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Distribution of Substance P (SP) and Vasoactive Intestinal Peptide (VIP) in pseudocapsules of uterine fibroids

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ABSTRACT

The authors examined the presence of Substance P (SP) and Vasoactive Intestinal Polypeptide (VIP) and their related fibers in the pseudocapsule of uterine fibroids (PUF) and in normal myometrium (NM) during myomectomies in 57 non-pregnant women. 4 samples were removed from the normal myometrium (NM) and from PUF. The samples were sent for histological and immune-fluorescent investigations. SP and VIP values were found non-significantly higher in PUF than in NM: SP values were 10.2 ± 0.1 conventional units (C.U.) in PUF at the fundus of the uterus (FU) vs. 8.1 ± 0.6 C.U. of NM in the FU ($p > 0.05$), and SP values were 25.1 ± 0.9 C.U. in PUF in the uterine body (UB) compared to. 23.2 ± 1.4 C.U. of NM in the myometrium of the UB ($p > 0.05$). VIP values were 11.5 ± 0.9 C.U. in the PUF in FU compared to 9.8 ± 1.4 C.U. of NM in the FU ($p > 0.05$), and VIP values were 33.9 ± 3.9 C.U. in the PUF in the UB vs. 32.6 ± 4.8 C.U. of the NM in the UB ($p > 0.05$). These findings show that SP and VIP neurofibers are present in the fibroid pseudocapsule, similar to the values in the normal myometrium of a non-pregnant uterus. An intracapsular myoma excision which respects the pseudocapsule permits a physiological healing process of the uterine scar, due to a neurotransmitter sparing at the hysterotomic site. In women planning pregnancy, the myomectomy should be preferably performed respecting the pseudocapsule in order to preserve the neurotransmission.

1. Introduction

When the architecture of the myometrium and its surrounding in the presence of fibroids was studied, a kind of pseudocapsule surrounding the fibroid was found. This pseudocapsule separates the fibroid from the normal myometrium.

The fibroid is attached to the pseudocapsule by connective bridges in continuation with the uterine collagen skeleton, but lacks its own vascular pedicle, since a vascular network surrounds the fibroids, so that the excision of a fibroid needs to be done inside the pseudocapsule separating this vascular network [27].

In the uterine structure different neurofibers and neuropeptides exist and among these, two estrogen-regulated neuropeptides, Substance P (SP) and the Vasoactive Intestinal Polypeptide (VIP), both involved in the musculature regulation.

The SP, in the genital tract, has afferent fibers which are involved in pain regulation and efferent fibers which are involved in microvasculature regulation. The SP plays an important role in the cervical ripening and in the perception of pain during labor [29]; in non-pregnant women, different studies described nerve fibers containing immunoreactivity to SP, associated either to cervical vascularization (particularly involved in vasodilatation) in the cervix and in the myometrium (for myoregulation) [7].

The VIP functions as neurotransmitter in the nervous control of the reproductive tract. In the female genital system, the nerve fibers

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containing VIP are found in blood vessels, non-vascular smooth muscles and in the epithelium lining [4,15].

There is a lack of experimental and clinical studies concerning the role of SP and of VIP related fibers in uterine fibroids and in its pseudocapsule.

The authors examined the SP and VIP distribution in the pseudocapsule and in the non-pregnant myometrium adjacent to the fibroids.

2. Material and methods

2.1. Materials

Between January 2005 and December 2009, 64 women were scheduled for single or multiple myomectomies in affiliated University Hospitals.

The indications for myomectomy were the following symptoms: severe pelvic pain, heavy menorrhagia not responding to conservative treatment or uncontrolled growth as verified by repeated ultrasounds.

Only 57 of the women agreed to sign an informed consent as approved by the local institutional research ethics committee and participated in the study. Prior to the surgery, pregnancy was excluded by a β-HCG test. All these women were Caucasians.

The exclusion criteria were: previous uterine scar (including cesarean section) and post-treatment of GNRH analogs as well as a history of gynecological tumors.

The exclusion criterion for women treated with GNRH analogs was due to the reported increased risk of recurrence and possible delay in the diagnosis of leiomyosarcoma as well as a risk of massive hemorrhage due to difficulties in entering the cleavage plane, and greater extent of hyalinization [7].

All fibroids were diagnosed using standardized transabdominal and transvaginal ultrasound myoma mapping by an expert technician; the fibroids were subserous, intramural, corporal and fundal and the ultrasound data were recorded for postsurgical evaluation.

All the fibroids in this study were single or multiple located in the fundus or the corpus. Pedunculated, cervical and intraligamentary fibroids were excluded as they show a different pseudocapsule and due to the absence of the normal myometrium around the fibroid.

All myomectomies were performed by laparotomy or laparoscopy, depending on their dimension and the surgeons' preference. The diameter of the fibroids was between 5 and 10 cm. All women were given a standard prophylactic antibiotic dosage of cefazolin 2 g I.V. All the operations were performed under general anesthesia with endotracheal intubation.

All surgical procedures were performed by experienced gynecologists or senor residents who master the correct methodology of removing fibroids from within the pseudocapsule as the reported evidence-based data show [26,27].

Fibroids are generally surrounded by vascular network, and the separation of the fibroids needs to occur in the inner aspect of the pseudocapsule, in order to prevent excessive bleeding and postoperative complications such as intra-myometrial hematomas and facilitate a favorable post-operative healing.

The laparotomy or endoscopic myomectomies were performed using a standardized method. The incisions were done longitudinally, preferably in the midline using a monopolar or bipolar coagulation gradually until opening the pseudocapsule enabling to enter the relatively bloodless plan between the pseudocapsule and the fibroid (Fig. 1). Once the surface of the fibroid was reached and its fiber bridges freed, the fibroid was hooked and extracted from its capsule by traction and pushing down the capsule.

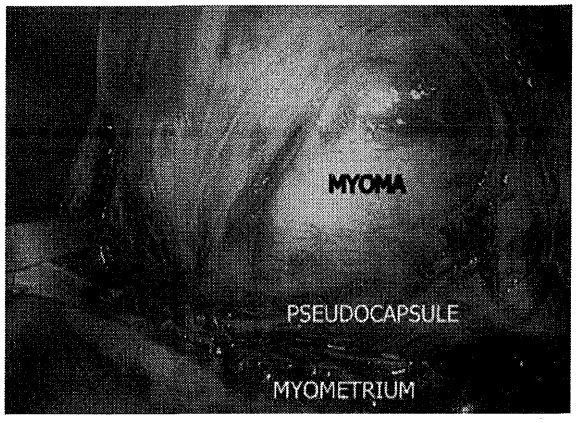

Fig. 1. Intraoperative laparoscopic picture of uterine myoma intracapsular removing; surgeons expose by scissors the myoma pseudocapsule and normal myometrium before sampling.

2.2. Experimental procedure

Samples were taken by scissors from the surface of the pseudocapsule as soon as good hemostasis was reached, and four samples of approximately 5 mm in depth, which included full thickness of the surrounding myometrium, were collected and sent to the laboratory in a dry-ice container for histological and immunefluorescent studies.

In the laboratory, the samples were washed by immersion in a cold Krebs-Ringer's solution, and examined through immunefluorescent techniques for detection of SP and VIP nerve fibers. Slides were prepared with cryostat to obtain sections of 40 μm. Each section was placed on a cover slide, to which it adhered due to the temperature difference. Each slide was checked for SP, according to the method described by Lorton et al. [19], and for VIP according to the methodology suggested by Gomariz et al. [9].

The analysis of the samples was carried out with a fluorescence Leitz Ortoplan microscope equipped with an epi-illumination system. The light source used was a mercury lamp (HB 100) combined with selective Leitz filters.

2.3. Analysis of SP and VIP

The density of SP and of VIP fibers was calculated by quantitative analysis using a Quantimet Leitz image analyzer which measures the following parameters:

1. number of SP and of VIP containing fibers counted in randomly chosen 10 fields;
2. percentage of the total area occupied by those fields;
3. number of observed varicosities;
4. number of crossings or intersections of the nerve fibers
5. the total perimeter of SP and of VIP structures in proportion to an average value (100 for each field).

Five consecutive serial sections were obtained by cryostat microtome, placed on five separate slides and prepared for the detection of each neurotransmitter and were placed in different batches.

In the first batch: primary or secondary antiserum omitted, denatured or previously absorbed by corresponding peptide in excess; in the second batch: primary or secondary antiserum replaced by a non-immune serum; in the third batch: sample previously fixed by immersion in a 4% solution of formaldehyde in PBS which did not preserve the immune-reactive sites; in the fourth batch: these samples were denatured with formaldehyde before or

Table 1
Distribution of Substance P and Vasoactive Intestinal Peptide in normal myometrium and in pseudocapsule.

	(A) Normal myometrium in fundus uteri	(B) Normal myometrium in uterine body	(C) Fibroid pseudocapsule of fundus uteri	(D) Fibroid pseudocapsule in uterine body	*p* Value
Substance P (SP)	8.1 U.C. ± 0.6	23.2 U.C. ± 1.4	10.2 U.C. ± 0.1	25.1 U.C. ± 0.9	$p > 0.05$
Vasoactive Intestinal Peptide (VIP)	9.8 U.C. ± 1.4	32.6 U.C. ± 4.8	11.5 U.C. ± 0.9	33.9 U.C. ± 3.9	$p > 0.05$

after treatment with primary antiserum or before treatment with secondary antiserum.

The samples were incubated for 18 ± 24 h at room temperature, so that the antibodies could completely penetrate all the thickness of the sections, with the polyclonal antiserum rabbit anti-VIP (RBI, Cambridge, UK) diluted 1:400 in PBS.

The samples were then washed in PBS and incubated with fluorescing isothyocyanate-conjugated antiserum; the goat anti-rabbit IgG (Nordic Immunological Reagents, Amersham, Netherlands) diluted 1:100 in PBS for 18 ± 24 h at room temperature allowing the complete penetration of the fluorescent IgG into the slides. Further details on each type of immunoassaying are reported [16,19]. The fresh cryostatic sections were not mounted during the staining. After the staining had been performed, the samples were washed in PBS and then immersed in Entellan (non-auto fluorescent) and examined using a Zeiss III photomicroscope (Carl Zeiss, Oberkochen, Germany).

Further details on each type of immunostaining are reported for VIP by Gomariz et al. [9].

Once the nerve fibers had been marked specifically (for SP and VIP), it became possible to identify under light microscopic examination the area of each of the types of nerve fiber marked by a specific fluorescent neurotransmitter. Morphometry has recently become a valid method for quantification of cell elements density in different pathological and physiological conditions such as normal and abnormal placentas [20].

Morphometrical quantification of the density of each type of nerve fiber was performed on photographs of stained samples using a Quantimet Leica 2000 image analyzer (Quantimet 500 Leica Microsystems Imaging Solutions Ltd., Cambridge, UK). The software provided with the Quantimet Leica analyzer is able to count and express these fluorescent areas in conventional units (C.U.), i.e. as percentages of the area occupied by a single type of nerve fibers related to the total observed area. By adding these values (single type of nerve fibers) it is possible to evaluate the sum of the areas occupied by the different types of nerve fibers.

The software also calculates the average values and translates them to a single value with standard deviation: this value can be read on the instrument display and is reported with standard error from the mean (SEM). Other details about the experimental procedures used in the morphometrical quantification by the Quantimet Leica are reported in the Manual of Methods.

2.4. Statistical analysis

Statistical analysis of the data were based on the results obtained from measurements from each sample by the Quantimet Leica analyzer software; and the data were averaged to obtain a median value per case. The mean ± SEM were then calculated and reported for each nerve fiber group. Repeated immune-fluorescent controls were made and the differences were calculated by Student's test, where the *p* was calculated as index of the significance.

3. Results

The fifty-seven women included had a mean age of 38.3 years, a mean BMI of 24.1, and a mean parity of 1.7.

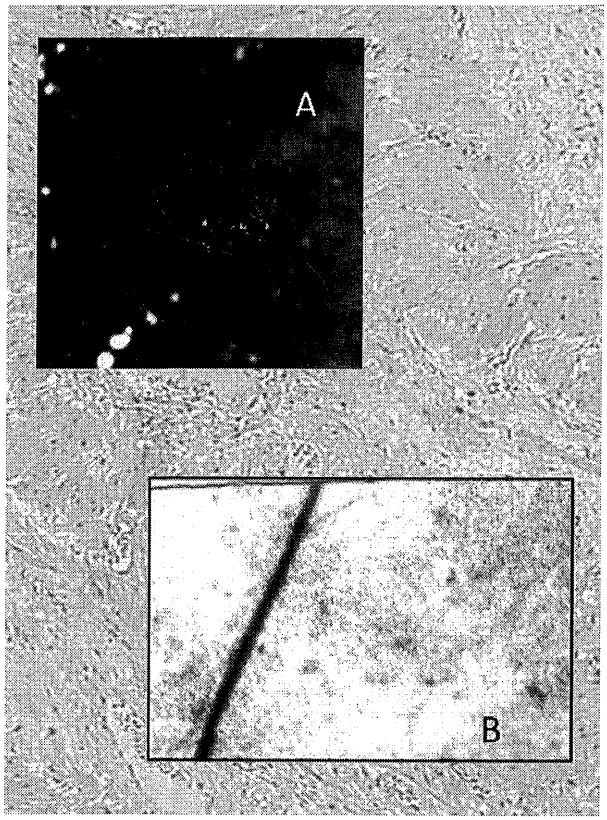

Fig. 2. Section of normal myometrium of fundus uteri hematoxylin–eosin stain. The upper boxed area corresponds to the Substance P fibers distribution ((A) magnification 600×), the lower boxed area corresponds to the VIP fibers distribution ((B) magnification 800×).

All of them had a normal postoperative recovery, without reported early or late complications on the first 15 days.

Before the immune-fluorescent evaluation of SP and VIP was performed, a histological examination was done either of the normal myometrium or of the pseudocapsule.

SP levels were 10.2 ± 0.1 C.U. in the fibroid pseudocapsule as compared to 8.1 ± 0.6 C.U. in the normal myometrium taken from the fundus (Fig. 2), and the SP levels were 25.1 ± 0.9 C.U. in the fibroid pseudocapsule of the uterine body as compared to 23.2 ± 1.4 C.U. in the normal myometrium of the uterine body (Fig. 3). The VIP levels were 11.5 ± 0.9 C.U. in the fibroid pseudocapsule from the fundus as compared to 9.8 ± 1.4 C.U. in the normal myometrium taken from the fundus (Fig. 4), and the VIP levels were 33.9 ± 3.9 C.U. in the fibroid pseudocapsule of the uterine body as compared to 32.6 ± 4.8 C.U. in the normal myometrium of the uterine body (Fig. 5). The SP and VIP quantitative distribution in the normal myometrium and the pseudocapsule, in the fundus and the uterine body, revealed a non-significant increase of SP and VIP levels in the pseudocapsule (see Table 1).

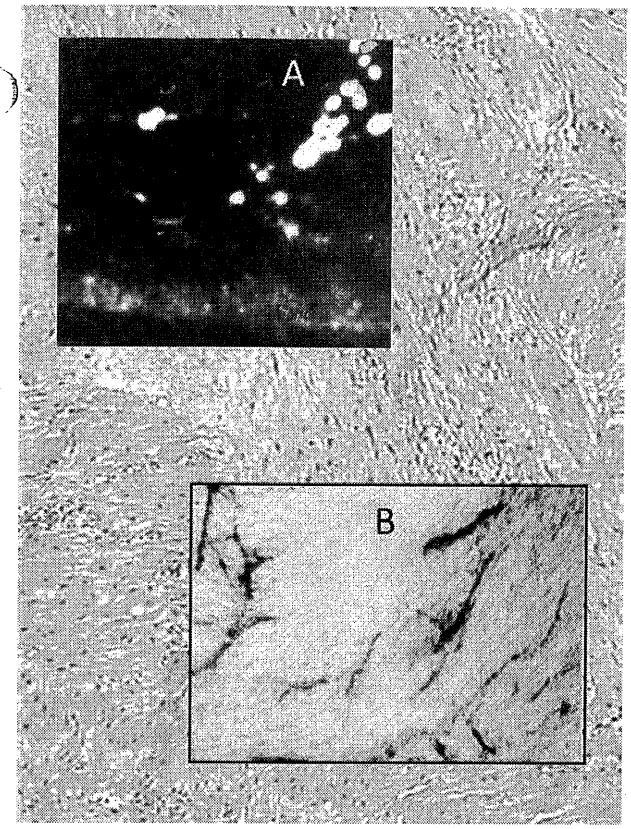

Fig. 3. Section of normal myometrium of uterine body. Hematoxylin–eosin stain. The upper boxed area corresponds to the Substance P fibers distribution ((A) magnification 600×), the lower boxed area corresponds to the VIP fibers distribution ((B) magnification 800×).

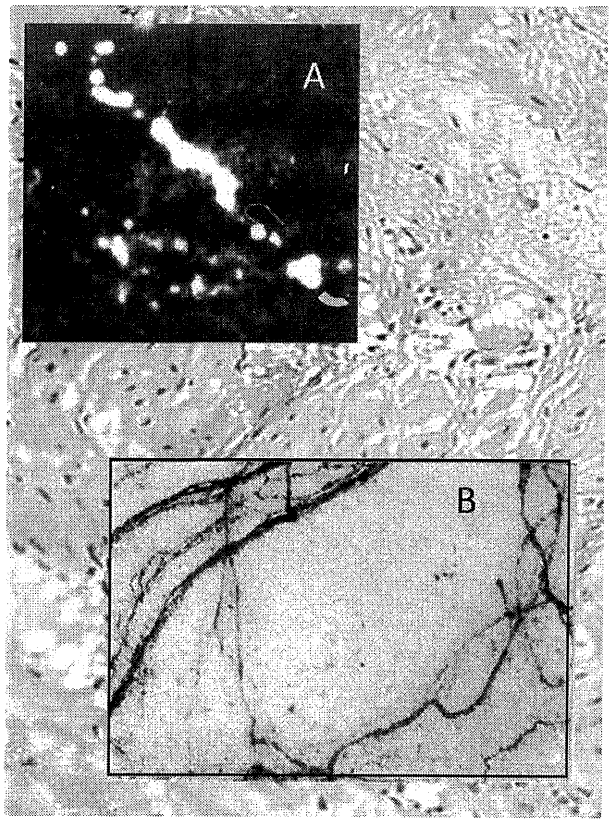

Fig. 4. Section of myoma pseudocapsule of fundus uteri. Hematoxylin–eosin stain. The upper boxed area corresponds to the Substance P fibers distribution ((A) magnification 600×), more present than the VIP fibers in the lower boxed area ((B) magnification 800×), respect normal myometrium.

4. Discussion

To better understand the role of SP and VIP in the myometrium and its pseudocapsule, the authors revised the existing clinical and experimental studies on uterine neurotransmitters.

Collins et al. [6] found an abundance of nerves accompanying the cervical vasculature and myometrial smooth muscle reacting immunologically to SP.

Gram and Ottesen [10] reported that SP may play a physiological role in the local nervous control of myometrial blood flow in the non-pregnant uterus.

Concerning the sensory part of SP neurofibers, there are studies in animals and in humans with endometriosis which show that in presence of endometriosis the SP neurofibers for evoked pain are altered [3,30]. Schmidt et al. [23] showed that SP acts via its preferred neurokinin 1 receptor (NK1-R); as its receptor is located on vascular cells.

A so-called "neurogenic inflammation reaction" is induced, which is characterized by an increase in vascular permeability, accompanied by the recruitment of leucocytes, and associated with vasodilatation [18,22].

The close association of SP immunoreactive nerve endings to the post-capillary cervical venules, and the localization of their receptors in the endothelium of these vessels [6], suggest that SP induced inflammatory-like responses may be mediated by the microvasculature. Indeed, SP administered to virgin rats invoked vasodilatation and vascular leakage, leading to the extravasation of plasma into the uterine cervix [6].

VIP has important biological functions in the female genital tract, including the relaxation of non-vascular and vascular smooth muscle as well as the ovarian steroid genesis and the induction of ovulation; the VIP is an important regulator of the smooth muscle cells of the circular and longitudinal musculature, particularly around the internal uterine orifice [21].

The regulatory role of VIP is morphologically confirmed by the presence of VIP-containing nerves in the uterus derived from the paracervical ganglion [1,11,14].

Pituitary adenylate cyclase activating polypeptide (PACAP) (a VIP-related peptide) immunoreactive nerve fibers have been detected in the human uterus suggesting a vascular regulatory role for this peptide [24]. Bajo et al. reported data on the expression and functionality of VIP receptors in the uterus and other parts of the genital tract. These findings strongly suggest an important role for this neuropeptide in normal and pathologic conditions. The fibroids seem to have a higher VIP receptor (VPAC2 receptor) protein level than the myometrium and the endometrium, whereas the tumor tissue exhibited low adenylyl cyclase activity as compared to the normal uterine tissues. This apparent discrepancy may be explained via variable degrees of receptor accessibility and/or differences in the extent of receptor/effecter coupling [2].

Houdeau et al. [13] report that the VIP coexists with acetylcholine and Stjernquist and Owman [25] affirmed that the VIP assists the relaxation of the myometrium through the inhibition of the excitatory activation of acetylcholine. The VIP is considered to be an important regulator of the uterine blood flow.

It is not known whether endothelium-derived relaxing factors contribute to the VIP action in the uterine arteries. There are reports that VIP produced a concentration-dependent relaxation in the uterine arterial rings of guinea pigs, both in the intact and the denuded endothelium [12].

Bredkjoer et al. [5] described a dense nerve fibers supply to the internal and external cervical uterine orifice which are immunore-

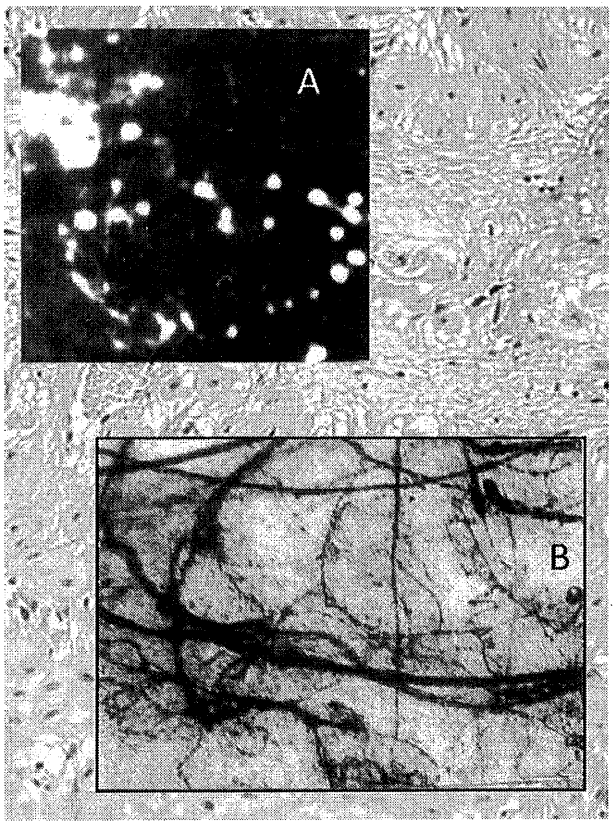

Fig. 5. Section of myoma pseudocapsule of uterine body. Hematoxylin–eosin stain. The upper boxed area corresponds to the Substance P fibers distribution ((A) magnification 600×), more present than the VIP fibers in the lower boxed area ((B) magnification 800×), respect normal myometrium.

active to all the VIP-derived peptides, especially those located around blood vessels.

The hyperinnervation in intestinal deep infiltrating endometriosis may explain why patients with this type of lesion have a higher degree of pain [31]. Immunoexpression of sympathetic and parasympathetic fibers (especially of VIP) is higher in women with deep pelvic endometriosis than in healthy women [17].

Moreover, the low estrogen level in postmenopausal women seems to increase the arterial vascular tone through a reduction of vasodilator neuropeptides and by an increase of vasoconstrictor peptides, in the arterial-wall endings of the autonomous system. These changes in the neuropeptide content in the arterial walls might probably lead to a new understanding of the mechanism underlying the negative effects of menopause on the cardiovascular system [8].

Currently, there are histological, ultrasonographic and electronic microscopically studies about various aspects of fibroid pseudocapsule [27], but there are no studies concerning the level of the neuropeptides connected to the neurotransmitter activities in them.

This study has shown that SP and VIP are present in the normal uterus, as well as they are increased in the fibroid pseudocapsule (Fig. 6); they could increase in the pseudocapsule due to the density rise of these neurotransmitters, which is similar to the normal myometrium pressed by the fibroid, the neoangiogenesis evoked by the growing fibroid, and because of the large dimension of fibroids, which compresses the pseudocapsule and myometrium, causing pain (associated with SP increasing). This happens also in fibroid degenerations.

Throughout the pregnancy, normal myometrium contains these neurofibers, who provide the environment for the growing fetus,

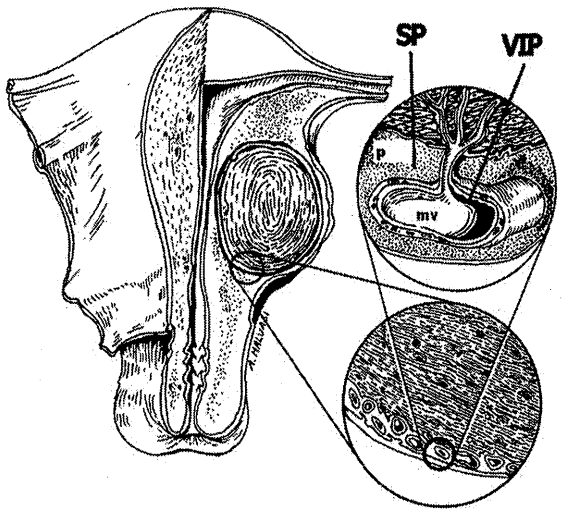

Fig. 6. Sagittal and frontal non-pregnant uterus in left midsection with corporal intramural myoma. The lower box area shows a pseudocapsule magnification. The upper box area shows the enlargement of myoma pseudocapsule vascularization. SP = Substance P, VIP = Vaso Intestinal Peptide, p = pain, mv = microvascularization (and/or neoangiogenesis).

while during labor, facilitate the cervix relaxing and uterine contracting, enabling the passage of the newborn. A lack of coordination of this transition leads to abnormal labor. Moreover, the estrogens acts directly on the uterus through the peripheral primary afferent neurons who innervates the uterine musculature, The birth process is therefore influenced by a combined neurons and hormones activity [28].

The presence of the neurotransmitters in the fibroid pseudocapsule shows that they are comparable to their presence in the myometrium, and they should be left in the uterus during myomectomy, by an intracapsular fibroid removal [26]. For these reasons, during the myomectomy, the fibroid pseudocapsule needs to be protected, avoiding destructive proceedings such as diathermocoagulation, who alters these neurotransmitters. In this way, a good healing and good functioning of the uterine wall is facilitated [26].

The authors suggest to avoid destructive proceedings during myomectomy such as extensive diathermy. This will alter the level of the neurotransmitters with negative effect on future pregnancies.

5. Conclusions

This study has shown that SP and VIP neuro fibers are present in the pseudocapsule of the fibroid as well as in the normal myometrium of the non-pregnant uterus. Each surgical method used for fibroid removal should respect the pseudocapsule, since it is rich in neurofibers, essentials for a correct muscular healing and myometrial functioning, particularly in future pregnancies.

The intracapsular fibroid excision enhances a correct healing process of the uterine scar, leading to a neurotransmitter sparing in the surgical site. For these reasons, in women planning pregnancy after myomectomy, the operation should be always performed using the intracapsular method, since this technique should preserve a possible future pregnancy, possibly avoiding uterine rupture during pregnancy and labor [26].

Conflict of interest

Authors certify that there is no actual or potential conflict of interest in relation to this article and they reveal any

financial interests or connections, direct or indirect, or other situations that might raise the question of bias in the work reported or the conclusions, implications, or opinions stated including pertinent commercial or other sources of funding for the individual author(s) or for the associated department(s) or organization(s), personal relationships, or direct academic competition.

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