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Choline Acetyltransferase (ChAT) Immunoreactive Structures in the Porcine Extrahepatic Bile Ducts

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ABSTRACT

***Background*:** Acetylcholine (ACh) as non-neuronal cholinergic system is known to be expressed in non-neuronal cells in animals and man, which are elements of a non-neuronal cholinergic system. Evidence for the synthesis of acetylcholine is the establishment of positive choline acetyltransferase (ChAT) immunoreactivity in noncholinenrgic system of humans and laboratory animals but no such information in domestic swine. Mast cells, neural and non-neuronal cholinergic systems in the gallbladder and extrahepatic bile ducts need a detailed morphological study to identify the type and localization of their structural components in domestic pigs. There are no data regarding porcine mast cells as well as about their involvement in various cholangiopathies. The aim of this study immunohistochemical was to qualitative and quantitatively analyze biliary cholinergic ganglia and nerves plexuses as well as non-neuronal cholinergic cells in gallbladder and extrahepatic bile ducts (EHBD) in healthy pigs.

***Materials, Methods & Results*:** In the present study were used tissue samples from the extrahepatic bile ducts and gallbladders of 6 male clinically healthy pigs. A part of the serial histological sections was stained with hematoxylin and eosin to rule out the presence of pathological findings. Another part of the sections was processed immunohistochemically to determine the expression of choline acetyltransferase and tryptase. Two primary antibodies were used: a polyclonal rabbit choline acetyltransferase and a monoclonal mouse antihuman mast cell tryptase. The number and dimensions of cholinergic ganglia, perikaryons and nerves forming ganglionic nerve plexuses in the propria, muscular and subserosal layers were estimated. The immunoexpression of choline acetyltransferase in biliary glandular cells and mast cells was also detected and analyzed.

***Discussion*:** The current study provided original information on the number and dimensions of intramural choline acetyltransferase positive nerves, ganglia, and perikaryons of ganglion neurons, showing that ganglia in porcine gallbladder and extrahepatic bile ducts are cholinergic. It was revealed that the localization of intramural nerves and ganglia in the porcine gallbladder and extrahepatic bile ducts was similar to that in man. The expression of choline acetyltransferase found by us for the 1st time in the mast cells of porcine gallbladder and extrahepatic bile ducts suggests, on the other hand, the possibility of its synthesis and release from these cells, and on the other hand, its involvement in modulating the interaction between mast cell and intramural neurons in the studied organs. Interactions between mast cells and neurons may potentially contribute to the pathogenesis of many diseases in gastrointestinal tract.

Keywords: mast cells, ganglia, choline acetyltransferase, tryptase, bile ducts, pigs.

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INTRODUCTION

Acetylcholine (ACh) as neurotransmitter is known to be expressed in non-neuronal cells in animals and man, which are elements of a non-neuronal cholinergic system [20,23,48]. Evidence for the synthesis of acetylcholine is the establishment of positive choline acetyltransferase (ChAT) immunoreactivity in noncholinenrgic system of humans and laboratory animals but no such information in domestic swine [20,23,48,49].

In human, canine and laboratory animal gallbladder (GB), the neurons of ganglia are cholinergic and [44-46,26-28]. In contrast to the gallbladder, in the sphincter of Oddi (SO) of the guinea pig, excitatory and inhibitory neurons constitute 2 distinct subpopulations: with nitric oxide synthase- and ChAT positive [6,8,35,47].

The role of the non-neuronal cholinergic system in respiratory system [1], gut [16] and immune cells [5,19,31] is known. Several authors found that cholinergic nerves regulate bile secretion in the biliary tract as well [9, 21,32, 37,41].

Data on AChE immunoreactivity in human and rodent mast cells are scarce [15,18,24,34,40,43] but no data regarding porcine mast cells as well as about their involvement in various cholangiopathies [11,13,14,17,22,38,39]

The aim of this study was to quantitatively analyze biliary cholinergic ganglia and nerves as well as non-neuronal cholinergic cells in gallbladder and extrahepatic bile ducts (EHBD) in healthy pigs.

MATERIALS AND METHODS

Animals

In the present study, tissue samples from the extrahepatic bile ducts and gallbladders of 6 male clinically healthy pigs (Bulgarian White × Landrace cross) aged 6 months (92-100 kg) were used. All procedures were approved by the Bulgarian Food Safety Agency with license № 174.

All animals were given time to acclimate to the new environment before slaughtering in slaughterhouse. Tissue samples from the common hepatic duct (near the terminal part of ductus cysticus), from the ductus cysticus near collum vesicae biliaris, from beginning of the ductus choledochus (DCHO) near the junction with the ductus cysticus, from the intramural part of ductus choledochus (DCHI) and papilla duodeni major (PDM), as well as from the bottom (fundus vesicae biliaris), body (corpus vesicae biliaris) and neck (collum vesicae biliaris) of the gallbladder were collected immediately after slaughtering the animals, then put in 10% aqueous formalin. Terminology is consistent with Nomina Histologica Veterinaria [42].

Histological methods

Tissue paraffin sections with thickness of 5 μm were placed on glass slides, then deparaffinized and rehydrated. A portion of the serial histological sections were stained with hematoxylin and eosin[1] to rule out the presence of pathological findings. Another part of the sections was processed immunohistochemically to determine the expression of choline acetyltransferase and tryptase.

Immunohistochemical method for determination of choline acetyltransferase and tryptase reactive structures

Serial sections were put in 0.1 M PBS, then in 1.2% hydrogen peroxide in methanol for 30 min. Antigen retrieval was performed in buffer (pH 9.0) for 20 min. Between steps, sections were washed with EnVisionFlex Wash Buffer[2], then incubated in a humid chamber overnight at 4°C with primary antibodies: a polyclonal rabbit choline acetyltransferase (ChAT, PA 1-4738)[3] and a monoclonal mouse antihuman mast cell tryptase[4] which is ready to use. After washing with PBS, the sections were incubated with an EnVision detection system (DAKO)[2] for 24 h at 4°C. Antibody-antigen reaction was visualized with diaminobenzidine. Serial sections were sequentially stained with choline acetyltransferase and tryptase. PBS instead primary antibody was used as a negative control.

Statistical analysis

The number of cholinergic nerves was counted on a field of view x400, and the number of ganglia was determined on sections of the examined organs using a light microscope[4] equipped with a digital camera (LEICA DFC 290) and quantitative analysis software (LAS V4.10.0 2016). The number of perikaryons per a ganglion was also determined. Endocrine cell density was determined by counting the number of endocrine cells per microscopic field x100 as well as per gland cross-section. Metric data were processed by GraphPad Prism 6 for Windows[5] by one-way ANOVA followed by Tukey-Kramer post-hoc test. *P*-values < 0.05 were

considered statistically significant. Data are presented as mean ± SD (Standard deviation).

RESULTS

Choline acetyltransferase positive nerve structures

Cholinergic nerves and ganglia in the GB and EHBD were identified by immunohistochemical expression of the enzyme choline acetyltransferase (ChAT). Choline acetyltransferase positive (ChAT+) structures in GB and EHBD were represented by nerves, perikaryons and outgrowths of ganglion neurons, mast cells, as well as immunopositive glandular cells were found in EHBD (Figure 1).

ChAT+ perikaryons and outgrowths of ganglion neurons with moderate enzyme reactivity were found mainly in the muscle and subserosal layers, less in the propria of the GB and EHBD, but in PDM - 1- and 2-neuron ganglia were located in the subglandular connective tissue layer (SGCT) of the propria adjacent to the sphincter of Oddi (SO) [Figure 1; Table 1-4].

The immunohistochemical examination showed that ChAT immunoreactive intramural nerves and ganglia formed ganglionic nerve plexuses (Figure 1). The most ganglia of muscle layer were found in the gallbladder's neck and body - 67% each, less in the bottom - 63%. These ganglia contained up to 8 perikaryons in the bottom, up to 10 in the body, and up to 12 in the neck of the gallbladder. Single ganglion cells were found in lamina propria of the bladder. Their number was the most in the propria of the body - 29%, followed by that in the neck - 22%, and the smallest number (12%) - in the bottom. The submucosal plexus is formed mainly by ganglion cells in the propria and by the ganglion outgrowths projected from muscular layer. The nerve fibers making up the submucosal plexus reached the basement membrane of the epithelium, but did not penetrate between the epithelial cells. In the fundus wall of the porcine GB, it was found that 25% of the ganglia were located in the subserosal layer near the muscle layer, 63% in the muscle layer, and 12% in the propria near the muscle layer. In the bladder`s body, 4% of the ganglia were found in the subserosal layer near the muscle layer, 67% in the muscle layer, 29% in the propria near the muscle layer.

The largest ganglia were located in collum vesicae biliaris, followed by the corpus and fundus vesicae biliaris (Table 1). In the wall of the ductus cysticus, the size of the ganglia was similar to the size of those located at fundus vesicae biliaris. The size of ganlia decreased in direction from GB to DHC, followed by intramural part of DCH, DC, extramural part of DCHT and the smallest ganglia were detected in PDM (Table 1).

The number of ganglia per cross section decreased from GB (more ganglia in body compared to neck and bottom) to PDM (Table 1). GB was followed by DC, DHC, DCHO, DCHT, PDM and DCHI (Table 1).

The diameter of the perikaryons in the ganglia decreased in direction from GB to PDM (Table 1). The largest perikaryons were identified in the ganglia of GB (with the largest diameter in the body, followed by the neck and bottom), followed by those in DC, DHC, DCHO, DCHT, DCHI and PDM.

The number of perikaryons per a ganglion was greatest in the GB (most perikaryons in the neck, followed by the body and bottom), followed by DHC, DCH, DC and PDM (Table 1).

Immunohistochemical expression of ChAT allowed for the 1st time to establish the localization of ChAT+ nerves in the muscular and subserosal ganglion nerve plexus, in the propria between the glands and near the superficial biliary epithelium as well as in the muscular layer and subserosa of porcine GB and EHBD (Figure 1).

The number and diameter of ChAT+ nerves were highest in the serosa and adventitia of the GB and EHBD, followed by the muscle layer and lowest in the propria (Tables 2 and 3). In the subglandular layer of DCHI and PDM, ChAT+ nerves were fewer but larger in diameter compared to nerves in the subepithelial layer.

Choline acetyltransferase positive glandular cells (ChAT+Cs)

Choline acetyltransferase positive cells (ChAT+Cs) with moderate to strong reactivity were found in the EHBD glands, but were absent in the GB. Their density was greatest in the glands of DCHI, followed by PDM, DHC, DCHO and DC (Table 4) [Figure 1].

The highest density of ChAT+Cs in the superficial glandular alveoli was observed in DCHO, followed by that in DHC, PDM, DCHI and DC. More ChAT+Cs were found in superficial glandular alveoli compared to deep glandular alveoli in DCHI and PDM,

whereas deep glands in DHC, DCHO and DC were immunonegative (Table 4).

Immunohistochemical localization of ChAT+ mast cells in GB, EHBD and PDM

To establish the presence of choline acetyltransferase positive mast cells (MCsChAT+), immunohistochemical identification of tryptase positive mast cells was performed on serial sections from the wall of GB, DC, DHC, extra- and intramural part of DCH, as well as from the wall of PDM. Both types of mast cells: MCsChAT+ and MCstr+, showed similar morphology (immunopositive granules in the cytoplasm) and similar localization in the propria, muscle and subserosal layers of the examined organs (Figure1).

MCsChAT+ and MCstr+ were found adjacent to capillaries, arterioles, and venules in the propria and basement membrane of the biliary epithelium of the mucosa of the GB, EHBD and majorduodenal papilla (Figure 1).

In the muscle layer of these organs, MCsChAT+ and MCstr+ were observed near blood vessels, superficial and deep glands, and between bundles of smooth muscle cells, but some mast cells were found adjacent to smooth muscle cells.

In adventitia or serosa of the organs, MCsChAT+ and MCstr+ were localized most often in the adventitia of the muscular-type arteries and adjacent to the nerves of the GB, DC, DHC and DCH.

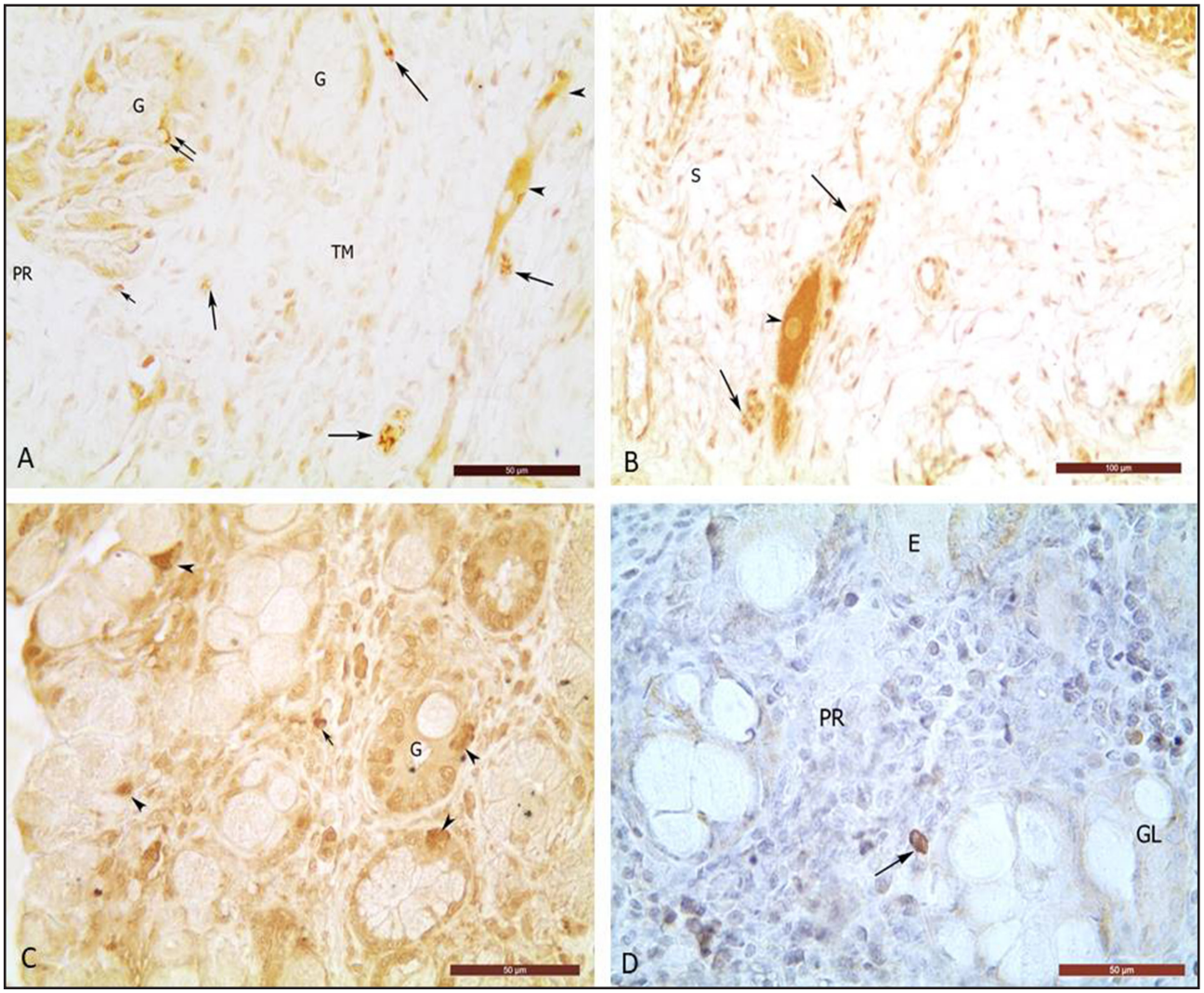

Figure 1. A- Moderate reactivity of ChAT+ perikaryons (arrowhead) of ganglia in the muscle layer, moderate to strong reactivity of ChAT+ nerves (large arrows) in the muscle layer, and moderate to strong reactivity of ChAT+ mast cells (small arrows) in the propria (PR) and muscular layer (TM) of ductus cysticus. G: glands with single ChAT+ cells [Bar = 50 µm]. B- Moderate reactivity of a ChAT+ neuron (arrowhead) and a ChAT+ nerve (arrows) located in the subserosal layer (S) of ductus cysticus [Bar = 50 µm]. C- Moderate to strong reactivity of ChAT+ glandular cells (arrowheads) as well as moderate to strong reactivity of ChAT+ mast cells (small arrows) in lamina propria of the intramural part of the ductus choledochus. D- moderate to strong reactivity of MCstr+ (arrow) in lamina propria of intramural part of ductus choledochus. GL: glands [Bar = 50 µm].

Table 1. Density (number of ganglia per cross section) and area (μm²) of ganglia in different parts of the gallbladder and in the wall of the ductus cysticus, ductus hepaticus communis, ductus choledochus and papilla duodeni major in 6-month-old pigs.

Localization	Number of ganglia	Ganglion area
Fundus of gallbladder	7.33 ± 0.74	3495.68 ±1946.16
Corpus of gallbladder	*** 8.16 ± 0.68	4021.58 ± 3188.62
Collum of gallbladder	*** 7.16 ± 0.69	*** 12956.70 ± 5823.72
Ductus cysticus	*** 4.66 ± 0.75	*** 3175.72 ± 2775.27
DHC	5.02 ± 0.75	12707.50 ± 14800.97
DCHO	5.11 ± 0.67 A1,B4,C4,D4,E2	3494 ± 1590 a4
DCHM	4.50 ± 0.51 F4,G4,H4,I0	1420 ± 196.6 b4
DCHT	3.50 ± 0.51 J0,K4,L0	3258 ± 163.3 c4
DCHIpr	3.56 ± 0.51 M4, N0	1067 ± 388.6 d4
DCHIm	2.44 ± 0.51 O4	11022 ± 8483 e4,f4,g4,h4,j4,k4
PDM	4.39 ± 0.50	2452 ± 1847

Data presented as mean ± SD (standard deviation). DHC: ductus hepaticus communis; DCHO: Ductus choledochus; DCHI: intramural part of ductus choledochus; PDM: papilla duodeni major. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ Statistically significant difference ("Ssd") compared to the upper row. DCHO: initial segment of DCH; DCHM: middle segment of DCH; DCHT: terminal segment of DCH; DCHIpr: propria in the intramural segment of DCH; DCHMm: tunica muscularis of the intramural segment of DCH. [$^1P < 0.05$; $^2P < 0.01$; $^3P < 0.001$ and $^4P < 0.0001$]. ^{A1}Ssd compared to DCHM; ^{B4}Ssd compared to DCHT; ^{C4}Ssd compared to DCHIpr; ^{D4}Ssd compared to DCHIm; ^{E2}Ssd compared to PDM; ^{F4}Ssd compared to DCHT; ^{G4}Ssd compared to DCHIpr; ^{H4}Ssd compared to DCHIm; ^{I0}Lack of Ssd compared to PDM; ^{J0}Lack of Ssd compared to DCHIpr; ^{K4}Ssd to DCHIm; ^{L0}No Ssd compared to PDM; ^{M4}Ssd compared to DCHIm; ^{N0}No Ssd compared to PDM; ^{O4}Ssd to PDM. ^{a4}Ssd compared to DCHIm; ^{b4}Ssd compared to DCHIm; ^{c4}Ssd compared to DCHIm; ^{d4}Ssd compared to DCHIm; ^{e4}Ssd compared to DCHIm; ^{f4}Ssd compared to DCHO; ^{g4}Ssd compared to DCHM; ^{h4}Ssd compared to DCHT; ^{j4}Ssd compared to DCHIpr.

Table 2. Diameter of Choline acetyltransferase positive (ChAT+) nerves in the layers of extrahepatic bile ducts and gallbladder of 6-month-old pigs.

Diameter of ChAT+ nerves in the tributaries of the EHBD and GB	DHC	DCHE	DCHI	PDM	DC	VB
1.LPM ChAT+ nerves	1.46 ± 0.42	1.26 ± 0.49	1.06 ± 0.23	1.01 ± 0.15	1.01 ± 0.12	0.83 ± 0.28
- SECT ChAT+ nerves			2.28 ± 0.33	1.79 ± 0.64		
-SGCT ChAT+ nerves						
2.TM ChAT+ nerves	3.23 ± 0.51	2.51 ± 0.32	2.08 ± 1.10		1.75 ± 0.52	1.72 ± 0.67
3.TS ChAT+ nerves	48.17 ±14.18	25.37 ± 0.48	36.03 ± 6.01		57.34 ± 8.62	67.62 ± 11.33
4. TA ChAT+ nerves	15.86 ± 0.44				53.71 ± 10.35	52.49 ± 7.21

Data presented as mean ± SD (standard deviation). EHBD: Extrahepatic bile ducts; GB: gallbladder; DHC: ductus hepsticus communis; DCHE: extramural part of ductus choledochus; DCHI: intramural part of ductus choledochus; PDM: papilla duodeni major; DC: ductus cisticus; VB: vesica biliaris; LPM: lamina propria mucosae; SECT: subepithelial connective tissue layer; SGCT: subglandular connective tissue layer; TM: tunica muscularis; TS: tunica serosa; TA: tunica adventitia. Choline acetyltransferase positive (ChAT+).

Table 3. Density (number of nerves/field of view x400 in LPM, SECT, CGCT, TM, but in TS and TA - number of nerves /1 cm) of ChAT+ nerves in EHBD and GB in in 6-month-old pigs.

Number of ChAT+, nerves in the layers of the EHBD and GB	DHC	DCHE	DCHI	PDM	DC	VB
1. LPM ChAT+ nerves						
- SECT ChAT+ nerves	7.40 ± 0.54	8.00 ± 1.00	11.00 ± 0.70 9.60 ± 0.54	10.80 ± 1.30 8.80 ± 1.64	10.50 ± 1.10	8.40 ± 0.54
- SGCT ChAT+ nerves						
2.TM ChAT+ nerves	10.40 ± 0.89	12.40 ± 0.89	14.20 ± 0.83		60.00 ± 2.73	27.00 ± 2.73
3.TS ChAT+ nerves	11.55 ± 1.10	13.00 ± 0.70	14.20 ± 0.83		12.30 ± 0.48	11.00 ± 1.00
4. TA ChAT+ nerves	10.45 ± 1.11				10.20 ± 0.44	10.10 ± 0.74

Data presented as mean ± SD (standard deviation). Choline acetyltransferase positive (ChAT+); EHBD: Extrahepatic bile ducts; GB gallbladder; DHC: ductus hepsticus communis; DCHE: extramural part of ductus choledochus; DCHI: intramural part of ductus choledochus; PDM: papilla duodeni major; DC: ductus cisticus; VB: vesica biliaris; LPM: lamina propria mucosae; SECT: subepithelial connective tissue layer; SGCT: subglandular connective tissue layer; TM: tunica muscularis; TS: tunica serosa; TA: tunica adventitia.

Table 4. Number of choline acetyltransferase positive cells (ChAT+ C) per microscopic field (ChAT+ C/F) or per gland cross-section (ChAT+ C/GL) in the gallbladder, ductus cysticus, ductus hepaticus communis, initial segment of the ductus choledochus , intraduodenal part of the ductus choledochus and ChAT+Cot in 6-month-old pigs.

Number of ChAT+ C	DHC	DCHO	DCHI	DC	PDM	VF collum
ChAT+ C /F	5.88 ± 1.23 A4,B4,C4	5.50 ± 1.20 D4,E4, F4	29.33 ± 1.28	1.94 ± 0.72	20.78 ± 2.15	0.00
ChAT+ C/GL	a4, A4,B4,C4	a4, D4,E4,F4	a1	a4	a4	
-SGls	4.72 ± 1.07	4.83 ± 0.98	2.0± 0.76	1.55± 0.51	2.50 ± 1.61	
-DGls	0.00	0.00	1.38 0.50	0.00	1.66 ± 0.48	

Data presented as mean ± SD (standard deviation). Choline acetyltransferase (ChAT); gallbladder (VF); ductus cysticus (DC); ductus hepaticus communis (DHC); initial segment of the ductus choledochus (DCHO); intraduodenal part of the ductus choledochus (DCHI); open type of endocrine cells (ChAT+Cot). [A]Statistically significant difference between DHC and DCHI; [B]Statistically significant difference between DHC and DC; [C]Statistically significant difference between DHC and PDM; [D]Statistically significant difference between DCHO and DCHI; [E]Statistically significant difference between DCHO and DC; [F]Statistically significant difference between DCHO and PDM; [a]Statistically significant difference between superficial glands (SGls) and deep glands (DGls); [1]$P < 0.05$; [2]$P < 0.01$; [3]$P < 0.001$; [4]$P < 0.0001$, respectively.

DISCUSSION

It is known that in gallbladder both internal and external innervation is present, which primarily participates in regulation of contractility of the smooth muscles and secretory activity of the organ. The internal innervation of the gallbladder is similar to the enteric nervous system (ENS) in the digestive tract and consists of neurons located in lamina propria, muscle layer and subserosa that form ganglion plexuses [8]. The number and precise localization of ganglion plexuses in the gallbladder wall depends on the animal species [26,30]. The findings of this study showed

that porcine gallbladder ganglion plexus localization resembles those of large animals [30] and that this type of plexus differs from those in rodents [25]. The current study provided original information on the number and dimensions of intramural ChAT+ nerves, ganglia, and perikaryons of ganglion neurons, showing that ganglia in porcine GB, EHBD and PDM are cholinergic. In addition, a similar localization of intramural nerves and ganglia was found in the porcine GB and EHBD to that in man [2]. The intramural nerves of the gallbladder, common hepatic and common bile ducts formed nerve plexuses in subserosa, muscular and submucosal layer, containing different numbers and dimensions of ganglia. In the human GB, subserosal ganglia are more numerous in the neck than in the body or fundus of the organ. However, in the pig the ChAT+ serosal ganglionated plexuses were most in the bottom - in all 3 layers of the bladder wall, followed by the neck and least in the body. The muscular nerve plexus of the human GB contains numerous small ganglia, consisting of 1 to 5 or 6 cells, throughout the corpus and fundus of the gallbladder. In the pig, the most muscular layer ganglia were in the neck and body, less in the bottom. Single ganglion cells were found in the lamina propria of the bladder in both man and pig. Similar to the human, the submucosal plexus was found to arise mainly from these cells and from the outgrowths of the cells in the muscular layer of GB. The fibers making up the submucosal plexus reached the epithelium but did not penetrate between the epithelial cells.

The present study identified ChAT immunoreactive ganglion perikaryons. The largest ganglia were located in the neck of the porcine GB, followed by its body and bottom. In the wall of the ductus cysticus, the size of the ganglia was similar to the size of those located at the bottom of the bladder. Immunohistochemical expression of ChAT allowed for the 1st time to establish the localization of ChAT+ nerves in the muscular and subserosal ganglion nerve plexus, in the interglandular propria and near the superficial biliary epithelium, in the muscular layer and subserosa of porcine GB and EHBD, which are most likely efferent nerve fibers because ChAT is a well-known marker of motor efferent nerve fibers [36]. Therefore, ChAT negative nerve fibers are afferent with a sensory role similar to those in the esophagus and intestine. A number of authors have found that primary afferent fibers serve as receptors for muscle stretch, mechanical and chemical mucosal stimuli, as well as mucosal distension [36]. Some authors revealed the absence of intramuscular cholinergic and adrenergic nerve fibers in the GB of guinea pig and cat [3,7]. In contrast to the mentioned authors, the present study found a well-defined network of intramuscular cholinergic nerves in the wall of the GB and EHBD in the domestic pig. According to another study [25], the ganglionic plexus of the gallbladder contains preganglionic vagus fibers that terminate in gallbladder neurons as well as sympathetic postganglionic fibers. Cholinergic nerves in lamina propria and muscular layer of the GB and EHBD in pigs were observed near the epithelial cells [12], which indicates the role of nerves in the regulation of secretory activity and muscle contractility of the gallbladder.

As described in alimentary canal, the mechanisms of neural regulation of the biliary epithelium in the intrahepatic bile ducts include direct nerve-cholangiocytic interaction, deposition of neurotransmitter in the peribular, extracellular environment [50]. The present micromorphometric study provided original data on the number and dimensions of intramural nerves, ganglia and ganglion perikaryons in GB and EHBD that could be used as reference values in the diagnosis of neuropathological processes [41] in these organs, where the amount of neural structures is known to be affected.

The immunohistochemical reactions carried out in this study to establish the expression of ChAT allowed for the 1st time the determination of non-neuronal cholinergic system elements in the GB and EHBD of the domestic pig, represented by neuroendocrine cells and ChAT+ mast cells.

Given ChAT+ endocrine cell distribution in the intramural biliary glands of the EHBD, we can hypothesize that, similar to the intestine, cholinergic endocrine cells may initiate both secretory and muscle activity in organs examined [4,29].

The current study for the 1st time provided data on the number and size of choline acetyltransferase immunoreactive mast cells (ChAT+M) in porcine GB and EHBD. Acetylcholine appears to mediate interaction between mast cells and acetylcholine neurons, which are localized in the intestine of mice and humans [34] similarly to porcine GB and EHBD as demonstrated in the present study. Neural regulation of mast cell degranulation is species specific. In humans, acetyl-

choline - inhibits [40], whereas in rats and rabbits, acetylcholine stimulates mast cell degranulation [24].

One of the 1st studies regarding MCs and primary biliary cirrhosis was done by Nakamura *et al*. [33], revealed the icreased number of mast cells around the portal tract in patients. The authors concluded that increased nerve stimulation induces mast cell migration and activation leading to fibrosis.

The expression of choline acetyltransferase defined for the 1st time in the mast cells of porcine GB, and EHBD and PDM suggests on the one hand, the possibility of its synthesis and release from these cells, and on the other hand, its involvement in modulating the interaction between mast cell and intramural neurons in the studied organs. This type of interactions in the GB, EHBD and PDM like in the gut, could also contribute to the pathogenesis of many diseases. Data of the present study are a solid basis for future studies aimed at elucidating the role of acetylcholine produced by mast cells in the physiological and pathological processes occurring in the gallbladder, extrahepatic bile ducts, and the large major duodenal papilla.

CONCLUSIONS

This immunohistochemical study allowed for qualitative and quantitative analysis of ChAT+ nerves, ganglionic and non-ganglionic nerve plexuses, contributing to the morphological knowledge of the cholinergic innervation of the GB, EHBD and PDM. At the same time, data were provided on the non-neuronal sources of acetylcholine: glandular cholinergic cells and cholinergic-type mast cells. The original data on the number and dimensions of intramural nerves, ganglia and ganglion perikaryons could be used as reference values in the diagnosis of neuropathological processes in GB and EHBD, which are known to affect the amount of neural structures. Clarifying the precise mechanisms of gallbladder smooth muscle contractility will contribute to the improvement of therapeutic strategies to prevent biliary stasis and biliary disorders.

MANUFACTURERS

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***Ethical approval*.** All procedures were in accordance with Ordinance 20 of 01.11.2012 on the minimum requirements for the protection and humane treatment of experimental animals and the requirements for facilities for use, breeding and/or supply, Directive 2010/63/EU of the European Union Parliament and the Council - September 22, 2010 for the protection of animals used for scientific purposes and approved by the Ethics Committee of the Bulgarian Food Safety Agency with license № 174 referring to the Scientific Project No. 13/2017 of Medical Faculty, Trakia University, Bulgaria.

***Declaration of interest*.** No conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results. The authors alone are responsible for the content and writing of the paper.

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