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*Original article*

# TRYPTASE- AND GHRELIN POSITIVE MAST CELLS IN THE INTERALVEOLAR SEPTA OF RAT'S LUNG

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**Summary**

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The mast cell mediators and distribution of lung mast cells in rats are often discussed in experimental studies on pulmonary fibrotic and allergic processes associated with changes in numbers of these cells, but information on the normal distribution of metachromatic and tryptase-positive mast cells in the interalveolar septa is scarce. There are no data on the presence of ghrelin in lung mast cells as well as the age-specific features of localisation and the number of mast cells in the interalveolar septa in rats of different ages. Therefore, the purpose of the present study was to determine the distribution of metachromatic, tryptase-, and ghrelin-positive mast cells in the interalveolar septa in 20 day-, 3 month- and 1 year-old rats. Tissue sections stained with toluidine blue had been taken from the left lung to visualise metachromasia and immunohistochemical expression of tryptase and ghrelin. The results showed that the amount of metachromatic mast cells in the interalveolar septa was significantly lower than that of tryptase- and ghrelin-positive cells. This allowed suggesting that mast cells were permanent occupants of the rat lung parenchyma and, on the other hand, the expression of ghrelin in their granules was most likely related to the synthesis of this protein. Our study showed that immunohistochemical identification by tryptase expression was more accurate than toluidine blue staining.

**Key words**: ghrelin, interalveolar septa, lung, mast cells, rat, tryptase

## INTRODUCTION

Rats are frequently used in experimental studies relating to pulmonary fibrosis and allergic processes associated with significant presence of mast cells (MCs) in the airway wall (Ahlstedt *et al*., 1983; Goto *et al*., 1984; Bachelet *et al*., 1988), but information about the normal distribution of metachromatic (toluidine blue stained mast cells – MCTB+) and tryptase positive (MCTr+) mast cells in the interveolar septa in the lung is scarce and contradictory. There is no evidence for the presence of ghrelin in mast cell granules in this organ. Mast cells are widespread cells in connective tissue of organs containing granules with pre-synthesised (chymase, tryptase, histamine, serotonin) and newly synthesised mediators (cytokines and

chemokines) with pro- and anti-inflammatory action. These cells participate in both acquired (Malaviya & Jakschik, 1993; Poncet *et al*., 1999; Lambrecht *et al*., 2000; Vermaelen *et al*., 2001; Stelekati*et al*., 2009) and innate immunity (Marshall, 2004; Hofmann & Abraham, 2009; Fukuda *et al*., 2013; Graham *et al.*, 2013). According to some authors (Ahlstedt *et al*., 1983; Goto *et al*., 1984; Bachelet *et al*., 1988), mast cells could not be found in the interalveolar septa, but according to other authors (Williams *et al*., 1977; Ivanova *et al*., 2019), although in small quantities, mast cells are often found there even in healthy rats.

Ghrelin is a peptide that stimulates appetite in mammals and is involved in many physiological processes, including control of gastric motility and acid secretion in animals and humans (Kojima & Kangawa, 2002; De Ambrogi *et al*., 2003; Gualillo *et al*., 2003). In contrast to the gastrointestinal tract, the expression and role of ghrelin in lung mast cells remain unclear.

There is no data on the presence of ghrelin positive mast cells (MCGhr) in the lung. For the first time, the expression of ghrelin in mast cells, localised in the stomach wall in rats, was demonstrated by Stefanov *et al.* (2017) using colocalisation of tryptase with ghrelin in double immuno-fluorescence staining. These data allowed the authors suggesting ability of MCs to produce, store and release this peptide (Stefanov *et al*., 2017).

The lack of data on age-related specificities regarding the distribution of metachromatic and tryptase-positive mast cells as well as the ability of these cells to synthesise and secrete ghrelin in the rat, motivated this study.

## MATERIALS AND METHODS

### *Experimental animals*

In this study, 18 male Wistar rats were used: 6 at 20 days of age (weighing 150–200 g), 6 at 3 months of age (weighing 290–320 g) and 6 at 1 year of age (weighing 400–450 g), provided from Project No. 13/2017, Medical Faculty, Trakia University. Animals were reared under a 12/12 h light/dark cycle, subjected to a standard diet and receiving water *ad libitum*. All procedures were carried out in accordance with the Bulgarian legislation regarding animal care (Ordinance 20 of 01.11.2012 on the minimum requirements for the protection and welfare of experimental animals and the requirements for the sites for use, breeding and/or delivery) and Directive 2010/63 / EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Animals were anaesthetised with ketamine and xylazine (90 mg/kg + 10 mg/kg, IP), then transcardially perfused with 4% paraformaldehyde in phosphate buffered saline (PBS). The lungs of animals were immediately removed, leaving the left lung in 4% paraformaldehyde for 24 hours, washed with PBS, dehydrated in alcohols, cleared in xylene, and embedded in paraffin.

### *Histological methods*

Serial tissue sections of 5 μm thickness were prepared from each animal, mounted on gelatin coated slides, then deparaffinised twice in xylene and rehydrated by a series of decreasing ethanol concentrations. The prepared sections were processed histochemically with toluidine blue to visualise gamma metachromasia and immunohistochemically for detection of tryptase- and ghrelin expression.

- Histochemical method with toluidine blue for visualisation of metachromatic granules in mast cells

Tissue sections were mounted on gelatinised slides, placed twice in xylene and rehydrated in decreasing ethanol concentrations. For visualisation of metachromasia, the sections were stained in a buffered solution of toluidine blue with pH 3.

- Immunohistochemical method 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 done for 20 minutes. Between steps, sections were washed with an EnVisionFlex Wash Buffer, then incubated in a humidified chamber overnight at 4 °C with primary antibodies: Monoclonal Mouse Anti-Human Serotonin (clone 5HT-H209, DAKO, Denmark) at a dilution of 1:100, Mouse antihuman ghrelin (2F4) at 1:50 dilution, Monoclonal Mouse antihuman mast cell tryptase – 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. 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 (LEIKA DM1000) equipped with a digital camera (LEIKA DFC 290).

Of the three serial sections used, two were stained with tryptase- and ghrelin antibodies (received from Project No. 13/2017, Medical Faculty, Trakia University), and the third was stained with toluidine blue for metachromasia in all age groups.

*Statistical methods*

The number of mast cells in the study was determined on three microscopic fields (×200 with an area of 0.163 $mm^2$) of sections of individual left lungs using a light research microscope (LEIKA DM1000) equipped with a digital camera (LEIKA DFC 290). Raw 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 values less than 0.05 were considered statistically significant. All reported data are presented as mean ± standard deviation (SD).

## RESULTS

Histochemical staining with toluidine blue and immunohistochemical reactions demonstrating tryptase and ghrelin, allowed a comparative study of the distribution of MCTB+, MCTr+, and ghrelin positive cells (GhrC+) in the interalveolar septa (Fig. 1–3).

The differences in the distribution of MCTB+ compared to that of MCTr+ and ghrelin positive cells were illustrated (Fig. 4). MCTB+ count in the interalveolar septa was significantly lower than MCTr+ and GhrC+ counts. In a comparative study on serial sections, a part of MCTr+ and GhrC+ did not show metachromasia. MCTBs+ counts at different ages showed no difference (0.83 ± 0.39 at the age of 20 days, 1.17 ± 0.72 at the age of 3 months, 1.33 ± 0.65 at the age of 1 year, $P>0.05$). However, a statistically significantly higher number ($P<0.0001$) of MCTr+ and GhrC+ was found in 1-year-old rats – 47.50±0.52 and 53.08±2.27, respectively, followed by 3-month-old (30.55±1.44 and 49.18±0.87, respectively) and 20-day-old

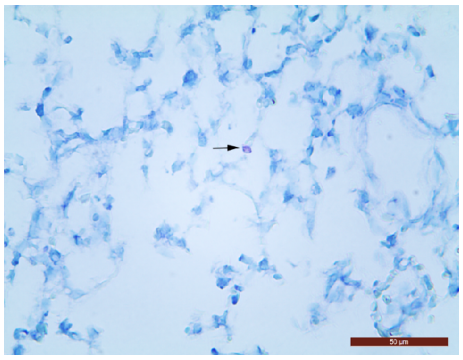

**Fig. 1A**. Single metachromatic (γ-metachromasia) cells (arrow) in interalveolar septa in 20-day-old rats. Bar=50 µm.

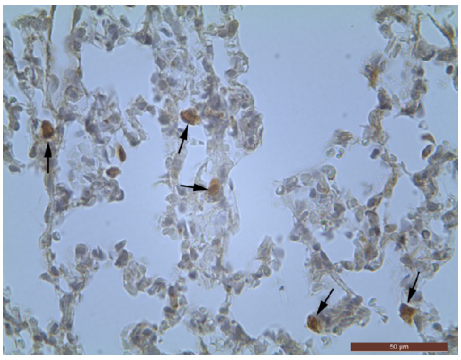

**Fig. 1B.** Tryptase positive mast cells (arrows) in the interalveolar septa in 20-day-old rats. Bar=50 µm.

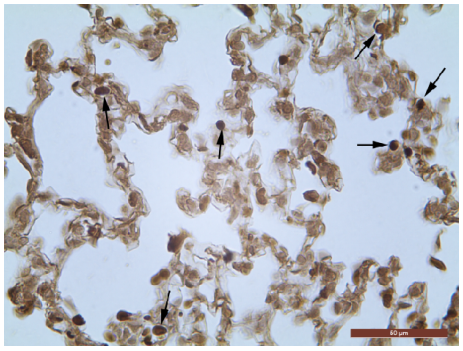

**Fig. 1C.** Ghrelin positive cells (arrows) in the interalveolar septa in 20-day-old rats. Bar=50 µm.

rats (21.50±0.52 and 40.42±1.78, respectively.

The percentage of MCTr+ and GhrC+ with manifested metachromasia varied across age groups. It was similar in all age groups – 4% and 2%, respectively in 20-month-old animals, 3% and 2.5% respectively in 3-month-old animals, and 4% and 2%, respectively in rats at 1 year of age. In studied structures, the age-related percentages of GhrCs positive for tryptase was as followed: at 20 days of age – 53%, at 3 months – 62%, at 1 year – 90%.

## DISCUSSION

The involvement of mast cells in lung fibrotic and allergic processes in rats, which are often used as experimental models, has been reported in a number of studies (Ahlstedt *et al*., 1983; Goto *et al*., 1984; Bachelet *et al*., 1988), but information on the normal distribution of metachromatic, tryptase-and ghrelin-positive mast cells in the intereveolar septa is scarce.

Based on previously reported results and our findings, we have attempted to elucidate several problems related to the distribution of lung mast cells in healthy

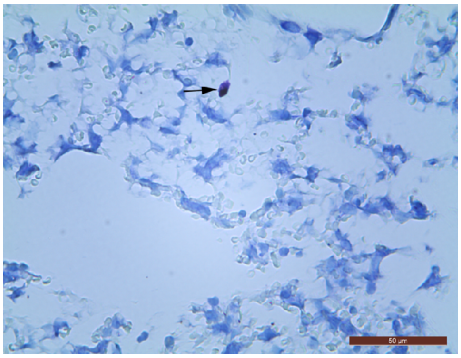

**Fig. 2A.** Single metachromatic (γ-metachromasia) mast cells (arrow) in interalveolar septa in 3-month-old rats. Bar=50 μm.

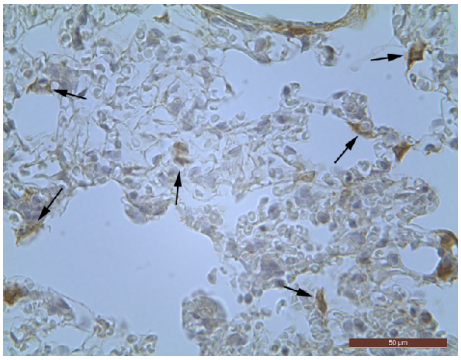

**Fig. 2B.** Tryptase positive mast cells (arrows) in the interalveolar septa in 3-month-old rats. Bar=50 μm.

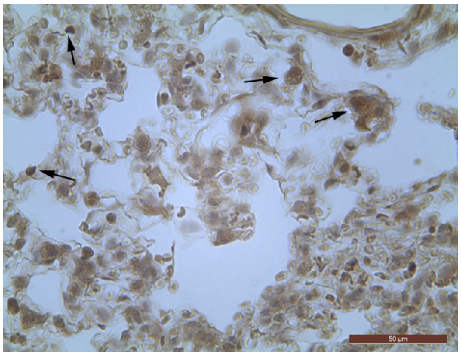

**Fig. 2C.** Ghrelin positive cells (arrows) in the interalveolar septa in 3-month-old rats. Bar=50 μm.

rats. The first is related to the establishment of normal distribution of tryptase- and ghrelin-positive mast cells in interalveolar septa, which resolutely resolve the controversial issue of whether MCs localise or not in rat interalveolar septa, the second problem is related to the quantitative characteristic of three types of mast cells (metachromatic, tryptase- and ghrelin positive) at various ages, and the third problem is related to determining the most accurate method for identifying those cells. The present study confirms the data from a previous study of ours (Ivanova *et al*., 2019), concerning the localisation of metachromatic mast cells in the interalveolar septa in rats. Also, new data were obtained regarding the distribution of metachromatic mast cells in the interalveolar septa in rats of different ages. The histochemical reaction with toluidine blue is known to be widely used to detect mast cells in various organs (Enerback *et al*., 1985).

Data on mast cell distribution in rat lungs are contradictory. Some authors did not observed mast cells in the interalveolar septa (Ahlstedt *et al*., 1983; Goto *et al*., 1984; Bachelet *et al*., 1988). The present study showed that single metachromatic mast cells are located in the interalveolar septa in the lung of rats of different ages confirming the results of other studies describing the same distribution of mast cells (Williams *et al*., 1977; Bienenstock *et al.,* 1985; Ivanova *et al.,* 2019).

Ivanova *et al.* (2019) found serotonin-positive cells in both the respiratory tract and in the interalveolar septa in sexually mature rats. These data support the results of Kushnir-Sukhov *et al.* (2007) on the migration of mast cells through the tissues of the lung, in relation to the ability of serotonin to induce mast cell attachment to fibronectin and the presence of serotonin 5-HT1A receptor in mast cells.

In the present study, the distribution of mast cells in the interalveolar septa was confirmed by toluidine blue staining and by immunohistochemical expression of tryptase. The results of the comparative study showed that the number of mast cells expressing tryptase was significantly higher than the number of metachromatic mast cells, which showed similar values in all three age groups. This suggests that immunohistochemical identification of mast cells by detection of tryptase in rat lung is more reliable than their histochemical identification by toluidine blue.

Co-localisation with tryptase on serial sections showed that most of the GhrC+ are MCTr+. In the interalveolar septa, GhrC+ number was significantly higher than that of MCTr+. It was found out that the percentage of Ghr-positive cells responding to tryptase wsa the highest in 1-year-old animals, followed by 3-month-old and 20-day-old rats (Ivanova, 2019). This finding could be explained, on one hand, by the expression of ghrelin by mast cells, thus confirming the data of Stefanov *et al.* (2017) who demonstrated ghrelin expression by mast cells, and on the other

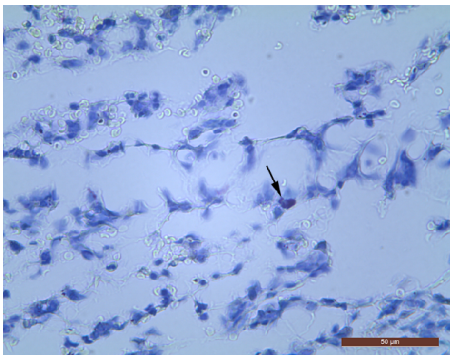

**Fig. 3A.** Single mast cells with γ-metachromasia (arrow) in the interalveolar septa, 1 year of age. Bar=50 μm.

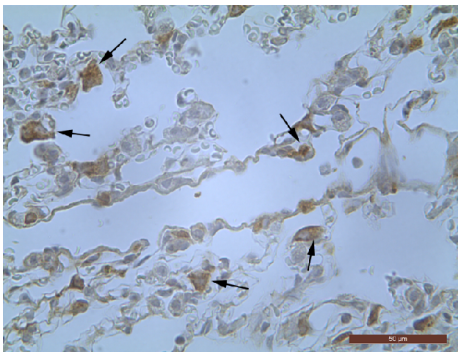

**Fig. 3B.** Tryptase positive cells (arrows) in the intervalveolar septa, 1 year of age. Bar=50 μm.

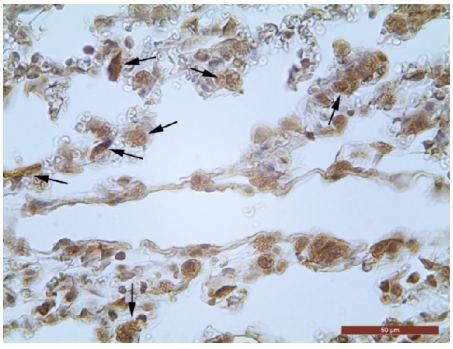

**Fig. 3C.** Ghrelin positive cells (arrows) are more numerous than tryptase positive cells, 1 year of age. Bar=50 μm.

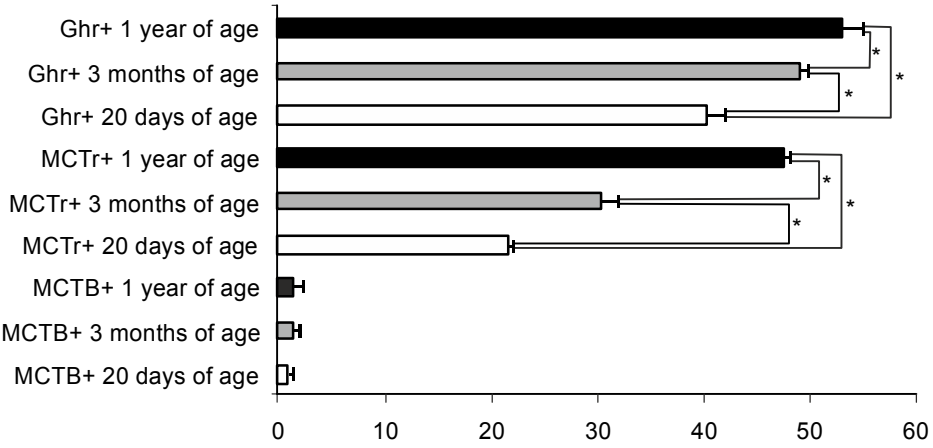


**Fig. 4.** Age dependent differences in number of metachromatic (MCTB+), tryptase positive mast cells (MCTr+) and ghrelin positive (Ghr+) cells in the caudal part of the left lobe of the lung alveolar septa. The data are presented as mean ± standard deviation (SD), n=6; * statistically significant difference at $P<0.0001$.

by the expression of ghrelin by cells other than mastocytes such as lymphocytes (B cells), T cells, monocytes and NK cells (Dixit *et al*., 2004; Hattori, 2009; Taub *et al*., 2010). Stefanov *et al.* (2017) suggested that the additional function of MCs in rat stomach is the expression of ghrelin, and that MCs may produce, store, and release ghrelin like other immune cells. Other studies described ghrelin synthesised by gastric endocrine cells as motilin-bound peptide in gastrointestinal contraction (Borchetta *et al*., 2004). By identifying ghrelin-producing cells in the gastric mucosa and the expression of ghrelin receptor in glandular and gastric muscle cells of human stomach and duodenum, Penkova *et al.* (2016) discussed the ability of ghrelin to directly affect the secretion and motility of these organs.

Based on the above studies and our results, it was hypothesised that ghrelin was involved in the maintenance of lung homeostasis, regulation of vascular contraction and epithelial function, and also in modulation of inflammatory processes.

## CONCLUSION

Histochemical and immunohistochemical studies have shown that mast cells are permanent residents of interalveolar septa in rats showing age-specific features and that the determination of their number depended on the method of study chosen. The obtained numerical data can be used as reference values in experimental studies using sexually mature or sexually immature rats.

The presence of tryptase and ghrelin-positive mast cells in the interalveolar septa outlined the role of these cells as one of the major sources of ghrelin and tryptase in lung tissue and therefore, their role as homeostasis-supporting cells.

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