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Original article

DISTRIBUTION OF NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE DIAPHORASE POSITIVE MAST CELLS IN THE NORMAL PORCINE GALL BLADDER

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Summary

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The aim of the current study was to determine the localisation and number of NADPH-d mast cells in the normal domestic pig gall bladder. The gall bladders of 6 male and 6 female pigs were examined by using light microscopic enzymohistochemical and immunohistochemical techniques. Enzymohistochemistry was performed to display NADPH diaphorase activity. Then, the avidin-biotin-peroxidase complex technique for detection of mast cell tryptase expression was performed in order to confirm that NADPH-d positive cells, observed in the studied organ, are actually mast cells. Light microscopy showed strong to medium NADPH-d reactivity in the mast cells granules. Mast cells number was the highest in the propria of gall bladder fundus, body and neck as well as in the same layer of the cystic duct *Ductus cysticus*, followed by fibromuscular layer. The smallest density of mast cells was observed in the subserosal layer. However, statistically significant difference between the number of these cells in the muscular layer and the tela subserosa of gall bladder fundus was not detected. The density and localisation of observed tryptase positive mast cells were the same. In conclusion, the results of this study gave us a reason to presume that NADPH-d positive cells observed in the wall of porcine gall bladder and its excretory duct possess metabolic pathway for nitric oxide synthesis. Based on obtained data it is suggested that NADPH-d positive mast cells are most probably involved in the regulation of the function of epithelium, smooth muscle layer and blood vessels of the organ.

Key words: gall bladder, mast cells, NADPH-diaphorase, pig, tryptase

INTRODUCTION

Nitric oxide (NO) is produced from the oxidation of the amino acid L-arginine by the enzyme nitric oxide synthase (NOS). This family of enzymes is generally divided into constitutive, calcium dependent (neuronal NOS, nNOS, NOS1 and endothelial NOS, eNOS, NOS3) or inducible, calcium independent (inducible NOS,

iNOS, NOS2) (Alderton *et al.*, 2001). NO is a potent mediator with diverse roles in regulating cellular functions, including nitrosylation of proteins, involved in signalling pathways (Alderton *et al.*, 2001; Stamler *et al.*, 2001). The role of NO in smooth muscle relaxation is also acknowledged (Cals-Grierson & Ormerod, 2004).

The ability of mast cells (MC) to produce NO is well known. MC arise from bone marrow haematopoietic precursor cells and mature and reside in tissues (Kulka & Befus, 2003). Gilchrist *et al.* (2004) demonstrated the expression of NOS isoforms and production of NO by various MC populations including rat peritoneal MC and human skin MC. The presence of enzymes such as NADPH-d and NOS involved in NO metabolism within the mast cells in the pigs kidney and canine paranal sinus wall suggests a possible metabolic pathway for NO synthesis in porcine and canine mast cells (Vodenicharov & Bozhilova-Pastirova, 2010; Stefanov & Vodenicharov, 2012; Stefanov *et al.*, 2012). Studies of human mast cells have been focused on the localisation of these cells that accumulate near and around intrahepatic large bile ducts and intrahepatic peribiliary glands (Koda *et al.*, 2000) and in the wall of common bile duct (Gulubova & Vlaykova, 2004). There are also many studies on NADPH-d expression in neuronal structures of gall bladder in different species (Sutherland, 1967) but no data concerning the NADPH-d positive mast cells in that organ in swine are available. At the same time, the physiological similarities between porcine and human extrahepatic bile ducts support the use of pigs in experimental models (Sand *et al.*, 1997).

These facts motivated us to undertake the current study to determine the distribution of NADPH-d mast cells in domestic pig's gall bladder. The presence of NADPH-d positive mast cells in the organ was demonstrated to prove that these cells possessed a metabolic pathway for NO synthesis. The obtained data could be used as background for further investigations to elucidate the role of NADPH-d positive mast cells in the regulation of the epithelium function and smooth muscle relaxation in the organ.

MATERIALS AND METHODS

Animals

The material was obtained from the wall of gall bladder fundus, body and neck and as well as from the wall of middle part of the cystic duct of 6 male and 6 female pigs (Landrace×Bulgarian White), 6 months of age, weighing 92–98 kg, slaughtered for meat consumption in a slaughterhouse in accordance with Bulgarian law.

Enzymohistochemical reaction for detection of NADPH diaphorase

Part of the samples was immediately immersed in 4% paraformaldehyde (Sigma Aldrich Chemie, Switzerland) in phosphate-buffered saline (PBS), pH 7.2, for 8 h at 4 °C. Then the samples were removed and soaked in solution of 30% sucrose in PBS overnight. Sections of 15 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.* (1983) 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 (+++).

Light microscopic immunohistochemistry

Another part of the same samples was used for immunohistochemical staining to detect mast cell tryptase. The avidin-biotin-peroxidase complex technique was performed on formalin-fixed and paraffin-embedded tissues. Then the tissues were cut to 5–6 μm thick serial sections, which were mounted on glass slides. The sections were deparaffinised twice in xylene, followed by absolute ethanol: 96 °C, 70 °C and 50 °C series, each for 10 min. The further immunohistochemical procedure was described earlier by Gulubova & Vlaykova (2006). In brief, the sections were rinsed in phosphate buffer saline (PBS), pH 7.4 and internal peroxidase was blocked by incubation in 1.2% hydrogen peroxide (Peroxidase block K0673, DAKO A/S, Glostrup, Denmark) in methanol for 30 min and rinsed in PBS with pH 7.4 for 15 min. Immunohistochemical reaction was carried out using primary monoclonal mouse anti-human mast cell tryptase antibody (N M7052, DAKO clone AA_1) with dilution 1:100. The sections were washed in PBS, pH 7.4, incubated with Dako REAL™ EnVision™ Detection System, Peroxidase/ DAB+, Rabbit/Mouse, K5007 for 60 min, then visualised with 3,3′ diaminobenzidine and counterstained with Mayer's haematoxylin. Sections incubated with non-immune sera instead of the primary antibodies were used as negative controls.

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

Statistical analysis

Cross sections of the wall of gall bladder fundus, body and neck and of its excretory duct of each animal were used. On serial sections from each part of the gall bladder, ten 1 mm^2 fields were investigated. Data for mast cells density (number/mm^2) are given as mean ± SD. Light microscope (ZEISS Primo Star, Germany), camera (Progres, Capture 2.6 – JENOPTIK) and software analysis programme (Soft Imaging System GmbH) were used. Statistical data processing was done by using Student's *t*-test. The difference was considered as significant at $P<0.05$.

RESULTS

The distribution of NADPH-d positive mast cells in the three layers – the lamina propria of the mucosa, the muscular layer and tela subserosa of the porcine gall bladder wall and the cystic duct was investigated. Light microscopic observation showed strong to medium enzyme reactivity in cytoplasmic positive granules of NADPH-d cells (Fig. 1). Nuclei of positive cells exhibited a negative reaction. In the propria of this organ, most NADPH-d mast cells were localised close to vessels of microcirculatory bed and near the basal membrane of biliary lining epithelium (Fig. 1). In the gall bladder neck mast cells were also found around the glands. Some of them were detected around the nerves in all layers of the organ (Fig. 2).

In the fibromuscular and subserosal layers the same cells were observed mainly in the vicinity of blood vessels (Fig. 3). NADPH-d positive mast cells were also located between smooth muscle bundles of the fibromuscular layer. In the wall of arteries and veins NADPH-d mast cells were localized mainly in the adventitial layer, followed by the tunica media.

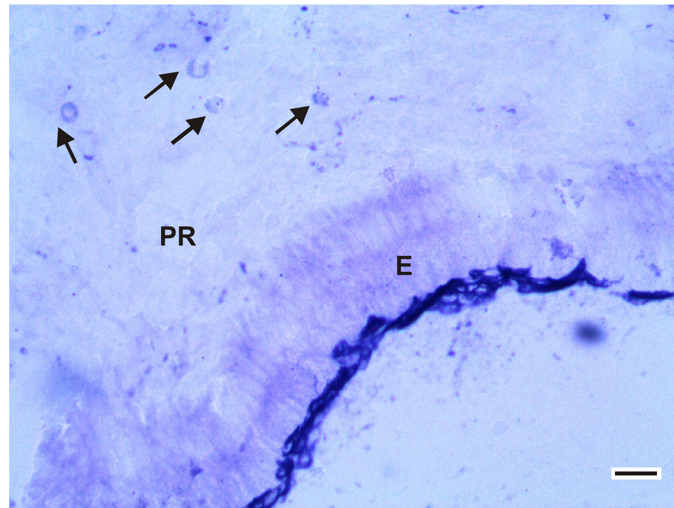

Fig. 1. Mast cells (arrows) containing NADPH-d positive granules and unstained nucleus, localised in the propria (PR) of gall bladder body. E – *lamina epithelialis mucosae*. Bar = 20 µm.

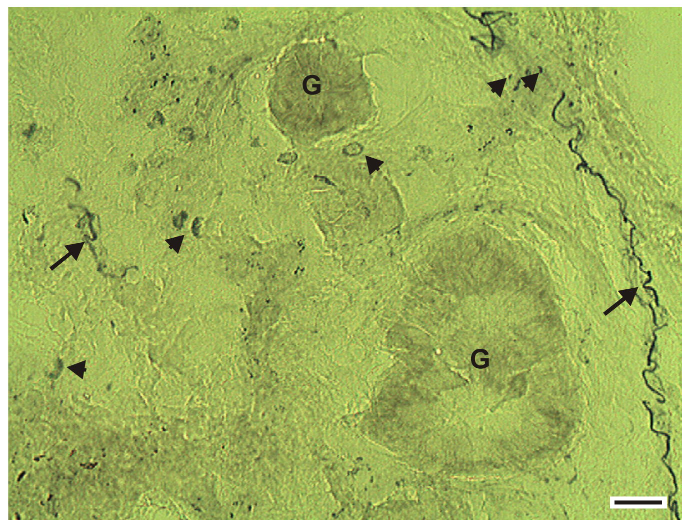

Fig. 2. NADPH-d positive mast cells (arrowheads) near the glands (G) and around the autonomic nitrergic nerve fibres (arrows) in the wall of gall bladder neck. Bar = 20 µm.

It is important to note that NADPH-d positive mast cells showed the same localisation as tryptase positive MC (Fig. 4).

The number of NADPH-d positive mast cells in the lamina propria of the mucosa of gall bladder fundus, body and neck and as well as in the same layer of the cystic duct was significantly higher than in the muscular layer and tela subserosa in both genders (Table 1).

In the different gall bladder parts and in the cystic duct, the number of mast cells

in the muscle layer was higher than in the subserosal layer. However, no statistically significant difference was observed between MC number in the muscular layer and tela subserosa of gall bladder fundus.

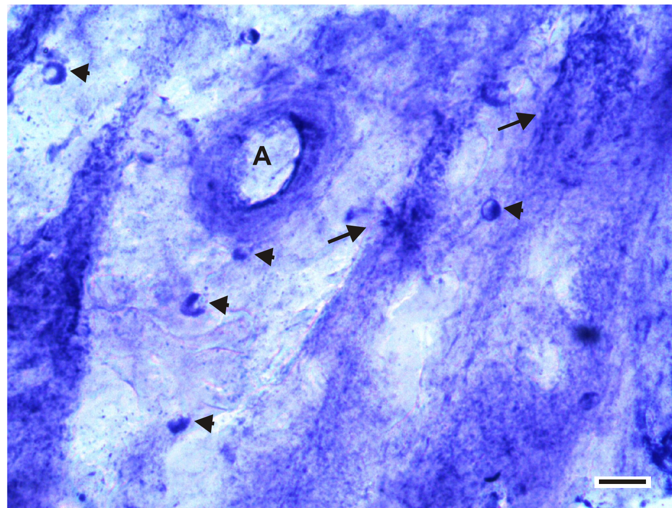

Fig. 3. Localisation of NADPH-d mast cells (arrowheads) near the media of small arteriae (A) and around smooth muscle bundles (arrows) in the fibromuscular layer of gall bladder neck. Bar = 20 µm.

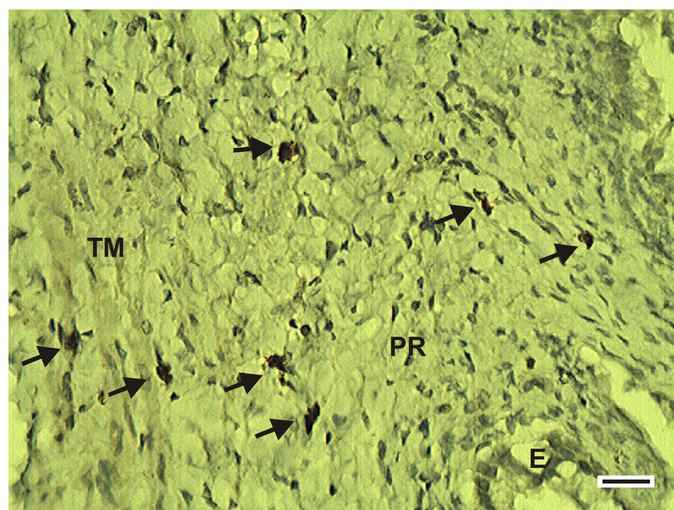

Fig. 4. Tryptase positive mast cells (arrows) localisation in the propria of the mucosa (PR) and near the smooth muscle cells (arrowheads) of the muscular layer (*tunica muscularis,* T) of gall bladder body. These cells showed the same localisation as NADPH-d positive mast cells. E – *lamina epithelialis mucosae.* Counterstaining with haematoxylin. Bar = 20 µm.

Table 1. Density (number/mm^2) of NADPH-d positive (NADPH-d MC) and tryptase positive mast cells (MCt) in different layers of the gall bladder fundus, body, neck and its excretory duct (*ductus cysticus*) in 6-month-old pigs. Data are presented as mean ± SD (n=6)

Localisation	NADPH-d MC, male pigs	NADPH-d MC, female pigs	MCt, male pigs	MCt, female pigs
Gall bladder fundus				
- propria	41.00 ±5.09	41.60± 5.29	40.95± 5.54	42.55 ±4.87
- muscular layer	21.30± 2.36***	22.08± 2.29***	21.80± 2.44***	22.35± 2.04***
- tela subserosa	21.65± 2.78	21.40± 2.28	22.25± 2.93	21.85± 2.43
Gall bladder body				
- propria	41.92± 4.39	41.95 ±4.27	42.00± 3.67	41.75± 4.47
- muscular layer	34.01± 9.29***	35.57± 5.62***	34.00± 8.93***	35.05± 5.69***
- tela subserosa	22.05± 2.37^	22.05± 2.24^	22.31± 2.60^	22.63± 2.37^
Gall bladder neck				
- propria	46.65 ±2.39	46.88 ±1.73	46.36± 2.13	47.10± 2.01
- muscular layer	32.40±10.12***	33.28± 9.16***	33.80± 7.85***	34.63± 8.38***
- tela subserosa	21.78±2.44^	21.70 ±1.95^	22.16± 2.33^	21.95± 2.43^
Ductus cysticus				
- propria	39.41±5.90	39.25± 7.87	40.26± 5.52	39.56 ±5.73
- muscular layer	30.16±5.63***	29.11 ±5.84***	29.95± 4.91***	29.10± 4.65***
- tela subserosa	20.96±4.36^	21.68 ±4.07^	21.61± 4.47^	21.65± 4.43^

***P<0.001 – statistically significant difference versus the number of mast cells in the propria; ^P<0.001 – statistically significant difference versus the number of mast cells in the muscular layer.

The density and localisation of NADPH-d positive mast cells were identical to those of mast cell tryptase positive cells in males and females, but a statistical significant difference was not established (Table 1).

DISCUSSION

NADPH-d positive mast cells localization and density in the different parts and layers of porcine gall bladder as well as its excretory duct were established for the first time. Our results showed that in the wall of porcine gall bladder NADPH-d positive cells had the same morphology and localisation like tryptase positive mast cells. According to Shaoheng *et al.* (1998) tryptase has been used as a marker for human mast cells. Based on this fact we proved that the NADPH-d positive cells in the studied organ are most probably mast cells. Other authors such as Jennings *et al.* (1995) established the distribution of histamine-containing mast cells in the mucosa and muscular/serosa layers of guinea pig gall bladder but there was no data about the presence of NADPH-d positive mast cells. We assumed that the localisation of mast cells near the basal membrane of the mucous epithelium and around the glands of gall bladder neck was connected with their role in regulation of epithelial function. Our findings correlate with the results of Castagliuolo *et al.* (1998) who directly demonstrated the participation of mast cells in colonic goblet cell discharge and prostaglandin E_2 secretion caused by restraint of mice. Their findings provide

direct evidence for a link between mast cells and the intestinal epithelium in the pathogenesis of stress-related responses. Similar investigation has been performed by Spanos *et al.* (1997) in rat urinary bladder who proved that psychological stress activated bladder mast cells, apparently via the action of at least some sensory neuropeptides.

The presence of enzymes such as NADPH-diaphorase involved in NO metabolism within the observed mast cells suggest a possible metabolic pathway for NO synthesis in porcine mast cells. Our results regarding the possibility of mast cells to produce NO support the investigation of Sekar *et al.* (2005) who established that mast cells express the three NOS isoforms (NOS1; NOS2 and NOS3) which is undisputed evidence that these cells produce NO. In previous studies, it was also demonstrated that canine mast cells express both NADPH-diaphorase and NOS (Stefanov & Vodenicharov, 2012; Stefanov *et al.*, 2012).

The relatively large amount of NADPH-d positive mast cells that are present around the blood vessels of this organ could reflect the well known role of nitric oxide as an active vasodilator (May *et al.*, 2002). Moreover, the mediators released by mast cells may play an important role in inflammation by causing hyperaemia, increasing microvascular permeability of plasma proteins and fluids, and enhanced flow of leukocytes at the injury site (Crowe & Perdue, 1992). The localisation of mast cells into the smooth muscle bundles of fibromuscular layer of porcine gallbladder can be explained with the findings of Cals-Grierson & Ormerod (2004) who established that endogenously synthesised NO was essential for smooth muscle relaxation. The localisation of NADPH-d positive mast cells around nitrergic nerves observed in current study is in agreement to the findings of Bienenstock (2002) who established the presence of bidirectional communication between the nervous and immune systems. In this relation, it deserves mentioning that while Stead *et al.* (1987) showed that mast cells and nerves were invariably approximated in rat intestinal villi, others have reported such associations in a variety of tissues in the humans (Stead*et al.*, 1987) as well as in many other species, including the frog (Chieffi *et al.*, 1998). Together with the obtained results, this is convincing evidence for the ability of gall bladder mast cells to produce NO, which is most probably involved in the communication between mast cells and nerves in the gall bladder wall.

CONCLUSION

In the current study the presence of NADPH-d positive mast cells in the wall of gall bladder indicated that these cells posses a metabolic pathway for NO synthesis. Based of the obtained data we can suggest that NADPH-d positive mast cells most probably participate in the regulation of the epithelium function and smooth muscle relaxation of gall bladder.

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