (P<0.01). Arrows indicate positive stained nerve fibers with PGP9.5.**
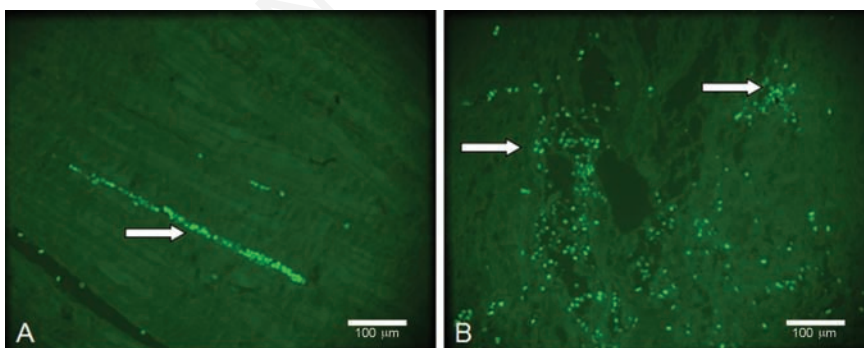


**Figure 4. Oxytocin-immunoreactive nerve fibers in the fibroid pseudocapsule (original magnification 400×). The chromaticity-positive nerve cells stained with oxytocin showed green fluorescence under fluorescence microscopy. Nerve fibers staining with oxytocin were irregularly (sparsely or densely) distributed along the smooth muscle and fibrous tissue as well as the vessels. The density of oxytocin-immunoreactive nerve fibers in the fibroid pseudocapsule was significantly higher in the posterior wall of the uterine isthmus (B) than that in the anterior wall of uterine body (A) (P<0.01). Arrows indicate positive stained nerve fibers with oxytocin.**

ronal marker labeling autonomic nerve fibers and also sensory nerve fibers.[20-23] PGP9.5-immunoreactive nerve fibers may be involved in the pathophysiology of uterine fibroids, and affect muscle contractility, uterine peristalsis and muscular healing.[4] Since pseudocapsule is a neurovascular bundle or fibrovascular network surrounding the fibroids,[2] damaging the pseudocapsule during the myomectomy procedure may affect reinnervation and revascularization of the uterine incision.[2, 4-6] As a neuropeptide, oxytocin has been shown to be involved in a variety of biological processes, not only enhancing uterine contractility, and modulating pain trigger and social behavior, but also possessing antiinflammatory, antioxidative stress and tumorigenic properties.[14-19, 30] Oxytocin exerts its biological functions by binding its receptor.[11-13] The abnormality of OXTR expression and abnormal response of OXTR to OXT may cause abnormal uterine contractility, leading to infertility or preterm birth.[31-33] Oxytocin or OXTR antagonists are believed to improve infertility treatment.[34,35] Obviously, oxytocin-immunoreactive nerve fibers in the pseudocapsule may play a role in the mechanisms of uterine fibroids and also influence on uterine muscle contractility and wound healing as well as future fertility after myomectomy if the pseudocapsule of uterine fibroids was damaged during the surgical procedure.

The pseudocapsule is a neurovascular bundle that separates fibroids from the adjacent normal myometrium.[2] Besides PGP9.5 and oxytocin immunoreactive nerve fibers in the pseudocapsule in our study, other nerve fibers such as neurotensin-, neuropeptide- tyrosine-, substance P- and vasoactive intestinal peptide-immunoreactive nerve fibers were also present in the pseudocapsule. [4,5] Interestingly, all these nerve fibers showed similar patterns of distribution in the pseudocapsule and the adjacent normal myometrium.[4,5] However, all these studies including ours did not investigate the correlation between fibroid pseudocapsule thickness and the density of nerve fibers.

Therefore, further studies with additional neural markers with different sites will be necessary to confirm or refute the role of nerves in the etiology of some patterns of fibroids.
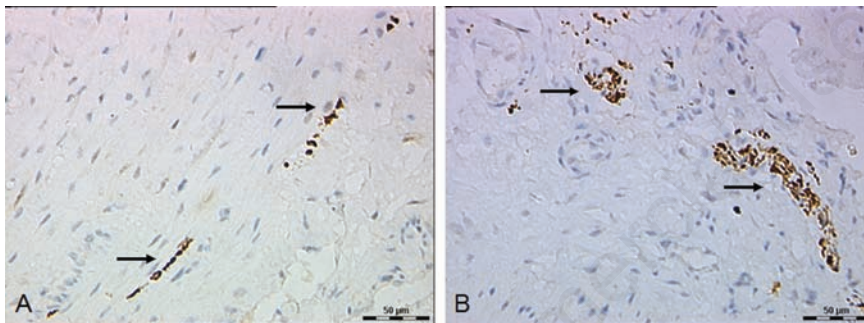
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