Bulgarian Journal of Veterinary Medicine, 2023 ONLINE FIRST
ISSN 1311-1477; DOI: 10.15547/bjvm.2023-0054

Original article

GLUCAGON AND INSULIN IMMUNOPOSITIVITY OF MAST CELLS IN PORCINE GALLBLADDER

I. S. STEFANOV[1,4], S. I. STEFANOV[2] & M. V. GULUBOVA[3,4]

[1]Department of Anatomy, Medical Faculty, Trakia University, Stara Zagora, Bulgaria; [2]Sixth year student at Medical Faculty, Trakia University, Stara Zagora, Bulgaria; [3]Department of General and Clinical Pathology, Medical Faculty, Trakia University, Stara Zagora, Bulgaria; [4]Department of Anatomy, Histology and Embryology, Pathology, Medical Faculty, Prof. Dr. Asen Zlatarov University, Burgas, Bulgaria

Summary

Stefanov, I. S., S. I. Stefanov & M. V. Gulubova, 2023. Glucagon and insulin immunopositivity of mast cells in porcine gallbladder. *Bulg. J. Vet. Med.* (online first).

It is well known that mast cells produce and release biologically active substances such as histamine, heparin, proteases, leukotrienes, cytokines, chemokines and growth factors. According to the available scientific literature on that topic this is the first time when gallbladder mast cells were found to have a capacity to produce insulin and glucagon. We aimed to perform an immunohistochemical study to determine glucagon- and insulin-positive mast cell existence and distribution in the wall of porcine gallbladder. The collocalisation of glucagon and insulin immunopositivity with metachromasy allowed us to detect the presence of glucagon and insulin immunoreactive mast cells in the bottom, body and neck of gallbladder. In addition, an immunohistochemical detection of tryptase as a better marker for mast cells than toluidine blue dye was used to compare the number of mast cells to glucagon and insulin immunoreactive cells. The ability of mast cells to produce hormones was confirmed by the chromogranin A immunopositivity of the same cells. The highest mast cell density was registered in in all layers of gallbladder's neck, followed by the body and bottom of gallbladder. In conclusion, we established histochemically and immunohistochemically the localisation and density of two new types of mast cells: glucagon and insulin positive mast cells.

Key words: chromogranin A, glucagon, insulin, mast cells, porcine gallbladder

INTRODUCTION

Domestic swine is considered as one of the most suitable animal models in morphological, physiological, biochemical, and genetic investigations as well as in xenotransplantation because pigs and humans share many similarities such as size, physiology, anatomy, metabolic profile, genome and chromosomal structure (Ribitsch *et al.*, 2020).

Several morphological studies established that mast cells (MCs) are localised predominantly in skin as well as at mucosal sides of gastrointestinal tract and respiratory system where they contact a variety

of antigens. These cells were observed in the connective tissue surrounding blood vessels, between smooth muscle bundles, near the epithelial cells and hair follicles (da Silva *et al.*, 2014). The role of MCs in maintaining homeostasis of the gut bacteria can be explained by the ability of the antigen to permeate through the epithelial layer of the gut mucosa by binding to IgE on mucosal type MCs (Cheng *et al.*, 2021). Then IgE antibody-antigen interaction causes activation of the MCs triggering an immune response. The activation of MCs stimulates their degranulation and release of inflammatory mediators leading to increase of vascular permeability, oedema of the gut epithelium and smooth muscle contraction accompanied by vomiting and diarrhoea.

Morphological and histochemical features of MCs in rodents and men were described in detail by Xu *et al.* (1993). Two mast cell types: mucosal-type (MMCs) and connective tissue type (CTMCs) were established in mice (Bienenstock *et al.*, 1982; Galli, 1990). In humans, on the basis of MCs reactivity with antibodies against proteases chymase and tryptase, two mast cell types were described: tryptase- and chymase-positive (MCstrc+, located mainly in the connective tissue and serosa, corresponding to CTMCs) and tryptase-positive, chymase-negative (MCstr+, localised predominantly in the intestinal and airway mucous tunics, corresponding to MMCs) subtypes (Irani *et al.*, 1986). CTMCs and MMCs were distinguished by the red staining with safranin depending on the heparin content in their secretory granules. For example, it was established that CTMCs contained heparin but MMCs – did not. CTMCs may be detected after fixation in 10% neutral buffered formalin, whereas MMCs are visualised by fixation in non-aldehyde non-aqueous solutions such as Carnoy's fixative (Irani & Schwartz, 1989). Xu *et al.* (1993) were the first to present detailed information for histochemical features of porcine CTMCs and MMCs in the organs of gastrointestinal tract but not in the bile ducts. Stefanov (2021) revealed that the staining results in porcine gallbladder were similar to those of porcine intestine (Xu *et al.*, 1993) because the granules of MCs in the mucosal and submucosal connective tissue of gallbladder (GB) and intestine were stained in blue in contrast to rat tissue where connective tissue mast cell granules stained dark purple and mucosal mast cell granules red (Enerback, 1966). It was reported that MC in the gastrointestinal tract, including the GB and the extrahepatic bile ducts (EHBD), exibited β-metachromasia. Stefanov (2021) also revealed that MCstr+ were localised in all layers of studied organs, which did not permit their differentiation into MMCs and CTMCs. In porcine GB, the immunolabelling with tryptase allowed detecting a higher number of MCs compared to other staining techniques – toluidine blue positive mast cells (MCstb+), alcian blue positive mast cells (MCsab2.5+ and MCsab1.0+ at pH 2.5 and pH 1.0, respectively) after formalin fixation.

Insulin producing β-cells and glucagon producing α-cells that are known as pancreatic endocrine cells, were also found to be localised in the murine EHBD (Dutton *et al.*, 2007). This phenomenon was explained by the common embryologic development of the extrahepatic bile ducts, gallbladder, and pancreatic primordia (Tomita & Hara, 2022). However, so far we have not found any data about the ability of mast cells to express insulin and/or glucagon. The presence of glucagon positive (MCsglu+) and insulin immunoreac-

tive (MCsins+) mast cells in the porcine gallbladder can be used for development of new approaches to the treatment of diabetes.

Considering the information above we aimed to establish the existence and distribution of glucagon and insulin positive mast cells in the wall of porcine gallbladder's neck, body and bottom.

MATERIALS AND METHODS

Animals

The present study used specimens from the bottom (*fundus vesicae biliaris*), body *(corpus vesicae biliaris*) and neck (*collum vesicae biliaris*) of normal gallbladders taken from 6 male pigs (crossbred Bulgarian White × Landrace) at the age of 6 months (92–100 kg), slaughtered for meat consumption at a slaughterhouse. The animals were delivered under Scientific Project No.13/2017, Medical Faculty, Trakia University. After slaughtering, the specimens were fixed in 10% aqueous solution of formalin.

Histological methods

Serial tissue sections of 5 µm thickness were prepared from each animal, mounted on gelatin coated slides, deparaffinised in xylene, rehydrated and stained with haematoxylin and eosin to exclude the presence of pathological findings. Another part of the sections was processed for histochemical staining with toluidine blue as well as for immunohistochemical detection of tryptase-, chromogranin A-, glucagon- and insulin expression.

Colocalisation of metachromatic and glucagon- and insulin positive mast cells

After immunohistochemical staining for glucagon and insulin expression, the same sections were used for toluidine blue histochemistry. For this purpose, the coverslips were removed from the already photographed slides, then were rehydrated and immersed in 0.1% toluidine blue in McIlvaine buffer (pH = 3) (Pearce, 1960) for demonstration of colocalisation of metachromatic mast cells and glucagon- and insulin positive mast cells.

Immunohistochemical methods

For visualisation of glucagon and insulin immunoreactive cells comparing to triptase and chromogranin A positive mast cells, tissue sections prepared for immunohistochemical staining were washed in 0.1M PBS and placed in 1.2% hydrogen peroxide in methanol then antigen recovery in buffer (pH 9.0) was done. Between steps, sections were washed with an EnVisionFlex Wash Buffer, then incubated in a humidified chamber overnight at 4 °C with following primary antibodies: glucagon mouse monoclonal antibody (C-11) SC-514592, Santa Cruz, Insulin mouse monoclonal antibody (2D 11-45), SC-8033, Santa Cruz, chromogranin A rabbit antibody (PA 0430) (Leica) and monoclonal mouse antihuman mast cell tryptase, which are ready to 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. 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). The serial sections were stained consequently with glucagon-, insulin-, tryptase- and chromogranin A antibodies. PBS replacing the primary antibody was used as a negative control.

Statistical analysis

The number of mast cells was estimated on the microscopic field ×200 (with an area of 0.163 mm^2) from the sections of the three parts of the gallbladder using a light microscope (LEICA DM1000) equipped with a digital camera (LEICA DFC 290). The data for mast cells density (number per field) were processed by GraphPad Prism 6 for Windows (GraphPad Software, Inc., USA) via one-way ANOVA followed by Tukey-Kramer's post-hoc test. P-values of less than 0.05 were considered statistically significant. The data are presented as mean ± SEM.

RESULTS

Tryptase immunolabelling and toluidine blue staining were used to confirm that the cells expressing glucagon and insulin were mast cells. The colocalisation of both tryptase and toluidine blue staining showed that tryptase immunoreactive mast cells (MCstr+) were more than metachromatic mast cells (Fig. 1A). In the propria, MCstb+ were only 35 % of the MCstr+, in the muscular trunic – 67%, in the serosal layer – 81%. The colocalisation of glucagon and insulin immunopositivity with metachromasia revealed that all metachromatic mast cells were immunoreactive for both hormones but some of glu+ and ins+ cells were not metachromatic (Fig. 1B, C). The immunohistochemical staining showed no statistically significant difference between the number of MCstr+, glu+ and ins+ cells. Therefore, the tryptase immunohistochemistry comparing to toluidine blue staining appeared to be more reliable technique for identifying all glu+ and ins+ mast cells.

It was established that mast cells expressing glucagon (MCsglu+), insulin (MCsins+) and tryptase (MCstr+) showed similar shape, density (Table 1) and localisation (Fig. 1D, E). The immunohistochemical staining for detection of chromogranin A (cgA) allowed identifying chromogranin A positive (cgA+) cells which showed similar shape and distribution to MCstr+, MCsglu+, MCsins+ (Fig. 1F).

The four types of mast cells were located near the blood vessels and nerves in the propria (*lamina propria mucosae*), muscular (*tunica muscularis*) and serosal *(tunica serosa*) tunics of gallbladder. In the muscle layer, all types of mast cells were also observed near the smooth muscle cells.

The most numerous mast cells were in the propria, followed by those in the muscle layer and the lowest number was found in the serous layer (Table 1). This difference was most pronounced in gallbladder's neck (P<0.0001). There was no significant difference between the number of MCstr+, MCsglu+, MCsins+ and McscgA+ in the same tunic of the gallbladder. In the gallbladder bottom, the density of the four mast cell types in the muscular and serosal tunics was similar. In all layers of gallbladder's neck, the mast cell density was the highest, followed by the mast cell density in the body and the bottom (Table 1).

CgA+ cells showed similar morphology, density (Table 1) and localisation as MCstr+, MCsglu+ and MCsins+.

DISCUSSION

This study is the first report on the existence, localisation and density of two new mast cell phenotypes expressing glucagon and insulin. The localisation and density of MCsglu+ and MCsins+ was determined in the wall of the three parts of porcine gallbladder – the neck, body, and bottom. To identify the MCsglu+ and MCsins+ immunohistochemical detection of MCstr+

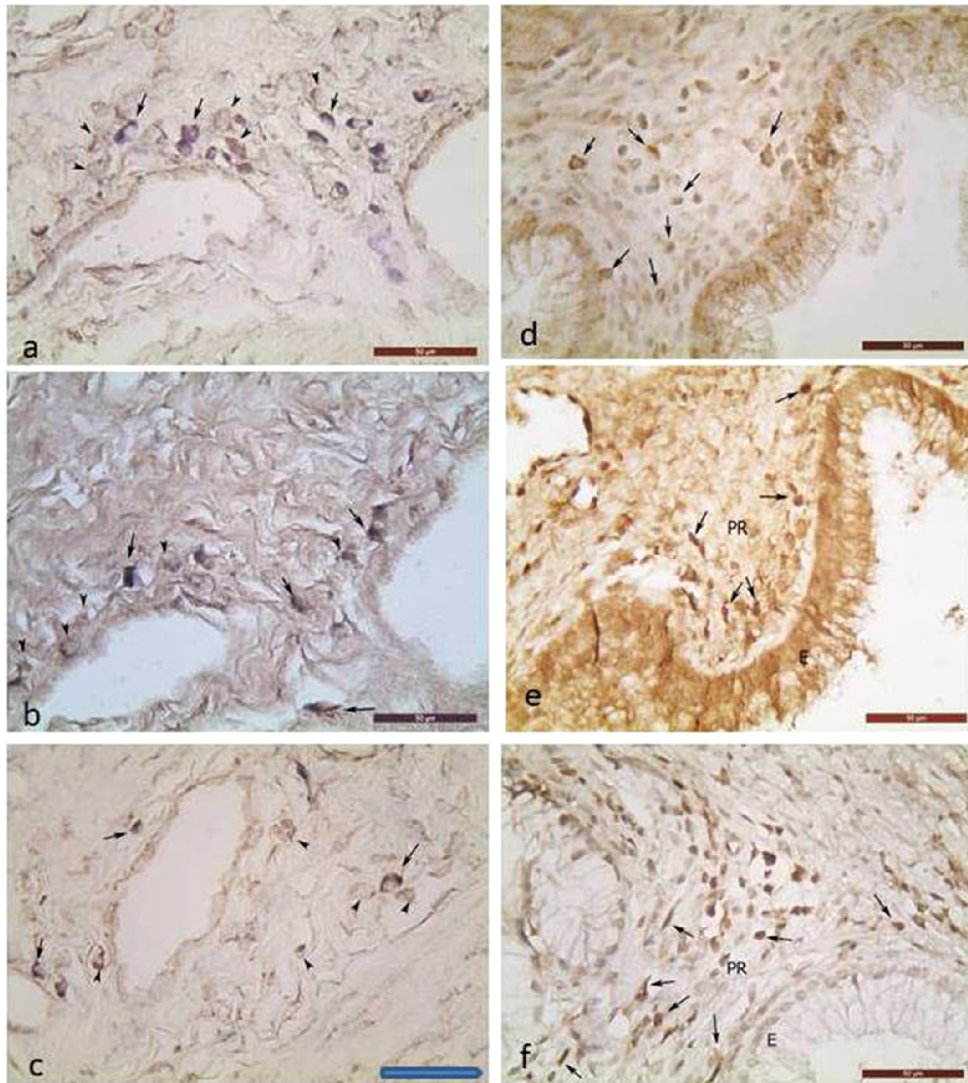

Fig 1. A) Colocalisation (arrows) of MCstr+ and metachromatic mast cells in *tunica muscularis*. Some of MCstr+ (arrowheads) did not show metachromasy. Bar=50 µm; **B)** Colocalisation (arrows) of MCsins+ and metachromatic mast cells in *tunica muscularis*. Some of insulin-positive mast cells (arrowheads) did not show metachromasy. Bar=50 µm; **C)** Colocalisation (arrows) of MCsglu+ and metachromatic mast cells *in tunica muscularis*. Some of glucagon positive mast cells (arrowheads) did not show metachromasy. Bar=50 µm; **D)** MCstr+ (arrows) in the lamina propria of gallbladder's body. Bar=50 µm; **E**) MCsins+ (arrows) in the lamina propria of gallbladder's body. Bar=50 µm; **F)** CgA+ mast cells (arrows) in the lamina propria of gallbladder's body. Bar=50 µm.

Table 1. Mast cell localisation and density (mean ± SEM MC number per microscopic field ×200) in the lamina propria, muscular and serosal tunics of the *collum, corpus* and *fundus vesicae biliaris* (VB).

MC localisation	Mast cells number per microscopic field ×200		
	Collum VB	*Corpus VB*	*Fundus VB*
MCtr+ in lamina propria	16.00±0.33 A4/B4/D4/E4	12.39±0.16 A1/B4/F1	11.28±0.18 A4/B4
MCtr+ in muscular tunic	13.50±0.23 C4/D4/E4	11.28±0.22 C4/F4	8.77±0.210
MCtr+ in serosal tunic	9.05±0.21	9.0±0.18	8.55±017
MCglu+ in lamina propria	15.44± 0.27 A4/B4/D4/E4	11.61±0.20 A1/B4/F1	10.72±0.24 A4/B4
MCTglu+ in muscular tunic	13.28±0.21 C4/ D4/E4	10.94±0.22 C4/F4	8.66±0.18
MCglu+ in serosal tunic	8.61±0.18	8.66±0.16	8.44±0.12
MCins+ in lamina propria	15.22±0.26 A4/B4/D4/E4	11.17±0.17 A1/B4/F1	10.28±0.19 A4/B4
MCins+ in muscular tunic	13.17±0.19 C4/ D4/E4	10.61±0.22 C4/F4	8.38±0.12
MCins+ in serosal tunic	8.33±0.16	8.44±0.22	8.22±0.17
MCcgA+ in lamina propria	16.17±0.35 A4/B4/D4/E4	12.22±0.19 A1/B4/F1	11.44±0.16 A4/B4
MCcgA+ in muscular tunic	13.39±0.22 C4/D4/E4	11.39±0.20 C4/F4	8.72±0.18
MCcgA+ in serosal tunic	8.94±0.19	9.16±0.15	8.72±0.16

Legend: A – statistical significant difference between the propria and muscular tunic; B – statistical significant difference between the propria and serosal tunic; C – statistical significant difference between the muscular tunic and serosal tunic; D – statistical significant difference between the neck and the body; E– statistical significant difference between the neck and the bottom; F – statistical significant difference between the body and the bottom. 1 – P<0.05, 4 – P<0.0001.

which is considered one of the most precise markers of these cells was used (Mohajeri *et al.*, 2019; Stefanov, 2021).

The other colocalisation of both tryptase and toluidine blue staining showed that tryptase positive mast cells (MCstr+) were more numerous than metachromatic mast cells. This colocalisation allowed estimating the percent of metachromatic mast cells that were positive for tryptase for example, in the propria MCstb+ were 35% of the MCstr+, in the muscular tunic – 67%, in the serosal tunic – 81%. Since the existence of statistically significant difference between the number of MCstr+ and glu+ and ins+ cells was not estab-

lished, it was assumed that toluidine blue staining was not able to mark all MCsglu+ and MCsins+. These findings support our previous study (Stefanov, 2021) demonstrating that compared to the toluidine blue and alcian blue stainings, the tryptase was the best marker for mast cells in formalin-fixed gallbladder neck because both mucosal type (MMC) and connective tissue type (CTMC) of mast cells expressed this enzyme. The current study revealed that MCsglu+ and MCsins+ showed similar morphology, localisation and density as MCstr+ in all parts of gallbladder – bottom, body, and neck.

Glucagon and insulin expression by mast cell granules defines a new, so far unknown role of these cells regarding their participation in glucose homeostasis regulation. Our results support the studies of several authors that reported involvement of mast cells in the pathogenesis of diabetes mellitus (Caughey, 2007). In T1DM, MCs present pancreatic T-cell antigens, allowing proinflammatory cytokines/chemokines to affect the function and survival of pancreatic β-cells (Caughey, 2007). The tumour necrosis factor involved in T1DM is a proinflammatory cytokine, involved in the acute phase of the reaction, with increased vascular permeability and can be generated and released by degranulated and non-granulated activated mast cells (Dudeck *et al.*, 2021).

MCs are also known to be crucial for the maturation of Th17 cells, which are key cells in T1DM (Milovanovic *et al.*, 2012). Diabetic mice have been found to decrease T regulatory (Treg) cells and reduce expression of IL-10, TGF-β and IL-6 in pancreatic tissue (Sia & Hänninen, 2010).

It was also reported that mast cell mediators, such as chemokines, cytokines, growth factors, heparin, histamine, proteases, chymase, and tryptase, contribute to pathogenesis of type 2 diabetes (T2DM) – the commonest type of diabetes. Tryptase is the major mast cell enzyme that stimulates fibroblast proliferation and collagen synthesis involved in diabetes-related vascular disorders, while the other mast cell enzyme – chymase, converts TGF-β from inactive to active form (Ikeda, 2003).

The release of glucagon by MCsglu+ can be explained by the findings of some authors related to the role of this hormone in the inhibition of gastrointestinal motility in humans. Pau (1973) found out that glucagon inhibits cholinergic motor activities not directly via either receptor on the smooth muscle cells but through postganglionic neurons in the canine small intestine as well as in the antrum, whereas glucagon activates cholinergic activity in the duodenum, jejunum and ileum.

Immunohistochemical detection of chromogranin A as a marker for endocrine cells was used to confirm the ability of mast cells to form hormone containing granules. According to Tomita (2020), β-, α-, δ- and PP islet cells in the pancreas were immunopositive for chromogranin A. Our study showed clearly that in porcine gallbladder the three types (MCsglu+, MCsins+ and MCstr+) of MC were chromogranin A-positive, which are new phenotypes of mast cells expressing insulin and glucagon similarly to β- and α-islet cells of the pancreas.

The presence of glucagon and insulin expressing cells in the gallbladder wall revealed in this study raises the need for performing experiments to clarify the exact mechanisms of the pharmacological effects of both hormones on gallbladder motility.

The immunoexpression of glucagon, insulin and chromogranin A by mast cells

allowed hypothesising that these cells are another extrapancreatic source of both hormones that probably participate in regulation of glucose level in normal and in diabetic conditions by a mechanism that needs to be established. We assume that there is a functional connection between the mast cells and the islets of Langerhans called a mast cell-islet axis, similar to the functional connection between the intestine and the islets of Langerhans called entero-islet axis by Fehmann *et al.* (1995). Mast cell glucagon and insulin may be used for development of new approaches to the treatment of diabetes by treatment with special substances influencing mast cell function especially the release of both hormones.

In conclusion, for the first time, the current study identified immunohistochemically and histochemically two new phenotypes of mast cells in mammals: glucagon and insulin immunoreactive mast cells. It also presents original data about their localisation and density in the tunics of porcine gallbladder's neck, body, and bottom. The presence of glucagon and insulin in mast cell granules may be due to the ability of mast cells to accumulate or to synthesise these hormones. In this respect, further investigations are needed to elucidate the mechanism of their synthesis and the clinical significance of the observed new mast cell types.

REFERENCES

Bienenstock, J., A. D. Befus, F. Pearce, J. Denburg, R. Goodacre**,** 1982. Mast cell heterogeneity: Derivation and function, with emphasis on the intestine. *Journal of Allergy and Clinical Immunology*, **70**, 407–412.

Caughey, G. H., 2007. Mast cell tryptases and chymases in inflammation and host defense. *Immunological Reviews*, 217, 141–154.

Cheng, L., Q. Q. Luo & S. L. Chen, 2021. The role of intestinal mast cell infiltration in irritable bowel syndrome. *Journal of Digestive Diseases,* **22**, 143–151.

da Silva, E. Z., M. C. Jamur & C. Oliver, 2014. Mast cell function: A new vision of an old cell. *Journal of Histochemistry and Cytochemistry*, **62**, 698–738.

Dudeck, J., J. Kotrba, R. Immler, A. Hoffmann, M. Voss, V. I. Alexaki, L. Morton, S. R. Jahn, K. Katsoulis-Dimitriou, S. Winzer, G. Kollias, T. Fischer, S. A. Nedospasov, I. R. Dunay, T. Chavakis, A. J. Müller, B. Schraven, M. Sperandio & A. Dudeck, 2021. Directional mast cell degranulation of tumor necrosis factor into blood vessels primes neutrophil extravasation. *Immunity*, **54**, 468–483.e5.

Dutton, J. R. & N. L. Chillingworth, D. Eberhard, C. Brannon, M. Hornsey, D. Tosh & J. M. W. Slack, 2007. Beta cells occur naturally in extrahepatic bile ducts of mice. *Journal of Cell Science*, **120**, 239–245.

Enerback, L. 1966. Mast cells in rat Gastrointestinal mucosa. 2. Dye-binding and metachromatic properties. *Acta Pathologica et microbiologica Scandinavica,* **66**, 303–312.

Fehmann, H. C., R. Göke & B. Göke, 1995. Cell and molecular biology of the incretin hormones glucagon-like peptide-I and glucose-dependent insulin releasing polypeptide. *Endocrine Reviews*, **16**, 390–410.

Galli, S. J., 1990. New insights into "the riddle of the mast cells": microenvironmental regulation of mast cell development and phenotypic heterogeneity. *Laboratory Investigation*, **62**, 5–33.

Ikeda, T., 2003. The pathogenesis of vitreoretinal diseases from the standpoint of molecular biology. *Nippon Ganka Gakkai Zasshi*, **107**, 785–812.

Irani, A. A., N. M. Schechter, S. S. Craig & L. Schwartz, 1986. Two types of human mast cells that have distinct neutral protease composition. *Proceedings of the National Academy of Sciences of the United States of America,* **83**, 4464–4468.

Irani, A. M. & L. B. Schwartz, 1989. Mast cell heterogeneity. *Clinical and Experimental Allergy,* **19**, 143–155.

Milovanovic, M., V. Volarevic & G. Radosavljevic, 2012. IL-33/ST2 axis in inflammation and immunopathology. *Immunologic Research*, **52**, 89–99.

Mohajeri, M., P. T. Kovanen, V. Bianconi, M. Pirro, A. F.G. Cicero & A. Sahebkar, 2019. Mast cell tryptase – marker and maker of cardiovascular diseases. *Pharmacology & Therapeutics,* **199**, 91–110.

Pau, F., 1973. Comparative studies on pancreas glucagon and secretion effect on motility of proximal and distal human gastrointestinal tract. *Verhandlungen der Deutschen Gesellschaft für Innere Medizin*, **79**, 841–843.

Pearce, A., 1960. Histochemistry, 2nd edn, J.& A. Churchill Ltd., London, pp. 472–889.

Ribitsch, I., P. M. Baptista, A. Lange-Consiglio, L. Melotti, M. Patruno, F. Jenner, Schnabl- E. Feichter, L. C. Dutton, D. J. Connolly, F. G. van Steenbeek, J. Dudhia & L. C. Penning, 2020. Large animal models in regenerative medicine and tissue engineering: To do or not to do. *Frontiers in Bioengineering and Biotechnology*, **8**, 972.

Sia, C. & A. Hänninen, 2010. Functional alterations of proinflammatory monocytes by T regulatory cells: Implications for the prevention and reversal of type 1 diabetes. *Review of Diabetic Studies*, **7**, 6–14.

Stefanov, I. S., 2021. Morphometric, histochemical and immunohistochemical characteristics of mast cells in the gallbladder and extrahepatic bile ducts in domestic pigs. Monograph, Medical Faculty, Trakia University Stara Zagora, KOTA Publishing House, pp. 1–100.

Strauss-Albee, D. M., A. Horowitz, P. Parham & C. A. Blish, 2014. Coordinated regulation of NK receptor expression in the maturing human immune system. *Journal of Immunology*, **193**, 4871–4879.

Tomita, T., 2020. Significance of chromogranin A and synaptophysin in pancreatic neuroendocrine tumors. *Bosnian Journal of Basic Medical Sciences*, **20**, 336–346.

Tomita, H. & A. Hara, 2022. Development of extrahepatic bile ducts and mechanisms of tumorigenesis: Lessons from mouse models. *Pathology International,* **72**, 589–605.

Xu, L. R., M. M. Carr, A. P. Bland & G. A. Hall, 1993. Histochemistry and morphology of porcine mast cells. *The Histochemical Journal*, **25**, 516–522.

Paper received 15.05.2023; accepted for publication 24.07.2023

Correspondence:
I. S. Stefanov
Department of Anatomy,
Medical Faculty,
Trakia University,
Armeiska Street 11,
6000 Stara Zagora, Bulgaria,
tel: 0886846035
e-mail: ivstefanov@abv.bg