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DISTRIBUTION OF NADPH-DIAPHORASE POSITIVE GANGLIONATED PLEXUSES IN PORCINE GALL BLADDER

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Abstract

The distribution and dimensions of nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) positive ganglia in the domestic pig's gall bladder and *Ductus cysticus* were studied for the first time. It was established that the number of ganglia was highest in the gall bladder's body, followed by those in the fundus and neck. The lowest number of ganglia was observed in *Ductus cysticus*. The largest ganglia were localized in the gall bladder's neck, followed by these in the body and fundus. The highest density of neurons per ganglion was detected in the neck, followed by body and fundus. Their number, however, was the lowest in the ganglia of *Ductus cysticus*. The largest neurons were established in the gall bladder's neck.

It was concluded that the NADPH-d ganglia as neuronal structures produce nitric oxide, which as a transmitter with neuronal origin most probably is involved in the control of the epithelial secretion and in the function of smooth muscle in the walls of both gall bladder and blood vessels as well.

Key words: NADPH-diaphorase, ganglionated plexus, porcine gall bladder

Introduction. Much progress has been made in recent years to increase our understanding of the organization and pathobiology of neural plexuses and the properties of neurons that directly innervate the biliary tree [1].

Morphological studies of the ganglionated plexus of the gall bladder have been carried out in several species. The first observation of intrinsic neurons in the gall bladder was made in birds about 140 years ago by Manz, four years before Auerbach described the myenteric plexus [2]. Further historical perspective is provided by Sutherland [3]. The anatomical and functional aspects of the innervation of the biliary tract are based on extensive studies in the guinea pigs [4–8] and the Australian brush-tailed possum [9, 10]. A nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) histochemical technique that relies

on certain neurons possessing an enzyme that catalyzes the NADPH-dependent conversion of a soluble tetrazolium salt to an insoluble visible formazan [11, 12] is used to identify neurons that utilize nitric oxide. Nitric oxide synthase positive (NOS) neurons have also been shown to stain for NADPH-d, and most NADPH-diaphorase containing cells also exhibit NOS-immunoreactivity [13–15]. In the gastrointestinal tract, all myenteric neurons that are NOS-immunoreactive have also been found to stain for NADPH-diaphorase [13]. The presence of NADPH-d positive ganglionated plexuses was established extensively in the gall bladder wall of guinea pigs [16].

The role of nitric oxide in the domestic pig gall bladder has not yet been examined. This fact motivated us to undertake the current study to determine the NADPH-d expression in the ganglionated plexuses of domestic pig gall bladder.

Materials and methods. ***1. Animals.*** The material was obtained from the wall of gall bladder of 6 castrated male and 6 female pigs (Landras X Bulgarian White), aged 6 months, slaughtered for meat consumption in a slaughterhouse.

2. Enzyme histochemical reaction for determination of NADPH-d. Samples were immediately immersed in 4% paraformaldehyde (Sigma Aldrich Chemie, Switzerland) in phosphate-buffered saline (PBS), pH 6.9, for 8 h at 4 °C. Then the samples were removed and soaked in a solution of 10% sucrose in PBS overnight. Sections of 15–20 µm thickness were prepared by means of a freezing microtome (Slee, Mainz, Germany). The free-floating sections were further processed according to the protocol of Sherer-Singler et al. [11] by incubation in a solution containing nitro blue tetrazolium (0.2 mg/ml, Sigma Aldrich Chemie GmbH, Germany), β-NADPH (Santa Cruz Biotech, Santa Cruz, CA, USA) and Triton X-100 (0.5%) (Merck Belgalabo, Overijse, Belgium) in PBS (0.1 M, pH 7.4) for 1–2 h at 37 °C.

Microscopic assessment of the reaction was scored as absent (0), weak (+), medium (++) and strong (+++).

3. Micromorphometrical investigation. In the wall of the gall bladder fundus, body and neck, and in the wall of *Ductus cysticus* as well, the number of NADPH-d-positive ganglia per transversal section and the number of NADPH-d-positive-neurons per ganglion were estimated. The area, width and length of the ganglia and the width and length of the neurons in these ganglia were also measured.

Statistics. Data for number and dimensions are given as mean ± SD. For that purpose were used a light microscope (ZEISS Primo Star, Germany), camera (Progres, Capture 2.6 – JENOPTIK) and software analysis programme (Soft Imaging Sistem GmbH).

Statistical data processing was done using Data Analysis tool and Student's t-test by means of the StatMost for Windows software and the difference was considered significant when P-values were less than 0.05.

Results. Light microscopic observations showed that the NADPH-d positive

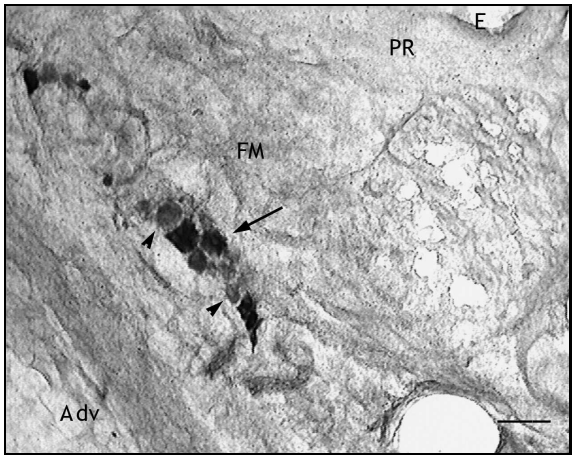

Fig. 1. NADPH-d positive ganglion in the fibromuscular layer (FM) near the adventitial layer (Adv) of gall bladder neck. Some of the neurons (arrow) showed strong NADPH-d reactivity but others – weak to medium reactivity (arrowheads). PR – *Lamina propria mucosae*, E – *Lamina epithelialis mucosae*. Bar = 100 µm

neurons were distinct due to purple formazan deposits in the neuronal perikarya and axons. Nuclei left unstained. Most neurons in NADPH-d positive ganglia exhibited strong enzyme reactivity while a few of them – weak NADPH-d reactivity (Fig. 1). The observation on topographical and structural features of the ganglia and nerves in the gall bladder and *Ductus cysticus* showed that the ganglionated nerve plexuses were located in the subserosal, adventitial, muscular and subepithelial (*Lamina propria mucosae*) layers. In the gall bladder, the subserosal plexus was composed of a network of irregular, triangular- or ovoid-shaped ganglia. Ganglia were interconnected by nitrergic nerve fibres, which were contiguous to paravascular nerve bundles that followed the extensive vascular distribution in this layer.

Nitrergic autonomic nerves with different dimensions were localized in the propria, muscular, adventitial and subserosal layer of all parts of gall bladder, following the vascular distribution where they formed para- and perivascular plexus. The same nerves were also observed around the glands of gall bladder neck.

The data of density, area, length and width of NADPH-d-positive ganglia in the wall of gall bladder fundus, body and neck, and of *Ductus cysticus* are given on Table 1. It is indicated that the number of ganglia is highest in the gall bladder body followed by fundus and neck. The lowest number of ganglia is observed in *Ductus cysticus*. The micromorphometric measurements showed that the largest ganglia were localized in the gall bladder neck, followed by body and fundus (Table 1). In the wall of *Ductus cysticus*, the size of ganglia was similar to the size of those located in the fundus. Statistical significance between the measurements made in different parts of gall bladder wall was established.

Density, length and width of NADPH-d-positive neurons in the ganglia of gall bladder fundus, corpus and neck and in the ganglia of *Ductus cysticus* were estimated as well (Table 2). The highest density of neurons per ganglion was detected in the neck, followed by body and fundus. Their number was lowest in

T a b l e 1

Density (number of ganglia per transversal section), area (μm^2), length (μm) and width (μm) of NADPH-d-positive ganglia in the different parts of gall bladder and in the wall *Ductus cysticus*

Parts of gall bladder	Number of ganglia	Area of ganglia	Length of ganglia	Width of ganglia
fundus				
♂	7.33 ± 0.74	3495.68 ± 1946.16	101.23 ± 53.76	61.50 ± 25.53
♀	7.41 ± 0.67	3471.79 ± 1947.95	100.32 ± 51.75	61.24 ± 25.32
body	* * *		* * *	*
♂	8.16 ± 0.68	4021.58 ± 3188.62	152.22 ± 86.39	53.54 ± 23.48
♀	8.18 ± 0.67	4320.30 ± 3373.93	172.20 ± 112.06	57.93 ± 28.42
neck	* * *	* * *	* * *	* * *
♂	7.16 ± 0.69	12956.70 ± 5823.72	295.87 ± 108.90	71.01 ± 28.62
♀	7.20 ± 0.67	13048.19 ± 5677.73	293.96 ± 104.97	71.96 ± 27.46
Ductus cysticus	* * *	* * *	* * *	* * *
♂	4.66 ± 0.75	3175.72 ± 2775.27	134.22 ± 107.24	44.62 ± 15.68
♀	4.73 ± 0.73	3088.15 ± 2584.28	130.96 ± 102.14	44.58 ± 17.95

♂– males, ♀– females
$^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, – statistically significant difference vs previous part of gall bladder wall (Student's t-test)

the ganglia of *Ductus cysticus*. The largest neurons (up to 70.9 μm in length and up to 41.2 μm in width) were established in the porcine gall bladder neck.

As statistically significant difference was not detected in the density, area, length and width of ganglia as well as in density, area, length and width of neurons in these ganglia in the porcine gall bladder between males and females, sexual dimorphism was not established.

In the fundus wall, 25% of ganglia were situated in the subserosal layer near the muscle layer, 63% – in the muscle layer and 12% – in the propria near the muscle layer. In the body wall, 4% of ganglia were detected in the subserosal layer near the muscle layer, 67% – in the muscle layer and 29% – in the propria near the muscle layer. In the neck wall, 11% of ganglia were detected in the subserosal layer near the muscle layer, 67% – in the muscle layer and 22% – in the propria between glands.

Discussion. In this study, for the first time, density, shape and dimensions of ganglionated nerve plexuses as well as dimensions and density of neurons per ganglion were estimated. The findings indicated that the topographical and structural organization of the ganglia and nerves in the gall bladder was similar to that in guinea pig and Australian brush-tailed possum. We found that the ganglionated nerve plexuses were located in the subserosal, muscular and subepithelial layers as in the Australian brush-tailed possum [1]. According to Balemba et al. [1], in guinea pigs the ganglionated nerve plexuses are situated in the subserosal

T a b l e 2

Density (number of neurons per ganglion), length (μm) and width (μm) of NADPH-d-positive neurons in the ganglia of different parts of gall bladder and in the wall of *Ductus cysticus*

Parts of gall bladder	Number of neurons	Length of neurons	Width of neurons
fundus			
♂	5.30 ± 3.45	33.43 ± 8.11	21.99 ± 6.51
♀	5.15 ± 3.45	32.81 ± 7.38	22.15 ± 28.42
body			
♂	6.53 ± 5.51	33.46 ± 11.64	24.70 ± 6.75**
♀	6.93 ± 5.20	34.12 ± 9.56	26.30 ± 5.84
neck			
♂	9.78 ± 4.69***	36.10 ± 12.19*	21.47 ± 7.14**
♀	8.55 ± 4.88	36.30 ± 12.37	21.46 ± 7.15
Ductus cysticus			
♂	4.03 ± 2.37***	31.80 ± 8.04**	25.18 ± 7.50**
♀	3.86 ± 2.36	33.30 ± 8.98	25.14 ± 7.73

♂– males, ♀– females
$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, – statistically significant difference vs previous part of gall bladder wall (Student's *t*-test)

and subepithelial layers but not in the muscular layer. These results indicate that the structural organization of the neural tissue in the porcine gall bladder is similar to that of gall bladder in the guinea pig and Australian brush-tailed possum, but there are some differences. In the gall bladder of guinea pig, the subserosal plexus is also composed of a network of small, irregular, triangular- or ovoid-shaped ganglia [2, 7]. According to some authors, in kitten, monkey and guinea pig these ganglia have an appearance similar to submucosal ganglia of the ENS, but the resident neurons, whose number range between 2 and 10 per ganglion, are larger and measure 25–60 μm in diameter [3, 7] versus 2 to 16 and 70.9 μm/41.2 μm in our study. Sutherland [2] reported that in monkeys, kittens and guinea pigs ganglia are small and scarce around the gall bladder neck, increasing in size and number over the body and fundus. The larger ganglia consist of several layers of cells arranged in an oval or triangular pattern, and are always closely associated with a rich blood supply. Unlike this author, we found that in the neck of porcine gall bladder ganglia were the biggest. Their number was lower than in the body of this organ but higher than in the *Ductus cysticus*. Based on our results, we can explain these differences with the dependence on species.

Talmage et al. [8] suggested that the VIP/NADPH d/NOS-containing neurons located in the subserosal and muscular plexuses of the gall bladder are intrinsic inhibitory motor neurons of the gall bladder and play a role in the active relaxation of the organ during filling. However, cholinergic gall bladder neurons

can be separated into two distinct populations. Neurons expressing SP, NPY and SOM are excitatory, whereas those expressing NOS, VIP and PACAP are inhibitory neurons [6, 8]. The observation of interganglionic communication in the gall bladder [5] suggests the existence of synaptic interactions among gall bladder neurons, which may serve to coordinate output to the smooth muscle.

Conclusion. The results about NADPH-d-positive ganglinated plexuses in all parts of the porcine gall bladder and in *Ductus cysticus* gave us a reason to suggest that the ganglia in gall bladder wall use nitric oxide as a nerve transmitter. Thus we presume that nitric oxide takes part in the regulatory mechanism of the gall bladder function in domestic pigs.

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