EFFECT OF BOTULINUM TOXIN (BTX) ON THE CHEMICAL CODING OF NERVE FIBRES SUPPLYING THE CANINE URINARY BLADDER

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Abstract

The objective of this study was to investigate the distribution and chemical coding patterns of nerve fibres supplying the canine urinary bladder before and after botulinum toxin (BTX) injection. The experimental material comprised six bitches. The injection of the BTX into the urinary bladder wall in dogs clearly altered the bladder's innervation pattern, indicating that BTX affects the components of both the sensory and parasympathetic nervous systems, and that degenerative changes are accompanied by restorative processes.

Key words: bitch, urinary bladder, botulinum toxin, innervation.

The results of research into the innervation of the urinary bladder in mammals indicate that the lower urinary tract comprises a unique system, which, from the neuroanatomical point of view, is controlled by both the central and peripheral nervous systems. The micturition centre is found in the pons section of the central nervous system. It is modulated by the cerebral cortex (17). Peripheral innervation, which is often classified as a system comprising all spinal cord centres, can be divided into sympathetic, parasympathetic, somatic, and sensory sections of urinary bladder innervation. Various types of neurotransmitters are found to coexist in the nerve endings of lower urinary tract tissue. Under pathological conditions, these neurotransmitters may also play the role of promoters and/or mediators of inflammatory processes (3, 6).

Sympathetic nerves (mostly of "pure" noradrenergic character) originate from the ganglia on the lumbar part of the sympathetic trunk, the posterior mesenteric ganglion, pelvic ganglia and single cells of intramural ganglia in the trigone of the urinary bladder (19). The efferent branch of sympathetic reflex arcs begins in the intermediolateral nuclei of the intermediate substance of the spinal cord at level Th10-L2 (humans), L1-L4 (dogs) and L2-L3 (cats), and preganglionic axons may pass out to postganglionic neurons in paravertebrate ganglia, posterior mesenteric ganglia, pelvic ganglia, or only in the ganglia of the target organ. They reach the above ganglia as hypogastric nerve branches (1, 19), releasing neurotransmitters such as NA (2, 12), NPY (2), SOM (15) GAL (28), or LENK (7).

The efferent branch of parasympathetic reflex arcs (composed of cholinergic nerve fibres) begins from the intermediomedial nuclei of the intermediate substance of the spinal cord at levels S2-S4 (humans), S1-S5 (cats and apes), and L6-S1 (rats) in the spinal micturition centre, which are formed by preganglionic cells (8, 21). As preganglionic nerves, their axons leave the spinal cord and, as pelvic nerve components, reach pelvic ganglia and the ganglia of the urinary bladder trigone. In pelvic ganglia, there is a synaptic link to parasympathetic postganglionic cells; alternatively, preganglionic fibres transit through those cells and pass directly to the intramural ganglia in the target organ (bladder, urethra), where they are linked with postganglionic neurons (11). The key neurotransmitters of the parasympathetic system are: ACh, NOS, SOM, NPY, and VIP.

The sensory innervation of the lower urinary tract is attributed to numerous afferent fibres, whose endings are found in all mural layers of target organs (bladder, urethra), and which are particularly abundant in the layer directly under the organ's epithelial lining. The active substances of sensory cells are: SP, CGRP, GAL, PACAP, SOM, and NOS, and in certain species, such as the cat – VIP (23). There are also somatic motor
nerves of the external urethral sphincter leading from the micturition centre (Onuf's nucleus). They are found in segments S2-S4 of the spinal cord, and they reach the target tissue via the pudendal nerve or the ventral roots of the spinal cord section S2-S4. ACh is the neurotransmitter of the somatic system (16).

Inflammations, mechanical damage originating outside (e.g., blunt injury of the abdominal cavity) or inside the bladder (e.g., cystolithiasis), and intravesical pharmacotherapy can affect bladder innervation, eliciting its plasticity. The term "nervous system plasticity" implies every adaptive modification of a developing or a fully-developed nervous system, which enables a nerve cell to adapt functionally to changed conditions (9, 26). Neurons of the peripheral nervous system respond to the damaged integrity of nerve processes by initiating a cascade of biochemical changes, which often modifies the type of the synthesised neurotransmitters. The synthesised neuropeptides may protect the damaged neurons by enabling them to survive damage or even successfully restoring their functions (18, 20).

Botulinum toxin (BTX) is a selective blocking agent of acetylcholine release from nerve endings, which contributes to the cessation of neural transmission. BTX type A is taken up by the neuromuscular junction, which is then destroyed by the toxin. BTX is bound by heavy chains to the presynaptic membrane of cholinergic nerve terminals. The resulting complex is internalised in the nerve ending by active endocytosis. Light chains of BTX are endopeptidases responsible for the degradation of proteins participating in the acetylcholine release process. After internalisation, light chains release SNAP-25 intracellular protein, damaging the secretory pathway (5). This change process is referred to as chemical denervation, which leads to muscular reinnervation (25). As shown by histological tests, a restorative process takes place through nerve fibre budding to produce axon branches without a myelin sheath. BTX inhibits exocytosis only temporarily, and muscular paralysis can persist for up to several months.

Since the chemical innervation pattern of the canine urinary bladder has not been described to date, the first task was to determine the number and coding pattern (the coexistence pattern of transmission substances) of nerve fibres in the urinary bladder of clinically-healthy animals. The distribution and chemical coding of nerve fibres supplying the canine urinary bladder before the injection of the toxin, as well as one week, one month, and six months after the injection, were investigated to broaden the existing knowledge on the mechanism of BTX action.

**Material and Methods**

The study was conducted on six clinically-healthy bitches of mixed breeds. Urinary bladder sections were sampled for immunohistochemical testing to determine the distribution and chemical coding of nerve fibres supplying the urinary bladder. BTX was injected into the bladder, and experimental material was sampled for further analyses 1 week, 1 month, and 6 months later. Chemical coding of nerve fibres was examined on frozen 10 µm, sections of the urinary bladder wall by means of routine immunohistochemical single-label staining.

The experiment was carried out with the use of primary antibodies of various species against Leu5-enkephalin (LENK), neuropeptide Y (NPY), calcitonine gene-related peptide (CGRP), vesicular acetylcholine transporter (VACHT), vasoactive intestinal peptide (VIP), nitric oxide synthase (NOS), galanine (GAL), substance P (SP), pituitary adenylate cyclase-activating peptide (PACAP), dopamine β-hydroxylase (DBH), and tyrosine hydroxylase (TH). Antigen-antibody complexes were visualised by using FITC- or CY-3-labeled secondary antibodies. Sections used for immunohistochemical assays were dried under cover at room temperature for around 45 min, and then rinsed in PBS for 15 min. After rinsing and straining buffer residue on the slide, sections were covered with a blocking agent (PAB). The sections were incubated in a moist chamber at room temperature for 1 h, rinsed three times in PBS for about 10 min, and covered with serum containing antibodies against the studied antigens. The serum was diluted with the PAB solution. The sections were incubated in a moist chamber at room temperature for 16-18 h and then rinsed with PBS (3 x 10 min). Afterwards, they were covered with secondary antibodies and incubated for 1 h at room temperature in a humidity-controlled chamber with the use of secondary antibodies to detect conjugates between the antibody and antigen. Staining (sIF) was performed with immunoglobulin G specific for the species from which primary antibodies were obtained and conjugated with fluorescein isothiocyanate (FITC) or CY-3. After 1 h of incubation, the sections were rinsed with PBS, 3 x 10 min. Cover glasses were placed on the sections using a glycerol solution and PBS. Stained sections were analysed under the Olympus BX51 fluorescent microscope equipped with a digital camera and AnalySIS v. 3.2 software.

**Results**

The chemical innervation pattern of the urinary bladder in healthy bitches was determined in the biopsy material collected before BTX injections. The density of bladder innervation in the studied dogs before and after BTX injections is presented in Table 1.

All of the analysed fibre types were observed in the muscular coat of the canine urinary bladder. The most numerous subset was formed by mesh-like arranged VACHT terminals, while long and varicose NPY-IR and VIP-IR nerves were moderate in number. Relatively numerous short and varicose fibres containing PACAP were found in the muscular coat and the submucosal layer. CGRP-, LENK- and SP-IR fibres were less abundant in number, while fibres containing NOS, GAL and TH were rarely observed. In the
The number of fibres containing the neurotransmitters varied significantly in the samples collected from clinically-healthy bitches 1 week after BTX injection. The toxin led to a distinct drop in the density of nerve processes containing VACHt, which were present in the form of fine single fibres. The number of CGRP-containing fibres decreased, but the relevant drop was not as high as in VACHt-IR fibres. A decrease in the number of fibres containing VIP with a simultaneous increase in the number fibres containing NPY and NOS was noted in all layers. NPY-IR and NOS-IR nerves were observed in the form of individual fibres with distinct varicosities. In the submucosal layer, NPY-IR fibres were most abundant, PACAP and NOS nerves were moderate in number, while only single LENK, CGRP, VIP, TH, and SP fibres were observed. A week after BTX administration, only varicose NPY-IR fibres were found under the urothelium.

Changes in the innervation of the canine urinary bladder were observed in the samples collected 1 month after BTX injections in comparison with the results noted before the treatment and 1 week after toxin administration. A minor decrease in NPY-IR fibres was reported in comparison with innervation density noted 1 week after BTX administration. The number of fibres containing VACHt and VIP was found to increase. One month after toxin injection, the number of NPY fibres was equal to that reported in clinically healthy animals. In the submucosal layer, fine single VACHt-IR fibres were observed, while the number of nerves containing nitric oxide synthetase decreased. There was a drop in the number of NPY fibres under the urothelium, and it was accompanied by the emergence of fibres containing the VIP neurotransmitter. VIP-IR fibre density increased in blood vessel area.

Table 1
A semi-quantitative analysis of the density of subpopulations of nerve fibres supplying the urinary bladder of bitches before the administration of botulinum toxin and 1 week, 1 month, and 6 months after BTX injections

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- absence of fibres, + single fibres, ++ moderate number of fibres, +++ numerous fibres, ++++ very numerous fibres
**Fig. 1.** The examples of nerve fibres subpopulation distribution in the urinary bladder wall of clinically-healthy dogs. Microphotographs a, b, c, e, f, h, i, and j concern muscular layer, microphotographs d and g submucosal layer. Magnification of a, d, and i is 20x, whereas the rest is 40x.
The innervation pattern of the urinary bladder wall was further remodeled 6 months after BTX treatment. In comparison with the results reported 1 month after toxin injections, a minor increase in the number of CGRP-fibres and a considerable increase in the number of VIP-IR fibres, found in all layers of the urinary bladder and around blood vessels, were noted. There was a decrease in the number of fibres containing the nitric oxide neurotransmitter. As regards VIP-IR fibres, their higher density with increased varicosity was reported in the submucosal layer. An insignificant increase in the number of TH-IR fibres was also observed in the muscular coat of the urinary bladder.

**Discussion**

The discussed changes in the distribution and chemical coding of nerve fibres after the administration of botulinum toxin have expanded the existing knowledge on the mechanism of toxin action in the urinary bladder. The results of this experiment indicate that nerve cell damage leads to dramatic biochemical changes in the cell.

Chronic cystitis increases PACAP-IR fibres in the spinal cord and dorsal sensory ganglion of the spinal cord (27). Studies conducted by us indicates that the spinal cord and dorsal sensory ganglion of the spinal cord (27). Studies conducted by us indicates that the bladder wall. The sensory fibers are activated in inflammatory and pathologic processes. Density of CGRP positive fibres increases in bitches with incontinence of urine, but also after the BTX injections. These fibres are activated when a high concentration of mechanical and biochemical stimuli are observed. This feature has already been used in the treatment of some urinary bladder diseases in human medicine (14). The investigations revealed that SP-IR fibres increased in all layers of the urinary bladder in bitches with urinary incontinence symptoms.

SP, CGRP, and PACAP-R fibres are afferent fibres, which take part in sensory conduction and they belong to C class fibres. After experimentally-induced inflammation in rats, all of v listed above increase their density in the urinary bladder wall (29).

Neuropeptide Y demonstrates a property to shrink the muscular layer of the urinary bladder. In sympathetic system neuropeptide Y coexists with NA, but it is released from synaptic vesicle in answer to high frequency stimuli. NPY intensifies and lengthens the shrinking property of NA when spoken about smooth muscles of blood vessels (4, 22).

In clinically-healthy animals, NPY-IR fibres were found in muscular and submucosal layers and under the urothelium. In animals of group II (animals with incontinence of urine symptoms) we observed that muscular layer contained much more NPY-IR fibres than in healthy animals; moreover their number increased a week after BTX injections, what may suggest that degenerative processes lead to inflammation but also to reconstruction of the tissue. Chemical sympathectomy causes disappearance of NPY-IR fibres in the submucosal wall of the urinary bladder (4, 22). BTX is a selective blocker of acetylcholine, which function is to block neuronal transmission. Our study proved that a week after BTX injections, VACHT-IR fibres disappeared, whereas these fibres showed great density in clinically-healthy animals. This fact confirms the mechanism of BTX action on urinary bladder wall. Due to BTX action on acetylcholinergic nerve fibres, the toxin can be used in the treatment of disorders of the lower urinary tract (10, 24).

The presented results suggest that the administration of BTX into the wall of the canine urinary bladder results in a clear remodeling of its innervation pattern already 1 week after BTX injection. BTX affects components of both the sensory and parasympathetic nervous system, while degenerative changes are accompanied by restorative processes (including inflammation-driven processes, as demonstrated by an increase in the number of NPY- and NOS-fibres).

**References**