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ORIGINAL ARTICLE

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Demonstration of nitrergic and cholinergic innervation in whole-mount preparations of rabbit, pig, and human upper urinary tract

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Abstract To investigate the distribution of nitrergic and cholinergic innervation in rabbit, pig, and human upper urinary tract, (UUT) whole-mount preparations and frozen sections were stained with nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase and acetylcholinesterase (AChE) histochemistry. NADPH-diaphorase and AChE staining demonstrated two neuronal plexuses in the submucous and muscular layer of the UUT in all three species. The presence of nitrergic and cholinergic neuronal networks in the normal UUT suggests that autonomic innervation may play an important role in the transmission of ureteral peristalsis.

Keywords Cholinergic · Nitrergic · Innervation · Upper urinary tract · Whole-mount preparation

Introduction

The mechanism of conduction of ureteral peristalsis has always been a debatable aspect of ureteral physiology. The ureter is a muscular conduit that contracts in response to stretch, and thus actively participates in the transport of urine from the kidney to the bladder. It is accepted that contractions are initiated and regulated by pacemaker tissue in the calyceal wall [1]. Contraction frequency and coupling between pelvic and ureteral peristalsis are dependent on urine production and urine flow [2]. Besides the existing theories about the generation and excitation of urine bolus transport, some authors have demonstrated nerve cells and fibres in the upper urinary tract (UUT) and suggested that the autonomic nervous system (ANS) may play a role in ureteral peristalsis [3–9].

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Nitric oxide (NO) is considered to be an important messenger of inhibitory nonadrenergic and noncholinergic nerves in the peripheral nervous system. NO synthase (NOS) colocalizes specifically in neurons and is identical with nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase [10]. Acetylcholine (ACh) is involved in peripheral autonomic neuroeffector transmission. Acetylcholinesterase (AChE) histochemical staining is an established technique to demonstrate the presence of cholinergic innervation [11].

Previous studies investigating the neuronal control of the UUT have used mainly conventional histological sections. The drawback of thin sections is that they fail to reveal clearly the overall organization of nerve fibres in the UUT wall. The whole-mount preparation technique provides three-dimensional (3D) morphology of the innervation and its relationship of branching and interconnecting nerve fibres to each other and to the neighbouring tissues. This method has been extremely useful for the morphological analysis of nerve distribution in luminal organs such as the gastrointestinal tract and UUT [12–14]. We designed this study to investigate the distribution and topography of nitrergic and cholinergic innervation in the rabbit, pig, and human UUT using whole-mount preparations.

Materials and methods

Normal UUTs were obtained from 10 rabbits (official license code: 1999-1998-561-64, TVV-798) and 5 young pigs (6–12 months old) from a slaughterhouse and from 4 adult humans without evidence of urinary-tract disease at autopsy. The samples were prefixed with Zamboni solution (4% formalin, 0.2% picric acid in 0.1 mol/l phosphate-buffer [pH 7.3; 900 OsM]) at 37 °C for 60 min and stored at –70 °C until they were used.

Specimens were rinsed in phosphate-buffered saline (PBS) with 10% sucrose, embedded in OCT-compound, and frozen in liquid nitrogen. The OCT-compound embedded specimens were cut into 8-μm serial sections.

Whole-mount preparation of each specimen was performed using fine-pointed forceps, microsurgical scissors, and a dissection microscope. The mucosa was removed, followed by separation of the submucosal and muscular layers. The separated layers were

fixed without stretching with fine-pointed pins on a Sylgard silicone elastomer tray (Dow Corning Europe, La Hulpe, Belgium).

For standard AChE histochemistry the specimens were incubated at 37 °C following the method of Karnovsky and Roots [15]. This involves two stock solutions (A and B). Solution A contained the following ingredients: acethylthiocholine iodide, 5.0 mg; 0.1 mol/l acetate buffer (pH 6.0), 6.5 ml; 0.1 mol/l sodium citrate, 0.5 ml; 30 mmol/l copper sulfate, 1.0 ml; distilled water, 1.0 ml and 4 mmol/l iso-octamethyl pyrophosphoramide, 0.2 ml. Solution B contained 5 mmol/l potassium ferricyanide, 1.0 ml. Slides with the mixture of solution A and B were incubated at 37 °C, rinsed in tap water for 10 s, treated with 6 mg p-phenylene diamine in 10 ml 0.01 mol/l PBS (pH 7.0) for 45 min at room temperature, washed in tap water, dehydrated, and mounted.

For histochemical staining with NADPH-diaphorase specimens were incubated in 1 mg/ml β-NADPH (Sigma, Dorset, UK), 0.1 mg/ml nitroblue tetrazolium (Sigma), and 0.3% Triton-X in 0.05 mol/l Tris-HCl buffer (pH 7.6) at 37 °C. When the specimens showed robust staining, they were mounted into Glycergel mounting medium (DAKO, Glostrup, DK) and investigated by conventional light microscopy.

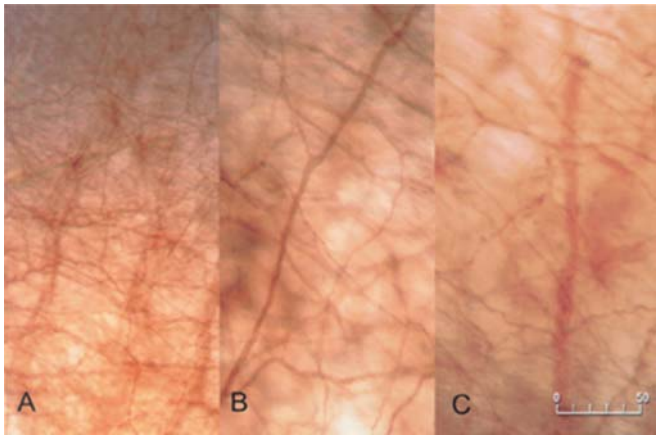

Fig. 2A–C. AChE-positive neuronal network in submucosal layer (whole-mount preparation) of upper urinary tract: **A** rabbit; **B** pig; **C** human

Results

Whole-mount and section preparations clearly demonstrated NADPH-diaphorase-positive nerve fibres forming dense, uniform neuronal networks in the submucosal layer in all three species. These neuronal plexuses were similar in shape, size, and distribution in all species. Positive fibres were also present in the muscular layer in rabbit, pig, and human UUT, forming a neuronal network (Fig. 1). The fibres mainly ran longitudinally, making interconnections with each other and with the submucous plexus. The thickness of the NADPH-diaphorase-positive fibres varied between 2 and 5 μm.

An AChE positive neuronal network was present in the submucosal layer of the UUT of all specimens. These networks contained thick nerve trunks (approximately 5–10 μm), running longitudinally and connected with finer nerve fibres (Fig. 2). In rabbit, pig, and human UUT AChE-positive cholinergic fibres were also present in the muscular layer, running mainly parallel with the muscle fibres, but also forming a neuronal network with interconnecting transverse nerve fibres. The section preparations revealed small submucosal AChE-positive fibres and larger AChE-positive nerve trunks in the muscle layer (Fig. 3). The size and shape of the submucosal and muscular plexuses remained unchanged throughout the pelvis and ureter. A single AChE-positive ganglion was found between muscular layers in the distal ureter of the rabbit (Fig. 4).

Discussion

Previous studies investigating the neuronal control of the UUT have used mainly conventional histological sections, i.e., paraffin or cryostat sections. UUT innervation is a complex 3-D structure that is difficult to appreciate on thin sections. Whole-mount preparation is an elegant technique for the visualisation of nerve

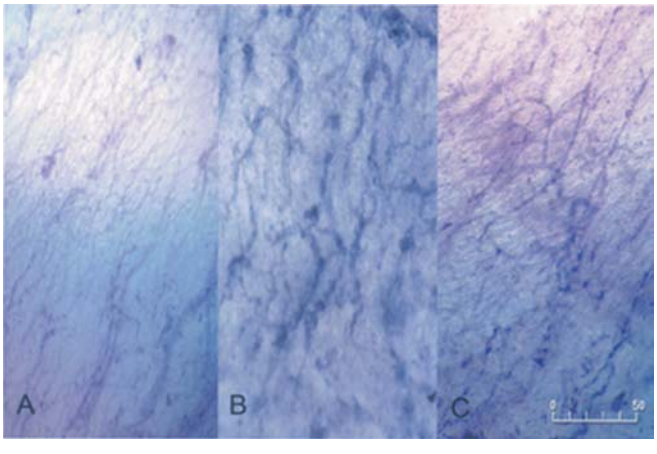

Fig. 1A–C. NADPH-diaphorase positive neuronal network in muscular layer (whole-mount preparation) of upper urinary tract: **A** rabbit; **B** pig, **C** human

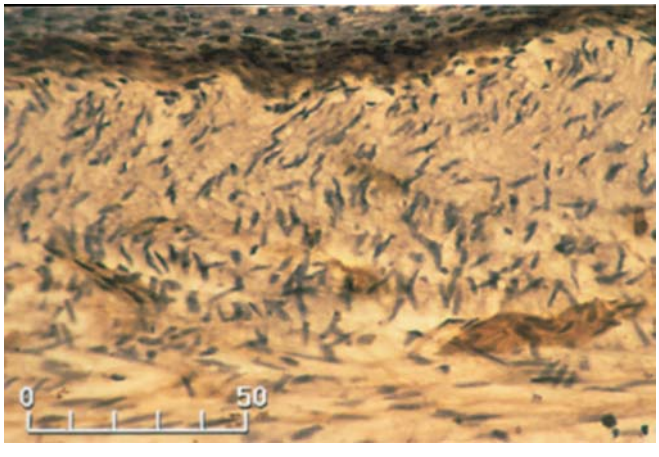

Fig. 3. AChE-positive submucosal and muscular nerve fibres in rabbit renal pelvis (cryostat section)

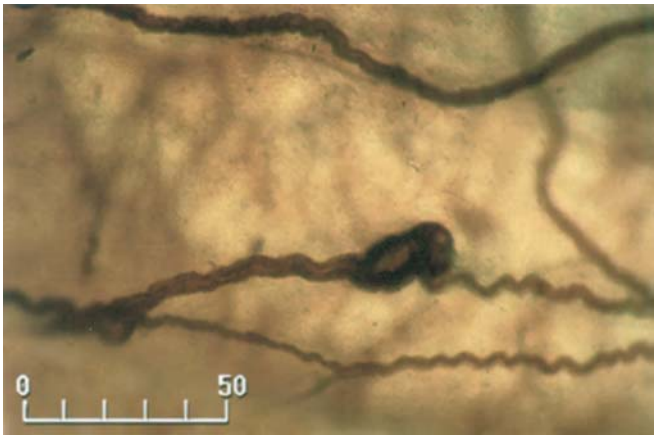

Fig. 4. AChE positive ganglia between muscular layers (whole-mount preparation) of distal rabbit ureter

plexuses because it produces 3D preparations that are more informative in assessing the topography and distribution of neuronal structures.

NOS is present in intrinsic neurons of various organs, suggesting an important role of NO in the peripheral ANS [10, 16, 17]. NO is a regulatory molecule acting both as a second messenger and a neurotransmitter [18, 19]. NOS and neuronal NADPH-diaphorase are identical in brain and peripheral tissues [20, 21]. Nitrergic nerves have been demonstrated in pig and sheep ureter and have been proposed to exert an inhibitory function on the ureter, mediating ureteral relaxation [22–24]. Previously, nitrergic fibres have been demonstrated mainly in the muscular and less frequently in the submucosal ureteral layer of various species [25–29]. Furthermore, it has been reported that nitrergic innervation is absent in the distal ureter [24]. The whole-mount preparation utilised in our study using NADPH-diaphorase histochemistry clearly demonstrated two well-formed, meshlike neuronal networks in the submucous and muscular layer throughout the renal pelvis and ureter in the three species studied. The presence of these two nitrergic plexuses suggests that nitrergic innervation has an important role in the relaxation of the ureter, propelling the urine to the bladder.

Evidence to suggest that cholinergic neurons play a role in modulating ureteral peristalsis is provided by the finding that the ureter and renal pelvis of humans, guinea-pigs, and rabbits contain considerable amounts of ACh which can be released by electric-field stimulation [29]. Further evidence is provided by studies, that show that ACh from cholinergic nerves increases the contractile activity of the ureter and may affect the rate of urine transport along the ureter and urine bolus volume [6, 30–32]. The AChE staining using whole-mount preparations in the present study demonstrated rich cholinergic innervation of the UUT in all three species studied. There were two well-formed cholinergic neuronal plexuses (submucous and muscular) in the ureteral wall. The presence of the well-formed nitrergic and cholinergic plexuses not only in the muscular layer, but also in the submucous layer, suggests that these plexuses have an important modulatory role in regulating the transport of urine in the UUT. Future studies of these neuronal networks should provide new insights into the pathophysiology of various anomalies of the UUT.

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