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Short communication

MAST CELL DENSITY IN DOMESTIC SWINE COMMON HEPATIC DUCT

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Summary

Stefanov, I., A. Vodenicharov, P. Atanassova, P. Hrischev, I. Vulkova, D. Stoyanov, N. Tsandev & H. A. Hristov, 2023. Mast cell density in domestic swine common hepatic duct. *Bulg. J. Vet. Med.,* **26**, No 2, 272–279.

Detailed mast cell classification is reported in humans and rats, however such classification is not available in porcine common hepatic duct. It is interesting to find out whether mast cells in common hepatic duct are able to produce ghrelin, which is involved in a series of biological functions including regulation of food intake, body weight, gastrointestinal motility, hormone secretion, glucose release, cardiovascular functions, enzyme release, cell proliferation and reproduction in pigs. Because the determination of the cellular mechanisms responsible for normal and pathological biliary tract motility is difficult in humans, swine appears to be one of the most suitable animal model for physiological and morphological studies related to ghrelin peptide. The lack of information about the distribution of mast cells in the wall of common hepatic duct (*Ductus hepaticus communis*) in domestic swine motivated us to undertake this study in order to gain a better understanding of ghrelin peptide. The aim of this study was to define the localisation and number of tryptase-, ghrelin- and toluidine blue positive mast cells in the layers of porcine common hepatic duct. In this respect, the immunohistochemical staining for detection of tryptase and ghrelin expression was performed. The toluidine blue staining allowed the visualisation of metachromatic mast cells. The comparative study showed that tryptase positive cells were more numerous than metachromatic cells in all layers of the studied organ but the density of ghrelin positive cells was the highest. Tryptase immunohistochemistry allowed distinguishing ghrelin expressing mast cells from all ghrelin positive cells. The highest number of ghrelin positive cells indicated that not only mast cells but other cells also express ghrelin. The results allowed concluding that studied mast cell types had different density in the three main layers of common hepatic duct. The highest density of mast cells in the muscular layer and the possibility of mast cells to express ghrelin define its role in regulation of common hepatic duct motility and glandular secretion.

Key words: common hepatic duct, ghrelin, mast cells, swine, tryptase

Mast cells were reported to mediate allergic reactions and stimulate wound healing, however, it has been found out that they may also be players in many autoimmune, inflammatory, infectious, and other disorders (Boyce, 2004; Beaven, 2009).

The classification of mast cells is dependent on their phenotypic characteristics and their anatomic locations. According to Reber *et al.* (2015) murine and human mast cells are classified as tryptase-positive (MCT), and tryptase and chymase-positive (MCTC). MCTC have an affinity for the small intestinal submucosa and muscularis mucosa, whereas MCT are localised in the mucosa of the stomach, small intestine, and colon. The first information about the presence of another mast cell phenotype in rat was given by Stefanov *et al.* (2017) who visualised ghrelin positive mast cells (MCgr). Data about the classification of mast cells in porcine extrahepatic bile ducts have not yet been reported.

Dong *et al.* (2009) reported that ghrelin (Gr) is involved in a series of biological functions including regulation of food intake, body weight, gastrointestinal (GI) motility, hormone secretion, glucose release, cardiovascular functions, enzyme release, cell proliferation and reproduction in pigs through binding to GHS-R 1a or unidentified receptors.

Because the determination of the cellular mechanisms responsible for normal and pathological biliary tract motility is difficult in humans, the pig appears to be one of the most suitable animal model for physiological and morphological studies related to ghrelin (Howard *et al.*, 1996; McKee *et al.*, 1997; Smith *et al.*, 1999; Dong *et al.*, 2009).

Ghrelin peptide, reported for the first time in rat stomach by Kojima *et al.* (1999), exerts its function using two main receptors: GHS-R1A and GHS-R1B. It is known that coupling of GHS-R1A to G-protein involves the 3rd intracellular rloop. The lack of a 3rd intracellular loop in GHS-R1B prohibits it from coupling to G-proteins (Smith *et al.*, 1999). GHS-R1B is unable to bind acyl or desacyl ghrelin (Howard *et al.*, 1996). GHS-R1B, considered in the past to be functionally inactive, is now believed to act as an important modulator in ghrelin-induced GHS-R1A signaling (Chan & Cheng, 2004, Chu *et al.*, 2007, Leung *et al.*, 2007, Chow *et al.*, 2012).

Animal experiments are focused predominantly on the gastrointestinal tract and have shown stimulating effects of ghrelin on gastric emptying and intestinal motility (Edholm *et al.*, 2004). A previous study of ours visualised ghrelin positive endocrine cells in glands and ghrelin positivity in some nerve fibres and ganglionic cells in gallbladder, cystic duct and common bile duct in domestic swine (Gulubova *et al.*, 2017). Stefanov *et al.* (2017) reported that mast cells in the rat stomach were positive for ghrelin. However, data about the ghrelin expression in common hepatic duct (*Ductus hepaticus communis*) (DHC) have not yet been reported.

Taking into acount a previous study of ours (Stefanov *et al.*, 2017) regarding the presence of Gr+ mast cells (MCGr+) in the rat stomach, we decided to undertake the current study in order to define the localisation and number of ghrelin immunopositive mast cells in the layers of porcine DHC using tryptase immunohistochemistry and toluidine blue staining.

- Animals

In the current study 6 male pigs at the age of 6 months were used. The middle part of the common hepatic duct of each pig was immediately removed after slaughtering in a slaughterhouse, put in 10% neutral formalin for 24 hours, washed with PBS, dehydrated in an alcohol, clarified in xylene, and included in paraffin. Serial tissue sections of 5 μm thickness from each animal were prepared, mounted on gelatin coated slides, deparaffinized in xylene and rehydrated by a series of decreasing ethanol concentrations. Then, tissue sections were processed by histochemical reaction with toluidine blue to visualise metachromasia and immunohistochemically to express tryptase and ghrelin.

- Toluidine blue staining for visualisation of metachromatic mast cells

Tissue sections were mounted on gelatinised slides, twice placed in xylene and rehydrated by decreasing ethanol concentrations. The sections were stained in a buffered solution of toluidine blue (pH=3).

- Immunohistochemical staining for visualisation of tryptase- and ghrelin-positive mast cells

The tissue sections were washed in 0.1M PBS and placed in 1.2% hydrogen peroxide in methanol for 30 minutes. Antigen recovery in buffer (pH 9.0) was performed, then incubated in a humidified chamber overnight at 4 °C with primary antibodies: monoclonal mouse ghrelin (2F4) (sc 293422, Santa Cruz Biotechnology) at 1:50 dilution, monoclonal mouse antihuman mast cell tryptase (IR 640, Dako, Denmark) – ready for use. After washing with PBS, the sections were incubated with EnVision detection system (DAKO) for 24 hours at 4 °C. The immune reaction was visualised with diaminobenzidine. PBS replacing the primary antibody was used as a negative control. The slices were dehydrated, washed, coated with glass slides and photographed with a research microscope (LEICA DM1000) equipped with a digital camera (LEICA DFC 290).

Of the three serial sections used, two were stained with tryptase and ghrelin antibodies, and the third was stained with toluidine blue for metachromasia.

- Statistical analysis

The number of mast cells was determined on the microscopic fields ×200 with an area of 0.163 mm^2 using a light research microscope (LEICA DM1000) with a digital camera (LEICA DFC 290). Mast cell density data (number / field of view) were processed using GraphPad Prism 6 for Windows (GraphPad Software, Inc., USA) for analysis of variations (one-way ANOVA), followed by Tukey-Kramer test. $P<0.05$ was considered significant. All reported data were expressed as mean ± standard deviation (SD).

The immunohistochemical detection of tryptase and ghrelin reactivity allowed identifying MCtr+ and ghrelin positive cell (Cgr+) localisation and their number per microscopic field on DHC slides (Table 1). Toluidine blue staining was used to visualise metachromatic mast cells (MCtb+). Co-localisation with tryptase on serial sections showed that most of the Cgr+ were MCTr+.

In DHC propria (*Lamina propria mucosae*), the three types of MC were observed predominantly near the blood vessels and biliary glands with a highest density of Cgr+ (Fig. 1–5). Co-localisation with tryptase allowed identifying MCGr+ which were less numerous than Cgr+ but more abundant than MCtb+ ($P<0.0001$).

Table 1. Number (mean±SD; n=6) of tryptase- (MCtr+) and toluidine blue (MCtb+) positive mast cells as well as of ghrelin positive cell (Cgr+) in the layers of the common hepatic duct of pigs.

Mast cell types	Common hepatic duct layers		
	Tunica mucosa	*Tunica muscularis*	*Tunica serosa*
MCtr+	8.11 ± 0.76	18.56 ± 1.10	9.22 ± 0.81
Cgr+	13.22 ± 0.73	25.67 ± 1.09	18.56 ± 1.25
MCtb+	2.22 ± 0.73	17.28 ± 1.23	6.72 ± 0.96

In DHC *Tunica muscularis*, MC were observed close to muscle cell bundles, in vicinity of blood vessels and around biliary glands. Cgr+ were most numerous (P<0.0001), followed by MCtr+ and MCtb+. MCtr+ were more numerous than MCtb+ (P<0.01). In this layer, the number of three types of mast cells was higher than those in the propria and serosa (Fig. 1–5).

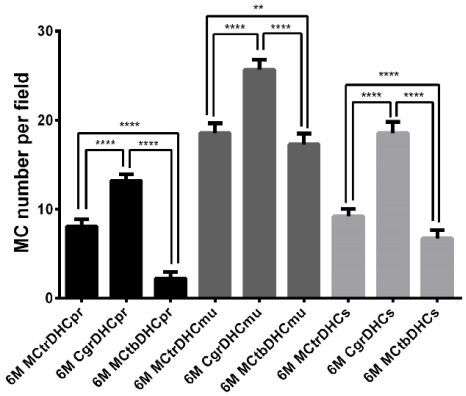

Fig. 1. Comparative quantitative analysis of tryptase- /toluidine blue positive mast cells (MCtr+, MCtb) and ghrelin positive cells (Cgr+) in the same layer of common hepatic duct: propria (pr), muscle (mu) and serous layer (s); **/**** - statistical significant difference at P<0.01/ P<0.0001.

In serosal layer (*Tunica serosa)* of DHC, MC were located near the vessels and nerves. Cgr+ were in highest number, followed by MCtr+ and MCtb+ (P<0.0001) (Fig. 1, 2).

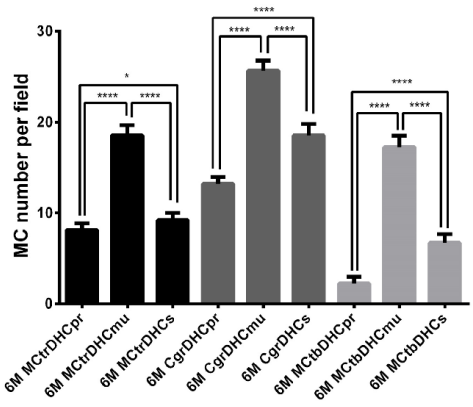

Fig. 2. Comparative quantitative analysis of tryptase- /toluidine blue positive mast cells (MCtr+, MCtb) and ghrelin positive cells (Cgr+) in propria (pr), muscle (mu) and serous layer (s) of common hepatic duct; */**** - statistical significant difference at P<0.05/ P<0.0001.

This study provides original information about the distribution of tryptase-, ghrelin- and toluidine blue positive mast cells in the wall layers of DHC. For the first time, ghrelin containg rat mast cells in the stomach were detected in a previous study of ours (Stefanov *et al.*, 2017). The current study adds new data about the presence of porcine ghrelin producing mast cells. For mast cell identification, tryptase immunopositivity (Walls *et al.*, 1990) and toluidine blue staining (Pearce, 1960) were used. The classification of mast cell phenotypes in human and rat is well known (Chan *et al.*, 2001; Zweifel *et*

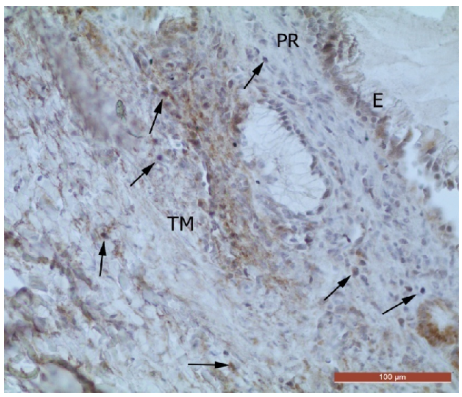

Fig. 3. Tryptase positive mast cells (arrows) in the propria (PR) and muscle layer (TM). E – *Lamina epithelialis mucosae*. Bar = 100 µm.

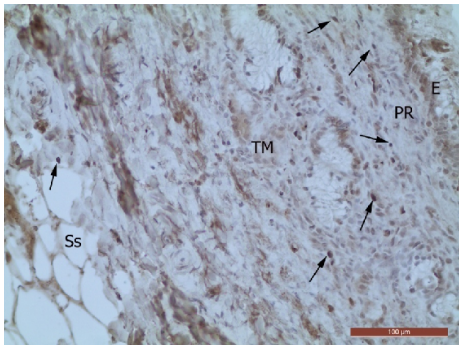

Fig. 4. Ghrelin positive mast cells (arrows) in the propria (PR), muscle layer (TM) and subserous layer (SS); E – *Lamina epithelialis mucosae*. Bar = 100 µm.

al., 2005; Reber *et al.*, 2015), however, detailed data about mast cell phenotype classification in porcine common hepatic duct are missing. The light microscopical histochemistry and immunohistochemistry used in the current study allowed the identification of three mast cell phenotypes exhibiting specific localisation in DHC. In propria, tryptase and grhrelin immunopositive mast cells dominated, yet no or only single metachromatic mast cells were present. Therefore, the mucosal mast cells were predominantly ghrelin- and

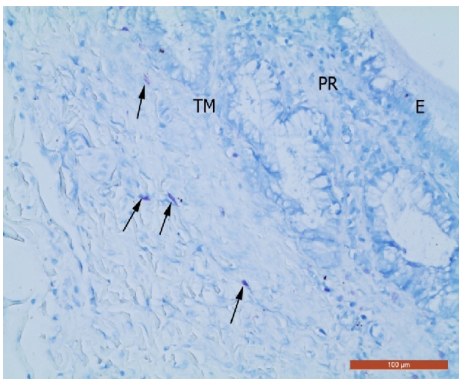

Fig. 5. Metachromatic mast cells (arrows) in the muscle layer (TM). E – *Lamina epithelialis mucosae*, PR – Lamina propria mucosae. Bar = 100 µm.

tryptase positive. We revealed that the density of the three mast cells types was highest in the muscle layer followed by that in the serosal layer of DHC, therefore these cells can be defined as connective tissue mast cells positive for tryptase, ghrelin and metachromatically stained by toluidine blue.

The localisation of the three types of mast cells in the vicinity of blood vessels is related to their role in controlling blood vessel permeability (McCauley *et al.*, 2005). The most important vascular effect of ghrelin is vasodilatation that seems to be endothelial and GH-independent, suggesting an action at smooth muscle level (Wiley & Davenport, 2002; Shimizu *et al.*, 2003; Henriques-Coelho *et al.*, 2004; Kleinz *et al.*, 2006). It is known that mast cells synthesise mediators such as serotonin (Keith *et al.*, 1987) and nitric oxide (McCauley *et al.*, 2005), responsible for smooth muscle contraction and relaxation. Similarly to what was reported in stomach and intestine (Penkova *et al.*, 2016), we suggest that ghrelin may regulate glandular secretion of common hepatic duct. Cgr+ were the most numerous, followed

by MCtr+ and MCtb+. This finding shows that some Cgr+ were MCgr+ but other immune cells (B lymphocytes (B cells), T cells, monocytes and NK cells) different from MC were also ghrelin immunoreactive, in support of investigations of other authors (Dixit *et al.*, 2004; Hattori, 2009; Taub *et al.*, 2010).

The localisation of mast cells in the muscular layer of DHC can be explaned by findings of Penkova *et al.* (2016) affirming that ghrelin takes part in the regulation of the gastrointestinal motility. Therefore, it can be assumed that this peptide has the same role in the common hepatic duct.

The localisation of mast cells near the nerves in the serosal DHC layer provides evidence for the existence of functional communication between mast cells and nervous system (Dimitriadou *et al.*, 1987).

In conclusion, ghrelin positive mast cells and their localisation around the biliary glands as well as the highest number of these cells in the muscle layer were related to their participation in regulation of the glandular secretion and motility of common hepatic duct.

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