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

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ABSTRACT

Ghrelin, the endogenous ligand for the GH-secretagogue receptor (GHS-R), has been found in the testes of many vertebrates, and in the cerebellum and gastrointestinal tract of African ostrich, but there is little known about its distribution in the testes of the African ostrich. In the present study, the distribution and morphological characteristics of ghrelin-immunopositive (ghrelin-ip) cells in the testes of African ostrich were investigated using immunohistochemistry. The testes of African ostrich consisted of a capsule and parenchyma. The capsule was divided into three annular layers, and the parenchyma consisted of tubular compartments and interstitial tissue. The tubular compartments were comprised of the seminiferous tubules, straight seminiferous tubules and rete testis, without any distinct boundary tissue between them. The interstitial tissue between the tubules contained many Leydig cells, tiny veins and connective tissue. Ghrelin-ip cells in the African ostrich testes were localized only in the parenchyma, and mostly in the tubular compartments. The ghrelin-ip cells in the tubular compartments were mainly spermatogenic cells and some Sertoli's cells. In the insterstitial tissue, the ghrelin-ip cells were identified as Leydig cells. The ghrelin-ip cells showed cytoplasmic staining, and were irregularly shaped. These findings indicate that ghrelin may participate in the spermatogenesis in the African ostrich. Further studies into this regulation will help clarify the physiological roles played by this hormone in the African ostrich.

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Authors' Contribution
LXY designed the experiments, performed the trials and wrote the manuscript. JXW designed the experiments, performed the analysis with constructive discussions. PL analysed the data. XTZ helped in performing the experiments.

Key words
African ostrich, Ghrelin, Immunohistochemistry, Testis.

INTRODUCTION

Ghrelin, a peptide hormone isolated from the stomach, is mainly secreted by X/A-like endocrine cells in the stomach and is an endogenous ligand of the growth hormone secretagogue receptor (Kojima *et al*., 1999; Ariyasu *et al*., 2001). Previous studies have shown that ghrelin has a regulatory effect on the secretion of growth hormone, energy balance and feeding behaviour (Wang *et al*., 2011; Sato *et al*., 2012; Mihalache *et al*., 2016). As it has a wide effect on the animals, ghrelin-ip cells not only have been found in most tissues and organisms in mammals (David *et al*., 2005; Raghay *et al*., 2013; Shi-Ping *et al*., 2013; Liu *et al*., 2017), but also have been identified in non-mammalian species (Neglia *et al*., 2005; Wei *et al*., 2010; Shao *et al*., 2010; Ma *et al*., 2015). Also previous studies have showed that ghrelin-ip cells were distributed in the seminiferous tubules and interstitial tissues of the testes of human, mouse and sheep (David *et al*., 2005; Gaytan *et al*., 2004; Ilhan and Erdost, 2013). Additionally, ghrelin is involved in the central endocrine system, following the hypothalamus-pituitary-gonadal axis in the regulation of the gonads (Miller *et al*., 2005). According to previous reports, ghrelin plays a significant role in the regulation of the testes in all physiological and pathological conditions (Gaytan *et al*., 2004), thus, it is of great significance to study the localization and distribution of ghrelin in testes. However, the distribution of ghrelin-ip cells in the testes of African ostrich has not been reported, although ghrelin-ip cells have been found in the intestine, brain and stomach of the African ostrich (Wang *et al*., 2009, 2012). In order to clearly understand the physiological role of the newly identified peptides, in this study, the distribution and morphological characteristics of ghrelin-ip cells in the testes of the African ostrich were investigated using immunohistochemistry.

MATERIALS AND METHODS

Animals

African ostriches (age, 8 months; weight, 75.68±3.25 kg) were used for this study. African ostrich (6 males) were obtained from the Ostrich Research Institute of Yangtze University in Hubei Province, China, where feed and water were made available and libitum. All of the birds were maintained in a heated room with slatted plastic flooring and were fed a starter diet for postnatal days 7, which was formulated according to the specifications of the Elsenburg

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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 testis were quickly removed, 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 on a Leica microtome (Nussloch Gmbh, Germany), 2 suit sections were prepared; one suit was stained by haematoxylin and eosin (H&E) to observe the cytoarchitecture of testis; the other was stained by immunohistochemistry (SABC) to observe the distribution and morphological characteristics of ghrelin-ip cells in the testis of the African ostrich.

Immunohistochemistry

Immunohistochemical detection of ghrelin-ip cells using rabbit anti-ghrelin was carried out by the 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. 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 (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 10 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 (5 µg/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 testis.

Morphometric analysis

For each testis tissue sample, 3 cross-sections were prepared after the samples had been stained with hematoxylin and eosin and SABC stain. Further, for each testis cross-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).

RESULTS

Cytoarchitecture of the testis in the African ostrich

The testes of Africa ostrich consisted of the capsule and parenchyma (Fig. 1A). The capsule was divided into three annular layers (from the parenchyma outwards): the tunica vasculosa, tunica albuginea and tunica serosa (Fig. 1B). The parenchyma included tubular compartments and interstitial tissue (Fig. 1A). The tubular compartments were comprised of the seminiferous tubules, straight seminiferous tubules and rete testis. The seminiferous tubules were composed of a myoid cell layer, basement membrane and seminiferous epithelium (Fig. 1C). The seminiferous epithelium consisted of the spermatogonia and Sertoli cells; Spermatogonia were round or oval with unclear boundaries and clear nuclei, while Sertoli cells were triangular or pyramidal with conical or elliptical nucleus (Fig. 1C). Each stage of spermatogenia cell was distributed from the basement membrane to the lumen, and the lumen was filled with a large amount of eosinophilous substance (Fig. 1C). The interstitial tissue contained many Leydig cells, tiny veins and connective tissue (Fig. 1D). Leydig cells were relatively large in the interstitial tissue, and were round or irregularly shaped with a round or oval asymmetrical nucleus (Fig. 1D).

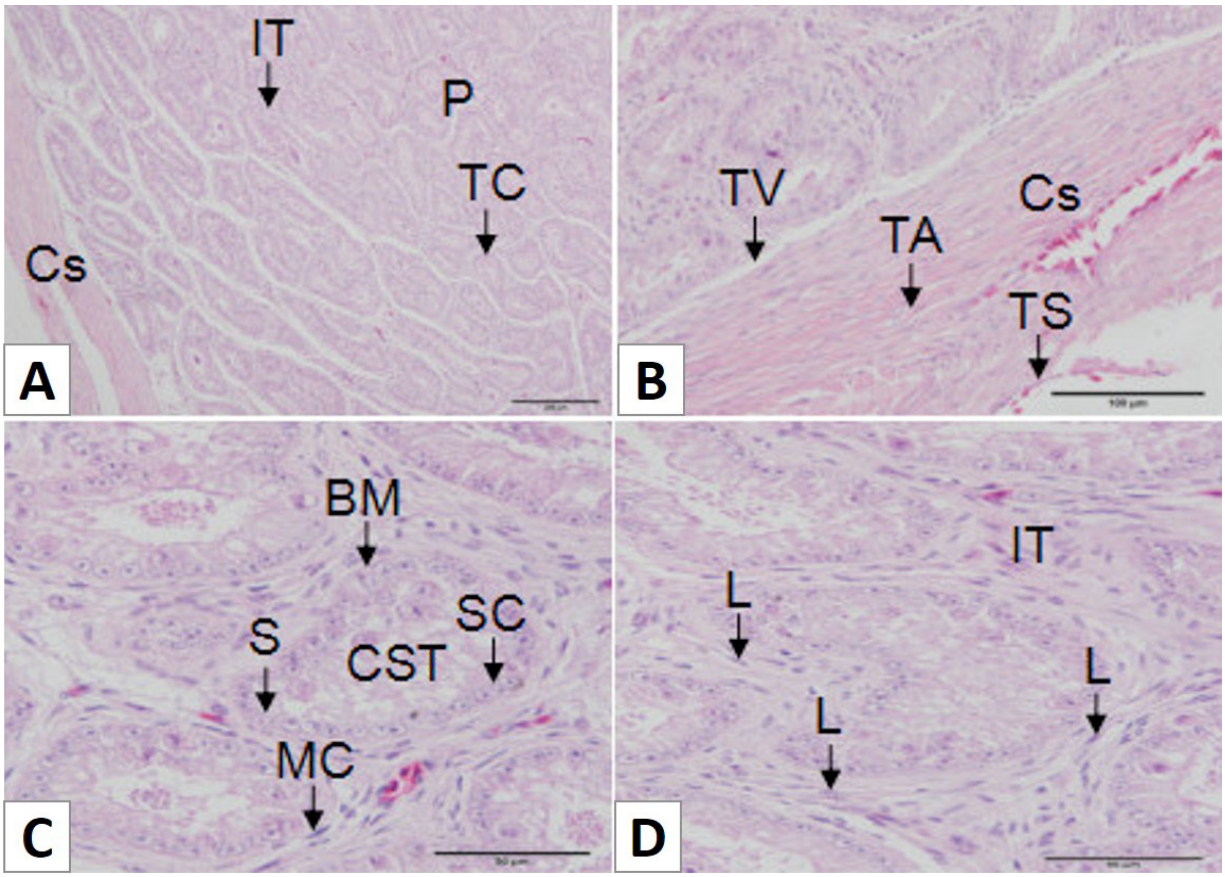

Fig. 1. Histology of the African Ostrich testes. A, the testis was divided into the capsule (Cs) and parenchyma (P); B, the capsule was divided into three annular layers: tunica serosa (TS), tunica albuginea (TA) and tunica vasculosa (TV); C, the structure of the seminiferous tubules (CST) includes the basement membrane (BM), spermatogonia (S), Sertoli cells (SC) and myoid cells (MC); D, the structure of the interstitial tissue (IT) includes Leydig cells (L), tiny veins and connective tissue. Scale bar: 200 μm (A), scale bar: 100 μm (B) and scale bar: 50 μm (C and D).

Distribution of ghrelin immunopositive cells in the testis of African ostrich

Immunohistochemical streptavidin-biotin-peroxidase complex (SABC) staining showed that ghrelin-ip cells were present in the tubular compartments and among the interstitial tissue in the testes of African ostrich (Fig. 2A). The positive cells in the tubular compartments were distributed in the spermatogenic cells and Sertoli cells of the seminiferous tubules (Fig. 2A); furthermore, they were irregular or oval in shape, showed cytoplasmic staining, in addition, their nuclear and cytoplasmic boundaries were blurred (Fig. 2B). Ghrelin-ip cells in the interstitial tissue were identified as Leydig cells, observed in clusters or singly, and oval in shape. They also showed cytoplasmic staining, and the nuclear and cytoplasmic boundaries were blurred (Fig. 2C). The control group of cells were negative for ghrelin (Fig. 2D).

DISCUSSION

Structure of the African ostrich testis

The results of the present study showed that the testis of the African ostrich is composed of a capsule and parenchyma, and the parenchyma includes tubular compartments and interstitial tissue, this structure is similar to that reported in other animal species (Wang *et al*., 2000, 2011; Fu *et al*., 2009; Yu *et al*., 2010; Bai *et al*., 2009; Zhang *et al*., 2002; Lebelo and Horst, 2017). The capsule is divided into three annular layers: tunica serosa, tunica albuginea and tunica vasculosa. The tunica albuginea and tunica vasculosa extend into the parenchyma of testes to form the stents, but without septa interlobulare, similar to other birds (Wang *et al*., 2000; Fu *et al*., 2009; Yu *et al*., 2010). In contrast, the testis in mammals generally have clear septa interlobulare (Bai *et al*., 2009; Wang *et al*., 2011; Zhang *et al*., 2002).

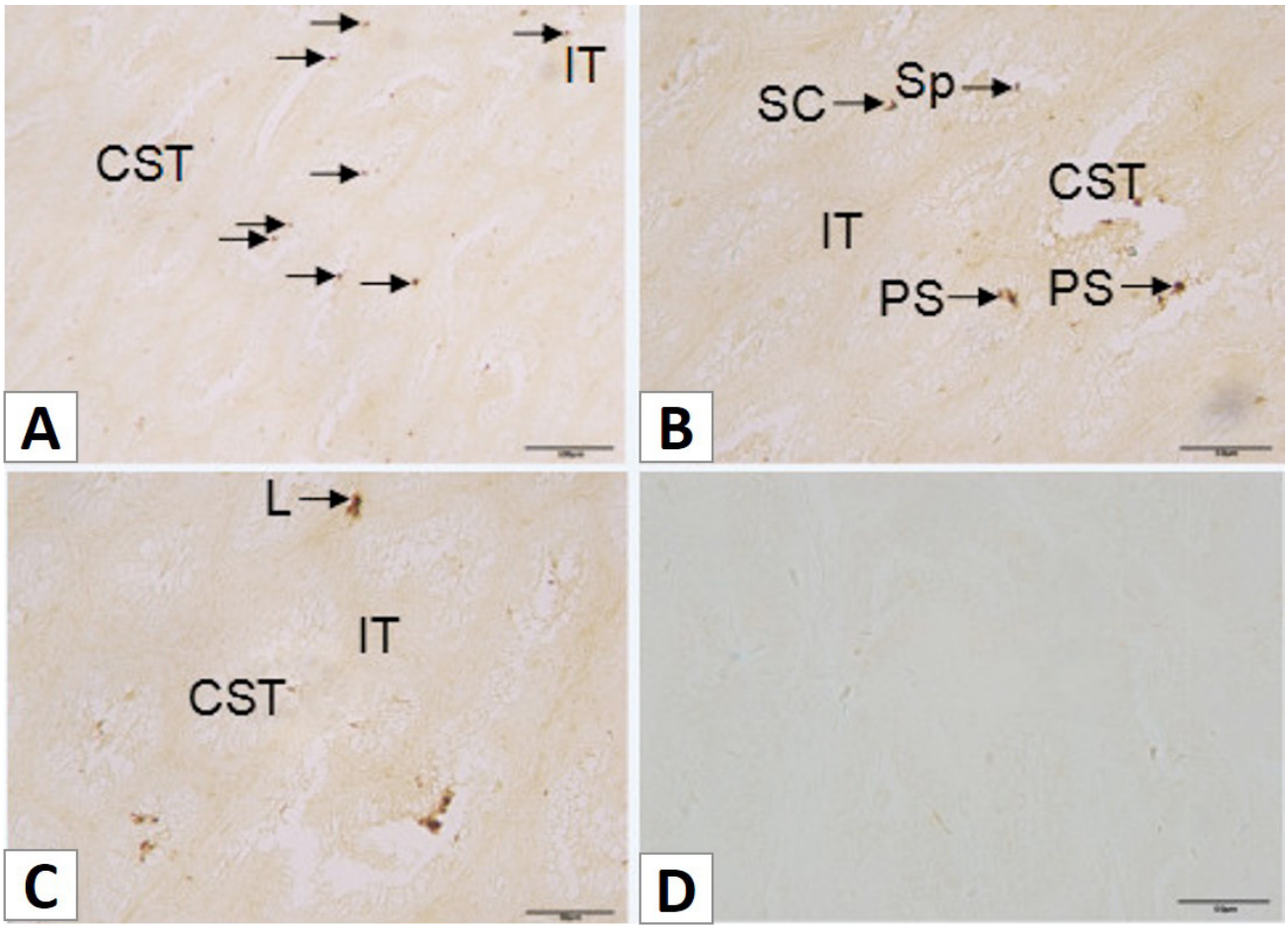

Fig. 2. Distribution of ghrelin-ip cells in the testes of the African ostrich. A, ghrelin-ip cells were found in the seminiferous tubules (CST) of the tubular compartments and interstitial tissue (IT) of the testis; B, ghrelin-ip cells were found in the sperm (SP), primary spermatocytes (PS) and Sertoli cells (SC) of the seminiferous tubules; C, ghrelin-ip cells were found in the Leydig cells (L) of the interstitial tissue; D, microphotograph of absorption test in the testes. Arrows indicate ghrelin-ip cells. Scale bar: 100 μm (A) and scale bar: 50 μm (B, C and D).

This indicates that the avian testes are at a lower stage of evolution than the mammalian testes.

In the seminiferous tubules, there were one to three layers of myoid cells surrounding the basement membrane, fewer than in the mammalian testes (Wang *et al*., 2011); this suggests that the ability to secrete sperm is relatively low in the African ostrich testes. The seminiferous epithelium was arranged tightly into one to two layers, a poor population compare to other birds (Wang *et al*., 2000; Fu *et al*., 2009; Yu *et al*., 2010), and this may contributes to the long maturation period the African ostrich.

There was much eosinophilous substance found in the lumen of the seminiferous tubules, dissimilar from most animals but similar to *Cygnus cygnus* (Yu *et al*., 2010). This is presumed to be secretions of the Sertoli cells or cytoplasmic fragments in the process of spermatogenesis, and it remains to be confirmed.

Distribution of Ghrelin immunoreactive cells in the testis of African ostrich

Previous studies have reported the distribution of ghrelin-ip cells in the testes of mammals (Gaytan *et al*., 2004; Barreiro *et al*., 2002; Miller *et al*., 2005; Zhang *et al*., 2015), but there are no reports currently available in birds. The results of the present study showed that ghrelin-ip cells were distributed in the tubular compartments and among interstitial tissue of the testis in the African ostrich, and it consistents with the results of previous studies. Thus, ghrelin-ip cells may be distributed in other bird testes in a similar manner.

Current knowledge of the distribution of ghrelin-ip cells in the testes is mainly limited to mammals (Gaytan

et al., 2004; Ilhan and Erdost, 2013; Barreiro *et al*., 2002; David *et al*., 2005). Gaytan *et al*. (2004) reported that in the human testes, most Leydig cells and tiny numbers of Sertoli cells were positive for ghrelin, but the spermatogenic cells were ghrelin-negative. In addition, Ilhan and Erdost (2013) found that ghrelin was located in the Leydig cells and Sertoli cells in the testes of mouse. Barreiro *et al*. (2002) found that ghrelin-ip cells were distributed only in the Leydig cells of the mouse testes. Furthermore, David *et al*. (2005) found that in the adult testes of sheep, ghrelin immunostaining was predominant in the germ and Sertoli cells, with the germ cells showing intense perinuclear staining; there appeared to be more intense ghrelin staining in the germ cells in all developmental stages prior to the first meiotic division. Lower level immunostaining was also observed in the Leydig cells. The results of the present study showed that ghrelin-ip cells were distributed in the tubular compartments and interstitial tissue of the testes of the African ostrich, and these positive cells were identified as spermatogenic cells, Sertoli cells and Leydig cells, much different from the distribution of ghrelin-ip cells in mammalian. Thus, the distribution of ghrelin-ip cells in the testes of the African ostrich is characteristic to this species. And the results of the present study may indicate that ghrelin may be related to spermatogenesis in the testis of Africa ostrich, and be involved in the proliferative activity of spermatogenic cells, as David *et al*. (2005) have showed that the ghrelin system was linked to the proliferative activity of germ and somatic cells in the testis of sheep, and Kheradmand *et al*. (2009) also have found that ghrelin has anti-proliferative effects on different testicular types and was a negative modulator of male reproductive system. However, the biological effect and mechanism of ghrelin on testis remains to be further studied.

CONCLUSION

In conclusion, this study provides a morphological basis for further studies on the regulation mechanism of ghrelin in the African ostrich testes, and contributes to new information about ghrelin in a species that has not been previously investig

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Statement of conflict of interest

The authors report no conflicts of interest. The authors are responsible for the content and writing of this article.

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