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• *BASIC RESEARCH* •

Immunohistochemical study on gastrointestinal endocrine cells of four reptiles

Xu-Gen Huang, Xiao-Bing Wu

Xu-Gen Huang, Xiao-Bing Wu, Key Laboratories on Conservation and Use of Important Biological Resources of Anhui Province, College of Life Science, Anhui Normal University, Wuhu 241000, Anhui Province, China
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Correspondence to: Professor Xiao-Bing Wu, Key Laboratories on Conservation and Use of Important Biological Resources of Anhui Province, College of Life Science, Anhui Normal University, Wuhu 241000, Anhui Province, China. wuxb@mail.ahnu. edu.cn
Telephone: +86-553-3836873 Fax: +86-553-3869298
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Abstract

AIM: To clarify the types, regional distributions and distribution densities as well as morphological features of gastrointestinal (GI) endocrine cells in various parts of the gastrointestinal track (GIT) of four reptiles, *Gekko japonicus*, *Eumeces chinensis*, *Sphenomorphus indicus* and *Eumeces elegans*.

METHODS: Paraffin-embedded sections (5 μm) of seven parts (cardia, fundus, pylorus, duodenum, jejunum, ileum, rectum) of GIT dissected from the four reptiles were prepared. GI endocrine cells were revealed by using immunohistochemical techniques of streptavidin-peroxidase (S-P) method. Seven types of antisera against 5-hydroxytryptamine (5-HT), somatostatin (SS), gastrin (GAS), glucagon (GLU), substance P (SP), insulin and pancreatic polypeptide were identified and then GI endocrine cells were photomicrographed and counted.

RESULTS: The GI endocrine system of four reptiles was a complex structure containing many endocrine cell types similar in morphology to those found in higher vertebrates. Five types of GI endocrine cells, namely 5-HT, SS, GAS, SP and GLU immunoreactive (IR) cells were identified in the GIT of *G. japonicus, E. chinensis* and *S. indicus*; while in the GIT of *E. elegans* only the former three types of endocrine cells were observed. No PP- and INS- IR cells were found in all four reptiles. 5-HT-IR cells, which were most commonly found in the pylorus or duodenum, distributed throughout the whole GIT of four reptiles. However, their distribution patterns varied from each other. SS-IR cells, which were mainly found in the stomach especially in the pylorus and/or fundus, were demonstrated in the whole GIT of *E. chinensis*, only showed restricted distribution in the other three species. GAS-IR cells, with a much restricted distribution, were mainly demonstrated in the pylorus and/or the proximal small intestine of four reptiles. GLU-IR cells exhibited a limited and species-dependent variant distribution in the GIT of four reptiles. SP-IR cells were found throughout the GIT except for jejunum in *E. elegans* and showed a restricted distribution in the GIT of *G. japonicus* and *S. indicus*. In the GIT of four reptiles the region with the highest degree of cell type heterogeneity was pylorus and most types of GI endocrine cells along the GIT showed the peak density in pylorus as well.

CONCLUSION: Some common and unique features of the distribution and morphology of different types of GI endocrine cells are found in four reptiles. This common trait may reflect the similarity in digestive physiology of various vertebrates.

Key words: Immunohistochemistry; Gastrointestinal tract; Endocrine cells; Distribution patterns; Four reptiles

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INTRODUCTION

Today more than 30 GI hormone genes and a multitude of GI hormones have been recognized, thus making the GIT the largest endocrine organ in the body[1]. GI hormones as regulatory peptides appear to be the major components of body integration and have important regulatory actions on physiological function of the GIT[2-6]. In recent years, the field of GI hormones expanding at a dazzling speed has become an important domain in gastroenterology. Some GI dysfunctions are related to GI hormones[7]. Some GI hormones have synergistic expression in gastric carcinoma and take part in the occurrence of gastric carcinoma[8,9]. Furthermore, some GI hormones in combination with their peptide analogs are helpful in developing new diagnostic and therapeutic strategies[10]. The physiological role of hormonal messengers, peptide receptors and their potential involvement in disease are under investigation.

GI endocrine cells, dispersed in epithelia and gastric gland of the GIT, are the anatomical units responsible for the production of GI hormones. Since pathological changes

of GIT also correlate with the changes of GI endocrine cells[11-15], the investigation of GI endocrine cells has been considered as an important part of GI hormone studies and can provide baseline data for basic research in gastroenterology.

It is generally accepted that GI endocrine cells are remarkably different in regional distribution, relative frequency, cell types, and each regional part of the GIT. Many studies have elucidated the regional distribution and relative frequency of different endocrine cells in the GIT of other vertebrates, such as fishes, amphibians[16,17], especially mammals[18-22]. Some studies on regional distribution and relative frequency or distribution density of GI endocrine cells of reptilian species have also been carried out[23-28], because they are centrally placed on the vertebrate phylogenetic tree.

The present study was conducted to clarify the types, regional distribution, distribution densities and morphology of GI endocrine cells in the GIT of *G. japonicus*, *E. chinensis*, *S. indicus* and *E. elegans* by specific immunohistochemical methods, and to provide baseline data not only for further morphological studies but also for physiological and pathological research on the digestive system of reptilians.

MATERIALS AND METHODS

Specimens and sections

G. japonicus (n = 5, body length: 130±14 mm) in the campus of Anhui Normal University, *E. chinensis* (n = 3, body length: 180±20 mm) in the campus of the 9th Middle School in Wuhu City, *S. indicus* (n = 5, body length: 190±18 mm) and *E. elegans* (n = 5, body length: 150±10 mm) in Langya Mountain were collected in summer and autumn. The animals were lightly anesthetized with ether and decapitated with their cardia, fundus, pylorus, duodenum, jejunum, ileum and rectum dissected and fixed in Bouin's fluid for 24 h. After being dehydrated through an ethanol-xylene series the specimens were embedded in paraffin. Sections were cut at 5 μm thickness and mounted on gelatin-coated slides.

Antisera and reagents

The details of the seven antisera and main reagents used in this study are listed in Table 1.

Immunohistochemical staining

Each representative section was deparaffinized, rehydrated and immunostained with the S-P method. Endogenous peroxidase activity was blocked by incubating the sections with 0.3% H_2O_2 in methanol for 10 min. The non-specific reaction was blocked with normal goat serum prior to overnight incubation at 4 ℃ with the primary antiserum (Table 1). After being rinsed in phosphate-buffered saline (PBS; 0.01 mol/L, pH 7.4), the sections were incubated for 15 min at room temperature with biotinylated goat anti-rabbit IgG serum for all antisera, while biotinylated goat anti-mouse IgG serum was used for insulin. They were then washed in PBS and incubated for 15 min with S-P. Peroxidase reaction was carried out in a solution of DAB containing 0.01% H_2O_2 in Tris-HCl buffer (0.05 mol/L, pH 7.6). After the being lightly counterstained with Mayer's hematoxylin, the sections were dehydrated and cover-slipped.

Experiment control

To investigate the specificity of the reactions, negative and positive controls were used. By replacement of the primary antisera with normal goat serum and PBS, negative control was set. A positive control was also established using tissue sections from the GIT of tortoise containing the hormones being studied. The positive reaction cells were colored brown.

Observation, photomicrograph and cell count

All animals were used for each of the four reptiles studied, seven specimens of each reptile were observed and photomicrographed under the Olympus BX-51 photomicroscope. The dark-brown positive reaction cells on the sections were counted under 10×40 times field. The average number of positive reaction cells from the 10 fields selected randomly in each specimen was the number of IR cells in each reptile. Then the average number of all the reptiles was again quantified as distribution density of IR cells.

Statistical analysis

Data were expressed as mean±SD and variance analysis was performed using SPSS 11.0 software. One-way analysis of variance was used for multiple comparisons, and Duncan's test was used for intra-group comparisons. $P<0.05$ was considered statistically significant.

RESULTS

Types, distributional feature of GI endocrine cells in GIT of four reptiles

Table 1 Details of antisera and main reagents used

Antisera and reagent	Donor	Code number	Working dilution	Source
Human 5-HT	Rabbit	ZA-0231	1:100	ZYMED Lab. Inc., USA
Human GAS	Rabbit	ZA-0115	1:50	Same as above
Human SS	Rabbit	ZA-0232	1:50	Same as above
Human PP	Rabbit	ZA-0211	1:50	Same as above
Human SP	Rabbit	ZA-0235	Working solution	Same as above
Human GLU	Rabbit	ZA-0119	1:100	Same as above
Human INS	Mouse	ZM-0155	1:50	Same as above
S-P Kit		SP-9001	Working solution	Zhongshan Biotech Co. LTD., Beijing, China
S-P Kit		SP-9002	Working solution	Same as above
DAB		ZLI-9030	0.6 g/L	Same as above

SP-9002 was only used in detecting INS-IR cells.

Five types of endocrine cells namely 5-HT-, SS-, GAS-, SP- and GLU-IR cells were identified in the GIT of *G. japonicus*, *E. chinensis* and *S. indicus*. However, in the GIT of *E. elegans* only 5-HT-, SS-, GAS-IR cells were found. PP- and INS-IR cells were not detected in the GIT of the four reptiles. The regional distribution and distribution density of GI endocrine cells in the GIT of four reptiles are listed in Tables 2-5. No positive reactions were detected in the negative control sections.

5-HT-IR cells detected throughout the whole GIT of four reptiles were the most predominant GI endocrine cells. These cells showed the highest density in pylorus of four reptiles except for *G. japonicus* in which the most abundant cells were observed in the duodenum. However, the distribution pattern of 5-HT-IR cells in the GIT varied considerably in four reptiles (Figure 1). SS-IR cells were demonstrated in the whole GIT of *E. chinensis*, but only showed restricted distribution in the other three. They were found in stomach and duodenum of *G. japonicus* and only in stomach of *S. indicus* and *E. elegans*.

Table 2 Distribution and density of endocrine cells in GIT of *G. japonicus* (cells/400× field, mean±SD)

	5-HT	SS	GAS	GLU	SP	PP	INS
Cardia	4.8±0.8	5.5±1.9	0	4.3±1.2	3.6±1.1	0	0
Fundus	5.6±1.4	6.0±1.2	0	11.7±0.7	14.6±1.8	0	0
Pylorus	4.1±1.5	9.6±1.0	0	16.3±2.2	11.2±2.2	0	0
Duodenum	8.6±2.2	1.0±0.5	3.8±0.8	0	0	0	0
Jejunum	7.0±2.9	0	1.4±0.5	0	0	0	0
Ileum	7.8±1.2	0	0	0	0	0	0
Rectum	7.0±1.6	0	0	0	0	0	0

Table 3 Distribution and density of endocrine cells in GIT of *E. chinensis* (cells/400× field, mean±SD)

	5-HT	SS	GAS	GLU	SP	PP	INS
Cardia	9.9±2.0	4.0±0.6	0	0	6.1±1.7	0	0
Fundus	10.4±1.4	6.8±1.4	0	0.5±0.5	5.1±0.9	0	0
Pylorus	13.2±2.4	14.0±0.6	6.6±3.5	0	5.6±2.6	0	0
Duodenum	11.8±2.3	1.6±0.3	0	0	2.1±0.7	0	0
Jejunum	9.0±3.0	0.3±0.4	0	0	0	0	0
Ileum	7.3±0.8	7.5±0.9	0	7.9±1.7	8.0±0.8	0	0
Rectum	5±1.1	2.7±0.9	0	3.8±1.4	3.3±1.4	0	0

Table 4 Distribution and density of endocrine cells in GIT of *S. indicus* (cells/400× field, mean±SD)

	5-HT	SS	GAS	GLU	SP	PP	INS
Cardia	4.1±1.5	2.8±1.0	0	0	0	0	0
Fundus	3.2±1.7	1.8±0.8	0	0	0	0	0
Pylorus	11.3±2.7	3.7±1.2	4.6±4.1	0	0	0	0
Duodenum	3.0±0.9	0	1.7±1.4	0	0	0	0
Jejunum	1.6±1.0	0	0	0	0	0	0
Ileum	3.1±1.1	0	0	1.4±0.7	0	0	0
Rectum	7.0±2.4	0	0	6.5±1.7	7.5±2.3	0	0

Table 5 Distribution and density of endocrine cells in GIT of *E. elegans* (cells/400× field, mean±SD)

	5-HT	SS	GAS	GLU	SP	PP	INS
Cardia	1.6±1.1	1.1±1.2	0	0	0	0	0
Fundus	4.2±1.0	2.3±0.7	0	0	0	0	0
Pylorus	9.1±1.7	4.6±1.9	21.4±11.6	0	0	0	0
Duodenum	3.1±0.9	0	1.3±1.1	0	0	0	0
Jejunum	1.4±1.4	0	0	0	0	0	0
Ileum	3.4±1.6	0	0	0	0	0	0
Rectum	5.2±2.0	0	0	0	0	0	0

SS-IR cells were most predominant in pylorus of four reptiles except for *G. japonicus* in which most SS-IR cells were detected in fundus (Figure 2). GAS-IR cells showed limited distribution in the GIT of four reptiles and were confined to pylorus of *E. chinensis*, pylorus and duodenum of *S. indicus* and *E. elegans*, duodenum and jejunum of *G. japonicus*, respectively. In the pylorus of *E. elegans*, GAS-IR cells showed the highest density (Figure 3). GLU-IR cells were detected in the whole stomach of *G. japonicus*, in ileum and rectum of *S. indicus*, in ileum and rectum, and rarely in fundus of *E. chinensis* (Figure 4). SP-IR cells distributed throughout the GIT except for jejunum of *E. elegans*, and were found in stomach of *G. japonicus* and in rectum of *S. indicus* respectively (Figure 5). In the GIT of four reptiles, the region with the highest degree of cell type heterogeneity was the pylorus and most types of GI endocrine cells in the GIT showed the peak density in pylorus as well.

Morphological features and localization of GI endocrine cells in GIT of four reptiles

The GI endocrine system of four reptiles contained many endocrine cell types similar in morphology to those found in higher vertebrates. The endocrine cells showed the characteristic form of both open and close types. They appeared as close-type cells as they did not possess lamina contact with their apical cytoplasmic processes and as open-type cells with apical cytoplasmic processes that reached the glandular or intestinal lumen. The GI endocrine cells were round, oval, triangular, spindle-, shuttle- or flask-like in shape. They were mostly found in gastric glands and intestinal epithelium, and occasionally in gastric epithelia. In gastric glands, the GI endocrine cells were located between

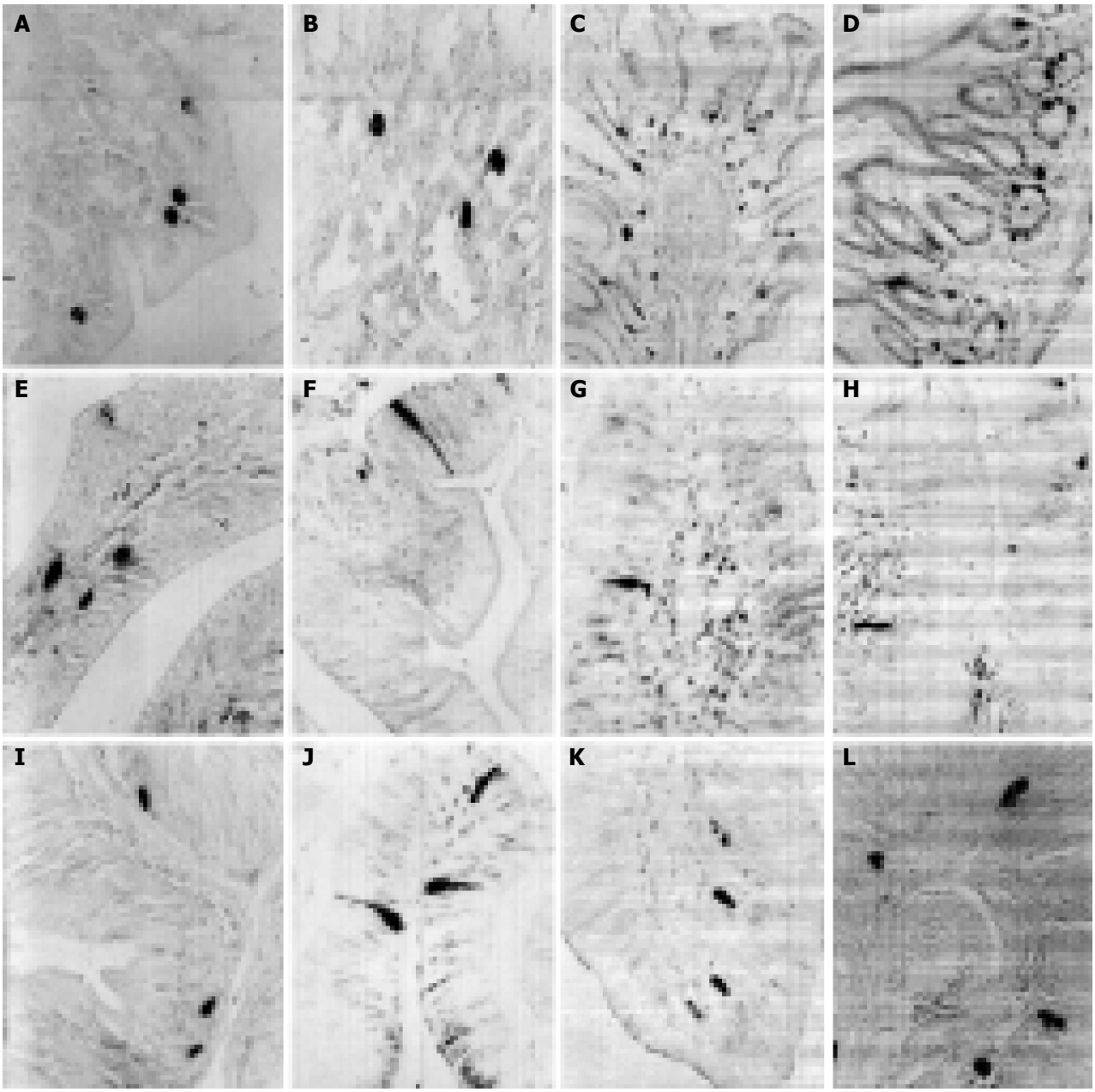

Figure 1 5-HT-IR cells in cardia of *E. chinensis* (**A**), fundus of *E. elegans* (**B**), pylorus of *S. indicus* (**C**) and *E. elegans* (**D**), duodenum of *G. japonicus* (**E**) and *S. indicus* (**F**), jejunum of *S. indicus* (**G**) and *E. elegans* (**H**), ileum of *S. indicus* (**I**) and *E. elegans* (**J**), rectum of *E. elegans* (**K**) and *E. chinensis* (**L**).

the glandular cells and the basement membrane at the basal portion of the glands. In the intestinal parts, most of these cells were situated in the basal portion of the epithelia.

DISCUSSION

The present study demonstrated that the GI endocrine system of four reptiles is a complex structure containing many endocrine cell types similar to those found in mammals except for PP-IR cells[19-22]. 5-HT-, SS-, GAS-, GLU-, SP-IR cells were identified in the GIT of four reptiles, except for *E. elegans*, in which only the former three types were found.

5-HT, a monoamine, which has a strong effect on regulation of digestive functions, is widely distributed in gastric epithelial endocrine cells, 5-HT-IR cells[4,23]. El-Salhy *et al*.[23], reported that 5-HT-IR cells are detected throughout the GIT of all species of vertebrates, suggesting that they have been established in the GIT at an early stage of vertebrate evolution. By using specific antiserum against 5-HT, these cells were detected throughout the GIT of four reptiles in the present study, further approving that 5-HT-IR cells have a wider distribution than other types of GI endocrine cells in GIT of vertebrates. Similarly to

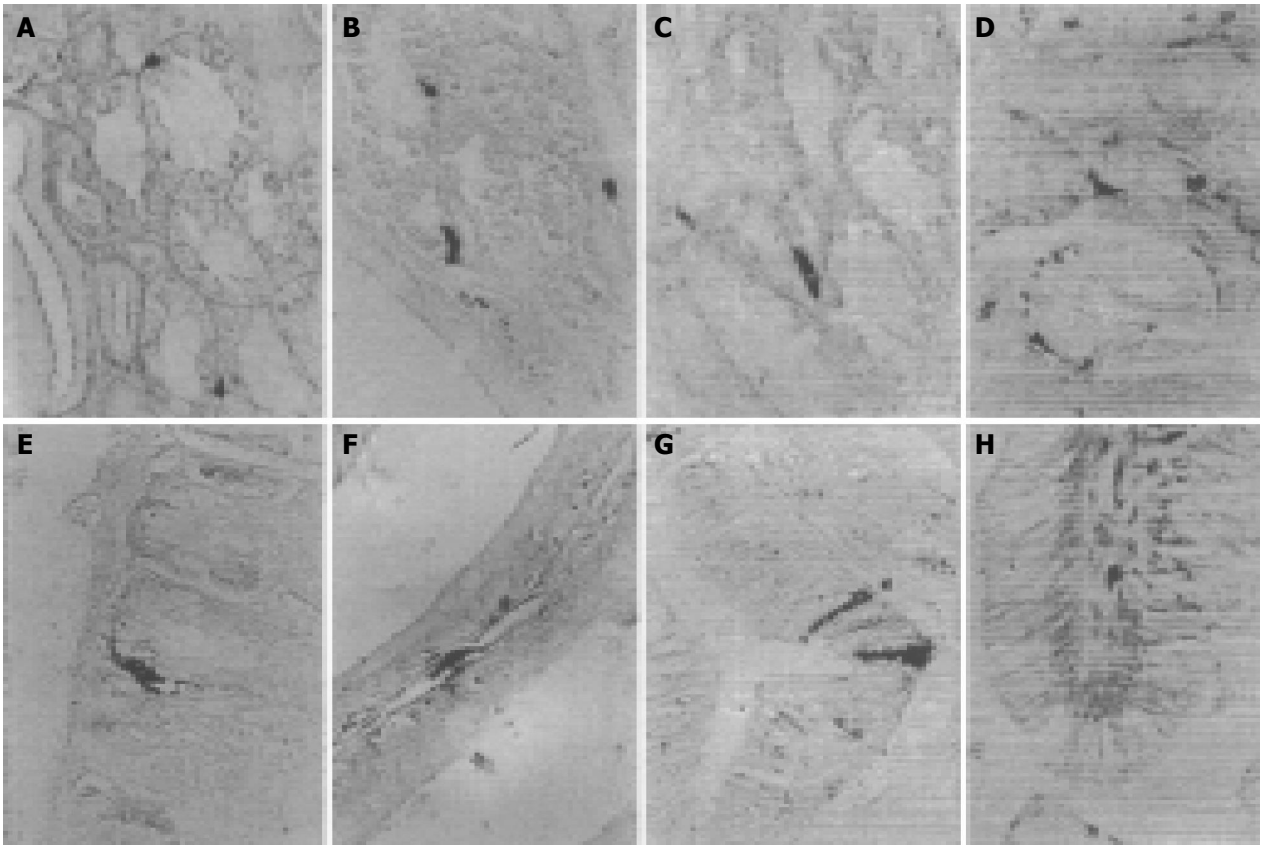

Figure 2 SS-IR cells in cardia of *S. indicus* (**A**) and *E. elegans* (**B**), fundus of *S. indicus* (**C**) and *G. japonicus* (**D**), pylorus of *G. japonicus* (**E**), duodenum of *G. japonicus* (**F**), ileum of *E. chinensis* (**G**), rectum of *E. chinensis* (**H**).

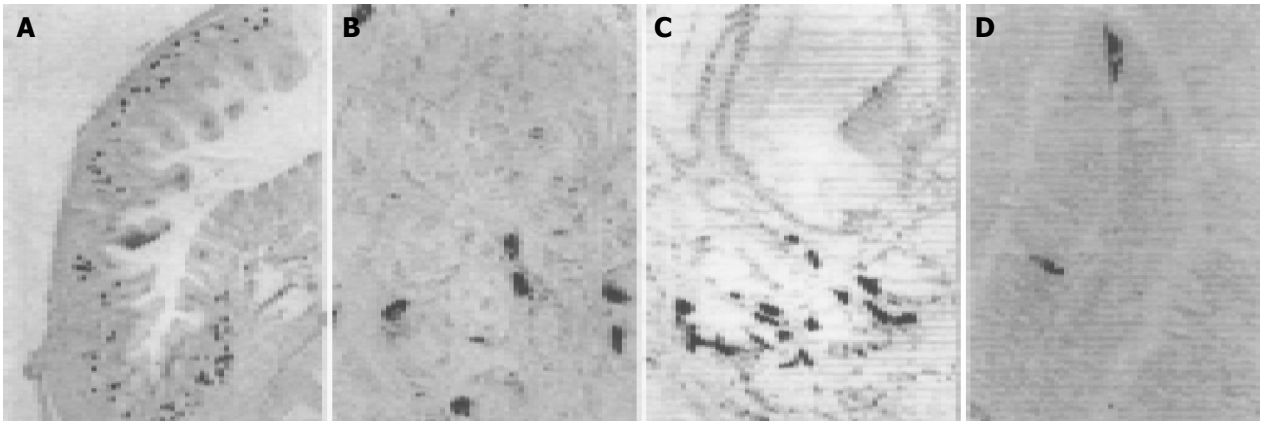

Figure 3 GAS-IR cells in pylorus of *E. elegans* (**A**) and *E. chinensis* (**B**) and *S. indicus* (**C**), duodenum of *G. japonicus* (**D**).

Ku *et al*.[29], and Arena *et al*.[30], 5-HT-IR cells are most predominant in pylorus of *E. chinensis*, *S. indicus* and *E. elegans*. In other studies[24,25,28,31,], the highest frequency of 5-HT-IR cells is in the duodenum and similar results are found in *G. japonicus*, suggesting that pylorus and/or duodenum are important parts in GIT for digestion and a great deal of 5-HT is needed to facilitate the digestive process. As for the distribution pattern (regional distribution and relative frequency or distribution density in GIT) of 5-HT-IR cells in the GIT of four reptiles, considerable variation was observed, and no one was similar to each other, suggesting that the distribution pattern of 5-HT-IR cells may vary from different species. In the present study, some 5-HT-IR cells in the stomach of four reptiles exhibited narrow apical cytoplasmic processes reaching adjacent cells or the gastric gland lumen, indicating that these cells can probably secrete 5-HT by paracrine pathway. In the intestines of four reptiles, 5-HT-IR cells with cytoplasmic processes reaching intestinal lumen were also observed, suggesting that 5-HT may be secreted by exocrine pathway to intestinal lumen.

In most mammals as well as some reptiles, SS-IR cells have a wide distribution in the GIT, except for the large intestine[29-33]. However, species-dependent variations in the distribution patterns of these IR cells have also been reported[24-28]. In the present study, SS-IR cells were demonstrated in the whole GIT of *E. chinensis*, which are in agreement with those reported in *Trimeresurus stejnegeri*, *Trionyx sinensis*, *Alligator mississippiensis* and *Caiman latirostris*[24-27]. In the other three reptiles, SS-IR cells showed restricted distribution. In the GIT of *G. japonicus*, SS-IR cells were demonstrated in stomach and duodenum, as in *Alligator sinensis*[28]. In the GIT of *S. indicus* and *E. elegans*, SS-IR cells are only restricted to the stomach, which differ from those of previous reports on other reptiles[29-33]. These studies suggest that the distribution pattern of SS-IR cells varies greatly in reptiles. On the other hand, common distribution features of SS-IR cells also exist. Studies show that in the GIT of other vertebrates especially mammals, SS-IR cells are found mainly in stomach[20,22], and this common place is also found in most reptiles[27-32]. In the present study SS-IR cells were most predominant in pylorus of *E. chinensis*, *S. indicus* and *E. elegans*. This finding is in line with those of most vertebrates[29,31]. Somewhat different from the above results, SS-IR cells were most abundant in fundus rather than in pylorus of *G. japonicus*, and this difference may be due to the different species. The results found in *G. japonicus* have also been reported in some mammals[18,19,22]. Larsson *et al*.[5], reported that in the stomach, SS-IR cells have cytoplasmic processes terminated on GAS-producing cells, and SS is secreted through cytoplasmic processes by paracrine pathway, inhibiting the secretion of GAS. In the present study, both SS- and GAS-IR cells showed the highest density in pylorus of four reptiles except for *G. japonicus*, suggesting that some relationships may exist between them. Some SS-IR cells with cytoplasmic processes extending to adjacent cells were also found, suggesting that SS-GAS paracrine regulation may exist in reptiles as in mammals.

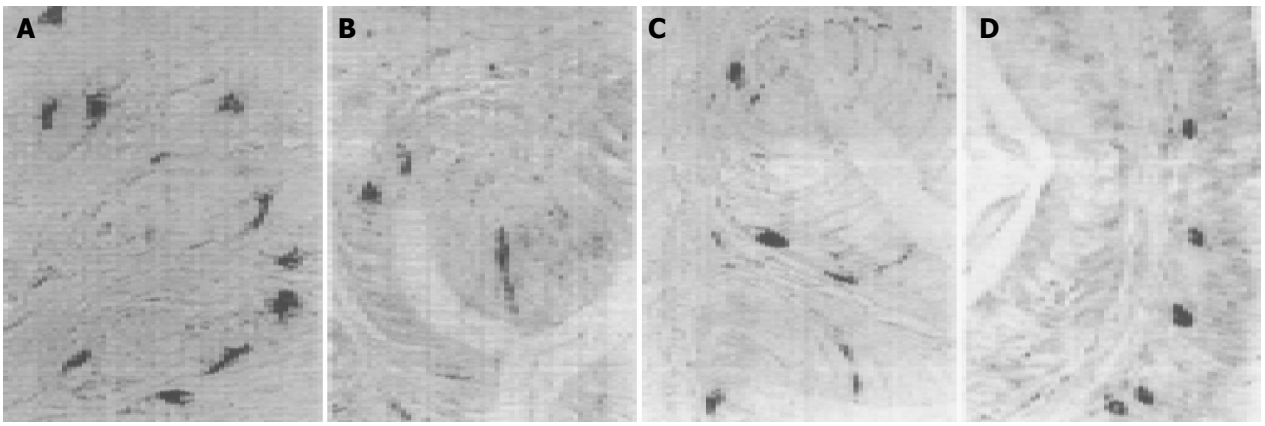

Figure 4 GLU-IR cells in fundus of *G. japonicus* (**A**), ileum of *E. chinensis* (**B**), rectum of *E. chinensis* (**C**) and *S. indicus* (**D**).

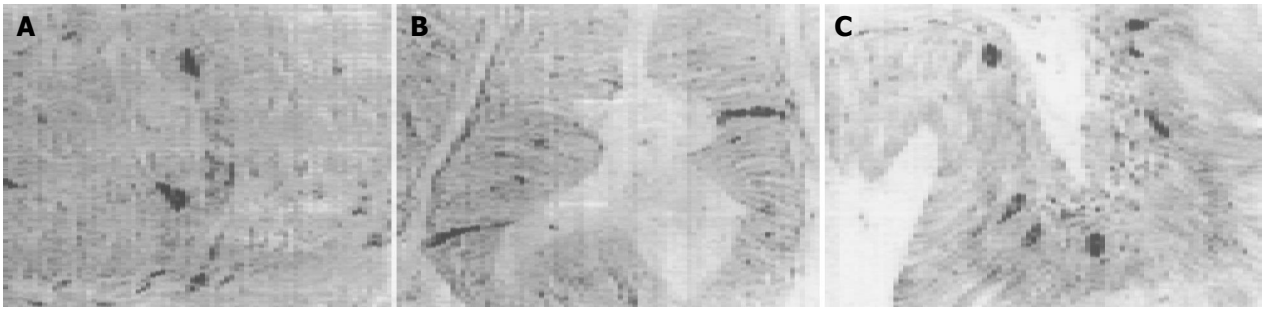

Figure 5 SP-IR cells in cardia of *G. japonicus* (**A**), ileum of *E. chinensis* (**B**), rectum of *S. indicus* (**C**).

In some reptiles, GAS-IR cells are observed in pylorus and whole small intestine of GIT[25,30-33], while in the present study, GAS-IR cells showed restricted distribution in the GIT of four reptiles and the results are similar to those reported in most mammals[18-22]. GAS-IR cells in pylorus and duodenum of *S. indicus* and *E. elegans* are reported in *Bubalus bubalis* and *Caiman latirostris*[20,27]. In the GIT of *E. chinensis*, GAS-IR cells are confined to the pylorus, corresponding to those found in C57BL/6 mice and SKH-1 hairless mice[18,19]. Interestingly, GAS-IR cells were not detected in the pylorus but in the duodenum and jejunum of *G. japonicus*, which differ from those of other three reptiles in which GAS-IR cells were numerous in the pylorus. However, this distribution is consistent with that in *Rana japonica japonica*[17]. In addition, GAS-IR cells are observed in the intestines of *Alligator sinensis* but not in the pylorus[28]. GAS secreted by GAS-IR cells, regulate gastric acid secretion[6]. In *E. chinensis*, *S. indicus* and *E. elegans*, like most vertebrates[30-32], the highest density of GAS-IR cells is found in the pylorus, and this distribution may be important for them to fulfill the regulation of digestive function. However, GAS-IR cells were detected in small intestine in *G. japonicus* but not in the pylorus. Gut hormones have been demonstrated to release into tissue spaces in a paracrine way, they can also release into the gut lumen in a partial exocrine manner or into the blood in an endocrine fashion[3]. In the present study, GAS-IR cells in the intestines of *G. japonicus* were often found with cytoplasmic processes either reaching intestinal lumen or extending to lamina propria, suggesting an exocrine or endocrine secretion of these cell types. Maybe in this way, GAS-IR cells in the intestine can regulate gastric acid secretion. Studies are required to elucidate the functional meaning of these findings.

GLU is synthesized in A cells of the pancreas and regulates serum glucose levels. GLU-IR cells have been demonstrated in various mammals and it is considered that the distribution pattern of these cells in GIT of mammals show species-dependent variation[18-22]. In the present study no GLU-IR cells were found in GIT of *E. elegans* and the findings are similar to those in *Trionyx sinensis*, *Alligator sinensis* and *Egernia kingii* and remarkably different from those in *Caiman latirostris* in which GLU-IR cells were detected throughout the GIT except for the cloaca[25,27,28,30]. In GIT of *G. japonicus*, GLU-IR cells are only found in the stomach, corresponding well with those in *Alligator mississippiensis*[26]. In GIT of *E. chinensis* and *S. indicus* GLU-IR cells are mainly localized in the ileum and rectum. The above studies suggest that the regional distribution of GLU-IR cells in the GIT varies in reptilians.

Up to date, reports on SP-IR cells in the GIT of reptiles are scarce[24,28,31,33,34]. The present study showed that SP-IR cells exhibited variant distribution patterns in GIT of four reptiles. In *E. chinensis* SP-IR cells were found throughout the GIT except for jejunum. Similar to *Lacerta lepida*[31], SP-IR cells are confined to the stomach in GIT of *G. japonicus*. The GIT of *E. elegans* is devoid of SP-IR cells, which is consistent with that in *Trimeresurus stejnegeri*, *Alligator sinensis*, *Testudo graeca* and *Mauremys caspica*[24,28,31]. In *S. indicus* SP-IR cells are only found in the rectum, which are quite different from those of previous studies[33,34].

In this study, the region with the highest degree of cell type heterogeneity was the pylorus. The distribution of most endocrine cell types in the GIT showed the peak density in the pylorus as well. The heterogeneity and concentration of endocrine cells in the pylorus may be attributed to the role of the cells in the feedback control of the function of the segment. In *G. japonicus* and *E. elegans* the spectrum of cell types in the intestine gradually narrowed. While in *E. chinensis* and *S. indicus* the spectrum gradually narrowed from duodenum to jejunum and then widened and got its minimum in jejunum and maximum in rectum.

In conclusion, some common features of the distribution and morphology of different types of GI endocrine cells are found in GIT of four reptiles. This common trait may reflect the similarity in digestive physiology of various vertebrates. Further physiological studies are required to elucidate the ways in which the distribution of GI endocrine cells may be related to the regulatory characteristics of the GIT of reptiles.

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