

Histological examination of testicular cell development and apoptosis in the ostrich chick

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Received: 06.06.2008

Abstract: The aim of this study was to observe the microstructure and ultrastructure of the testis, demonstrate testicular cell development and apoptosis, and elucidate regularity of development and apoptosis in ostrich chick testes. By employing light microscopy 3 obvious development characteristics were detected with ostrich age increasing: first, many primordial germ cells and a few spermatogonia were found while seminiferous tubule integrity was not evident in testes of 1-day-old ostrich chicks nor were primordial germ cells. In the 30-day-old ostrich chicks spermatogonia were completely differentiated, but very few primary spermatocytes were observed in testes at 45 days of age. Second, the quantity of mitochondria in spermatogonia and lipid droplet in Leydig cells increased gradually with the increasing age of ostrich. Third, testicular cell apoptosis was observed and the number of apoptotic testicular cells showed a peak in the testis of 45-day-old ostrich chicks ($P < 0.05$), as testicular cells were prone to apoptosis at that age.

Key words: Ostrich chicks, testis development, histological structure, apoptosis

Introduction

In birds, as in mammals, the testes are responsible for the production of spermatozoa and the secretion of androgen. Spermatocytogenesis consists of the mitotic divisions involving proliferation and maintenance of spermatogonia (1). Germ cell transfer is becoming an important technique for the study of spermatogenesis and potentially can be used for the production of transgenic progeny (2). Spermatogonia then undergo meiosis to form primary spermatocytes and then secondary spermatocytes, which differentiate into spermatids. Morphological and histochemical characterization of the seminiferous epithelial (SE) and Leydig cells

of the turkey were observed (3). The morphological characteristics of the testes in the same species always change according to age and sexual activity cycle. The cytological relationships of the cells comprising the SE of several avian species have already been examined, with the most comprehensive studies done on Japanese quail (4,5). The rete testis and the efferent ducts of the ostrich (6,7) as well as the morphological characteristics of the ostrich testes have been studied (8,9).

The ostrich, belonging to the class Aves, order Struthioniformes, family Struthionidae, genus *Struthio*, is the biggest bird existing in the world, and its adaptability is very strong. Ostrich meat

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possesses the characteristics of high protein, and low fat, cholesterol, and calories. The leather made from ostrich skin is soft and durable, and has good permeability. Therefore, ostrich husbandry is becoming more widespread and important. Although the ostrich has assumed significant economic importance as a farmed bird, the reproductive biology of this primitive avian species remains unknown. Still ostrich reproduction is dogged by problems of infertility.

The reproductive cell continuously proliferated and at the same time it degenerated during the course of differentiation and development (10). Cell apoptosis, the process of cellular self-destruction, also involves active processes of intracellular synthesis, and is controlled by cellular genes (11,12). Cells with a high regeneration and division rate, or under endocrine control, are particularly susceptible to apoptosis (13). Although there is growing knowledge on the apoptosis of the testes in mammals (14-16), very little information is currently available pertaining to the apoptosis of the testes in avian species.

In the present study, we attempted to find the morphological characteristics of testis and testicular cell apoptosis by employing light microscopy, transmission electron microscopy, and terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL). Compared to the studies published on other birds and mammals, the morphological features and apoptosis of the ostrich testes from birth to 90-days old were examined to provide morphological evidence for the comparative histology, the developmental biology, and the reproductive biology, as well as to provide data for the formation and prevention of disease in ostrich chicks.

Materials and methods

Animals

Twelve male ostrich chicks, 1, 30, 45, and 90 days of age, were procured from the Ostrich Farm in Guangdong province, China. These birds were deeply anesthetized with 10% urethane (1 g/kg body

weight) and blood was collected from the arteria cervicalis, and the testes of the birds were obtained. All procedures involving animals were carried out in accordance with the Animal Protection Regulations of China.

Light microscopy

The testis tissue samples at different developmental phases were obtained and fixed in 4% paraformaldehyde phosphate buffered solution. The fixed tissue blocks were made and paraffin sections (6 μm) prepared from the paraffin-embedded material and serial sections were stained with hematoxylin-eosin for light microscopy.

Electron microscopy

The fixed tissue blocks by 3% glutaraldehyde were subsequently processed by standard methods through secondary fixation in 1% osmium tetroxide, dehydrated through a graded series of ethanol, and subsequently embedded in resin and ultra-thin sections were cut and stained conventionally with uranyl acetate and lead citrate and viewed and photographed with an electron microscope.

Apoptosis detection by TUNEL

After being fixed in 4% paraformaldehyde phosphate buffered solution at 4 °C, the testes tissue were embedded in paraffin by the standard method and cut by microtome into 6 μm thick sections. After deparaffining, they were then stained for identification of apoptotic cells by TUNEL method using an in situ cell detection kit (MK1020 Boster Ltd, Wuhan). Sections were then counterstained with hematoxylin. Control staining showed negative results.

Statistical analyses

For each sample, the brown positive testicular cells were numbered and quantitative histology index was measured by image analysis system from 10 views on each slice, and calculated average value of per view and 5 slices were selected from every testis. Testicular cells involved in apoptosis were examined for statistical significance by calculating the mean \pm S.E.M. The sections were examined under an Olympus light microscope (400 \times).

Results

Microstructure of the testis of ostrich chicks at different ages

In the testis of 1-day-old ostrich chicks (Figure 1A), some tubiform structures similar to seminiferous tubules were found, which were surrounded with many primordial germ cells and a few spermatogonia, but a complete seminiferous tubule did not emerge. Primordial germ cells showed a larger spherical nuclear size than any of the other cell types and with a higher nuclear-cytoplasm ratio, and the nucleus was basophilic, with light staining and clear flocci. The number of spermatogonia was low, and they showed small nuclei which were stained dark. There was redundant interstitial tissue in the testis of 1-day-old ostrich chicks and interstitial tissue comprised of generous desmocyte and small amounts of Leydig cells.

In the testis of 30-day-old ostrich chicks (Figure 1B), integrity seminiferous tubules were observed, but their shapes were irregular. At this age, primordial germ cells differentiated into spermatogonia completely. Spermatogonia were cubiform with spherical nuclei near the basement layer, and the seminiferous epithelium comprised a few Sertoli's cells and spermatogonia. There was less interstitial tissue in the testis of 30-day-old ostrich chicks than in that of 1-day-old chicks.

In the testis of 45-day-old ostrich chicks (Figure 1C), regular seminiferous tubules emerged, and the tubal wall was basement layer and seminiferous epithelium. Seminiferous epithelium was composed of Sertoli's cells and 1-2 layers of spermatogenic cells, and covered with wrap basal membrane comprising myoid cells and collagen fibers. Spermatogonia were predominantly cuboidal to low cuboidal and restricted

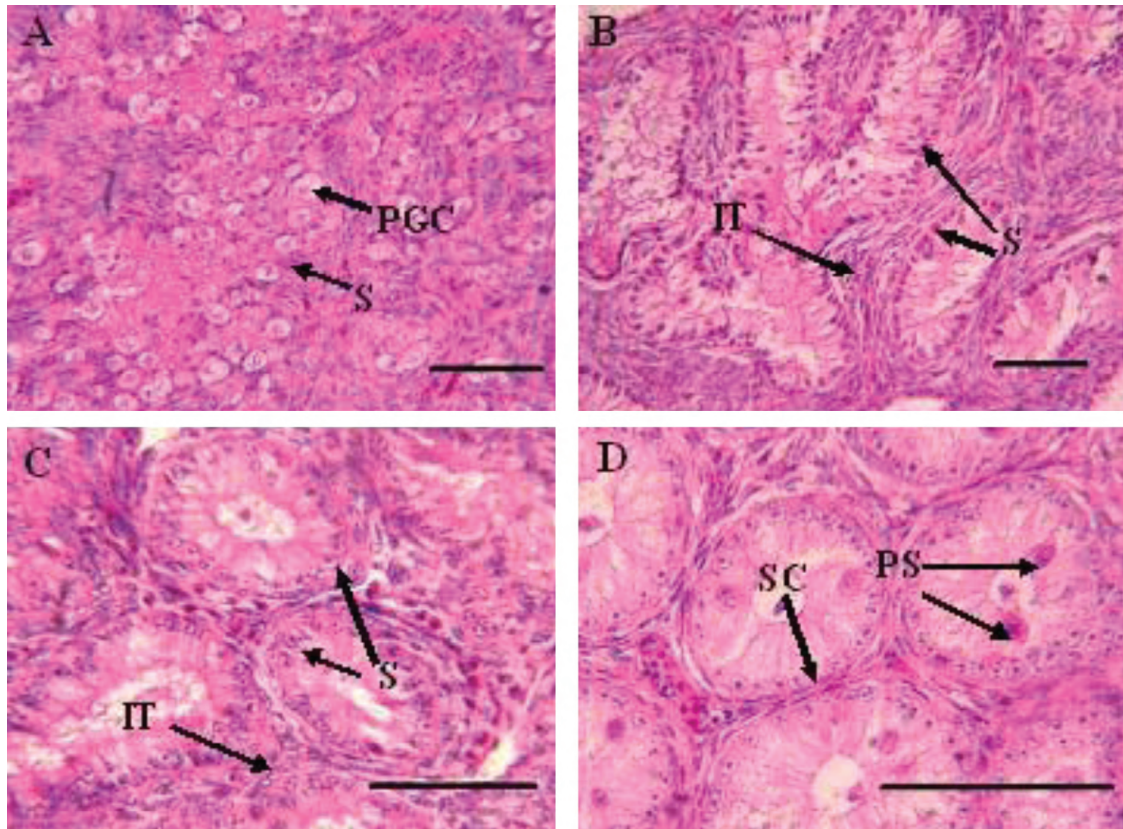


Figure 1. Histological section of ostrich chick testes from day 1 to day 90 of age. Figure 1A. Primordial germ cell (PGC) and spermatogonium (S) at day 1. Bar = 50 μ m. Figure 1B. Spermatogonium (S) and interstitial tissue of testis (IT) at day 30. Bar = 50 μ m. Figure 1C. Spermatogonium (S) and interstitial tissue of testis (IT) at day 45. Bar = 60 μ m. Figure 1D. Primary spermatocyte (PS) differentiated by spermatogonium and Sertoli cell (SC) at day 90. Bar = 75 μ m.

to contact with or close proximity to the inner wall of the seminiferous tubule. Spermatogonia showed large round or elliptic nuclei and were arranged regularly, and the nucleus was stained dark. At that stage, a scanty part of the spermatogonia population assumed a more irregular elongated appearance with their nuclei repositioned closer to the central lumen and differentiated into primary spermatocyte while the phase of cleavage was obvious. The number of primary spermatocytes was few and their nuclei were round. However, the other kinds of cells of the seminiferous epithelium were Sertoli's cells, which were conoid or columnar, and the number of them was few. The root of Sertoli's cell was wide and the shape of nuclei was triangular.

In the testis of 90-day-old ostrich chicks (Figure 1D), the diameter of seminiferous tubule was larger than that of 45-day-old ostrich chicks, the spermatogonia also scale up gradually, some of them had 2 nucleoli, and the nucleoli were stained dark. The number of primary spermatocytes located in the inside of spermatogenous cells increased and they were near the cavosurface of the seminiferous tubule. At this age, second spermatocyte was not present.

Ultrastructure of the testis of ostrich chicks at different ages

Electron micrographs of the testis of 1-day-old ostrich chicks showed primordial germ cell (Figure 2A, 2B) and desmocyte (Figure 2A, 2C). The nucleus of primordial germ cell was very big with low electro-dense chromatin. Chromatin was distributed uniformly in the nucleus of spermatogonium and had a high electro-density. Desmocyte was irregular in shape with a jagged nucleus; organella in cytoplasm was not redundant.

Also, electron micrographs showed that in the testis of 45-day-old ostrich chicks the nuclei of myoid cells in spermatogenic epithelium were fusiform (Figure 2D), while organelles in myoid cells were not prosperous. Spermatogonium in testis of 45-day-old ostrich chicks had cytoplasmic features resembling those of spermatogonium in testis of 30-day-old ostrich chicks, but it had slightly developed mitochondria (Figure 2D, 2E). Spermatogonium was very regular in shape and located basally. The terminal line of spermatogenous cells was unsharp, while the nucleoli were obvious. The nucleus

was surrounded by double layer nucleolemma, and there were many nuclear pores in the nucleolemma. Chromatin in cytoplasm was divided into euchromatin and heterochromatin, and the heterochromatin was located around nucleolemma and nucleus with exhibiting electron dense bodies (Figure 2E). The nucleus of Leydig cell was bigger with obvious nuclear pore in the nucleolemma (Figure 2F). Cytoplasm of Leydig cell comprised many lipid droplets and less other organelles, while the nuclei of fibrocyte were fusiform in shape and located aside. Sertoli's cell was irregular in shape and located basally (Figure 2G). The contact surface with Sertoli's cells and membrana basilaris was bigger, and the top of Sertoli's cell extended into the lumina of the convoluted seminiferous tubule.

Finally, electron micrograph of testis of 90-day-old ostrich chicks showed spermatogonium and myoid cell (Figure 2H). In the testis of 90-day-old ostrich, cytoplasmic features of myoid cell had no obvious change, but spermiogonium had developed mitochondria and rough endoplasmic reticulum (Figure 2I), and some spermatogenous cells had 2 nucleoli (Figure 2H). All of them showed that it was at the differentiating stage. Organelles in Sertoli's cell increased as the age of ostrich increased, and there was plentiful heterochromatin in the nuclei and many free ribosomes in the cytoplasm of Sertoli cells of testis from 90-day-old ostrich chicks (Figure 2J), but there were few mitochondria in them. The verge was clear with Sertoli cell, spermiogonium and primary spermatocytes, and cytoplasmic bridge corpuscle and gap junctions were visible. The construction features of primary spermatocytes differentiated by spermatogonium (Figure 2K) was similar with those of spermatogonium, but primary spermatocytes were much bigger than the spermatogonium, and the cytoplasm increased and contained redundant round or oval mitochondria, free ribosome and endocyttoplasmic reticulum. Many lipid droplets were observed in the cytoplasm of Leydig cell, the nucleus of which was squeezed aside by lipid droplets with intensive heterochromatin (Figure 2L), but there were less other organelles.

Apoptosis of testicular cells

Apoptosis of testicular cells took place at any developmental period from 1-day-old to 90-day-

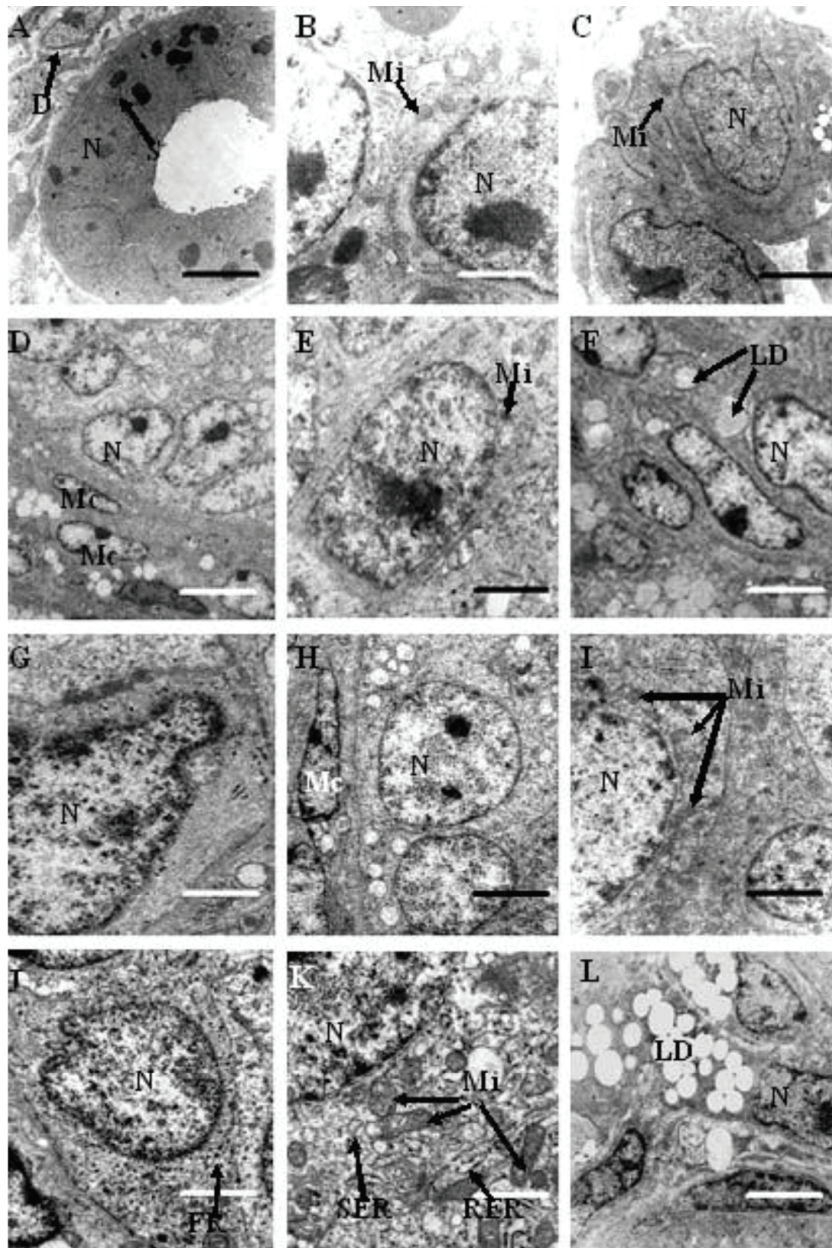


Figure 2. Electron micrographs of the testis of the ostrich chick. Figure 2A. Primordial germ cell (PGC), spermatogonium (S) and desmocyte (D) of testis of 1 day ostrich: N, nucleus of primordial germ cell; S, nucleus of spermatogonium; D, nucleus of desmocyte. Bar = 15 μ m. Figure 2B. Primordial germ cell of testis of 1 day ostrich: N, nucleus; Mi, mitochondria. Bar = 3 μ m. Figure 2C. Desmocyte of testis of 1 day ostrich: D, nucleus of desmocyte; Mi, mitochondria. Bar = 5 μ m. Figure 2D. Spermatogonium and myoid cell of testis of 45 day ostrich: nucleus of spermatogonium; MC, nucleus of myoid cells. Bar = 5 μ m. Figure 2E. Spermatogonium of testis of 45 day ostrich: N, nucleus; Mi, mitochondria. Bar = 2 μ m. Figure 2F. Leydig cells of testis of 45 day ostrich: N, nucleus; LD, lipid droplet. Bar = 3 μ m. Figure 2G. Sertoli's cell of testis of 45 day ostrich: N, nucleus; Mi, mitochondria. Bar = 1 μ m. Figure 2H. Spermatogonium and myoid cell of testis of 90 day ostrich: nucleus of spermatogonium; MC, nucleus of myoid cells. Bar = 3 μ m. Figure 2I. Spermatogonium of testis of 90 day ostrich: N, nucleus; Mi, mitochondria. Bar = 1 μ m. Figure 2J. Sertoli's cell of testis of 90 day ostrich: N, nucleus; Mi, mitochondria. Bar = 2 μ m. Figure 2K. Primary spermatocytes of testis of 90 day ostrich: N, nucleus of primary spermatocyte; Mi, mitochondria; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum. Figure 2L. Leydig cell of testis of 90 day ostrich: N, nucleus; LD, lipid droplets. Bar = 3 μ m.

old (Figure 3A, 3B, 3C, 3D), but more apoptosis testicular cells were observed at day 45 (Figure 3C). Most of apoptosis testicular cells were spermatocyte and Leydig cells (Figure 3C, 3D), which could almost not be detected in desmocyte and myoid cells. There were mainly primordial germ cells in testis of 1-day-old ostrich chicks, and apoptosis primordial germ cells were not observed; only a few apoptosis Leydig cells were detected (Figure 3A). The number of apoptotic cells decreased at the period from 1 day to 30 days ostrich age, then increased after day 30, which was the biggest at day 45 (Figure 4). Apoptosis was mainly detected in Leydig cells and primary spermatocyte but not in spermatogonium, although there were few primary spermatocytes. After day 45, apoptotic cells decreased gradually. Apoptosis was detected in primary spermatocyte and Leydig cells, but the number was less at day 90.

Discussion

Characterization of microstructure and ultrastructure of ostrich chick testis

Ostrich testis underwent a complicated course, including spermatogonium proliferation and differentiation by mitosis, formation of primary spermatocyte, and so on. At the same time, some changes took place in the morphologic structure of testis, for instance, with increasing age of ostrich, primordial germ cell gradually differentiated, seminiferous tubule was formed at early developmental phase especially at day 1-30, and primary spermatocyte presented at later phase at days 45-90.

In addition, the convoluted seminiferous tubules of the ostrich varied with the increasing of age. The diameter of seminiferous tubule increased gradually

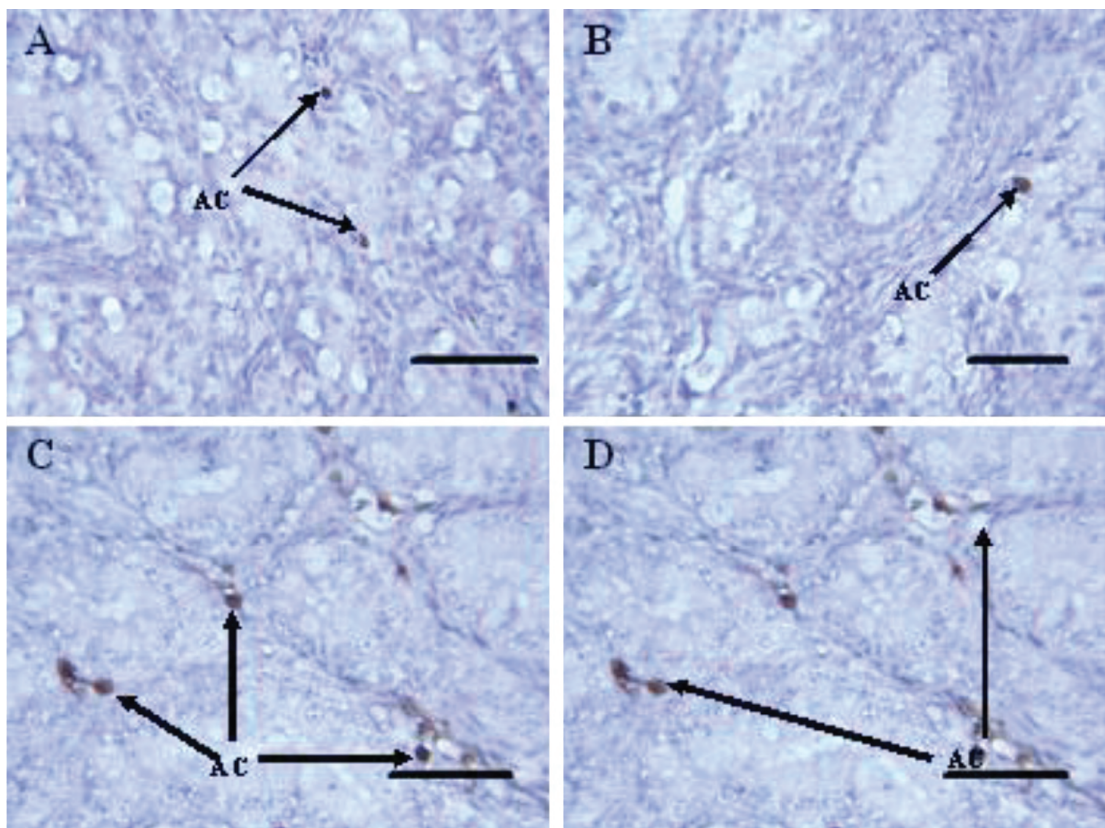


Figure 3. Germ cell apoptosis detected by TUNEL. Figure 3A. Apoptotic cells (AC) showing darker brown staining by day 1. Bar = 50 μ m. Figure 3B. Apoptotic cell by day 30. Bar = 50 μ m. Figure 3C. Apoptotic cells by day 45. Apoptosis cells could mainly be detected in Leydig cells. Bar = 60 μ m. Figure 3D. Apoptotic cells by day 90. Apoptosis cells could mainly be detected in primary spermatocyte. Bar = 75 μ m.

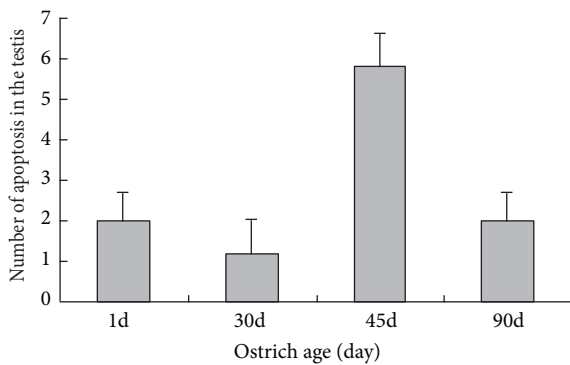


Figure 4. Column diagram showing the average number of apoptotic testicular cells per view (400 \times) from 1 day to 90 days ostrich age by TUNEL. Number of apoptosis testicular cells is most at 45 days among the 4 developmental periods.

from day 30 to day 90 of ostrich testis, but the diameter of 1-day-old ostrich testis was between 30-day-old and 45-day-old in size, and the reason could be that primordial germ cell was bigger than spermatogonium in size. Some tubiform structures being similar to seminiferous tubule were found in the testis of 1-day-old ostrich chicks, which were surrounded with many primordial germ cells and a few spermatogonia. At day 45 of ostrich age, spermatogonium began to differentiate into primary spermatocyte, and spermiogonium and primary spermatocytes were observed by turns from basal membrane to lumens of seminiferous tubule, but the number of primary spermatocytes was less and the secondary spermatocyte and the spermatozoon were not found in convoluted seminiferous tubule. Nevertheless, spermatozoa were found in convoluted seminiferous tubule of the testis of 80-day-old fowl (17). This might be due to the difference of the testes developmental feature of the ostrich and the fowl, and was also probably because sexual maturity of the ostrich was much later and the male ostrich reached sexual maturity at the age of 2.5 to 3 years.

According to the morphology and size of nuclei, density of chromatin, and shade of chromatin being stained in nuclei, the spermatogonia of domestic animal were divided into 3 types: type A, type B, and middle type spermatogonium (18). In adult turkeys, 3 types of spermatogonia were defined based on their chromatin distribution and nuclear morphology: the dark type A (Ad), the pale type A (Ap), and the type B (19). While spermatogonia of

ostrich chicks testes from 1-day-old to 90-day-old chicks were not defined the 3 types of spermatogonia, because the phenomenon of categorization is not visible if the development of animals does not reach some degree. The nuclei of spermatogonia were big and round, with more heterochromatin and less euchromatin. There was little cytoplasm in spermatogonia, and organelles in cytoplasm increased gradually with increasing age, which indicated the function activity of spermatogonium also increased. After the nutritive substance in some spermatogonia increased endlessly and the volume of the spermatogonia accreted gradually, they were differentiated into primary spermatocyte. There were more mitochondria and rough endoplasmic reticulum in cytoplasm of primary spermatocyte than of spermatogonium. The feature may explain that the function activity of the period spermatogonium was intensive and just differentiated into primary spermatocyte. There were cytoplasmic bridge corpuscle and gap junction with Sertoli cells and spermatogonium, primary spermatocyte and the structure feature can help to transport nutritive substance among them. The number of lipid droplet in Leydig cells can act as a morphological index that measures the function of Leydig cells at some degree.

Apoptosis cells in ostrich chick testis

Mammalian male germ cells undergo apoptosis at 37 °C internal body temperature; birds, however, are unique among homeothermic animals in developing spermatogenesis at the elevated avian internal body temperature of 40~41 °C (3). This study described the apoptotic features of testicular cells in ostrich chick testis from day 1 to day 90. Apoptosis of testicular cells took place at any developmental period from 1-day-old to 90-day-old ostrich age, but more apoptosis testicular cells were observed at day 45. That is to say, apoptosis varied depending on the developmental stage. Leydig cells proliferation reached a peak at postnatal 2 weeks, and the number of apoptosis increased gradually at the developmental stage (20). The number of apoptosis testicular cells was the highest at day 45, probably because in Leydig cells proliferation of ostrich testis mainly centralizes at about 45-day-old.

This study described the morphologic and apoptotic features of testicular cells of ostrich.

Development of 1-90 day old ostrich testis underwent a complicated course, including primordial germ cell differentiation by meiosis, spermatogonium proliferation by mitosis, formation of primary spermatocyte and testicular cells apoptosis and so on. At the same time, great changes took place in morphologic structure of testis, with increasing the ages of ostrich. We presumed our observations provided the morphologic and apoptotic aspects of 1-90 day old ostrich testis and evidence that apoptosis was associated with testicular cells in the testis of the ostrich.

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Acknowledgements

We would like to thank Dr. Liu Huazhen of Department of Anatomy, Histology and Embryology, College of Animal Science and Veterinary Medicine, Huazhong Agricultural University, for her valuable comments on the experiments. This study was supported by the National Natural Science Fund Project of China, No.30471249 and No.39970547 and No. 30972152, and Specialized Research Fund for the Doctoral Program of China Higher Education. No. 200805040023.

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