HARRAN ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

Development Status of The Hindlimb Extremity Bones of The Watchdog Hybrid Fetus (40 Days Old)

Şükrü Hakan ATALGIN[1,a], Mustafa KORKMAZ[1,b,*], Kerem CABAS[2,c]

[1]Department of Anatomy, Faculty of Veterinary Medicine, Balıkesir University, Balıkesir, Türkiye.

[2]Ilgı Veterinary Clinic, Balıkesir, Türkiye.

[a]ORCİD: 0000-0001-9436-6270

[b]ORCID: 0000-0002-4493-0643

[c]ORCID: 0009-0004-0779-4656

Received: 22.08.2024

Accepted: 18.03.2025

How to cite this article: Atalgin ŞH, Korkmaz M, Cabas K. (2025). Development Status of The Hindlimb Extremity Bones of The Watchdog Hybrid Fetus (40 Days Old). Harran Üniversitesi Veteriner Fakültesi Dergisi, 14(1): 1-05. DOI:10.31196/huvfd.1537265.

***Correspondence:** Mustafa Korkmaz

Department of Anatomy, Faculty of Veterinary Medicine, Balıkesir University, Balıkesir, Türkiye.

e-mail: sukruhakan@balikesir.edu.tr

Available on-line at: https://dergipark.org.tr/tr/pub/huvfd

Abstract: Hindlimb extremity bones of 40-day-old watchdog hybrid fetuses were examined. Knowing the normal formation of the extremities is important for understanding possible disorders and their treatment. The developmental processes of the movement system do not occur as sequentially as expected. Alizarin red and alcian according to Inouye technique bones of 40-day-old animals were stained with alcian blue. Dissections of bones preserved in appropriate solutions were performed. Stereomicroscopic and normal photographs were taken with a digital camera. Measurements were taken from the dissected legs with a 150 mm Mitutoyo brand caliper. Hindlimb bones of 40-day-old guard dog hybrid fetuses were observed to have primary ossification centers, while some bones had no ossification centers. A primary ossification center was observed in the corpus of the femur, tibia, fibula, and metatarsus bones of the hind limb, while no ossification center was observed in the proximal and distal ends. Also, patella, ossa tarsi, and ossa digitorum pedis no ossification centers were observed.

Keywords: *Bone, Fetus, Hind limb, Watchdog hybrid.*

Bekçi Köpeği Melezi Fetüsünün (40 Günlük) Arka Ekstremite Kemiklerinin Gelişim Durumu

Özet: 40 günlük bekçi köpeği melezi fetüslerinin arka bacak ekstremite kemikleri incelendi. Ekstremitelerin normal oluşumunu bilmek, olası bozuklukları ve tedavilerini anlamak için önemlidir. Hareket sisteminin gelişim süreçleri beklendiği gibi sırayla gerçekleşmez. Inouye tekniğine göre 40 günlük hayvanların kemikleri alizarin red ve alcian blue ile boyandı. Uygun solüsyonlarda saklanan kemiklerin diseksiyonları yapıldı. Stereomikroskop ve dijital kamera ile fotoğrafları çekildi. Diseke edilen bacaklardan 150 mm'lik Mitutoyo marka kumpas ile ölçümler alındı. 40 günlük bekçi köpeği melez fetüslerinin arka bacak kemiklerinde birincil kemikleşme merkezleri gözlenirken, bazı kemiklerde hiç kemikleşme merkezi olmadığı görüldü. Arka ekstremite kemiklerinden femur, tibia, fibula ve metatarsus kemiklerinin gövdelerinde birer tane primer ossifikasyon merkezi gözlenirken, proksimal ve distal uçlarda ossifikasyon merkezi gözlenmedi. Ayrıca, patella, ossa tarsi ve ossa digitorum pedis'te ossifikasyon merkezi gözlenmedi.

Anahtar Kelimeler: *Arka ekstremite, Fetus, Kemik, Bekçi köpeği melezi.*

Introduction

Understanding the typical development of the foot is crucial for identifying potential disorders and their treatment. The development of the movement system doesn't follow a strictly sequential pattern as one might expect. That's why it should be investigated. Most of the mammalian skeleton, except the skull bones, is formed by endochondral ossification, which is the replacement of cartilage by bone tissue (Wang et al., 2023; Zhang et al., 2011). The vertebral column, pelvis, and extremity bones are formed by endochondral ossification. In this type of bone formation, cartilage tissue forms first and is eventually replaced by bone tissue (Ko and Sumner, 2021; Serra-Vinardell et al., 2020). The first sign of endochondral is a local expansion of bone formation with hypertrophy of the chondrocyte in the middle (diaphysis) of the cartilage model in the long bone (Kume et al., 2012). A miniature model is formed from hyaline cartilage, followed by bone tissue replacing the cartilage model. Until the bones take their final shape, new bone is made on one hand, and a part of the bone is destroyed (remodeling). On the other hand, in the cartilage model the diaphyseal region, the mesenchyme cells in the inner layer of the perichondrium covering the cartilage divide and differentiate into osteoprogenitor cells, which in turn differentiate into osteoblasts (Stevens, 2008). A cylindrical bone cuff is formed in the diaphysis of the cartilage model by intramembranous ossification. The membrane surrounding the bone cuff is now called periosteum (Topaloğlu et al., 2017). Ossification begins in the embryonic stage and continues throughout postnatal life. While primary ossification centers (POC), which form the diaphysis of the bone, first appear during the pregnancy phase, secondary ossification centers (SOC), which may be one or more, appear in the epiphysis of long bones after birth (Getty, 1975; Song, 2022). Ossification in long bones begins towards the end of the embryonal period. First, diaphyses are formed from the POC. In humans, the first foci of ossification of all bones become visible in the 12th week. Ossification is observed in the clavicle before all bones in the mammals' body, followed by the femur (Arıncı and Elhan, 2001; Williams and Dyson, 1989).

The part that ossifies from the second ossification center is the epiphysis of the bone. The femur from the second ossification center tibia with its distal the proximal part forms in the 9th month of intrauterine life in humans. Although most of the second ossification centers are formed after birth, the first ossification center is present at birth (Arıncı and Elhan, 2001).

The diaphysis formed from the first ossification center does not immediately fuse with the epiphysis formed from the second ossification center. This union does not occur until the normal bone dimensions of an adult person are reached. During bone growth, there is a cartilage growth plate between diaphysis and epiphysis. (Doğuer and Erençin, 1962; Williams and Dyson, 1989).

It is important to know the normal development and ossification stages of bones for the diagnosis and treatment of intrauterine anomalies, developmental disorders, and genetic bone tissue diseases (Barone, 1986; Dyce et al., 1987; Atalgın and Çakır, 2006). The formation of the lower extremities begins with bud-like formations of the lower lateral coccyx wall in the late embryonic stages (Gardner et al., 1959). These regions also depend on the variation of elongation and adjustment of the spatial position of the bones, and they do not show a constant growth mode. The relationships between specific foot regions also change considerably throughout prenatal life. Unlike many other body regions, the human foot must undergo several important changes during the fetal period due to its limited and highly specialized functions in postpartum life (Debrunner and Jacob, 1998; Pisani, 1998). The medial foot shows a size maximum relative to other foot regions and the medial foot grows faster than the lateral one (Gruber et al., 2001).

Gruber et al. (2001) state that the human fetal foot has an irregular growth mode and that growth priorities within the foot skeleton vary with age and region. Although the growth of the fetal foot skeleton is irregular, it is not unconnected. The result of this peculiar growth mode is to form the foot and is therefore functionally directed toward its specific purposes. In postnatal life, research has shown that various aspects of the human lower limb undergo irregular changes, influenced by specific growth-priority areas that correspond to different age groups (Schilling, 1985). Bone is very sensitive to external stimuli (Rogers et al., 2021). Increasing knowledge about bone development and bone repair has important therapeutic implications for the treatment of bone disease and aging-associated degeneration (Salhotra et al., 2020).

Nowadays, various techniques are applied to visualize the ossification stages, including single and double staining techniques, radiography, ultrasonography, MRI, and various histological staining methods. Especially double staining techniques give successful results in experimental studies on bone development of animals (Atalgın and Çakır, 2006; Atalgın et al., 2007; Atalgın and Kürtül, 2009). Therefore, we aimed to apply the alizarin red and alcian blue double staining technique to reveal the ossification stages by imaging the hind limb ossification centers of fetuses of an approximately 40-day-old watchdog hybrid.

Materials and Methods

In this study, the hind limbs of 40-day-old watchdog hybrid fetuses were examined. Four dead fetuses were used as research material. Their ages were confirmed using crown rump length (CRL) measurements suggested in the work of Evans and Sack (1973). It was found to be approximately 40 days old and 100 mm long. Samples obtained from the watchdog were kept in 10% formaldehyde solution and washed with distilled water. They were then stored in containers filled with 95% ethanol.

To observe the mineralization stages, the materials were stained in a final solution containing alcian blue (300 mg alcian blue and 100 ml 70% ethanol) and alizarin red (100 mg alizarin red and 100 ml 95% ethanol). This solution was prepared by adding 100 ml glacial acetic acid and 1700 ml 70% ethanol. The hind limbs were placed in mixed staining solution in an etuve at 40 °C for four days and then washed under running water for 2 hours. After washing, they were stored in a container containing 2% KOH. Additionally, the materials were cleaned with 20% glycerin and 1% KOH and stored in 50% and 80% glycerin for 7 days. Finally, they were preserved in 100% glycerin solution. A digital caliper was used to measure the cartilaginous outlines and ossified parts of the bones. Since dead material is used, ethics committee approval is not required.

Results

Femur: The total length of the femur was measured as 12 mm, its body was mostly ossified, and primary ossification centers (POC) were observed in the diaphyseal region. The center of primary ossification measured 5,8 mm. However, no secondary ossification center (SOC) was observed in the epiphyseal region (Figure 1.).

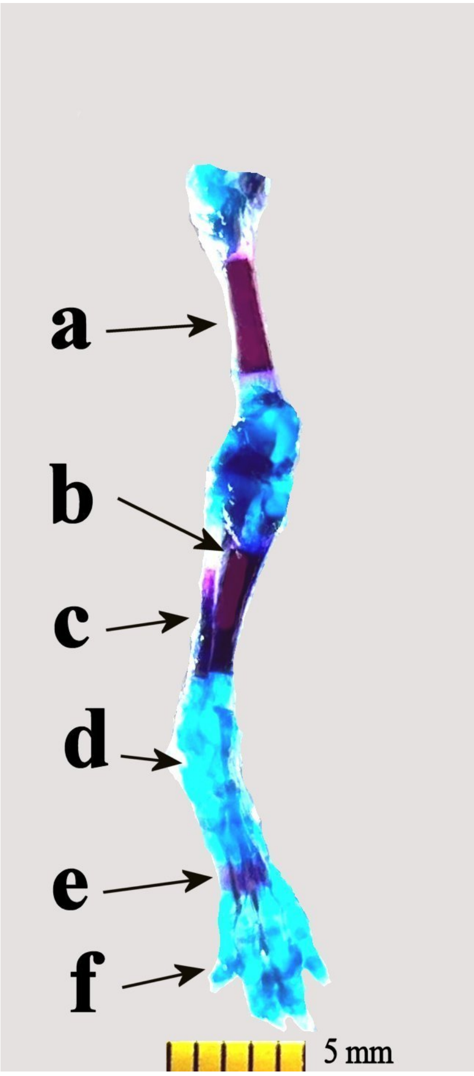

Figure 1. Appearance of the hind limb bones of a 40-day-old watchdog stained according to the Inouye technique. a) Femur b) Tibia c) Fibula d) Ossa tarsi e) Metatarsus f) Phalanx.

Patella: The bone model is entirely cartilaginous (Figure 1.).

Ossa cruris: The total length of the tibia was calculated to be 9,3 mm. The fibula, which is also close in size, measured 12 mm, and while the diaphyseal part of both bones had primary ossification centers, no secondary ossification centers were observed in the epiphyseal parts. While the POC in the fibula had a length of 5 mm, the POC in the tibia was found to be 5.8 mm long (Figure 1.).

Ossa tarsi: The total length of the ossa tarsi was measured as 5 mm. Os tarsi fibulare, os tarsi tibiale (talus) and os tarsale no POC or SOC were found in the quartum bones. The bone model is entirely cartilaginous (Figure 1.).

Os metatarsale: Ossa the total length of the metatarsale was measured 5 mm, and the POC was calculated to be 1 mm long in total. Neither primary nor secondary ossification centers were found in the bone models (Figure 1. and Figure 2.).

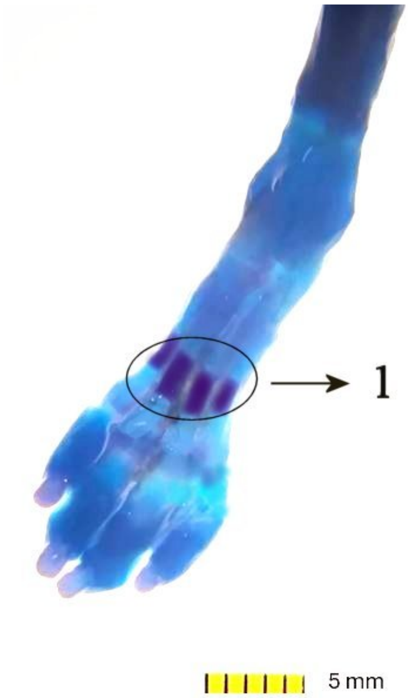

Figure 2. Appearance of the metatarsus of a 40-day-old watchdog stained according to the Inouye technique. 1)Metatarsus

Phalanx: the phalanx was determined 4 mm. Phalanx proximalis, medialis and distalis no POC or SOC was found in the medialis (Figure 1.).

Discussion and Conclusion

Generally, in domestic mammals, the femur consists of one primer ossification center and four secondary ossification centers. The first ossification in the femur occurs in the corpus in intrauterine life. It has been reported that ossification at the distal end begins earlier than the proximal end (Arıncı and Elhan, 2001; Barone, 1986; Willams and Dyson, 1989).

The earliest of these centers is diaphysis; It is observed that it is observed on the 60th or 70th days of intrauterine life. Generally, our findings in 40-day-old fetuses are consistent with this study.

The femur in dogs develops from five ossification centers (Hare, 1961). The distal end of the femur develops from a single center. According to literature corpus ossis femoris, is formed at birth, the ossification center of the caput ossis femoris was observed in the 1st, 2nd or 3rd weeks after birth (Chapman, 1965). Our findings in 40-day-old fetuses are consistent with this study.

In dogs, the ossification centers united at the proximal end merge with the corpus of the femur at 30-36 months after birth. He reported that the ossification center at the distal end merges with the corpus in the 30th to 37th weeks (Chapman, 1965). In the study, no secondary ossification center was observed in 40-day-old fetuses.

In dogs, the ossification centers joined at the proximal end merge with the body at 30-36 months after birth. He reported that the ossification center at the distal end merges with the corpus of the femur in the 30th to 37th weeks (Chapman, 1965). The observation of only the POC in 40-day-old fetuses is compatible with these data.

Atalgın and Çakır (2006) reported that in newborn rabbits, the patella was observed to be oval and had a cartilage outline without an ossification center. In the study, it was observed in the form of cartilage in the patella too, and no growth centers were observed.

The tibia generally develops from 4 ossification centers (Getty, 1975), one of these centers forms the diaphysis, the other two form the proximal end, and the other forms the distal end (Barone, 1986). In the same literature, it was reported that this center was seen in the 2nd month after birth in carnivora (Barone, 1986). He stated that ossification at the distal end occurred on the 25th day in dogs (Chapman, 1965). But Hare (1961) reported that the tibia in dogs ossifies in 5 centers. In the same literature, it is stated that the diaphysis is well-developed at birth (Hare, 1961).

Ossification at the distal end is observed a little later than the proximal end, at the 1st month in carnivora. This center is seen at the earliest in the 2nd month of carnivora. According to Barone (1986), the closure of the growth cartilage occurs at the distal end towards the 10th month in dogs (Barone, 1986). Generally, the growth cartilage closes around the 8th month. At the proximal end, the closure of the growth cartilage occurs a little later.

The study conducted is in accordance with (Baron, 1986; Chapman, 1965). In this study, while primary ossification centers were observed in the diaphysis region of the tibia, no growth centers were found in the proximal and distal epiphyseal regions.

The fibula in domestic mammals develops from three centers (Barone, 1986; Chapman, 1965). When the ossification center of the tibia is seen, the corpus fibula is observed at approximately the same time (Barone,1986). Being the same size in the same period in our study shows that it is compatible with our data.

In dogs, the ossification center of diaphysis is well-developed at birth. In the same literature, it was reported that the ossification center at the proximal end was observed in the 2nd and 3rd months, while the centers at the distal end were observed in the 4th, 5th, 6th and 7th weeks. It was stated by (Barone, 1986; Chapman, 1965) that in carnivora, the distal epiphysis closes at the end of the 2nd month, while the proximal epiphysis closes later than this. The study conducted is in accordance with Chapman (1965), and Baron (1986).

In dogs, tarsus bones develop from a single ossification center, except for the calcaneus. One of these centers forms the body of the calcaneus, the other the tuber calcanei. He reported that the tarsal bones except the calcaneus showed the POC in the 2nd, 3rd, 4th and 5th weeks, and that the ossification center of all tarsal bones was seen at the end of 2 months. The study was not in accordance with Hare (1961). The reason for this situation is that the dog fetus used in the study was approximately 5.5 weeks old and no growth center was detected in the tarsal bones.

It has been stated that there are two ossification centers in dogs; one of these centers forms the corpus metatarsale, while the other forms the distal end (Chapman, 1965; Hare, 1961).

The fact that the dog fetus in the study was approximately 5.5 weeks old and no growth centers were detected in the tarsal bones contradicts Hare's findings, which are thought to be due to environmental effects, nutrition, and genetic factors.

Phalanxes develop from two ossification centers each. One of them is at the diaphysis and the other is at the distal end. The ossification center in diaphysis is present at birth in dogs (Hare, 1961). Secondary ossification centers at the proximal end are seen on the 30th day in dogs (Chapman, 1965; Hare, 1961). Only primary ossification centers were observed in the study.

These areas are also influenced by the differences in bone elongation and the adjustment of their spatial positioning, and they do not follow a consistent growth pattern. The connections between specific regions of the foot also undergo significant changes throughout prenatal development. The fact that the dog fetus in the study was approximately 5.5 weeks old and no growth centers were detected in the tarsal bones contradicts Hare's (1961) findings, which is thought to be due to environmental effects, nutrition and genetic factors.

As a result, a POC was formed and measured in the hind limb bones of the fetuses of the 40-day-old watchdog hybrid. No SOC has been formed. The data obtained were compared with existing literature data and contributed to the lack of literature on the subject. To date, the causes of many skeletal abnormalities remain unknown, leading to largely empirical approaches in the treatment of foot deformities. The goal of our study is to create a foundation for a morphologically accurate and standardized treatment approach.

Ethical Approval

This study is not subject to HADYEK permission in accordance with Article 8 (k) of the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees". (In this case, the "Ethics Statement Form" or

"Informed Consent Form" must be filled in, signed by all authors and uploaded to the system.)

(Note: If animals were used in the study, the research should be approved by the ethics committee and the relevant document should be uploaded to the system. For studies that do not use animals, but that were carried out by collecting data, the permission document obtained from the relevant institutions and organizations, indicating that they obtained the data, information and documents within the framework of academic and ethical rules, "Informed Consent Form". or "Ethics Declaration Form" must be filled in and uploaded to the system.)

Similarity Rate

We declare that the similarity rate of the article is 12% as stated in the report uploaded to the system.

Conflict of Interest

The authors stated that they did not have anyreal, potential or perceived conflict of interest.

Author Contributions

Motivation / Concept: ŞHA
Design: ŞHA, MK
Control/Supervision: ŞHA
Data Collection and/or Processing: ŞHA, MK, KC
Analysis and / or Interpretation: ŞHA, MK
Literature Review ŞHA, MK, KC
Writing the Article: ŞHA, MK, KC
Critical Review: ŞHA, MK,

References

Arıncı K, Elhan A, 2001: Kemikler, Eklemler, Kaslar, İç organlar Ders Kitabı. Güneş Kitabevi. Ankara. 1-30.

Atalgın H, Kürtül İ, Bozkurt EU, 2007: Postnatal osteological development of hyoid bone in the New Zealand White Rabbit. *Vet Res Commun*, 31, 653-.660.

Atalgın ŞH, Çakır A, 2006: Postnatal osteological develeopment of the os coxae and femur of the New Zealand rabbit (Oryctolagus cuniculus L.). *Vet J Ankara Univ*, 53 (3), 155-159

Atalgın ŞH, Kürtül I, 2009: A morphological study of skeletal development in Turkey during the prehatching stage. *Anat Histol Embryol,* 38, 23-30.

Barone R, 1986: Anatomie Comparee Des Mammiferes Domestiques. Osteologie Vigot freres. Paris. 542-589.

Chapman WL, 1965: Appearance of ossication centers and epiphysial closures as determined by radyographic techniques. *J.A.V.M.A*, 147, 138-141.

Debrunner HU, Jacob HAC, 1998: Biomechanik des FuBes. *Beih Z Orthop*, 49-55.

Doğuer S, Erençin Z, 1962: Evcil Hayvanların Komparativ Osteolojisi. Ankara Üniversitesi Basımevi, Ankara.

Dyce KM, Sack WD, Wensing CJG, 1987: Textbook of Veterinary Anatomy, WB; pp. 67-76, Saunders Company, London.

Evans HE, Sack WO, 1973: Prenatal development of domestic and laboratory mammals: Growth curves, external features, and selected references. *Anat Histol Embryol*, 2, 11-45.

Gardner E, Gray DJ, O'Rahilly R, 1959: The prenatal development of the skeleton and joints of the human foot. *J Bone Joint Surg*, 41, 847-876.

Getty R, 1975: The Anatomy of the Domestic Animals, WB. Stunders Company. London 22-23, 271-296.

Gruber H, Brenner E, Schmitt O, Fritsch H, 2001: The different growth zones of the fetal foot. *Anat Anz*, 183 (3), 267-273.

Hare WCD, 1961: The ages at which the centers of ossification appearr roentgenographically in the limb bones of the dog. *Am J Vet Res*, 90, 825-835.

Ko FC, Sumner DR, 2021: How faithfully does intramembranous bone regeneration recapitulate embryonic skeletal development? *Dev Dynam*, 250 (3), 377-392.

Kume K, Satomura K, Nishisho S, Kitaoka E, Yamanouchi K, Tobiume S, Nagayama M, 2012: Potential Role of Leptin in Endochondral Ossification. *JHC,* 50 (2), 159-169.

Pisani G, 1998: Ontomorphogenese. In: Fugchirurgie. Georg Thieme, Stuttgart, New York, pp 53-84.

Rogers CW, Gee EK, Dittmer KE, 2021: Growth and Bone Development in the Horse: When Is a Horse Skeletally Mature? *Animals*, 29; 11(12):3402. https://doi.org/10.3390/ani11123402

Salhotra A, Shah HN, Levi B, Longaker MT, 2020: Mechanisms of bone development and repair. *Nat Rev Mol Cell Biol*, 21 (11), 696-711.

Schilling FW, 1985: Wachstum und Entwicklung des Fuges im Kindesalter. Habilitationsschrift, Mtinster.

Serra-Vinardell J, Roca-Ayats N, De-Ugarte L, Vilageliu L, Balcells S, Grinberg D, 2020: Bone development and remodeling in metabolic disorders. *JIMD,* 43 (1), 133-144.

Song L, 2022: Effects of exercise or mechanical stimulation on bone development and bone repair. *Stem Cells Int*, 2022 (1), 5372229.

Stevens MM, 2008: Biomaterials for bone tissue engineering. *Mater Today Energy*, 11 (5), 18-25.

Topaloğlu U, Ketani MA, Güney BS, 2017: Kemik doku ve kemikleşme çeşitleri. *Dicle Üniv Vet Fak Derg*, 10 (1), 62-71.

Wang JS, Tokavanich N, Wein MN, 2023: SP7: from Bone Development to Skeletal Disease. *Curr Osteoporos Rep,* 21, 241–252. https://doi.org/10.1007/s11914-023-00778-7

Williams PL, Dyson M, 1989: Gray's Anatomy. The Both Press, London, 279-312.

Zhang X, Siclari VA, Lan S, Zhu J, Koyama E, Dupuis HL, Iwamoto ME, Beier F, Qin L, 2011: The Critical Role of the Epidermal Growth Factor Receptor in Endochondral Ossification. *JBMR*, 26 (11), 2622–2633.