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# Distribution and Morphology of Ghrelin-Immunopositive Cells in the Pancreas of the African Ostrich

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**JW planned the study, preformed the trials and wrote the manuscript. PL analyzed the data.**

**Key words**
**African ostrich, Ghrelin, Pancreas, Immunohistochemistry.**

## ABSTRACT

Ghrelin, the endogenous ligand for the growth hormone secretagogue receptor, has been identified in the pancreas of many vertebrates. While ghrelin has been found in the cerebellum and gastrointestinal tract of African ostrich chicks, little is known about its distribution in the pancreas of the African ostrich. In the present study, the distribution and morphological characteristics of ghrelin-immunopositive cells in the African ostrich pancreas were investigated using immunohistochemistry. Our results indicate that the pancreas is divided into two sections: the exocrine and endocrine portion.The exocrine portionis comprised of peripheral gland cells and centroacinar cells; the endocrine portion consists of islet cells. Ghrelin-immunopositive (ghrelin-ip) cells were found in both the pancreatic exocrine portion and islets. The greatest number of ghrelin-ip cells was found in the islets, and the cell density decreased gradually from the pancreatic islets to the extrainsular regions. The distribution of ghrelin-ip cells was observed mostly at the periphery of the islet, a few ghrelin-ip cells were found in the central portion of the pancreatic islets. The ghrelin-ip cells were fusiform or irregular polygons, and their cytoplasm was stained intensely. These results demonstrate the presence of ghrelin-ip cells in the pancreas of the African ostrich. Thus, it is speculated that ghrelin may have a physiological function in the pancreas.

## INTRODUCTION

Ghrelin is a brain–gut peptide that has been isolated as an endogenous ligand for the growth hormone secretagogue receptor in the rat stomach (Kojima *et al*., 1999). Ghrelin is predominantly produced by X/A-like endocrine cells in the oxyntic mucosa of the stomach, which is the major source of circulating ghrelin (Kojima *et al*., 1999; Alamri *et al*., 2016). In mammals, ghrelin-immunopositive (ghrelin-ip) cells have been identified in the intestine, pancreas, heart, liver, hypothalamus, cerebrum, cerebellum, thymus gland, hypothalamic arcuate nucleus, kidneys, and pituitary (Kojima and Kangawa, 2005; Ghelardoni *et al*., 2006). In addition to mammals, ghrelin has also been identified in several non-mammalian (Kaiya *et al*., 2008; Fang *et al*., 2008; Wei *et al*., 2010; Suzuki and Yamamoto, 2011).

In non-mammalian, ghrelin-ip cells have also been found in the stomach, intestines, pancreas, heart, liver, hypothalamus, cerebrum, cerebellum, thymus gland, and bursa of Fabricius (Kaiya *et al*., 2008; Fang *et al*., 2008; Wei et al., 2010; Suzuki and Yamamoto, 2011). In the pancreas, ghrelin-producing cells represent an independent cell population within the pancreatic islets and ghrelin is thought to be potentially involved in glucose homeostasis (Granata and Ghigo, 2013). The ghrelin system (ghrelin and its receptor) also exists in pancreatic acinar cells, and ghrelin could be implicated in the stimulation of pancreatic enzyme secretion (Jaworek and Konturek, 2014). Yada *et al*. (2014) showed that the ghrelin originating from islets restricts insulin release and thereby upwardly regulates the systemic glucose level. Muller *et al*. (2015) also demonstrated that ghrelin could also modulate the release of insulin and glucagon. Furthermore, exogenous ghrelin administration was found to decrease glucose-induced insulin release and increase glucose levels in both humans and rodents (Alamri *et al*., 2016). In general, to understand or hypothesize the physiological role of identified peptides, it is important to determine the morphological characteristics of the producing cell and its distribution. However, in the African ostrich, only the stomach, intestines, and cerebellum have been studied for the distribution of ghrelin (Wang *et al*., 2009, 2012). There are no existing studies on the distribution of ghrelin in the pancreas of the African ostrich. Therefore, in this study, the distribution and morphological characteristics of ghrelin-

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producing cells in the pancreas of the African ostrich were investigated using immunohistochemistry.

## MATERIALS AND METHODS

*Animal*

African ostriches (age, 12 months; weight, 90.78±3.25 kg) were used in this study. African ostrich (2 females and 2 males) were obtained from the Ostrich Research Institute of Yangtze University in Hubei Province, China, where feed and water were made available ad libitum. All of the birds were maintained in a heated room with slatted plastic floor and were fed a starter diet for postnatal days 7, which was formulated according to the specifications of the Elsenburg Ostrich Feed Database (Brand, 2000). All procedures were approved by the Animal Care and Welfare Committee of our Institute.

*Tissue preparation*

The ostriches were deeply anesthetized with 10% urethane (Caoyang Secondary Chemical Plant, Shanghai, China) at a dose of 1 g/kg BW, and perfused initially with 1000 mL of 0.85% normal saline (containing 0.075% sodium citrate) and thereafter with 1500 mL of 4% paraformaldehyde phosphate-buffered solution (0.1 mol/L, pH 7.4) at 4 °C. The abdomen was cut open and the entire pancreas were quickly taken, and gently flushed with 0.85% normal saline to remove the content, then be postfixed for more than 24 h with 4% paraformaldehyde. After immersion, the tissues were embedded in paraffin. Serial sections (5 µm) were cut with a Leica microtome (Nussloch Gmbh, Germany), 2 suitsections were prepared; one suit was stained by haematoxylin and eosin (H&E) to observe the cytoarchitecture of pancreas; the other was stained by immunohistochemistry (SABC) to observe the distribution, morphological characteristics of ghrelin-producing cells in the pancreas of the African ostrich .

*Immunohistochemistry*

Immunohistochemical detection of ghrelin cells using rabbit anti-ghrelin was carried out by streptavidin-biotin-peroxidase complex (SABC) method. The production and specificity of the anti-human ghrelin serum used in this study were previously reported (Wang *et al*., 2009); it is established that this antiserum recognizes both N- and C-terminial of human ghrelin. Immuno-histochemical staining was performed according to the following procedure. The sections were deparaffinized with xylene and rehydrated with decreasing concentrations of ethanol, then treated with 3% hydrogen peroxide ($H_2O_2$) to block endogenous peroxidase for 10 min at room temperature. After rinsing with distilled water, the sections were incubated with a citrate buffer (pH 6.0) and placed in a microwave oven until the water boiled to fully expose the antigen. After rinsing with phosphate-buffered saline (PBS), the sections were incubated with 5% normal goat serum for 20 min. After removing superfluous liquid, the sections were incubated with rabbit anti-ghrelin serum (BA1619; Boster Corporation) diluted 1:100 in PBS for 12 h in a humid chamber at 4 °C. After washing with PBS for 6 min, a second incubation with biotin-conjugated anti-rabbit IgG serum (SA1022; Boster) was carried out for 20 min, and this was followed by further washing with PBS. Finally, the sections were incubated for 20 min with an SABC solution prepared according to the manufacturer's instructions. After washing with PBS for 20 min, the sections were reacted in a diaminobenzidine-tetrachloride kit (DAB kit, AR1022, Boster Corp) for 30 min to detect immunostaining. After washing with distilled water, the sections were dehydrated with a graded ethanol series, cleared in xylene, mounted with a coverslip, and viewed under a light microscope (BH-2; Olympus, Japan). All of the incubations were carried out in a humid chamber at room temperature. Control sections were prepared using the same method, omitting the primary antibody. To examine the specificity of rabbit anti-human ghrelin antiserum, the diluted antiserum (1:100) was incubated with human ghrelin (5µg/ml) at room temperature for 10 h, and mixtures were centrifuged at 12000 rpm for 25 min at 4°C. The supernatant was used as the primary antiserum for absorption tests.

*Morphometric analysis*

The densities of ghrelin cells in various regions of the African ostrich pancreas were estimated. For each pancreastissue sample, 3 cross-sections were prepared after the samples had been stained with hematoxylin and eosin and SABC stain. Further, for each pancreascross-section, 10 intact, well-oriented units were selected for experiments conducted in triplicate (30 measurements for each sample). After taking digital photographs under a light microscope with a digital camera (COOLPIX4500; Nikon, Japan), the number of ghrelin-ip cells in each section was counted using a computerized image analysis program: HMIAS-2000 High-definition Chromatic Color Medical Science Figure Analysis Program (Qianping, Wuhan, China). The ghrelin-ip cells density was calculated as the number of ghrelin-ip cells per unit area.

*Statistical analyses*

Results are expressed as means ± standard errors on the mean (means± S.E.). Statistical analysis was done using analysis of variance statistics software (SAS Institute, 2000) with Duncan's multiple range test where

appropriate. Differences of $p < 0.05$ were considered significant.

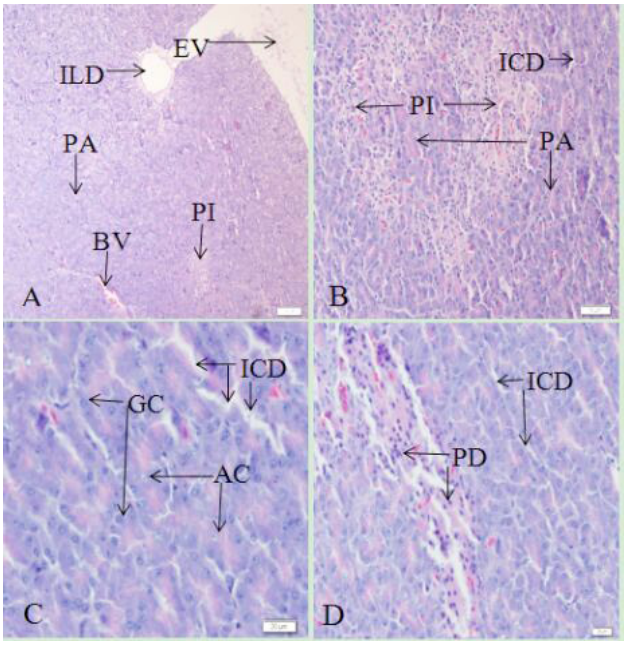


Fig. 1. Histological structure of the African Ostrich pancreas (HE staining). A, the pancreas; B, the pancreatic parenchyma; C, the pancreatic exocrine portion; D, the pancreatic parenchyma. EV, envelope; PA, pancreatic acinus; PI, islet; BV, blood vessels; ILD, intralobular duct; ICD, intercalary duct; GC, pancreatic gland cells; AC, centroacinar cell; PD, islet cells. (A) Scale bar: 100 μm; (B) scale bar: 50 μm; and (C and D) scale bar: 20 μm.

## RESULTS

All results presented are those obtained from both female and male chicks; no gender-specific effects were observed.

*Cytoarchitecture of the pancreas*

The pancreas of the ostrich was found to be gray-white in color, the outer surface contained a thin seromuscular envelope, and the pancreatic parenchyma was divided into many lobules by outer membrane; however, the lobules were not obvious (Fig. 1A). The pancreatic parenchyma was composed of both an exocrine and endocrine portion. The exocrine portion consisted of compound tubuloacinar glands composed of pancreatic acinar and ducts (Fig. 1B). The pancreatic acinus were tubular or vesicular, and formed the peripheral gland cells, as well as the middle centroacinar cells. Additionally, the gland cells consisted of a serous and irregular cone shape, and the cytoplasm was rich in red-stained secretory granules; the nucleus was round or oval, located in the basal part of the cell, and one or several nucleus were prominent (Fig. 1C). The centroacinar cells were located in the gland cavity which was surrounded by the gland cells that were lighter in color, contained a kernel that was not obvious, and there were no secretory granules in the cytoplasm (Fig. 1C). The ducts divided into the intercalary duct, intralobular duct, interlobular duct, and pancreatic duct (Fig. 1A, B). The endocrine portion consisted of the pancreas islets, which were comprised of round or oval cell clusters that were formed by a large number of islet cells irregularly interspersed between the exocrine acin. The islet cells were arranged in clusters, light in color, round or oval with a round nucleus located in the center of the cell. The cytoplasm was relatively dilute and the difference was obvious when compared with the surrounding gland cells. Moreover, there was an abundance of capillaries among the cells (Fig. 1D).

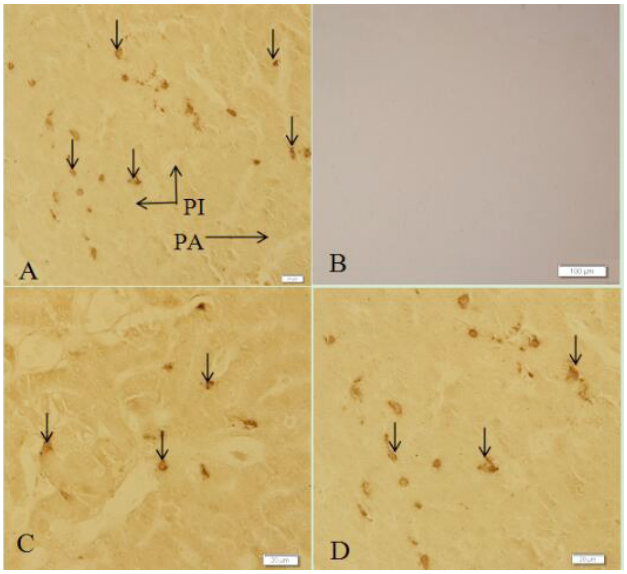


Fig. 2. Histological structure of pancreas showing ghrelin-immunopositive cells in the pancreas of the African ostrich (SABC staining). A, a large number of ghrelin cells (arrows) were found within the pancreas; B, microphotograph of the absorption testing in the pancreas; C, ghrelin-ip cells (arrows) amound the Pancreatic acinar cells; D, ghrelin-ip cells (arrows) within the islets. PA, pancreatic acinus; PI, islet. (A, C and D) Scale bar: 20 μm; (B) scale bar: 100 μm.

*Distribution of ghrelin-immunopositive cells*

African ostrich ghrelin-ip cells were identified in the pancreatic exocrine portion and islets, and tended to be restricted to a single cell (Fig. 2A). These staining profiles were not observed when the sections were processed with only the supernatant that was used as the primary antiserum

for absorption tests (Fig. 2B). In the exocrine portion, ghrelin-ip cells were located among the pancreatic gland cells, the cytoplasm was stained, and the cells were spindle, cone, triangular or polygonal, round, or oval in shape (Fig. 2C). In the pancreatic endocrine portion, ghrelin-ip cells were located among the pancreatic islet cells, and and the distribution of ghrelin-ip cells was observed mostly at the periphery of the islet, a few ghrelin-ip cells were found in the central portion of pancreatic islets. In addition, similar to the exocrine portion, the cells exhibited astained cytoplasm,and were spindle, cone, triangular or polygonal, round or oval in shape (Fig. 2D).

*Morphometric analysis*

The morphometric analysis revealed that the ghrelin-ip cells were localized preferentially within the pancreatic islets ($P < 0.05$) (Fig. 3). The cell density decreased gradually from the pancreatic islets to the extrainsular regions ($P < 0.05$) (Fig. 3).

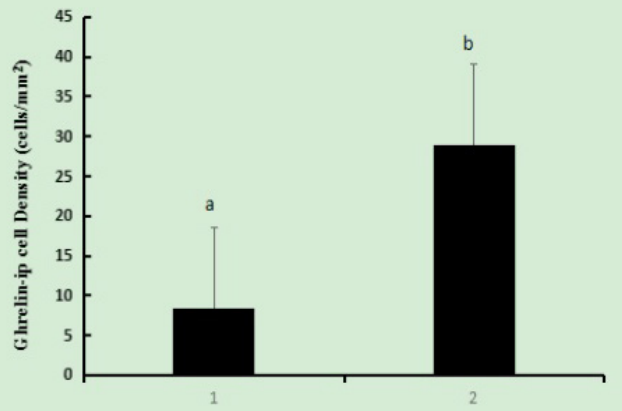


Fig. 3. Histogram showing the densities of ghrelin-immunoposive cells (cells/mm$^2$) in the 334 day-old African ostrich pancreas. The number of ghrelin-immunopositive cells were a steady decrease from pancreatic islet to pancreatic acinus. Ghrelin-immunoposive were present throughout the pancreas. a-b, Different letters within the same column indicate significant differences among segments according to Duncan's multiple range ($P \leq 0.05$). 1, pancreatic acinar; 2, islet.

## DISCUSSION

The pancreas is an important digestive and endocrine gland in the body, and the location, shape, color, and structure differ between species. In the present study, we found that the pancreas of the ostrich was gray-white in color, the surface was covered with a thin membrane, which divided the pancreas into multiple leaflets. However, the boundaries were not obvious, similar to the results observed in Bustards (Liu *et al*., 2002), Syrrhaptes Paradoxus (Wang *et al*., 2013), Quai (Wang *et al*., 2007), and Wanxi White Geese (Fang *et al*., 2005); however, the pancreatic gland leaflets of Red Pandas (Mi, 2004) and Xinjiang donkey (Zhang *et al*., 2015) are very obvious, which is different from the African ostrich. In Bustards, the parenchyma of the pancreas is divided into the exocrine portion and endocrine portion. The exocrine portion consists of multiple pancreatic acini cells; however, centroacinar cells are not found within the pancreatic acini, which is consistent with Syrrhaptes Paradoxus (Liu *et al*., 2002; Wang *et al*., 2013). In contrast, in African ostriches, the exocrine portion is composed of peripheral gland cells and centroacinar cells in the middle, similar to that of Quai, Wanxi, White Geese, Red Pandas and Xinjiang donkey (Mi, 2004; Fang *et al*., 2005; Wang *et al*., 2007; Zhang *et al*., 2015). In addition, the endocrine portion contained islets composed of round or oval cell clusters formed by a large number of islet cells irregularly interspersed between the exocrine acini. These findings are in accordance with Quai, Wanxi, White Geese, Red Pandas and Xinjiang donkey (Mi, 2004; Fang *et al*., 2005; Wang *et al*., 2007; Zhang *et al*., 2015). The above results reveal that the pancreatic structure differs between animals. Future studies should investigate whether such differences result in different functionality of the pancreas.

The ghrelin cell was discovered 11 years ago as a novel cell type that resides within the islets of the human pancreas (Wierup *et al*., 2014). Subsequent studies have identified the presence of islet ghrelin cells in several animals, including mice, rats, gerbils and fish (Wierup *et al*., 2014). In this study, ghrelin-ip cells were also found to be located within in the pancreas of the African ostrich; these results suggest that ghrelin cells may be present in the pancreas of many animals. Moreover, we also found that the ghrelin-ip cells were located in both the pancreatic exocrine portion and pancreatic islets of the African ostrich; however, several ghrelinimmunor-eactive cells were also found among the pancreatic islet cells, and ghrelin-ip cells were located mostly at the periphery of the islet, a few ghrelin-ip cells were found in the central portion of the pancreatic islets. These distribution features of ghrelin-immunopositive cells were similar to the pancreas of Xenopus, chicken, Wanxi and White Geese (Fang *et al*., 2008; Wei *et al*., 2010; Suzuki and Yamamoto, 2011), but differs from that of the rat. In the rat pancreas, ghrelin-immunoreactive cells were found in the middle of the pancreatic islets, similar toglucagon-producing-cells (Date *et al*., 2002). Kageyama *et al*. (2005) found that ghrelin-immunoreactive cells were not detected among the exocrine acini orcentroacinar cells. Moreover, ghrelinimmun-oreactivy was primarily detected in the periphery of the islets of Langerhans where the cell

population consists mainly of α cells. However, Raghay *et al*. (2013) found that ghrelin-immunopositive cells were located in the central portion of the rat pancreatic islets. In the human pancreas, ghrelin immunoreactivity exhibited the same expression patterns as rats (Date *et al*., 2002); but Grönberg*et al*. (2008) and Raghay *et al*. (2013) revealed a limited number of ghrelin immunoreactive cells in the periphery of the pancreatic islets. Instead, a few scattered ghrelin-immunoreactive cells were detected inconnection to the exocrine pancreatic ducts, similar to that observed in the African ostrich.

The pancreatic islets vary in size and cellularity but are composed of four main cell types: α (20% of the total), β(68%), δ(10%), and PP (2%) (Freychet, 1990). Insulin-producing β-cells are located within the center of the islets, glucagon-producing α-cells are found at the periphery, and somatostatin-producing δ-cells are interposed between the two (Date *et al*., 2002). In this study, ghrelin-ip cells were almost located in the periphery of the pancreatic islet, a few ghrelin-ip cells were detected in the central portion of the pancreatic islets, suggesting that ghrelin expression is almost localised in glucagon-producing cells (α cells), and a bit in Insulin-producing cells (β-cells) in African ostrich's pancreas.

Ghrelin may be implicated in the stimulation of pancreatic enzyme secretion, and modulate the release of insulin and glucagon (Jaworek and Konturek, 2014; Muller *et al*., 2015). Furthermore, exogenous ghrelin administration has been shown to decrease glucose-induced insulin release and increase glucose levels in both humans and rodents (Alamri *et al*., 2016). In African ostrich, ghrelin may have a physiological function in the pancreas. Therefore, it is important to perform further studies on the function of these peptides within this location.

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*Statement of conflict of interest*

Authors have declared no conflict of interest.

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