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Original article

# The influence of botulinum toxin type A (BTX) on the immunohistochemical characteristics of noradrenergic and cholinergic nerve fibers supplying the porcine urinary bladder wall

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## Abstract

Botulinum toxin (BTX) belongs to a family of neurotoxins which strongly influence the function of autonomic neurons supplying the urinary bladder. Accordingly, BTX has been used as an effective drug in experimental therapies of a range of neurogenic bladder disorders. However, there is no detailed information dealing with the influence of BTX on the morphological and chemical properties of nerve fibres supplying the urinary bladder wall. Therefore, the present study investigated, using double-labeling immunohistochemistry, the distribution, relative frequency and chemical coding of cholinergic and noradrenergic nerve fibers supplying the wall of the urinary bladder in normal female pigs (n=6) and in the pigs (n=6) after intravesical BTX injections. In the pigs injected with BTX, the number of adrenergic (D $\beta$ H-positive) nerve fibers distributed in the bladder wall (urothelium, submucosa and muscle coat) was distinctly higher while the number of cholinergic (VAcHT-positive) nerve terminals was lower than that found in the control animals. Moreover, the injections of BTX resulted in some changes dealing with the chemical coding of the adrenergic nerve fibers. In contrast to the normal pigs, in BTX injected animals the number of D $\beta$ H/NPY- or D $\beta$ H/CGRP-positive axons was higher in the muscle coat, and some fibres distributed in the urothelium and submucosa expressed immunoreactivity to CGRP. The results obtained suggest that the therapeutic effects of BTX on the urinary bladder might be dependent on changes in the distribution and chemical coding of nerve fibers supplying this organ.

**Key words:** botulinum toxin, urinary bladder, nerve fibres, immunohistochemistry, pig

## Introduction

The proper urine storage process depends, among other things, on an undisturbed transmission in the autonomic nerves supplying the urinary bladder wall. The smooth urinary bladder detrusor muscle has

double, acting contradictory, sympathetic-parasympathetic innervation. The sympathetic innervation takes place via the hypogastric nerve coming from the intermediolateral nucleus of the lumbar segments of the spinal cord. The parasympathetic innervation is carried out by pelvic nerve coming from the inter-

mediolateral nucleus of the sacral segments of the spinal cord (el-Badawi and Schenk 1966).

One of substances which strongly influence the function of the autonomic nervous system supplying the urinary bladder wall is Botulinum toxin (BTX), produced by the Gram-negative, rodshaped anaerobic bacteria *Clostridium botulinum* (van Ermengem 1897). This potent neurotoxin acts by inhibiting acetylcholine (ACh) release at the presynaptic cholinergic neuromuscular junction (Arnon et al. 2001). Currently, seven immunologically distinct forms of botulinum toxin are distinguished, including A, B, C, D, E, F and G. Only botulinum toxin type A (BOTOX®, Dysport®) and B (Myobloc®/Neurobloc®) have been approved for use in the treatment of conditions that are characterized by excessive or inappropriate muscle contractions. Botulinum toxin type A (BoNT/A) was first investigated for its effects on the parasympathetic nervous system in the 1920s (Dickson and Shevky 1923). Recently, BTX has been used in medicine as an effective drug in experimental therapy of a range of neurogenic urinary bladder disorders. The literature in the field contains many contributions regarding a possible clinical use of BTX-toxin in urology (for instance: Schurch et al. 2000, Reitz et al. 2004, Grosse et al. 2005). However, there is no data concerning the influence of BTX on the chemical coding of autonomic nerves supplying the urinary bladder wall, whereas, this matter seems to be of a great interest considering the mechanism of the toxin action and the clinical results obtained. Therefore, the present study was aimed at investigating the distribution, relative frequency and chemical coding of cholinergic and noradrenergic nerve fibers supplying the wall of the urinary bladder in normal female pigs and in the pigs after intravesical BTX injections.

## Materials and Methods

The study was performed on 12 juvenile (8-12 weeks old, 15-20 kg body weight, b.w.) female pigs of the Large White Polish race. The animals were kept under standard laboratory conditions with free access to water. All Surgical procedures were performed under deep barbiturate anaesthesia according to the guidelines of the Local Ethics Committee. The pigs were divided into two groups. Six animals were used as a control group. Six other pigs were injected with botulinum toxin type A (100 IU per animal, Botox) into the urinary bladder wall using cystoscope. Before the BTX injections, the animals were pretreated with atropine (Polfa, Poland; 0.04 mg/kg b.w., s.c.) and propionylpromazine (Stresnil, Janssen Pharmaceutica, Belgium; 0.5 mg/kg b.w., i.m.), and after thirty minutes, sodium pentobarbital (Tiopental, 0.5 g per animal) was given intravenously in a slow, fractionated

infusion. One week after the BTX injections, all the pigs were deeply anaesthetized with sodium pentobarbital and transcardially perfused with 4% buffered paraformaldehyde (pH 7.4). The samples of the urinary bladder corpus were collected, placed in the fixative (10 minutes), washed several times in 0.1 M phosphate buffer and stored in 18% buffered sucrose at 4°C. The samples were cut with a cryostat on 10- $\mu$ m-thick sections which were processed for double-labelling immunofluorescence.

## Immunohistochemical procedure

Ten  $\mu$ m-thick cryostat sections of the tissue samples were processed for double-labeling immunofluorescence (according to an earlier described method; Kaleczyc et al. 1999) to study the distribution of the intramural nerve fibres and their chemical coding using antibodies (listed in Table 1) against dopamine  $\beta$ -hydroxylase (D $\beta$ H; marker of noradrenergic fibres), vesicular acetylcholine transporter (VAcHT; marker of cholinergic fibres), NOS (nitric oxide synthase), NPY (neuropeptide Y), VIP (vasoactive intestinal polypeptide), GAL (galanin), L-ENK (Leu<sup>5</sup>-enkephalin), PACAP (pituitary adenylate cyclase-activating polypeptide), SOM (somatostatin), SP (substance P) and CGRP (calcitonin gene-related peptide). D $\beta$ H-antiserum was applied in a mixture with antisera against NOS, NPY, VIP, GAL, L-ENK, PACAP, SOM, SP or CGRP, respectively. VAcHT-antiserum was applied in a mixture with antisera against CGRP, GAL, L-ENK, NOS, PACAP, SOM, SP or VIP, respectively.

The sections were studied with an Olympus BX51 microscope equipped with epifluorescence filter and an appropriate filter set for CY3 and FITC. Micrographs were made using a digital camera connected to a PC, analyzed with AanalySIS software (version 3.02, Soft Imaging System, FRG) and printed on a wax printer (Phaser 8200, Xerox, USA).

## Results

### Distribution of VAcHT-immunoreactive nerve fibers and their chemical coding

#### Control animals

In the control animals, a very dense network of VAcHT-IR nerve fibers was distributed in the muscle coat (Fig. 1a, 2a). Many of these nerve terminals were observed around blood vessels. A moderate number of the cholinergic nerve endings were found in the submucosa and only single axons were encountered beneath the urothelium (Fig. 2a).

Table 1. List of primary antisera and secondary reagents used in the study. (FITC fluorescein isothiocyanate).

Antigen	Code	Dilution	Species	Supplier
Primary antibodies				
CGRP	T-5027	1:400	Guinea pig	Peninsula
	AB5920	1:8000	Rabbit	Chemicon
D $\beta$ H	MAB 308	1:300	Mouse	Chemicon
	D21020	1:4000	Rabbit	Biomol
GAL	T-5036	1:1000	Guinea pig	Peninsula
	4600-5004	1:4000	Rabbit	Biogenesis
L-ENK	4140-0355	1:800	Mouse	Biogenesis
	EA1149-002	1:600	Rabbit	Biomol
NOS	N2280	1:400	Mouse	Sigma
	AS2143	1:17000	Rabbit	Euro-Diagnostic
NPY	NA1233	1:8000	Rabbit	Biomol
	ZO5869	1:100	Rat	Biomol
PACAP	40119	1:200	Guinea pig	Phenix
	T-4465	1:20000	Rabbit	Peninsula
SOM	11180	1:30	Rabbit	Inc-Cappel
	T-1608	1:30	Rat	Bachem
SP	8450-0505	1:100	Rat	Biogenesis
VACHT	H-V006	1:6000	Rabbit	Phoenix
VIP	VA1285	1:6000	Rabbit	Biomol
	9535-0504	1:1000	Mouse	Biogenesis
Secondary reagents				
Biotinylated antiserum	E 0432	1:800	Rabbit	Dako
CY3	711-165-152	1:8000	Rabbit	Jackson I.R.
FITC-conjugated anti-mouse IgG	715-096-151	1:400		Jackson I.R.
FITC-conjugated anti-rat IgG	712-095-153	1:400		Jackson I.R.
FITC-conjugated anti-guinea pig IgG	706-095-148	1:600		Jackson I.R.

In the muscle layer, the vast majority of VACHT-IR nerve fibers were immunopositive to SOM or NPY, and many VACHT-IR axons contained immunoreactivity to NOS. Solitary cholinergic axons located in the muscle coat were also CGRP or VIP-positive.

Most of the cholinergic nerve terminals surrounding blood vessels exhibited immunoreactivity to SOM, and single VACHT-IR axons expressed also immunoreactivity to CGRP or NOS.

In the submucosa, the majority of the cholinergic nerve fibers showed immunoreactivity to SOM. A moderate number of axons were also VIP-positive and single nerve terminals stained for NOS.

The vast majority of the cholinergic nerve terminals penetrating beneath the urothelium were SOM-immunopositive and single VACHT-IR axons revealed immunoreactivity to VIP or CGRP.

The cholinergic nerve fibers were GAL, L-ENK-, PACAP-, SP- and D $\beta$ H-immunonegative.

## Animals after BTX injections

After BTX injections, VACHT-IR fibers were unevenly distributed in the muscle coat (Fig. 1b). Only single nerve terminals were found in areas located close to the submucosa (Fig. 2b), whereas in the remaining, external part they formed a very dense network, comparable to that observed in the control animals. Single VACHT-IR nerve endings were distributed around blood vessels and in the submucosa. No VACHT-IR fibers were found beneath the urothelium.

Double-labeling immunofluorescence revealed that the chemical coding of cholinergic axons in BTX treated pigs was similar to that observed in the control animals.

VACHT-IR nerve fibers were GAL, L-ENK-, PACAP-, SP- and D $\beta$ H-immunonegative.

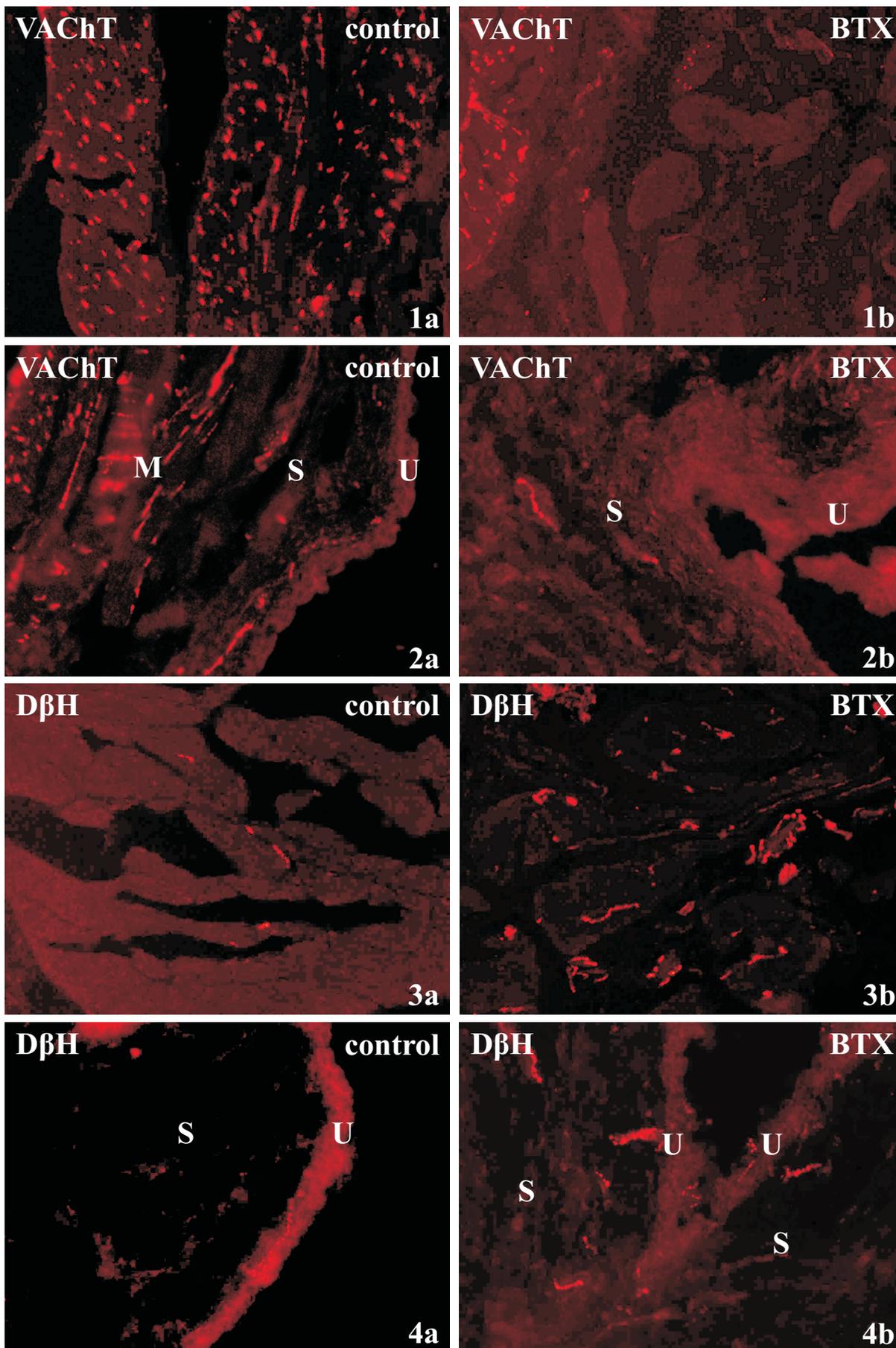


Table 3. The distribution and relative frequency of VACHT-IR nerve fibers supplying the porcine urinary bladder wall.

Part of the urinary bladder wall	Control pigs	Pigs after BTX injection	
Muscle layer	++++	+++	↓
Submucosal layer	++	+/-	↓
Urothelium	+	-	↓
Around blood vessels	+++	+/-	↓

+/- – single fibres; + – few fibres; ++ – moderate number of fibres; +++ – many fibres

### Distribution of D $\beta$ H-immunoreactive (D $\beta$ H-IR) nerve fibers and their chemical coding

#### Control animals

In the urinary bladder of the control animals, a small number of D $\beta$ H-IR nerve fibers was distributed in the muscle coat (Fig. 3a). Blood vessels were densely supplied with these axons. A moderate number of D $\beta$ H-IR nerve terminals was observed in the submucosa and only single fibers were found beneath the urothelium (Fig. 4a).

Double-labeling immunohistochemistry disclosed that in the muscle layer, a moderate number of D $\beta$ H-IR axons stained for SOM or L-ENK and many of the adrenergic nerve fibers revealed also immunoreactivity to NPY. Solitary D $\beta$ H-IR nerve terminals were CGRP-IR.

Most of the adrenergic axons associated with blood vessels stained for NPY, and single D $\beta$ H-IR nerve terminals expressed immunoreactivity to SOM or L-ENK.

Only single D $\beta$ H-IR axons stained also for SOM, NPY or L-ENK in the submucosa and beneath the urothelium.

The adrenergic nerve terminals were GAL-, NOS-, PACAP-, SP-, VIP- and VACHT-immunonegative.

#### Animals after BTX injections

In the urinary bladder wall of the pigs after intravesical BTX injections, the number of D $\beta$ H-IR nerve fibers in the smooth muscle layer was significantly

higher than that found in the control animals (Fig. 3b). Many of these axons were also observed around blood vessels. A large number of the fibers was distributed in the submucosa and a few axons were found to penetrate under the epithelium (Fig. 4b).

Double-labeling investigations revealed that the chemical coding of the adrenergic nerve terminals after BTX treatment was basically very similar to that found in the control group. However, in the muscle layer, the number of D $\beta$ H-IR nerve terminals containing also immunoreactivity to NPY was slightly higher (Fig. 5). Some distinct differences were also observed with regard to adrenergic nerve fibers which exhibited immunoreactivity to CGRP. The number of these axons was slightly higher in the muscle layer. Moreover, in contrast to the findings obtained from the control group, many D $\beta$ H-IR nerve fibers found in the submucosa (Fig. 6) and beneath the urothelium expressed immunoreactivity to CGRP, and single D $\beta$ H/CGRP-IR nerve terminals were associated with blood vessels. D $\beta$ H-positive nerve terminals were GAL, NOS, PACAP, SP, VIP, and VACHT-immunonegative.

### Discussion

The present study has revealed that application of BTX causes significant changes in the distribution, relative frequency and chemical coding of adrenergic and cholinergic nerve fibers supplying the wall of the porcine urinary bladder.

The present results dealing with adrenergic and cholinergic innervation pattern of urinary bladder wall

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Fig. 1. Distribution of VACHT-positive nerve fibers (red; labelled with CY3) in the muscle layer of the urinary bladder in the normal (a) and BTX-treated (b) pig. In the BTX-injected animal, the nerve fibres were less numerous than in the normal pig; x20.

Fig. 2. Distribution of VACHT-positive nerve fibers (red; labelled with CY3) in the urinary bladder wall of the normal (a) and BTX-treated (b) pig. In the BTX-injected animal, the nerve fibres were less numerous than in the normal pig; U, urothelium; S, submucosa; M, muscle layer; x20.

Fig. 3. Distribution of D $\beta$ H-positive nerve fibers (red; labelled with CY3) in the muscle layer of the urinary bladder in the normal (a) and BTX-treated (b) pig. In the BTX-injected animal, the nerve fibres were more numerous than in the normal pig; x20.

Fig. 4. Distribution of D $\beta$ H-positive nerve fibers in the urinary bladder wall of a normal (a) and BTX-treated (b) pig. In the BTX-injected animal, the nerve fibres were more numerous than in the normal pig; U, urothelium; S, submucosa; x20.

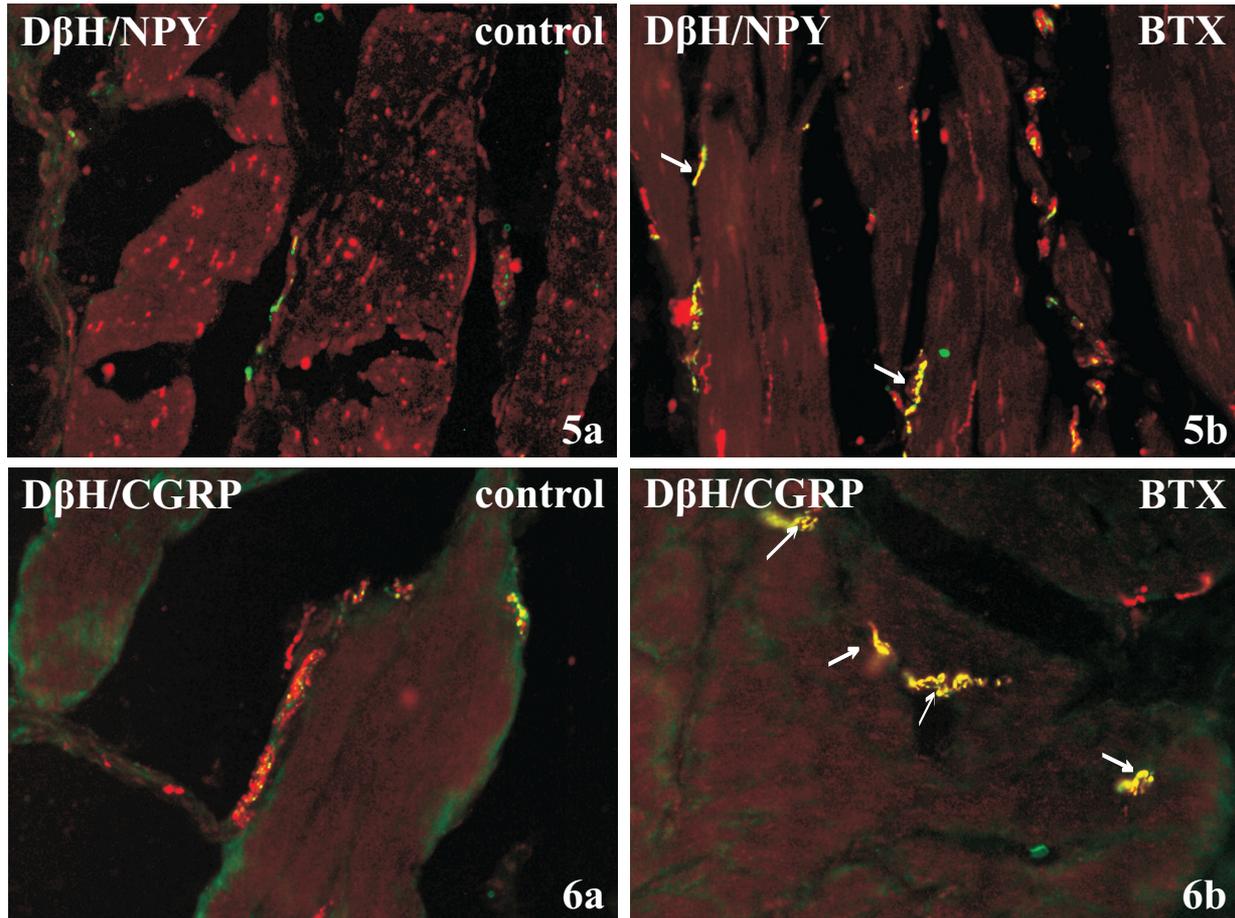


Fig. 5. Distribution of D $\beta$ H- (green; labelled with FITC) and NPY-positive red (labelled with CY3) nerve fibres in the muscle layer of the urinary bladder in the normal (a) and BTX-treated (b) pig. Red and green channels were digitally superimposed. Double-labelled (D $\beta$ H/NPY-positive) fibres are yellow to orange. Note that the number of D $\beta$ H/NPY-positive nerve terminals (arrows) was slightly higher in the BTX-injected animal; x20.

Fig. 6. Distribution of D $\beta$ H- (red; labelled with CY3) and CGRP-positive (green; labelled with FITC) nerve fibres in the submucosa of the urinary bladder in the normal (a) and BTX-treated (b) pig. Red and green channels were digitally superimposed. Double-labelled (D $\beta$ H/CGRP-positive) fibres are yellow to orange. Note that the number of D $\beta$ H/CGRP-positive nerve terminals (arrows) was slightly higher in the BTX-injected animal; x20.

Table 2. The distribution and relative frequency of D $\beta$ H-IR nerve fibers supplying the porcine urinary bladder wall

Part of the urinary bladder wall	Control pigs	Pigs after BTX injection	
Muscle layer	+	+++	↑
Submucosal layer	++	+++	↑
Urothelium	+/-	+	↑
Around blood vessels	++++	+++	↓

+/- – single fibres; + – few fibres; ++ – moderate number of fibres; +++ – many fibres

in control pigs generally correspond well with findings obtained by other authors, in both pigs (Crowe and Burnstock 1989, Persson et al. 1995) and in other mammalian species studied, including humans (Dixon et al. 2000) or rat (Alm et al. 1995), even though the methods used in the above mentioned investigations to visualize autonomic nerves were different to those used in the present study.

It is well known that peripheral autonomic neurons are highly plastic under the influence of diverse physiological and pathological factors (Sharkey and Kroese 2001, Csillik et al. 2003). The plastic changes of neurons include modifications of their chemical phenotype and/or alterations in the density of nerve fibers. These have been confirmed also in pigs in studies performed under physiological

(Łakomy et al. 1986a,b) or experimental (Kaleczyc 1994) conditions.

Probably the most radical factor evoking plastic changes is axotomy, which deprives injured neurons of growth factors, specifically nerve growth factor (NGF) – an important biomolecule for the survival of adrenergic neurons (Hamburger 1992, Levi-Montalcini et al. 1996). Wąsowicz (2003) has revealed that the total or partial extirpation of the uterus in the pig triggers dramatic changes in the chemical coding of uterus-projecting neurons in the porcine inferior mesenteric ganglion. Besides axotomy, there are many other pathological agents or conditions influencing the peripheral autonomic innervation of the urogenital tract. In this paper, only few examples will be mentioned including some investigations concerning the urinary bladder. Immunohistochemical characteristics of nerve fibers supplying the female rat urinary bladder may be affected for instance by: the chronic cystitis (Dickson et al. 2006), the x-ray irradiation (Crowe et al. 1996) or the treatment with biologically active substances like capsaicin or resiniferatoxin (RTX) (Avelino and Cruz 2000). The plastic changes of neurons were also observed in men with a form of the bladder neck obstruction, called bladder neck dys-synergia (Crowe et al. 1995).

The present findings suggest that BTX is a factor evoking very strong adaptational changes in neurons supplying the urinary bladder wall. It is impossible to discuss these results with data obtained by other authors, as no morphological investigations dealing with the distribution and chemical coding of autonomic nerves supplying the urinary bladder wall after BTX injections have been performed so far. However, it has been found that BTX may influence sensory neurons, because significant differences in amounts of CGRP released from the urinary bladder tissues were observed between rats treated with this toxin and the control animals (Rapp et al. 2006). Using a radiochemical method, it has also been demonstrated that BTX influences the release of ACh and norepinephrine from the rat bladder and urethra tissues (Smith et al. 2003).

The paucity of data dealing with the influence of BTX toxin on the chemical coding of nerve fibers supplying the urinary bladder wall is in a striking contrast to the large number of contributions focusing on the possible clinical use of this toxin in urology.

It is well known, that BTX binds to and enters the presynaptic end plate of cholinergic neurons by receptor-mediated endocytosis and selectively cleaves synaptosomal-associated protein 25 (SNAP 25). In that way, BTX prevents normal vesicle docking and fusion to the presynaptic plasma membrane, and inhibits ACh release at the presynaptic cholinergic neuromuscular junction (Haferkamp et al. 2004). Probably due to the toxin mechanism of action, the

most distinct changes observed in this study after BTX treatment concerned the number of VChT-immunopositive fibers. A marked decrease in the number of these fibers was observed in all layers of the urinary bladder wall. In the muscular layer, however, the number of VChT-IR fibers was reduced only in areas neighboring to the submucosa. It should be noted that the samples of the urinary bladder wall were collected one week after the application of the toxin, whereas the temporal clinical effect of the BTX treatment begins within 5 to 7 days and lasts up to 6 months (Schurch and Reitz 2004). Therefore it can be assumed that after a longer period, a decrease in the number of VChT-IR fibers would be observed over the entire width of the muscle layer, but this hypothesis needs further experiments. The parasympathetic innervation, through the release of ACh, activates muscarinic receptors of the detrusor muscle and mediates the contraction of the detrusor muscle (Thuroff 1982) and, thus, causes bladder emptying and micturition (Levin et al. 1986). Therefore a decrease in the number of cholinergic nerve fibers after BTX injections observed in this study can be considered as a very promising finding from the perspective of the treatment of the urinary bladder disorders involving the overreactivity of the bladder detrusor, and may be a foundation for good clinical results. There are two possible rationales for the above mentioned changes in the cholinergic innervation pattern of the urinary bladder wall: first, they could reflect the diminish of the neurotransmitter in some nerve fibers; second, they may reflect the reduction in the general number of the nerves fibers.

Unexpectedly, BTX injections caused also a distinct increase in the number of DβH-IR nerve fibers in all three layers of the urinary bladder wall, especially in the muscle layer. The function of sympathetic innervation is opposite to that of parasympathetic innervation. The release of sympathetic neurotransmitter noradrenalin causes that the detrusor is blocked by inhibitory β-adrenergic receptors and both the bladder neck and the smooth-muscular urethra are tonicized by excitatory detrusor α-adrenergic receptors, thus achieving continence (Nergardh and Boreus 1972, Edvardsen and Setekleiv 1968, Salimi et al. 1969, Raezer et al. 1973, Awad et al. 1974, Levin and Wein 1979). Therefore, the observed increase in the number of adrenergic nerve fibers could be a factor which additionally decreases the spasticity of the overreactive bladder and for that reason improves the treatment.

The present study has revealed that BTX treatment also induces an increase in the number of adrenergic nerve terminals that are immunopositive to CGRP. Generally CGRP is considered as a good marker of sensory nerve fibers since it is expressed in more than 80% of bladder afferents (Gabella and

Davis 1998, Cervero and Laird 2004). The reason for the increased expression of CGRP in the D $\beta$ H-IR axons after BTX injections is not clear. Dickson et al. (2006) observed an overall increase in the number of CGRP-IR nerve terminals especially in the submucosa of the rat urinary bladder in a chronic model of cyclophosphamide-induced cystitis. It is possible that an increase in the number of DBH/CGRP-positive axons observed in the present study may be caused by a temporary cystitis induced by the BTX injection technique. On the other hand Rapp et al. (2006) have found that the incubation of the isolated rat bladders with BTX-A solution inhibits evoked CGRP release, thus the amount of CGRP may be increased in the nerve fibers, because it is accumulated in axons.

In conclusion, the present study has revealed for the first time the existence of profound differences in the distribution, relative frequency and chemical coding of cholinergic and adrenergic nerve fibers supplying the wall of urinary bladder in normal female pigs and in female pigs after intravesical BTX injections. The results obtained suggest that the therapeutic effects of BTX on the urinary bladder might be dependent on changes in the distribution and chemical coding of nerve fibres supplying this organ.

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