Morphological changes of cholinergic nerve fibers in the urinary bladder after establishment of artificial somatic-autonomic reflex arc in rats

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Abstract: Objective To establish an artificial somatic-autonomic reflex arc in rats and observe the following distributive changes of neural fibers in the bladder. **Methods** Adult Sprague-Dawley rats were randomly divided into three groups: control group, spinal cord injury (SCI) group, and reinnervation group. DiI retrograde tracing was used to verify establishment of the model and to investigate the transport function of the regenerated efferent axons in the new reflex arc. Choline acetyltransferase (ChAT) in the DiI-labeled neurons was detected by immunohistochemistry. Distribution of neural fibers in the bladder was observed by acetylcholine esterase staining. **Results** DiI-labeled neurons distributed mainly in the left ventral horn from L3 to L5, and some of them were also ChAT-positive. The neural fibers in the bladder detrusor reduced remarkably in the SCI group compared with the control ($P < 0.05$). After establishment of the somatic-autonomic reflex arc in the reinnervation group, the number of ipsilateral fibers in the bladder increased markedly compared with the SCI group ($P < 0.05$), though still much less than that in the control ($P < 0.05$). **Conclusion** The efferent branches of the somatic nerves may grow and replace the parasympathetic preganglionic axons through axonal regeneration. Acetylcholine is still the major neurotransmitter of the new reflex arc. The controllability of detrusor may be promoted when it is reinnervated by the pelvic ganglia efferent somatic motor fibers from the postganglionic axons.

Keywords: urinary bladder; acetylcholine esterase; innervation

1 Introduction

Restoring normal voiding function in spinal cord injury (SCI) patients is a great challenge for neurology^[1]. Researches on restoring functional micturition have been mainly focused on electrical stimulation for decades and with progresses, but it is not yet the definitive solution for majority of SCI patients^[2]. The ideal therapy is repair of the injured spinal cord.

Attempts on bladder reinnervation were initiated by

Kilvington *et al.*^[3], though their experiments on dog did not demonstrate any bladder contraction relating to the nerve reinnervation from lumbar (L) to sacral (S) level. Early in 1990, Xiao *et al.* proposed to establish an artificial “skin-CNS-bladder” reflex pathway below the spinal cord lesion level, as a means of restoring the controllable micturition for patients post-SCI^[4]. This reflex pathway which transfers somatic motor impulses to the bladder was designed to allow SCI patients to initiate voiding by scratching the skin, and was tested first in rats^[5]. The distal end of left L4 ventral root was microanastomosed intradurally to the proximal end of left L6 ventral root, which innervate the bladder and external urethral sphincter while maintain the L4 dorsal root intact as a starter of micturition. There were many subsequent experiments focused on the underlying mechanism

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of this artificial arc, but the somatic-autonomic reflex pathway for micturition remains undetailed.

Cholinergic nerve fibers are both the preganglionic fiber of autonomic nerve and the postganglionic fiber of parasympathetic nerve to dominate urinary bladder and urethra. In the present study, we established an artificial somatic-autonomic reflex arc in rats to observe the distributive changes of the cholinergic nerve fibers, and intended to explore roles of the cholinergic nerve fiber regeneration in micturition.

2 Materials and methods

2.1 Animals and surgery Adult female Sprague-Dawley rats were supplied by the laboratory animal centre of the Institute of Surgery in Daping Hospital, Third Military Medical University of China. Fifteen rats were divided randomly into three groups ($n = 5$ for each group): control group, SCI group and reinnervation group. Animals in the reinnervation group were anaesthetized with 0.3% pentobarbital (10 mL/kg, i.p.). The left dorsal root (DR) ganglia from L4 to L6 was exposed by lateral hemi-laminectomy under sterile condition. L4 and L6 ventral roots (VR) were transected, and the proximal end of the L4 root was anastomosed to the distal end of the L6 root. Other roots remained intact and the wound was closed. Three months later, rats in the SCI group and reinnervation group were performed laminectomy at T8-T9 level, and their spinal cords from T10 to T11 were transected completely. The rats were then maintained for another three months. Rats in the control group did not receive any surgery or treatment.

2.2 Retrograde tracing technique Three months after the model establishment, fifteen microlitre of 1,1'-diiododecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI, 4 mg/mL; Sigma) was injected into the left major pelvic ganglia (MPG) of the reinnervation rats and maintained for 72 h. After rats were perfused with 4% paraformaldehyde (PFA, Sigma), the spinal cord at L2-S2 level was excised and post-fixed for 12 h in the same fixative. Then the specimens were placed in 30% sucrose at 4 °C until they sank to the bottom of the containers. All of the specimens were sectioned at 20 μ m with a cryostat (Leica).

2.3 Immunofluorescence assay After retrograde tracing, the sections of spinal cord segments from L2 to S2 were rinsed with PBS for 5 min, then incubated with rabbit polyclonal choline acetyltransferase (ChAT) primary antibody (1:1 000; Chemicon) for 1 h at room temperature, and

washed in PBS again. Next, the sections were incubated with goat-anti rabbit fluorescein isothiocyanate (FITC)-labeled secondary antibody (Sigma) for 1 h, and finally visualized under a fluorescence microscope (Olympus).

2.4 Stretched preparation The animals of all groups were anaesthetized with 0.3% pentobarbital. The urethral orifice was clamped. When feeling the bladder was fully distended with urine by abdominal palpation, the following procedure was performed. The abdomen was opened and the urethra and the ureters were ligated. The bladder was excised and filled with acetone solution. After fixation for 12-24 h at 4 °C, the bladder was cut open along the dorsal midline from the dome to the urethra, and prepared for mounting as a single specimen until a reasonably flat preparation was obtained under a dissecting microscope. In the reinnervation group, only the anastomosis side of the bladder was used for preparation.

2.5 Acetylcholine esterase staining For histochemical detection of the acetylcholine esterase, the sections were transferred to phosphate-buffered solution for 12 h, then stained with acetylcholine esterase according to the Karnovsky-Roots method^[6] with a little modification by Baker *et al*^[7]. At the end of incubation (usually about 8 h), bladders were opened by radial cuts, allowing them to be flattened with minimal folds. The bladders were mounted to slides with p-xylene-bis-pyridinium bromide (DPX) after dehydration.

2.6 Data analysis and statistics Morphometric data were collected by number-counting under a light microscope. The number of fluorescence-labeled cells was counted using a magnification of $\times 100$ and the number of nerve fibers was counted using a magnification of $\times 40$. Quantitative measurements are given as mean $\pm$ SEM. Multiple comparisons were analyzed by analysis of variance. $P < 0.05$ was considered statistically significant.

3 Results

3.1 DiI retrograde tracing The DiI-positive neurons, with nucleolus, prominent processes and clear shape, could be found in the left ventral horn from L3 caudal to L5 cephalic level under a fluorescence microscope.

3.2 Immunofluorescence assay On the sections pretreated by DiI retrograde tracing, the indirect immunofluorescence was done by using ChAT antibody. Part of ChAT-like immunopositive substances distributed coincidentally with the DiI-labeled neurons. Double staining revealed that

Fig. 1 ChAT and DiI staining. A: ChAT-positive neurons (green); B: DiI retrograde tracing, labeled the locus of left ventral horn (red); C: Merged, the double staining cells. Scale bar, 100 μ m.

Fig. 2 Acetylcholine esterase stained preparations. A: Control group, normal innervation of the parasympathetic fibers; B: SCI group, faintly stained nerves. C: Reinnervation group, the fibers stained more heavily than the SCI group. Scale bar, 100 μ m.

about 68% (8/12) ChAT-positive neurons showed DiI-positive (Fig. 1).

3.3 Acetylcholine esterase staining For the normal innervated bladder in the control group, the stretched preparation consisted of entire extent of the opened and flattened bladder and entire thickness of its wall. All of the nerve fibers and nerve trunks in the wall including those small trunks adjacent to the muscle bundles were stained symmetrically by acetylcholine esterase, but small nerve bundles and individual nerve fibers were not stained. Some musculatures including that of blood vessels were also weakly stained. Ventral nerves mainly distributed to the ventral part of the bladder, while the nerves reaching the bladder dorsolaterally to the ureters innervate the dorsolateral part. The left and right ventral nerves run approximately parallel to each other, while the dorsolateral nerves radiated out from the locus where they reached the bladder.

In the reinnervation group, the large-diameter ($> 5 \mu$ m) fibers decreased dramatically (about 62% of normal bladder) in number and staining intensity in the bladder. Sections from the proximal segments of both the ventral and dorsolateral nerves showed that there remained surviving axons with weak-staining on both sides.

As a consequence of anastomosis, there was a shift in the main course of the remaining nerves. The nerves in the

Fig. 3 Numbers of the acetylcholine esterase-positive nerves in the bladders of different groups. The nerve fibers in the SCI group reduced significantly compared with the control. Following the somatic-autonomic anastomosis, the amount of bladder nerve fibers in the reinnervation group increased significantly compared with the SCI rats, though still much less than that in the control. * $P < 0.05$ vs control group, ** $P < 0.05$ vs SCI group.

right side still showed reduced amount and weaker staining. There remained some visible nerves in the longitudinal muscle due to partial cross-innervation, despite total lack of axon staining in the proximal portion of the ventral nerve. Some remaining dorsolateral nerves were demonstrated on the right side, and some branches crossed over from the left to the right side. The nerve fibers in the anastomosis (left) side seemed different. The new nerve fibers without main nerve trunks regenerated more chaotically than in the right side. Nerves were well away from the ventral midline

of the bladder with a reasonable increase (about 30% to injury bladder) in number. No axonal degeneration was found on the anastomosis side. (Fig. 2)

The nerve fibers in the bladder reduced remarkably in the SCI rats (99.5 ± 6.65) compared with the control rats (153.8 ± 6.06) ($P < 0.05$). After establishment of the artificial reflex arc in the reinnervation group, the average number of bladder nerve fibers (123.0 ± 7.09) were significantly higher than that in the SCI group ($P < 0.05$), though still significantly less than that in the control group ($P < 0.05$). (Fig. 3)

4 Discussion

Neurogenic bladder caused by SCI is a major issue to be resolved for neuropathists. SCI-caused spino-bulbo-spinal pathway (connecting the lumbar-sacral segment of spinal cord to the brainstem) damage, whether complete or incomplete, can result in serious disruption of coordination of the pelvic organs and sphincters and lead to uninhibited (parasympathetic) pelvic reflexes. The ascending afferent activity from the bladder or urethra could be blocked^[8], and the descending excitatory modulating influences from the lateral pontine nucleus to the Onuf's nucleus in the sacral cord could be cut off^[9]. In bladder, this result is commonly termed detrusor hyperreflexia or neurogenic detrusor-over-activity. Detrusor hyperreflexia is often exacerbated by uncoordinated viscera-somatic reflexes which are resulted from vesicourethral function impairment, and subsequently leads to obstructed voiding, high bladder pressures and incontinence^[10].

In this study, DiI neural tracing demonstrated that somatic motor axons successfully regenerated into the pelvic nerve and the DiI-labeled neurons in the anastomosed side of the spinal cord were partly co-located with the ChAT-positive cells. The main neurotransmitter released by new reflex arc was still the acetylcholine. The results suggested that the somatic motor fibers may replace the autonomic preganglionic fiber. The same transmitter of preganglionic fibers and postganglionic fibers in the major pelvic ganglion (MPG) may be the other reason that the bladder control could be recovered after establishing the artificial reflex arc.

We also found that the artificial reflex affected the course of ipsilateral intramural nerves of the bladder, which distributed chaotically, not as regularly as those under the normal pattern showed. According to Uvenius B *et al.*, in normal pattern, the main nerves from the pelvic ganglia reach the bladder with 1-4 trunks on each side. The nerves

usually go the shortest way from the ganglion to the bladder, ventrally and caudally to the ureterovesical junction^[11]. Our results indicate that the bladder reinnervation can change the distribution of bladder intramural nerves.

Acetylcholine esterase is the enzyme to hydrolyze the acetylcholine, and can be used as an indicator for the excitability of parasympathetic nerve fibers in the urinary bladder. Previous researches demonstrated that total ChAT activity per bladder increased by 250% in the female rats following a 6-week obstruction^[12]. After removal of one pelvic ganglion, the ChAT activity in the bladder fell by about 50% within 3 d^[13]. In our study, nerve fibers innervating the bladder were stained faintly in the SCI group, suggesting the complete axonal degeneration and acetylcholine esterase inactivity. The nerve fibers in the reinnervation group stained more heavily than in the SCI group. But within the reinnervation group, the pattern of innervation of the detrusor was different between the anastomosis side and the intact side. It showed that the axons from the anastomosis side reinnervate the previously denervated side. The cholinergic innervation seems mostly recovered and the ChAT activity increased significantly in the surgery bladder compared with the SCI bladder.

After the L4-L6 anastomosis, Waller's degeneration of the preganglion fibers occurred, and the bladder intramural fibers were subsequently degenerated. While when the new preganglion fibers regenerated into the previous autonomic preganglionic axons and reached the MPG, the number and the function of the bladder nerve fibers could be recovered.

In summary, the results suggest that the neurotransmitter of the artificial reflex is not changed and the previously damaged nerves could, to some extent, reinnervate the bladder after the new reflex establishment. It also indicated that the efferent motor branches of somatic nerves can grow into the parasympathetic fibers of autonomic nerves through axonal regeneration, similar to the results of de Castro *et al.*^[14] and Eugene *et al.*^[15]. In the new reflex, somatic motor excitation evoked by the cutaneous stimuli is probably transmitted to the bladder through those anastomosed efferent motor nerves, resulting in the autonomic bladder contraction. Speculatively, there may be a high degree of plasticity in the bladder innervation following the artificial reflex establishment.

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人工反射弧建立后大鼠膀胱胆碱能神经的形态学变化

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摘要: **目的** 观察体神经-内脏神经人工反射弧建立后, 大鼠膀胱肌间神经丛分布的改变以及神经肌肉接头处的变化。 **方法** Sprague-Dawley 大鼠随机分为三组: 对照组、脊髓横断组和手术重建组。手术重建组大鼠术后饲养3个月, 与脊髓横断组大鼠一起进行脊髓横断, 再继续饲养3个月, 对照组不做任何处理。DiI 进行逆行神经追踪; 免疫荧光的方法显示 DiI 阳性标记细胞中的胆碱乙酰转移酶(choline acetyltransferase, ChAT); 改良的Karnovsky-Roots法检测膀胱铺片中神经纤维的分布。 **结果** DiI阳性标记细胞主要分布于脊髓L3尾部至L5头侧前角, ChAT 阳性细胞和 DiI 阳性标记细胞部分重叠。手术重建组和对照组相比, 膀胱肌间神经纤维的数量较少, 染色浓度也较浅($P < 0.05$); 而手术重建组神经纤维密度较脊髓横断组增大, 染色浓度增强($P < 0.05$), 且出现明显的神经再分布。 **结论** 人工体内脏神经反射弧建立后, 新的传出支为体神经, 可以长入副交感神经纤维, 传出神经元的递质仍为乙酰胆碱, 膀胱内胆碱能神经纤维再生和乙酰胆碱活性增强且出现神经再分布, 这可能在膀胱的控制性排尿中起作用。

关键词: 膀胱; 乙酰胆碱酯酶; 神经分布