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Distribution and relative frequency of immunohistochemically detected endocrine cells in the stomach of New Zealand White rabbit (*Oryctolagus cuniculus*)

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Summary

Background: Gastrointestinal (GI) endocrine cells produce many GI hormones that perform various physiological functions of the digestive system. **Aims:** We aimed to investigate the presence and distribution of immunoreactive (IR) endocrine cells to glucagon, somatostatin, cholecystokinin-8 (CCK-8), serotonin, secretin and histamine in the stomach of adult male New Zealand White rabbit (*Oryctolagus cuniculus*). **Methods:** For immunohistochemical staining, peroxidase anti-peroxidase (PAP) method was applied to stomach samples. **Results:** Glucagon-IR cells of closed- and open type were found throughout all the stomach parts examined. Somatostatin-IR cells of closed- and open type in the cardiac and oxyntic glands were localized to deep portions of foveola gastrica. CCK-8 IR cells that were not observed in the cardia and fundus were mostly localized to the glands and lamina epithelialis in the pyloric part near the duodenum. Oval-shaped open and closed type serotonin-IR cells were mostly dispersed throughout the fundic and pyloric glands. Secretin-IR cells were rare in the pyloric and cardiac region although they were not observed in the fundic glands. Histamine-IR cells were rarely found in the cardia, fundus and pylorus. **Conclusion:** Our findings show that glucagon, histamine, somatostatin, secretin and serotonin might be produced by all the stomach regions while pyloric region had only CCK-8 IR. These distribution patterns also provide further evidence of species-specific differences, which might be important from the evolutionary aspect of the digestive tract in relation to evolutional niches and nutrient resources.

Key words: Endocrine cells, Histamine, Immunoreactivity, New Zealand White rabbit, Serotonin

Introduction

The endocrine cells found throughout the digestive tract produce multiple gastrointestinal (GI) hormones that perform a key role in various physiological functions, including gut motility, nutrient absorption and intestinal blood flow (Bell, 1979; Budipitojo *et al*., 2016). The examination of GI endocrine cells would be useful for phylogenetic studies (D'Este *et al*., 1994). Many studies have shown that the relative frequencies and regional distributions of these endocrine cells vary depending on feeding habits and animal species. These studies reveal interspecific variations, suggesting endocrine cell distribution is linked to feeding habits (Solcia *et al*., 1975; Lee *et al*., 2014; Firmiano *et al*., 2017).

Several types of endocrine cells have been displayed in the GI tract of many vertebrates using immunohistochemical methods. Up to now, the distribution of GI endocrine cells has been broadly investigated in many vertebrate species (Cerrarelli *et al*., 1985; Krause *et al*., 1985; Castaldo and Lucini, 1991; Mimoda *et al*., 1998; Nisa *et al*., 2005). In addition, the distribution and relative frequencies of these cells have been studied in various vertebrate species, including the gerbil (Lee *et al*., 2000), hairless mouse (Ku *et al*., 2002), BALC/c mouse (Ku *et al*., 2004), Anatolian ground gerbil, *Spermophilus xanthoprymnus* (Timurkaan *et al*., 2009), thin dogfish, *Oligosarcus hepsetus* (Vieira-Lopes *et al*., 2013), sunda porcupine, *Hystrix javanica* (Budipitojo *et al*., 2016). However, little is known about the distribution and relative frequency of endocrine cells in New Zealand White rabbit (*Oryctolagus cuniculus*), which is commonly being used in many researches. In this study, we sought to find out the relative frequency and distribution of immunoreactive (IR) endocrine cells to glucagon, somatostatin, cholecystokinin-8 (CCK-8), serotonin, secretin and histamine in the stomach of New Zealand White rabbit using immunohistochemistry.

Materials and Methods

Tissue preparation

Stomachs of ten adult male New Zealand White rabbits with a weight range of 2-6 kg purchased from a local rabbit breeder in Isparta, Turkey were used as the material in this study. The study was conducted in accordance with the ethical approval of Experimental Animal Production and Experimental Research Laboratory, Süleyman Demirel University (SDU-HÜDAL B.30.2.SDÜ.0.05.06.00-186). The animals were anesthetized with ketamine/xylazine (80/12 mg/kg). The samples from cardia, fundus and pylorus were fixed in Bouin's solution, dehydrated in ethanol, cleared in

xylene and embedded in paraffin as previously described (Beyaz *et al*., 2017).

Immunohistochemistry

Stomach samples were cut into sections of 5-6 µm thickness using rotary microtome (RM2125RT, Leica, Nussloch, Germany) and placed on gelatin-coated slides. Immunohistochemical staining was performed using the peroxidase anti-peroxidase (PAP) method as described previously (Öztop *et al*., 2018). Briefly, the slides were dewaxed with xylene, and rehydrated through descending series of ethanol. After rinsing with phosphate buffered saline (PBS: 0.01 M, pH = 7.2), the slides were incubated for 20 min in 3% methanolic H_2O_2 to quench endogenous peroxidase activity, and then rinsed in PBS for 5 min three times. Non-specific binding was blocked with normal goat serum at 1:10 dilution (G9023, Sigma, USA) for 30 min. Following drainage of excess serum from the slides, primary antibodies were left to incubate with the sections overnight at 4°C (Table 1). After washing in PBS for 5 min three times, the slides were incubated with goat anti-rabbit IgG antibody at 1:200 dilution (A0545, Sigma, USA). After rinsing in PBS for 5 min three times, the slides were treated with PAP at 1:200 dilution (P1291, Sigma, USA). The resulting signal was developed with 0.05% 3,3 diaminobenzidine (DAB-Plus substrate kit, Invitrogen, Camarillo, CA) for 10 min. Finally, the slides were cleared, dehydrated and mounted in entellan (107960, Merck, Darmstadt, Germany). Immunostaining was evaluated as the presence of a brown color detection chromogen (DAB) and observed under a light microscope (Olympus CX41, Tokyo, Japan). The primary antibodies that we used, together with related information, are listed in Table 1. Dilutions were as specified by the respective manufacturer's instructions. Phosphate buffered saline was substituted for primary antibody as the negative control. All protocols were performed in a humidified chamber at room temperature unless stated otherwise.

Table 1: Antisera used in this study

Primary antibodies *	Catalogue No.	Dilution	Supplier
Glucagon	sc-13091	1:200	Santa Cruz
Somatostatin	sc-13099	1:200	Santa Cruz
CCK-8	C2581	1:200	Sigma
Serotonin	S5545	1:200	Sigma
Secretin	sc-20938	1:200	Santa Cruz
Histamine	H7403	1:200	Sigma

* All primary antibodies were raised in rabbits and were polyclonal. CCK-8: Cholecystokinin-8

Semi-quantitative evaluations

Stained and control sections were observed under a light microscope (Olympus CX41, Tokyo, Japan) equipped with a DP72 digital camera (Olympus, Tokyo, Japan). Distributions of cells IR to glucagon, somatostatin, CCK-8, serotonin, secretin, and histamine in cardia, fundus and pylorus were evaluated semiquantitatively from five separate fields of each slide selected. Immunoreactive endocrine cells with discernible nuclei were taken into consideration only. As described previously (Özbek *et al*., 2018), the existence of each type of IR cell was graded as:

- : No positive cells
+ : A few positive cells but not in every field investigated
++ : Rare in number
+++ : Moderate
++++ : Numerous

Then, photographs were taken from relevant parts.

Results

Immunohistochemistry revealed that there were significant differences in both distribution and frequency of the IR endocrine cells. These cells are generally oval shaped and their cytoplasm cannot reach the lumen. These cells are called as closed-type. However, cytoplasm of fewer cells reaches the lumen. These cells are called as open-type. Table 2 shows regional distribution and relative frequencies of the endocrine cells in the cardia, fundus and pylorus of the New Zealand White rabbit.

Table 2: Regional distribution and relative frequencies of the endocrine cells in the cardia, fundus and pylorus

Antibodies	Cardia		Fundus		Pylorus	
	LE	G	LE	G	LE	G
Glucagon	+	++	+	+	++	+
Somatostatin	+	++++	+	+++	+++	++++
CCK-8	–	–	–	–	++	++++
Serotonin	+	+++	+	+++	+	+++
Secretin	+	+	–	+	–/+	+
Histamine	–/+	–/+	–/+	+	+	–/+

- : No positive cells, + : A few positive cells but not in every field investigated, ++ : Rare in number, +++ : Moderate, and ++++ : Numerous. LE: Lamina epithelialis, and G: Gland

Labeled glucagon cells

Glucagon-IR cells were found throughout all the stomach parts investigated. Most of the glucagon-IR cells (closed-type) present in the cardia and fundus could not reach the lumen. On the contrary, most of the glucagon-IR cells (open-type) in the pylorus could reach the lumen. While glucagon-IR cells were typically spindle- and oval-shaped in the cardiac, fundic (or oxyntic) and pyloric glands, they were spindle-shaped in their lamina epithelialis (Figs. 1A-C).

Labeled somatostatin cells

Somatostatin-IR cells with oval to spindle-shaped in the cardiac and oxyntic glands were localized to deep portions of foveola gastrica. Most of the somatostatin-IR cells in these glands could not connect to the lumen but few somatostatin-IR cells could reach the lumen. In the pylorus, these cells were located in the lamina epithelialis and the glands. They were both of open and closed type (Figs. 2A-C).

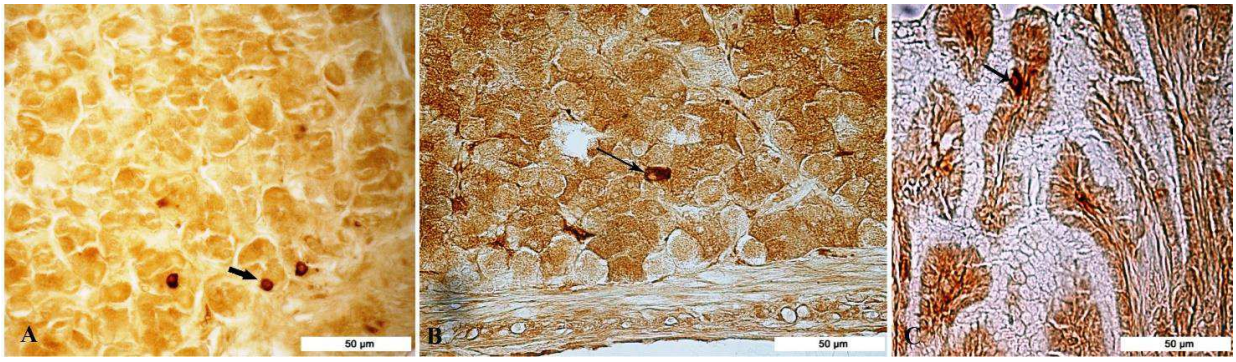

Fig. 1: Glucagon immunostained cells. Glucagon-IR closed-type cells in the cardia (**A**), fundus (**B**), and open-type cell in pylorus (**C**) of New Zealand White rabbit. Arrows indicate immunoreactive cells for glucagon (scale bars, 50 µm; PAP method)

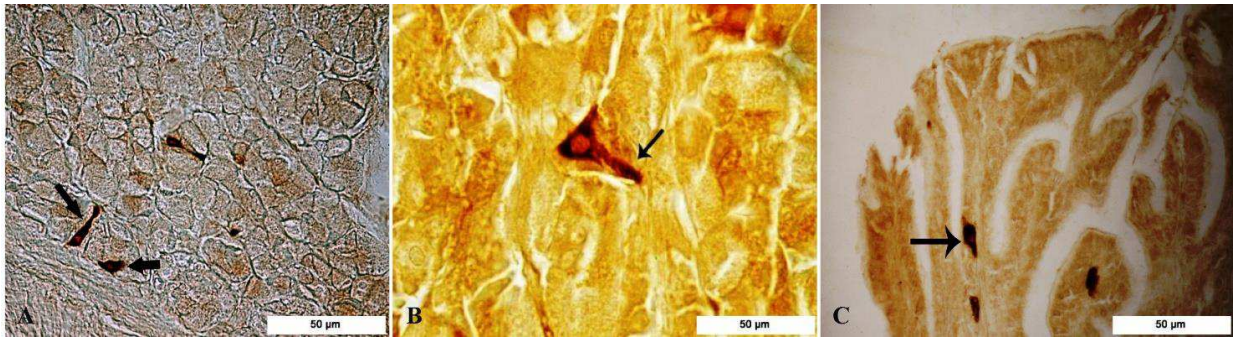

Fig. 2: Somatostatin immunostained cells. Somatostatin-IR open-type cells in the cardia (**A**), fundus (**B**), and pylorus (**C**) of New Zealand White rabbit. Arrows indicate immunoreactive cells for somatostatin (scale bars, 50 µm; PAP method)

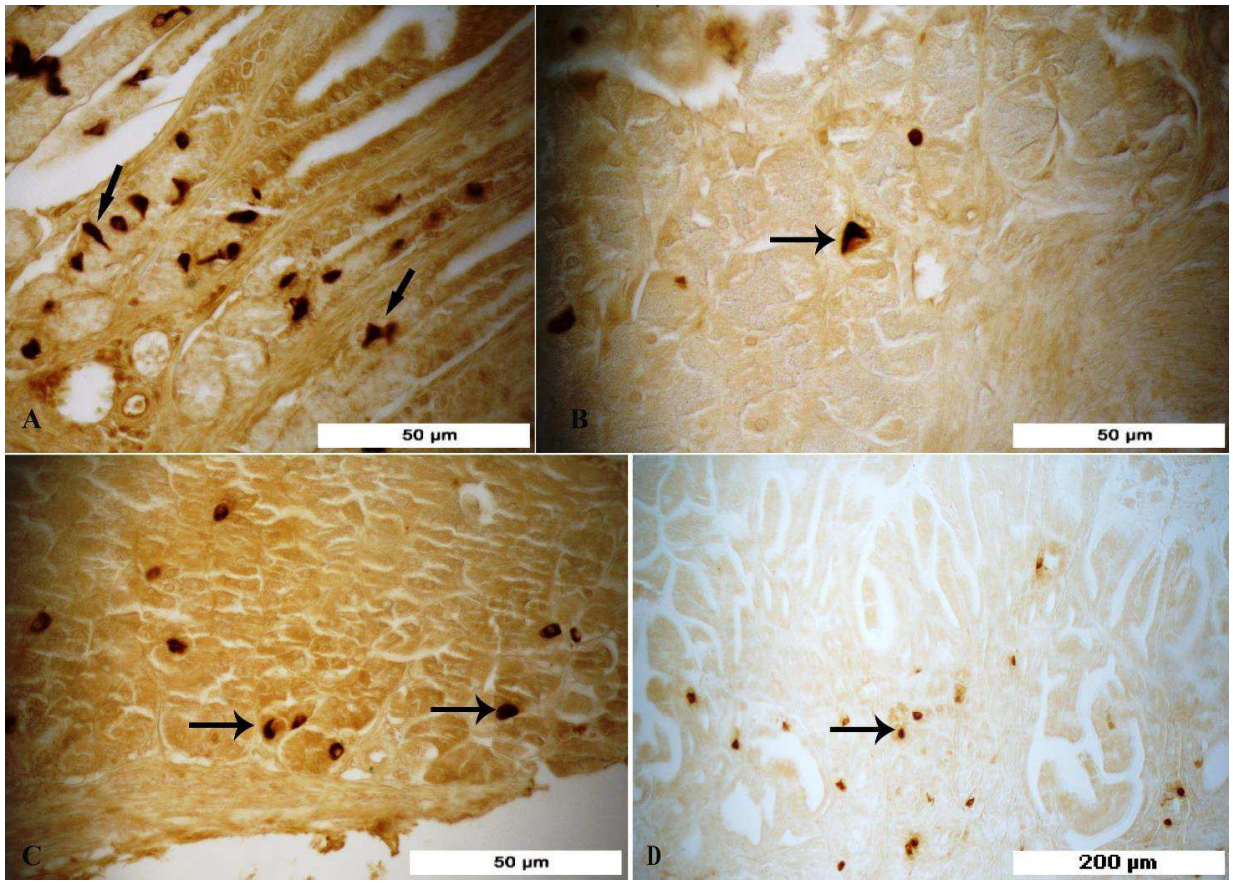

Fig. 3: CCK-8 and serotonin immunostained cells. CCK-8 IR open-type cells in the fundus (**A**), serotonin-IR open-type cells in the cardia (**B**), closed-type cells in the fundus (**C**), and pylorus (**D**) of New Zealand White rabbit. Arrows indicate immunoreactive cells for CCK-8 and serotonin (scale bars, 50 µm (**A**, **B** and **C**), and 200 µm (**D**); PAP method)

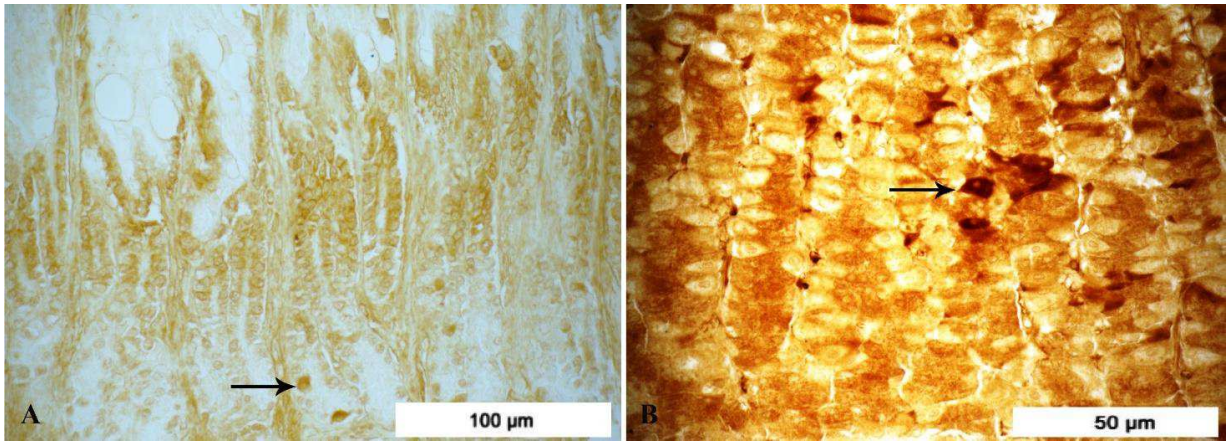

Fig. 4: Secretin immunostained cells. Secretin-IR cells in the fundus (**A**), and pylorus (**B**) of New Zealand White rabbit. Arrows indicate immunoreactive cells for secretin (scale bars, 100 µm (**A**), and 50 µm (**B**); PAP method)

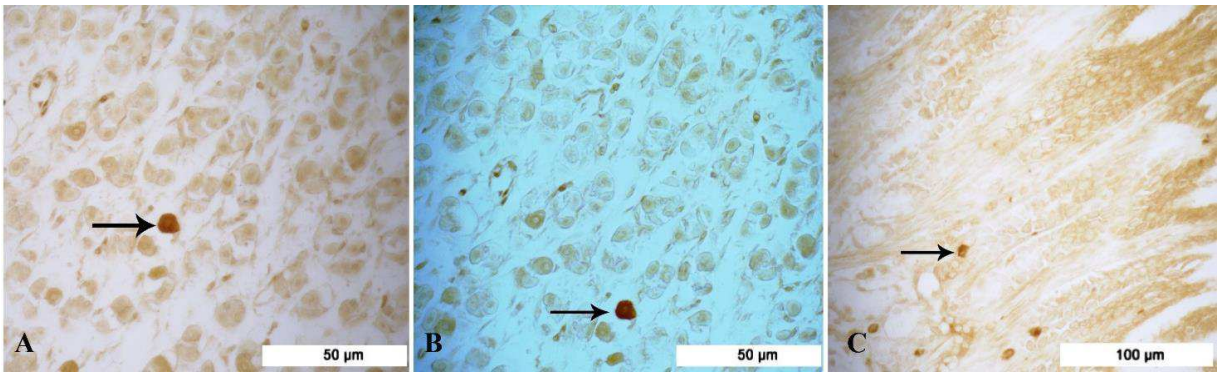

Fig. 5: Histamin immunostained cells. Histamin-IR cells in the cardia (**A**), fundus (**B**), and pylorus (**C**) of New Zealand White rabbit. Arrows indicate immunoreactive cells for histamin (scale bars, 50 µm (**A** and **B**), and 100 µm (**C**); PAP method)

Labeled CCK-8 cells

CCK-8 cells that were not observed in the cardia and fundus were mostly localized to the glands and lamina epithelialis in the pyloric part near the duodenum. These cells of open and closed type were oval and spindle in shape (Fig. 3A).

Labeled serotonin cells

In the cardia, most of the serotonin-IR cells with oval-shape in the lamina epithelialis and the glands could not open into the lumen but few IR cells could reach the lumen. Oval-shaped open and closed type serotonin-IR cells were mostly distributed throughout the fundic and pyloric glands (Figs. 3B-D).

Labeled secretin cells

Secretin-IR cells were rare in the pyloric and cardiac region although they were not observed in the fundic glands. These cells had oval and spindle-shape (Figs. 4A-B).

Labeled histamine cells

Oval and spindle-shaped histamine-IR cells were rarely found in the cardia, fundus and pylorus. In addition, histamine-IR connective tissue mast cells were observed in lamina propria of the pylorus region (Figs. 5A-C).

Discussion

We identified six types of endocrine cells IR for glucagon, somatostatin, CCK-8, serotonin, secretin and histamine in the cardia, fundus and pylorus of the New Zealand White rabbit. The cell types and shapes observed here were similar to those reported in other mammal and non-mammalian species (Agungpriyono *et al*., 1994; Baltazar *et al*., 1998; Agungpriyono *et al*., 2000; Nisa *et al*., 2005).

Glucagon-IR cells have been shown in many mammalian and non-mammalian species. Few glucagon-IR cells were found in cardiac and fundic glands of Korean tree squirrel (Lee *et al*., 1991), in the pylorus and fundus of babirusa, *Babyrousa babyrussa* (Agungpriyono *et al*., 2000), red-eared slider, *Trachemys scripta elegans* (Ku *et al*., 2001), C57BL/6 mice (Ku *et al*., 2003), insectivorous bat (Santos *et al*., 2008), and ddN mice (Ku *et al*., 2010) and in the oxyntic glands of musk shrew, *Suncus murinus* (Kitamura *et al*., 1990). Our finding showing the closed-type glucagon-IR cells in the fundus are consistent with those reported for musk shrew (Kitamura *et al*., 1990), hairless mice (Ku *et al*., 2002), C57BL/6 mice (Ku *et al*., 2003), Balb/c mice (Ku *et al*., 2004), Balb/c-nu/nu mice (Ku *et al*., 2006), Anatolian ground squirrel *S. xanthoprymnus* (Timurkaan *et al*., 2009) and ddN mouse (Lee *et al*., 2010). It has

been reported that closed type glucagon-IR cells were present in the pyloric region of ddN mouse (Lee *et al*., 2010), while open type glucagon-IR cells were dispersed in the pyloric glands of insectivorous bat (Santos *et al*., 2008) in a similar way to our findings. Taken together, distribution of glucagon-IR cells in the stomach regions of mammals and non-mammals exhibits species-dependent variation.

Yaman *et al*. (2007) found that somatostatin-IR cells were not present in the stomach regions of porcupine, *Hystrix cristata,* although moderate somatostatin-IR cells had been demonstrated in the stomach regions of the common tree shrew, *Tupaia belangeri* (Yamada *et al*., 1999) and the babirusa *B. babyrussa* (Agungpriyono *et al*., 2000). Consistent with these reports, we found evidence of moderate to numerous-IR cells throughout all the glands. Although somatostatin-IR cells were rarely found in the fundus of the musk shrew, *S. murinus* (Kitamura *et al*., 1990), Balb/c mouse (Ku *et al*., 2004) and the barking deer, *Muntiacus muntjak* (Adnyane *et al*., 2011), moderate-IR cells were located in the same region in the red-eared slider, hairless mice, Anatolian ground squirrel and ddN mice (Ku *et al*., 2001; Ku *et al*., 2002; Timurkaan *et al*., 2009; Lee *et al*., 2010). As in the present study, numerous-IR cells were distributed throughout the fundus of C57BL/6 mice (Ku *et al*., 2003) and Balb/c-nu/nu mice (Ku *et al*., 2006). Similar to our findings, numerous somatostatin-IR cells were detected in the pylorus of the Manchurian Chipmunk, *Tamias sibiricus barberi* (Lee *et al*., 1998) and the Grass Lizard, *Takysromus wolteri* (Lee and Ku, 2004). But these cells were rarely seen in the pylorus of the Mongolian gerbil, *Meriones unguiculatus* (Lee *et al*., 2000). Our findings are consistent with the studies in which open and closed somatostatin-IR cells were detected in the fundus of C57BL/6 mice (Ku *et al*., 2003) and Anatolian ground squirrel (Timurkaan *et al*., 2009). On the other hand, it has been reported that closed-type cells were localized to the fundus of hairless mice (Ku *et al*., 2002) and Balb/c-nu/nu mice (Ku *et al*., 2006).

It has been established that while few CCK-8 IR cells were present in the fundus and pylorus of the red-eared slider (Ku *et al*., 2001) and the barking deer, *M. muntjak* (Adnyane *et al*., 2011), these cells were detected in the same regions of the babirusa, *B. babyrussa* at a moderate frequency (Agungpriyono *et al*., 2000). Consistent with our findings, however, absence of CCK-8 IR cells has been reported in the fundus of hairless mice (Ku *et al*., 2002), Balb/c mice (Ku *et al*., 2004), insectivorous bats (Santos *et al*., 2008) and ddN mice (Lee *et al*., 2010). These findings show that CCK-8 immunoreactivity may vary from species to species, and relate to their feeding habits. Our finding showing numerous CCK-8 IR cells in the pylorus is similar to those reported for the same region of Korean tree squirrel, *Sciurus vulgaris*, wild boar, gerbil, hairless mice, Balb/c mice, the grass lizard, *T. wolteri* (Lee and Ku, 2004), insectivorous bats and ddN mice (Lee *et al*., 1991; Dall'Aglio *et al*., 1998; Lee *et al*., 2000; Ku *et al*., 2002; Ku *et al*., 2004; Santos *et al*., 2008; Lee *et al*., 2010). But these cells were not found in the stomach regions of the musk shrew, *S. murinus* (Kitamura *et al*., 1990), the common tree shrew, *T. belangeri* (Yamada *et al*., 1999) and the porcupine, *H. cristata* (Yaman *et al*., 2007). This study also revealed open and closed type CCK-8 IR cells localized in the pylorus, similar to the findings obtained in the wild boar (Dall'Aglio *et al*., 1998) and ddN mice (Lee *et al*., 2010). On the other hand, open type CCK-8 IR cells were found to be localized to the pylorus of insectivorous bats (Santos *et al*., 2008) and the barking deer, *M. muntjak* (Adnyane *et al*., 2011).

Numerous serotonin-IR cells were detected in the stomach regions of common tree shrew, *T. belangeri* (Yamada *et al*., 1999), the babirusa, *B. babyrussa* (Agungpriyono *et al*., 2000), red-eared slider (Ku *et al*., 2001), C57BL/6 mice (Ku *et al*., 2003), Balb/c mice (Ku *et al*., 2004), the porcupine, *H. cristata* (Yaman *et al*., 2007), insectivorous bats (Santos *et al*., 2008) and the barking deer, *M. muntjak* (Adnyane *et al*., 2011). Moderate serotonin-IR cells were scattered throughout the stomach regions of the musk shrew, *S. murinus* (Kitamura *et al*., 1990), the Philippine carabao, *Bubalus bubalis* (Baltazar *et al*., 1998), the manchurian chipmunk, *T. sibiricus barberi* (Lee *et al*., 1998), wild boar (Dall'Aglio *et al*., 1998), hairless mice (Ku *et al*., 2002), the porcupine, *H. cristata* (Timurkaan *et al*., 2005) and ddN mice (Lee *et al*., 2010), as numerous-IR cells detected in the present study. Closed-type serotonin-IR cells were dispersed in the fundus of hairless mice (Ku *et al*., 2002), C57BL/6 mice (Ku *et al*., 2003), Balb/c mice (Ku *et al*., 2004), Balb/c-nu/nu mice (Ku *et al*., 2006) and the barking deer, *M. muntjak* (Adnyane *et al*., 2011), while our study revealed both open- and closed-type serotonin-IR cells in the same region. The present study found evidence of both the open- and closed-type serotonin-IR cells in the pyloric region, parallel to the findings obtained from wild boar (Dall'Aglio *et al*., 1998), hairless mice (Ku *et al*., 2002), C57BL/6 mice (Ku *et al*., 2003) and the barking deer, *M. muntjak* (Adnyane *et al*., 2011). On the other hand, open-type serotonin-IR cells were observed in the pylorus region of Balb/c mice (Ku *et al*., 2004), the porcupine, *H. cristata* (Timurkaan *et al*., 2005) and Balb/c-nu/nu mice (Ku *et al*., 2006).

This study showed few secretin-IR cells expressed by the stomach regions. However, secretin-IR cells were not detected in the stomach of the musk shrew, *S. murinus* (Kitamura *et al*., 1990), the king's skink, *Egernia kingii* (Arena *et al*., 1990), the common tree shrew, *T. belangeri* (Yamada *et al*., 1999), the babirusa, *B. babyrussa* (Agungpriyono *et al*., 2000), red-eared slider (Ku *et al*., 2001), hairless mice (Ku *et al*., 2002) and the grass lizard, *T. wolteri* (Lee and Ku, 2004).

Tahara *et al*. (2000) reported that numerous histamine-IR cells were present in the lamina epithelialis of the stomach, being moderate-IR cells in the gastric glands of guinea pig. However, histamine-IR cells were not found in rat stomach (Fan and Iseki, 1999). Hakanson *et al*. (1986) found that histamine-IR cells were numerous in the rat gastric mucosa and were

moderate in the gastric mucosa of pig, mouse, hamster, guinea pig, hedgehog, rabbit and cat.

To conclude, the regional distribution and relative frequency of the endocrine cells in the cardia, fundus and pylorus of the New Zealand White rabbit was similar to those observed for other mammalian and non-mammalian species with the same feeding habits, but interspecific differences were noted. These differences could be important in grasping the evolution of the digestive tract in relationship to the partitioning by evolutional niches and nutrient resources. These findings might also contribute to future studies to clarify whether there is any mechanistic and functional link between feeding habits, endocrine cells' frequency and regional distribution. It would seem that species' feeding habits have an important impact on the distribution of these endocrine cells that are highly important in the integrated regulation and maintenance of the GI functions.

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Conceived and designed the experiments: S. Türk, K. Çınar. Performed the experiments: S. Türk. Analyzed the data: S. Türk, K. Çınar, M. Öztop. Contributed reagents/materials/analysis tools: S. Türk, K. Çınar, M. Öztop. Wrote the paper: M. Öztop. This study was supported by the Süleyman Demirel University Scientific Research Projects Coordination Unit Presidency (Project No.: 3297-D1-12).

Conflict of interest

No potential conflicts of interest were disclosed.

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