Bulgarian Journal of Veterinary Medicine (2013), **16**, No 1, 7–12

NADPH-DIAPHORASE POSITIVE MAST CELLS IN THE WALL OF PORCINE COMMON HEPATIC DUCT

I. S. STEFANOV

Department of Veterinary Anatomy, Histology and Embryology,
Faculty of Veterinary Medicine, Trakia University,
6000 Stara Zagora, Bulgaria

Summary

Stefanov, I. S., 2013. NADPH-diaphorase positive mast cells in the wall of porcine common hepatic duct. *Bulg. J. Vet. Med.*, **16**, No 1, 7–12.

There are no data about the presence of NADPH-d positive mast cells in the porcine common hepatic duct. That is why we aimed to establish the expression of NADPH-d activity in mast cells and their number in its mucosal, fibromuscular and subserosal layers. The material was obtained from the common hepatic duct of 6 male and 6 female crossbred pigs. Tissue pieces were taken immediately after the slaughter of the animals and fixed in 4% paraformaldehyde. Sections of 15–20 μm thickness were prepared by means of a freezing microtome. The NADPH-d histochemical expression was investigated according to the method of Sherer-Singler. NADPH-d positive cells were found in all layers of the organ. They were located mainly in the vicinity of blood vessels, around the peribiliary glands and near the basal lamina of the surface epithelium. The obtained data showed that NADPH-d positive cells observed in the wall of common hepatic duct are mast cells which obviously produce nitric oxide. The results gave us a reason to suggest that they are most probably involved in the regulation of the function of epithelium and blood vessels.

Key words: common hepatic duct, mast cells, NADPH-d, pig

INTRODUCTION

Most studies on human mast cells have been focused on cells that accumulate near and around intrahepatic large bile ducts and intrahepatic peribiliary glands, termed peribiliary mast cells (Koda *et al.*, 2000). Mast cells were found in normal livers to congregate under the biliary lining and throughout the ductal walls and periductal tissue (Koda *et al.*, 2000). It is acknowledged that nitric oxide (NO) is a messenger molecule (Moncada *et al.*, 1991). The investigations of Gilchrist *et al.* (2004) have demonstrated the ability of mast cells to synthesise NO. It regulates the mast cell phenotype but is also produced by mast cells. Studies of many authors are focused on relationships between mast cells and autonomic nerves in human and animal gastrointestinal tract (Gottwald *et al.*, 1995, 1997; De Jonge *et al.*, 2003; Schemann *et al.*, 2005), in porcine kidney (Vodenicharov & Bozhilova-Pastirova, 2010), and canine paranal sinus (Stefanov, 2012).

Since the role of NO in the domestic pig's common hepatic duct has not yet been examined we aimed to determine the localisation of NADPH-d positive mast cells in that organ.

MATERIALS AND METHODS

Animals

The material was obtained from the wall of common hepatic duct of 6 male and 6 female pigs (Landrace×Bulgarian White), aged 6 months, slaughtered for meat consumption in a slaughterhouse in accordance with Bulgarian laws.

Enzyme histochemical determination of NADPH diaphorase positive mast cells

Part of the samples was 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 solution of 10% sucrose in PBS overnight. Sections of 15–20 μm 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 (+++).

Histochemical detection of metachromasia in mast cells

The samples were fixed in Carnoy's liquid at room temperature for 4 h and further they were processed for serial paraffin sections (5–6 μm) that were stained with 0.1% solution of toluidine blue in McIlvane's buffer, pH 3 for assessment of mast cells' metachromasia (Pearce, 1960).

Statistical analysis

Data for density (number/mm^2) are given as mean ± SD. For that purpose a light microscope (ZEISS Primo Star, Germany), camera (Progres, Capture 2.6 – JENOPTIK) and software for analysis (Soft Imaging System GmbH) were used. Statistical data processing was done using the Data Analysis tool and t-test (StatMost for Windows software) and the difference was considered significant when P values were less than 0.05.

RESULTS

Light microscopic observation showed that cytoplasmic positive granules of NADPH-d cells showed predominantly medium and strong enzyme reactivity. Nuclei of positive cells always exhibited a negative reaction. NADPH-d positive cells were present in all layers of the hepatic duct wall. These cells were predominantly localised in the propria under the biliary lining epithelium and close to small vessels, including capillaries of the inner layer of the peribiliary vascular plexus (Fig. 1). NADPH-d positive cells were also detected around the peribiliary glands in the propria. In the fibromuscular and subserosal layers, the same cells were observed mainly in the vicinity of blood vessels and autonomic nerves.

On toluidine blue-stained paraffin sections from corresponding areas, mast cells with the same localisation and of similar dimensions exhibited a marked metachromasia (Fig. 2).

In both genders, the number of NADPH-d positive mast cells in *Lamina propria mucosae* was significantly higher than respective numbers in *Tunica muscularis* and *Tela subserosa* (Table 1). There was not a statistically significant

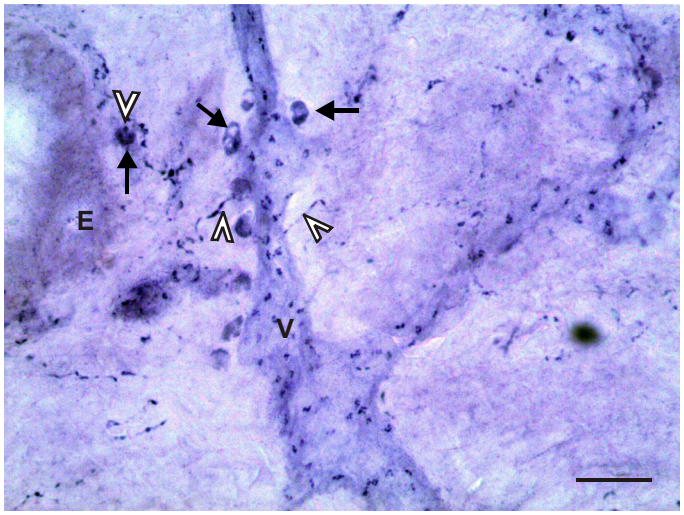

Fig. 1. NADPH-d positive mast cells (arrows) in the propria next to the lining epithelium (E), blood vessels (V) and nitrergic nerves (arrowheads). Bar = 30 µm.

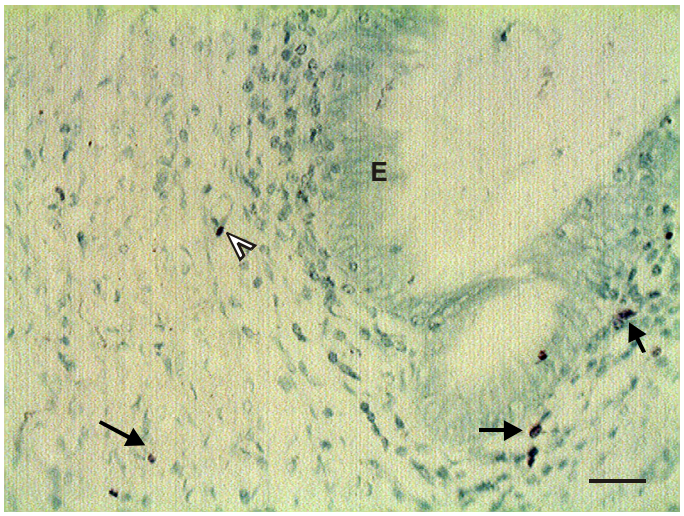

Fig. 2. Mast cells with metachromasia (arrows and arrowhead) near the epithelium and capillary showed similar localisation like NADPH-d positive cells. Bar= 20 µm.

difference between densities in *Tunica muscularis* and *Tela subserosa.*

Sexual dimorphism has not been established.

DISCUSSION

The presence of enzymes involved in NO metabolism within the mast cells in the porcine common hepatic duct suggests a possible metabolic pathway for NO

Table 1. Density (number/mm^2) of NADPH-d-positive mast cells (NADPH-d MC) in the different layers of common hepatic duct in 6-month-old pigs. Data are presented as mean ± SD (n=6).

Animals	NADPH-d MC in *Lamina propria mucosae*	NADPH-d MC in *Tunica muscularis*	NADPH-d MC in *Tela subserosa*
Male pigs	39.40 ± 7.61***	34.83 ± 7.04	33.95 ± 4.86
Female pigs	40.13 ± 6.78**	36.75 ± 5.73	35.30 ± 5.11

**p< 0.01, ***p< 0.001, – statistically significant difference vs *Tunica muscularis* and *Tela subserosa* (Student's *t* test).

synthesis in them. The mast cells identified on the toluidine blue-stained preparations of the porcine common hepatic duct showed a similarity in shape and position with the NADPH-d positive cells in this organ. This finding suggests both metachromasia and NADPH-positivity of these mast cells. This colocalisation corresponds with similar findings in porcine kidneys (Vodenicharov & Bozhilova-Pastirova, 2010). The present study is the first to establish the mast cells' localisation in the propria, fibromuscular and subserosal layers of porcine common hepatic duct. A similar investigation was performed by Jennings *et al.* (1995) and reported the distribution of histamine-containing mast cells in the mucosal, muscular and serosal layers of guinea pig gall bladder. Gulubova & Vodenicharov (2001) detected the localisation of single tryptase poitive mast cells under the surface epithelium of human normal common bile duct. There are however not data about the mast cells distribution in the wall of common hepatic duct.

One of the most important findings in this study is the localisation of mast cells next to neuron of the ganglia which allows suggesting an interconnection between mast cells and autonomic nerves. The first unambiguous morphological evidence for the close apposition between mast cells and enteric nerves has been provided in a German research (Stach, 1973), and subsequently, in many morphological (Stead *et al.*, 1987; 1989; Cooke, 1994; Bauer & Razin, 2000; De Jonge *et al.*, 2003; Barbara *et al.*, 2004; Stead *et al.*, 2006) and functional (Newson, 1983; Gottwald *et al.*, 1995; 1997; Mori *et al.*, 2002; Schemann *et al.*, 2005; Stead *et al.*, 2006) studies providing conclusive evidence for a bidirectional crosstalk between mucosal mast cells (MMC) and nerves in the gastrointestinal tract wall. Cited studies showed that mast cells not only released mediators influencing the activities of extrinsic and intrinsic neurons, but in turn, transmitter release from these extrinsic and intrinsic neurons may affect mucosal mast cells as well. In addition, stress-related research has clearly revealed that the central nervous system influences both mediator release from mast cells as well as transmitter release in the gastrointestinal tract. It is known that postganglionic sympathetic nerves are suspected to provide tonic inhibition of intestinal MMC mediator release (Stead *et al.*, 2006). Mouse and human mast cell populations express adrenoceptors, preferentially the β2-adrenoceptor subtype (Williams *et al.*, 1995). Vagal stimulation causes an increase in the number of MMCs expressing histamine and serotonin in the rat jejunum (Gottwald *et al.*, 1995; Stead *et al.*, 2006).

Our findings about the localisation of mast cells near the blood vessels confirm the data reported by May *et al.* (2000) and give us a reason to suggest that observed mast cells take part in the regulation of vascular tone in the studied organ. The relatively large amount of NADPH-d positive mast cells that are present around the blood vessels could reflect the well known role of nitric oxide as an active vasodilator (May *et al.*, 2000). 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. After activation, mast cells are able to produce all important signs of acute inflammation (Crowe & Perdue, 1992) and therefore the prevention of mast cell reactivity could offer a potential therapeutic approach in inflammation control.

In conclusion, based on the results, it can be assumed that NADPH-d mast cells in the wall of common hepatic duct produce nitric oxide and are probably involved in the regulation of the function of epithelium, fibromuscular layer and blood vessels.

REFERENCES

Barbara, G., V. Stanghellini, R. De Giorgio, C. Cremon, G. S. Cottrell, D. Santini, G. Pasquinelli, A. Morselli-Labate, E. Grady, N. Bunnett, S. Collins & R. Corinaldesi, 2004. Activated mast cells in proximity to colonic nerves correlate with abdominal pain in irritable bowel syndrome. *Gastroenterology*, **126**, 693–702.

Bauer, O. & E. Razin, 2000. Mast cell–nerve interactions. *News in Physiological Sciences*, **15**, 213–218.

Cooke, H. J., 1994. Neuroimmune signalling in regulation of intestinal ion transport. *American Journal of Physiology*, **266**, 167–G178.

Crowe, S. & M. Perdue, 1992. Gastrointestinal food hypersensitivity: Basic mechanisms of pathophysiology. *Gastroenterology*, **103**, 1075–1095.

De Jonge, F., L. Van Nassauw, D. Adriaensen, F. Van Meir, H. Miller, E. Van Marck & J.-P. Timmermans, 2003. Effect of intestinal inflammation on capsaicin-sensitive afferents in the ileum of *Schistosoma mansoni*-infected mice. *Histochemistry and Cell Biology*, **119**, 477–484.

Gilchrist, M., S. McCauley & A. Befus, 2004. Expression, localization and regulation of nitric oxide synthase (NOS) in human mast cell lines: Effects on leukotriene production. *Blood,* **104**, 462–469.

Gottwald, T. P., B. R. Hewlett, S. Lhotak & R. H. Stead, 1995. Electrical stimulation of the vagus nerve modulates the histamine content of mast cells in the rat jejunal mucosa. *NeuroReport*, **7**, 313–317.

Gottwald, T. P., S. Lhotak & R. H.Stead, 1997. Effect of truncal vagotomy and capsaicin on mast cells and IgA positive plasma cells in rat jejunal mucosa. *Neurogastroenterology and Motility*, **9**, 25–32.

Gulubova, M. & A. Vodenicharov, 2001. Structural examination of triptase-, and VIP-positive mast cells in the common bile duct of patients with lithiasis. *Acta Histochemica*, **103**, 437–452.

Jennings, L. J., G. Salido, M. Pozo, J. Davison, K. Sharkey , R. Lea & J. Singh, 1995. The source and action of histamine in the isolated guinea-pig gallbladder. *Inflammation Research*, **44**, 447–453.

Koda, W., K. Harada, K. Tsuneyama, N. Kono, M. Sasaki, O. Matsui & Y. Nakanuma, 2000. Evidence of the participation of peribiliary mast cells in regulation of the peribiliary vascular plexus along the intrahepatic biliary tree. *Laboratory Investigation*, **80**, 1007–1017.

May, A., M. Fusbs, M. Scheib & E. Lutjen-Drecoll, 2002. Characterization of nitrergic neurons in the porcine and human

ciliary nerves. *Investigative Ophthalmology and Visual Science*, **43**, 581–586.

Moncada, S., R. Palmer & E. Higgs, 1991. Nitric oxide: Physiology, pathophysiology and pharmacology. *Pharmacological Reviews*, **43**, 109–142.

Mori, N., R. Suzuki, T. Furuno, D. M. McKay, M. Wada, R. Teshima, J. Bienenstock & M. Nakanishi, 2002. Nerve-mast cell (RBL) interaction: RBL membrane ruffling occurs at the contact site with an activated neurite. *American Journal of Physiology*, **283**, C1738–C1744.

Newson, B., A. Dahlstrom, L. Enerback & H. Ahlman, 1983. Suggestive evidence for a direct innervation of mucosal mast cells. *Neuroscience,* **10**, 565–570.

Schemann, M., K. Michel, M. Ceregrzyn, F. Zeller, S. Seidl & S. C. Bischoff, 2005. Human mast cell mediator cocktail excites neurons in human and guinea-pig enteric nervous system. *Neurogastroenterology and Motility*, **17**, 281–289.

Sherer-Singler, U., S. Vincent, H. Kimura & E. McGeer, 1983. Demonstration of a unique population of neurons with NADPH-diaphorase histochemistry. *Journal of Neuroscience Methods*, **9**, 229–234.

Stach, W., 1973. Über die Nervengeflechte der Duodenalzotten. Lichtund elektronenmikroskopische Untersuchungen. *Acta Anatomica*, **85**, 216–231.

Stead, R. H., M. Tomioka, G. Quinonez, G. T. Simon, S. Y. Felten & J. Bienenstock, 1987. Intestinal mucosal mast cells in normal and nematode-infected rat intestines are in intimate contact with peptidergic nerves. In: *Proceedings of the National Academy of Sciences of the United States of America*, **84**, 2975–2979.

Stead, R. H., M. F. Dixon, N. H. Bramwell, R. H. Riddell & J. Bienenstock, 1989. Mast cells are closely apposed to nerves in human gastrointestinal mucosa. *Gastroenterology*, **97**, 575–585.

Stead, R. H., E. C. Colley, B. Wang, E. Partosoedarso, J. Lin, A. Stanisz & K. Hillsley, 2006. Vagal influences over mast cells. *Autonomic Neuroscience*, **125**, 53–61.

Stefanov, I. S., 2012. NADPH-diaphorase positive cells (mast cells) around and within the autonomic nerves in the periphery of subglandular connective tissue layer of dog paranal sinus (*Sinus paranalis*). *Revue de Médecine Vétérinaire,* **163**, 250–253.

Vodenicharov, A. & A. Bozhilova-Pastirova, 2010. NADPH-d cells (mast cells) around and within the autonomic nerves of porcine renal hilus. *Tissue and Cell,* **42**, 195–197.

Williams, R. M., H. R. Berthoud & R. Stead, 1997. Vagal afferent nerve fibres contact mast cells in rat small intestinal mucosa. *Neuroimmunomodulation*, **4**, 266–270.

Paper received 27.08.2012; accepted for publication 07.11.2012

Correspondence:

Dr. I. S. Stefanov
Department of Veterinary Anatomy,
Histology and Embryology,
Faculty of Veterinary Medicine,
Trakia University,
6000 Stara Zagora, Bulgaria,
phone: +359 42 699 650,
e-mail: iv_stefanov@uni-sz.bg