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The Morphology Study on the Development of African Ostrich Tibia

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Abstract: The research was carried out on 1-90 days old African ostrich chicks to study the morphological characteristics of tibia development. The length, weight and Bone Mineral Density (BMD) and the developmental characteristics of microstructure and ultrastructure were measured and analyzed in the research. Results showed: ostrich chicks <90 days grew fast and the length, weight and BMD of the tibia all increased significantly and continuously. In the tibia of 1 day old bird, transitional trabeculae constituted the primary cancellous bone which constituted the early formation of backbone. Lots of osteoblasts cling to the surface of cartilage formed thin primary cancellous bone; transitional bone trabeculae were very thin. In the tibia of 45 days old bird, resorption was very active, transitional bone trabecula was absorbed by osteoclasts and then rebuilded. The calcification process of cartilage matrix was active. In the tibia of 90 days old bird, rudiment of new born compact bone and osteons had already existed but the mature bone was still not formed. The study provides foundation for the further study on the influencing factor about tibia development and its molecular mechanism. Simultaneously, it gives the theoretical basis for breeding disease prevention and treatment.

Key words: African ostrich, cancellous bone, bone trabeculae, compact bone, osteons

INTRODUCTION

Ostrich is on the list of endangered species which has high ornamental and economic value (Huo *et al*., 2008). African ostrich is the largest living bird in the world that has long tibias. Although, their skeleton is similar to other birds in terms of structure and appearance, highly distinctive with respect to the amazing running speeds of these birds and non-ability to fly (Yao *et al*., 2006). It has thus been speculated that ostrich legs would have distinct tissue structures and physiological functions. But further reports about tibia development of African ostrich chicks had not been previously studies in detail. The formation and development of bone is a complex process with a lot of inorganic element, chemical compound and hormone taking part in regulating it (Wang *et al*., 2007) such as parathormone, calcitonin, glucocorticoid, growth hormone, estrogen and so on (Coin *et al*., 2012; Hamed *et al*., 2011). The bones of African ostrich chicks <90 days old are still immaturity and easy to suffer fracture. Seriously, this situation leads numbers of the chicken to becoming defective birds and even dying. The trouble decrease livability of ostrich chicks obviously and make ostrich industry suffering great losses of economic benefit (Qiang *et al*., 2001). The observations of the developmental characteristics and bone density determination of 1-90 days African ostrich chicks' tibia might provide a theoretical basis for novel ostrich breeding management and disease prevention strategies and also offer valuable data for the further study on the influencing factor about tibia development and its molecular mechanism.

MATERIALS AND METHODS

Animals and sampling: American ostrich chicks divide by age 1, 45 and 90 days into 3 groups, 4 males and 4 females in each group, purchased from Zhengzhou Jin Lu Ostrich Farm in Henan Province. The birds were deeply anesthetized with 15% urethane (Caoyang Secondary Chemaical Plant, Shanghai, China) at a dose of 1.2 g kg^{-1} body weight and arteria cervicalis bleed to death. Took the crura at once, peeled off the skins and muscles, amputated the fibulas and keep tibias.

Determination of length, weight and BMD: The tibia was exposed completely and measured length and weight of tibia. Bone Mineral Density (BMD) of proximal tibia was measured by Dual-energy X-Ray absorptiometry (Lunar PIXImus2 densitometer, General Electric Medical Systems, USA) immediately.

Samples for electronic microscope observation: The 3 mm^3 bone blocks were cut from 1/4 of the length closed

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to proximal tibia fixed in 2% paraformaldehyde-2.5% glutaraldehyde solution for 2 h in 4°C. And the blocks were washed using sodium cacodylate solution (pH 7.2) for 30 min. Then, fixed in 1% osmic acid for 2 h in 4°C and washed by ultrapure water for 30 min. After all the treatment including dehydration through graded ethanal, replacement by isoamyl acetate, critical point drying and plating film of gold, the bone blocks were observed the ultrastructure of the inside surfaces of the tibia necks and taken photomicrography by scanning electron microscopy (Quanta 200, FEI Company, Holland).

Samples for paraffin slices and HE staining: The 0.5 cm thickness fan shaped bone slices were sawn off at the 1/4 of the length closed to proximal of each tibia fixed in 4% neutral paraformaldehyde for 72 h. Bone slices were treated in microwave on 250 W output for decalcification: Put the bone slices into the inner layer of double vessel with neutral 13% EDTA while ice water mixture in the outer layer and then put the double vessel into microwave field. It is important to make sure the temperature of EDTA solution is lower than 15°C by replacing ice water mixture frequently during the microwave decalcification treatment. The 3 h later when the bone slices turned a little softer, cut them into 0.5 cm^3 blocks and went on treated in microwave field. After 15 h when the bone blocks were soft enough and elastic, the decalcification had been done. After dehydration, vitrification and waxdip, samples were embedded in paraffin and 9 μm sections were cut and stained with Hematoxylin-Eosin (HE) staining. Sections were observed under a bright field optical microscope and photomicrography is taken by OLYMPUS DP2-BSW imaging system (Japan).

Data analysis: All data files were uploaded into a data acquisition and analysis program (SPSS Version 11.5). Analysis is including the length and weight of the African ostrich chicks' tibia. Statistical results were expressed as M±SD (Mean±Standard Deviation). The t-test was performed to detect significance of difference between groups (significance level applied was $p<0.05$).

RESULTS AND DISCUSSION

Basic index of ostrich chick tibia: The tibias of <90 days ostrich chicks grew rapidly. The length of 1, 45 and 90 days ostrich chicks' tibias was, respectively 6.38±0.35, 15.28±0.63, 25.04±0.34 cm (Fig. 1), the difference among each stage was significant ($p<0.01$). The weight of 1, 45 and 90 days ostrich chicks' tibias was, respectively, 9.34±0.21, 52.34±0.56, 122.94±1.18 g (Fig. 2), the difference among each stage was significant ($p<0.01$).

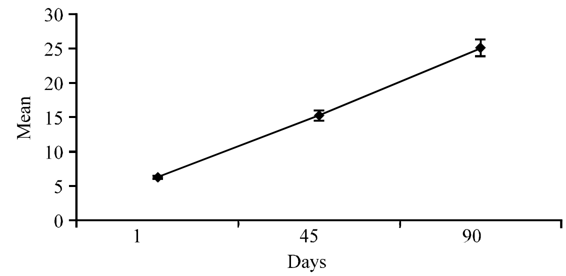

Fig. 1: Line chart showing the mean (±SD) of length of 1 day = 6.38 cm, 45 days = 15.28 cm and 90 days = 25.04 cm ostrich chick tibias (tibia length in cm)

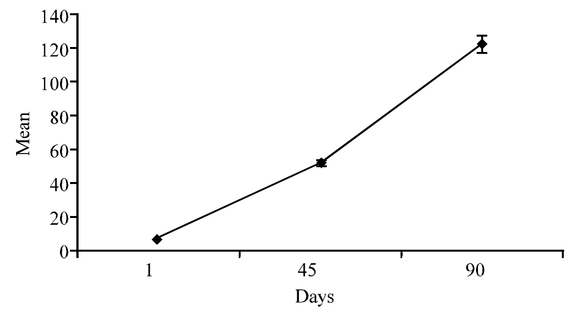

Fig. 2: Line chart showing the mean (±SD) of weight of 1 day = 9.34 g, 45 days = 52.34 g and 90 days = 122.94 g ostrich chick tibias (tibia weight in g)

BMD of ostrich chick tibia: The tibia of 1 day ostrich chick was so tiny that its BMD could not be measured by the instrument. The BMD of 1/4 of length closest to proximal tibia of 45 days ostrich chick's tibia was 0.28-0.32 g cm^{-2}. The BMD of 1/4 of length closest to proximal tibia of 90 days ostrich chick's tibia was 0.56-0.61 g cm^{-2} as the BMD of backbone was 0.56 g cm^{-2}. BMD grew rapidly, the difference of BMD between 45 and 90 days ostrich chick was significant (Fig. 3).

Microstructure of ostrich chick's tibia: The tibias of 1, 45 and 90 days ostrich chicks grew wider and thicker rapidly as well as continuously with the diameter of their marrow cavity increased (Fig. 4a).

In the tibia of 1 day bird, transitional trabeculae constituted the primary cancellous bone which constituted the early formation of backbone. From the outside to the inside of the structure were: external periosteum that consisted of Outer external Periosteum (OP) and Inner external Periosteum (IP), Transitional Trabeculae (TT), Primary Marrow Cavity (PMC) which are full of Primary bone Marrow (PM) (Fig. 4b). Lots of Osteoblasts (OB) attached to the surface of cartilage formed thin primary cancellous bone, transitional trabeculae were very thin.

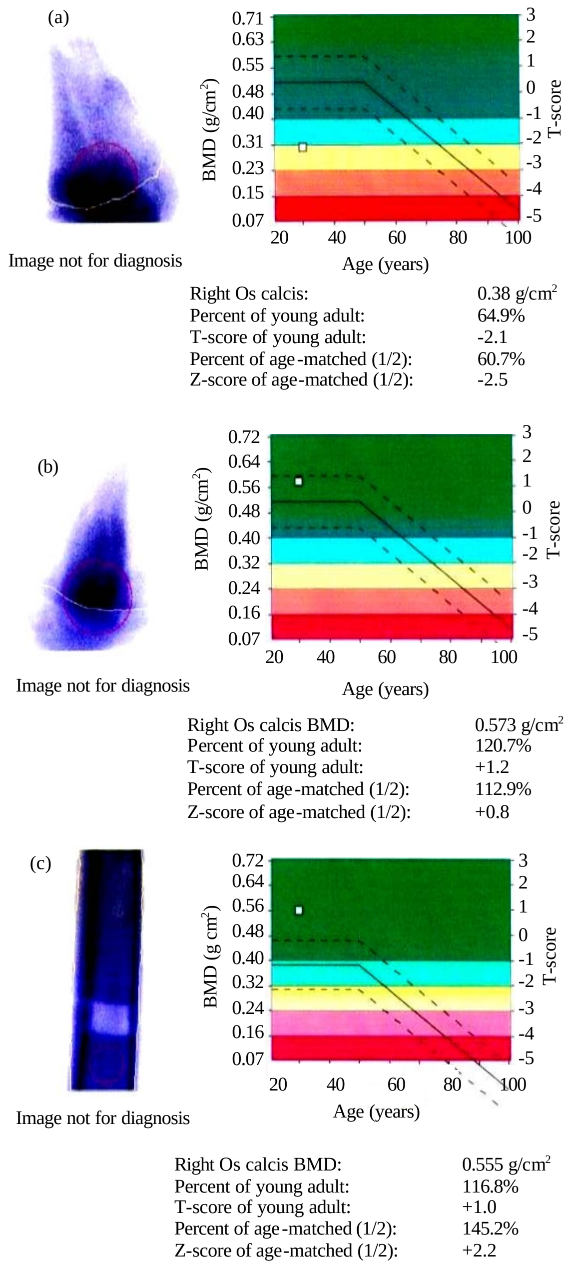

Fig. 3: Dual-energy X-Ray absorptiometry (Lunar PIXImus2 Densitometer System) output showing Bone Mineral Density (BMD) of 45 and 90 days ostrich chick tibias; a) BMD of proximal tibia of 45 days ostrich chicks is 0.308 g cm^{-2}; b) BMD of proximal tibia of 90 days ostrich chicks is 0.573 g cm^{-2}; c) BMD of middle tibia diathesis in 90 days ostrich chicks is 0.555 g cm^{-2}

The calcification in the middle of trabeculae was unconspicuous (Fig. 4c). In the tibia of 45 days bird, microstructure showed that: the reticular structure of Transitional Trabeculae (TT) was scattered (Fig. 4d). Chondrocyte became vacuolated or nuclear condensation or degradation and death. Osteoblasts (OB) and Osteoclasts (OC) attached to trabeculae surface, organic in different sizes can be observed and there were some osteocyte appeared too (Fig. 4e).

In the tibia of 90 days bird, Transitional Trabeculae (TT) and its outer layer got thicker; mesh pore in the trabeculae became dense and narrow (Fig. 4f). Rudiments of new born compact bone and osteons had already existed. An eosinophilic stained ring located in the peripheral regions of osteons-like structure (Fig. 4). Calcification of basophilic Cartilage Matrix (CM) could be observed in the regions inside the transitional trabeculae. Chondrocyte were degenerated or dead, the ones left large cartilage lacuna (Fig. 4h).

Ultrastructure of ostrich chick's tibia: The honeycomb transitional bone trabecula could be observed in 1 day ostrich chick tibia through electron microscope which had flake bone trabeculae in honey-comb shape (Fig. 5a) and was covered by the newly generated degenerate bone tissue on surface (Fig. 5b). A great number of osteoclasts in large volume without protuberance were 30-100 μm in diameter. Those edges with dark irregular folds could be observed by scanning electron microscope in 45 days ostrich chick tibia (Fig. 5c). Many immature osteocytes attached to the surface of 90 days ostrich chick tibia tissue could be observed with scanning electron microscope which were embeded into osteoid to become osteocytes. These immature osteocytes had similar morphology with mature osteocytes but sizes are smaller, deplanate and much more protuberance (Fig. 5l).

BMD and bone heath: Bone health in later years may rely on the bone mass accumulation during growth and development. Recent research shows that bone mass of individual after puberty is still closely related to the initial bone mass (Perez-Lopez *et al*., 2010). Children who experience their first fracture at a young age have high rates of risking fracture later (Yeh *et al*., 2006).

A 3 years observational study found that weight-bearing physical activity could promote bone mass of prepubertal teenagers but had no effect on those in late adolescence (Slemenda *et al*., 1994). Simultaneously, many studied confirmed that weight in childhood were an important factor to determine the bone mass in adulthood (Schlussel *et al*., 2010; Rawad *et al*., 2010; El Rawad *et al*., 2011). Ostriches' weight is relatively large and may be considered as weight-bearing physical activity to their legs which may be an important factor to promote the bone mineral density increasing rapidly in their childhood.

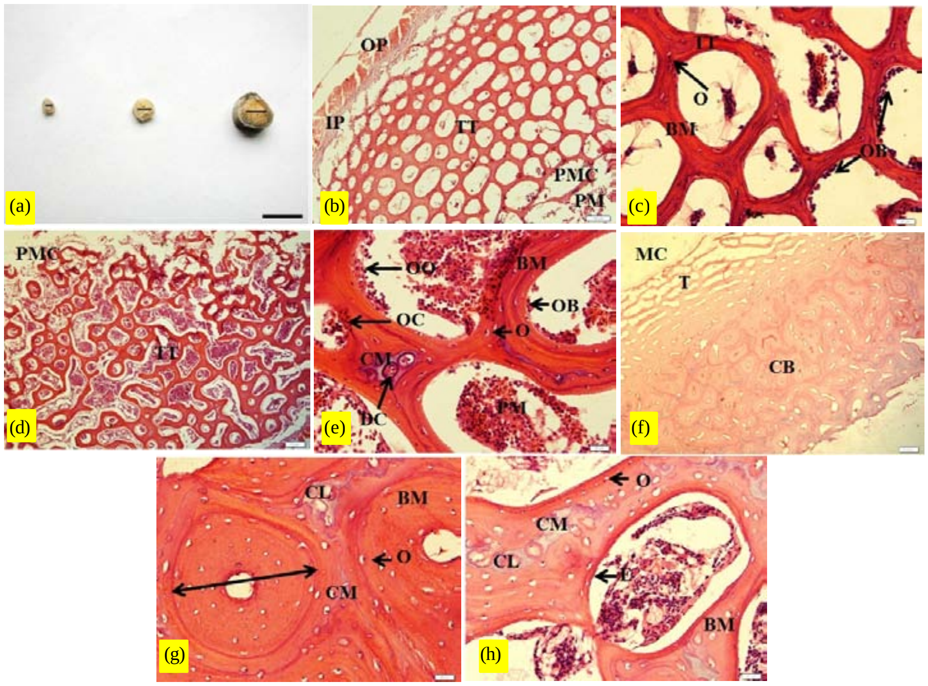

Fig. 4: Digital photographs and microphotographs showing the structure of 1, 45 and 90 days ostrich chick tibias; a) Cross-section comparison of 1, 45 and 90 days ostrich chick tibias, black lines indicate the bone marrow cavity turning larger; b) Microstructure of 1 day ostrich chick tibia showing Transitional bone Trabecular (TT), Primary bone Marrow Cavity (PMC), Primary bone Marrow (PM), Outer Periosteum (OP) and periosteum inlayer (IP); c) Microstructure of 1 day ostrich chick tibia showing Transitional bone Trabecular (TT), Bone Matrix (BM), Osteoblasts (OB) and Osteocytes (O); d) Microstructure of 45 days ostrich chick tibia showing Transitional bone Trabecular (TT) with active reabsorption process and Primary bone Marrow Cavity (PMC); e) Microstructure of 45 days ostrich chick tibia showing strong basophilic calcified Cartilage Matrix (CM), Degenerate Chondrocytes (DC), Osteoclasts (OC), Osteoblasts (OB), Osteocytes just turned from Osteoblasts (OO), Osteocytes (O), Bone Matrix (BM) and Primary bone Marrow (PM); f) Microstructure of 90 days ostrich chick tibia, showing Compact Bone rudiment (CB), bone Trabecula (T) and bone Marrow Cavity (MC); g) Microstructure of 90 days ostrich chick tibia compact bone showing Osteocytes (O), Bone Matrix (BM), calcified Cartilage Matrix (CM), larger Cartilage Lacunae (CL) and new born osteon-shape structure (two-way arrow); h) Microstructure of 90 days ostrich chick tibia bone trabecula showing calcified Cartilage Matrix (CM), larger Cartilage Lacunae (CL), Osteocytes (O), Bone Matrix (BM), Endosteum (E). Amplification: a) bar = 2 cm; b) bar = 100 μm, 100x; c, e, g, h) bar = 20 μm, 400x; d, f) bar = 200 μm, 40x

The results are significant for future studies about effects of trace elements and cytokines on ostrich bone mineral density and provide a theoretical basis for studying pathogenesis of African ostrich chick bone diseases.

Bone Mineral Density (BMD) is bone mineral content within a unit area of bone tissue which is an important factor affecting as well as the most direct and common indicator evaluating bone strength. It is used as a main standard to diagnose osteoporosis (Watts *et al*., 2010). This reference diagnose for osteoporosis should be based on BMD was given by the World Health Organization (WHO) published in 1994 (Kanis *et al*., 2008). Therefore, it is essential to measure BMD for researching growth and development of the ostrich chick tibia. It may indicate whether the bone developed healthy or not.

Characteristics of developmental African ostrich tibia: The sections of the 1/4 of length closest to proximal tibia were chosen to study the developmental characteristics of microstructure and ultrastructure of tibia. They were not only the location of calcification but also position of the edge of secondary ossification center, so that could observe the morphological characteristics both of new generated mature and immature bone tissue.

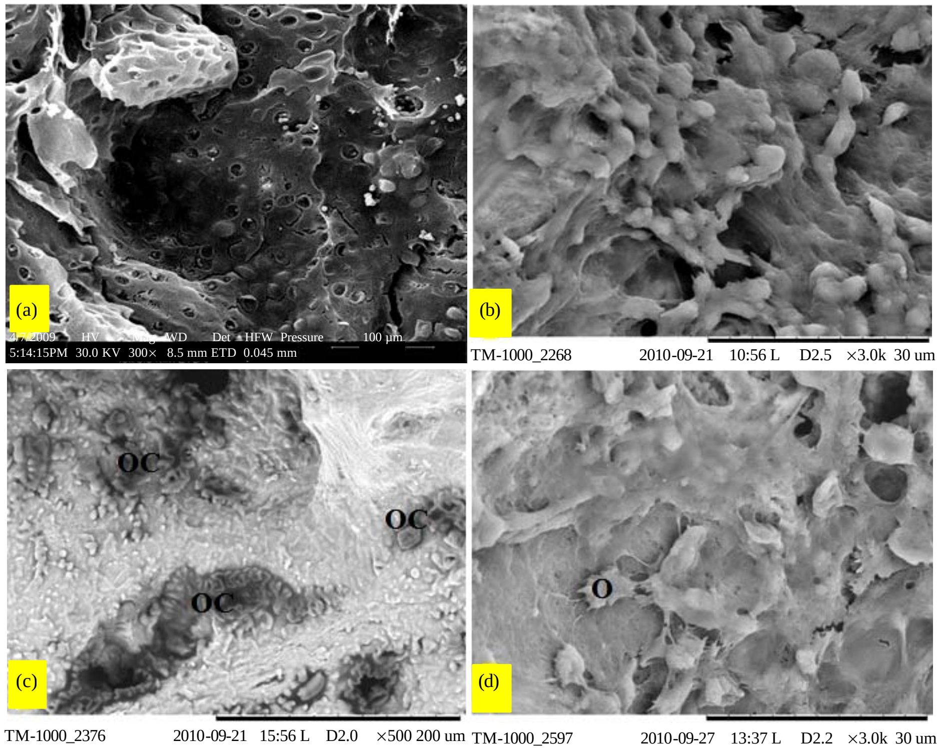

Fig. 5: Microphotographs obtained from scanning electron microscopy ultrastructure of 1, 45 and 90 days ostrich chick tibias; a) In 1 day ostrich chick tibia, flake bone trabeculae are in honey comb shape; b) In 1 day ostrich chick tibia, new bone tissues are in granular; c) In 45 days ostrich chick tibia, ruffled border exist on the surface of Osteoclasts (OC); d) In 90 days ostrich chick tibia, oblate Osteocytes (O) covered on the surface of bone with lots of gracile protuberance. Amplification; a) bar = 100 µm; b, d) bar = 30 µm; c) bar = 200 µm

In the tibia of 1 day bird, transitional trabeculae constituted the primary cancellous bone which constituted the early formation of backbone. Lots of osteoblasts attached to the surface of cartilage formed thin primary cancellous bone.

In the tibia of 45 days bird, resorption and rebuilding were very active, transitional trabeculae were absorbed by osteoclasts and then rebuilded. Furthermore, the observation of ultrastructure structure support that the calcification process in the middle of trabeculae was active correspondingly. The thick primary cortical bone area and osteons-like structure had been formed. By contrast, it would take 1 year to form mature osteons during bone development of human beings. It seems that the development process of ostrich bone may be much faster than that of human. In the tibia of 90 days bird, the eosinophilic stained ring is considered to play a role just like the bone cement line of mature osteon which is bone matrix containing much more mineral salt and less ossein fiber. Newly formed osteons-like structure may be boost the bone tissue grew very fast, absorbed and decomposed old bone tissue to form new bone tissue constantly, it was essential to keep this balance to complete thickening and growth of backbone.

According to the developmental morphology of 1, 45 and 90 days ostrich chicks tibias, researchers can presume the formation process of osteon: osteoblasts attached to the trabeculae and formed new osseous tissue continuously as the beginning of the formation of osteon, the trabeculae got thicker and closed that they formed a hollow ring attached by many osteoblasts. Then, osseous tissue would become much stronger because of bone canalicules did not cross bone cement line so that much more bone mineral could be deposited here. Rudiments of osteons had already formed as osteoblasts formed bone matrix and the middle channel tapered continuously. The balances between the function of osteoblasts and that of osteoclasts keeps bone healthy (Lee *et al*., 2006). The function of them is conversely with each other and both of them have vital function to growth and development of bone. Bone formation and resorption, the two opposite processes, occur during the process of bone development alternately and continuously. The balance between these two processes is strictly controlled since the homeostasis is closely related to bone mass.

As above, the growth and formation of tibias was supposed to approach to endochondral ossification, chondrocyte became mature to make cartilage matrix calcification in process of bone formation. The calcified cartilage matrix blocked nutrient supply which resulted in degradation and death of cartilage cells and left the big cartilage lacuna. Blood vessel, lots of osteoprogenitor cells and osteoclasts intruded into cartilage lacuna to dissolve and absorb calcified cartilage matrix around the calcified cartilage fragments, osteoprogenitor cells differentiated into osteoblasts continuously and osteoblasts attached to the calcified cartilage fragments formed transitional trabeculae.

CONCLUSION

It is essential to make reasonable ration formula to meet the needs of growth and development in each period of growing ostrich in this industry. Studying the processes of resorption and rebuilding would provide foundation for the further study on the influencing factor of tibia development and its molecular mechanism, it also gives the theoretical basis for disease prevention and treatment for ostrich farming.

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