

Development of forelimb bones in indigenous sheep fetuses

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Abstract

The study included detection of the sites of ossification centers and their sequence of appearance in the forelimb bones of indigenous sheep fetuses by using double staining method with younger specimens and radiography or maceration methods with old specimens, as well as, histological study with some ages. The results showed that the primary ossification centers of the forelimb in indigenous sheep fetuses appeared firstly in the diaphyses of radius and ulna, humerus, scapula, metacarpus, phalanges and lastly in the carpal bone at an estimated age of 43, 45, 46, 47, 49 - 56 and 90-118 days old respectively. The results of statistical analysis of the total lengths of scapula, humerus, radius, ulna and metacarpus with the lengths of their ossified parts through the 7th - 15th weeks of fetus age, showed presence of significant differences in the average of these measurements among most of studied weeks. Also there was a significant differences in the average of relative increase in the total length and length of ossified part of diaphysis of studied bones during the 7th week in comparison to the same average in the other studied weeks (8th-15th week) of indigenous sheep fetuses age.

Keywords: Forelimb, Bone, Sheep, Development, Ossification

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تطور عظام القائمة الأمامية في أجنة الأغنام المحلية

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فرع التشريح، كلية الطب البيطري، جامعة الموصل، الموصل، العراق

الخلاصة

تضمنت الدراسة تحديد مواقع مراكز التعظم وتسلسل ظهور كل منها في عظام القائمة الأمامية لأجنة الأغنام المحلية باستخدام طريقة الصبغة المزدوجة مع العينات الصغيرة وطريقة التصوير الشعاعي أو التعطين مع العينات الأكبر كما وأجريت دراسة نسيجية لبعض الأعمار. أظهرت نتائج الدراسة بان مراكز التعظم الابتدائية لعظام القائمة الأمامية لأجنة الأغنام المحلية ظهرت أولاً في اجسام عظام الكعبرة والزند في عمر تقديري ٤٣ يوم وفي العضد بعمر ٤٥ يوم وفي اللوح بعمر ٤٦ يوم وفي مشط اليد بعمر ٤٧ يوم وفي سلاميات أصابع اليد بعمر ٤٩ - ٥٦ يوم وفي عظام رسغ اليد بعمر ٩٠ - ١١٨ يوماً. أظهرت نتائج تحليل التباين على الطول الكلي لكل من عظام اللوح والعضد والكعبرة والزند ومشط اليد وأطوال أجزائها المتعظمة خلال الأسابيع السابع إلى الخامس عشر من عمر الجنين وجود فرق معنوي في معدل هذه القياسات بين اغلب الأسابيع المدروسة وتبين وجود فرق معنوي ملحوظ في معدل الزيادة النسبية للطول الكلي وطول الجزء المتعظم من اجسام العظام المدروسة خلال الأسبوع السابع من عمر الجنين مقارنة مع المعدلات ذاتها في بقية الأسابيع المدروسة (٨-١٥) من عمر جنين الأغنام المحلية.

Introduction

The natural morphological development of bones and joints is very important for the diagnosis of skeletal diseases in young (1). The study of the appearance of limb ossification centers provide great aids in age estimation during prenatal life and assessment of fetal bone maturation and helps in the detection of some fetal abnormalities (2, 3). Some researches depend on limb bone lengths to estimate the late fetal and prenatal age (4).

Limbs grow outward from body wall somatopleure as limb buds. As the limb buds elongate the regions of the limbs develop in proximodistal order and the shoulder appears first, while the manus is the last to be added (5).

Long bones of the limbs ossify by endochondral ossification and the main important sources relating to first appearance of prenatal ossification centers in human are based on the specimens cleared in diluted potassium hydroxide. Some studies use bone dye (alizarin red) which demonstrate the calcification of cartilaginous matrix as a step of the endochondral ossification (6). Other works depend on radiography and double staining methods in studying the ossification centers in human fetus limbs (7). Some researchers chose to study the ossification centers in femur and humerus of human fetus dissection and dryness then compared the symmetrical bones of both sides (8).

Demonstration of ossification centers within the bones of animal fetuses such cat, pig, sheep and goat depend on the radiography alone, (9, 10, 11, 12, 13, 14, 15), or on the staining of skeleton with 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 than the radiography or potassium hydroxide -maceration for the fetal bones of older ages (16, 17, 18, 1, 19, 20, 21).

In human, ultrasonography is used to measure and early visualize the ossification centers in embryo and fetal limb bones (22, 23).

The aim of this work was detect the time of the first appearance of the primary ossification centers in the forelimb bones of indigenous sheep fetuses and to follow up the further bone development of the body of scapula, humerus, radius, ulna and metacarpus bones.

Materials and Methods

Thirty-five fetuses were collected from uteri of the pregnant Awassi ewes slaughtered in Mosul. The crown-rump lengths of these fetuses were measured to find out the estimated ages (24), which ranged between 40-130 days.

The forelimbs were separated from the fetuses' skeletons. Limbs of fetuses with 10 cm. crown-rump length (70-days old) or less were prepared by skinning and fixing in 90% ethyl alcohol then staining with modified double-

staining method by using a mixed alcoholic solutions consist of 1 part of 0.14% alizarin red S and 1 part of 0.3% alcian blue. They were macerated by using 2% potassium hydroxide and cleared by using gradual concentrations of glycerin in distilled water (25).

The stained limbs were examined by dissecting microscope to detect the primary ossification centers appeared with each age, these centers identified by their red colour, while the cartilaginous substance of bone appeared blue. The diagnosis of each bone in the early stages depend on it's position in the limb's skeleton and the bone's characters such as triangular shape of scapula, longitudinal spiral groove of the humerus, dorsopalmer flatness of the radius, hypertrophy of proximal end of ulna with the narrower of it's distal end (18), and the dorsopalmer flattened body of the metacarpus with convex dorsal and concave palmer surfaces (26).

The forelimbs of the older fetuses with more than 10 cm. crown-rump length were exposed to x-ray under exposure factors of 60KV and 100mA and 0.1 seconds. Other samples were macerated after skinning and removing of loose connective tissue by putting them in diluted solution of potassium hydroxide 2-10% for about 6-24 hours according to the age of the fetus, the older ages need more time and more concentrated solution of potassium hydroxide about 8- 10%potassium hydroxide for 20-24 hours (27, 28).

The growth of bones was studied in the diaphyses of scapula, humerus, radius, ulna and metacarpus by using the vernier to measure the total length of each bone as well as the length of ossified part of the diaphysis (Fig 1). The measurements were taken directly from the stained limb skeleton in young fetuses and from the radiographs in older ones and directly from the bone of macerated limbs. Analysis of variance and Duncan test (29) were applied on these measurements to detect significant differences in the average of the relative increase of these measurements among the five studied bones as well as among the nine studied weeks (7th - 15th).

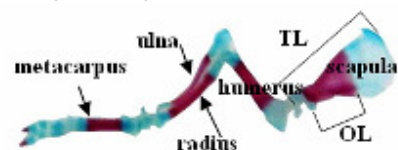


Fig. 1: Forelimb of indigenous sheep fetus with an estimated age 50 days prepared with double staining method (alcian blue & alizarin red) show the bones of the limb with the total length (TL) and length of ossified part of diaphysis (OL) of studied bone

To supplement the results, histological examinations were done on the forelimb bones by using histological

sections for samples from serial ages (30), fixed with buffered formalin and decalcified by 10% formic acid then processed and stained routinely with hematoxylin and eosin (31).

Results

The results showed that the primary ossification centers in the forelimbs of the indigenous sheep fetuses, firstly appeared in the diaphyses of radius and ulna at 43 days old while they appeared in the middle phalanx at 56 days old of intrauterine life (Table 1).

The ossification occurred rapidly in diaphyses of the five large studied bones, but it delayed in the carpal bones

and it was very slow in the epiphyses of the long limb bones (Fig2).

The results of histological examination of humerus showed that using of alizarin red in demonstration of a primary ossification center gave an indication of calcification just prior to early ossification, where the center appeared as a red locus by this stain. Con-currently, the histological picture of this bone at the same age showed proliferation and maturation of chondrocytes and calcification of the cartilaginous matrix (Fig5). After one day, the ossification appeared (Fig6), followed by the formation of bony spicules and trabecules (Fig7 and Fig8). The study showed that these results applied also to the other bones of the forelimb.

Table 1: Sequence of appearance of the ossification centers in forelimb bones of indigenous sheep fetuses

Number of specimens	Crown –rump length (cm)	Estimated age (day)	Bones in which ossification centers appeared
3	3.52, 3.61, 3.68	43	Diaphysis of radius and ulna.
3	4.48, 4.58, 4.62	45	Diaphysis of humerus.
3	5.02, 5.08, 5.12	46	Caudal edge of scapula.
3	5.47, 5.52, 5.58	47	Diaphysis of 3rd and 4th metacarpal bones.
4	6.35, 6.41, 6.79, 6.97	49-50	Junction of parietal and solar border of 3rd phalanx. (Fig2)
3	8.31, 8.39, 8.46	53	Diaphysis of 1st phalanx.
4	9.23, 9.4, 9.63, 9.78	55-56	Palmer surface of 2nd phalanx. (Fig2)
3	25.92, 26.02, 26.05	90	Radius, ulna, intermediate carpal bones.
3	30.68, 30.74, 30.82	100	3rd, 4th carpal bones, distal extremity of radius. (Fig4)
3	34.44, 34.5, 34.58	108	Distal extremity (condyles) of humerus, distal extremity of 3rd, 4thmetacarpals, proximal extremity of radius. (Fig3)
3	39.2, 39.32, 39.38	118	Accessory carpal bone.

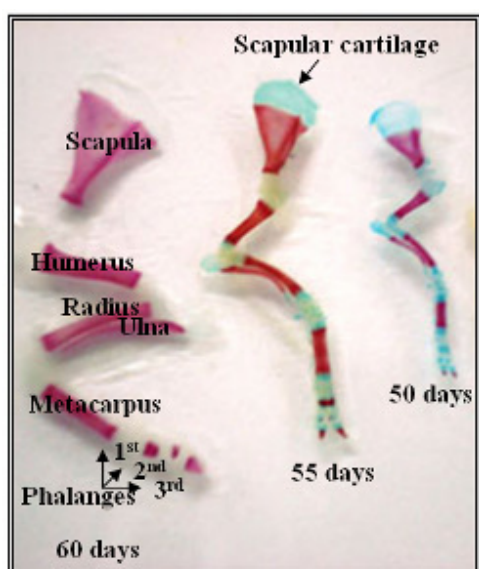


Fig. 2: Forelimbs of indigenous sheep fetuses with an estimated age of 50, 55, 60 days prepared with double staining method. Notice the successive bony development in the forelimb bones (X 1.5).

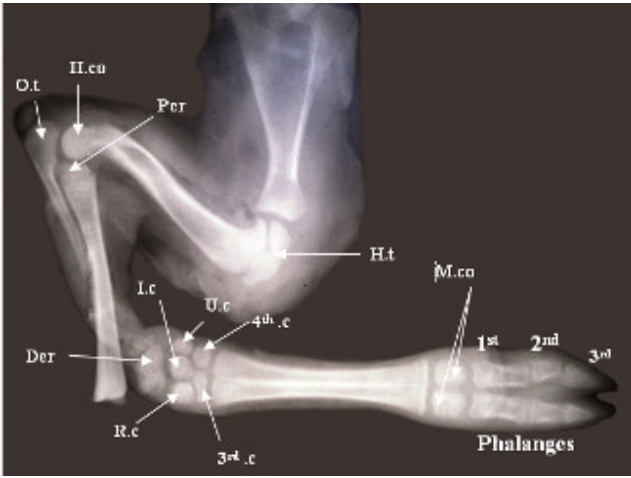


Fig. 3: x-ray image to forelimb of indigenous sheep fetus with an estimated age of 128 days old. Notice carpal bones: Radial (Rc), Intermediate (Ic), Ulnar (Uc), Third (3rd), Fourth (4th) carpal bones) radial (RC)}, Humerus tuberosity (Ht), Humerus condyle (Hco), Olecranon tuberosity (Ot), Metacarpus condyles (Mco), condyles (Mco), Proximal & Distal extremities of radius (Per, Der).

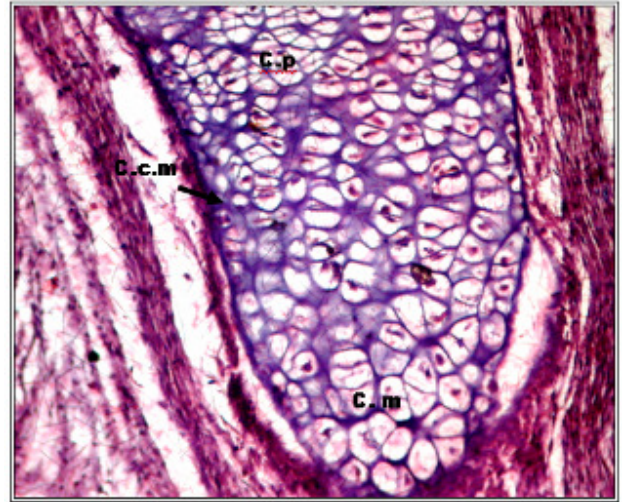


Fig. 5: Humerus diaphysis (Longitudinal section) of indigenous sheep fetus with an estimated age of 45 days stained with hematoxyline and eosin. Notice the proliferation (Cp) and maturation (Cm) of chondrocytes and calcification of matrix (Ccm), (X 200).

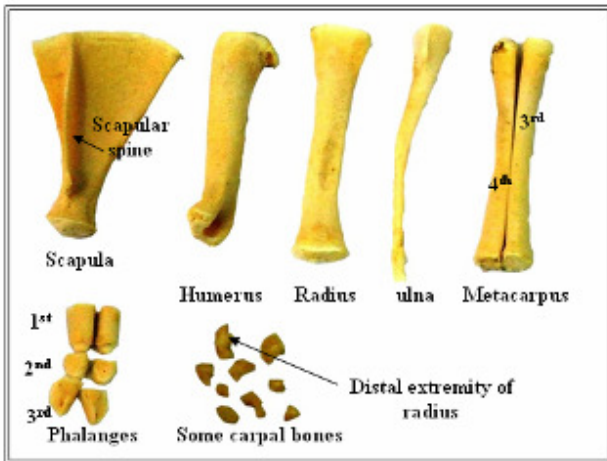


Fig. 4: Forelimb bones of indigenous sheep fetus with an estimated age of 105 days using diluted KOH for maceration (X 1.3).

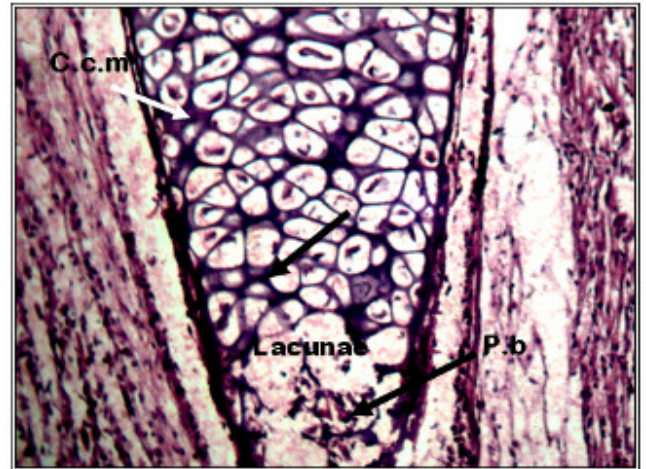


Fig. 6: Humerus diaphysis (Longitudinal section) of indigenous sheep fetus with an estimated age of 46 days stained with hematoxyline and eosin. Notice calcified cartilaginous matrix (Ccm) and the periosteal bud (Pb) that contains osteoblasts, (X 200).

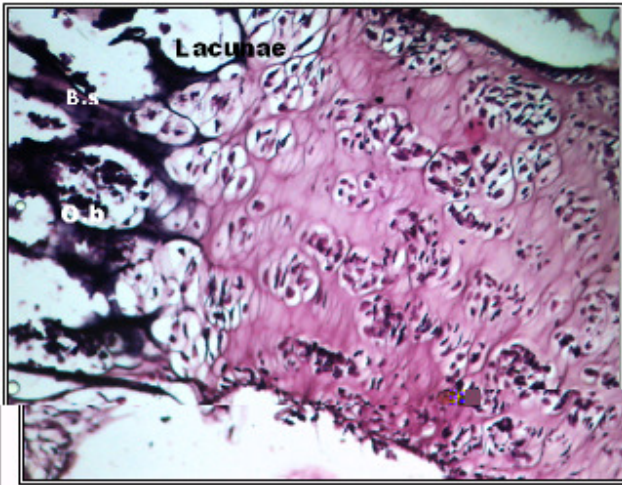


Fig. 7: Humerus diaphysis (Longitudinal section) of indigenous sheep fetus with an estimated age of 48 days stained with hematoxyline and eosin. Notice lacunae, osteoblasts (Ob), bony spicules (Bs) of primary ossification center of this bone (X 200).

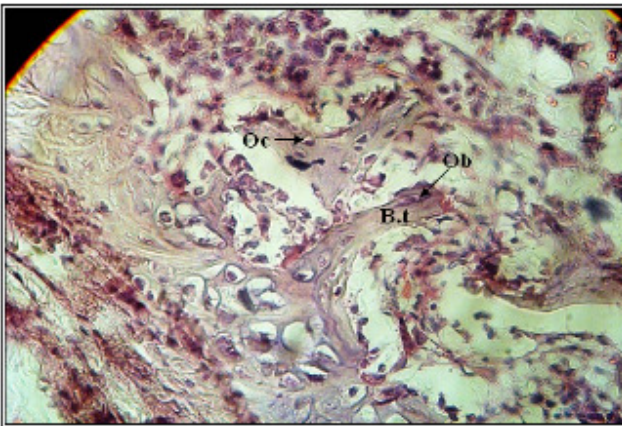
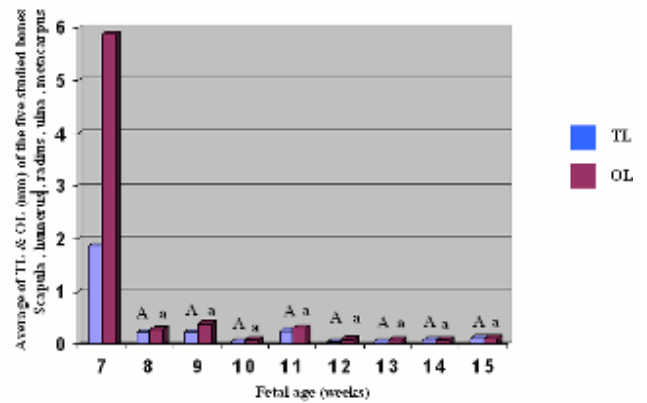


Fig. 8: Humerus diaphysis (Longitudinal section) of indigenous sheep fetus with an estimated age 50 days stained with hematoxyline and eosin. Notice bony trabecule (Bt), osteocytes (Oc), osteoblasts (Ob), (X 240).

The results of analysis of variance done on the measurements of total length and length of the ossified part of scapula, humerus, radius, ulna and metacarpus diaphyses during the nine studied weeks (7th-15th weeks) of indigenous sheep fetuses age showed presence of significant differences in both of average of total length and average of length of ossified part of diaphysis to each of the five studied bones among most of weeks during the nine studied weeks of fetal age at $p < 0.05$ (table 2).

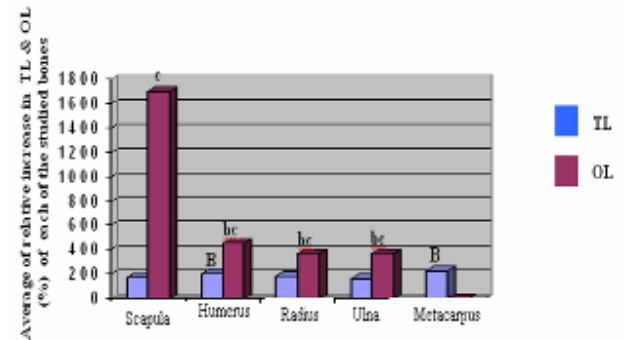
The statistical results showed a significant differences in both the average of total length and the average of length of ossified part of diaphysis of the five studied bones during

the seventh week and the same averages in the other studied weeks (8th-15th weeks) of indigenous sheep fetuses age at $p < 0.05$ (Fig 9), while there was no significant changes among the later weeks (8th -15th weeks). There is a significant differences between metacarpus and ulna, radius and scapula in the relative increase of total length, and also between scapula and the other bones and between metacarpus and the other bones in the relative increase of the ossified part of diaphysis at $p < 0.05$. (Fig 10).



A, a significantly differ from the value of TL, OL respectively of the 7th week at $p < 0.05$.

Fig. 9: The total length (TL) and length of ossified part of diaphysis (OL) in the five studied bones (scapula, humerus, radius, ulna, and metacarpus) of indigenous sheep fetuses during the 7th-15th weeks of its developmental age.



B,b: significantly differ from the value of relative increase in TL and OL respectively of scapula at $P < 0.05$. c: significantly differ from the value of relative increase in OL of metacarpus at $P < 0.05$. b,c: significantly differ from the value of relative increase in OL of scapula and metacarpus at $p < 0.05$

Fig. 10: The relative increase in the total length (TL) and length of the ossified part (OL) of studied bones (scapula, humerus, radius, ulna, and metacarpus) in the 7th week of indigenous sheep fetuses age.

Table 2: Total length (TL) and length of ossified part (OP) of diaphysis in mm of five forelimb bones of indigenous sheep during the fetal age

age (week)	Scapula		Humerus		Radius		Ulna		Metacarpus	
	TL	OP	TL	OP	TL	OP	TL	OP	TL	OP
7	7.880 ±1.415	2.920 ±1.051	6.860 ±1.239	2.760 ±0.630	6.160 ±0.993	3.560 a ±0.738	7.300 ±1.237	3.900 a ±0.822	4.500 ±0.899	0.720 ±0.388
8	12.850 ±0.506	6.950 a ±0.444	10.950 ±0.568	5.825 a ±0.272	10.750 a ±0.877	6.575 ab ±0.502	12.650 a ±0.780	7.075 ab ±0.189	8.125 a ±0.320	3.950 a ±0.584
9	17.200 ±0.987	10.050 ab ±1.187	15.150 ±1.374	8.075 a ±0.896	13.150 a ±1.053	9.425 b ±0.777	15.700 a ±1.229	10.675 b ±1.310	9.800 ab ±0.548	6.675 a ±0.706
10	20.225 ±0.470	13.575 b ±0.239	18.900 ±0.332	12.200 ±0.416	18.100 ±0.545	13.200 ±0.385	19.850 ±0.499	14.500 ±0.238	13.475 b ±0.596	10.100 ±0.372
11	26.625 ±1.273	19.975 ±3.129	26.600 ±1.324	17.550 ±0.377	23.950 ±1.385	17.200 ±0.906	27.925 ±1.434	20.725 ±1.446	19.925 ±2.302	12.625 ±1.195
12	30.500 ±1.167	23.700 c ±0.714	30.400 ±0.906	21.225 ±1.099	28.375 ±1.229	22.475 ±0.936	34.350 ±0.359	23.875 ±0.448	25.175 ±0.193	15.600 ±0.286
13	37.800 ±0.495	27.250 cd ±0.466	36.650 ±0.953	28.625 ±0.904	35.350 ±0.690	29.175 ±0.864	38.800 ±0.804	29.275 ±0.730	27.475 ±0.850	19.900 ±0.567
14	41.950 ±1.093	30.475 d ±0.844	41.475 ±0.694	33.300 ±0.634	39.825 ±0.831	33.125 ±1.171	43.975 ±0.771	36.400 ±1.521	33.775 ±1.616	27.425 ±1.146
15	48.875 ±0.862	37.775 ±2.833	50.025 ±2.293	38.77 ±1.737	47.900 ±2.696	41.800 ±2.714	55.775 ±3.425	49.975 ±2.585	46.950 ±3.475	41.200 ±2.740

Values are mean ± SD

Note: Within the column, the data without letters show significant differences, whereas data with the same letters show no significant differences.

Discussion

The first appearance of primary ossification centers in the forelimb occur in the diaphyses of radius and ulna (approximately at the middle of the forelimb. The sequence of appearance of the limb bones' ossification centers continue to occur in proximodistal direction and it grew proximally faster than distally.

The primary ossification centers in the forelimb bones appeared in a limited period range from 43-56 days old in the studied fetuses, but the centers of ossification in carpal bones delayed to appear till 90-108 days old, while the accessory carpal bone extended up to 118 days old. These results agreed with the results of other study on sheep fetuses, where the primary ossification centers appeared with a period ranged from 50-60 days old, while the ossification centers in the carpal bones delayed to appear till 90-98 days old, except the accessory carpal bone which appeared lastly in the period ranging between 113-128 days old (11).

The sequence of appearance of appendicular ossification centers during the prenatal life showed a varying degree of conformity with the theory of proximo-distal developmental direction mentioned by (31). The early appearance of the ossification in the distal (3rd) phalanx attributed to the fact that the claw (hoof) including this bone is the weight bearing part of the limb (1). The study showed that the secondary ossification centers firstly appeared in the distal end of the radius at 100 days old with the centers of carpal bones, then in the distal ends of both 3rd and 4th metacarpal bones, distal end of humerus and the proximal end of the radius at 108 days old followed by olecranon tuberosity of ulna at 110 days old and lastly the center of accessory carpal bone at the age of 118 days old. These results relatively agreed with results of (11) on sheep fetuses whom said that the secondary ossification centers appeared firstly in the distal end of the radius, humerus condyle and distal ends of both 3rd and 4th metacarpal bones, while the ossification centers of humerus head, proximal end of radius, greater tuberosity of humerus, the proximal end of 1st phalanx and the olecranon tuberosity of ulna present at 112 days old, however, the ossification center of the accessory carpal bone appeared at 113 days of the fetal life (11).

The results of histological studies revealed that the appearance of red colour of alizarin red in the diaphysis of fetal bone stained with double staining method, alizarin red s and alcian blue, doesn't mean ossification. Histological sections of humerus bone of 45 days of intrauterine life showed no features of real ossification but only calcification of the cartilaginous matrix, while, one day after (46 days) gave the first initiative sign of ossification, when the periosteal bud that contains osteogenic cells, osteoblasts and capillaries appeared. After that the bony

spicules and bony trabecules were shown. These results agreed with (32) who stated that, when the periosteal bud reach the midsection of cartilage model, they are said to constitute a center of ossification. Similarly (33) mentioned that the histological examination is necessary to detect the ossification of the skeletal development.

The statistical results showed presence of significant differences in the average of total length (TL) and length of ossified part of diaphysis (OL) among most of studied weeks, and presence of significant differences in the average of (TL) and (OL) in the 7th week in comparison to same average in the other studied weeks (8th-15th). These results agreed with the study on forelimb bones development in black goat fetuses (20).

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