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NADPH-diaphorase in glandular cells and nerves and its relation to acetylcholinesterase-positive nerves in the male reproductive tract of man and guinea-pig

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Abstract The presence of NADPH-diaphorase activity and acetylcholinesterase in the testis, epididymis, vas deferens, seminal vesicle, pelvic plexus, prostate and urethra of man and guinea-pig was investigated with the nitro blue NADPH technique and the thiocholine method, respectively. In human material NADPH-diaphorase activity was found in the Leydig cells, Sertoli cells and the epithelial linings of the rete testis, the excretory ducts, seminal vesicle, prostate and urethra. The guinea-pig material showed staining of the Leydig cells and spermatozoa and similar epithelial staining of the tract as man. Nerves beneath the epithelium and in the muscle layers of cauda epididymis, vas deferens, seminal vesicle, prostate and urethra were also stained. NADPH-diaphorase-positive nerve cells were seen in the pelvic plexus. Some cells also displayed acetylcholinesterase activity but others showed activity for only one of the enzymes or no activity for either enzyme. In the cauda epididymis, vas deferens, seminal vesicle, prostate and urethra acetylcholinesterase-positive nerve fibres formed a plexus beneath the secretory cells. It is concluded that NADPH-diaphorase, generally accepted as a nitric oxide synthase, is present in glandular cells of the male genital tract. The enzyme is also present in nerves, where it is partly co-localized with acetylcholinesterase.

Key words Acetylcholinesterase · Autonomic nerves · Glandular cells · Male genital tract · NADPH-diaphorase · Nitric oxide synthase

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Introduction

NADPH-diaphorase (NADPH-d) staining was introduced by Vincent [25], who showed stainable neurons in the cerebellum. However, the great interest in this staining technique came when it was realized that NADPH-d was a nitric oxide synthase (NOS) [26]. Consequently the technique has been widely used for the detection of nitrergic nerves in various tissues [2, 18, 23, 26]. The smooth muscle of the urogenital tract is supplied with nitrergic nerves, which seem to constitute the main myoinhibitory innervation of these organs [1, 15, 23].

Ca^{2+}-dependent, that is constitutive, NOS activity is prominent in many human male genital organs, especially the seminal vesicle [8]. The relative activity of the enzyme does not, however, follow the typical distribution of a constituent of muscle nerves such as noradrenaline (NA) [20], but rather suggests a relation to the proportion of secretory compartments of the reproductive organs. This prompted us to investigate whether the enzyme was also localized in secretory cells or nerves close to them.

Since secretory cells of the dog prostate [16] and guinea-pig seminal vesicle [22] have a cholinergic secretomotor innervation, we also found it of interest to combine the NADPH-d staining with acetylcholinesterase (AChE) staining. In addition to human material we added material from the guinea-pig, which is a suitable animal for experimental studies of seminal vesicle secretion [22]. While this work was in progress Burnett et al. [3] reported on NOS activity of the glandular cells and nerves in the human prostate. The same group also showed NOS activity and NOS immunoreactivity in subepithelial nerves and various structures in the male genital tract of rat [4, 5]. A preliminary account of our study has been presented [9]. In a previous study [10], we concentrated on the function of glandular NOS in the seminal vesicle, whereas this study is confined to the localization of NADPH-d/NOS and AChE.

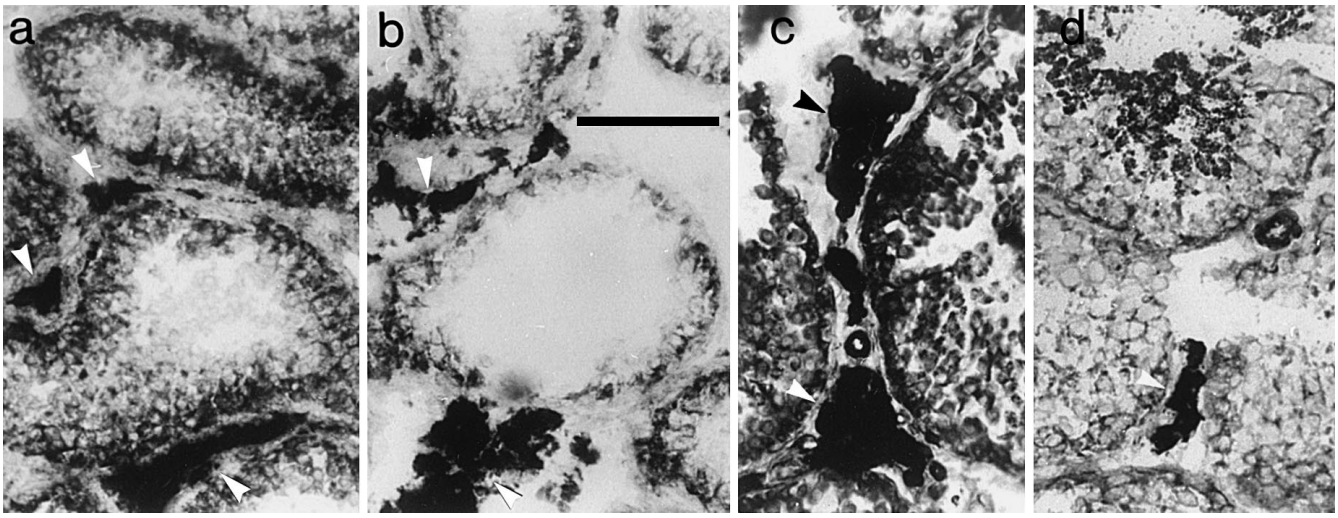

Fig. 1a–d NADPH-diaphorase (NADPH-d) staining of the testis. The strongly stained interstitial cells are indicated by *arrowheads*. **a** Stained Sertoli cells are seen in the seminiferous tubules of a 71-year-old man. **b** In a 54-year-old man treated with oestrogen and progesterone only dark Sertoli cells are seen in the tubules. **c** Untreated guinea-pig. **d** Reduced Leydig cells in a testosterone-treated guinea-pig. Fixed sections. Scale bar represents 100 μm

Materials and methods

Histochemistry

Testes, epididymis, scrotal and funicular parts of the vas deferens were obtained from nine men (27–54 years) subjected to transsexual operations and 1 man (71 years) subjected to ablation because of prostatic cancer. The men undergoing transsexual surgery had been treated with female sex hormones for months [21] but this was not the case with the tenth man. Abdominal parts of the vas deferens, the ampulla ductus deferentis, the prostate and the seminal vesicle as well as vesicular parts of the pelvic plexus were obtained from six men (54–80 years) subjected to radical prostatectomy or cystectomy due to cancer. Prostatic parts of the urethra were also obtained from these patients, while cavernous parts of the organ were obtained from the men undergoing transsexual operations.

Material from guinea-pigs was obtained from ten testosterone-treated [10] and four untreated guinea-pigs weighing 774–1028 g. The part of the pelvic plexus investigated was that at the end of the hypogastric nerve and close to the male genital organs [20, 22].

Human material was transported within 30 min to the laboratory in ice-cold Ringer solution. Guinea-pig material was removed immediately after stunning and bleeding.

Tissue specimens were dissected free, cut in blocks (3–9 mm^3) and either placed immediately on chilled chucks, frozen (−30°C) and cut in a cryostat (−30°C) or fixed in ice-cold formaldehyde solution before freezing on the chucks. Specimens that had been fixed were either rinsed in "Gum-sucrose" (0°C) [12] and stored in this medium for 1–5 days (0°C) or directly rinsed in phosphate buffer (0°C) and placed on chilled chucks. In preliminary studies two formaldehyde solutions (10% in 2% $CaCl_2$ and 4% in phosphate buffer) and different fixation times (30 min to 8 h) were tested. The best pictures were obtained with 1 h fixation in 10% formaldehyde in 2% $CaCl_2$, which became the standard procedure. Sections from unfixed blocks were either stained directly or fixed in 4% neutral formaldehyde (0°C) for 20 min before staining. The sections, 8–15 μm thick, were either investigated immediately or stored for 1–10 days at −30°C. NADPH-d staining was accomplished by 1 h (37°C) in the NADPH-nitro blue medium recommended by Vincent [26]. AChE staining was performed by incubation for 1 h (37°C) in the acetylthiocholine–metal salt medium recommended by Karnovsky and Roots [14]. When double stainings were performed the sections were first incubated for 1 h in the medium for NADPH-d staining, then washed in phosphate buffer and incubated in the medium for AChE staining for 2 h (after 1 h the staining was too weak). The opposite procedure, i.e. starting with AChE staining, never resulted in full NADPH-d staining. For general staining eosin was used in some preparations. The histochemical material comprised with respect to each investigated organ or organ level, tissue specimens from four to nine men and guinea-pigs, respectively. From each tissue specimen between three and 12 blocks were investigated. From each block four to 14 sections were cut for AChE staining and NADPH-d staining, respectively. In about two-thirds of the blocks, sections were also cut for combined staining. Two small pelvic ganglia in the vicinity of the seminal vesicle of one man were captured and fixed. The guinea-pig material of the pelvic plexus comprised 20 blocks, derived from 10 animals. In the case of material from neural tissues, sections were always cut for combined staining.

Drugs

NADPH staining: NADPH, nitro blue tetrazolium, Triton-X, TRIS buffer (all Sigma).

AChE staining: Acetylthiocholine iodide, isoompa (Sigma). Eosin, phosphate buffer, sodium citrate, copper sulphate, potassium ferricyanide (all Merck).

For fixation: Formaldehyde, $CaCl_2$, phosphate buffer (all Merck).

Results

General

Stained delicate nerve terminals were seen in unfixed sections. Following fixation, terminals – if present at all – usually appeared as coarse stainable fragments. Nerve cell bodies and glandular cells showed good stainability in fixed as well as unfixed sections. NADPH-d positive glandular cells were evident even after 8 h of formaldehyde fixation.

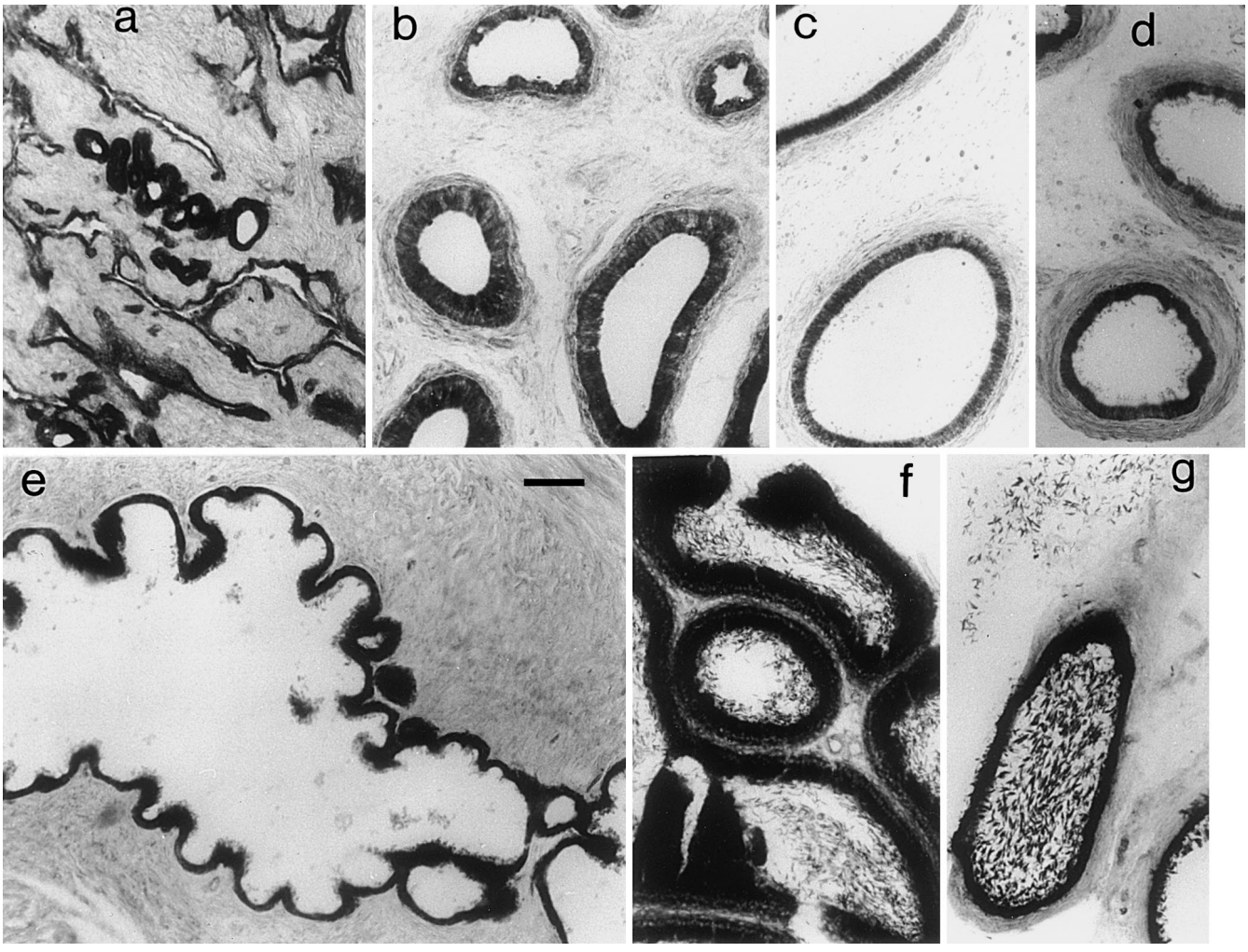

Fig. 2a–g NADPH-d staining of epithelial linings of: **a** rete testis, **b** caput epididymidis (upper ducts are ductuli efferentes, lower ducts are epididymal duct), **c** corpus epididymidis, **d** testicular segment of cauda epididymidis and **e** prostatic segment of cauda epididymidis of a hormone-treated man. **f** caput epididymidis and **g** cauda epididymidis of guinea-pig. Fixed sections. Scale bar represents 100 μm

Testis, caput and corpus epididymidis

In the human testis, the Sertoli cells and the interstitial cells of Leydig showed strong NADPH-d stainability (Fig. 1a, b). In the guinea-pig material the Leydig cells showed strong reaction (Fig. 1c, d) while the staining of the Sertoli cells was less prominent. The spermatozoa were – in contrast to human spermatozoa – stained (Fig. 1c, d; Fig. 2f, g). The epithelial linings of the human rete testis, ductuli efferentes and epididymal duct were NADPH-d positive (Fig. 2a–c). Similar stainability was seen in the guinea-pig (Fig. 2f). AChE-positive nerves and a few NADPH-d-positive nerves were seen perivascularly and in the walls of blood vessels. Vascular endothelium was NADPH-d positive.

Cauda epididymidis, vas deferens, ampulla ductus deferentis and seminal vesicle

The cells of the secretory linings, folds and crypts showed prominent NADPH-d staining (Fig. 2d, e, g; Fig. 3). In the human material NADPH-d-positive nerves, concentrated beneath the epithelium, were also observed in about a half to a third of the sections from the ampulla ductus deferentis, seminal vesicle and prostatic end of the vas deferens (Fig. 3e–g). Such nerves were rare in the cauda epididymidis and the epididymal part of the vas deferens. In the guinea-pig material only few NADPH-d-positive nerves were detected. AChE staining revealed a prominent subendothelial nerve plexus and also nerves in the muscle coat (Fig. 5a–d). In some nerves and terminals in the human material AChE co-localized with NADPH-d (Fig. 6f). Co-localization (Fig. 6g) was very rare in the guinea-pig material.

Prostate

NADPH-d activity was seen in both secretory cells and nerves of the human prostate (Fig. 4a). The NADPH-d staining was usually weaker in prostatic secretory cells

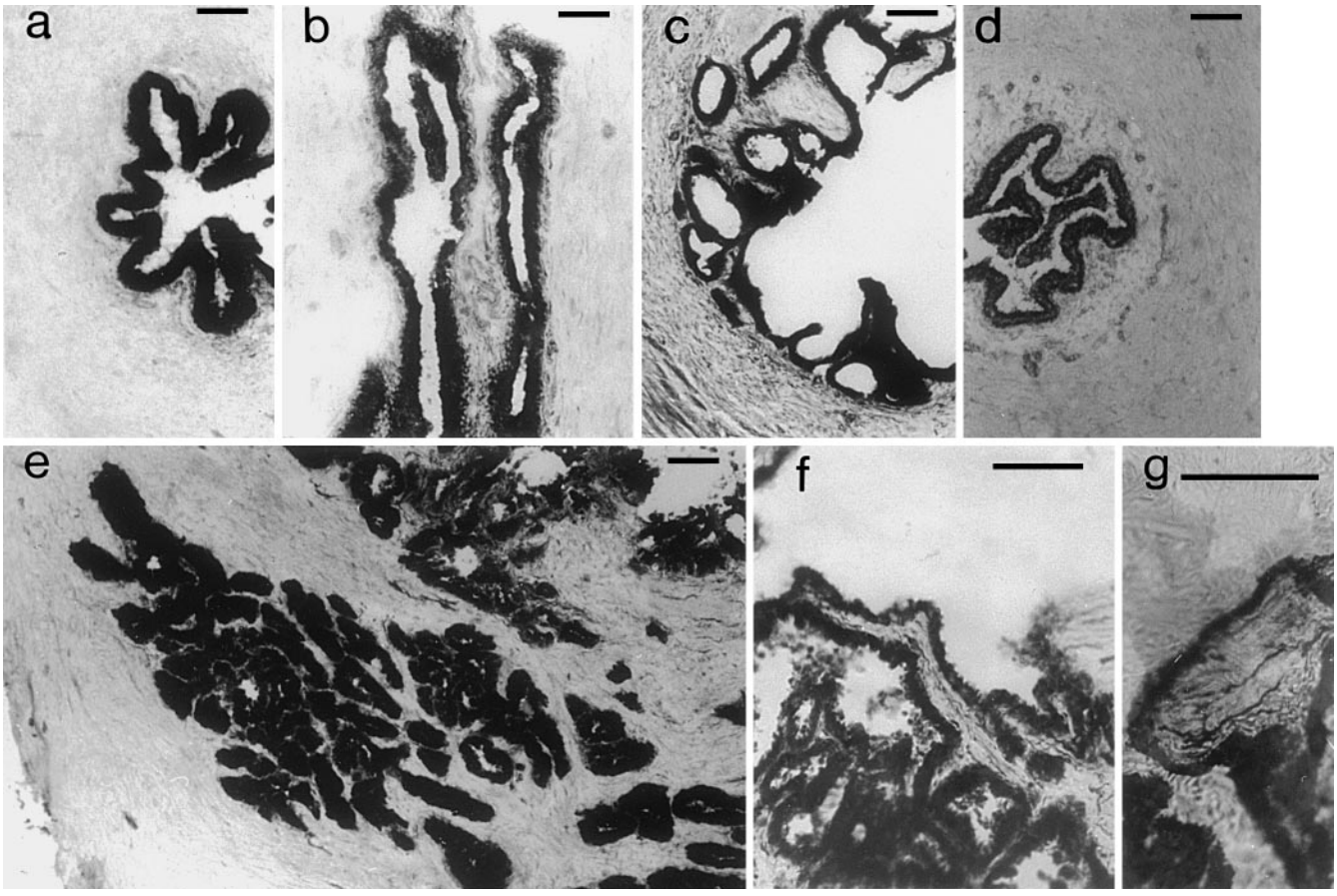

Fig. 3a–g NADPH-d staining of epithelium in vas deferens, ampulla ductus deferentis and seminal vesicle. **a** Scrotal segment of human vas deferens (transverse section). **b** Longitudinal section from funicular segment from the same patient (age 71 years). **c** Human ampulla ductus deferentis. **d** Middle segment of guinea-pig vas deferens. **e–g** Human seminal vesicle. **e** Glandular cells as well as nerves are stained. **f, g** Fold of mucosa with stained nerve fibres in the fold. Fixed sections. Scale bars represent 100 μm

than in those described above. AChE-positive nerves were noted in the stroma and close to the secretory cells (Fig. 5e). Some fibres were double stained. The coagulating gland, and dorsal and ventral prostates of guinea-pig also had NADPH-d stainable glandular cells (Fig. 4b–d), but as in man the colouring was weaker than in the excretory ducts and seminal vesicle. NADPH-d-positive nerves were rare, but subendothelial AChE-positive terminals were frequent.

Urethra

The epithelial cells of man as well as guinea-pig were darkly stained with nitro blue (Fig. 4e, f). NADPH-d-positive nerves as well as AChE-positive fibres were seen beneath the epithelium and in the muscle of human urethra. Some fibres showed co-localization of NADPH-d and AChE. NADPH-d-positive nerves were rare in the guinea-pig urethra.

Pelvic plexus

Pelvic ganglia of man and guinea-pig had nerve cells that showed very strong NADPH-d reaction but others showed no or only weak reaction (Fig. 6a, d). NADPH-d-positive terminals (Fig. 6d) were also seen in the ganglia. Some cells showed strong AChE staining but many of the cells displayed rather weak or no reaction (Fig. 6b). Strongly AChE-positive terminals were seen around many nerve cells (Fig. 6e). Using combined staining we could identify four types of nerve cells: (1) cells showing no or only faint reaction to the stains, (2) cells essentially positive to NADPH-d only, (3) cells essentially positive to AChE only, and (4) cells showing reactions to both the enzymatic stains (Fig. 6c, e). The most frequent cell type was the one that stained – although to a varying degree – with both stains. Almost as frequent was the essentially unstained type. A minor fraction was positive to AChE only. The rarest type (only seven cells noted) was that which stained with nitro blue only. This type was not observed in the scarce human material, where most cells were AChE-positive, although the reaction was very weak in some cells. In the pelvic plexus of guinea-pig unstained or weakly coloured cells sometimes occupied the largest area in a ganglion and strongly stained cells were lacking or essentially located at the edges of, or in cells strands within, the ganglion (Fig. 6d, e). In other ganglia stained cells

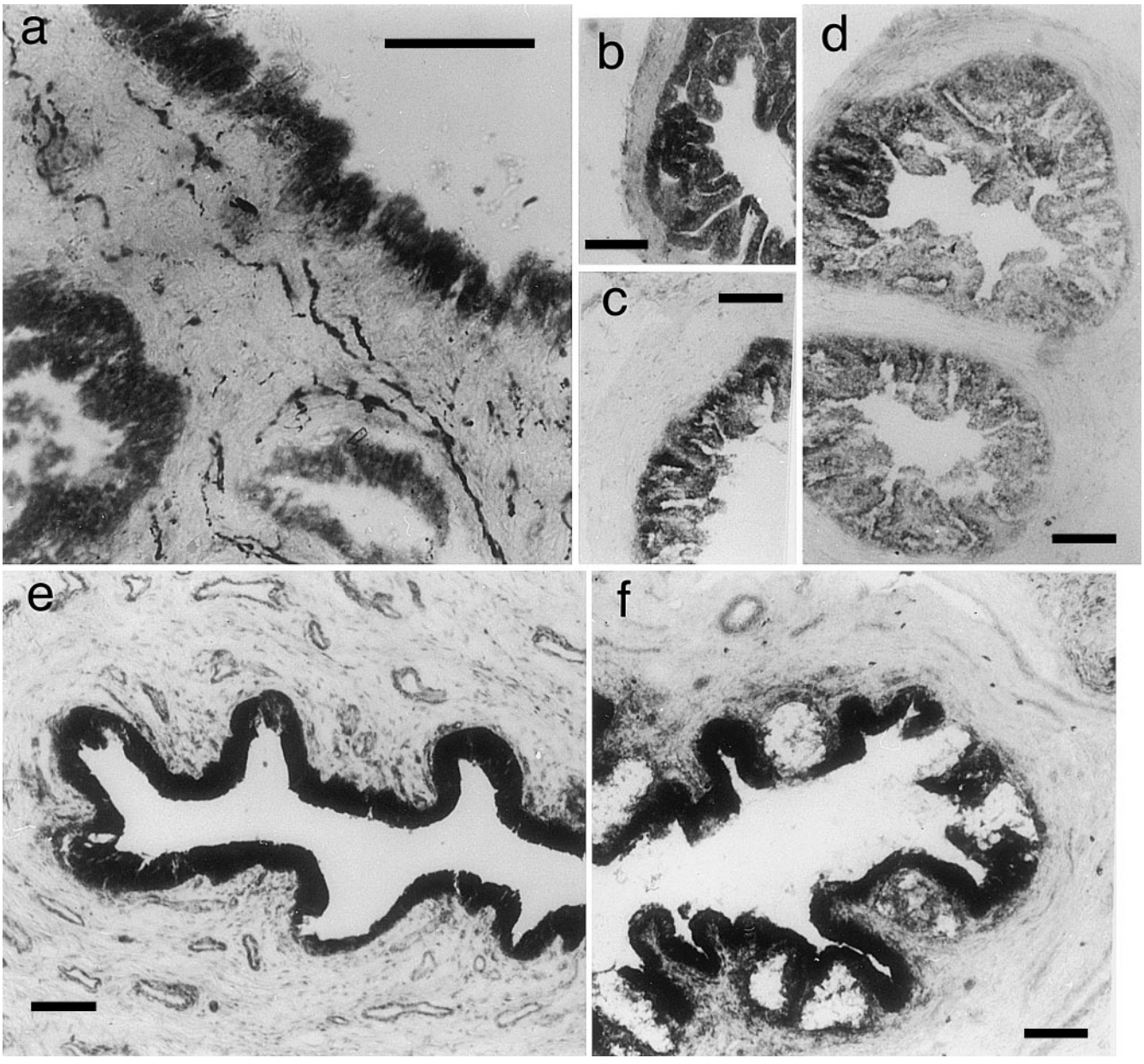

Fig. 4a–f NADPH-d staining of prostate and urethra. **a** Human prostate with stained secretory cells and nerve fibres. **b** Ventral prostatic lobulus. **c** Dorsal prostatic lobulus and **d** lobuli of coagulating gland of guinea-pig. **e** Urethra close to glans penis from a hormone-treated man (note coloured endothelium in sinusoidal vessels). **f** Urethra from a guinea-pig (clear parts are mucous glands). Fixed sections. Scale bars represent 100 μm

dominated or were the only cell type. From the ganglia nerve trunks with fibres stainable with one or both stains, or unstained fibres, emerged (Fig. 6a, b).

Discussion

Burnett and coworkers [3, 4] have reported on NOS in epithelial cells and subendothelial nerves in the human prostate and parts of the rat male genital tract, using immunohistochemistry and in some rat organs also the NADPH-d technique. However, according to these authors the seminal vesicle has little NOS activity. Davidoff et al. [7] found NADPH-d in Leydig cells and to some extent also in Sertoli cells of man. Our main contribution concerns the NADPH-d positivity of epithelial cells throughout the male tract.The similarity between man and guinea-pig concerning general epithelial NADPH-d stainability is also noteworthy and suggests that the guinea-pig may be the experimental animal of choice for studies of secretion in the male genital tract. The presence of NADPH-d positivity together with AChE positivity in some nerves confirms to findings in the lower urinary tract of pig [17]. In agreement with earlier findings [26] the NADPH-d staining of cell bodies showed marked resistance to formaldehyde fixation. However, as regards nerve terminals fixation usually

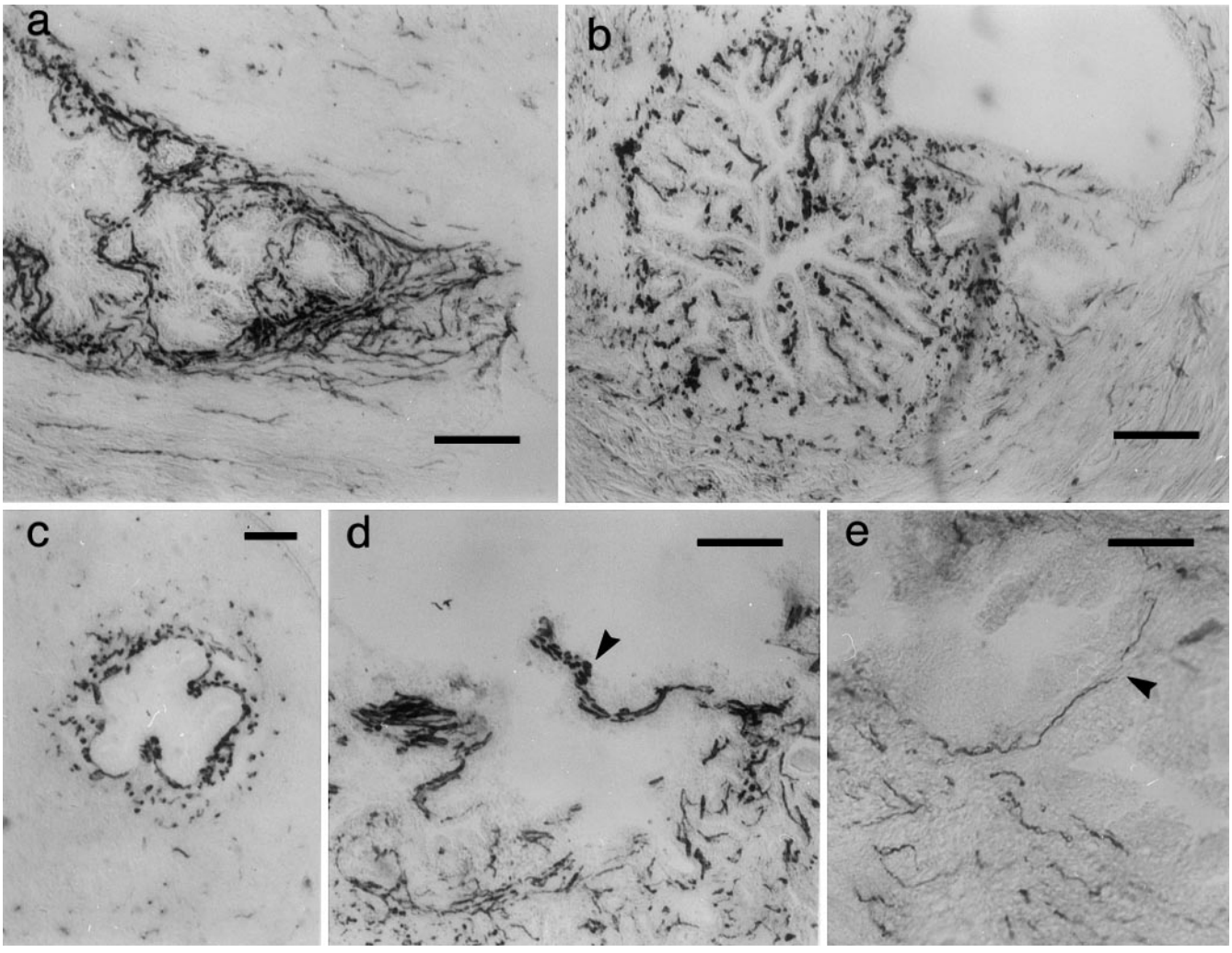

Fig. 5a–e AChE-positive nerves to secretory epithelium of human internal male genital glands. **a** Abdominal vas deferens close to ampulla ductus deferentis (longitudinal section). Nerves run in the folds. **b** Transverse section from ampulla ductus deferentis. Folds with nerves and nerves around a gland (*upper right*). **c** Scrotal vas deferens. **d** Folds (*arrowhead*) in the seminal vesicle are strongly innervated. **e** Nerves around glandular acinus (*arrowhead*) and in stroma of prostate. All sections are fixed except **c**, which is unfixed. Scale bars represent 100 μm

hampered the enzymatic stainings. The distribution of AChE-positive nerves correlates with earlier findings [6, 11]. These nerves are probably secretomotor [22]. Subendothelial nitrergic nerves may also be secretomotor [10]. Those in the smooth muscle may be myoinhibitory, but at present evidence of myoinhibitory innervation of smooth muscle of internal male genital organs has been confined to the rabbit, canine and human prostate [19, 24].

Our finding of the differential staining of nerve cells in the pelvic plexus confirms the recent concept that autonomic nerve cells are much more variable with respect to mediators than was believed a decade ago and consequently may have more than one transmitter or at least the capacity for forming more than one mediator (see quotations and discussion in Sjöstrand and Klinge [23]). It is possible that the nerve cells that were not stained by either stain were adrenergic, but further investigations are needed for establishing the neuronal relation of NOS and catecholamines. Thus, recently NOS and tyrosine hydroxylase were found to be co-localized in some ganglionic cells of the pelvic plexus and nerves of the genitourinary organs of children 2 months to 3 years old [13].

It would be premature to speculate on possible functions of the glandular NADPH-d, presumably NOS, in this paper. But in a previous report [10] we presented results indicating that glandular formation of nitric oxide is a prerequisite for muscarinic, that is, neurogenic secretion in the guinea-pig seminal vesicle. If similar mechanisms operate also in other secretory cells of the male genital tract, then a new aspect of formation of seminal fluid and male secretion will be established.

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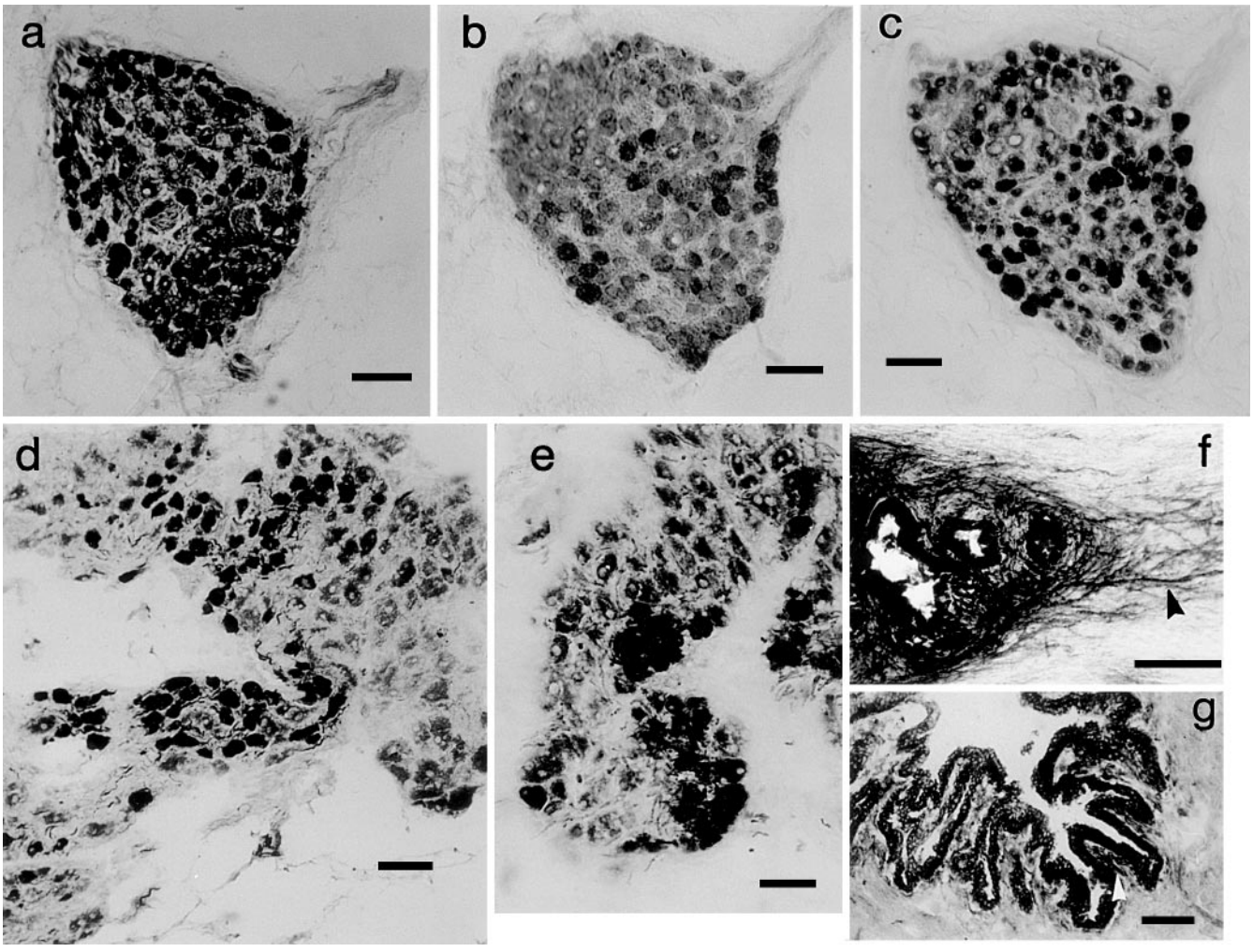

Fig. 6a–c Small ganglion in connective tissue of human seminal vesicle. **a** NADPH-d staining. Some nerve cells are darkly stained blue. Note the trunk with stained fibres at the edge of the ganglion. **b** Parallel section, AChE staining. Cells are brown, stained with varying intensity. A few cells are unstained. Note the nerve trunk. **c** Double staining. Some cells are almost black, stained with both stains, while others are weakly stained and some cells are brownish, stained only for AChE. **d** NADPH-d staining of a ganglion from pelvic plexus of guinea-pig. Darkly stained cells are mingled with weakly stained or unstained cells. Note stained nerve terminals. **e** Double staining of a similar ganglion. Many blackish, double-stained nerve cells, some unstained cells and a few brownish cells positive for AChE only are seen. Note brownish or blackish terminals. **f** Double-stained human vas deferens close to the ampulla, parallel section to Fig. 5a. NADPH-d positive blue glandular cells are seen. Nerves are AChE-positive (brownish) or double stained (blackish); *arrowhead* indicates a double-stained long fibre. **g** Guinea-pig seminal vesicle. Double staining. NADPH-d positive blue secretory cells and AChE-positive brown nerves are visible. *Arrowhead* denotes a black, double-stained fibre. Fixed sections. Scale bars represent 100 μm

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