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A study of primary ossification centers in the hind limbs of Awasi sheep fetuses by modified method of double stains and radiography

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Abstract

Forty Local Awasi sheep fetuses were collected from several areas in Nineveh Governorate, further than local slaughter house. Their estimated ages calculated depending on crown-rump length with the aid of Richardson formula. The study aimed to detect the site and the time of appearance of centers of ossification in the bones of hind limb of Awasi sheep by using modified method of double stains to demonstrate cartilage with bone, or by using radiography. The first ossification centers appeared in the hind limb bones was the primary center of the tibia and femur, metatarsus with ilium then ischium at 45, 46, 48, 50, 52 days of fetal age respectively. The ossification centers of pes phalanges appeared in the following sequence; distal phalanx, followed by the proximal phalanx and end by middle phalanx, at 58, 60, 64 days respectively. Ossification centers of tarsal bones began to appear in calcaneus at 64 days, talus at 77 days, central and 4th tarsal bones at 96 days, followed by centers of 3rd and 2nd tarsal bones at 100 days while, the center of 1st tarsal bone appeared lastly at 105 days. Femur, tibia and metatarsus studied statistically by follow up the relative increase of their total lengths with ossified portion length of their diaphyses. The very important finding of our work is that the maximum relative increment in the length of the long bones and their ossified diaphysis is happening within the 7th to 8th weeks of gestation making this time to be considered as the most critical period for bone development especially long bones so it is essential to avoid giving any medication or therapy during that time which could affect or even retard this vital process.

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Introduction

Sheep is an essential part of livestock in many countries of the world, and plays an important role in human life, because it is an important source of food represented by meat and milk, as well as other products of high economic value such as wool and leather (1,2). The skeleton represents the main axis of the body in animals that have skeletons since it forms the most solid part of their bodies, thus support and protect the sensitive parts of body organs and holds its various parts, this lead to maintain the general shape of animals' bodies, besides the bone marrow generates blood cells, skeleton consider as a source of calcium and phosphorus (3). The bones of the hind limbs represent an important part of the body skeleton owing to pushing the body forward, supporting the muscles, and maintaining the important sensitive organs inside the pelvic cavity (4). The limbs arise externally from the lateral body wall, the somatopleure, as a terminal bud that begins to appear in sheep embryos at the end of the third week of pregnancy, hind limb arise in the lumbosacral region. The forelimbs precede the hind limbs by a day or two. The bony and cartilaginous matter and the connective tissues of the stature arise from the somatic mesoderm of the terminal bud, while the dermis and skeletal muscle come from the dermatome and myotome migrating to the terminal bud (5). There are

some studies on our local animals as the study of development of the double facial bones of local sheep (6) and ossification of centers in vertebrae of local sheep and black goats' fetuses (7), and bone development in the fore-limb of local sheep (8), as well as bone development in forelimb and hind limb of local black goat's fetuses (9,10). The bone development may be studied with the help of ultrasound, as the study on the local sheep embryos for estimating age and fetal development (11), but yet there is no information about bone development in hind limbs of indigenous sheep fetuses. As these studies provide information for workers in other medical fields to discover bone deformities resulting from the effect of a particular substance or a nutritional or drug elements on the bone growth of the fetus. The current study aims to provide a database for bone growth in hind limb of local Awasi sheep fetuses by determining the duration and location of primary ossification centers in each bone of the hind limb, as well as following up the relative increase of bone growth in diaphysis of long bones during gestation.

Material and methods

Samples

Forty local Awasi sheep fetuses of both sexes were collected from several areas in Nineveh governorate besides local slaughter house.

Fetuses measurements and identification

For each fetus, the crown-ramp length (C.R.L) was measured using either a vernia machine (Figure 1) or a measuring tape (7). Furthermore, the estimated age was measured following Richardson formula (12). Estimated age (day) = 2.1[C.R.L (cm.) +17].

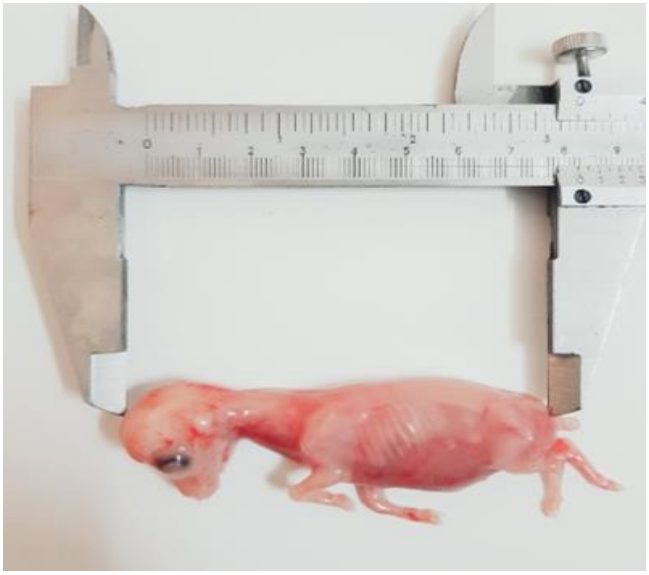

Figure 1: A photo of using vernia in measuring of crown-rump length in Awasi sheep fetus.

Fetus preparations

At the beginning, the fetus hind limbs were separated from the rest of body, then skinning and removing of the soft tissues and pelvic viscera. The hind limbs were fixed using immersion with 95% ethyl alcohol for at least 72 hours, more time with the second use of the fixative (10).

Fetus staining method

Modified double staining method was used for ten fetuses of 45-70 days. The procedure started with the conversion of the fetuses from ethyl alcohol to the double staining solution and left within this solution for 24 hours, the time increased with older fetuses and with repeated using of the staining solution. At the end of staining, samples of hind limbs were transferred and left in maceration solution represented by 2-5 % sodium hydroxide solution for 12-72 hours (7) then immersed in clearing solution for 24 hours in a mixture solution consisting of 70% ethyl alcohol solution, glycerin and benzyl alcohol in a ratio of 2:2:1 (7,13). In addition to the whole above procedures, and to make bones and cartilages clear and visible, the limb samples were cleared through passing them in gradual concentrations of glycerin 25, 50, 75 and 87% for appropriate periods until the soft tissues become transparent and the bones and cartilage can be seen through (8,10).

Staining preparations

One volume of 0.3% alcian blue dissolved in 70% ethyl alcohol and filtrated added to one volume of 0.12% filtrated alizarin red-s dissolved in 90% ethyl alcohol and filtrated, then one volume of glacial acetic acid and 17 volumes of 70% ethyl alcohol added to them the specimens were washed in distil water at the end of the staining process (7,14).

Stained fetus storage

To avoid contamination and rotting, the stained limbs were stored in airtight glass or plastic bottles containing 87% concentrated glycerin, supplied with few crystals of thymol to further avoidance of fungal contamination and putrefaction (15,16).

Radiographic preparations

Awasi sheep fetuses with an estimated age older than 70 days have been studied using radiographic imaging. These samples were x-rayed using 43 K.V technology and M.A current of 100 and an exposure time of Mas-sec 0.020 of a second (7). The x-ray machine that used was manufactured by Won solution co. Ltd / Toshiba/ Japan, with an attached software of taking measurements on each image .

Studying bone development

Bone development within three long bones of both stained and radiographed samples have been followed up by measuring the total length and the length of ossified portion of diaphysis (Figure 2) with the aid of vernia, in order to detect the relative increment in the total length of these bones

with the length of the ossified parts of their diaphyses and then to compare the results among these three bones and among the studied periods using Duncan's Multiple Range Test (MRT) (17).

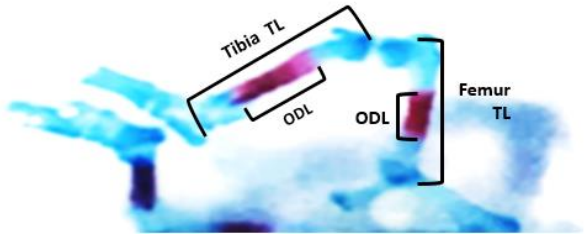

Figure 2: A photo of hind limbs of fetus of Awasi sheep with 47 days old prepared with modified method of double stains show the total length (TL) and the ossified diaphysis length (ODL) of femur and tibia.

Results

The first center of ossification formed in both hind limbs was the primary center of the tibia and femur, which appeared at 45, 46 days respectively, then the primary centers of 3rd and 4th metatarsal bones accompanied with that of both ilial bones, followed by the ossification centers of both ischial bones (Figures 3 and 4).

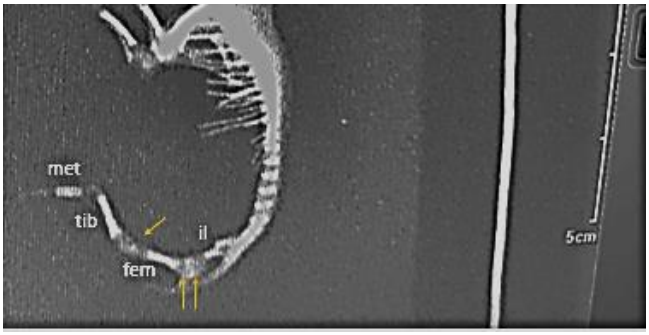

Figure 3: X-ray film for the right hind limb of Awasi sheep fetus with an estimated age 50 day, show the ossification centers of tibia (tib), femur(fem), the large metatarsus (met), ilium (il), beginning of the ossification centers of ischium (two arrows) and proximal epiphysis of fibula bone (arrow).

The first ossification centers of phalanges began to appear in the distal phalanx at 58 day followed by the center of proximal phalanx, while the last primary center in digit appeared at middle phalanx at 64 days old (Figures 5 and 6).

The ossification centers of tarsal bones began to appear in calcaneus at 64 days, followed by talus then central and 4th tarsal bones, followed by centers of 3rd and 2nd tarsal bones while, the center of the 1st tarsal bone appeared lastly at 105 days (Table 1).

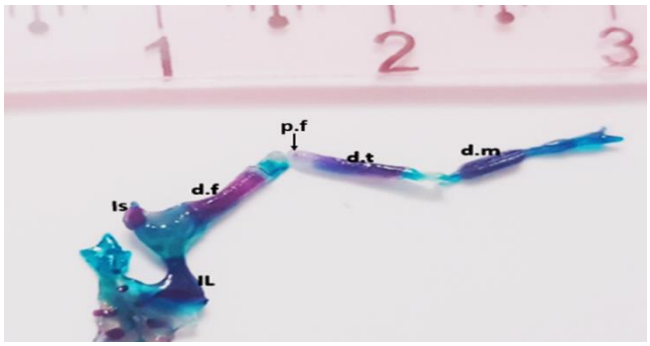

Figure 4: Left hind limb of Awasi sheep fetus with 50 days old colored with modified method of double stains, show the ossification centers at diaphysis of femur (d.f), Ilium (IL), Ischium (Is), diaphysis of tibia (d.t), proximal epiphysis of fibula (p.f), diaphysis of 3rdand 4th metatarsal bones (d.m).

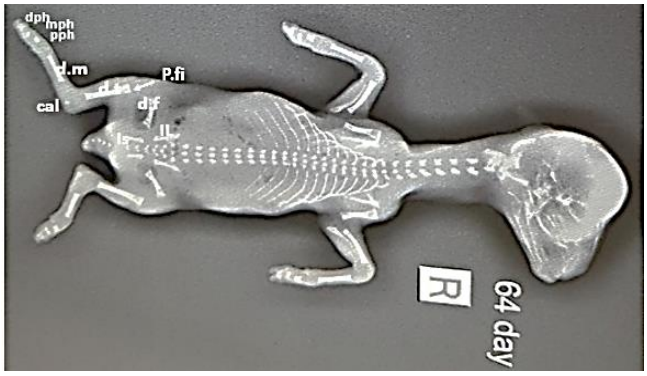

Figure 5: X-ray film of Awasi sheep fetus with 64 days old show the ossification centers of diaphysis of femur (d.f), Ilium (IL), Ischium (Is), diaphysis of tibia (d.t), proximal part of fibula (p.fi), calcaneus bone (cal), diaphysis of 3rd and 4th metatarsal bones (d.m), first phalanx (pph), second phalanx (mph) and third (dph) phalanx.

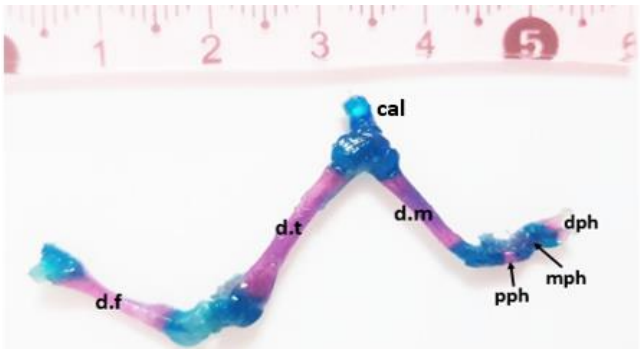

Figure 6: Hind limb of Awasi sheep fetus with 64 days old colored with modified method of double stains show the ossification centers of diaphysis of femur (d.f), diaphysis of tibia (d.t), calcaneus bone (cal), diaphysis of 3rd and 4th metatarsal bones (d.m), first phalanx (pph), second phalanx (mph) and third (dph) phalanx.

Table1: Appearance sequence of primary centers of ossification in hind limb bones of Awasi sheep

Fetal age (days)	Ossification centers sites
45	Tibia diaphysis
46	Femur diaphysis
48	diaphysis of large metatarsal bones and body of ilium.
50-52	Ischium body, Proximal head of fibula
58	3rd phalanx
60	1st phalanx
64	2nd phalanx**,** and calcaneus
77	Talus bone
96	Pubis**,** middle raw of tarsal bones (central & 4th)
100	Proximal sesamoid bones**,** distal raw of tarsal bones (2nd and 3rd)
105	First tarsal bone

The results show that the primary ossification centers of long bones have been appeared in the mid diaphysis of each long bone, and the growth extend in both proximal and distal directions of each bone, it has been noticed also that the ossification in these bones was quickly in comparison with the other bones of limb like the tarsal bones and phalanges (Figure 7).

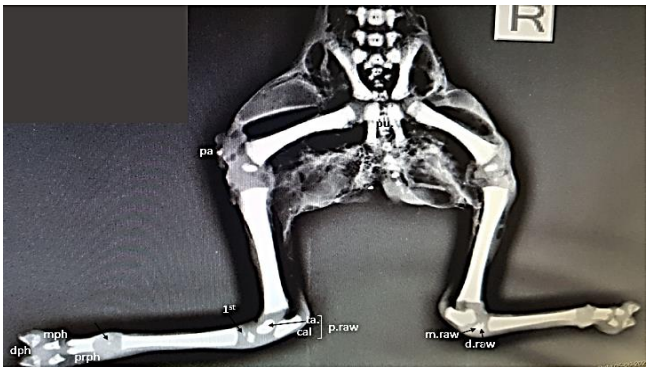

Figure 7: X-ray film of fetus of Awasi sheep with 105 days old, show the ossification centers of pubis (pu), patella (pa), proximal raw of tarsal bone (p. raw) include calcaneus (cal), talus (ta), middle raw of tarsal bones (m. raw), distal raw of tarsal (d. raw), first tarsal bone (1st), proximal phalanx (prph), middle phalanx (mph) and distal phalanx (dph).

The analysis of variance conducted on the total length measurements with the ossified portion length of femur, tibia, and metatarsus diaphysis over the seven studied fortnightly periods of fetal age in local sheep from 46th to 150th days revealed significant variance, through all studied periods (Figure 8). According to the statistical results in Figure 8 and Figure 9, there is a significant difference in total length and length of ossified portion between the three measured bones of the femur, tibia, and metatarsus through studied periods. From figure 8, it is clear that the tibia is the longest among the measured bones, and that it significantly differs from both other bones in all studied periods, followed by the femur, and then the metatarsus, as there is no significant difference between these two bones at a significant level except in some periods, as shown in the figure.

The length means of the diaphysis ossified portion of the three measured bones yields the same results as the total length mean, indicating that the bone development in tibia diaphysis is the highest among the measured bones (Figure 9).

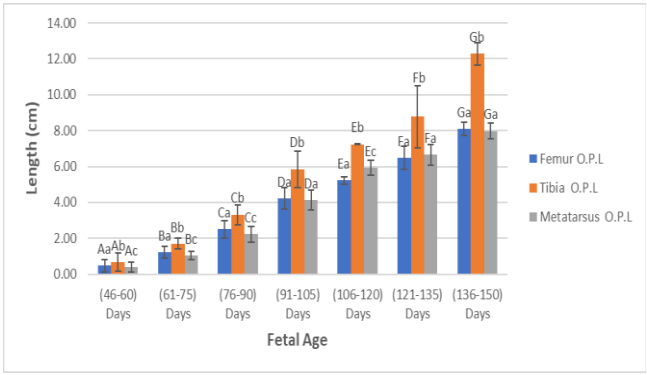

Figure 8: A histogram of the mean and standard deviations of total length of the three measured bones showing the significant differences within the same periods and among studied periods. A, B, C, D, E, F, G different letters among age groups indicate significant differences at P≤0.05. a, b, c different letters among bones in the same age indicate significant differences at P≤0.05.

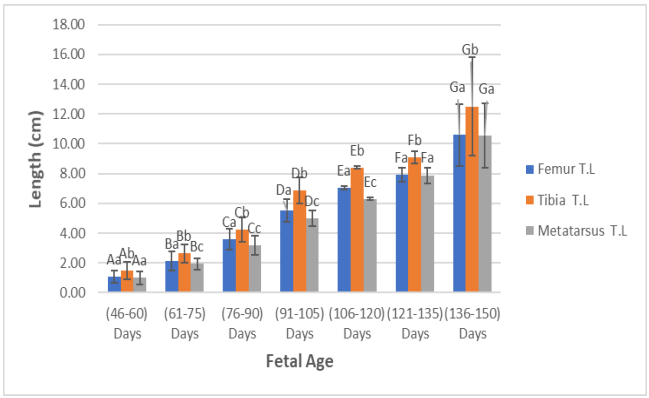

Figure 9: A histogram of the mean and standard deviations of length of diaphysis ossified portion length of the three measured bones showing the significant differences within the same periods and among studied periods. A, B, C, D, E, F, G different letters among age groups indicate significant differences at P≤0.05. a, b, c different letters among bones in the same age indicate significant differences at P≤0.05.

The statistical results reveal presence of high significant variance between the relative increment average in the total length of femur, tibia and metatarsus in the first studied period 46-60 days and the other next six studied fortnightly periods (Figure 10). The same results clearly noticed with the relative increase average in length of diaphysis ossified portion of the three measured bones (Figure 11).

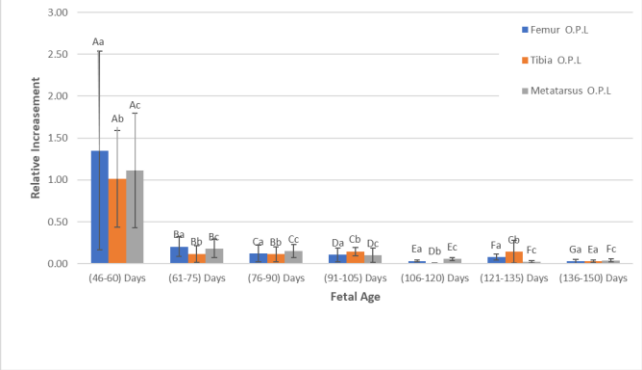

Figure 10: A histogram of the relative increment in the total length average of femur, tibia and metatarsus of Awasi sheep fetuses in seven successive fortnightly periods of gestation showing the significant differences within the same periods and among studied periods. A, B, C, D, E, F, G different letters among age groups indicate significant differences at P≤0.05. a, b, c different letters among bones in the same age indicate significant differences at P≤0.05.

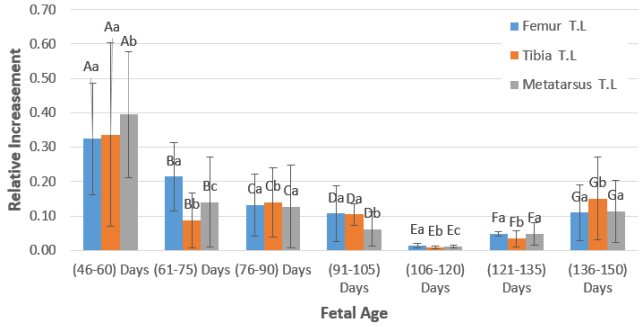

Figure 11: A histogram of the relative increment in the length average of diaphysis ossified portion of femur, tibia and metatarsus of Awasi sheep fetuses in seven successive fortnightly periods of gestation showing the significant differences within the same periods and among studied periods. A, B, C, D, E, F, G different letters among age groups indicate significant differences at P≤0.05. a, b, c different letters among bones in the same age indicate significant differences at P≤0.05.

Discussion

The primary ossification centers of the diaphysis of the long bones tibia and femur, which appeared at 45 and 46 days, respectively, are the first centers appeared in the hind limb of Awasi sheep fetuses, followed by the primary centers of the 3rd and 4th metatarsal bones, followed by the primary centers of the ilium bones, and finally the ischium bones. This is consistent with the results of Wenham (18) in terms of sequence, although the time of appearance differed by a few days .

It was clear in our findings, that the ossification centers of both tibia and femur appeared first, while the appearance was delayed for pelvic bones especially pupis, this variation could be explained from many points, first of all, it could be due to the differences in the celluar origin of these bones, tibia and femur originated from lateral plate mesenchyme while pelvic bones are originated from ectomesenchyme, secondly, the mechanism for bone formation of large bones is through endochodral ossification that is different from the intramembranous process for flat bones (19). An extra clarification for such differences in the time of development of ossification centers between long and flat bones is the fact that their cartilages don't replaced by bone as flat bones but by marrow and vascular tissue (20) Another explanation could be arising from the difference in the future physiological function and works of long bones that make them bearing the heaviest body weight and stress in compare to other bones especially pelvic bones.

Our findings are consistent with those reported by Majeed and Ahmed (10), who conducted a study on bone growth in the hind limb of local black goat fetuses and found that the primary ossification centers appeared in the diaphysis of long bones approximately in the same sequence, beginning with the tibia at an estimated age of 42 days, followed by the femur center at 43 days, and finally the center of the ilium bones at 44 days. The same sequence was also mentioned by Ahmed (7) and Majeed and Ahmed (8) in their studies on forelimb bone growth of local sheep and black goat fetuses. The diaphyses of the long bones radius and ulna, as well as the humerus, were the first to appear, followed by the scapula, metacarpus, phalanges, and finally the carpal bones appeared .

Duck embryo bones develop in a sequential pattern as well Salih (21) with the primary ossification center appearing in the diaphysis center of femur, tibia, and fibula on the eleventh day of incubation and the ossification process in the wing and limbs being completed on the 22nd day of incubation, with the exception of the carpal bones of the wing. The ossification process start in diaphysis of forelimb long bones of quail's embryo at 8th day of incubation (22). The onset of ossification in chick's embryo begin in forelimb before the hind limb begin to ossify, some signs of ossification of the tarsal and carpal bones appear to be delayed before hatching in some cases, in addition to presence of clearly increasing lengths in the limbs in accordance with the growth (23).

This research discovered that the first ossification centers of phalanges began to appear at the distal phalanx at 58 days, followed by the proximal phalanx, while the last primary

center in digit phalanges appeared in the middle phalanx at the end of the second month. All of these findings are in accordance with the topographical picture of sheep foot ossification described by Ahmed (7), according to which the ossification of the phalanges begins at the age of 50 to 56 days of prenatal development, progresses through to the proximal phalanx, and is completed by the middle phalanx. Ossification centers of tarsal bones first appeared in the calcaneus at 64 days, then in the talus at 77 days, then in the central and 4th tarsal bones at 96 days, then in third and second tarsal bones at 100 days, and finally in the first tarsal bone at 105 days, according to the findings .

Our findings are consistent with those of Majeed and Ahmed (9), who found that the ossification center of the talus bone appeared at 75 days of goat gestation, while the third tarsal bone began to ossify at 95 days, followed by the fourth with central tarsal bones that begin ossification at 96 days, and that by the age of 155 days, the ossification of the tarsal bones, which included the first and second tarsal bones, was complete.

These variations in the time of developing of ossification centres of tarsal bones that were documented in our data, could be due to the genetic background that control every cell activity during endochondral ossification (24). Another study mentioned that embryonic bone development including the geometric properties like medullary area and thickness and even the mechanical characters such as power to fracture and ultimate load is a completely and genetically programmed event that is modified by epigenetic factors (25).

The statistical analysis reveals a significant difference between the total length mean and the ossified portion mean of the diaphysis of the three measured bones within the same period and among studied periods for the three measured bones. The statistical results show that there is a high significant variance between the average of relative increment in total length and the average length of ossified portion of femur, tibia, and metatarsus in the first studied period 46-60 days in comparison with the other six studied next fortnightly periods. This result corroborated the findings of Ahmed (6) ,on her study on the development of the vertebral column in local sheep and black goat fetuses, as well as the findings of Ahmed (7), Majeed and Ahmed (8), on their studies on the ossification of fore limb bones in both indigenous sheep and black goat, and with Majeed and Ahmed (9) , on their study on the ossification of the hind limb bones. All of these studies show a high significant variance in the relative increase average at seventh and eighth weeks in comparison with the next successive weeks of gestation in both indigenous sheep and black goat fetuses.

Conclusions

Initiation of ossification begin in the mid diaphysis of long bones, in the middle of the hind limb of Awasi sheep fetuses, followed by extension in both proximal and distal directions. Bone development proportionally increased along the gestational age, and give high significant relative increase during the second half of the second month of gestation, so this period considers as critical period for bone development especially long bones so it is essential to avoid giving any medication or therapy during that time which could affect or even retard this vital process.

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Conflict of interest

Authors declared that there is no conflict of interests. regarding the publication of this manuscript.

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دراسة لمراكز التعظم الابتدائية في القوائم الخلفية لأجنة الأغنام العواسية بطريقة الصبغة المزدوجة المحورة والتصوير الشعاعي

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

الخلاصة

تم جمع أربعين جنيناً من أجنة الأغنام العواسية من بضعة مناطق من محافظة نينوى بالإضافة إلى مجزرة المدينة وتم تقدير أعمارها اعتماداً على الطول التاجي- المنبتي وعن طريق معادلة ريتشارد وتهدف الدراسة إلى تحديد موقع وموعد ظهور مراكز التعظم في عظام القائمة الخلفية لأجنة الأغنام العواسية باستخدام طريقة الصبغة المزدوجة المحورة أو باستخدام التصوير الشعاعي. كانت أول مراكز التعظم التي ظهرت في القائمة الخلفية لأجنة الأغنام العواسية هي مراكز التعظم الابتدائية لعظام القصبة والفخذ ومشط القدم والحرقفة ثم الورك وبعمر تقديري ٤٥، ٤٦، ٤٨، ٥٠ و ٥٢ يوماً وعلى التوالي. ظهرت مراكز تعظم سلاميات القدم بالتسلسل التالي: السلامية القاصية وتتبعها السلامية الدانية ثم السلامية الوسطى وبعمر تقديري ٥٨، ٦٠ و ٦٤ يوماً وعلى التوالي. بدأت مراكز التعظم في عظام رسغ القدم بالظهور في عظم العقب بعمر ٦٤ يوماً، وفي القنزعي بعمر ٧٧ يوماً، وفي العظمين المركزي والرابع بعمر ٩٦ يوماً، وفي العظمين الثالث والثاني بعمر ١٠٠ يوم، وأخيراً يظهر مركز تعظم العظم الرسغي الأول بعمر ١٠٥ يوماً. ولقد درست عظام الفخذ والقصبة ومشط القدم إحصائيا من خلال تتبع الزيادة النسبية للطول الكلي للعظام وطول الجزء المتعظم في محاور هذه العظام وأظهرت النتائج أن أقصى زيادة نسبية في طول العظم وفي طول المحور المتعظم للعظام الطويلة حدثت في الأسبوعين السابع والثامن من الحمل، مما يجعلها فترة حرجة للنمو العظمي وخاصة في العظام الطويلة، لذلك من الضروري تجنب إعطاء النعاج الحوامل أي مستحضرات دوائية تؤثر على سير هذه العملية الحيوية أو تتسبب في تأخيرها.

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