



## MORPHO-HISTOLOGICAL STUDY OF SKULL BONES DEVELOPMENT IN INDIGENOUS GOOSE EMBRYO (*ANSER ANSER DOMESTICUS*)

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### ABSTRACT

Morphogenesis of bones of the skull in an indigenous goose embryo including the morphological (anatomical) and histological changes accompanied the process of bone creation, and transitional changes of the chondrofication, ossification and growth patterns of bones of different parts of the skull 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 appear deformities. Observations of most of elements of different parts of the skull 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. Some of the chondral skull elements start showing blue staining at interval between 9-15 days and start ossifying at interval between 14-24 days, and other membranous skull elements showed direct red staining at interval between 11-20 days of incubation. Almost all components of the hyoid apparatus remained cartilaginous except the ceratobranchial at hatching.

**KEY WORDS:** morphogenesis, bone, skull 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 investigate and 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 teratogenic consequences of new drugs (Hashizum *et al.*, 1993). This is important for the study of factors which could modify the skeletal development, and for evaluation

of its modifications 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 of this research, we keenly felt the necessity for study of sequences of the normal embryonic skeletogenesis of skull bones of the indigenous Iraqi Greylag strain goose (*Anser anser domesticus*) as a normal control.

The endochondral and intramembranous ossification sequences are involved together not only in long bones formation, but also in the formation of skull and facial bone formation and repair (Shapiro, 2008).

### 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 cleared by glycerin after fixation in ethyl alcohol and 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 is as following (Whitaker and Kathleen, 1979; Erdodan *et al.*, 1995):-

Complete skinning by remove skin, eyes, thoracic and abdominal viscera and adipose tissue.

- A. Fixation of embryos in absolute ethyl alcohol for a minimum of 3 days at early stage and maximum of 7 days at late stages.
- B. 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, in which the stained skeletal elements 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 the skull 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 sequenes 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).

**RESULTS**

**Anatomical study**

**Whole mount staining:**

Developmental features of bone elements of geese embryos from the 7<sup>th</sup> day throughout the 28<sup>th</sup> 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 are illustrated in tables-1 and Figs.1, 2, and 3.

**TABLE -1:** Transitional Chondrofication and Ossification of Skull Bones (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
Occipital																		
Basioccipital	-	-	-	-	-	B	B	B	B	R	R	R	R	R	R	R	R	R
Exoccipital	-	-	-	B	B	B	B	R	R	R	R	R	R	R	R	R	R	R
Supraoccipital	-	-	-	-	-	B	B	B	B	B	R	R	R	R	R	R	R	R
Sphenoid																		
Basisphenoid	-	-	B	B	B	B	B	B	R	R	R	R	R	R	R	R	R	R
Parasphenoid	-	-	-	B	B	B	B	B	B	B	B	R	R	R	R	R	R	R
Orbitosphenoid	-	-	B	B	B	B	B	B	B	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
Temporal																		
Squamasal	-	-	-	-	-	R	R	R	R	R	R	R	R	R	R	R	R	R
Prootic	-	-	-	-	-	-	B	B	B	B	B	B	B	B	B	R	R	R
Epiotic	-	-	-	-	-	-	B	B	B	B	B	B	B	B	B	R	R	R
Opisthotic	-	-	-	-	-	-	-	-	B	B	B	B	B	B	B	R	R	R
Parietal	-	-	-	-	-	-	-	-	-	R	R	R	R	R	R	R	R	R
Prefrontal	-	-	-	-	-	-	-	R	R	R	R	R	R	R	R	R	R	R
Frontal	-	-	-	-	-	-	-	-	R	R	R	R	R	R	R	R	R	R
Trabeculae	-	-	B	B	B	B	B	B	B	B	B	B	B	B	R	R	R	R
Maxilla	-	-	-	-	-	-	-	-	R	R	R	R	R	R	R	R	R	R
Premaxilla	-	-	-	-	-	-	-	-	-	R	R	R	R	R	R	R	R	R
Vomer	-	-	-	-	-	-	-	R	R	R	R	R	R	R	R	R	R	R
Palatine	-	-	-	-	-	-	R	R	R	R	R	R	R	R	R	R	R	R
Pterygoid	-	-	-	-	-	-	R	R	R	R	R	R	R	R	R	R	R	R
Jugal	-	-	-	-	-	-	-	R	R	R	R	R	R	R	R	R	R	R
Quadrato	-	-	B	B	B	B	B	B	B	B	R	R	R	R	R	R	R	R
Quadratojugal	-	-	-	-	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Nasal	-	-	-	-	-	-	-	-	-	R	R	R	R	R	R	R	R	R
Mandible																		

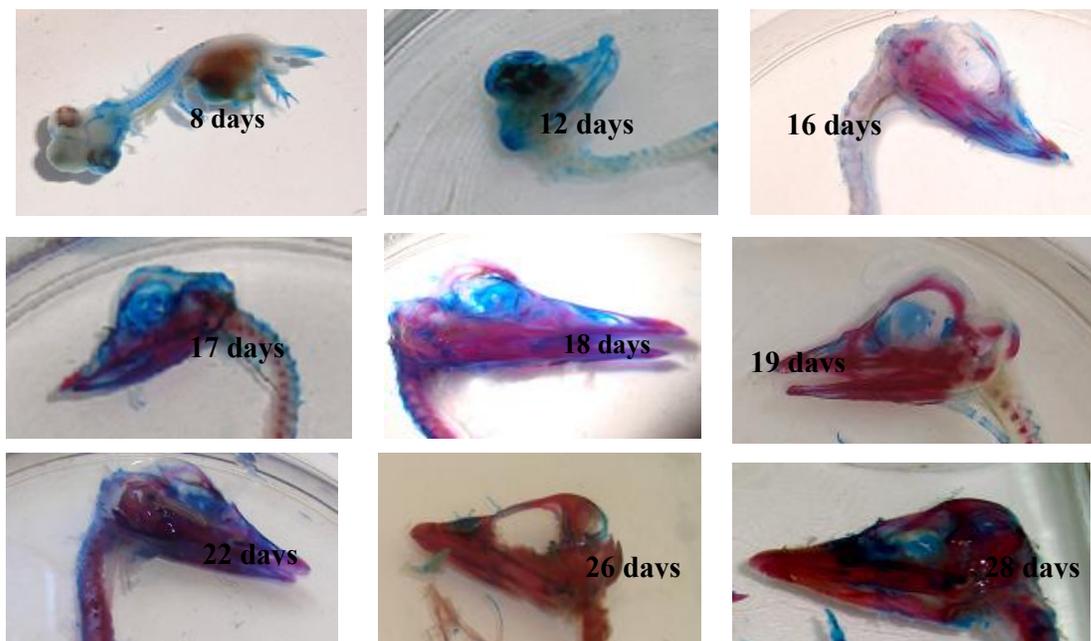
Dentary	-	-	-	-	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Angular	-	-	-	-	-	-	-	-	-	-	-	-	R	R	R	R	R	R
Supra-angular	-	-	-	-	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Articular	-	-	-	B	B	B	B	B	B	B	B	B	B	B	R	R	R	R
Splénial	-	-	-	-	-	-	-	-	-	-	-	R	R	R	R	R	R	R
Hyoid apparatus																		
Ceratobranchial	-	-	B	B	B	B	B	B	B	R	R	R	R	R	R	R	R	R
Entoglossal	-	-	-	-	B	B	B	B	B	B	B	B	B	B	B	B	B	B
Basihyal	-	-	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
Urohyal	-	-	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
Epibranchial	-	-	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B

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

At 7-8<sup>th</sup> days of Incubation , there was no signs of blue staining of bone elements of the skull.

At 9<sup>th</sup> day of Incubation, a slight blue staining of some skull elements including basisphenoid, orbitosphenoid of the sphenoid, trabeculae, quadrate, very thin small hyoid apparatus (basihyal, urihyal, ceratobranchial, epibranchial) were stained blue.

By the 10<sup>th</sup> day of incubation , there was obvious chondrofication, which observed by blue staining of some skeletal elements of the skull including exooccipital element of the occipital, parasphenoid of the sphenoid, and articular part of the mandible.



**Figure 1:**

Skulls of goose embryos of different ascending stages prepared by double staining method of Alizarin Red-S and Alcian Blue, illustrates serial development of skull bones.

At 11<sup>th</sup> day of incubation, all components of the hyoid apparatus were stained blue.

At 12<sup>th</sup> day of incubation, the skull elements including the basioccipital and supraoccipital of the occipital were stained blue. Whereas the squamosal element of the skull stained red. This indicates beginning of ossification of this element, which is the first membranous skull element that stained (not turned) red, which directly, begun ossifying without chondrotic intermediate. This can be determined by histological trails, because the whole mount staining is focusing the gross appearance of the ossification only.

At 13<sup>th</sup> day of incubation, there was blue staining of the epiotic element of the temporal as a part of the skull forms the roof of the otic capsule and fused with the squamosal and the other two otic elements. The palatine and pterygoid membranous

At 14<sup>th</sup> day of incubation, the prootic of the otic capsule stained blue. Otherwise, the exooccipital of the occipital turned red, at the same time the vomer, jugal, and prefrontal elements of the skull were partly stained red.

At 15<sup>th</sup> day of Incubation, the chondrofication of all three elements of the otic capsule were completed by blue staining of its opisthotic element, which forms the caudal

wall of the otic capsule. On the other hand basisphenoid of the sphenoid have turned red, at the same time the frontal and maxilla elements of the skull began partly ossifying.

At 16<sup>th</sup> day of Incubation, the chondrofication of all skeletal components of the goose embryo was entirely completed. On the other hand, the ossification of several parts of the skull, including the basioccipital of the occipital, orbitosphenoid (laterosphenoid) of the sphenoid, and ceratobranchial of the hyoid apparatus were partly turned red. At the same time the parietal, premaxillary and nasal elements of the skull showed partly red staining. The ossification of the ceratobranchial of the hyoid apparatus and cartilaginous remaining of the other parts including basihyal, urihyal, entoglossal, and epibranchial.

At 17<sup>th</sup> day of Incubation, no new blue staining was observed, while supraoccipital of the occipital and quadrate were turned red.

At 18<sup>th</sup> day of Incubation, parasphenoid part of the sphenoid, and the splenial element of the mandible began ossifying.

At 19<sup>th</sup> of Incubation, angular element of the mandible showed beginning of ossification by partial red staining.

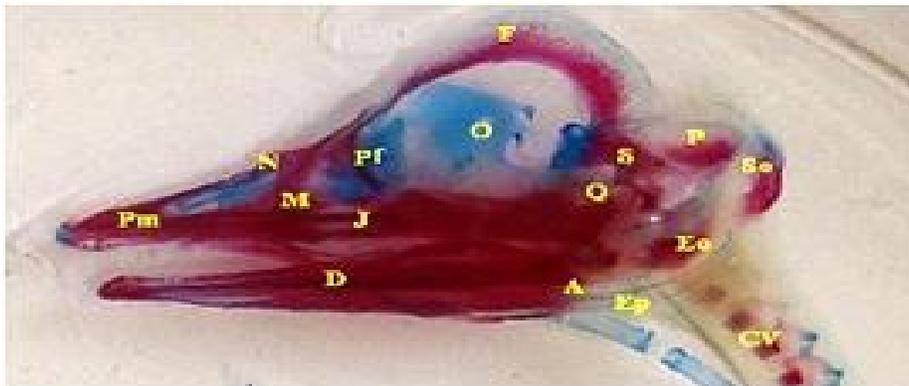
At 20<sup>th</sup> day of Incubation, there was no transitional changes in the skull elements. In this stage of the study.

At 22<sup>th</sup> day of incubation, the articular element of the mandible and the trabecular element of skull began ossifying.

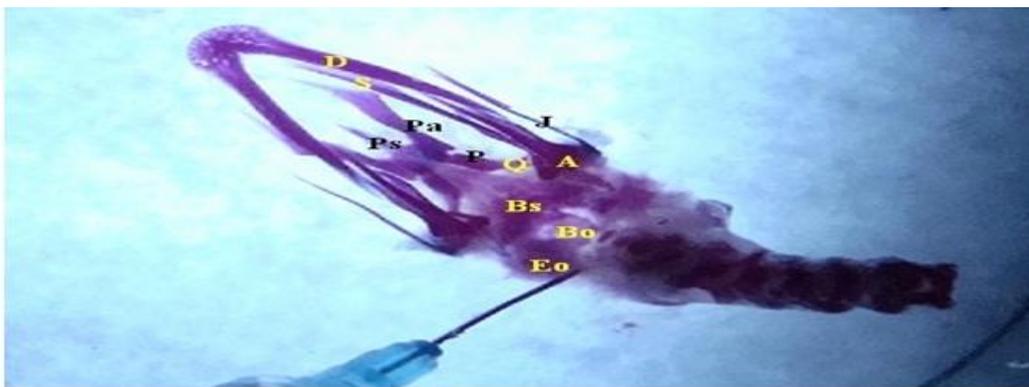
At 24<sup>th</sup> day of incubation, temporal elements including prootic, epiotic, and opisthotic were lastly began ossifying.

At 26<sup>th</sup> day of incubation, there was no new ossification centers of the skull element have seen at this stage of this study, and the red regions of each skull bone were expanded. After this time of incubation, the ossified skeletal components of the skull described previously were continued to improve sufficient development until the day 28 of incubation, where the hatching takes place.

**FIGURE 1:** Skulls of goose embryos of different ascending stages prepared by double staining method of Alizarin Red-S and Alcian Blue, illustrates serial development of skull bones.



**FIGURE 2:** Lateral view of skull of goose embryo at 19<sup>th</sup> of incubation, stained with Alcian blue and Alizarin Red-S double stain. Chondroified EP, epibranchial. Ossified A, angular ; CV, cervical vertebrae ; D, dentary ; Ex, exooccipital; F, frontal; J, jugal ; M, maxilla; N, nasal ; O, Orbit ; P, parietal ; Pf, prefrontal; Pm, premaxilla; Q, quadrate ; S, squamosal ; So, supraoccipital.



**FIGURE 3:** Ventral view of skull of goose embryo at 28<sup>th</sup> of incubation, stained with Alcian blue and Alizarin Red-S double stain. Ossified A angular ; Bo, basioccipital ; Bs, basisphenoid ; D, dentary ; Eo, exooccipital ; J, jugal ; P, pterygoid ; Pa, palatine ; Ps, parasphenoid ; Q, quadrate ; S, splenial

### Histological study

Light microscopy study of wax histology of equivalent stages confirmed the double staining of alizarin red and

alcian blue results. The observation of the histological feature of the skull development revealed that most of their skeletal elements formed by intramembranous type of ossification, in which the mesenchymal cells were differentiated to osteoblasts, without cartilaginous scaffolding. At 7-8<sup>th</sup> day of incubation, the cellular condensation of some skull bones including the frontal, squamosal, parietal, prefrontal, frontal, maxilla, premaxilla, vomer, palatine, pterygoid, jugal, quadratojugal, nasal, dentary, angular, supraangular, and splenial. These elements observed just visible, appeared as small areas of more cellular density of the mesenchyme(Figs.4).

These mesenchymal condensations developed in different areas of the skull, were observed within the deep vascular layer of the mesenchyme(fig.6).The cells of the deepest layer of the mesenchyme were stellate, with thin and long process extend in a wide intercellular spaces seen in the mesenchyme of the head.

At 9<sup>th</sup> day of incubation(Fig.5), the cells of the condensation were clearly distinguished than surrounding parts in the deep avascular layer of the mesenchyme, as a central core of rounded cells surrounded by a sheet of elongated cells. At 10 day of incubation of , numerous small capillaries were identified in the deep, previously avascular mesenchyme, some of which penetrated the area of the condensations of some elements of the skull. The central part of the condensation showed rounded cells separated from each other by matrix, which was excreted by differentiated typical osteoblasts. This matrix is osteoid and the cells created were osteoblasts. Later in this stage there was detection of the mineralization of the matrix for the first time, by differentiated osteoblasts, and there was clear vascularization of the bone, with small capillaries observed at the periphery at the condensation.

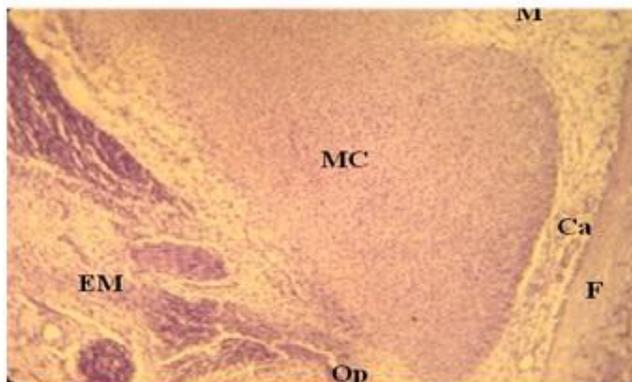
From day 11 and above throughout day 19 of incubation, in the recent study, there was subsequently appearance of ossification at first time, demonstrated the characteristics

of newly formed primary membrane bones of several elements at different times listed in (Table-1) confirmed the onset of ossification of these elements, which were demonstrated by double staining of Alizarin red-S and Alcian blue of whole mounts mentioned previously.

Small capillaries observed in the mesenchyme, osteoblasts lined the osteoid secreted by them. In the site of the resolutions, osteoblasts trapped in the matrix secreted by them, differentiating into osteocytes(fig.7), which situated in spaces of lacuna. And bony spicules formed. These spicules interconnected to form trabeculae, interposed by marrow cavities, represented the newly formed woven or cancellous bone at 18<sup>th</sup> day of incubation. The newly formed woven or cancellous bones of the skull at this stage of embryonic development is termed immature bone of more cellular proportionally were seen in this study (Figs.8).

There was observable gradual enlargement of the skull after day 14 of incubation in this study . This due to rapidly absorption of the spongy bone which formed, while simultaneously new bone is laid down by the subperiosteal osteoblasts, and it turned to lamellar bone , which infested by haversian canals(Fig.9). This is to provide rapid growth in the size of the skull that is required for the growing of the brain.

On the other hand there were other skull skeletal elements of the goose showed endochondral model of ossification. These elements included; Basisoccipital, Exoccipital, Supraoccipital, Sphenoid, Basisphenoid, Parasphenoid Orbitosphenoid, Prootic, Epitotic, Opisthotic, Quadrate, Articular, and hyoid apparatus(Table-1). These parts of the skull starts with the formation of a cartilaginous template, which eventually is being replaced by bone. At interval between (14-24<sup>th</sup>) days of incubation of the goose embryo, there was clear signs of primary ossification center observed in the site of resolution in the different positions of these chondral bones.



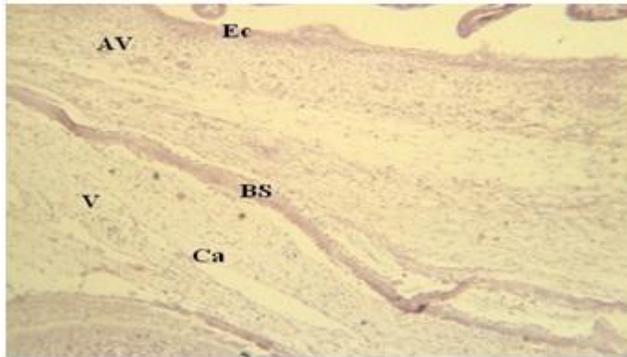
**FIGURE 4:** Histological section of the head of the goose of 8<sup>th</sup> day of incubation. Ca, capillaries ; EM, eye muscles; F, floor of the cranium; M, undifferentiated mesenchymal; MC, mesenchymal condensation; OP, optic nerve (H and E)X100



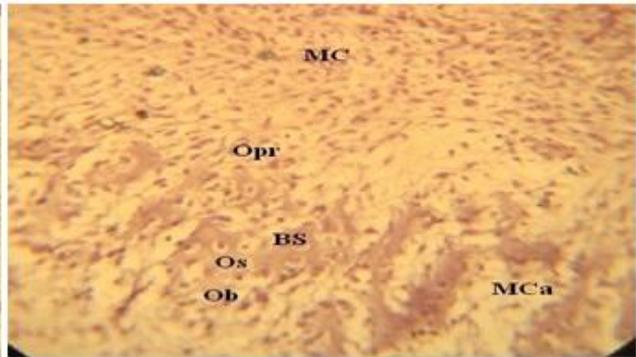
**FIGURE 5:** Histological section through the packed cells of the mesenchyma condensation and AM, adjacent undifferentiated mesenchymal as a part of the head of 10<sup>th</sup> day of incubation; Ca, capillaries; Mt, matrix (H and E)X400.

**FIGURE 6:** Histological section of the frontal bone of 13<sup>th</sup> day of incubation. Avascular layer (AV) ; BS, bone spicule; Ca,

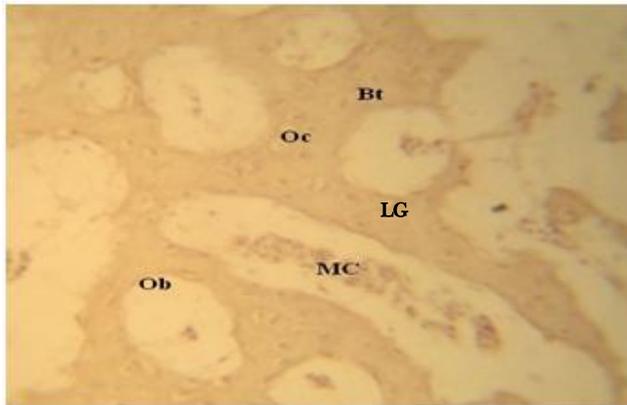
**FIGURE 7:** Histological section of the squamosal of the goose embryo at 18<sup>th</sup> day of incubation showing the initial spicule



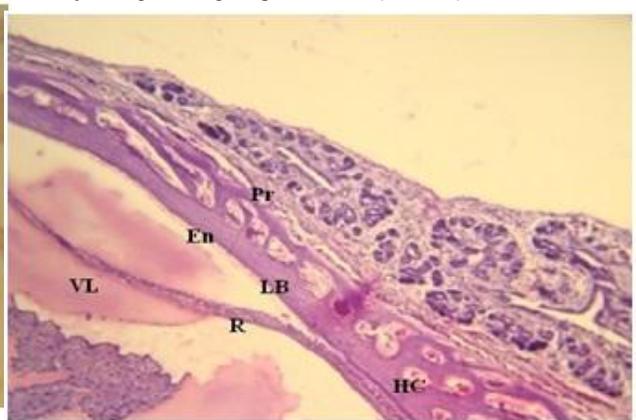
capillaries; Ec, ectoderm; V, vascular layer.(H and E stain) X40.



formation. BS, bone specules ; MC,undifferentiated mesenchymal cells; MCa. Marrow cavity ; Ob, osteoblasts; Oc, osteocytes; Opr, osteoprogenitor cells (H and E)X100 .



**FIGURE 8:** Histological section of the squamasa of the goose embryo at 20<sup>th</sup> day of incubation showing obvious woven bone formation. Bt, bone trabeculae ; MC, marrow cavity; Ob, surface osteoblasts; Oc, osteocytes(H and E stain) X100.



**FIGURE 9:** Histological section of the frontal bone of the goose embryo at 28<sup>th</sup> day of incubation showing lamellar bone formation. En, endosteum ;HC, haversian canal. LB, lamellar bone; LG, lacrima gland; Pr, periosteum; R, retina and VL, vitrous liquid of the eye (H and stain) X100.

## DISCUSSION

Absence of signs of blue staining of some chondral bone elements of the skull at 7-8<sup>th</sup> days and appearance of blue staining at 9<sup>th</sup> day of incubation and ended at 15<sup>th</sup> day by blue staining of the Opisthotic, was in parallel to the same feature of chondrofication of these elements of the turkey embryo of the same incubation period(28 days), which occurred firstly at 9<sup>th</sup> day, while ended at 16<sup>th</sup> day of incubation (Atalgin and Kurtul, 2009).

On day 11 of incubation of this study, first appearance of calcification which was determined grossly in some skull elements including dentary, suprangular and quadratojugal which stained red. Remarkable observations about the time of appearance of calcification of these skull elements in this stage was disagreed with the membrane bones beginning for ossification from 9 days, though there are discrepancies in the exact times given by different authors in chick embryos. Most of the skull bones of the chick embryo have undergone at least some ossification by day 14 according to table mentioned by Romanoff, (1960). While Sawad *et al.*, (2009) were disagreed with the previous studies that they mentioned the ossification centers in the skull elements become clear at 10<sup>th</sup> day and completed at 14<sup>th</sup> day of incubation in chick embryo. While in quails, although there is high range of variation in incubation period than that of chick, the ossification

starts at 9-10 days of incubation (Nakane and Tsudzuki, 1999).

Direct red staining of some bone element and red turning of other previous blue stained cartilaginous elements, indicates involvement of bone creation of the skull of the goose via both of an intramembraneous and endochondral ossification processes. This observation was in agreement with Nakane and Tsudzuki, 1999; Bellairs and Osmond 2005; Atalgin and Kurtul, 2009 in the quail, chick and turkey embryos previously.

Intramembraneous ossification during embryonic development, involved in the development of flat bones of the cranium, various facial bones, parts of mandible, were by mesenchymal cells differentiate directly to osteoblasts (Marks and Hermy, 1996; Roland,2008).

The cellular condensation of some membrane skull bones which observed just visible, appeared as small areas of more cellular density of the mesenchyme developed in different areas of the skull, as an initial precursor elements of bones of future in the skull. This observation was compatible with Bellairs and Osmond, (2005) who mentioned that the membrane bones provide most of the vault and sides of the skull, the jaws and palate bones of neural crest origin based on data from Couly *et al.*, (1993).

Description of cellular content and their shape of the mesenchymal condensation at 9<sup>th</sup> days of incubation in this study in the deep avascular layer of the mesenchyme, as a central core of rounded cells surrounded by a sheet of elongated cells. was in a similar pattern with Brighton and Roberts, (1991) who noted that the changes in the morphology of the MSCs in this stage, the cell body become larger and rounded. And the cells within the aggregate display the characteristic of an osteoprogenitor cell.

At 10<sup>th</sup> day of incubation of this study, numerous small capillaries were identified in the deep avascular mesenchyme, and rounded cells separated from each other by matrix, which was excreted by differentiated typical osteoblasts were parallel to Thompson *et al.*, (1989) who noted these changes in chick embryo of 10 days. And detection of the mineralization of the matrix for the first time, by differentiated osteoblasts at this stage of some skull bones development was parallel to that observed by Netter, (1987) who noted the osteoblast, which lines the nodule continue to form osteoid at the center.

Subsequently appearance of ossification at first time in membrane bones at interval between (11-19<sup>th</sup>) days of, and in chondral bones at interval between (14-24<sup>th</sup>) days incubation in goose embryo) confirmed the onset of ossification of these elements, which were demonstrated by double staining of Alizarin red-S and Alcian blue of whole mounts mentioned previously in this study. The site in which an initiate ossification takes place is known as "primary ossification center" (Shapiro, 2008). This observation was in agreement with Romanoff, (1960) who mentioned that the most of the skull bones have undergone at least some ossification at day 14.

Events at the different sites of the skull elements appeared to be showing first the transition from loose mesenchyma to orientated cells, and formation of condensation and matrix production and finally bony trabeculae. As growth continue, trabeculae interconnected and woven bone formed (Thompson *et al.*, 1989; Brighton and Robert, 1991; Ross *et al.*, 1995).

There was observable gradual enlargement of the skull after day 14 of incubation in this study. This due to rapidly absorption of the spongy bone which formed, while simultaneously new bone is laid down by the subperiosteal osteoblasts This is to provide rapid growth in the size of the skull that is required for the growing of the brain (Carleton and Short, 1953). In order to maintain the appropriate shape in relation to size, remodeling will occur by continuous growth through resorption of localized area of bone tissue by osteoclasts (Ross *et al.*, 1995).

The process of endochondral bone formation of some chondral skull bones characterized by replacement of cartilage by bone, during which chondrogenesis and osteogenesis are coordinated in time and space (Ungil *et al.*, 2001). This process allow the mesenchymal precursor cells to expand resulting in the development of a structure similar to that of long bones. Vascular invasion of the cartilage template structure is concomitant with hypertrophic differentiation of the chondrocytes. Subsequently, these chondrocytes in the central region of the cartilage undergo hypertrophy and synthesize an

extracellular matrix (Iyama *et al.*, 1991). The mature hypertrophied chondrocytes stops secreting collagen and begin secreting alkaline phosphatase, an enzyme essential for mineral deposition, then calcification of the matrix and apoptosis of hypertrophied chondrocytes occurs (Shapiro, 2008). The apoptotic chondrocytes lay down a mineralized cartilage scaffold, which become a template for the bone of the future by osteoblasts and osteoclasts by invaded blood vessels. The osteoblasts differentiate on this scaffold template in between hypertrophic chondrocytes forming primary trabeculae bone (Maes *et al.*, 2002). In order to maintain the appropriate shape in relation to size, remodeling will occur by continuous growth through resorption of localized area of bone tissue by osteoclasts (Ross *et al.*, 1995). Ossification and replacement of cartilage by bone continue in the cartilage model until all the cartilage has been replaced by bone (Seely *et al.*, 1992 ; Ross *et al.*, 1995).

Vascular Endothelial Growth Factor (VEGF) Involved in early blood vessel development is secreted by the hypertrophic chondrocytes inducing angiogenesis from the perichondrium leading to the recruitment of osteoblasts and haematopoietic cells. These sequences of events leads to development of the "primary ossification centers". The hypertrophic matrix within these centers then degrades, and the recruited osteoblasts replaced the degraded cartilage with trabecular bone and a bone marrow is thus formed (Collin-Osdoby, 1994; Gerber and Ferrara, 2000; Hall *et al.*, 2006).

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