GLOBAL JOURNAL OF BIO-SCIENCE AND BIOTECHNOLOGY

www.scienceandnature.org

MORPHO-HISTOLOGICAL STUDY OF LIMBS BONES DEVELOPMENT IN INDIGENOUS IRAQI GOOSE EMBRYO (*Anser anser domesticus*)

Bayatli, K. A. & Fadhil S. Mohammed
Department of Anatomy and Histology, College of Veterinary Medicine, University of Baghdad – Iraq

ABSTRACT
Morphogenesis of bones of the fore limb(wing) and hind limb in an indigenous Iraqi goose embryo including the anatomical and histological changes accompanied the process of bone creation, and transitional changes of the chondrofication, ossification and growth patterns of different bones of limbs were investigated and analyzed to enable assessment of the developmental status and evaluation of the experimental effects on bone development (Teratological studies) and skeletal mutations, which leads to deformities. Observations of most elements of different parts of the fore and hind-limbs performed under continuous serial pursuit to evaluate sequence of the developmental changes were occurred, with special attention to the timing of chondrofication and ossification of bones. Serial histological feature of the developmental sequences fortified these observations by study of the structural and transitional changes occurred during bone formation. The skeletal elements of both fore and hind-limbs showed later appearance of cartilage at interval between 9-16 days, while the onset of ossification initiated in the diaphyseal parts of the femur, tibia and fibula of the hind-limb had ossified at 11th day of incubation. Chondrofication and ossification were occurred in the mid-diaphyseal regions and progressed to the proximal and distal regions in all bones. Ossification of all elements of the fore and hind-limbs completed at 22 day of incubation except carpals and 2nd phalanx of the 2nd digit of the fore limb and tarsals in addition to the patella of the hind limb were remained cartilaginous without ossification during hatching.

KEY WORDS: morphogenesis, bone, limbs and goose.

INTRODUCTION

The study of the natural development of bones has economic importance in diagnosis of skeletal disorders, which are significant in the poultry industry. An excellent review published by (Sullivan, 1994), on some of terminology associated with various skeletal anomalies in poultry and their considerable losses to the poultry industry. In addition although, there are dissimilarities between human and avian bone developments, the avian is considered a valuable model for human skeletal defects (Cook, 2001).

A list of skeletal development of the skull is thought to be indispensable as a normal control in avian experiments, because for example, in field of avian researches the skeleton seems to be valuable indicator to judge whether cultured embryos develop normally under artificial conditions. In teratological test the skeleton is also an essential indicator to investigate the teratogenic effects of specific materials. Researches in the field of experimental embryology of avian species have advanced extraordinarily through focusing specially on natural skeletal development, teratological testing and developmental engineering in avian species. These researches and tests are designed to investigateand analyze embryonic skeletogenesis (Hashizum *et al*., 1993), skeletal mutations (Tsudzuki *et al*., 1998), and development of cultured embryos under artificial conditions (Naito *et al*., 1990) and to reveal the teratologenic consequences of drugs (Hashizum *et al*., 1993).This is important for the study of factors which could modify the skeletal development, and for evaluation of that in importance and time of onset of ossification(Baeriswyl ,1980). There have been several researches accumulating on the ossificatory developmental stages of bones in various avian species including chicken (Hamburger and Hamilton, 1951, Bellairs and Osmand, 2005; Sawad *et al*., 2009), quail (Nakane and Tsudzuki, 1999), and turkey (Atalgin and Kurtul, 2009) embryos. It had documented that the ossification centers of either partial or whole fetal skeletal components to contribute significant basic knowledge to studies in experimental embryology to acquire more precise and efficient data (Hamilton, 1952; Jollie, 1957; Atalgin and Kurtul, 2009). In the course this research, we keenly felt the necessity for study sequences of the normal embryonic skeletogenesis of limbs bones of the indigenous Iraqi Greylag strain goose (*Anser anser domesticus*) as a normal control.

MATERIALS AND METHODS

126 goose embryos obtained from Tuz-Khormato; city in the middle of Iraq from 7 to 28 days (hatching) of incubation were used in this study. 108 embryos used for morphological and (18) embryos used for histological studies. Embryos of morphological study were stained by double staining of alizarin-red and alcian blue for cartilage and ossified parts detection, respectively. Principle steps of the procedure of double staining of bone and cartilage with Alizarin Red-S and Alcian blue ar as following (Whitaker and Kathleen, 1979; Erdodan *et al*., 1

A. Complete skinning by remove skin, eyes, thoracic and abdominal viscera and adipose tissue.
B. Fixation of embryos in absolute ethyl alcohol for a minimum of 3 days at early stage and maximum of 7 days at late stages.

C. Staining of embryos for 4 days at (37-40 C) in the following solution:

a. 1 volume 0.3% (300 mg) filtered Alcian Blue in 70% ethyl alcohol (100ml).
b. 1 volume 0.1% (100mg) filtered Alizarin Red-s in 95% ethyl alcohol (100ml).
c. 1 volume glacial acetic acid (100ml).
d. 1 volume 70% ethyl alcohol (1700ml).

Solution (a) and (b) were mixed, and then (c) and (d) were added. At least 100ml of the resulting staining solution was used per full-term embryo.

D. Washing: Specimens were washed for 2 hours in tape water.

E. Maceration: Embryos were placed in aqueous potassium hydroxide (KOH) solution of gradual concentration of minimum 0.5% and maximum 2% for gradual increase of time of exposure between 16-24 hours.

F. Clearing and Storing: Macerated, stained specimens cleared by aqueous solution of ascending gradual concentration of glycerol(20,50,80%) diluted with distilled water , for 3 days for each step, then transferred into 100% glycerol to which a few crystals of thymol crystal have been added to avoid mold proliferation, kept and stored until they were examined and photographed. They may store for years without loss of stain properties of specimens (Miller and Tarpley, 1996).

Observations of most of elements of different parts of limbs performed under continuous serial pursuit to evaluate sequence of the developmental changes, with special attention to the timing of chondrofication and ossification of bones. Serial histological feature of the developmental sequences fortified these observations by study of the structural and transitional changes occurred during bone formation. Embryos used for histological study were processed by routine histological techniques for light-microscopic histology to establish their histogenesis, after fixation in buffered formal-saline(Luna, 1968). Linear measurements were made by measuring the total length of primary elements of the wing, as well as of the hind limb by using of dissecting microscope for embryos of early stages and Vernier Caliper for embryos of late stages of incubation.

RESULTS

Anatomical study

Whole mount staining

Developmental features of bone elements of geese embryos from the 7th day throughout the 28th day of incubation were described during continuous pursuit of ascending serial stages of the embryonic development. Transitional developmental changes of both chondrofication and ossification processes of bones of limbs are illustrated in table-1 and Figs.1, 2, and 3

.

TABLE 1: Transitional Chondrofication and Ossification of Bones in the Fore and Hind-Limbs.

Time of incubation (days)

Name of bones	7	8	9	10	11	12	13	14	15	16	17	18	19	20	22	24	26	28
Scapula	-	-	B	B	B	B	B	B	B	R	R	R	R	R	R	R	R	R
Coracoids	-	-	B	B	B	B	B	R	R	R	R	R	R	R	R	R	R	R
Clavicle	-	-	-	-	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Humerus	-	-	B	B	B	R	R	R	R	R	R	R	R	R	R	R	R	R
Name of bones	7	8	9	10	11	12	13	14	15	16	17	18	19	20	22	24	26	28
Radius	-	-	B	B	B	R	R	R	R	R	R	R	R	R	R	R	R	R
Ulna	-	-	B	B	B	R	R	R	R	R	R	R	R	R	R	R	R	R
Carpi radiale	-	-	-	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
Carpi ulnare	-	-	-	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
Metacarpal II	-	-	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
Metacarpal III	-	-	B	B	B	B	B	B	R	R	R	R	R	R	R	R	R	R
Metacarpal IV	-	-	B	B	B	B	B	B	R	R	R	R	R	R	R	R	R	R
Second digit																		
First phalanx	-	-	B	B	B	B	B	B	B	R	R	R	R	R	R	R	R	R
Second phal.	-	-	-	-	B	B	B	B	B	B	B	B	B	B	B	B	B	B
Third digit																		
First phalanx	-	-	-	B	B	B	B	B	B	B	R	R	R	R	R	R	R	R
Second phal.	-	-	-	-	-	B	B	B	B	B	B	R	R	R	R	R	R	R
Fourth digit																		
First phalanx	-	-	-	-	B	B	B	B	B	B	B	B	B	R	R	R	R	R

Name of bones	7	8	9	11	12	13	14	15	16	17	18	19	20	22	24	26	28
Hind-limb																	
Ilium	-	-	-	B	B	B	B	B	R	R	R	R	R	R	R	R	R
Ischium	-	-	-	B	B	B	B	B	R	R	R	R	R	R	R	R	R
Pubis	-	-	-	B	B	B	B	B	B	R	R	R	R	R	R	R	R
Femur	-	-	B	B	R	R	R	R	R	R	R	R	R	R	R	R	R
Tibia	-	-	B	B	R	R	R	R	R	R	R	R	R	R	R	R	R
Fibula	-	-	B	B	R	R	R	R	R	R	R	R	R	R	R	R	R
Patella	-	-	-	-	-	-	-	-	B	B	B	B	B	B	B	B	B
Tarsal I and II	-	-	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
Metatarsus I	-	-	-	B	B	B	B	B	B	B	B	R	R	R	R	R	R
Metatarsus II	-	-	B	B	B	B	R	R	R	R	R	R	R	R	R	R	R
Metatarsus III	-	-	B	B	B	B	R	R	R	R	R	R	R	R	R	R	R
Metatarsus IV	-	-	B	B	B	B	R	R	R	R	R	R	R	R	R	R	R
Digits																	
First digit																	
First phalanx	-	-	-	-	B	B	B	B	B	R	R	R	R	R	R	R	R
Second phalanx	-	-	-	-	-	B	B	B	B	B	B	R	R	R	R	R	R
Second digit																	
First phalanx	-	-	-	B	B	B	B	B	B	R	R	R	R	R	R	R	R
Second phalanx	-	-	-	-	B	B	B	B	B	B	R	R	R	R	R	R	R
Third phalanx	-	-	-	-	-	-	-	B	B	B	B	B	R	R	R	R	R
Third digit																	
First phalanx	-	-	-	B	B	B	B	B	R	R	R	R	R	R	R	R	R
Second phalanx	-	-	-	-	B	B	B	B	B	R	R	R	R	R	R	R	R
Third phalanx	-	-	-	-	-	B	B	B	B	B	B	R	R	R	R	R	R
Fourth phalanx	-	-	-	-	-	-	B	B	B	B	B	B	R	R	R	R	R
Fourth digit																	
First phalanx	-	-	-	B	B	B	B	B	R	R	R	R	R	R	R	R	R
Second phalanx	-	-	-	-	B	B	B	B	B	R	R	R	R	R	R	R	R
Third phalanx	-	-	-	-	-	B	B	B	B	B	B	B	B	R	R	R	R
Fourth phalanx	-	-	-	-	-	-	-	B	B	B	B	B	R	R	R	R	R
Fifth phalanx	-	-	-	-	-	-	-	-	-	B	B	B	R	R	R	R	R

(-) : Not stained with Alcian blue or Alizarin red-S, (B): Stained blue with Alcian blue, (R) : Stained red with Alizarin red-S.

At 7thday of incubation(Fig.1) ,at the tip of the limb-buds of both limbs, the ectoderm becomes thickened and is known as the apical ectodermal ridge.

At 8th day of incubation (Fig.2), there was precursor of cartilaginous, establishments of the proximal elements of the limb buds at this stage of this study, including humerus, ulna and radius, derived from the wing bud. Likewise, the precursor of the femur, tibia, and fibula has developed from the leg bud.

At 9th day of incubation, the developmental features of the shoulder girdle revealed blue staining of the scapula and coracoids.

At 10th day of incubation (Fig.3), chondrotic drafts of carpal elements including carpiradiale and carpiulnare, and 1st phalanx at 3rd digit of the fore limb were stained blue. Likewise the pelvic girdle elements including the ilium, ischium, and pubis, in addition to chondrotic drafts of metatarsal I and 1st phalanges of the 2nd, 3rd and 4th digit were stained blue in this study.

At 11th day of incubation(Fig.4) ,there was blue staining of the 1st phalanges of 1st and 4th digits and 2nd phalanx of the 2nd digit of the forelimb and 1st phalanx of the 1st digit and 2nd phalanx of the 2nd, 3rd and 4th digits of the hind limb. On the other hand the initial occurrence of calcification in the long bones of the hind-limb observed in the mid-diaphysis of the femur, tibia, and fibula at this stage, which was being of half length with 1/4 thickness of the tibia in this study.

At 12th day of incubation, the 2nd phalanx of the 3rd digit of the forelimb and 2nd phalanx of the 2nd digit and the 3rd phalanx of the 3rd and 4th digits were stained blue. Observation of red turning of the humerus, at this stage, which occurred later in relation to the femur.

At 13th day of incubation the mid-diaphyseal portion of the tarso- metatarsals II, III, and IV were partly turned red at this stage of this study. The tarsometatasal element was formed by fusion of the distal tarsal, in which the proximal ones fused with the tibia forming the tibiotarsal element.

At 14th day of incubation (Fig.5), the coracoids element of the pectoral girdle at its mid-diaphyseal portion turned red.

At 15th day of incubation, The metacarpals III and IV (metacarpal majus and minus) of the fore limb in this

study were slightly turned red staining at their mid-diaphyseal as a beginning of ossifying, the metacarpal III (majus) bone was thicker than the IV (minus) one, while metacarpal II which chondrofied together with other metacarpals, but it had no chance for ossification identically during hatching time. The ilium and ischium of the innominate of the hind limb were turned red in this stage of this study,

At 16th day of incubation the scapula of the pectoral girdle and the pubis of the pelvic girdle began ossifying, by which two girdles elements were ossified entirely(Fig.6).

At 17th day of incubation(Fig.7), the 1st phalanx of the 3rd digit of the fore limb and the 2nd phalanx of the 2nd and 4th digits of the hind limb were began ossifying. This study revealed that the chondrofication of all skeletal elements of the goose embryo were completed at 16 days of incubation, though there was no any blue staining element seen at this stage.

At 18th day of incubation(Fig.8), the metatarsal I also began ossifying (digit majus), and the metatarsal bones along with the tarsal I and II united at this stage to form the tarsometatarsal element. The sequence of developmental pattern of metatarsus in this study revealed that the ossification of metatarsals II, III and IV occurred on day 13, whereas metatarsal I was began ossifying on day 18 of incubation.

At 19th day of incubation (Fig.9), 1st phalanx of the 4th digit of the fore limb, and 3rd phalanx of the 2nd digit, and 4th digit of the 3rd digit began ossifying at this stage of incubation. At this stage of incubation, in this study, all of the skeletal elements of the fore limb, were ossified except the non-fused carpal elements including carpi radial and carpi ulnari, and 2nd phalange of the alular (2nd) digit. On the other hand, all of the phalanges of digits of the fore limb showed serial ossification beginning from day 16 until this stage (day 19) of incubation, except the 2nd phalanx of the 2nd digit (alular digit).

At 20th day of incubation the 4th and 5th phalanges of the 4th digit began ossifying. These phalanges ossified before ossification of the 3rd phalanges of the 4th digit, which was in contrary to the linage of sequences of the phalangeal developmental feature.

At 22th day of incubation, the 3rd phalanx of the 4th digit of the hind limb showed beginning of ossification, by which all of the skeletal componenets of the hind limb were ossified except the patella which remained cartilaginous at hatching.

At 24th day of incubation, the ossification of limbs elements was terminated. Continuous serial developmental sequences of the different skeletal elements of limbs of the goose to improve sufficient development until the day 28 of incubation (Fig.10, 11 an12), when the hatching happened. We concluded that each element of limbs has specific time for both of chondrofication and ossification. It is interested to mention that there are some skeletal elements remained cartilaginous at hatching, including; carpals, metacarpal II , 2nd phalanx of the 2nd digit of the fore limb and the patella and tarsals I and II of the hind limb.

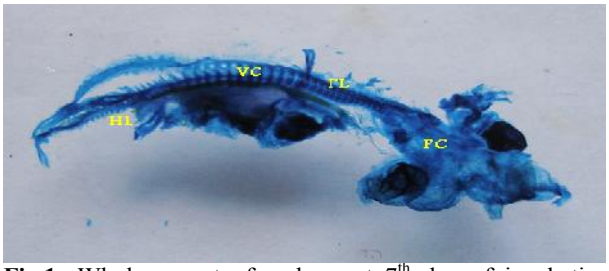


Fig.1: Whole mount of embryo at 7th day of incubation stained with Alcian blue and Alizarin Red-S double staining method. Chondrodrafte of VC, vertebral column; FL, fore-limb ; HL, hind-limb ; PC, parachordal cartilage.

Fig.2: Whole mount of embryo at 8th day of incubation stained with Alcian blue and Alizarin Red-S double staining method. , FL, fore-limb ; HL, hind-limb ; S , Scapula.

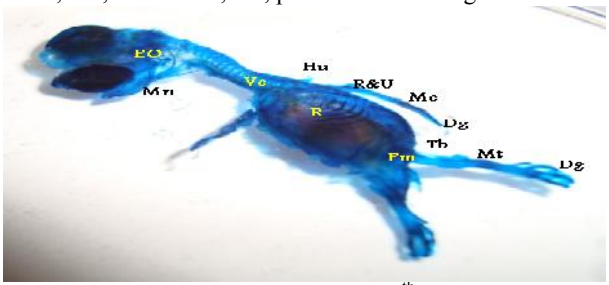


Fig.3: Whole mount of embryo at 10th day of incubation stained with Alcian blue and Alizarin Red-S double staining method. Chondrofied DG, digits ; Fm, femur ; Hu, humerus ; Mc, metacarpus ; Ma, mandible; Mt, metatarsus; R&U, radius & ulna; Tb, tibia.

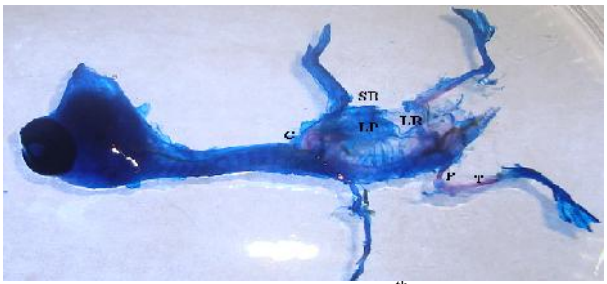


Fig.4: Whole mount of embryo at 11th day of incubation stained with Alcian blue and Alizarin Red-S double staining method. Ossified parts of C, clavicle ; F ,femur ; T, tibia.

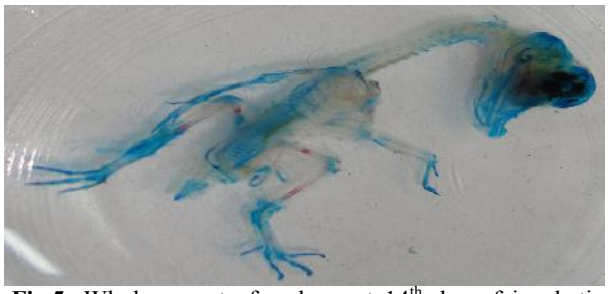


Fig.5: Whole mount of embryo at 14th day of incubation stained with Alcian blue and Alizarin Red-S double staining method. Ossified parts of Tm, tarsometatarsus .

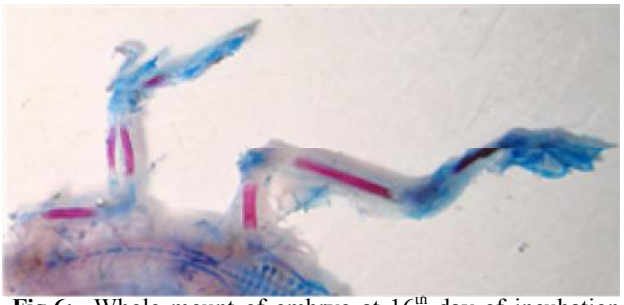


Fig.6: Whole mount of embryo at 16tn day of incubation stained with Alcian blue and Alizarin Red-S double staining method. P, pubis ; ; S, scapula

Fig.7: Whole mount of embryo at 17th day of incubation stained with Alcian blue and Alizarin Red-S double staining method. A, ventral view; B, dorsal view. Ossification of 1 PH, 1st phalanx of 3rd digits.

Fig.8: Whole mount of embryo at 18th day of incubation stained with Alcian blue and Alizarin Red-S double staining method. A, ventral view; B, dorsal view. Ossified parts of P2, 2nd phalanx of 3rd digit.

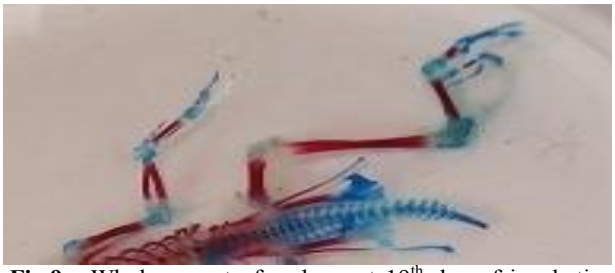


Fig.9: Whole mount of embryo at 19th day of incubation stained with Alcian blue and alizarin Red-S double staining method. A, vevtral view; B, dorsal view. Ossified parts of P1, 1st phalanx of 2nd digit of fore-limb; P3, 3rd phalanx of 2nd ; P4, 4th phalanx of 3rd digit.

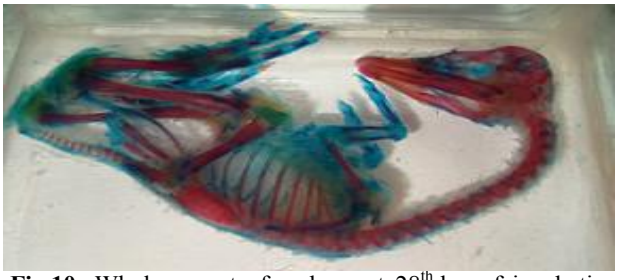


Fig.10: Whole mount of embryo at 28thday of incubation (hatching time), stained with Alcian blue and Alizarin Red-S double staining method. A,ventral view; B, lateral view. Non-ossified Ca, cartilaginous carpals; Pa, patella .Ossified 1, 2, 3, and 4, 1st,2nd.3rd,and 4thdigits of the hind limb.

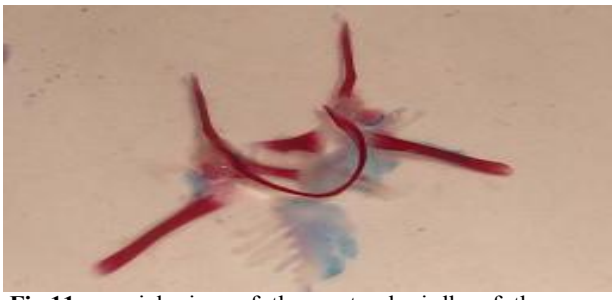


Fig.11: cranial view of the pectoral girdle of the goose embryo at 28th day of incubation, stained with Alcian blueand Alizarin Red–S double staining method. Ossified CL, clavicle ; Co, corocoid; H, humerus ; S,scapula ; St rternum ; SR, sternal ribs**.**

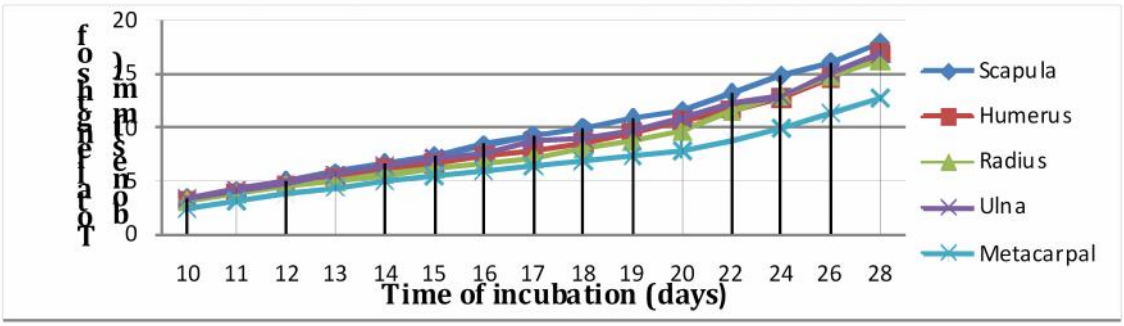
Fig.12: Long bones of the hind-limb of an indigenous goose stained with embryo at 28th of incubation, stained with Alcian blue and Alizarin Red-S double staining method. Ossified F, femur; Fi, fibula ; M. metatarsaus 2 , 3 , and 4; P, non-ossified patella ; T, tibia.

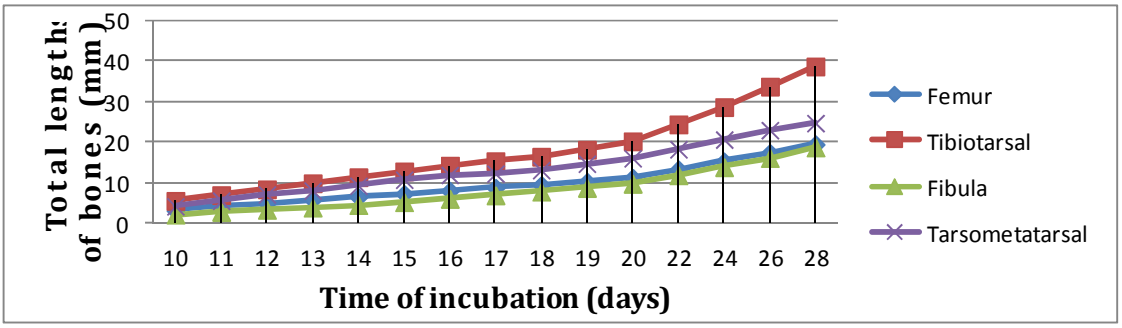
Lengths of long bones

The data acquired regarding the development of the total length of bones of the fore limb including scapula, humerus, radius, ulna and metacarpus, likewise long bones of the hind limb including femur, tibia, fibula and tarsometatarsal, and the length of the ossified portion of these bones beginning from the initial appearance of ossification throughout the hatching, against the embryonic periods were measured and displayed in curves (Fig.12and 13). There was eminently higher growth rate in the femur and tibia than that of the humerus, radius and ulna with increase of the time (Figs.13 and 14).

Fig.13: Growth curves of total lengths of the fore-limb bones.

Fig.14: Growth curves of the total lengths of the hind-limb bones





Histological study

In this study, the creation of long bones in both of fore and hind limbs showed distinguishable difference than that of flat bones of the skull. There was initiation of the cartilaginous model, contributes to longitudinal growth and is gradually replaced by bone through a complex process known as endochondral ossification. Early on day 8 of incubation, in the embryonic limb bud of the fore and hind limbs at 8th day of incubation, there was distinguishable sites of condensations (Figs.15 and 16), composed of aggregation of mesenchymal cells appeared as a rod-like, at the site of some elements; i.e. scapula, humerus of the fore limb and ilium, ischium, pubis, femur of the hind limb. The morphological characteristic of these mesenchymal cells before beginning of developmental condensation are small cells bodies.

On day 9 of incubation in this work, the mesenchymal cells in the center of condensations of precursors or templates including scapula and humerus of the fore limb and femur of the tibia of the hind limb become elongated of newly formed bones, transversely to the long axis, with distinct osteogenic layer around the

central cartilage rod, responsible for formation of bone collar(Figs.17).

At day 10 of incubation, the appearance of vasculature elements, associated with the differentiation of the osteogenic precursor represented by the osteoprogenitor cells, which was differentiated to osteoblasts as the first step of initiation of osteogenesis without cartilaginous intermediate were distinguished (Figs.18,19 and 20).

On day 11 of incubation(Fig.21), there was observable collar mineralized bone surrounding the central cartilaginous core of long bones of limbs. This bone generated from the periosteum after differentiation of the osteoprogenitor cells into osteoblasts which secrete calcified matrix. This periosteal bone is of membranous type, because it originate from mesenchymal cell without cartilage mediation. The central part of the cartilaginous core of the mid-diaphyseal region showed polyhedral shape of large hypertrophied chondrocytes interspersed in calcified matrix stained intensely with hematoxylin, surrounded by smaller proliferated chondrocytes. There was observable mononucleated, basophilic osteoblasts of polygonal-shape around the bony spicules of the collar around the cartilage core. These osteoblasts were arranged on the surface of the trabeculae.

At day 12 of incubation (Figs.22), there was initial first layer of the collar in the humerus, ulna and radius. At the same time there was a second layer of ossified tissue had laid down and deposited on the first layer of collar bone at the mid-diaphyseal region of the femur, tibia and fibula (Figs.21), with vascular development of the capillaries invaded between the two collar layers through the osteogenic layer. In this stage of long bone development of the goose, there was clear signs of primary ossification center observed in the site of resolution in the mid-

diaphysis of the femur, tibia and fibula, coordinated by vascular invasion through the periosteal bud brings all of fundamentals essential to establish the ossification center, including osteoprogenitor cells, osteoblasts and hemopoietic cells from the periosteum toward the hypertrophied cartilaginous center penetrating the bone collar which appeared eroded by buds.

On day 13 of incubation (Fig.23), there was calcification in the collar of II, III and IV metatarsals of the hind limb. But in previously calcified bones like femur, there was more than two layers of deposition in the bony collar interconnected by radically arranged spicules giving rise to form trabecular appearance of woven bone contained osteoblasts on the surface, with trapping of some of them in its secreted matrix into osteocytes of star-shape. The layers became thicker and contain osteocytes . At day 14 of incubation(Fig.24), the bone collar layers became thickening due to more ossified deposition, and resorption in the cartilage core progressed proximo-distally from the mid-diaphyseal region toward epiphysis. This associated with obvious invasion of the periosteal buds along the resoluted parts of the diaphysis.

At 15^{th} - 16^{th} days of incubation, there was observable initial collar bone formation in the mid-diaphysis of the of femur, humerus, tibia, radius and ulna there was three or more layers of cortical bone in the mid-diaphysis, which were interconnected by radial arranged spicules. The cortical bones progressed toward proximal and distal epiphysis of these elements. In addition to obvious sites of resolution in their shafts which showed well formed spicules and trabeculae and marrow cavities between them, with blood vessels as a beginning of the woven bone formation. There was continuous hypertrophy of the chondrocytes facing the cavity(Figs.25).

At the period from 17^{th} day of incubation and above throughout hatching, long bones of limbs showed consequence of developmental changes led to observable longitudinal and radial growth of the diaphysis. The radial growth extends parts with direct a position of the cortical bone by osteoblasts from the inner layer of the periosteum(Fig.27) .

It is interested to mention that After day 22 of incubation ,there was round or oval-shaped structures of newly formed Haversian canals, interspersed in cross sections of long bones, and became obvious with enlargment of the embryo. These canals were lined with osteoblasts, and filled with bone marrow(Fig.26). The considerable histological feature of epiphysis had been noted during bone development in the cartilaginous proximal and distal epiphysis of all long bones of limbs even short bones of digits were invaded by a network of vascular cartilage canals at 17^{th} day of incubation of this study . These canals increased branching gradually with aging of embryos (Figs.28 and 29). The high magnification of the canal showed blood capillaries carried blood cells and osteogenic cells including osteoprogenitor cells and osteoblasts in addition to haemopoitic cells
(Figs.28). Continuous replacement of cartilage by bone until majority of cartilage portion of the long bones approximately (75-86%) replaced by bone during hatching.

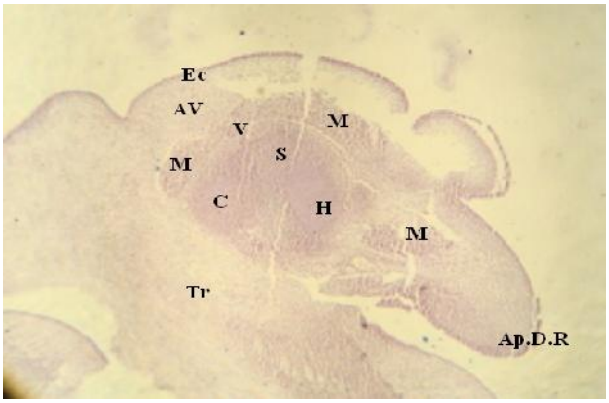


Fig.15: Histological section of the fore-limb bud of the goose embryo at 8^{th} day of incubation. AV,avascular layer; Ap.D.R, apical dermal ridge, Ec, ectoderm; M, muscles; H, precartilaginous precursor of the humeru; S, scapula;and C, corocoid ; Tr, trunk of the embryo ;V ,vascular layer.(H and E stain)X40.

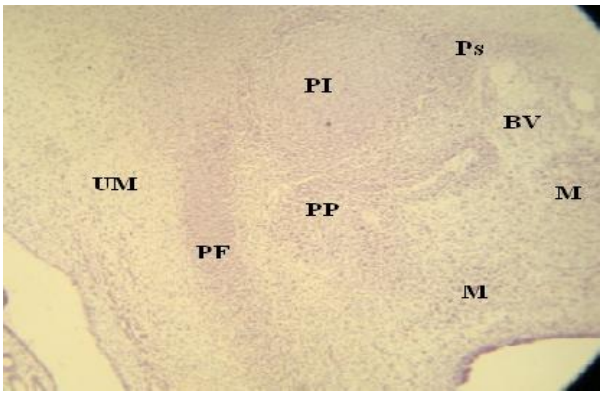


Fig.16: Histological section of hind-limb bud of the goose embryo at 8^{th} day of incubation. BV, blood vessels; M, muscles, PF,PI,and PP precursor of the femur, ilium, ischum,and pubis. UM, undefferen- tiated mesenchymal cells(H and E)X40.

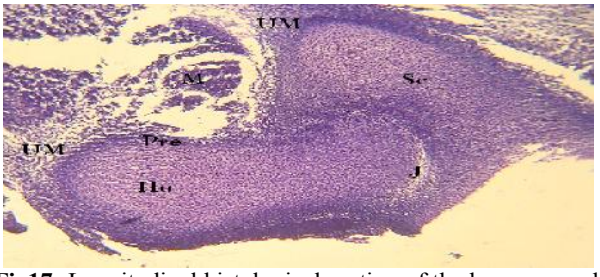


Fig17: Longitudinal histological section of the humerus and part of scapula of the goose at 9th day of incubation. Hu,humerus; J, shoulder joint; M, muscles; Pre, prechondrium ; Sc, scapula ; UM, undifferentiated mesenchymal cells.(H and E stain)X40.

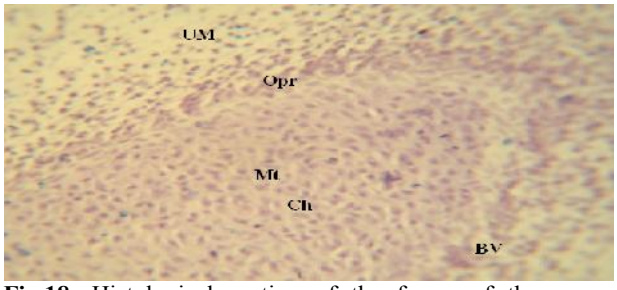


Fig.18: Histological section of the femur of the goose embryo at 10th day of incubation. BV, blood vessel; Ch, chondrocytes; Opr, progenitor cells ; UM, undifferent -iated mesenchymal cells ; Mt, matrix.(H and E stain)X100.

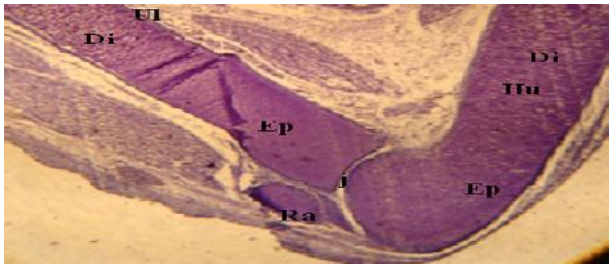


Fig.19: Longtudinal histolagical section the Hu, humerus;Ul,ulna and Ra, radius at 10th day of incubation. Di, diaphysis; Ep, epiphysis; J, shoulder joint(H and E) X40 .

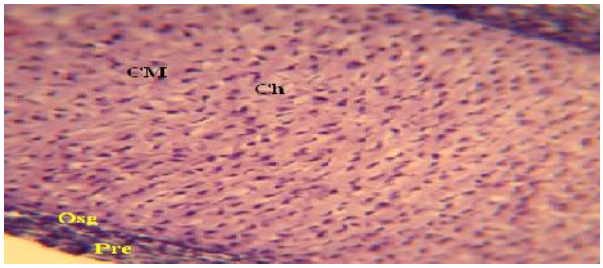


Fig.20*:* Magnified histological section across the diaphysis of the humerus of the 10th day goose embryo to show Ch, chondrocytes ; CM, calcified matric ; Osg, inner osteogenic layer(H and E)X100.

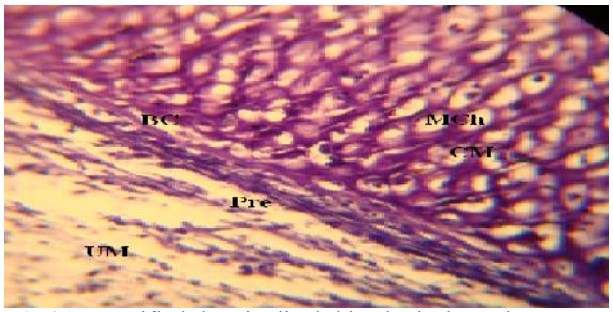


Fig.21:Magnified longitudinal histological section across the diaphysis of humerus of 11th day goose embryo. BC, bone collar; MCh, mature chondrocytes, CM, calcified matrix, Ob,osteoblast; Pre, perichondrium; UM, undifferentiated mesenchymal cells(H and E stain) X400.

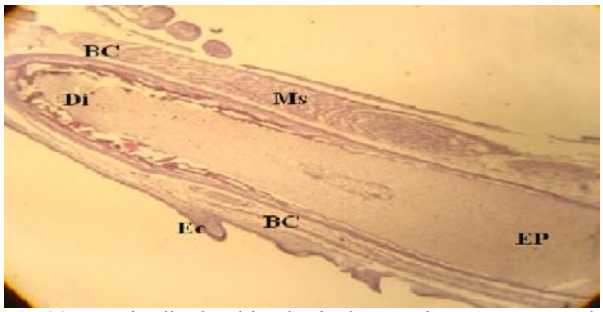


Fig.22:Longitudinal histological section across the diaphysis of the femur of 12th day goose embryo,to show a second layer of ossified tissue of BC, bone collars; Di, mid-diaphyseal ; Ec, ectoderm; Ep, epiphysis; Ms, muscles.(H and E stain) X40**.**

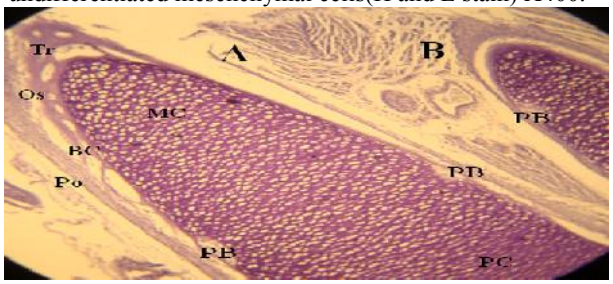


Fig.23: Longitudinal section of the ulna(A)and radius(B) of 13th day goose embryo,to show the BC, bone collar ; MC, mature calcified chondrocytes; Os,osteo- progenitor cells layer; PB, periosteal buds ; PC, proliferating chondrocytes; Po, fibrous periosteum; Tr, trabeculae. (H and E stain)X100.

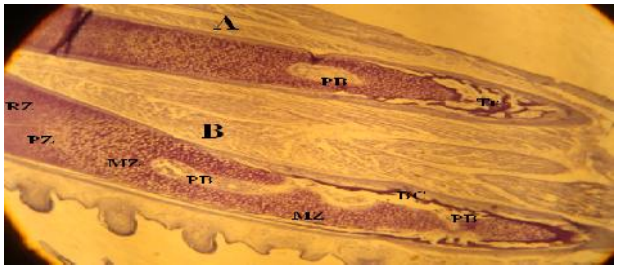


Fig.24: Longitudinal section of the ulna (A)and radius (B) at 14th day goose embryo,to show BC, bone collar layers; PB, periosteal buds; RZ, PZ, MZ, rest , proliferating, and mature zones of the growth plate; Tr,trabeculae.(H and E stain)X40.

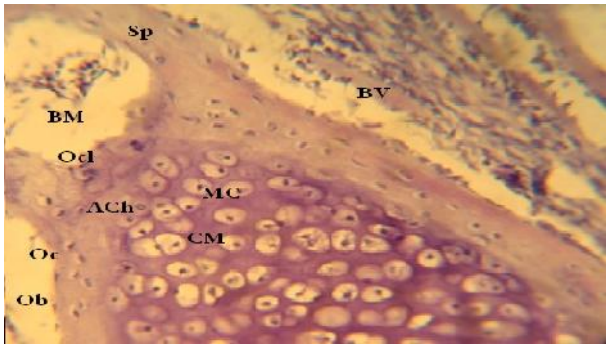


Fig.25: Magnified histological section through the diaphysis of humerus of 16th day goose embryo to show Ach, apoptotic chondrocytes;BM, bone marrow; BV,blood vessel; CM, calcified matrix ; MC, mature chondrocytes ; Ob, osteoblasts ; Oc, osteocytes ; Ocl,oste- oclasts;Sp,bone specule(Hand E)X100.

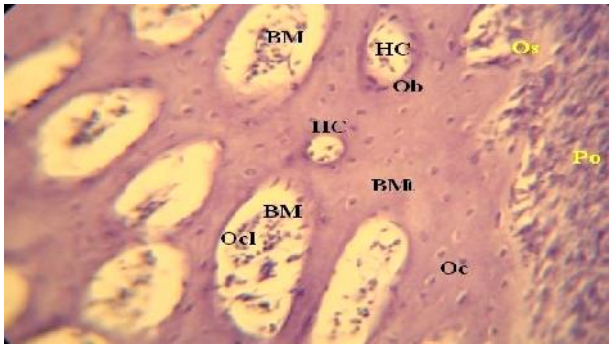


Fig.26: Histological section through the radius of the goose embryo at 24th day to show HC, Haversian canals; BMt, bone matrix ; Ob, osteoblasts; BM, bone marrow ; Ocl, osteoclasts. Po, outer fibrous; Os, inner osteogenic layers of the periosteum; Oc, osteocytes trapped in lacunae.(H and E stain) X400.

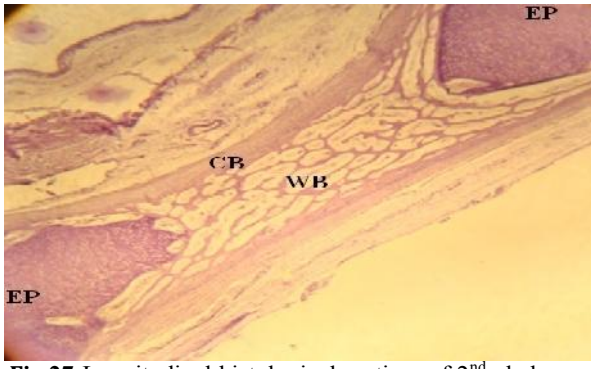


Fig.27: Longitudinal histological section of 2nd phala -nx of 3rd digit of the goose embryo at 26th day to show an increase of both length and width of bones by the central WB,woven and peripheral CB, compact bones respectively; EP, epiphyseal plate (H and E)X40.

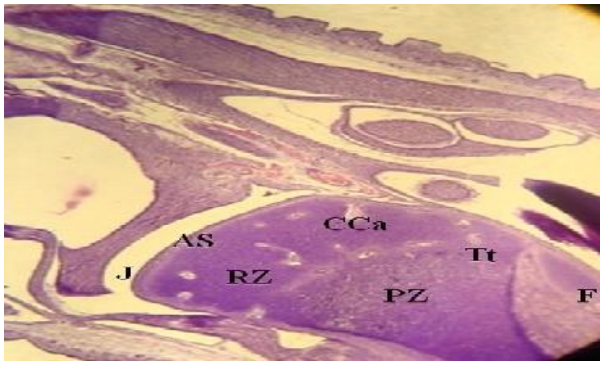


Fig.28: Histological section of the proximal exterimity of Tt, tibiotarsus and F, fibula of 28th day goose embryo to show the AS, articular surface ; CCa cartilage canals ; J, joint cavity; RZ, rest and PZ,proliferating zones of the epihpyseal plate(H and E stain)X40.

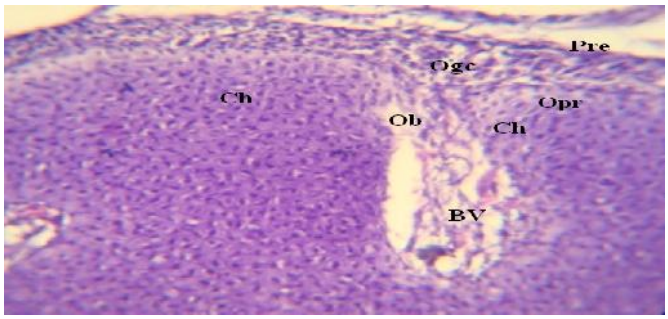


Fig.29: Magnified histological section through the proximal epiphysis of the tibiotarsus of 28th day goose embryo showing the catilage canal contains BV, blood vessels carries Ogc, osteogenic cells from the Opr, inner osteoproginator layer of the Pre, periosteum; Ch, chondrocytes; Ob, osteoblasts.(H and E stain)X100**.**

DISSCUSSION

Observation of the apical ectodermal ridge of limbs of 7th day goose embryo was compatible with (Saunders, 1957). establishment of the wing and leg buds of the pre-cartilaginous elements of chick embryo at 8th day of incubation (Bellairs and Osmond, 2005). Blue staining of the scapula and coracoids, with no appearance of the clavicle chondrofication at 9th day of incubation was in consistence with Chevallier, (1977) who found out that by H.H. stage 29(6 days) of chick embryo.

At 10th day of incubation, blue staining of chondrotic drafts of some of the proximal elements of the fore limb and pelvic girdle elements of the hind limb was approximately parallel to that of turkey except pelvic girdle and digits occurred at day 11 (Atalgin and Kurtul, 2009).

At 11th day of incubation , blue staining of the some phalanges of both fore and hind limbs and initial occurrence of ossification in the mid-diaphysis of the femur, tibia, and fibula was in agreement with Archer *et*

al., (1982). Observation of red turning of the humerus, which occurred later in relation to the femur was typically parallel to accumulating data in turkey embryo, but varied in the timing, which occurred in the fore and hind limbs at 12 and 13 days of incubation respectively(Atalgin and kurtul, 2009).

The earliest onset of ossification of long bones of hind limb including femur and tibia rather than that of fore limb may be led to our speculation that in the domestic goose of this study, the newly hatched goslet has ability for swimming soon after hatching. This needs gaining giant muscle and more developed skeletal elements, responsible for provide an energy requirement for specific movement of the legs essential for swimming.

The pattren of development of tarsometatarsals II, III, and IV at 13th day of incubation of this study, was similar to that noted by Lansdown, (1967); Nakane and Tsudzuki, (1999) in the quail, Kurtul *et al*., (2009) in the chick.

Red turning of metacarpal and metatarsal elements in addition to the ilium and ischium rather than pubis at 14th day of incubation was in disagreement with Atalgin and Kurtul, (2009) who noted occurrence of that at 15th and 16th in the ilium and ischium in turkey.

Ossification of scapula and pubis at 16th day of incubation of the goose, while Bellairs and Osmond, (2005), mentioned that the ossification starts on day 12 and 13 in the scapula and pubis, respectively, in the chick embryo. While Sawad *et al*. (2009) noted the ossification of the scapula starts at 10th day, and the pubis at 14th day of incubation in the domestic chick(Gallus gallus) embryo. This seems to be obviously natural because the eggs used in the later study are from the broiler, which gains giant muscle mass at a very short period. This variation explained by Baeriswyl,(1980) who determined by double staining the appearance time of primary ossification centers in the limb skeleton, and demonstrated that there are appreciable chronological differences between the Hubbard and White Leghorn stains.

Chondrofication of all skeletal elements of the goose embryo were completed at 16th day, though there was no new blue staining element seen at 17th day of incubation. This was similar to that occurred in the turkey embryo (Atalgin and Kurtul, 2009).

At 19th day of incubation in this study, ossification of almost of the skeletal elements of the fore limb, except the non-fused carpal elements. On the other hand, ossification of almost of the phalanges of digits of the fore limb except the 2nd phalanx of the 2nd digit (alular digit), which observed cartilaginous during hatching, was in agreement with Atalgin and Kurtul,(2009) in the turkey. While Nomina Anatomica Avium, (1993); Kurtul *et al*. (2009) mentioned that the alular digit of the Hubbert strain chick embryo showed very late ossification before hatching, whereas Nakane and Tsudzuki, (1999) noted red turning of this element at 11th day of incubation in the quail e mbryo.

At 20th day of incubation, 4th and 5th phalanges of the 4th digit phalange began ossifying s before the 3rd of the 4th digit, which was in contrary to the linage of sequences of the phalangeal developmental feature. The same pattern occurred in the turkey embryo in which the 5th phalanx ossified before the 4th phalanx of the 4th digit (Atalgin and Kurtul, 2009). Ahmed, (2008) observed the ossification of the distal(3rd)phalanx at 50 days and the proximal(2nd)phalnax at 56 days old indigenous sheep fetus. Similarly in man, the distal phalanges ossify before the proximal phalanges in the digits of the hand. This indicates that the pattern of ossification does not follow a clear proximo-distal sequence(Noback and Robertson, 1951).

The observation about the carpal elements was compatible with Lansdown, (1967) who noted the two proximal cartilaginous carpal elements in the wing of the quail embryo, where adjacent to the distal extremities of the radius and ulna respectively, are radiale and ulnare. And two distal cartilaginous carpal elements, are in the process of fusion to form a cartilage close to the proximal extremity of the metacarpal complex and ultimately fuse with it. He concluded that no ossification was seen in the carpal region in the embryonic period of hatching. Holder, (1978) suggested that the carpal elements of the wrist In the chick embryo do not ossify until hatching. This supported by Atalgin and Kurtul, (2009) in turkey embryo.

The results of the histological studies revealed that the distinguishable sites of condensations early on day 8 of incubation, in the embryonic limb, which composed of aggregation of mesenchymal cells appeared as a rod-like, of some elements; i.e. scapula, humerus of the fore limb and ilium, ischium, pubis, femur of the hind limb was compatible with Ross *et al*., (1995). They noted the occurrence of endochondral ossification of long bones, where a rod of cartilage was seen to be develope in the expected final portion of the bone.Differentiation of the mesenchymal precursors into pre-cartilage condensation seen by Gerber and Ferrara, (2000).They noted this processes to allow the precursors cells to expand resulting in the development of a structure similar to that of long bones of the future.

The pattern of initiation of bone formation on day 9 of incubation and elongation of newly formed bones, transversely to the long axis, formation of bone collar. was compatible with Pechak *et al*. (1986) and Caplan, (1988) whom noticed that the critical mass of cells that initiate the process of development not the cartilage model, but a group of cells arranged as a stack in the mid-diaphyseal region as a collar which lie around a cartilaginous center, which will develop later.

At 10th day of incubation, the appearance of vasculature elements, associated with the differentiation of the mesenchymal cells into osteoblasts as the first step of initiation of osteogenesis without cartilaginous intermediate osteoblasts from osteoprogenitor cells in the was compatible with Caplan, (1988) who noted differentiation of stalk cells into osteoprogenitor cells and further into osteoblast, which secrete the collagen fibrils of the matrix with developing vasculature. The description of the osteoprogenitor cells with the development of the capillaries was in conistent with that observed by Ross *et al*. (1995). Ehrlich and Lanyon, (2002) mentioned that osteoblasts cease to generate osteoid and meniralized matrix of collagen fibrils, and arising of periosteum was agreed with Dippolito *et al*. (1999). On day 11 of incubation. polyhedral shape of large hypertrophied chondrocytes interspersed in calcified matrix of the central cartilaginous core surrounded by the collar mineralized bone of long bones of limbs and

formation of the periosteal bone of membranous type, because it originate from mesenchymal cell without cartilage mediation speculated by Caplan, (1988) that the rigid collar forms a barrier for nutrients diffusion into a vascular cartilage core, and further that these physical limitations may initiate the observed hypertrophy of core chondrocytes. Holder, (1978) noted that the osteogenesis in long bones programmed by early stage, in which osteoblasts begin to produce a bone matrix at specific time giving rise to a subperiosteal collar of bone in the central diaphyseal regions of the skeletal elements of both the developing wing and leg. Ross *et al*. (1995) revealed that during the early stage of osteogenesis, a collar of bone forms around the core of the center of the cartilage model. This bone called periosteal bone, because it develops from the calcified cartilage. Thus both of interamembranous and endochondral ossification sequences are involved together in long bones formation (Shapiro, 2008).The same process occurs for flat bones and on the periosteal surface of the diaphysis of long bones (Summerlee, 1992).

The appearance of red color of alizarin red-S in the diaphysis of the femur, tibia and fibula of the hind limb elements with double staining procedure of alizarin red and alcian blue at 11th day of incubation was contributed to the osteoid of the collar bones and calcified matrix of the cartilaginous center, while there was no ossification in the central cartilaginous core of the diaphyseal region of these elements. This observation was in consistance with Ahmed, (2008) who observed the humerus bone of 45 days of intrauterine life of sheep embryo showed no feature of real ossification but only calcification of the cartilaginous matrix, and he revealed that one day after (46 days) gave the first initiative sign of ossification.

The histological features of osteoblasts and their arrangement on the surface of the trabeculae was agreed with Ross *et al*., (1995) and Salentijin, (2007). At day 12 of incubation, initial first layer of the collar in the humerus, ulna and radius and laying down a second layer of ossified tissue deposited on the first layer of collar bone at the mid-diaphyseal region of the femur, tibia and fibula with vascular development of the capillaries invaded between the two collar layers through the osteogenic layer was parallel to that observed by Pechak *et al*. (1986). The initiation of capillary at the time of osteoblasts formation speculated by Caplan, (1988) who noted that fundamental to this process is the relationship between the capillary endothelium and the osteoblasts. Highly secretory active secretary process carried out by osteoblasts is clearly related to the transport across the cell from the blood.

Vascular invasion of the cartilage template structure in this study in consistence with Iyama *et al*., (1991)whom described that as concomitant with hypertrophic differentiation of chondrocytes in the diaphysis of the bone. Subsequently, these chondrocytes in the central region of the cartilage undergo hypertrophy and synthesis an extracellular matrix.

On day 13 and 14 of incubation, thickening of the bone collar layers due to more ossified deposition, and proximo-distal progressing of the resorption in the cartilage core from the mid-diaphyseal region toward epiphysis were associated with obvious invasion of the periosteal buds along the resoluted parts of the diaphysis. This developmental pattern was parallel to that observed by Hosseini and Hogg, (1991) at day 10 of incubation in the chick embryo.

At 15th - 16th days of incubation, observable initial collar bone formation of three or more layers of cortical bone in the mid-diaphysisof some long bones, which were interconnected by radial arranged spicules ended by formation trabeculae progressed toward proximal and distal epiphysis of these elements. These events were parallel to that found on day 12 of incubation in chick embryo studied by Hosseini and Hogg, (1991) and Eriksen, *et al.* (1994).

Observable longitudinal and radial growth of the diaphysis At the period from 17th day of incubation and above throughout hatching,. The radial growth extends parts with direct a position of the cortical bone by osteoblasts from the inner layer of the periosteum was in agreement with Shapiro, (2008). While the longitudinal growth occurred at this period through an observable degree of maturation and degeneration and calcified projections of cartilage mass extend deep along the diaphysis at each end of long bones, acting as scaffolding arranged along tunnels or plates of calcified chodrocytes in lacunae compatible with Howlett , (1979) . These events of developmental sequences in mid-embryonic stages of prehatching period was compatible with Shapiro, (2008) who revealed that a developing long bone consisting of the epiphysis and metaphysis at each end, and the diaphysis in between. Four distinguishable zones of cartilage cells developmental stages of different characters and arrangements, of the growth plate was in agreement with Difiore, (1981); Dellmann and Brown, (1987); and Seeley, *et al.* (1992).

Oval-shaped structures of newly formed Haversian canals, interspersed in cross sections of long bones on day 22 of was in agreement with Holder, (1978) and Sawad et al. (2009) in the domestic chick embryo at 10th day of incubation.

Vascular cartilage canals at 17th day of incubation and their branching gradually with aging of embryos, which was the considerable histological feature of epiphysis, explained the excessive vascular requirements for establishment of newly formed bone tissues. Lutfi, (1970^{a}) and Blumer *et al.* (2005) revealed that the epiphyseal cartilaginous anlagen are penetrated by a complex canal network which extends centrally from the perichondrium. Cartilage canals are tubes containing vessels that are found in the hyaline cartilage prior to the formation of the secondary ossification centers Blumer *et al*. (2005).

Continuous replacement of cartilage by expects the articular surface of both epiphysis was in agreement with Seeley *et al*., (1992).

REFERENCES

Ahmed, N.S. (2008) Development of fore limb bones in indigenous sheep fetuses. Iraqi Journal of Veterinary Science, Vol.22, No.2. P 87-94.

Archer, C.W., Rooney, P. and Wolpert, L. (1982) Cell shape and cartilage differentiation of early chick limb bud cell in culture. Cell Differentiation 11, 245-251.

Atalgin, H. and Kurtul, A. (2009) A morphological study of skeletal development in turkey during the pre-hatching stage. Anat. Histol. Embryol, 38:23-30.

Baeriswyl, F. (1980) Morphometric development of ossification in the chick leg from the 7th to 17th day of incubation. Bull. Assoc. Anat. (Nancy) 64:183-198.

Bellairs, B. and Osmond, M. (2005) The Atlas of Chick Development. Elsevier academic press, California USA P:91-99.

Blumer, M.J.F., Longato, S., Richter E., Perez M.T., Konakci, K.Z. and Fristach, H. (2005). The role of cartilage canals in endochondral and perichondral bone formation: are there similarities between two processes. J. Anat. 206 (4): 359-372. Available in http://www. pubmedcentral. nih.gov/ articlerender .fcgi? artid= 1571487.

Caplan, A.I. (1988) Bone development. In Caplan A.I. Pechak D.G., editors. Cell and Molecular Biology of Vertebrate Hard Tissues, Ciba Foundation Symposium (New series), 3-21.

Chevalier, A. (1977) Origin des ceintures scapulaires of pelvinnes ches I'embryo d'oiseau. J. Emryol, exp. Morph. 42, 275-292. .

Cook, M.E. (2001) Skeletal deformities and their causes: Introduction. Poult. Sci., 79: 982-984.

Delmann, H.D. and Brown, E.M. (1987) Textbook of Veterinary Histology. 3rded

Difiore, M.S.H. (1981) Atlas of Human Histology, 5thed. Lea and Fefiger, Philadelphia.

D'ippolito, G., Schiller,P. C., Recordi, C. R. , Bernard, A. and Howard, G.A. (1999). "Age-related osteogenic potential of mesenchymal stromal stem cells from human vertebral bone marrow". Journal of bone and mineral research 14 (7): 1115-1122.

Ehrlich, P.J. and Lanyon, L.E. (2002) Mechanical strain and bone cell function: A review. Osteoporosis international /13 (9): 688-700.

Erdodan, D. ,Kadiodlu, D. and Peker, T. (1995) Visualisation of the fetal skeletal system by double staining with Alizarin Red and Alcian Blue. Gazi Tip Fakultaesi-Gazi Medical Journal. 6:55-58.

Eriksen, E.F.,Axelrod, D.W. and Melsen, F.(1994) Bone Histomorphometry. Raven Press, New York, pp13-20.

Gerber, H.P. and Ferrara, N. (2000) Angiogenesis and bone growth. Trends cardiovasc Med. 10: 223-228.

Hamilton, H.L. (1952) Lillie's Development of the Chick. An Introduction to Embryology. New York. Holt & Rinchart. Winston.

Hashizume, R., Noda, A., Itoh, M., Yamamoto, Y., Masui, S. and Oka, M. (1993) A method for detecting malformations in chicken embryos. Jap poult sci. 30: 298-305.

Holder, N. (1978) The onset of osteogenesis in the developing chick limb. J. embryo. Exp. Morph. 44:15-29.

Hosseini, A. and Hogg, D.A. (1991) The effects of paralysis on skeletal development in the chick embryo. II effects on histogenesis of the tibia. J. Anat. 177: 169-178.

Howlett, C.R. (1979) The fine structure of the proximal growth plate of the avian tibia. J. Anat. 128(2): 377-399.

Iyama, K. , Ninomiya, Y. , Olsen, B.R. , Linsenmayar, T.F. , Trelstad, R.L. and Hayashi, M. (1991) Spatiotemporal pattern of type X collagen gene expression and collagen deposition in embryonic check vertebrae undergoing endochondral ossification. Anat. Rec. 229: 462-472.

Jollie, M.T. (1957) The head skeleton of the chicken and remarks on the anatomy of this region in other birds. J. morphol. 100: 389-436.

Kurtul, I., Atalgin, S.H., Aslan, K. and Buzkurt, E.U. (2009) Ossification and growth of the bones of the wing and legs in prehatching period of the Hubbert strain broiler. Kafkas Univ. Vet Fak Derg 15(6): 869-874.

Lansdown, A.B.G. (1967) An investigation of the development of the wing skeleton in the quail (Coturnix C. Japanica). J. Anat. 105: 103-114.

Luna, L. (1968) Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology 3rd Edn., McGraw-Hill Book Comp.

Lutfi, A.M. (1970[a]) mode of growth, fate and function of cartilage canals. Acta Anat. 106: 135-145.

Miller, D.M. and Tarpley, J. (1996) An Automated Double Staining Procedure for Bone and Cartilage. Biotechnic and Histochemistry. 71(2): 79-83.

Naito, M. , Sasaki, E., Ohtaki, M. and Sakurai, M. (1994) Introductions of exogenous DNA into somatic and germ cells of chickens by microinjection into the germ disc of fertilized ova. Mol. Reprod. Dev. 37: 167-171.

Nakane, Y. and Tsudzuki, M. (1999) Development of the skeleton in Japanese quail embryos. Dev. Growth. Differ, 41: 523-534.

Noback, C.R. and Robertson, G. (1951) Sequences of appearance of ossification centers in the human skeleton during the first five prenatal months. Am. J. Anat. 89, 1-28.

Nomina Anatomica Avium (1993). Handbook of Avian Anatomy Published by the Nuttol Ornithological Club. No.: 23, Cambridge, Massachusetts.

Pechak, D.G., Kujawa, M.J and Caplan, A.I. (1986) Morphological and histochemical events during first bone formation in embryonic chick limbs. Bone 7(6): 441-458.

Ross, M.H., Romrell, L.J. and Kaye, G.I. (1995) Histology. A text and Atlas. 3rd ED. Williams and Wilkins, New York USA. P: 150-186.

Salentijn, L. (2007) Biology of mineralized tissues: Cartilage and bone. Columbia university college of dental medicine post-graduate dental lecture series.

Saunders, J.W., Cairns, J.M. and Gasseling, M.T. (1957).The role of the apical ridge of ectoderm in the differentiation of the morphological structure and inductive specificity of limb parts of the chick. J. Morphol. 101: 57-88.

Sawad, A.A., Balsam, A.H. and Anwar, N. (2009) Morphological study of the skeleton development in chick embryo. International Journal of poult. Sci. 8(7): 710-714.

Seeley, R.R. , Stephens, T.D. and Tate, P. (1992). Anatomy and Physiology 2nded Mosby year book, Philadelphia USA.

Shapiro, F. (2008) Bone development and it's relation to the fracture.The role of mesenchymal osteoblasts and surface osteoblasts.European cells an materials.15:51-76

Sullivan, T.W. (1994) Skeletal problems in poultry: Estimated annual cost and describtion. Poult. Sci. 73: 879-882.

Summerlee, J.S. (1992) Bone formation and development. In memory of Richard N. Smith. Available online:

Tsudzuki, M. , Nakane, Y. and Wada, A. (1998) Hereditary multiple malformationin Japanese quail: A possible powerful animal model for morphogenetic studies. J. Hered, 89: 24-31.

Whitaker, J. and Kathleen, M.D. (1979) Double staining technique for rat foetus skeletons in teratological studies. Laboratory Animals, 13: 309-310.