80 GÜNLÜK YABAN DOMUZU FÖTÜSLERİNDE ÖN VE ARKA EXTREMİTELERİN KEMİKLEŞME VE GELİŞİMLERİ

OSSIFICATION CENTERS IN THE BONE DRAFTS OF THE FORE- AND HIND- LIMBS OF 80 DAYS OLD FETAL SIBLINGS OF A WILD PIG

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ÖZET

GİRİŞ ve AMAÇ:: Normal kemik gelişim verileri kemik gelişim çalışmalarını yorumalamak ve açıklamak için vazgeçilmez parametrelerdir. Bu çalışmada 80 günlük yaban domuzu fetüslerine ait 32 ekstremite kullanılmış, extremitelerinin normal kemik gelişiminin ortaya çıkartılması amaçlanmıştır.
YÖNTEM ve GEREÇLER: Materyaller % 5'lik formaldehit solusyonunda tespit edildikten ve % 95'lik etanolde bekletildikten sonra saf aseton ile muamele edildi. Materyaller alizarin red alcian blue kombinasyonu ile boyandı. Bu boyama tekniği kıkırdak dokuları maviye, kemik dokuları ise kırmızıya boyadı. Bu teknik gelişen kıkırdak dokuları ve kemiklerdeki erken gelişen kemikleşme merkezlerinin lokalizasyonunu gösterdi. Stereomikroskop altında kıkırdaklaşma ve kemikleşme zamanları dikkatle izlendi.
BULGULAR: Büyük kemiklerin herbirinin bir adet primer kemikleşme merkezine (POC) sahip olduğu gözlendi. Ossa carpi, ossa tarsi ve patella dışındaki tüm kemiklerde gövdedeki kemikleşmenin görüldüğü belirlendi. Görülen kıkırdak modellerin gelişmesini tamamlamış olanlara benzediği saptandı.
TARTIŞMA ve SONUÇ: Sadece radius'a ait bir örnekte distal kısımda sekonder kemikleşme merkezi (SOC) belirlendi.

Anahtar Kelimeler: *Fötüs, kemik, kemikleşme, yaban domuzu*

SUMMARY

OBJECTIVE: Having information about the normal skeletogenous stages is essential in order to comment on experimental bone development studies. With this regard, we aimed at revealing the normal ossification status of the 32 extremities of the 80 days old wild pig fetuses.
METHODS: The materials were fixed in 5% formaldehyde solution and 95% ethanol, followed by pure acetone incubation. Then, samples were stained with an alcian blue -alizarin red combination. As a result of the staining procedure, the cartilaginous tissue was stained in blue and the osseous tissue was stained in red. By using this method, it is possible to display the development of the cartilaginous components and localization of the early centers of the ossification areas in the bones. Chondrification and ossification of the bones were observed attentively under a stereo microscopy.
RESULTS: Each big bone has only one primary ossification body center (POC). The ossification centers in all bones have been shown whereas there are no ossification centers in the carpal, tarsal bones and patella. The extremities with the blue stained cartilaginous and red osseous tissues have resembled to the gross structural shapes of the mature stages.
DISCUSSION AND CONCLUSION: Only one secondary ossification center (SOC) was observed in the distal part of radius of one specimen.

Keywords: *Bone, fetal, ossification, wild pig*

INTRODUCTION

In mammals, intramembranous ossification occurs in both long and short bones while intracartilaginous ossification takes place in flat bones[1,2]. Ossification begins at the embryonic stage, and continues throughout the postnatal life. Primary ossification centers (POC), which form the diaphysis of the bone, appear first at the gestational stage while secondary ossification centers (SOC), which may be one or more, are seen in the epiphysis of long bones after the birth. This cartilage mass, which is very essential for the longitudinal bone growth, ossifies in time and complete the closure of the cartilage in long bones[1].

It is important to know about the normal development and ossification stages of bones in order to diagnose and treatment of the intrauterine anomalies, development deterioration, and genetic bone tissue diseases[3,4,5,6]. Several techniques including single and double staining techniques, radiography, ultrasonography, MR, and various histological staining methods are being performed to visualize ossification stages. Particularly, double staining techniques have been successfully used in experimental studies of the bone development of fishes, birds, mice, rabbits and reptiles[3,7,8] . On the other hand, studies performed on the bone development of the pig, which has been recently used increasingly as an experimental model, seem to be limited. However, radiological and histological techniques have been commonly used in studies performed especially with domestic pigs[9] . Therefore, we aimed to apply alizarin red and alcian blue double staining technique in order to reveal the ossification stages via visualizing ossification centers in the fore- and hind- limbs of approximately 80 days old fetal siblings of a wild pig. This technique possesses affirmative advantages in terms of depicting the details of ossification centers.

MATERIAL AND METHOD

The fore- and hind- limbs of 8 fetal siblings of a wild pig, which were hunted by villagers, were included in this study. Their ages were determined by the use of Crown-rump length (CRL) measurements as suggested by the studies of Evans, Sack[10] and Henry[11] . They were approximately 80 days old and 160-210 mm long. The samples obtained from the pigs were kept in 10% formaldehyde solution and washed with distilled water. Then they were stored in containers filled with 95% ethanol.

In order to observe the mineralization stages, the extremities were stained in a final solution of alcian blue (300 mg alcian blue and 100 ml 70% ethanol) and alizarin red (100 mg alizarin red and 100 ml 95% ethanol) which was prepared by adding 100 ml glacial acetic acid and 1700 ml 70% ethanol. The extremities were incubated in a mixed staining solution for four days at 40 °C in an oven and, then they were washed for 2 hours under the running water. Upon the washing process, they were stored in a container including 2% KOH. Furthermore, extremities were cleaned by 20% glycerin and 1% KOH and they were incubated in 50% and 80% glycerin for 7 days. Finally, they were kept in 100% glycerin solution. A digital caliper was used to measure the cartilage drafts of the bones and the ossified parts. Dead material was used so no ethics committee approval was received.

This staining procedure has been further modified by adapting the staining protocol which was previously developed[3,12].

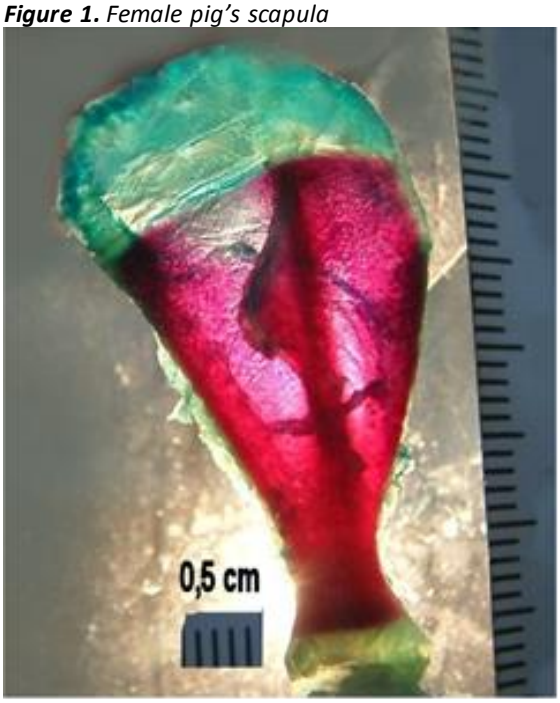
RESULTS

Forelimbs

Scapula (Fig.1)

Scapula was mostly mineralized, which indicated the presence of the POC. However, no SOC was observed at this stage. The boundary of the cartilage of the scapula was clearly observed. The spine of the scapula showed its characteristic resemblance as it can be seen in the mature stage and its edge was cartilage.

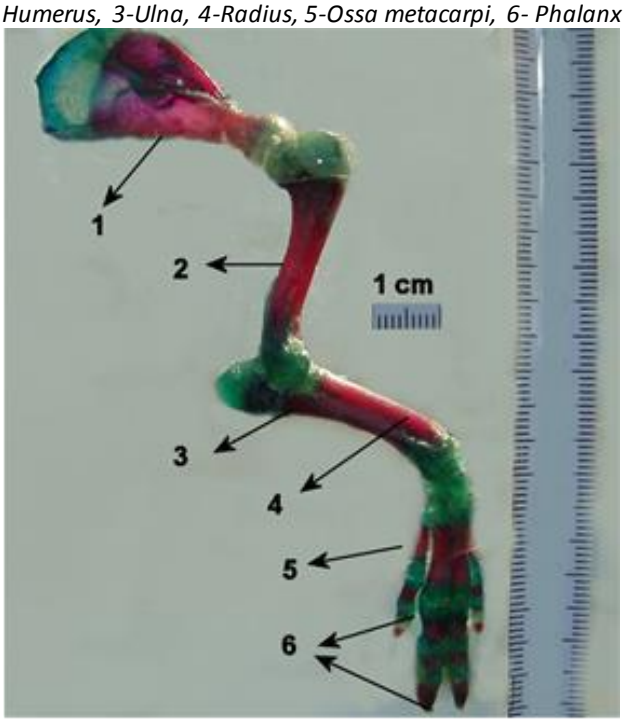
Figure 1. *Female pig's scapula*



3.1.2. Humerus (Fig.2.2)

In each fetus, the POC of the humerus was present and structurally forming the draft body of the bone. However, no SOC was observed at both edges.

***Figure 2**. Forelimb of male wild pig. 1- Scapula, 2- Humerus, 3-Ulna, 4-Radius, 5-Ossa metacarpi, 6- Phalanx*



3.1.3. Antebrachium (Fig.2.3-4)

In each of the fetuses, each POC was visible in the bodies of the radius (Fig.2.4) and ulna (Fig.2.3), respectively. The intensity of the mineralization and the extension was observed much more in the radius. There was no fusion between the two bone body models due to the ossification extension. In one of the fetuses, there was only one SOC located in the distal edge of the radius.

3.1.4. Ossa Carpi

Cartilage models were present in all the carpal bones (Fig.3.3); but, the ossification was determined in only certain ones. A small POC was observed in each of the radial carpal, intermedial carpal as well as ulnar carpal bone drafts. A wider POC was also indicated in the quartal carpal bone draft of the lower row.

3.1.5. Ossa Metacarpi (Fig.3.4)

A POC was displayed in each of the metacarpal II, III, IV, and V. Those, present in the metacarpal III and IV, were larger; but, there was no fusion.

3.1.6. Phalanx (Fig.2.6; Fig.3.5)

Each of the phalanges had a POC and they showed similar pattern with the phalanges of the hind limbs. A POC was present in the entire phalangeal drafts. The drafts of the 3rd and 4th phalanges were wider compared to the 2nd and 5th ones.

Measurements of forelimbs of the female and male fetuses are displayed in Table 1.

***Figure 3.** Forelimb of female wild pig. 1-Radius, 2- Seconder ossofication center of radius, 3- Cartilage draft of ossa carpi, 4- Ossa metatarsi, 5- Phalanx*

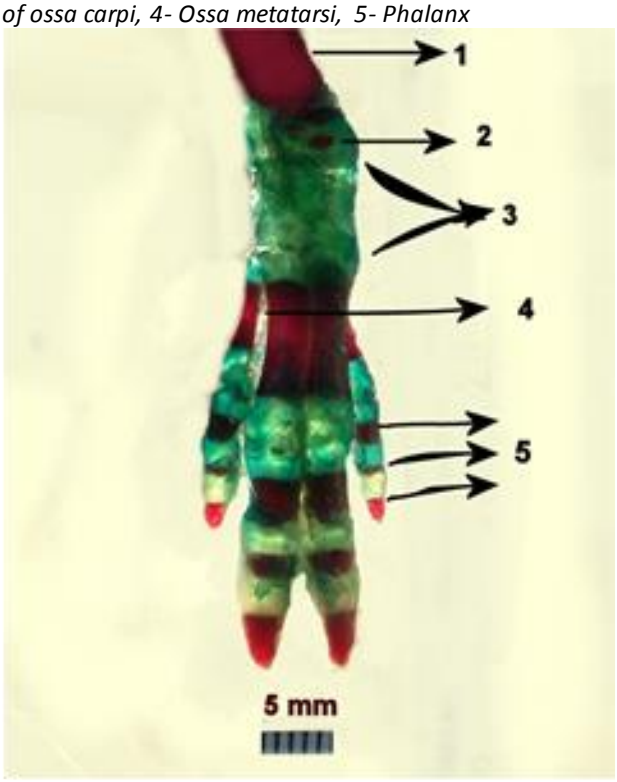
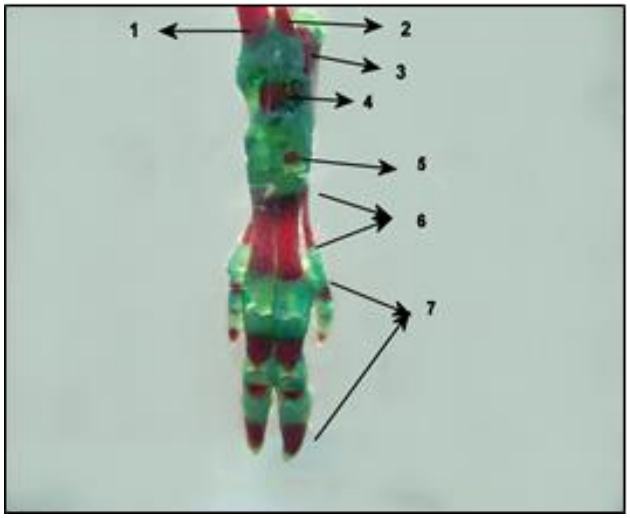


***Figure 4.** Hindlimb of male wild pig. 1. Tibia, 2. Fibula, 3- Talus, 4- Calcaneus, 5- Ossification center of os tarsale quartum (os cuboideum), 6- Ossa metatarsi, 7- Phalanx*



3.2. Hindlimbs

3.2.1. Femur

Body of the femur was mostly mineralized and the POC was clearly observed. However, there was no SOC determined. The bone draft of the patella was completely in cartilage structure.

3.2.2. Ossa Cruris:

Each tibia of the cadavers possessed a distinct POC; thus, it did not have SOC. Likewise, each fibula had a clearly observed POC.

3.2.3. Ossa Tarsi:

Os Tarsi Fibulare (Calcaneus): Each fibular tarsal bone draft had a POC which resembled to the fully developed bone.

Os Tarsi Tibiale (Talus): Each tibial tarsal bone draft consisted of POC.

Os Tarsale Quartum:POC was observed in the quartal tarsal bone draft in only one of the cadavers.

3.2.4. Os Metatarsale:

POC was displayed in the 2nd, 3rd, 4th, and 5th metatarsal bone drafts of all the samples.

3.2.5. Phalanges:

Each of the phalanges had POC and they showed similar pattern with the phalanges of the forelimbs.

Measurements taken from the hind limbs of the female and male fetuses are displayed in Table 2.

Table1. *Mean lengths of the mineralized portion of the bone drafts in the forelimbs of fetuses*

Forelimb bone drafts	*Mean mineralized length, female♀, mm*	*Mean mineralized length, male♂, mm*
Scapula	*23.78*	*24,99*
Humerus	*22.85*	*24,21*
Radius	*17.86*	*18,48*
Ulna	*21.31*	*23,52*
Osmetacarpale III	*7.9*	*8,13*
Osmetacarpale IV	*8.08*	*9,86*

Table 2. *Mean lengths of the mineralized portion of the bone drafts in the hind limbs of fetuses*

Forelimb bone drafts	*Mean mineralized length, female♀, mm*	*Mean mineralized length, male♂, mm*
Femur	*23,66*	*24,81*
Tibia	*25,66*	*25,73*
Fibula	*22,28*	*23,65*
Osmetatarsale III	*9,18*	*9,37*
Osmetatarsale IV	*9,58*	*9,75*

DISCUSSION

Alizarin red and alcian blue double staining technique has frequently been used in studies performed on bone development and skeletal anomalies[1,2,9] . Moreover, histological techniques have also been used to validate the results of the studies evaluating ossification centers[13,14,15]. It has been stated that radiological imaging techniques, which are used to determine the ossification centers during the development, reveal indefinite results particularly about SOC. Even though having also been employed to determine ossification centers at developmental stage, radiological visualization techniques have been suggested to give indefinite results

particularly on SOC[13] . Therefore, double staining histological techniques have also been applied for the final validation of the ossification centers, as it was employed in this study. The results acquired hereby have confirmed this idea.

In a study performed with the elbow joint, the SOCs were observed in all of the humeral and radial bones of one day old domestic boars[14], However, in this study there was no SOC in these bones of the 80 days old fetuses. On the other hand, it is important to specify that only one SOC was observed in the distal edge of the radius of a female fetus.

In a study[15] performed with domestic boar fetuses, the pregnancy ages were determined via measuring the length of the femur by using the MR technology. Three fetuses were less than 40 days old (the femur lengths were shorter than 6 mm), seven fetuses were 40-80 days old (the femur lengths were between 6-20 mm), and five fetuses were 80-100 days old (the femur lengths were between20-32 mm). Moreover, studies have revealed that the ossification center of the femur was in circle shape in the end of the second phase of the pregnancy while it was in semi-circle shape at the last period of the pregnancy[14,15]. In our study, the average length of the femur was 23 mm in male and 24 mm female fetuses which indicated that they were between 80-100 days old fetuses. Our results were consistent with the findings of the literature.

In a radiological study performed by Paul C. Hodges[14] fetuses with the 35 mm CRL possessed pronounced POC in the humerus. POCs of the scapula, radius and ulna have already been seen in the fetuses with the 40 mm CRL. However, POCs of II., III., IV., and V. metacarpals were observed in the fetuses with 58 mm and 73 mm CRL lengths, respectively. POCs of III.-IV. proximal and distal phalanges were also detected in approximately 58-65 mm long fetuses. The shape of the II-V. distal phalanges were observed in 58 mm long fetuses while it had a distinct shape in 85 mm long ones. Likewise, the POCs of the II. and IV. medial and II.-V. proximal phalanges were seen in 82 mm and 100 mm long fetuses. The findings of our study showed a great consistency with the literature summarized above.

In a study, SOC was clearly marked in the distal edge of the humerus of fetuses with the length of 180-250 mm[14] . On the other hand, we did not observe SOC in the 160-200 mm long fetuses. There can be variances between studies since a variety of several factors may affect the growing period of the ossification centers[3,4,6]. Hodges (Paul & Hodges, 1953) has revealed the existence of the SOC in the distal edge of the radius in 180 mm and 220 mm long fetuses. Likewise, we detected an oval shaped SOC in the distal edge of the radius in female fetus with 210 mm in length. Our findings are consistent with the study performed by Paul & Hodges[9] which also verified the CRL.

In the same study, Paul & Hodges[9] has also reported the presence of the POCs in the cartilages of the radiocarpal, intermediocarpal and ulnocarpal bones in 210 mm long fetuses. Similarly, hawse also detected tiny ossification centers on the upper sequence of the cartilages of the carpal bones. They measured the lengths of the POCs in the bony cartilages of the hind limbs as follows; femur 35 mm, tibia 38 mm, fibula 38 to 53 mm. They have also shown ossification areas in metatarsale III-V and phalanx distale III-IV in 58 mm and 88 mm long fetuses. In our study, we indicated the presence of the POCs in metatarsal II-V and proximal phalanges of the III and IV digits at the same period. The POCs of the medial phalanx of the III and IV digits has been observed at 86 mm long fetuses. Finally, we showed the SOC in the distal edge of the femur of the 180 mm long fetuses. On the other hand, no SOC has been detected even in the 210 mm long fetuses. The SOC is normally present at the proximal edge of the tibia in 230 mm long fetuses[9]. However, it has not been observed in our study. Likewise, the SOCs is observed in the fibula and metatarsal III and IV at 240 mm long fetuses which was not the case in our study. However, we showed the POC of the fibulotarsal bony cartilage in 100 mm and 230 mm long fetuses.

All in all, or study which was performed to reveal the ossification centers in the fetal siblings of a wild pig has matched mostly with studies done on the domestic pigs. Conclusively, it is important to indicate that various factors including dietary habits, hormones, environmental effects and individual differences affect the ossification.

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