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Partial urethral obstruction enhances NADPH-diaphorase activity in the monkey (*Macaca fascicularis*) bladder: light and electron microscopic studies

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Summary. The effect of partially obstructing the urethra on the nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) activity in neurons of the intramural ganglia of the monkey (*Macaca fascicularis*) bladder was examined by light and electron microscopy. Partial urethral ligation was done in adult male monkeys. The animals were sacrificed 2, 4 weeks after partial urethral obstruction. This was compared to controls (normal and sham operated). Urethral obstructed animals were observed to have increased urinary frequency and decreased urinary flow rate. Two weeks after urethral obstruction, the overall NADPH-d activity in the intramural ganglia of the bladder base was enhanced compared to control animals. The frequency of intensely stained NADPH-d positive neurons was increased compared to the control animals. About one-third of intensely stained NADPH-d positive neurons appeared to undergo degenerative changes. At 4 weeks after urethral obstruction, a wide occurrence of NADPH-d positive neurons in advanced stages of degeneration in the bladder base was observed. Cellular debris was strewn among normal looking ganglion cells and along the nerve processes. The proportion of intensely stained NADPH-d positive neurons was relatively lower than the controls. The total number of NADPH-d positive neurons and the nerve fibres in the entire bladder was significantly reduced when compared to control animals. Electron microscopy showed some NADPH-d activity in intramural ganglion cells in 2 weeks after partial urethral obstruction. NADPH-d reaction product (formazan) was deposited on the membranes of the rough endoplasmic reticulum, and the outer membranes of some mitochondria in the intramural neuron. At 4 weeks after urethral obstruction, NADPH-d was present in the membrane of the mitochondria and some mitochondria appeared swollen with disrupted cristae. Present results show that NADPH-d activity in neurons of the intramural ganglia of the monkey (*Macaca fascicularis*) urinary bladder was increased after two weeks and reduced after 4 weeks of partial urethral obstruction. It is speculated that the increased NADPH-d activity associated with partial urethral obstruction would lead to neuronal damage and death, which may contribute to detrusor overactivity. However, it warrants further investigation to understand the mechanism of neuronal cell death after partial urethral obstruction.

Key words: NADPH-diaphorase; electron microscopy; partial urethral obstruction; monkey

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Introduction

Bladder outlet obstruction (BOO) is a common urological problem in elderly male patients (McGuire, 1984). BOO secondary to benign prostatic hyperplasia (BPH) induces various bladder dysfunctions. Increases in urinary frequency, urgency and decreased urinary stream are typical signs of the early stages of BPH (McGuire, 1984; Steers and De Groat, 1988). During the later stages of BPH, patients suffer from increasing amounts of residual urine and are subject to overflow incontinence due to retention (McGuire, 1984; Steers and De Groat, 1988). These symptoms may be related to the lack of ability of the detrusor to compensate for BOO and result in impaired detrusor contractility (Saito et al., 1993). BOO can lead to many anatomical and physiological changes in neural pathways to the urinary bladder (Steers et al., 1990; Zhou and Ling, 1999). The morphological and functional changes associated with BOO have been investigated in a variety of animals including the dog (Rohner et al., 1978), rat (Steers and

De Groat, 1988), rabbit (Kitada et al., 1989), pig (Sibley, 1985), cat (Radzinski et al., 1991), guinea pig (Zhou and Ling, 1997; Hu et al., 2004) and sheep (Nikesh et al., 2003) using the techniques of urethral ligation. It has been reported that obstruction in the rat model produces morphological and functional changes similar to those found in patients with severe BOO (Steers and De Groat, 1988). Using a rat model, investigators have documented increased residual urine, bladder volume, voiding pressures and compliance, as well as urodynamic evidence of an unstable detrusor muscle (Malmgren et al., 1987; Steers and De Groat, 1988). The latter dysfunction was attributed to partial denervation in the bladder wall (Speakman et al., 1987), changes in the peptide contents in the bladder nerves (Andersson et al., 1988), and alterations of responses of detrusor muscle to autonomic transmitters (Steers et al., 1990). Experimental models of fetal urinary tract obstruction have exploited the sheep as a model because ovine fetuses can be manipulated relatively early in gestation without mortality (Peters et al., 1992; Levin et al., 2001; Nyirady et al., 2002; Nikesh et al., 2003). The possible mechanism is the triggering of growth by mechanical distention of the developing fetal bladder by the increasing volume of urine (Baskin et al., 1994). Among the various neurochemicals, nitric oxide (NO) has been implicated as a non-adrenergic non-cholinergic (NANC) inhibitory neurotransmitter at various sites in the nervous system (Bredt and Snyder, 1992; Schuman and Madison, 1994). Its function has been documented in muscular and sphincter relaxation in the urogenital systems (Burnett et al. 1992; Mevorach et al., 1994). NO-containing neurons have been detected histochemically by localization of nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) in bladder afferent and postganglionic efferent neurons of the rat (Vizzard et al., 1993). NADPH-d positive neurons and their processes have been localized in the intramural ganglia in the guinea pig urinary bladder (Saffrey et al., 1994; Smet et al., 1996; Zhou et al., 1997). In recent years, some authors have reported that increased intravesical pressure and BOO are associated with decreased bladder blood flow (Lin et al., 1998; Greenland and Brading, 2001). Recent study has investigated the changes in density and morphology of the intramural NADPH-d positive neurons of the porcine urinary bladder during development using whole-mount preparation (Pirker et al., 2005). Drake et al. (2003) reported that increased pressure fluctuations and enhanced modular activity in the partial urethral obstruction rat. The above study suggested that micturition dysfunction in rats with partial urethral obstruction may contribute to the development of detrusor overactivity (DO). In view of these observations, it is hypothesized that the increased NADPH-d activity associated with partial urethral obstruction would lead to neuronal damage and death, which may be involved in the bladder dysfunction such as DO. This study will therefore examine the involvement of NO containing neurons in neuronal damage and death in the partial urethral obstruction monkey (*Macaca fascicularis*) bladder.

Materials and methods

Animals

A total of 16 adult male monkeys (*Macaca fascicularis*) weighing 3 to 4 kg was used for this study. Of these, 8 animals underwent partial urethral ligation, 4 underwent sham operation and the remaining 4 served as normal controls. The animals were divided into 4 groups: 4 each for 2 weeks urethral obstruction, 4 weeks urethral obstruction, normal and sham operation. Two animals in each group were used for light and electron microscopy studies. Throughout the experiment, the animal was treated in accordance with the Guide for Care and Use of Laboratory Animals (National Institutes of Health) and the Institutional Animal Care and Use Committee (IACUC, National University of Singapore).

Surgical procedures

The experimental animals were deeply anaesthetized by an intraperitoneal injection of sodium pentobarbital (30 mg/kg body weight). The detailed surgical procedures have been reported previously (Steers and De Groat, 1988; Mostwin et al., 1991). Under aseptic conditions, the outer circumference of the urethra was reduced to about 2.0 mm by tying a 4-zero silk suture around the urethra using an extraluminally placed polyethylene tubing 2.0 mm in diameter. Control monkeys underwent sham surgery in which the urethra were circumferentially dissected but not ligated. All experimental animals operation was successful and showed signs of successful ligation of urethra included increased urinary frequency and decreased urinary flow rate. None of these signs were observed in the sham operated controls.

Tissue preparations

All animals were deeply anaesthetized with overdose of sodium pentobarbital before perfusion. The animals were sacrificed at 2 weeks and 4 weeks postoperatively for both light and electron microscopic studies (including sham operated). In addition, normal animals served as normal controls for both light and electron microscopic studies.

NADPH-diaphorase histochemistry

Following deep anesthesia, all animals were perfused with Ringer's solution at room temperature (37°C) for a brief period followed by an ice-cold fixative containing of 4% paraformaldehyde (PF) in 0.1M phosphate buffer (PB, pH 7.4). The bladder was given an injection of the same fixative into the lumen during

perfusion to ensure adequate fixation. After perfusion, the bladder was removed and postfixed for 2 hours in the same fixative. Under the dissecting microscope, the urothelium of the bladder was stripped off from the muscular layers by using a fine pair of forceps. The tissue samples were kept overnight in 0.1M PB (pH 7.4) containing 20% sucrose overnight at 4°C. The tissue samples were rinsed in 0.1M Tris buffer (TB, pH 7.6) for 10 min and then incubated in NADPH incubating medium in the dark room temperature (37°C) for 60 min. The incubating medium contained 0.3mM nitroblue tetrazolium (NBT), 1.2mM ß-NADPH, and 0.3% Triton X-100 (all from Sigma, USA). After incubation, the tissue samples were rinsed in 3 changes of TB (pH 7.6). The whole mount preparations were air-dried overnight at room temperature and then cleared in xylene and mounted in Permount.

Transmission electron microscopic (TEM) studies

After deep anesthesia, animals were perfused with Ringer's solution followed by a fixative containing 4% PF and 3.0% glutaraldehyde in 0.1M PB (pH 7.4). After that, the bladder was removed and post fixed for 2 hours in the same fixative and then kept in 5% sucrose in 0.1M PB overnight at 4°C. The whole-mount tissue preparations were incubated in the dark room at 37°C for 2 hours in a medium containing BSPT (Sigma, USA) which was dissolved with several drops of dimethylformamide in 0.1M PB (pH 7.6). The reaction was stopped by washing in PB. The areas of the whole-mount preparations containing NADPH-d reactive neurons were identified and trimmed into small pieces of tissue sample under the light microscope. The tissue samples were post fixed in 1% OsO_4 in 0.1M PB for 1 hour, then dehydrated in a graded series of ethanol and finally embedded in Araldite. Ultrathin sections were double stained with lead citrate and uranyl acetate and viewed under a Philips CM120 or JOEL 1220 electron microscope.

For specificity control in both light and electron microscopy, NADPH was omitted from the incubation medium.

Quantitative study

Cell counts were carried out in whole mount preparations of the entire bladder in normal, sham operated and urethral obstructed animals. Since the number of NADPH-d positive neurons and staining between the normal and sham operated animals did not appear to differ significantly, data on these animals were pooled as a single control group. Cell counts were performed in whole bladder under the light microscope at magnification x100. All profiles of positively stained cells were identified and counted in each group of animals. Identification of cell profiles was sometimes confirmed by adjusting the focal depths of the objective. The total number (mean ± SD) of NADPH-d positive neurons in the entire bladder between different groups of animals was compared statistically using ANOVA test (analysis of variance). The proportions of intensely stained NADPH-d positive neurons against the total population of NADPH-d positive neurons in the bladder as reference (100%) between control and urethral obstructed groups were also compared with Student's *t*-test (Table 1). Differences were considered significant at $p < 0.05$.

Results

All experimental and sham-operated animals survived. Partial urethral obstruction animals were observed to have increased urinary frequency and decreased urinary flow rate. All of these symptoms are consistent with clinical observations in patients with BPH. Sham-operated animals did not show any change in the micturition pattern.

Cell counting

Two weeks after urethral obstruction, the total number of intensely stained NADPH-d positive neurons in the entire bladder was increased. At 4 weeks, the number of intensely stained NADPH-d positive neurons was significantly reduced when compared with control animals (Table 1).

NADPH-diaphorase histochemistry

In sham operated animals, NADPH-d activity in neurons of the intramural ganglia of the bladder was comparable to the normal animals. In the control groups, NADPH-diaphorase positive neurons were widely distributed in the muscular lamina (Fig. 1A,B), but were negligible in the urothelium of the bladder that was stripped off (Fig. 1C). The majority of the NADPH-d positive cells were localized in ganglia consisting of different sizes of neurons (Fig. 1A). On average, ganglia lying at the base of the bladder contained more neurons while ganglia situated at the dome and body of the bladder contained less neurons. These NADPH-d positive neurons showed a marked gradation of activity for the enzyme, about 32.2% of them are intensely

Table 1. Number (mean ± SD) and percentage of intensely stained NADPH-d positive neurons against total population of NADPH-d positive neurons in the control and urethral obstructed animals.

Groups of animals	n	No. and % of intensely stained NADPH-d neurons	Total No. of NADPH-d neurons
Control	8	767±142 (32.2%)	2379±221
2 Wks	4	1184±145 (46.4%)*	2553±250
4 Wks	4	382±157 (23.4%)*	1633±303$

$: $p < 0.01$ (ANOVA test), *: $p < 0.01$ (Student's t-test).

stained while the remainders are moderately or weakly stained (Table 1; Fig. 1A). NADPH-d positive nerve fibres were denser in the muscular layer, often showing varicosities and tended to occur in bundles linking the ganglia into the bladder wall (Fig. 1B). Two weeks after urethral obstruction, the overall NADPH-d activity in the intramural ganglia of the bladder base was enhanced (Fig. 2A). The frequency of intensely stained NADPH-d positive neurons was increased to 46.4% (Table 1; Fig. 2A,B). The neurites of some neurons were grossly dilated (Fig. 2C). About one-third of intensely stained NADPH-d positive neurons in this group appeared to undergo degenerative changes (Fig. 2D). At 4 weeks the outline of the intensely stained NADPH-d positive cells became extremely irregular due to eruption of cytoplasm from the cell surface. The neurons in the bladder base showed advanced stages of degeneration (Fig. 3A-C). Cellular debris was strewn among normal looking ganglion cells and along the nerve processes. The proportion of intensely stained NADPH-d positive neurons was relatively lower than the controls. The total number of NADPH-d positive neurons and the nerve fibres in the entire bladder was significantly reduced when compared to control animals (Table 1; Fig. 3B,C).

Transmission electron microscopic study

The intramural ganglia of the bladder base in the sham-operated control animals were located between nerve bundles, and usually lay nearer to the serosa than the mucosa. Ultrastructurally, the intramural neurons had a large, round pale nucleus (Fig. 4A) bearing a single nucleolus. The neuronal perikarya were surrounded by glial cells which had a smaller and elongated nucleus showing dense chromatin masses (Fig. 4A). The cytoplasm contained several profiles of Golgi apparatus located in the perinuclear region. The cisternae of rough endoplasmic reticulum were present as short profile (Fig. 4B). Mitochondria and lysosomal dense bodies including lipofuscin granules were abundant and randomly distributed (Fig. 4B). At 2 weeks after partial outlet obstruction, NADPH-d reaction product (formazan) was deposited on the membranes of the rough endoplasmic reticulum (rER), and the outer membranes of some

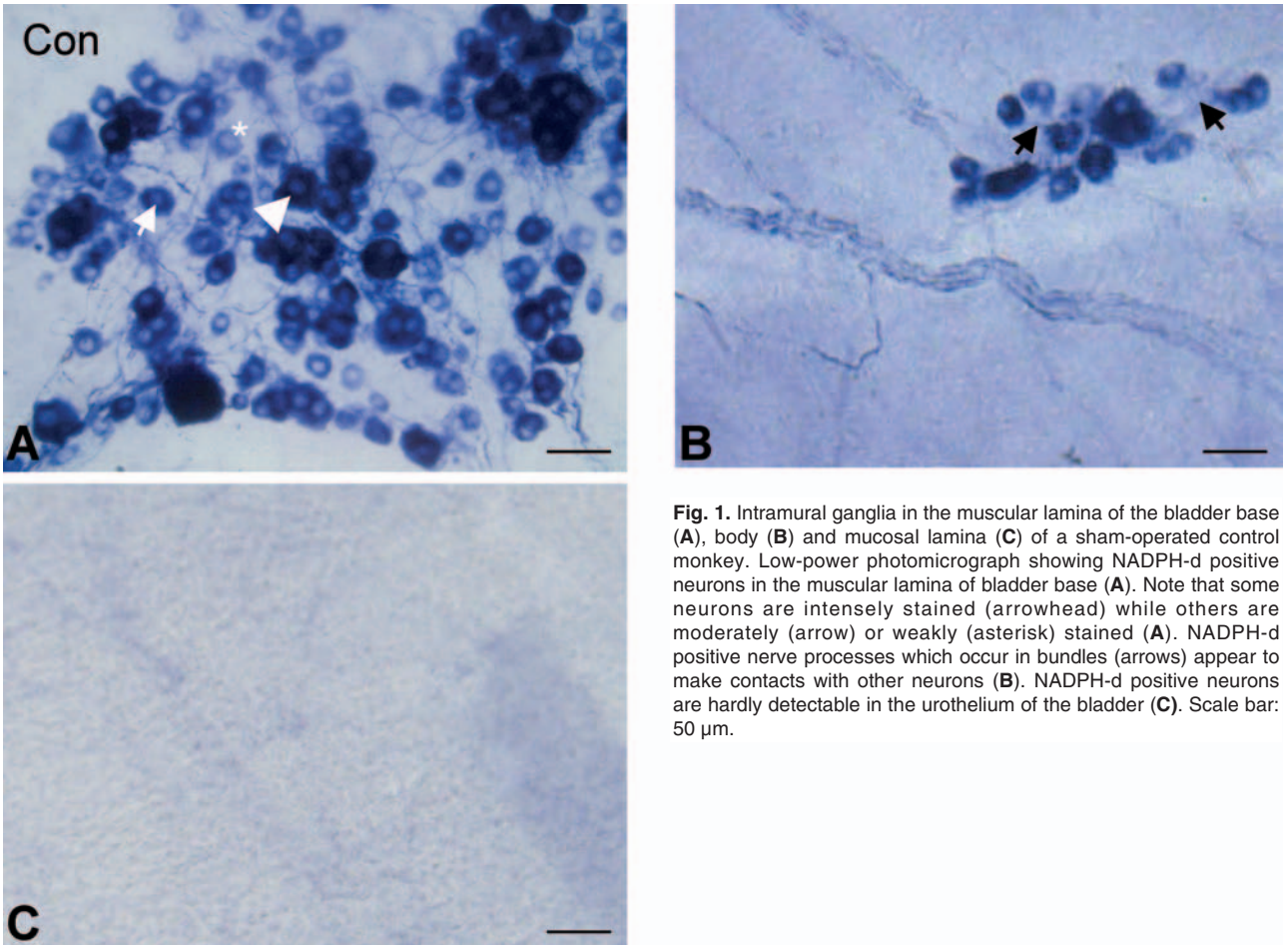

Fig. 1. Intramural ganglia in the muscular lamina of the bladder base (**A**), body (**B**) and mucosal lamina (**C**) of a sham-operated control monkey. Low-power photomicrograph showing NADPH-d positive neurons in the muscular lamina of bladder base (**A**). Note that some neurons are intensely stained (arrowhead) while others are moderately (arrow) or weakly (asterisk) stained (**A**). NADPH-d positive nerve processes which occur in bundles (arrows) appear to make contacts with other neurons (**B**). NADPH-d positive neurons are hardly detectable in the urothelium of the bladder (**C**). Scale bar: 50 µm.

mitochondria in the intramural neuron (Fig. 4C). At 4 weeks after partial outlet obstruction, NADPH-d was present in the membrane of the rER and mitochondria, and some mitochondria appeared swollen with disrupted cristae (Fig. 4D). There was no evidence of NADPH-d positive cell death at the various time points examined.

Discussion

The pelvic ganglia have been considered to be the sites where sympathetic and parasympathetic information is integrated for regulating the function of the bladder (Fletcher and Bradley, 1978). The intramural ganglia embedded in the bladder wall constitute part of the pelvic ganglia (Lincoln and Burnstock, 1993) and they show considerable species variation, being numerous in the guinea pig (Gabella, 1990), fewer in cats, rabbits, ferrets and absent in rats and mice (Lincoln and Burnstock, 1993). Their existence in the human bladder has also been reported (Gilpin et al., 1983). The intramural nervous control of the bladder is more complex than has been hitherto assumed (Crowe et al., 1986). The urinary bladder of several species has been shown to receive adrenergic and cholinergic innervation (Alm and Elmer, 1975) and quinacrine positive nerves (a possible marker for purinergic nerves) (Burnstock et al., 1978). Immunohistochemical studies of the guinea pig urinary bladder have demonstrated the presence of peptide-containing neurons, including those labeled for substance P, vasoactive intestinal polypeptide, somatostatin, met-enkaphalin, and neuropeptide Y (Crowe et al., 1986; James and Burnstock, 1989). In recent years, some authors have identified the enzyme nitric oxide synthase or in an indirect form, the presence of NADPH-d activity in nerves supplying the bladder and urethra (Saffrey et al., 1994; Smet et al., 1996; Zhou et al., 1997; Hu et al., 2004; Pirker et al., 2005).

To date, no experimental studies were made on the NADPH-d activity in neurons of the intramural ganglia of the monkey urinary bladder. The present results confirmed the earlier study (Zhou et al., 1997) which showed that the intramural ganglion cells in the guinea pig urinary bladder express NADPH-d activity. Recently, the changes in density and morphology of the intramural

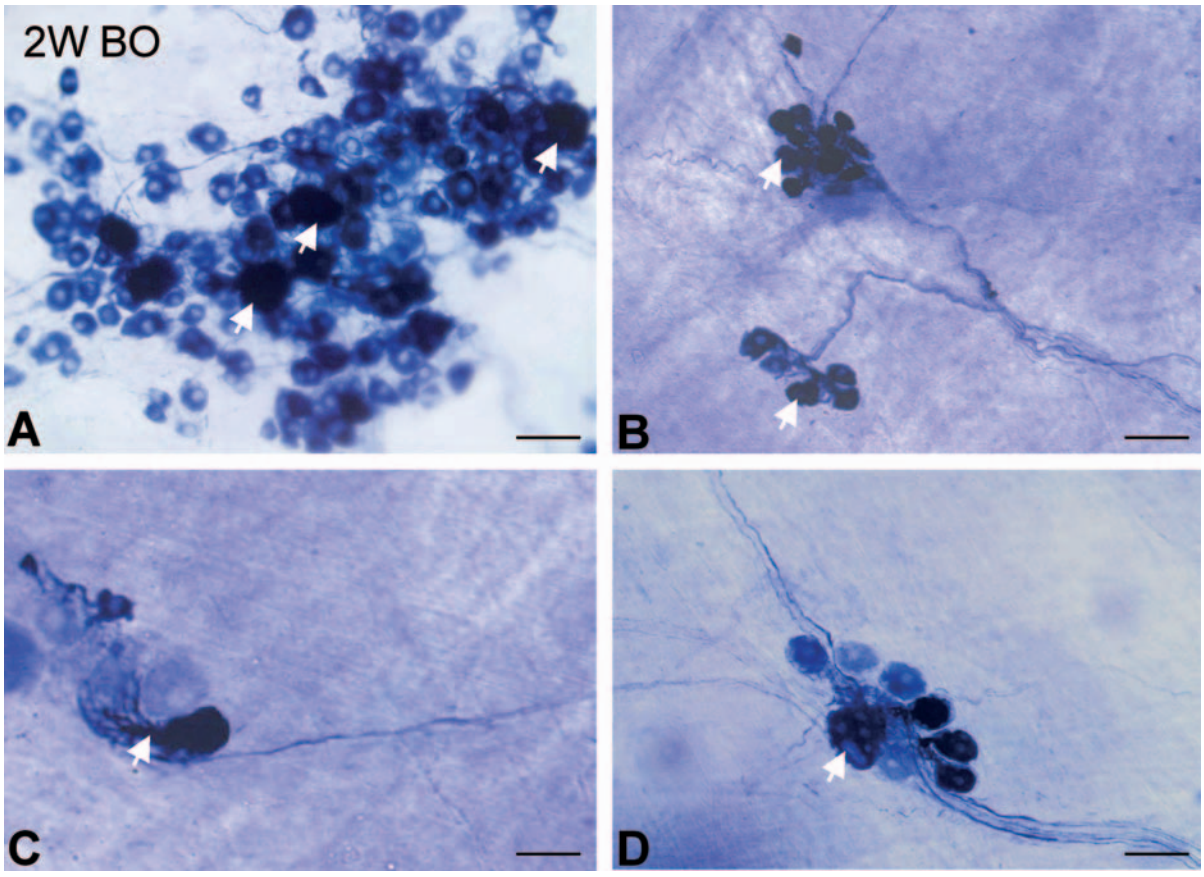

Fig. 2. Intramural ganglia in the bladder base of 2 weeks after partial urethral obstruction. The great majority of cells exhibit intense staining for NADPH-d; some cells are so intensely stained for NADPH-d that their profiles are masked (**A, B**; arrows). Neurites of some neurons are markedly dilated (**C**; arrow). In (**D**), the intensely stained NADPH-d positive cells appear to undergo degenerative changes (arrow). Scale bar: 50 μm.

NADPH-d positive neurons of the porcine urinary bladder during development were demonstrated using whole-mount preparation (Pirker et al., 2005). The current study confirmed the earlier study (Pirker et al., 2005) that the majority of NO-containing neurons, as revealed by NADPH-d are distributed in the entire bladder. Since the majority of the NADPH-d positive neurons were concentrated in the bladder base, it is suggested that NO might be involved in the relaxation activity in the base during micturition. This would be consistent with previous pharmacological studies which demonstrated that NO was a mediator for the neurogenic dilation of the bladder neck during the micturition reflex in different species (Dokita et al., 1991; Persson and Anderson, 1992; Ehren et al., 1994; Zhou et al., 1997).

Acute urinary retention is a common urological problem (Christensen and Bruskewitz, 1990; McConnell et al., 1998). It has been reported that 152 of the 1503 men in the placebo group (10%) and 69 of the 1513 men in the finasteride group (5%) underwent surgery for BPH. Acute urinary retention developed in 99 men in the placebo group (7%) and 42 men in the finasteride group (3%) (McConnell et al., 1998). A striking feature in the present study was the increased number of NADPH-d positive neurons and the proportions of intensely stained NADPH-d positive neurons in the intramural ganglia after 2 weeks of partial urethral obstruction. At 4 weeks, the number of intensely stained NADPH-d positive neurons was significantly reduced in the intramural ganglia when compared to control animals. The present study also shows that some intensely stained NADPH-d positive neurons appeared to undergo degenerative changes or showed signs of complete disintegration or total lysis. The decrease in the number of NADPH-d positive neurons may be due to upregulation of the NADPH-d expression (Verge et al., 1992; Kitchener et al., 1993). Clinically, Gosling et al. (1986) have shown a 56% reduction in the autonomic innervation of the urinary bladder in patients with BOO. The significant denervation following urethral obstruction may markedly impair the neuromuscular control of the bladder (Gosling et al., 1986) and is usually associated with the detrusor overactivity (Steers et al., 1990). The urethral obstructed-animals were observed to have increased urinary frequency and decreased urinary flow rate which is often seen in patients with BOO (Sibley, 1985; Speakman et al., 1987; Steers et al., 1990). Vesical blood flow has been shown to be decreased in the BOO (Kitada et al., 1989; Lin et al., 1998; Greenland and Brading, 2001). The possible explanation for the

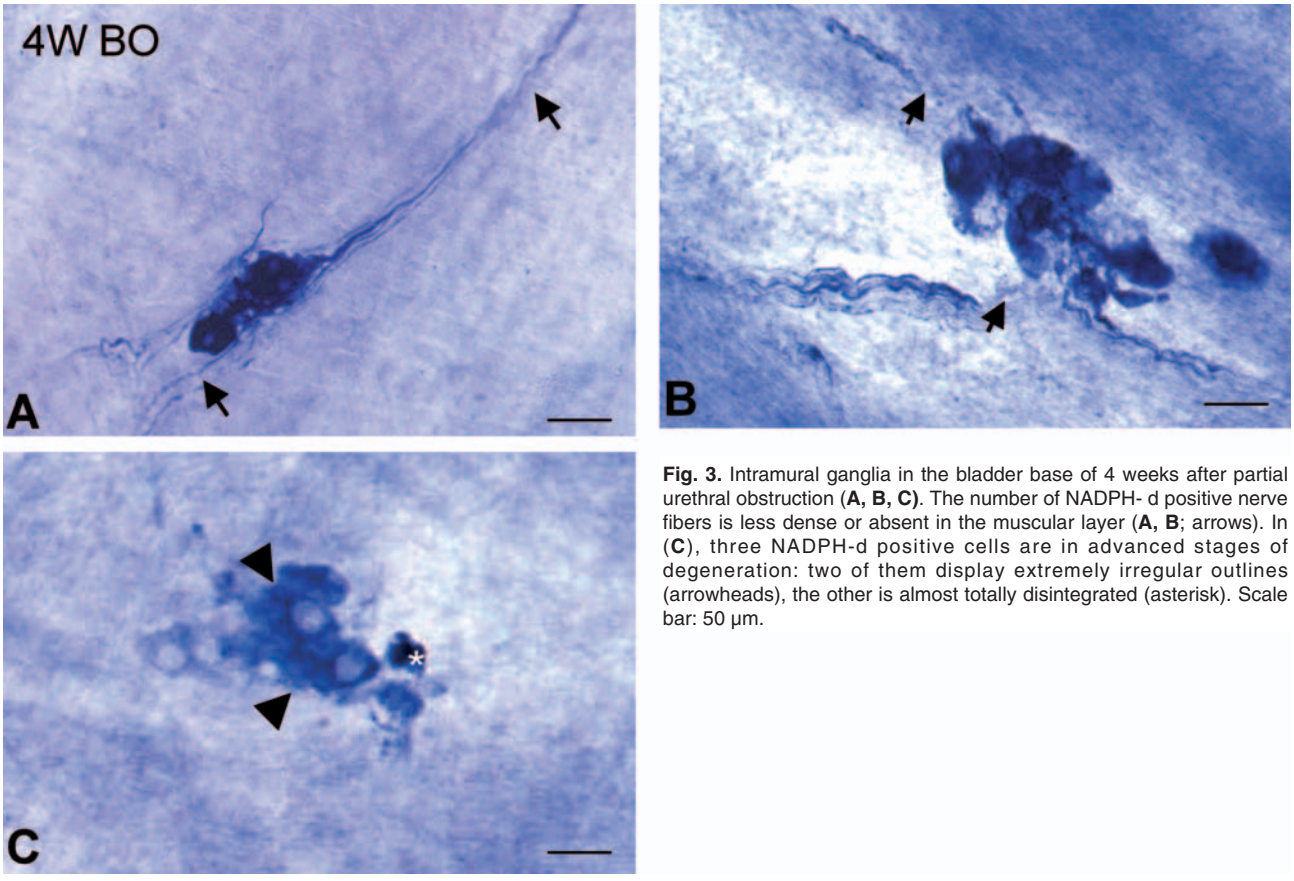

Fig. 3. Intramural ganglia in the bladder base of 4 weeks after partial urethral obstruction (**A, B, C)**. The number of NADPH- d positive nerve fibers is less dense or absent in the muscular layer (**A, B**; arrows). In (**C**), three NADPH-d positive cells are in advanced stages of degeneration: two of them display extremely irregular outlines (arrowheads), the other is almost totally disintegrated (asterisk). Scale bar: 50 µm.

decrease in NADPH-d positive neurons may be induced as result of reduced blood flow as well as mechanical damage due to high intravesical pressure (Kitada et al., 1989; Hu et al., 2004).

In regard to the alterations in the NADPH-d expression, previous studies have shown the considerable plasticity of neuronal NADPH-d in different pathological events. For example, increased NADPH-d activity was observed in the lumbar spinal cord neurons after inflammation of the rat hindpaw (Solodkin et al., 1992), in the cortical pyramidal neurons after stab lesions in the rat (Kitchener et al., 1993), in the rat vagal motor neurons after axotomy (Wang et al., 1996) and in the lumbar spinal cord in rats following

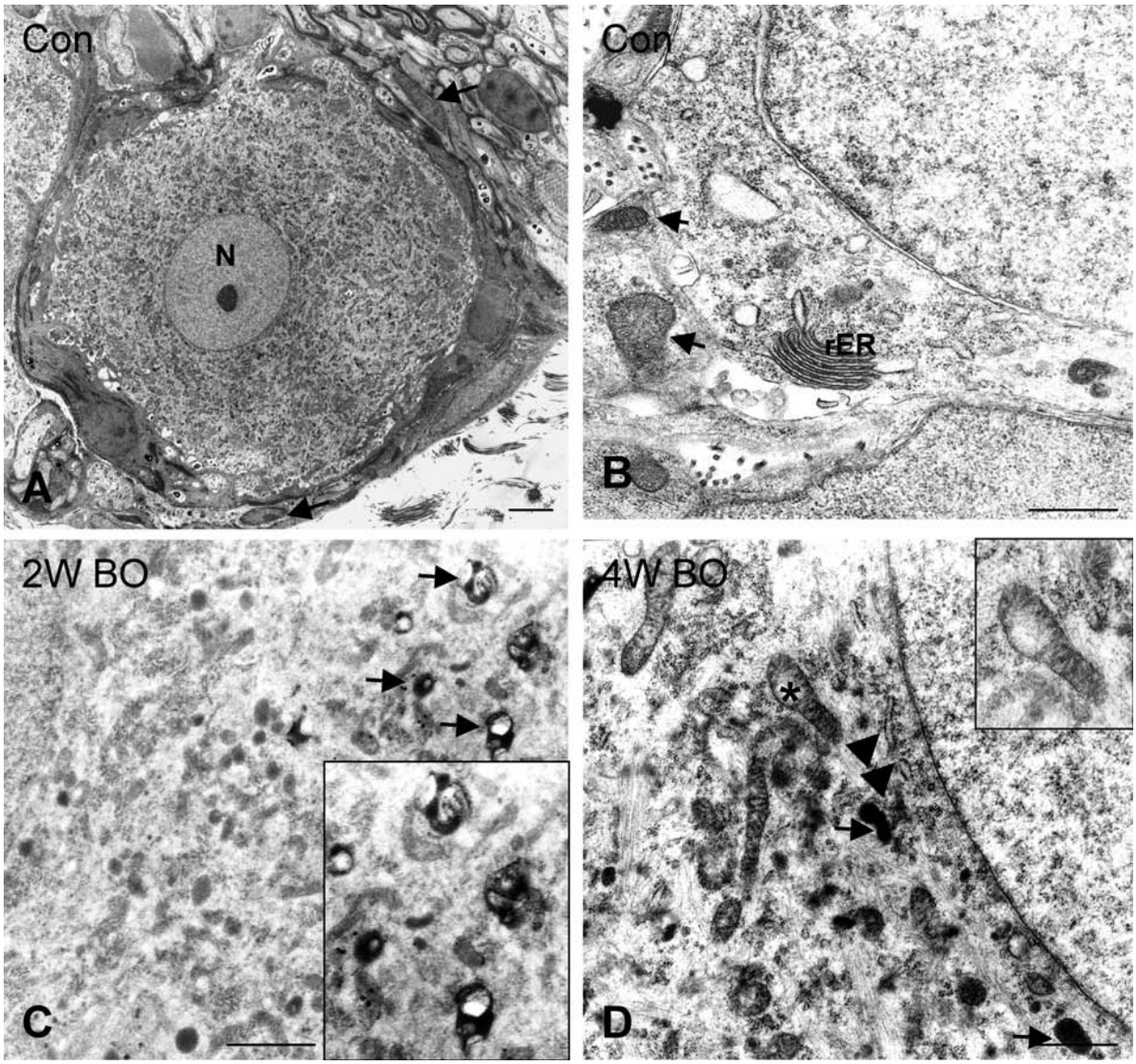

Fig. 4. Electron micrograph showing an intramural neuron of the bladder base in the sham-operated control (**A, B**), 2 weeks after partial urethral obstruction (**C**) and 4 weeks after partial urethral obstruction (**D**). In control tissues, the neuron shows a large, round pale nucleus (N) bearing a prominent nucleolus and the neuronal soma is surrounded by glial cells (arrows; **A**). In control tissues, the neuronal cytoplasm contains Golgi apparatus, slender mitochondria (arrows), and cisternae of rough endoplasmic reticulum (rER) (**B**). At 2 weeks after partial urethral obstruction, the NADPH-d reaction products (formazan) are deposited on the membranes of the rER and the outer membranes of some mitochondria (arrows) in the intramural neuron (**C**). Inset shows the mitochondria at higher magnification. At 4 weeks after partial urethral obstruction, NADPH-d is present in the membrane of the rER (arrowheads) and mitochondria (arrows), and some mitochondria appear to be swollen (asterisk; **D**). Inset shows the high magnification of swollen mitochondria. Scale bars: A, 10 μm; B-D, 0.5 μm.

rhizotomy. It has been suggested that the upregulated NADPH-d expression after nerve lesion was related to both neurodegenerative and neuroregenerative effects (Verge et al., 1992; Kitchener et al., 1993). In this connection, it has been reported that small amounts of NO might be beneficial, whereas larger amounts of NO might be detrimental as it is known to medicate neuronal death (Verge et al., 1992). The present results suggest that the increased NADPH-d activity in the intramural ganglion cells after partial urethral obstruction may be a harbinger to consequent neuronal degeneration and cell death.

The present description of the ultrastructural localization of NADPH-d activity in the intramural ganglion cells of the monkey bladder is consistent with previous observation in the submucous ganglion cells in the intestines (Wang et al., 1995) and the intramural ganglion cells in the bladder (Zhou and Ling, 1997) of guinea pigs. The present results further confirmed that NADPH-d is a membrane-associated protein with a widespread subcellular distribution being localized chiefly in the rough endoplasmic reticulum and mitochondria as reported previously (Valtschanoff et al., 1992). The present study has also firstly demonstrated the ultrastructural localization of NADPH- d activity in the intramural ganglion cells of the monkey bladder. The earliest degenerative changes seemed to affect the mitochondria which appeared swollen as detected ultrastructurally at 4 weeks after partial urethral obstruction. A feature worthy of note was the lack of neuronal cell death in the intramural ganglion at the ultrastructural levels at all the time intervals after partial urethral obstruction. A possible explanation for the lack of neuronal cell death ultrastructurally may be due the fact that relatively small amounts of tissues were sampled. This along with previous findings (Zhou and Ling, 1997) further strengthens the view that NADPH-d activity in neurons of the intramural ganglion is plastic which could be upregulated by pathological conditions.

Conclusion

The present results has shown that NADPH-d activity in the intramural ganglion cells of the monkey (*Macaca fascicularis*) urinary bladder is plastic and can be upregulated after two weeks and reduced after 4 weeks of partial urethral obstruction. It is speculated that the increased NADPH-d activity associated with partial urethral obstruction would lead to neuronal damage and cell death, which may contribute to detrusor overactivity. However, further investigation is necessary to understand the mechanism of neuronal cell death after partial urethral obstruction.

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