The Potential Role of Endometrial Nerve Fibers in the Pathogenesis of Pain During Endometrial Biopsy at Office Hysteroscopy

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Abstract

We aimed to evaluate whether nerve fibers are present in the endometrial layer of patients submitted to office hysteroscopy and their potential contribution to the pathogenesis of pain during that procedure. Through a prospective case-control study performed in tertiary centers for women's health, endometrium samples were collected during operative office hysteroscopy from 198 cycling women who previously underwent laparoscopy and/or magnetic resonance imaging investigation for infertility assessment. Samples were classified according to the degree of the pain patients experienced and scored from values ranging from 0 (absence of discomfort/pain) to 10 (intolerable pain) on a 10-cm visual analog scale (VAS). The presence of nerve fiber markers (S100, NSE, SP, VIP, NPY, NKA, NKB, NKRI, NKR2, and NKR3) in the endometrium was also evaluated by morphologic and immunohistochemical analyses. We found that S-100, NSE, NKRI, NK-A, NK-B, VIP, and NPY, were immunolocalized in samples of endometrium, in significantly ($P < .01$, for all) higher levels in samples collected from patients with VAS score > 5 (group A) than ≤ 5 (group B) and significantly ($P < .0001$ for all) positively correlated with VAS levels. A statistically significant ($P = .018$) higher prevalence of endometriosis and/or adenomyosis was depicted in patients of group A than group B. Data from the present study led us to conclude that nerve fibers are expressed at the level of the functional layer of the endometrium and may contribute to pain generation during office hysteroscopy, mainly in women affected by endometriosis and adenomyosis.

Keywords

office hysteroscopy, pain, neuropeptides, endometriosis, adenomyosis

Introduction

A plethora of data have assessed that office hysteroscopy represents to date the gold standard for the evaluation of the uterine cavity in relation to both abnormal uterine bleeding investigation and adequate endometrial sampling for histopathological diagnosis,^{1,2} with a diagnostic success rate close to 100%.³⁻⁸ However, the physicians fear of causing discomfort and pain to their patients during the procedure still limits the widespread use of office hysteroscopy,⁹ and, therefore, the number of gynecologists performing such a procedure continues to be low.^{10,11}

On this regard, one should keep in mind that pain during hysteroscopy may be caused by the introduction of hysteroscope through cervical canal, especially when it is pushed through the internal uterine ostium. Other well-known possible causes of pain may be the myometrial contractile activity caused by the distension of the uterine cavity by means of distension medium and the direct stimulation of the uterine

walls through the tip of the hysteroscope.¹⁰ Since the endometrium has been described as lacking terminal innervation under normal conditions,¹² its role in the etiology of pain during hysteroscopy was judged as irrelevant, and the evidence that most women feel only a minimal discomfort/pain during procedures that stimulate the endometrium (eg, endometrial biopsy) do support that concept. However, recent studies have reported nerve endings in the endometrium of women affected

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by estrogen-dependent gynecological pathologies, such as endometriosis, uterine fibromatosis, and adenomyosis.¹³⁻¹⁶ These evidences and the fact that some women show intense hyperalgesia during surgical stimulation of the endometrium together have suggested that sensitive nerve terminals of the endometrium can play a role in the genesis of this pain during hysteroscopy.

The present study aimed to evaluate (1) whether nerve fibers are present in the endometrial layers of patients submitted to hysteroscopy; (2) their potential contribution to the pathogenesis of pain during that procedure and, finally; (2) whether their expression changes in presence of estrogen-dependent gynecological pathologies, such as endometriosis and adenomyosis.

Materials and Methods

Patients

Clinical details and endometrial samples were prospectively collected from fertile women who underwent laparoscopy and/or magnetic resonance imaging investigation for infertility assessment, in whom hysteroscopic evaluation of the uterine cavity was performed from December 2007 to April 2009 at our tertiary centers for women health care. Prepubertal or postmenopausal women, patients with systemic diseases, active infections of the genital tract, immunodeficiency, or who had used any hormonal medication or intrauterine contraceptives 6 months prior to the study, or who had been diagnosed with reproductive cancer were excluded from the study.

The purpose of the study—that was approved by the local Human Investigation Committee—and complete information regarding it were clearly explained, after which a consent form was signed by all patients enrolled.

Office Hysteroscopy

Office hysteroscopy was performed during the early proliferative phase of the menstrual cycle. All procedures were performed by expert hysteroscopists by means of a 5-mm, continuous-flow office hysteroscope (Bettocchi Office Hysteroscope “size 5” Karl Storz, Tuttlingen, Germany) with a 2.9-mm rod optic lens. The vaginoscopic approach, without speculum and tenaculum,¹⁷ was used and no analgesia or anesthesia was administered to the patient.² Distension of the uterine cavity was obtained with saline solution, and the intrauterine pressure was automatically controlled by an irrigation-suction electronic device (Endomat; Karl Storz, GmbH & Co, Tuttlingen, Germany) set at ≤ 50 mm Hg and an intrauterine flow set at ≤ 300 mL/min; the aspiration pressure was regulated at values ≤ 0.2 bar. Illumination was supplied by a 300 W xenon source of light. Images were visualized by a high-resolution color monitor, using an integrated circuit video camera, and all procedures were recorded from introduction to extraction of the hysteroscope.

Soon after uterine cavity inspection, using the “grasp biopsy” technique, an endometrial biopsy was performed to obtain an adequate endometrial sample for histological analysis. Open jaw biopsy forceps were positioned at the endometrium level in order to penetrate the tissue for about 0.5 to 1 cm, thus avoiding stimulation and/or lesion of the subjacent myometrium. All operative procedures (eg, polypectomy, myomectomy, adhesiolysis) were always preceded by endometrial biopsy. The patient was asked not to watch the procedure on the monitor and was requested to quantify the degree of discomfort/pain during the endometrial biopsy. Accordingly, values ranging from 0 (absence of discomfort/pain) to 10 (intolerable pain) were fixed on a 10-cm visual analog scale (VAS). A second operator, next to the patient, quizzed the patient during the procedure. On the basis of her discomfort/pain during this step of the hysteroscopic procedure each patient was then assigned either to group A (VAS score > 5) or group B (VAS score ≤ 5).

Morphologic and Immunohistochemical Analyses

The endometrial tissue was immediately fixed in Bouin solution and then embedded in paraffin for the immunohistochemistry study. To evaluate the presence of myelinic and amyelinic fibers, the antigens against S100, NSE, SP, VIP, NPY, NKA, NKB, NKR1, NKR2, and NKR3 were used.¹⁸

For immunohistochemistry analysis, 5- μ m thick serial sections were employed using as primary antibody the following monoclonal and polyclonal antibodies according to these dilutions: anti-S100 mouse, 1:250 (Sigma, St Louis, Missouri); anti-SP rabbit, 1:500 (RPMS, London, United Kingdom); anti-NPY rabbit, 1:500 (Sigma); anti-VIP rabbit, 1:500 (BiomedCorp, Forster City, California); anti-NK1R mouse, 1:300 (Sigma); anti-NKA mouse, 1:250 (Santa Cruz Biotechnology, Inc, Santa Cruz, California); anti-NKR2 mouse, 1:400 (Sigma); anti NKB rabbit, 1:500 (Santa Cruz Biotechnology); anti NKR3 mouse, 1:350 (Sigma). In addition, as secondary antibodies, biotin-marked anti-immunoglobulin G (anti-IgG) mouse and anti-IgG rabbit, 1:200 and 1:20,000 diluted, respectively, were used.^{18,19} The avidin-biotin-peroxidase immunoenzymatic method was used to identify the antigen-antibody complex (Avidin Biotin Complex/Horseradish Peroxidase [ABC/HRP]; Vector Laboratories, Burlingame, California), as already published.²⁰ Negative control sections were incubated with normal rabbit serum, while unrelated antisera such as monoclonal mouse anti-insulin 1:1500 (Sigma) and polyclonal rabbit antiglucagon 1:3000 (Chemicon, Milan, Italy) were used to stain pancreatic tissue as a positive control for the immunoenzymatic reaction. Nickel sulfate enhancement with di-amino-benzidine chromogen in the presence of 0.03% H_2O_2 was used to reveal the antigens enzymatically. Sections were counterstained with hematoxylin.

The individual intensity of immunostaining of tissue section was scored on a subjective scale of 0 (no staining) to 5 (maximal staining) by 3 independent assessors, blinded to the diagnosis

Table 1. Clinical and Anthropometric Details of Enrolled Women.^a

Characteristics	Group A ^b (n = 56)	Group B ^b (n = 142)	P
Age, years	38.84 ± 7.8	40.33 ± 7.44	ns
Body mass index (BMI)	24.6 ± 2.8	24.8 ± 2.7	ns
Parity	1.52 ± 1.16	1.36 ± 0.83	ns
Operative procedures, n (%)			
Polipectomy	36 (65)	98 (69)	ns
Myomectomy	14 (25)	31 (22)	ns
Metroplasty	6 (10)	13 (9)	ns
Pain at hysteroscopy (VAS score)	7.7 ± 1.28	2.75 ± 1.17	<.001
Adenomyosis/endometriosis, n (%)	28 (50)	36 (25)	.018

Abbreviations: VAS, visual analog scale.

^aData are reported as mean ± SD, when not indicated.

^bGroup A: VAS score > 5—group B: VAS score ≤ 5.

Figure 1. A and B, Immunostaining for S100 in a patient of group A and group B, respectively. C and D, Immunostaining for neuron-specific enolase (NSE) in a patient of group A and group B, respectively. The marking of fibers (arrows) is localized mainly around glands (A-D: ABC/HRP method, original magnification ×400).

of patients. Values were reported as mean ± standard deviation (SD).

Statistical Analysis

Statistical analysis was performed using the SPSS 14.0 for Windows (SPSS Inc, Chicago, Illinois). The Shapiro-Wilks assessment was used to evaluate whether values had a Gaussian distribution, in order to choose between parametric and non-parametric statistical tests. The chi-square test was used to evaluate statistical significance between the categorical variables; the Mann-Whitney *U* test was used for the evaluation

of the continuous variables. Correlation between pain (as evaluated by using the VAS scale) and immunostaining intensities of myelinic and amyelinic fibers was calculated using the Spearman rank test. Statistical significance was set at $P < .05$.

Results

Clinical Findings

Of the 208 patients meeting the criteria, 198 agreed to be enrolled in the study (Table 1). On the basis of the VAS score during hysteroscopic assessment of uterine cavity, patients were divided in group A, composed by patients with VAS

Figure 2. Immunostaining for neurokinin receptor I (NKR-I; A), neurokinin A (NK-A; B), and neurokinin B (NK-B; C) in a patient of group A. The marking is prevalently periglandular and peristroma and in subepithelial layer. Immunostaining for neurokinin receptor I (NKR-I; D), neurokinin A (NK-A; E), neurokinin B (NK-B; F) in a patient of group B (A–F: ABC/HRP method, original magnification $\times 200$).

score > 5 ($n = 56$; mean $\pm$ SD: 7.7 ± 1.28), and group B, composed by patients with VAS score ≤ 5 ($n = 142$; mean $\pm$ SD: 2.75 ± 1.17 ; Table 1). No significant differences were retrieved according to age, parity, and major demographic traits as well as with respect to hysteroscopic procedures (endometrial biopsy, polypectomy, myomectomy, metroplasty). A statistically significant ($P = .018$) higher prevalence of endometriosis and/or adenomyosis was depicted in group A (28 [50%] out of 56 patients) than group B (36 [25%] of 142 women; Table 1).

Immunohistological Findings

Figures 1 to 3 show immunohistological localization of for S-100, NSE, NKR1, NK-A, NK-B, VIP, and NPY in samples of endometrium collected at the time of hysteroscopy from

both groups of patients. In detail, general immunostaining of S-100 and NSE nerve fiber markers was localized mainly in samples collected from patients belonging to the group A (patients with VAS score for pain during hysteroscopy > 5) around mucous glands. Endothelial cells of vessel walls, epithelial glandular, and stromal cells were devoted to staining of sensory neuropeptides as VIP, NPY, NK-A, NK-B, and their receptors like NKR-1. Furthermore, weak, aspecific background or no signal was detected in the majority of tissues collected from patients who experienced low pain during hysteroscopy (VAS score ≤ 5 , group B). On the contrary, no immunostaining was found in any sample regarding SP, NKR2, and NKR3 (Figure 4).

With respect to differences in staining intensity, it was judged to be significantly ($P < .01$, for all) higher in endometrial samples collected from group A (S100: 4.17 ± 0.69 ;

Figure 3. A and B, Immunostaining for vasoactive intestinal peptide (VIP) in a patient of group A and group B, respectively. C and D, Immunostaining for neuropeptide Y (NPY) in a patient of group A and group B, respectively (A-D: ABC/HRP method, original magnification $\times 200$. The staining is evident in periglandular region).

NSE: 4.16 ± 0.73 ; VIP: 4.1 ± 0.77 ; NPY: 3.67 ± 0.71 ; NKA: 3.66 ± 0.72 ; NKR1: 3.92 ± 0.82) than group B (S100: 2.38 ± 0.84 ; NSE: 2.1 ± 1.0 ; VIP: 1.88 ± 0.92 ; NPY: 2.02 ± 0.83 ; NKA: 2.08 ± 1.04 ; NKR1: 2.33 ± 0.91) patients (Figure 5).

We therefore evaluated the putative correlation between pain intensity, as per VAS score evaluation, and the intensity of staining for nerve fiber markers both in patients who presented with endometriosis/adenomyosis and those who did not. With respect to women not affected by those 2 diseases, a statistically significant ($P < .0001$, for all) positive correlation was found between intensity of staining for nerve fiber markers and VAS levels (S100, r^2 : .58; NSE, r^2 : .68; VIP, r^2 : .88; NPY, r^2 : .67; NKA, r^2 : .57; NKR1, and r^2 : .61). A statistically significant ($P < .0001$ for all with the exception of NPY) positive correlation was also found between intensity of staining for nerve fiber markers and VAS levels in women affected by endometriosis/adenomyosis (S100, r^2 : .48; NSE, r^2 : .65; VIP, r^2 : .79; NKA, r^2 : .57; NKR1, and r^2 : .72; data not shown).

Discussion

The present study first refers on the correlation between pain experienced during office hysteroscopy and both the presence and level of expression of markers for nerve fibers in the endometrium. Indeed, we found that S-100, NSE, NKR1,

NK-A, NK-B, VIP, and NPY fibers were present mainly in and around the endometrial mucous glands from women who experienced intense pain at hysteroscopy and that those patients had a higher expression of nerve fiber markers, with a positive correlation between VAS score and immunostaining of nerve fiber markers. Moreover, we also found a higher incidence of endometriosis/adenomyosis—known causes of pelvic pain—in patients belonging to the group of women experiencing higher pain during hysteroscopy.

The present findings would merit discussion, since in the last decade several efforts have been provided on maximizing the chance of a successful hysteroscopic procedure by minimizing patient discomfort. And therefore, the use of saline as distension medium, the availability of high-resolution rigid and flexible mini endoscopes, and the introduction of the vaginoscopic approach, developed by Bettocchi and Selvaggi,¹⁸ were demonstrated to be effective to that aim. Nevertheless, the anatomical characteristics of the uterus whose sensitive innervation is reported to begin from the myometrium, while lacking in the endometrium, in fibrotic tissue and lesions eventually associated^{12,21} would support the concept that most of hysteroscopic procedures can be performed easily without use of analgesia or anesthesia.

However, clinical experience in office hysteroscopy has taught that while most women subjected to procedures that stimulate the endometrium (eg, endometrial biopsy) do not feel any discomfort/pain, a significant minority of them feel

Figure 4. Immunostaining for substance P (SP; A-D), neurokinin receptor 2 (NKR-2; B-E) and neurokinin receptor 3 (NKR-3; C-F) in a patient of group A and group B, respectively. All samples are completely negative for staining (A–F: ABC/HRP method, original magnification $\times 200$).

intense hyperalgesia when operative instruments *trigger* the endometrium, thus disavowing the concept that the endometrium is devoted of sensitive innervation. Findings of the present study do suggest that sensitive nerve terminals are present in the endometrium and they may have a potential role in generating discomfort/pain during hysteroscopy. Since the nerve fiber markers we evaluated, with the exception of S100 and NSE, are specific for small sized myelinated nerve fibers, we suggest that C-type sensitive fibers may innervate the functional layer of the endometrium. On the other hand, the high immunopositive reactivity for S100 and NSE, which are neuromediators independent of fiber diameter and degree of myelination, suggest that other sensitive terminal endings are present in the functional layer of the endometrium. The positive correlation between expression of nerve fibers and pain intensity further reinforce this hypothesis.

We used endometrial biopsy samples obtained from patients who underwent office hysteroscopy for immunohistochemically exploring the occurrence of specific neuronal markers. Among the various ways of endometrial stimulation (mechanical stimulation by means of the hysteroscope tip, polypectomy, curettage via Novak cannula or Pipelle), we preferred to use hysteroscopic endometrial biops, due to its easy and fast execution, the good compliance in the majority of the patients, and the possibility to obtain enough material for an appropriate immunohistochemical analysis. Among the various steps of the hysteroscopic examination, we focused only on the endometrial biopsy since from a theoretical standpoint an eventual biopsy forceps-elicited pain would be associated with the presence of nerve endings in the endometrium. Indeed, the other steps of the hysteroscopic examination were not evaluated because the VAS score could be influenced by a

Figure 5. Staining intensity of markers of nerve fibers in the endometrium was significantly ($P < .001$) higher in samples collected from group A than group B. The box plots represent the medians and inter-quartile ranges.

number of factors (passage of the hysteroscope through the cervical channel, over distension of the uterine cavity, myometrium stimulation).

Another evidence that merits discussion refers to the fact that the higher expression of markers of nerve fibers—S-100, NSE, NK1, NK-A, NK-B, VIP, and NPY—was found to be associated mainly with endometriosis and adenomyosis, 2 well-known causes of pelvic pain^{22,23}: the percentage of patients affected by uterine endometriosis and/or adenomyosis was significantly higher in women belonging to group A (VAS score > 5). The present finding leads us to hypothesize, according also other reports,¹³⁻¹⁵ that the endometrium of women affected by endometriosis exhibits C-type sensitive fibers and other sensitive terminal endings in the functional layer of the endometrium. These evidences may have clinical usefulness, since the experience of intense pain in patients undergoing hysteroscopic evaluation of the uterine cavity may help in revealing the presence of the anatomical extent and dimension of endometriosis.²⁴

In conclusion, results of the present study do suggest that it is conceivable that the presence of nerve fibers at the level of the functional layer of the endometrium may contribute to pain generation during office hysteroscopy. This finding could result from the occurrence of estrogen-dependent gynecological pathologies such as uterine endometriosis and adenomyosis.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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