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Morpho- histological study of supraoccipital bone development in domestic rabbit fetuses *Oryctolagus cuniculus*

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Summary

The developmental study of supraoccipital bone has been done in the rabbit fetuses, which including detection the timing primary appearance and pattern of ossification by using double staining method as well as, histological study which squired for each stages of present study. The double staining technique which are furthering by histological examination for each age, showed the supraoccipital bone was ossify by intramembranous method. The results showed that the primary ossification centers of supraoccipital appeared firstly at(22) day of gestation, and showed direct red staining, at(24) day of gestation, these centers become fused. The supraoccipital bone appear its completely intramembranous ossification at (30) day of gestation and form the roof of foramen magnum.

دراسة مظهرية – نسيجية لتطور العظم القفوي العلوي في أجنة الأرانب المحلية

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الخلاصة

أجريت دراسة تطور العظم القفوي العلوي في أجنة الأرانب المحلية, والتي تضمنت تحديد زمن ظهور مركز التعظم ونمط تكوينه باستخدام الصبغة المزدوجة عيانيا ودراسة المقاطع النسيجية المصاحبة لكل فترة من مراحل الدراسة. خلال هذه الدراسة تمت المتابعة المستمرة للمراحل المتعاقبة لنمو العظم القفوي العلوي مع تسليط الضوء على الجزء الغشائي وتحوله الانتقالي إلى العظم (Osteogenesis).أظهرت نتائج الدراسة إنالجزء العلوي من العظم القفوي تتكون بطريقة التعظم الغشائي.بينت نتائج استخدام تقنية الصبغة المزدوجة والمعززة بنتائج الفحص النسيجي إن مراكز التعظم الابتدائية بدأت بعمر (22) يوم من فترة الحمل والتي أظهرت بالصبغة الحمراء مباشرة, ثم بدأت هذه المراكز بالاندماج بعمر (24) يوم من فترة الحمل.أصبح العظم القفوي العلوي كامل التعظم بعمر (30) يوم من فترة الحمل ويكّون الجزء العلوي من الثقب الكبير(foramen magnum).

Introduction

The bones of rabbit skulls consist from neurocranium enclosing the brain and major sense organs (nose, eye and ear), and facial skeleton (viscerocranium) supporting parts of the digestive and respiratory systems, which forms the skeleton of the face. The membranous part of craniumis includingfrontal bones, parietal bones, interparietal bone, squamous part of temporal bone, supraoccipital part of occipital bone (1). The timing of ossification and the growth rates of different components of the skeleton have long been the objects of study, not least for their importance in determining the age of an individual who is undergoing normal growth, but also for assessing abnormal rates of growth. Studies on the human skeleton have been numerous and detailed (2,3,4) much comparable work has also been carried out in the rat (5,6) and morpho-histological study of skeletal development have been studied completely in indigenous goose (7), though relatively few studies have been carried out in the rabbit.

Demonstration of ossificationcenters within the dermal bones of animal fetuses such as cat, pig, sheep and goat depend on the radiography alone,(8,9,10,11) or on the staining of skeletonwith bone dye (alizarin red) alone or with cartilage dye (alcian blue)in a double staining method for demonstration of the ossification centers of bones in young fetuses further more than the radiography(12, 13, 14, 15).There are no sufficient studies about the occipital parts in rabbit, so the present investigation describes the ossification of supraoccipital bone,

during the prenatal development of Iraqi local breed rabbit, because it has economical importance in our country and also in scientific researches.

Materials and Methods

To investigate the aim of this study, thirty two fetuses of rabbits were collected from uteri of the local breed pregnant does in estimated ages, eight fetuses prepared for every stage beginning from (16, 22, 24, and 30 days by which gestation occurred). These fetuses divided as four for whole mount transparency technique to demonstrate cartilage and the bones in different stages and four for routine histological procedure. All fetuses ages were estimated according to the days assumed to have elapsed from copulation (16). The crown-rump length (CRL) will measure for corrections. CRL is the measurement from the vertex of the skull to the midpoint between the apices of the buttocks for prenatal only (17). The CRLs at each stage are summarized in the table (1). The body weight was recorded for each prenatal fetus by using sensitive balance. The body weight was recorded before the fetuses were sacrificed. The mean weight at each stage is summarized in the table (2).Procedure of double staining of bone and cartilage with alizarin red-s and alcian blue is as following(18, 19). (a) Complete skinning by remove skin, muscles, thoracic and abdominal viscera and adipose tissue. (b) Fixation of embryos in absolute ethyl alcohol for minimum of 3 days at early stage and maximum of 7 days at late stages.(c) Staining of embryos for 4days at (37-40 C) in the following solution: 1 volume 0.3% (300mg) filtrated alcian blue in 70% ethyl alcohol (100 ml), 1 volume 0.1% (100mg) filtrated alizarin red-s in 95% ethyl alcohol (100 ml), 1 volume glacial acetic acid (100ml) and 1volum 70% ethyl alcohol (1700ml).(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 16-24houres. (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 steps, the transferred into 100% glycerol to which a few crystals of thymol have been added to avoid mold proliferation. They may store for years without loss of stain properties of specimens(20).To supplement the results, histological examinations were done on thesupraoccipital bone by using histological sections for samples from serial ages, fixed with buffered formalin and decalcified by 10% formic acid thenprocessed and stained routinely with hematoxylin and eosin (7).

Results

1- At day16 of gestation.

Midway through gestation, the double staining procedure of alizarin red and alcian blue for bone and cartilage differentiation of specimen of this stage revealed that supraoccipital part of occipital region are still as membranous and exhibited an almost complete absence of mineralized bone tissue in this part(figure 1). The light microscopy study of proportionate stages confirmed the double staining alizarin and alcian blue results. The histological examination of the occipital region showed by using haematoxylin and eosin gave an indication of thesupraoccipital formed by intramembranous type of ossification, in which the mesenchymal cells were differentiated to osteoblasts, without cartilaginous scaffolding and this part appear as membranous structure. At this day of gestation, the area of caudal cranium roof including supraoccipital which visible initially consist from the mesenchymal cells. These mesenchymal cells appear hyperblastic and crowded with homogenous fluid ground substance. Some of these cells become more fibroblast-like cells, and collagen fiber are randomly scattered among these cells, and other mesenchymal cells differentiate into osteoprogenitor cells (figure2).

2- At day22 of gestation :- Double - stained skull preparations show the bilaterally primary intramembranous ossification centers of thesupraoccipital part of the occipital bone which

appear as red color (figure 3). At this time of gestation, the histological examination byhaematoxylin and eosin staining section of supraoccipital, we noted the supraoccipital part of occipital bone (dermal bone) began ossify by intramembranous ossification. Organic bone matrix first appears at the site as small irregularly shaped spicules. Bone spicules are bright pink in H&E stained sections and are covered with osteoblasts (figure 4).

3- At day 24 of gestation.

The result of double – stainingtechnique at this prenatal age, the two ossification centers of supraoccipital become merged and this part form the roof of foramen magnum (figure 5). The histological examination of supraoccipitalis showing advanced intramembranus ossification become more clearly in form (figure 6). Subsequently the events in previous stage, bone spicules surrounded by layer of osteoblasts.When more spicules form in some location and increase in thickness by the process of oppositional growth, these spicules become interconnected forming a trabecular of cancellous bone. The trabeculae grow out radially from the center of osteogenesis, in the curved plane of the developing skull.

4- At age 30 day of gestation.

The supraoccipital bone is completely ossified by intramembranous method at this time of gestation period and this part of occipital bone form the dorsal border of foramen magnum (figure 7). The coronal sections through cranium vault at this fetal stage demonstrate ossification in the membranous supraoccipital segment very well. This part of occipital bone separated from interparietal bone by the connective tissue suture. The lattice like arrangements of anastomosing trabeculae arethat characterizes this type of woven bone may be seen in this section. On other hand, the fibrous layer of periosteum is covering the fronts of bone tissue (figure 8).

Table (1): Crown-rump length of the prenatal fetuses

Gestation period / day	crown-rump length /mm
16	18.74 ± 0.15 A
22	38.37±0.28 B
24	49.08± 0.73 D
30	95.05 ±1.34 E

Table (2): The body weight of the prenatal fetuses.

Gestation period/ day	Weight of prenatal fetuses/ gm
16	0.56 ± 0.01 A
22	3.60±0.08 B
24	13.17± 0.31 C
30	31.29 ± 0.68 D

Values represent mean ±S.E. Different capital letters mean significant differences at($P<0.05$) results between different periods.

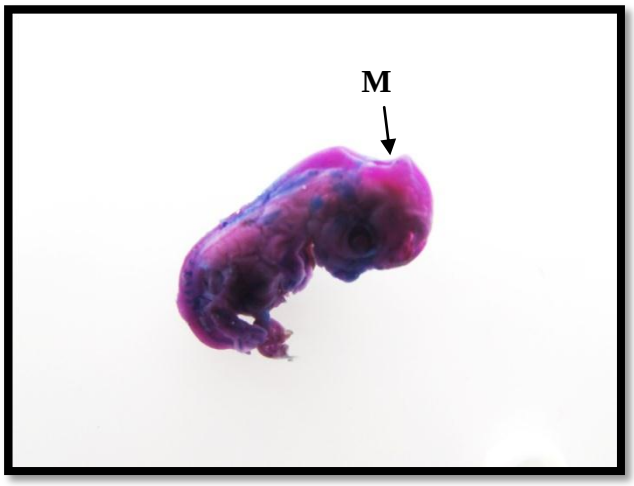

Figure (1): Whole mount of embryo at 16 day of gestationstained with alcian blue andthe alizarine red -s. The supraoccipital part of occipitalbone still as membranous (M).

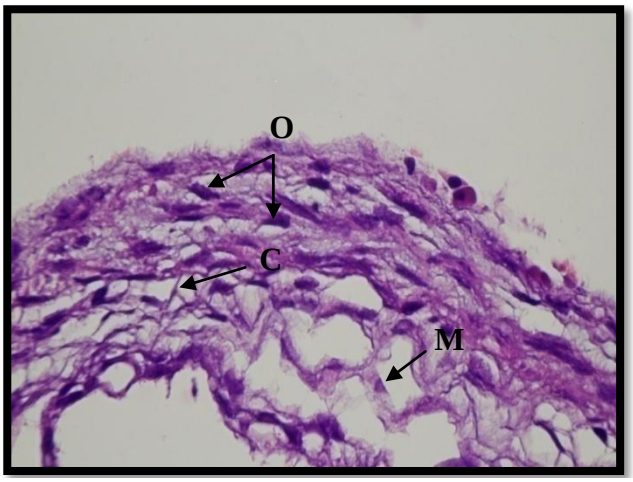

Figure (2): Photomicroscope ofsupraoccipital part at 16 day of gestation illustrates the mesenchymal tissue. Mesenchymalcells (M), osteoprogenitor cells (O), collagen fibers (C) (H&E stain, X 100).

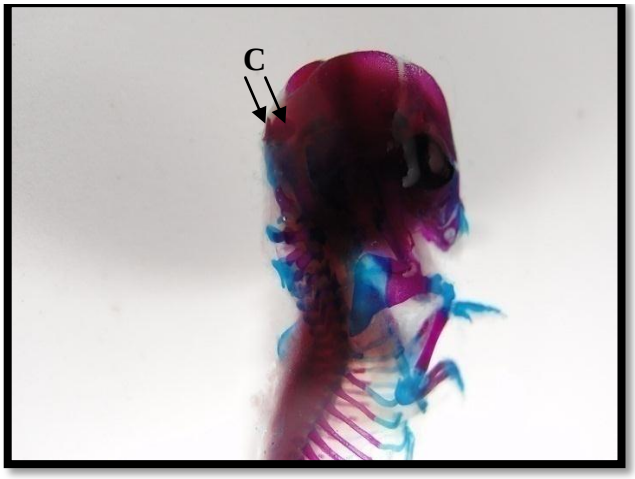

Figure (3): Whole mount of embryo at 22dayof gestation stained with alcianblue and alizarin red- s.The primary ossification of supraoccipitalappears as two centers (C).

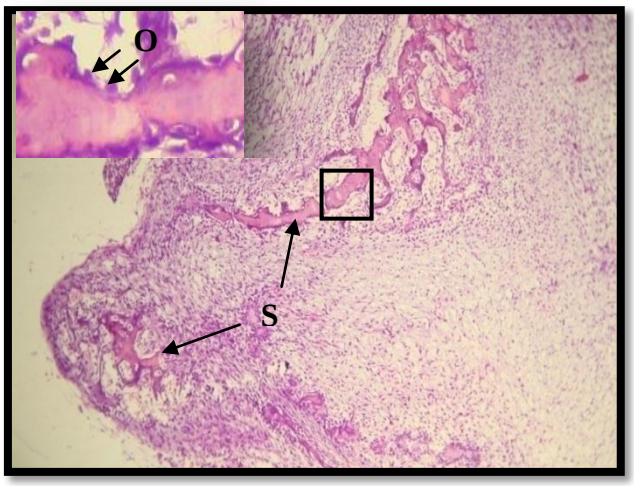

Figure (4):Photomicroscope illustrates the intramembranous ossification of supraoccipital Part at 22daysof gestation.The irregularly shaped spicules (S) covered with osteoblasts (O)(H&E stain, X20, X40).

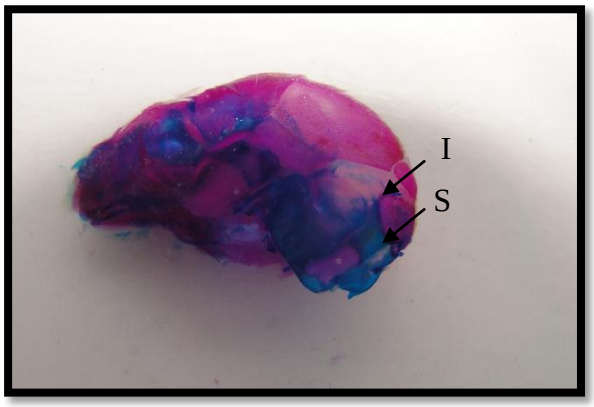

Figure (5):Whole mount of craniumof embryoat 24 day of gestation stained with alcian blueand alizarin red- s. Supraoccipitalbone (S) appearing separated from interparietalbone (I) cranially and form the roof offoramen magnum.

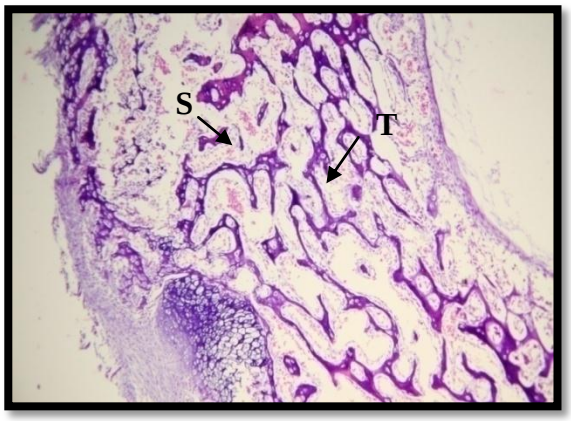

Figure (6): Photomicroscope of the supraoccipital bone illustrates the progress intramembranous ossification, the spicules (S) become inter-connected forming a trabecular (T) of woven bone. (H&E stain, X20).

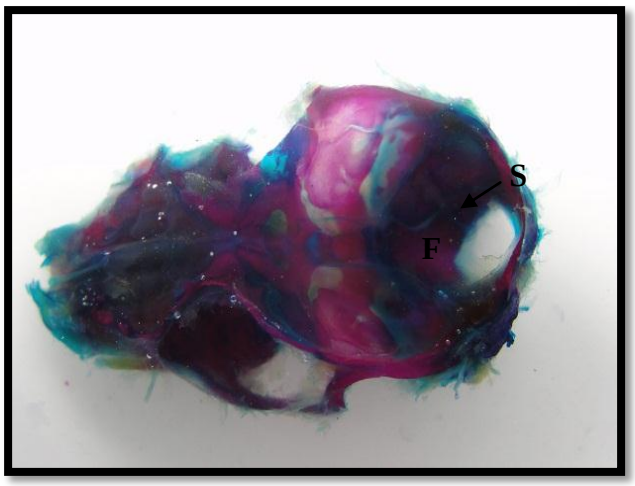

Figure (7): Whole mount of cranium of embryo at 30 day of gestation stained withalcian blueand alizarin red- s.The supraoccipital bone (S) is completely ossified and form the dorsal border of foramen magnum (F).

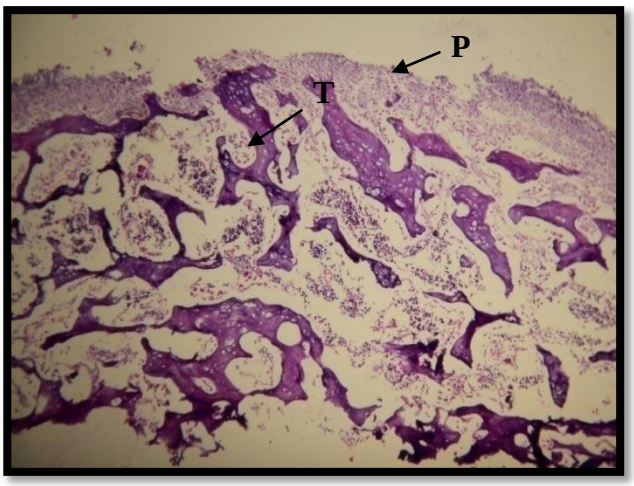

Figure (8): Photomicroscope of supraoccipital bone, illustrates the anastomosingtrabeculae (T) of this woven bone. The fibrous layer of periosteum(P) is covering the fronts of bone (H&E stain, X 20).

Discussion

During 16 day of gestation, the result of present work shows the supraoccipitalparts of occipital region are still as membranous.These result agreed with results of other study in albino mice (21) at 15 day of gestation and in cat(22) at 19-21 day of gestation. The perimordium of dorsal parts of cranium is mainly membranous, only in basal parts cartilaginous. Because of variation in the gestation period between the different species including mice, cat and the species model of present study, we are stabilizing these developmental features of this stage in rabbit fetuses. The results of histological study revealed that thesupraoccipital formed by intramembranous type of ossification, in which the mesenchymal cells were differentiated to osteoblasts, without cartilaginous scaffolding. Our observation was in agreement with (23,24, and 25)in skull of mice; the dermal roof lacks a cartilaginous precursor and instead arises viaintramembranous ossification of osteogenicmesenchymal cells. At this stage, the area of caudal cranium roof including

supraoccipitalwhich visible initially consist from the mesenchymal cells, This observation parallel to(26) in cat at 28 day of gestation and (21) in mouse at 15 day of gestation, they noted the histological structure of all dorsal cranium elements which consist from mesechymal tissue. The histological results of present work shows these mesenchymal cells appear hyperblastic and crowded with homogenous fluid ground substance. Some of these cells become more fibroblast-like cells, and other mesenchymal cells differentiate into osteoprogenitor cells. This observation agreed with(27, 28) as they described osteogenesis of dermal bone in general.

The results of double – staining technique at 22 day of gestation shows the bilaterally primary intramembranous ossification centers of the supraoccipital part of the occipital bone which appear as red color, the pattern ossification of this part of cranium in this study was parallel with that in house mouse at 17 day of gestation(29), in albino mouse at 16 day of gestation(21) the primary ossification of supraoccipital appear separated as two centers and (30) in japans musk shrew, noted primary ossification of supraoccipital appear at 22 day of gestation through intramembranous method. The result of this stage disagree with (31), reported that the mammalian supraoccipital bone was a cartilaginous bone, and that it constituted the dorsal border of the foramen magnum. In contrast, the dorsal border in rabbit was occupied not with the cartilaginous supraoccipital but with the above mentioned membranous bone.(31) discussed the interchangeability between membrane bone and cartilage bone, whether membranes bone in one vertebrate was homologous with cartilage bone in another. On the other hand, some reports(32, 33) suggest that the chondrocranial elements are interchangeable with the membranous bony elements in ontogenetic cranial formation. These result disagreed with(34) they noted, in human, by intracartilaginous ossification the supraoccipital segment ossified from a single focus at 9 weeks of gestation. Through embryogenesis of the human skull bones, the occipital bone has a dual origin from cartilage and membrane, the union of four primary cartilaginous centers laid down in the chondrocranium around the foramen magnum (basioccipital anterior to the foramen magnum, the lateral or exoccipitals on each side of the foramen magnum, and the supraoccipital posterior to the foramen) and from a fifth membranous element gives rise to the interparietal bone.

The results of histological examination at this age revealed thatsupraoccipital appear its primary intramembranous ossification centers, through this ossification the bone spicules are bright pink in H&E stained sections and are covered with osteoblasts. The histological result of present work was conformable with(34)they noted the sipcules and trabeculaes of dermal supraocipital bone at 10 week of human cranium embedded in paraffin and stain by hematoxelin and eosin, whereas (30), they were recorded in japans musk shrew the previous histological elements of primary fuci of intramembranous ossification in horizontal section of supraoccipital which stained by hematoxlin and eosin at 22 day of gestation. However, we are setting up these events of dermal bones formation firstly in rabbit fetuses at 22 day of gestation period.

At 24of gestation, The result of double – staining technique showsthe two ossification centers of supraoccipital become merged and this part form the roof of foramen magnum, thisfinding were parallel to observation of (35) in house mouse, the primary ossification of supraoccipital appear at 17 day of gestation, as two separated centers while at 18 day these centers of supraoccipital have fused.Therefore we fixed these newly data in rabbit fetuses.

The results of histological studies revealed thatsupraoccipital is showing advanced intramembranus ossification become more clearly in formed bone spicules become interconnected forming a trabecular of cancellous bone. These result were properly compatible with several recent reviews (36) and a new books(27, 37) have veryeloquently described for formation of woven (cancellous) bone throughosteogenesis of flat bone of skulls in most animals and human.

At stage of the mature fetuses (31)day of gestation just before or during birth,the supraoccipital bone is completely ossified by intramembranous method and this part of occipital form the dorsal border of foramen magnum. In considering the findings of this study agree with (30) who noted through development of cranium in japans musk shrew, the supraoccipital bone was a membranous bone, and that it constituted the dorsal border of the foramen magnum. In contrast, (31) was reported in human, the dorsal border was occupied not with the membranous supraoccipital but with the cartilaginous bone. On the other hand, he discussed the interchangeability between membrane bone and cartilage bone: whether a membrane bone in one vertebrate was homologous with cartilage bone in another. Although he described many possible instances of interchangeability, conclusive proof was lacking. In this respect, (31) reported the dermosupraoccipital bone as constituting a part of dorsal border of the foramen magnum in some teleosts which lack a tectumposterius.The histological result at this time of gestation demonstrates ossification in the membranous supraoccipital bone as well. This part of occipital bone separated from interparietal bone by the connective tissue suture. The lattice like arrangement of anastomosing trabeculae isthat characterizes this type of woven bone. On other hand, the fibrous layer of periosteum is covering the fronts of bone tissue. These results are parallel to some others (27, 37) a distinct fibrous periosteal layer is seen around the approximating bone fronts of membranous bones in rat and other mammals respectively.

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