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Effects of Exogenous Ghrelin on the Tissue Structure and Somato-Statin Secretion in the Pancreas of African Ostrich Chicks

Efectos de la Grelina Exógena en la Estructura del Tejido y la Secreción de Somato-Estatinas en el Páncreas de los Polluelos de Avestruz Africanos

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SUMMARY: This study aimed to investigate the effect of exogenous ghrelin on pancreatic growth and development in African ostrich chicks. Sixteen 40-day-old African ostrich chicks (male or female) were randomly divided into four groups and injected intravenously metatarsal vein with saline (control) or ghrelin (10, 50, and 100 μg/kg) for 6 days. Body and pancreas weight were determined, structural characteristics were observed using HE staining, somatostatin-immunopositive cells were detected using immunohistochemistry. The results were as follows: 1. The 50 and 100 μg/kg groups showed lower relative pancreas weight than the control group ($P < 0.05$). 2.The islet area per unit area of the pancreas was higher in the 10, 50, and 100 μg/kg groups than in the control group ($P < 0.05$). The number of islets per unit area of the pancreas was lower in the 10 μg/kg group than in the control ($P < 0.05$) and slightly higher in the 50 and 100 μg/kg groups than in the control ($P > 0.05$. Moreover, compared with the control, the islet cells in treatment groups were loosely arranged and showed reduced cytoplasm. In the exocrine pancreas, the volume of acinar cells in the 10, 50, and 100 μg/kg groups all decreased to varying degrees. 3. Somatostatin immunopositive cells were mainly located around the periphery of the islets and sporadically distributed in the center. The density of the somatostatin immunopositive cells in the 10, 50, and 100 μg/kg groups was higher than that in the control ($P < 0.05$). These findings suggest that exogenous ghrelin increases the area and number of islets and number of somatostatin immunopositive cells but reduces relative pancreas weight and effects the morphological and structural development of the pancreas, which may inhibit the pancreatic growth and development in African ostrich chicks.

KEY WORDS: African ostrich; Ghrelin; Pancreas; Growth and development.

INTRODUCTION

Ghrelin—the endogenous ligand for the growth hormone secretagogue receptor (GHS-R)—was first isolated from the rat stomach; it is mainly secreted by X/A-like endocrine cells (Kojima *et al*., 1999; Ariyasu *et al*., 2001). Ghrelin can regulate feeding and energy balance (Klok *et al*., 2007), promotes growth hormone release (Kojima *et al*.), regulates cell proliferation, participates in immune anti-inflammatory activities, and improves cardiovascular function. Studies have shown that ghrelin-immunopositive cells are widely distributed in African ostrich chicks (Ye *et al*., 2017; Wang *et al*., 2017a). Wang *et al*. (2017b) reported that ghrelin-immunopositive cells are also found in the pancreas of African ostrich chicks; during the 1–90-day brooding period, the number of ghrelin-immunopositive cells decreased gradually with increasing age, which suggests that ghrelin acts as a signal that can influence developmental processes in the pancreas of African ostrich chicks.

In the pancreas, somatostatin is produced by delta cells of the pancreatic islets, where it exerts local effects, inhibiting insulin and glucagon secretion (Kasacka *et al*., 2012). In addition, the interaction of somatostatin and Somatostatin receptors is very important during normal pancreas development, and is associated with many pancreatic diseases such as diabetes and pancreatic cancer (Ballian *et al*., 2006). Previous studies have shown that ghrelin has a certain regulatory effect on somatostatin, for example, Adriaenssens *et al*. (2016) reported that ghrelin acts specifically on delta cells within pancreatic islets to elicit somatostatin secretion, which in turn inhibits insulin and glucagon release. However, to our knowledge, the effect of exogenous ghrelin on the pancreas structure and somatostatin secretion of African ostriches has not been reported. Therefore, the present study investigated the effect of exogenous ghrelin on the weight, pancreatic tissue structure

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and the distribution of somatostatin immunopositive cells in the pancreas of African ostrich chicks to provide a theoretical basis for further study on the effect of ghrelin on the development of ostrich pancreas.

MATERIAL AND METHOD

Animals. Seven-day-old African ostrich chicks were obtained from the Ostrich Research Institute of Yangtze University, Hubei, China. The birds had free access to water and the feed prepared according to the specifications of the Elsenburg Ostrich feed database (Brand, 2010). After 33 days, the birds were weighed, and 16 African ostrich chicks (male or female) with similar weight (average weight: 3.65 ± 0.53 kg) and growth status were selected for the experiment. The experimental procedures and ostrich treatment procedures have been approved by the Animal Protection and Welfare Committee of our institute.

Tissue preparation. The 16 ostrich chicks were divided into four groups containing four chicks each—one control and three experimental groups. The ostrich chicks were injected intravenously (metatarsal vein) with saline (control) or ghrelin (experimental) at 8 am every day for 6 days; the total injection dose was 500 μl per bird. The three experimental groups were treated with 10 μg/kg, 50 μg/kg, and 100 μg/kg, respectively, of ostrich ghrelin (synthesized by Shanghai Qiangyao Biological Technology Co., Ltd.) diluted with normal saline. After 6 days of treatment, the birds were weighed and anesthetized with 20 % urethane (1 g/kg China Caoyang No. 2 Middle School Chemical Factory). The abdominal cavity of each bird was opened, the pancreas was immediately removed and weighed, and the pancreas weight relative to the total body weight (BW) was calculated. Then postfixed for more than 24h with 4 % paraformaldehyde. After immersion, the tissues wereembedded in paraffin, the paraffin blocks were sectioned (4 μm) using a Leica microtome (Nussloch Gmbh,Wetzlar, Germany),and the sections were used for hematoxylin and eosin (HE) staining and immunohistochemical staining.

Immunohistochemistry. Immunohistochemical detection of somatostatin-immunopositive cells using rabbit polyclonal anti-somatostatin was carried out by the Streptavidin-biotin-peroxidase complex (SABC) method. Immunohistochemical 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(H2O2) 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 % BSA blocking solution for 30 min. After removing the excess liquid, the sections were incubated with rabbit polyclonal anti-somatostatin (BA0124, Boschde, Wuhan, China) in PBS at a dilution of 1:150 in a humid chamber at 4°C for 12 hours. After washing withPBS for 6 min, a second incubation with HRP-labeled goat anti-rabbit IgG (BA1054; Boschde, Wuhan, China) was carried out for 30 min, and this was followed by further washing with PBS. Finally, the sections were incubated for 30 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 diaminobenzidinetetrachloride kit (DAB kit, AR1022, Boster Corporation, Wuhan, China) for 30 min to detect immunereactivity. After washing with distilled water, the sections were dehydrated with a graded ethanol series, cleared in xylene, mounted with a cover slip, and viewed under a light microscope (BH-2; Olympus, Japan).

Morphometric analysis. After taking digital photographs under a light microscope (BH-2; Olympus, Japan) with a digital camera (COOLPIX4500; Nikon, Japan), the number of somatostatin immunopositive cells and islets 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). In addition, the area of the islets is also calculated. The somatostatin-immunopositive cell density was calculated as the number of somatostatin-immunopositive cells per unit area. The islet area is calculated as the sum of islet area per unit of pancreas area.

Statistical analysis. Results are expressed as means ± standard errors on the mean (means ± S.E.). Statistical analysis was done using analysis of variancestatistics software (SAS Institute, 2000) with Duncan's multiple range test where appropriate. Differences of $P < 0.05$ were considered significant.

RESULTS

Effects of exogenous ghrelin on the Pancreas weight of African ostrich chicks. Gross anatomy showed that the experimental groups treated with exogenous ghrelin showed higher BW than the control group ($P < 0.05$) (Table I), The pancreas weight was higher in the 10 μg/kg group than in the control ($P < 0.05$) (Table I), and slightly higher in the 50 and 100 μg/kg groups than in the control ($P > 0.05$) (Table I). But there was no difference in the relative pancreas weight

between the 10 μg/kg group and control group (P > 0.05). The relative pancreas weight in the 50 μg/kg and 100 μg/kg groups was lower than that in the control group (P < 0.05) (Table I). The relative pancreas weight in the 10 μg/kg group was higher than that in the 50 μg/kg and 100 μg/kg groups (P < 0.05) (Table I). There was no difference in the relative pancreas weight between the 50 μg/kg and 100 μg/kg groups (P > 0.05) (Table I).

Table I Effects of exogenous ghrelin on pancreas weight of African ostrich chicks

Exogenous ghrelin (ug/kg)	Body weight (kg)	Pancreas weight (g)	Pancreas weight/Body weight (g/100g BW)
0	4.6±0.71[a]	7.87±1.86[a]	17.11±2.41[a]
10	6.8±0.41[b]	11.80±2.03[b]	17.35±3.63[a]
50	6.7±0.52[b]	9.17±1.41[a]	13.69±1.93[b]
100	6.5±0.67[b]	8.11±1.98[a]	12.48±2.05[b]

The data is expressed as mean±standard deviation (n=4). a-b Different letters in the same column indicate significant differences (P < 0.05).

Effects of exogenous ghrelin on Pancreatic histology of African ostrich chicks. Light microscopy showed no morphological difference in the islet cells in the 10 μg/kg, 50 μg/kg, and 100 μg/kg groups, but the cell arrangement was loose and the cytoplasm was reduced in comparison with the control group (Fig. 1). In the exocrine pancreas, the 10 μg/kg group showed a significantly lower volume of acinar gland cells, cell atrophy, reduced cytoplasm, and a higher number of nuclear contractile gland cell in comparison with the control group (Fig. 2B). The acinar cells in the 50 μg/kg group showed the most marked difference, with cell atrophy, cytoplasm reduction, nuclear shrinkage, and loose arrangement of glandular cells (Fig. 2C). The 100 μg/kg group showed no significant changes in the tissue structure of the exocrine pancreas; only the volume of glandular cells decreased slightly (Fig. 2D).

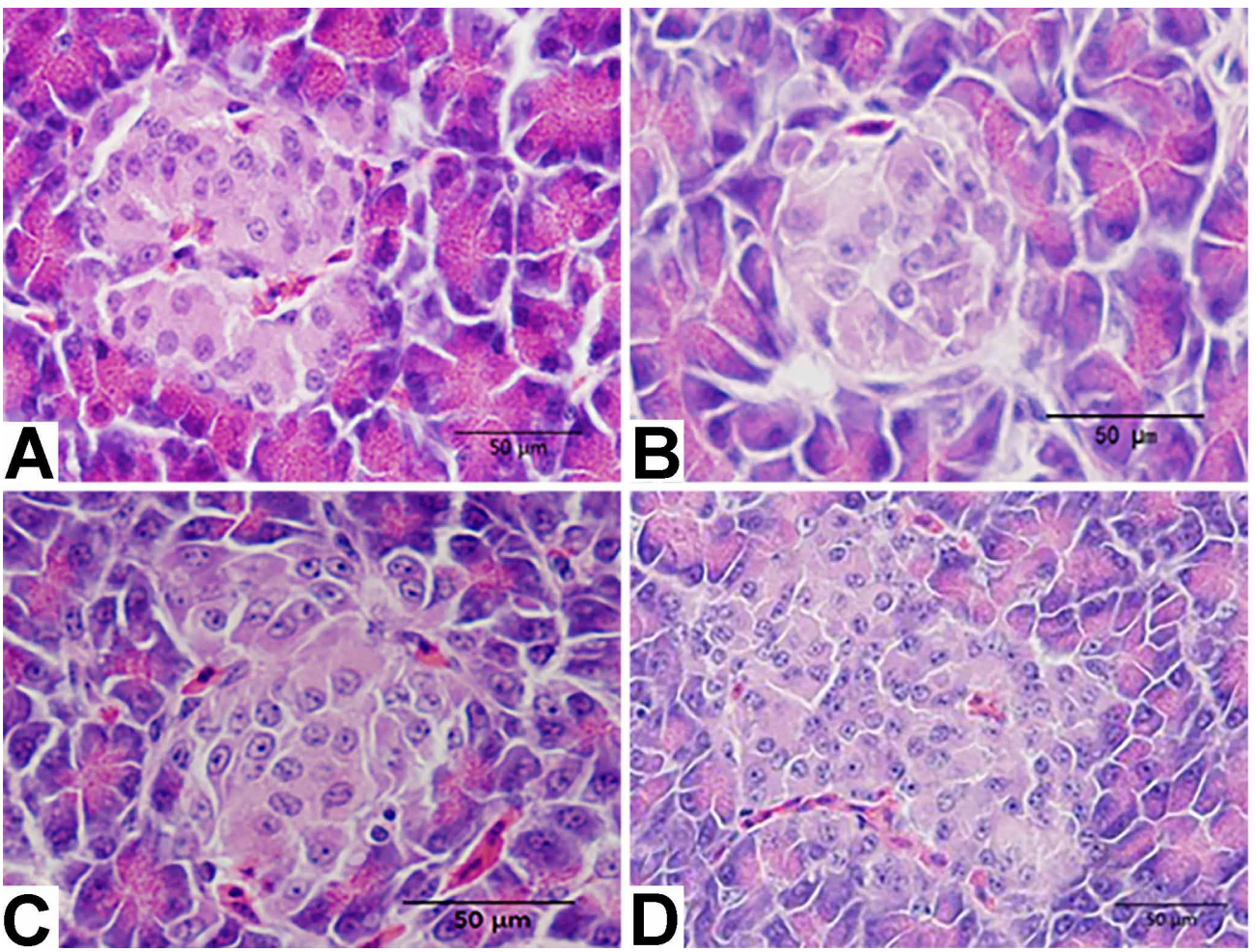

Fig. 1. The effects of exogenous ghrelin on the tissue structure of pancreatic islets of African ostrich chicks (HE Staining). (A) 0 μg/kg dose group (control group). (B) 10 μg/kg dose group. (C) 50 μg/kg dose group. (D) 100 μg/kg dose group. Scale bar: 50 μm.

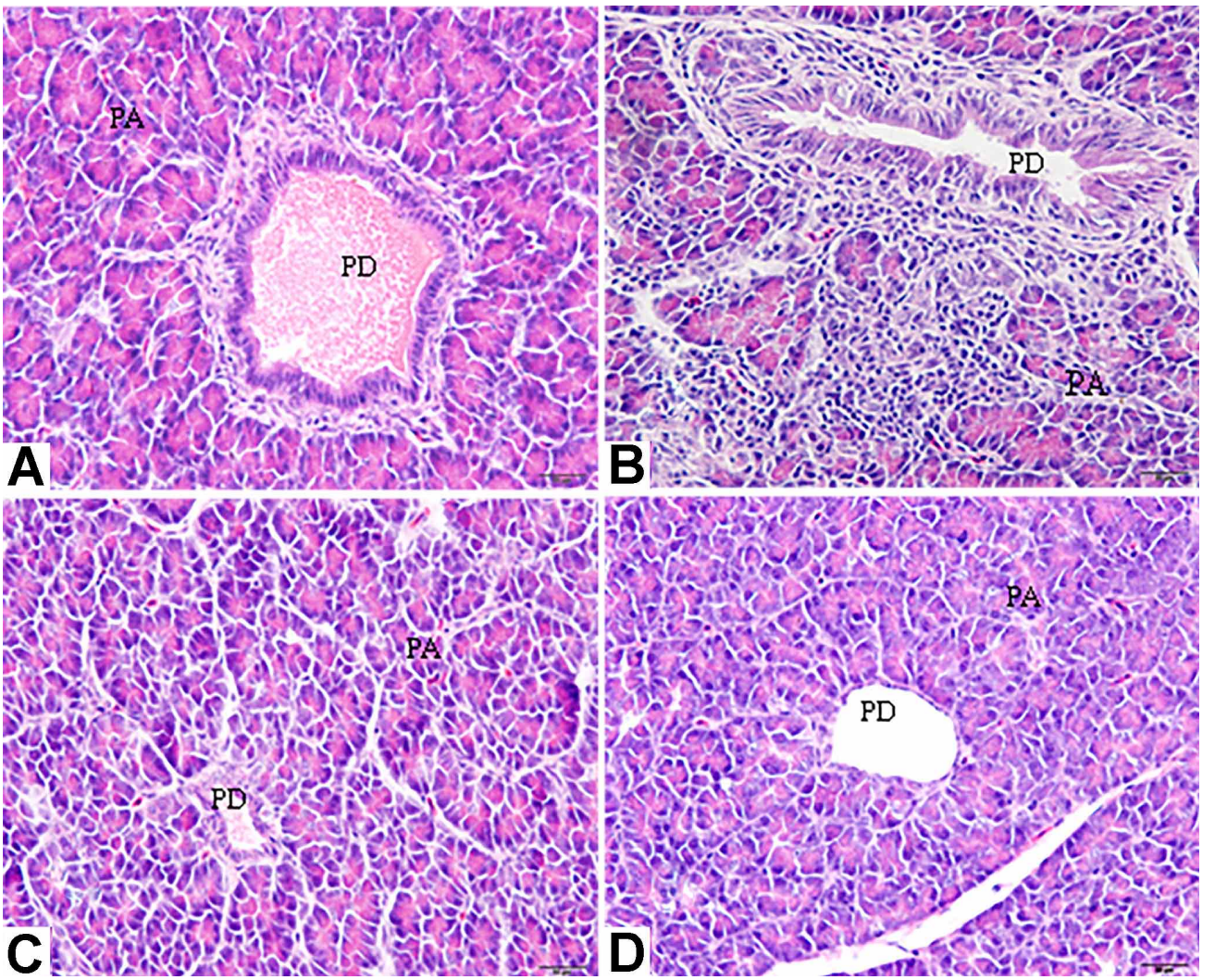

Fig. 2. The effect of exogenous ghrelin on the tissue structure of pancreas exocrine in African chicks (HE staining). (A) 0 μg/kg dose group (control group). (B) 10 μg/kg dose group. (C) 50 μg/kg dose group. (D) 100 μg/kg dose group. PD, pancreatic duct of the pancreas of African ostriches; PA, pancreatic acinus of the pancreas of African ostriches. Scale bar: 50 μm.

Table II Effect of exogenous ghrelin on the area of pancreatic islets in African ostrich chicks

Exogenous ghrelin (μg/kg)	pancreatic islets area ($\mu m^2/mm^2$)
0	4923.51 ± 182.96^a
10	7614.73 ± 206.42^b
50	28461.68 ± 218.75^c
100	10952.97 ± 197.03^d

The data is expressed as mean±standard deviation (n=4). a-d Different letters in the same column indicate significant differences($P < 0.05$).

Table III Effects of exogenous ghrelin on the number of pancreatic islets in African ostrich chicks

Exogenous ghrelin (μg/kg)	Pancreatic islets number (number /mm^2)
0	$2.7\pm0.36_a$
10	$1.9\pm0.75_b$
50	$3.2\pm0.99_a$
100	3.1 ± 0.83^a

The data is expressed as mean±standard deviation (n=4). a-b Different letters in the same column indicate significant differences($P < 0.05$).

Morphometric analysis showed that the 10 μg/kg, 50 μg/kg, and 100 μg/kg groups had higher islet area per unit area of the pancreas than the control group ($P < 0.05$) (Table II). The islet area also differed among the groups; the 50 μg/kg group showed a higher islet area than the low-dose and high-dose experimental groups ($P < 0.05$) (Table II), and

the 10 μg/kg group had the smallest islet area (Table II). In addition, the number of islets per unit area of the pancreas in the 10 μg/kg group was lower than that in the control group ($P < 0.05$) (Table III), whereas the number of islets in the 50 μg/kg and 100 μg/kg groups was slightly higher than that in the control group ($P > 0.05$) (Table III). Among the experimental groups, the number of islets in the 10 μg/kg group was lower than that in the other two groups ($P < 0.05$) (Table III).

Effects of exogenous ghrelin on the Somatostatin immunopositive cells in the pancreas. Immunohistochemical staining was used to locate the Somatostatin- immunopositive cells. The results showed that somatostatin-immunopositive cells were distributed around the islets, and there was sporadic distribution in the center. The positive cells showed cytoplasmic staining, and the cells were diverse in shape (for example, polygonal, circular, and elliptical; Fig. 3). The density of somatostatin-immunopositive cells in the pancreas

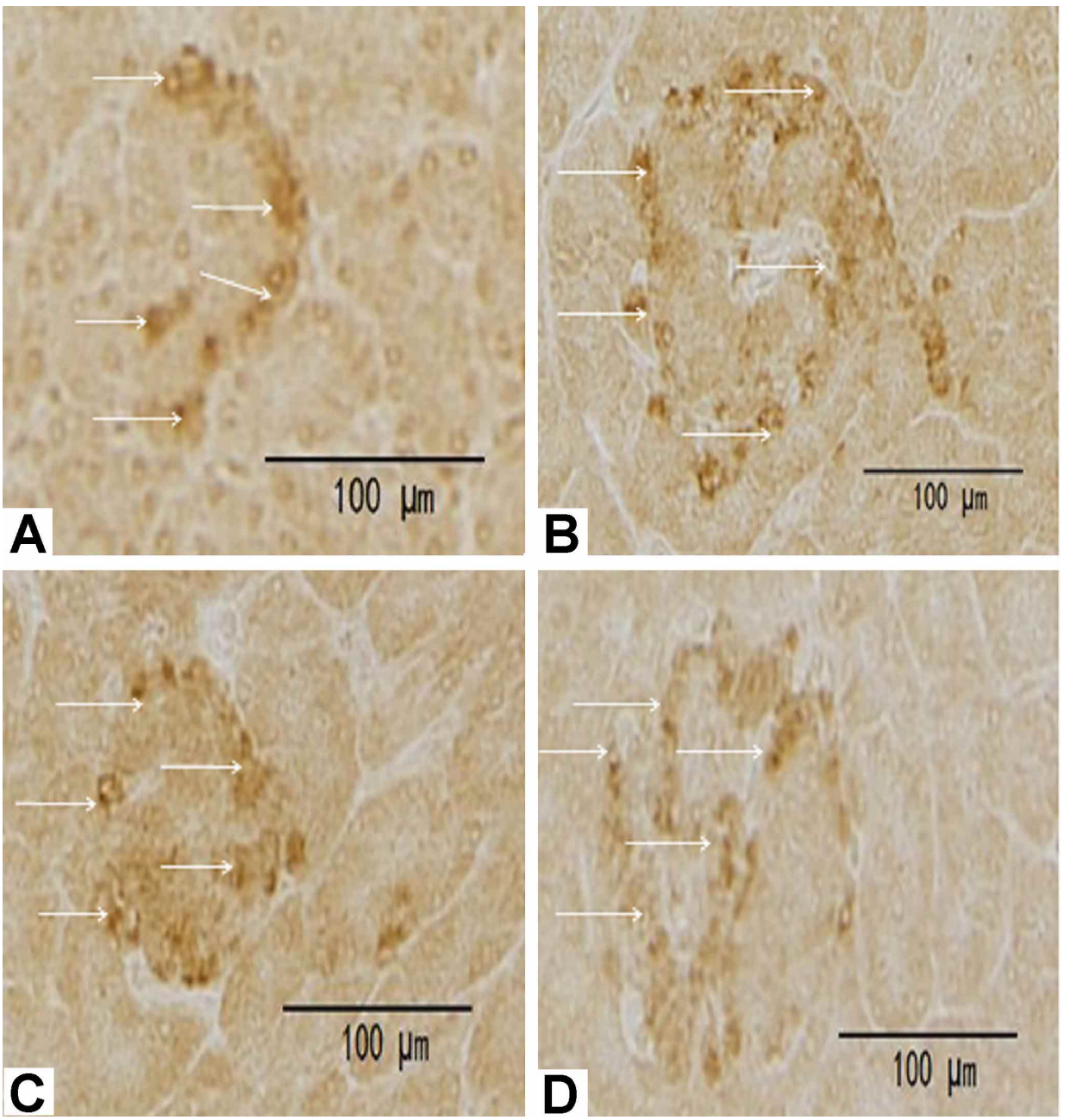

Fig. 3. Effects of exogenous ghrelin on the distribution of somatostatin immunopositive cells in pancreatic islets of African ostriches chicks (SABC staining). somatostatin immunopositive cells (arrows) were observed in each dose group. (A) 0 μg/kg dose group (control group). (B) 10 μg/kg dose group. (C) 50 μg/kg dose group. (D) 100 μg/kg dose group. Scale bar: 100 μm.

in the 10 μg/kg, 50 μg/kg, 100 μg/kg dose groups was higher than that in the control group ($P < 0.05$). The density of somatostatin-immunopositive cells in each experimental group differed, but the difference was not significant ($P > 0.05$) (Table IV).

Table IV Effect of exogenous ghrelin on the density of somatostatin immunopositive cells in the pancreatic islets of African ostrich chicks

Exogenous ghrelin (μg/kg)	Density of somatostatin immunopositive cells (number /mm^2)
0	6.42±0.43^a
10	17.23±0.65^b
50	14.98±0.97^b
100	16.71±0.28^b

The data is expressed as mean±standard deviation (n=4). a-b Different letters in the same column indicate significant differences($P < 0.05$).

DISCUSSION

Effects of exogenous ghrelin on the Pancreas weight of African ostrich chicks. Ghrelin is currently the only known gut-derived hormone that regulates feeding through central or peripheral stimulation (Wren *et al*., 2001). It plays an important role in increasing the growth rate of animals and increasing the daily weight gain of tissues and organs during growth in many organisms (Du *et al*., 2013). However, other studies suggest that ghrelin does not always promote growth and development of animals, tissues, and organs. Dembinski *et al*. (2005) found that ghrelin reduced pancreatic growth in suckling rats and increased pancreatic growth in weaned and 7-week-old rats. This biphasic effect of ghrelin on pancreatic growth in young animals seems to be related to age-dependent changes in the anabolic insulin-like growth factor 1 (IGF-1) secretion. The results of the present study indicate that the body weight of the African ostrich chicks and the pancreas weight was higher in the experimental groups than in the control group. But there was no significant difference in the relative pancreas weight between the 10 μg/kg dose group and the control group, whereas the 50 μg/kg dose group and the 100 μg/kg dose group showed a lower relative pancreas weight than the control group. These findings are contrary to most of the existing research results, indicating that the effect of exogenous ghrelin on pancreatic growth and development in African ostrich chicks is related to the ghrelin concentration, and the high doses of exogenous ghrelin may inhibit the growth of pancreas. Further research is required to investigate whether this finding is associated with the effect of ghrelin on IGF-1 synthesis.

Effects of exogenous ghrelin on Pancreatic histology of African ostrich chicks. In the present study, HE staining and histological assessment was used to further investigate the effects of ghrelin on pancreatic growth and development in African ostriches. The results indicate that ghrelin significantly affected both the endocrine and exocrine parts of the pancreas. In the exocrine pancreas, the volume of acinar cells in different dose groups has been reduced to varying degrees, these findings suggest that ghrelin not only inhibited the growth of the exocrine pancreas but possibly also affected the secretion of pancreatic juice. In the endocrine pancreas, According to previous studies, ghrelin is essential for the development and differentiation of islet cells during mouse embryonic development (Hill *et al*., 2009), and it promotes the proliferation of pancreatic beta cells and islet cells in humans and inhibits their apoptosis (Granata *et al*., 2007). In addition, Kerem *et al*. (2009) showed that the intraperitoneal injection of ghrelin increases the number of islet cells and promotes islet development after 90 % pancreatectomy in rats. Turk *et al*. (2012) also showed that islet area increased after ghrelin treatment in streptozotocin-induced-diabetic rats. The results of the present study indicate that exogenous ghrelin can significantly promote the growth of pancreatic islets in African ostrich chicks, which is consistent with the results of previous studies (Granata *et al*.; Kerem *et al*.; Warzecha *et al*., 2010; Turk *et al*.). And in each dose group, the strongest promoting effect on the growth of pancreas islets in African ostriches was caused by the 50mg/kg dose, indicating that the intensity of its effect is closely related to the circulating ghrelin level.

Effects of exogenous ghrelin on the Somatostatin immunopositive cells in the pancreas. Numerous studies have indeed identified an interaction between ghrelin and somatostatin. Shimada *et al*. (2003) found that somatostatin inhibited ghrelin secretion in rat stomach. Ghrelin also has a regulatory effect on the secretion of somatostatin, and some studies have shown that direct injection of ghrelin into the brain stimulates the expression of somatostatin mRNA (Seoane *et al*., 2003). Another study showed that gastric mucosal cells isolated from piglets treated with a certain concentration of ghrelin showed significantly increased somatostatin mRNA expression (Du *et al*., 2006). In the present study, the effect of ghrelin on somatostatin in African ostrich chicks was investigated using immunohistochemical staining. The results showed that somatostatin immunopositive cells were widely distributed in islets; the density of somatostatin immunopositive cells was significantly higher in the 10 μg/kg, 50 μg/kg, and 100 μg/kg groups than in the control group, indicating that different ghrelin concentrations significantly promote somatostatin expression in the pancreas of African ostrich chicks. It is well known that somatostatin can inhibit insulin

secretion. Luethy *et al*. (2019) found that intravenous and subcutaneous administration of somatostatin inhibits insulin secretion and increases glucose concentration in horses. Córdoba-Chacón *et al*. (2013) also found that endogenous somatostatin can regulate arginine-stimulated insulin secretion. In addition, DiGruccio *et al*. (2016) found that ghrelin promotes somatostatin release likely by engaging the canonical Gaq/11 and Ca2+ cascade in delta cells, which then inhibit insulin secretion via beta cell somatostatin receptors coupled to Gai. Therefore, this suggests that ghrelin may inhibit insulin release by promoting the secretion of somatostatin in the pancreas of African ostrich chicks. However, this needs further study.

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RESUMEN: Este estudio tuvo como objetivo investigar el efecto de la grelina exógena sobre el crecimiento y desarrollo del páncreas en polluelos de avestruz africana. Dieciséis pollos de avestruz africana de 40 días (machos o hembras) se dividieron al azar en cuatro grupos y se inyectaron por vía intravenosa con solución salina (control) o grelina (10, 50 y 100 μg / kg) durante 6 días. determinadas, se observaron las características estructurales mediante tinción Hematoxilina-Eosina, se detectaron células inmunopositivas a somatostatina mediante inmunohistoquímica. Los resultados fueron los siguientes: ¨Los grupos de 50 y 100 μg / kg mostraron un menor peso relativo del páncreas que el grupo de control (P <0,05). El área de islotes por unidad de área del páncreas fue mayor en los grupos de 10, 50 y 100 μg / kg grupos que en el grupo de control (P <0,05). El número de islotes por unidad de área del páncreas fue menor en el grupo de 10 μg / kg que en el control (P <0,05). Además, en comparación con el control, las células de los islotes en los grupos de tratamiento estaban dispuestas de forma holgada y mostraban un citoplasma reducido. En el páncreas exocrino, el volumen de células acinares en los grupos de 10, 50 y 100 μg / kg disminuyó en diversos grados. Las células inmunopositivas de somatostatina se ubicaron principalmente alrededor de la periferia de los islotes y se distribuyeron esporádicamente en el centro. La densidad de las células inmunopositivas a la somatostatina en los grupos de 10, 50 y 100 μg / kg fue mayor que la del control (P <0,05). Estos hallazgos sugieren que la grelina exógena aumenta el área y el número de islotes y el número de células inmunopositivas a la somatostatina, pero reduce el peso relativo del páncreas, lo que puede inhibir el crecimiento y desarrollo pancreático en los polluelos de avestruz africana.

PALABRAS CLAVE: Avestruz africana; Grelina; Páncreas; Crecimiento; Desarrollo.

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