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PRENATAL DEVELOPMENT OF THE SCAPULA OF RABBIT

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ABSTRACT

In this study, we investigated the spatiotemporal patterns of the scapular development in rabbit embryos using light microscopic examination. Tissue sections were prepared from specimens collected at the embryonic day (E) 11, 14, 17, 21 and 28 days, and stained with hematoxylin and Eosin, Safranin-O, Periodic Acid-Schiff, von Kossa's stains and Fast green as well as double staining of Alcian blue with Alizarin Red. We observed the following characteristic chronological sequences: 1) At E11, the limb bud was formed of undifferentiated mesenchymal cells, marginal vein, apical ectodermal ridge and progress zone. 2) At E14, the scapular blade appeared as a condensation of mesenchymal cells differentiated into chondrocytes. 3) At E17, endochondral ossification was induced after the invasion of blood vessels and formation of three different types of chondrocytes arranged in morphologically distinct zones. 4) At E21, appearance of primary ossification center within the scapula. There were two primary ossification centers; one within the center of scapular blade and the second one in the scapular spines. Ossification extended bidirectionally in both sides and elongation of the scapula continuous until taken mostly the shape of the future scapula. 5) At E28, remodeling and resorption processes of scapula occurred due to a substantial increase in the number of osteoclast and osteocytes. We conclude that the prenatal pattern of scapular development is peculiar in rabbit, and that our results provide better understanding of bone growth in the flat bone and are useful for teratological and clinical studies of regeneration and healing of flat bone.

KEYWORDS: Rabbit - Scapula - Development – Ossification.

1. INTRODUCTION

Rabbits (Oryctolaguscuniculus) are increasing in popularity as house pets and popular model in laboratory animal medicine due to its relatively large size and docile nature. They are model for numerous medical experiments especially experimental toxicology and teratology. All these facts place rabbits in the focus of research and require more knowledge of the precise structures as well as their development.[1] The natural morphological development of bones and joints is very important for the diagnosis of skeletal diseases in young.[2] Bones in mammals develop via two distinct processes. Intramembranous bone formation produces many of the craniofacial bones directly from mesenchymal condensations.[3] Conversely, endochondral ossification represents the principal process responsible for forming the most of the mammalian skeleton and generates bone via a cartilage intermediate.[4] The scapula is a thin, compact bone that serves as the bony attachment site for numerous muscles between the head, neck and forelimb.[5] Despite extensive literature on the scapula in human[6-7], primates[8], marsupial mammals[9], there is a lack of information relating to its pre- and postnatal growth profile. Chondrification of the mesenchymal scapular plate is followed by ossification of the plate, and cavitation of the glenohumeral joint.[10] Ossification expands bidirectionally and reaches the level of the base of the spine and the glenoid.[8, 11-12] This pattern of ossification leads to the proximal (vertebral) and distal (glenoidal) epiphyseal formation at the end of radiating cones of endochondral. Growth rates of human scapula are accelerated in the vertebral cone, which results in a greater expansion of the medial border compared to that for the lateral mass.[12, 13] In the last years, advanced molecular researches focused on the origin and the development of scapula as well as the description of pectoral girdle in extinct animals has shown that the development of scapula is more complex than ever thought.[14-16] Correlation of the histologic events with molecular mechanisms during the development of the scapula requires knowledge of the precise sequence of these histologic events and the times at which they occur. Morphogenesis and histochemistry of fetal skeletal system of rabbits received little attention. Thus, the purpose of our study is to determine the sequence events involved in the formation of scapula from the primitive limb-bud stage until the time of birth.

MATERIALS AND METHODS

2.1. Animals preparation and tissue collection

Five pregnant female rabbits (20-22 weeks old) were purchased from the farm of Alexandria University Faculty of Agriculture (Alexandria, Egypt) and used for this investigation. The day after mating was designated as the embryonic day 1 (E1). Dams anesthetized by chloroform, surgically sacrificed and then perfused transcardially with 0.1 M phosphate buffered saline (PBS, pH 7.4) for 1 minute followed by 4% paraformaldehyde (PFA) in 0.1 M PBS for 10 minutes using peristaltic pump. A total of forty embryos were collected at E11, E14, E17, E21 and E28. Fetuses were washed by phosphate buffer saline then skinned and then the abdomen was opened to eviscerate the internal organs without damaging the ribs and then fixed in 10% neutral buffered formalin. Meanwhile, the embryos used for double staining technique were fixed in 95% ethyl alcohol for at least 7 days, and E28 specimens were decalcified with 5% EDTA to assist sectioning. All animal experiments were reviewed and approved by the Damanhour University research committee prior to the experiment.

2.2. Light microscopic examination

Samples either whole embryo or separated forelimb were then dehydrated in ascending grades of ethanol, cleared in xylene, impregnated with melted paraffin wax. Finally, paraffin blocks were prepared. About 5 µm-thick sections were cut and processed for stained with haematoxylion and eosin (H and E), Saffranin-O, PAS (Periodic Acid-Schiff), Alizarin red after Cortés-Delgado et al[17], Von Kossa's, Alcian blue and Fast Green Food (FGF) after Bancroft and Gamble.[18]

2.3. Double-Staining technique

Alizarin Red protocol after is convenient for staining ossified bone. Fetuses were kept in 95% Ethyl alcohol for at least 7 days then placed in acetone for 2 days for fat removal. Maceration process was performed with potassium hydroxide (KOH) 2% to remove muscle. For staining the bone and cartilage by Alizarin Red and Alcian blue, we performed the protocol described by Menegola et al.[19] Specimens were completely immersed in a mixture of Alcian blue for 2 days, rehydrated in degrading series of ethanol, and transferred to alcohol for 24 hours. Then, they were processed for transparency through four successive steps according to Soysal et al.[20] Firstly, stained fetuses were put in 1% KOH for one day. Secondly, they were put in 80 cc 1% KOH and 20 cc 20% glycerin for 5 days. Thirdly, they were put in 50 cc 1% KOH and 50 cc 50% glycerin for 5 days, and finally, stained fetuses were put in 20 cc 1% KOH and 80 cc 80% glycerin for 5 days. The stained sections were dehydrated with a graded series of ethanol, cleared in xylene, cover slipped with Permount (Fisher Scientific, Fairlawn, NJ) and examined with light microscopy. Whole mounts of stained embryos were examined using stereo microscopy at a magnification of 30x and were photographed.

2.3. Photography

All photographic images were captured on CCD camera mounted on microscope and stored on optical disks for offline investigations. Images were edited by Adobe Photoshop CC (Adobe Systems, San Jose, CA). Contrast was adjusted as needed for all figures.

RESULTS

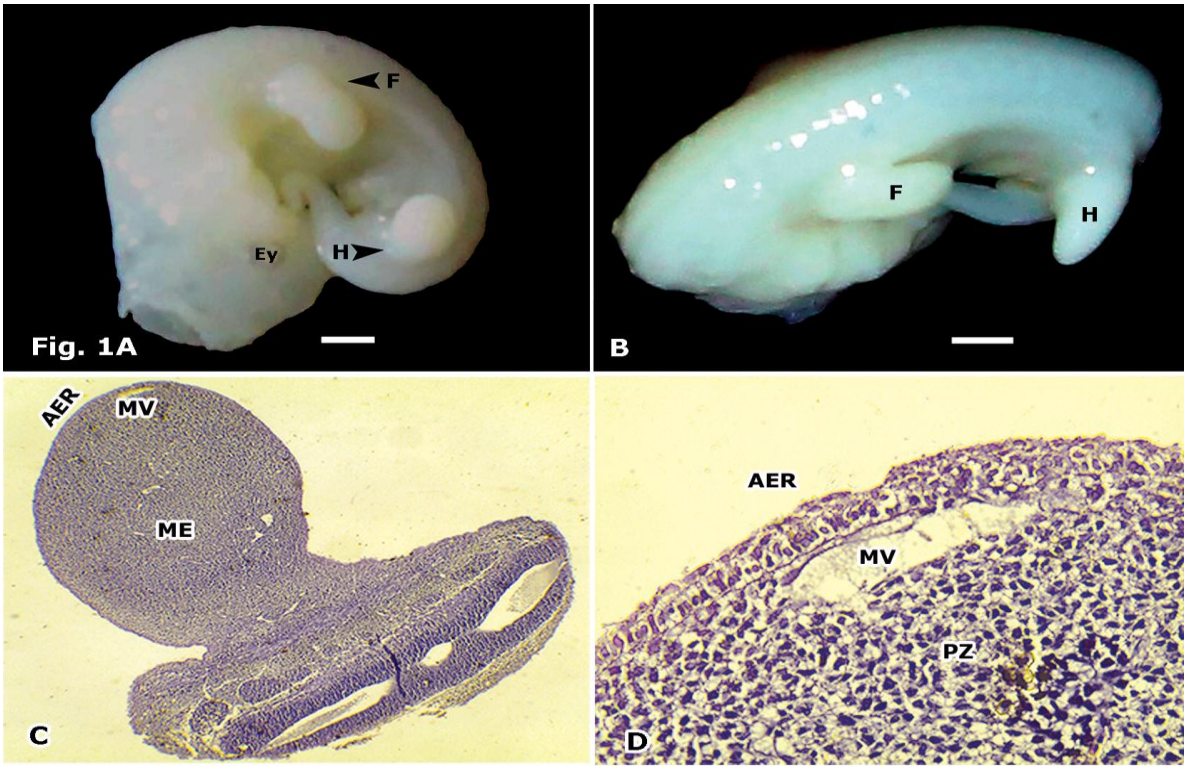
At E11, the forelimb bud appeared as a protrusion from the ventrolateral abdominal wall and directed caudoventrally at the proximal third of the body **(Fig.1A-B)**. the bud was composed of a mass of undifferentiated mesenchymal tissue covered by a single layer of cuboidal surface epithelium **(Fig.1C-D)**. At the distal border of the limb, it formed a marked thickened band composed of 2-3 layers of cuboidal cells. This band is known as the apical ectodermal ridge (AER). The bulk of the bud, adjacent to the AER, was formed of a population of undifferentiated and rapidly proliferating mesenchymal cells called the progress zone At E14, the bud shows an evidence of differentiation into two distinct parts by a well-defined constriction **(Fig.2A)**. The proximal part corresponds to the primitive shoulder girdle region and arm region while the distal part is more peripheral and circular forming a paddle shaped hand plate. Chondrogenesis has just started at this stage and was the condensed mesenchymal cells of the limb bud differentiated into flattened chondrocytes **(Fig. 2B-C**). These chondrocytes began to secret cartilage matrix that appeared orange with Safranin-O stain and magenta or pink with PAS stain **(Fig. 2 D-E)**, respectively. The primitive scapula was formed with nascent chondrocytes, which stained blue with Alcian blue and Alizarin red. The acromion and metaacromion were developed **(Fig.2F)**, meanwhile the scapular spine was not formed yet.

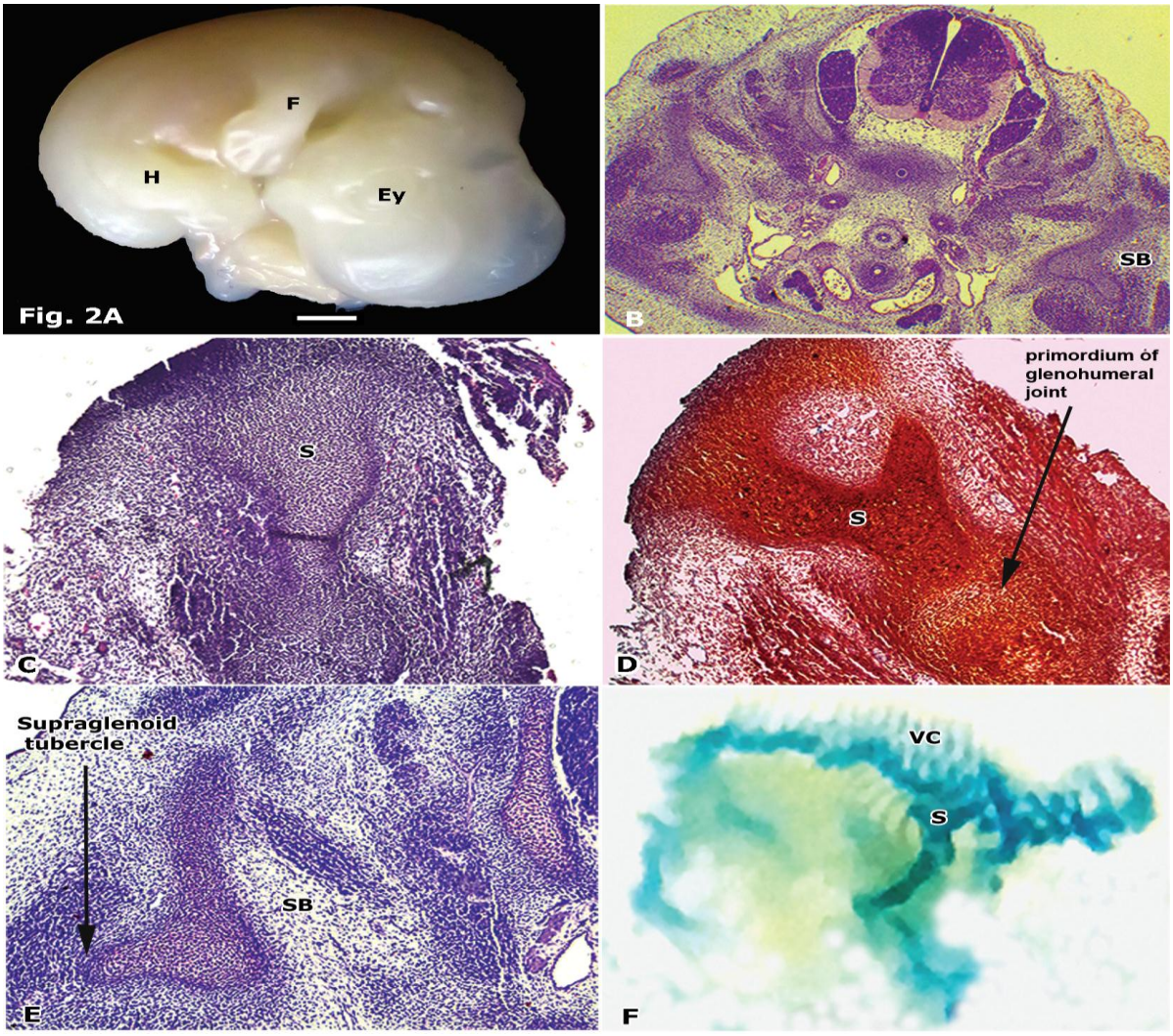
At E17, invasion of blood vessels occurred within the developing scapula and differentiation of chondrocyte into 3 distinctive zones. This vascular invasion considers the initiative signal of ossification of the developing scapula where the principal primary centers of ossification were obvious in the body of scapular blade and scapular spine **(Fig. 3A, C)**. The endochondral ossification was induced after the invasion of blood vessels closer distally toward the glenoid than proximally toward the vertebral border. We observed three different types of chondrocytes arranged in morphologically distinct zones along the infraspinous fossa around to the nutrient foramen, which was located close to the glenoid. The farthest zone from the ossification center is the zone of resting chondrocytes, followed by the zone of proliferation where the round proliferating chondrocytes became flattened as they were packed into multicellular clusters. Following the proliferation zone, the chondrocytes increased in volume and become hypertrophic chondrocytes **(Fig. 3B)**. Double staining confirmed the complete chondrification of the primitive scapula. Elongation of the scapular cartilage template and the scapular spine appeared as hock like structure at

the distal end and joined with acromion and metaacromion processes while the proximal end was incompletely developed along the scapular blade. Therefore, the demarcation between the supraspinous and infraspinous fossae was faint **(Fig. 3D)**.

At E21, the appearance of the primary ossification center within the scapula was established where two primary ossification centers could be recognized. One center appeared within the center of scapular blade and the second within the scapular spine. The ossification extended bidirectionally in both sides and the elongation of the scapula continued until it took mostly the shape of the definite scapula **(Fig. 4A)**. We detected the formation of five layers within the developing scapula according to their distance from the primary ossification center. The farthest layer was the zone of resting cartilage formed of round chondrocytes. The second layer, adjacent to the resting zone was the zone of proliferating cartilage, which was firstly formed of round chondrocytes became flattened and packed into multicellular clusters. The third layer was the zone of hypertrophic cartilage where the cytoplasm of the chondrocytes was ballooned and appeared clear with abundant glycogen increasing their volume dramatically, at the same time secreting extracellular matrix, which eventually became mineralized forming the fourth zone of cartilage degeneration with the cartilage matrix mineralization and finally the zone of woven bone formation on the mineralized cartilage matrix **(Fig. 4B)**. The endochondral ossification continued in cone shape from the glenoid and the vertebral border but more rapid in the vertebral cone, which leaded to greater expansion of the vertebral border than the caudal border of the scapula. The space between the vertebral and glenoid cones was filled by perichondral ossification given flat appearance of the scapula. During this stage, the head and vertebral border were still cartilaginous as appeared with Safranin-O **(Fig. 5A),** while the most of the scapula was well developed and appeared as a bony structure. Calcification occurred at this stage was confirmed by Von Kossa and Alizarin Red with Fast green food stains **(Fig. 5B, C)** respectively. Morphological appearance of the scapula showed the spreading of primary ossification centers and calcification of the most of scapula with changing in the shape of the spine from horizontally into longitudinally along the entire length of the scapula. The acromion and the metaacromion process were still cartilaginous **(Fig. 5D).**

At E28, the scapula showed woven bone that appeared hypercellular, with lacunae arranged in a haphazard pattern. The woven bone trabeculae appeared hypercellular, with lacunae arranged in a haphazard pattern. These trabeculae were covered by osteoblasts. The periosteum separated the collar bone from the surrounding connective tissue **(Fig. 6A)**. At the scapular blade, a plenty number of osteoclasts appeared as multinucleated, large cells with foamy cytoplasm on the surface of the bone in a pit like; Howship's lacunae **(Fig. 6B)**. With double staining, most of the scapula was ossified including the acromion process meanwhile the glenoid articular surface, coracoid process and vertebral border were still cartilaginous **(Fig. 6C).**

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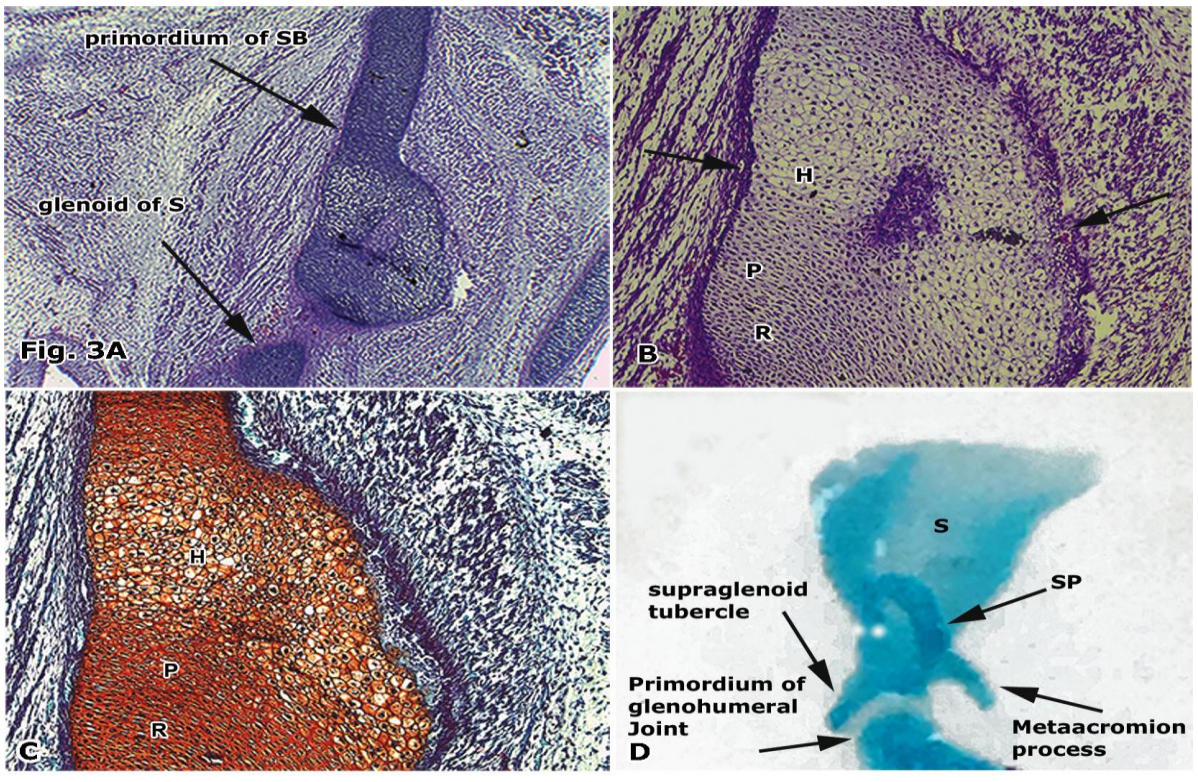
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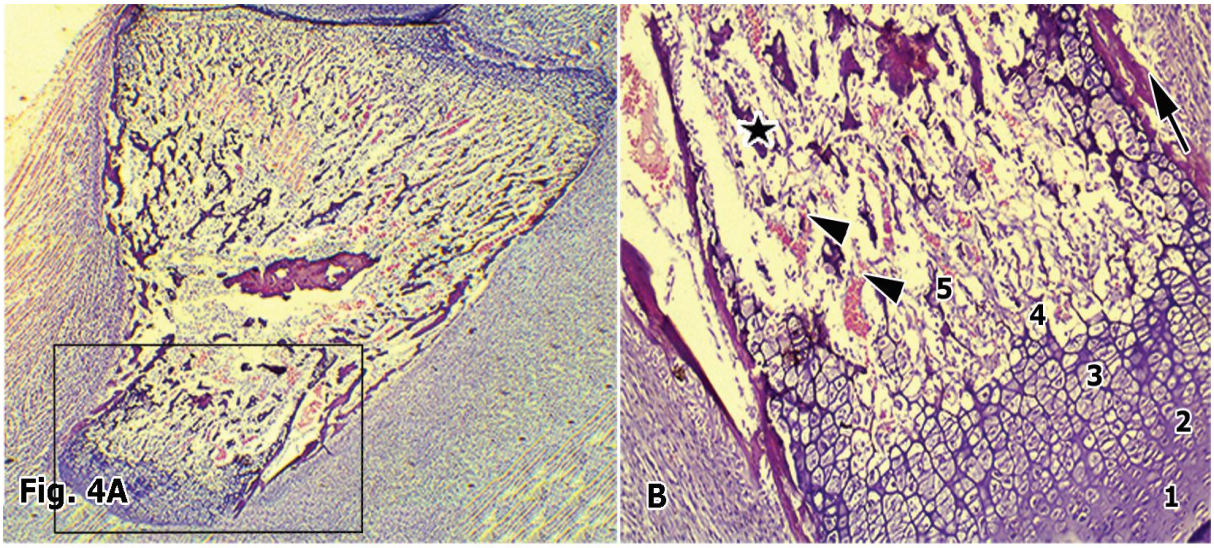
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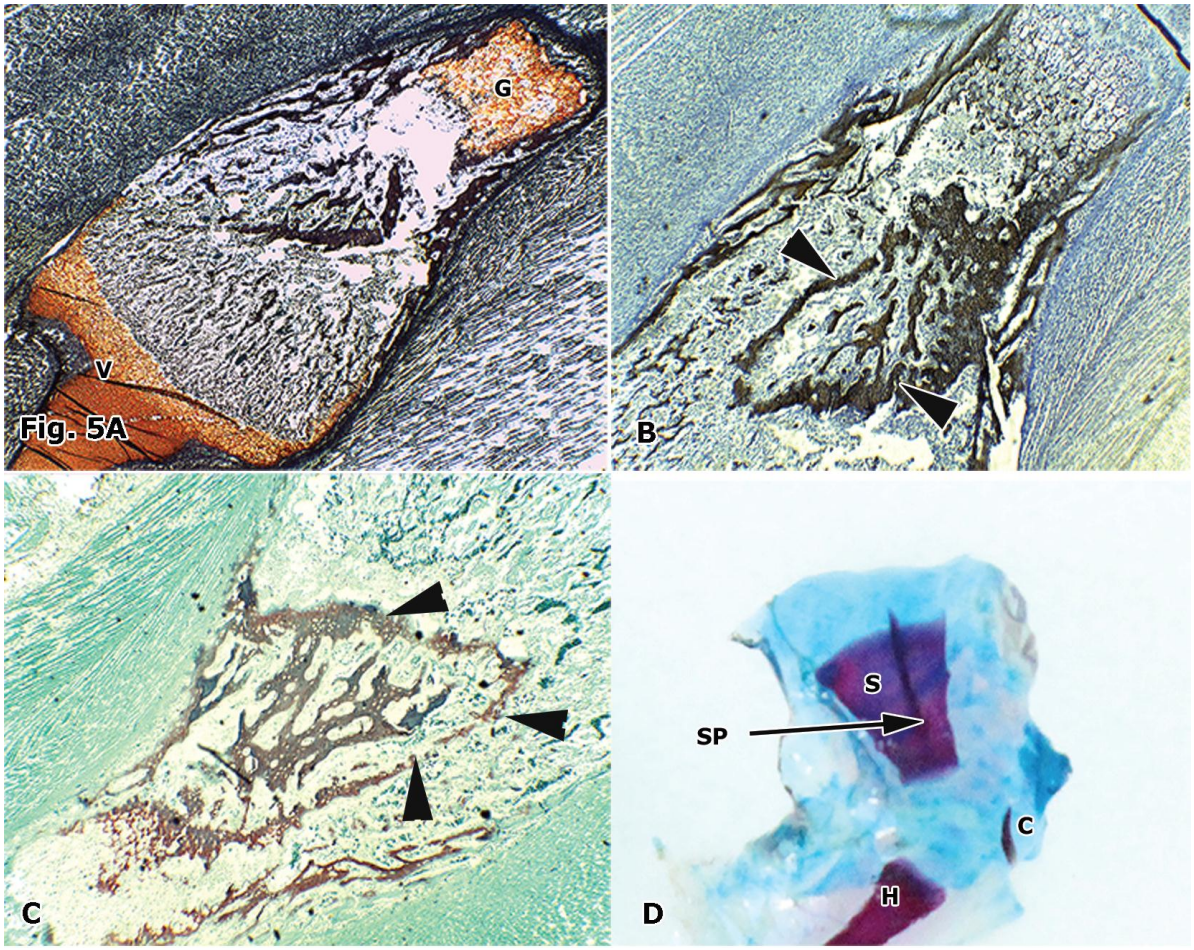
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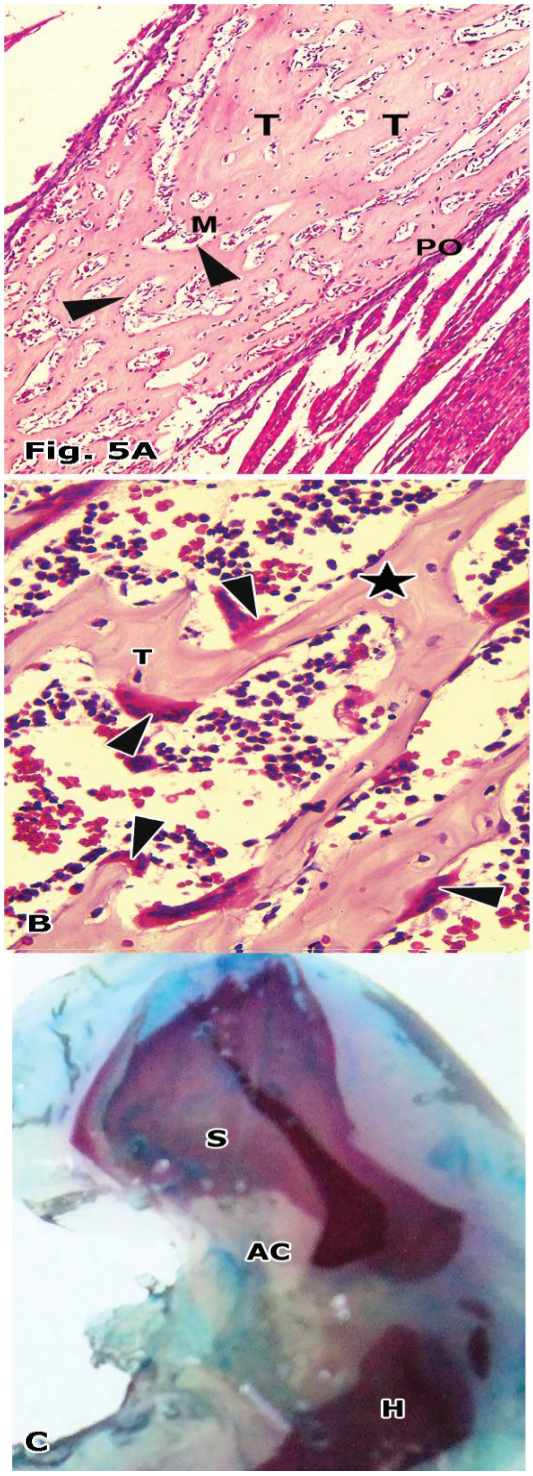

LEGEND OF FIGURES

Fig.1: Gross and microscopical features of the forelimb bud of E11 rabbit embryo. **A-B.** The forelimb bud appeared as a protrusion from the ventrolateral abdominal wall. **C-D.** Medio-lateral view of the forelimb bud. It is composed of a mass of undifferentiated mesenchymal cells (ME), marginal vein (MV), apical ectodermal ridge and the progress zone (PZ). F: forelimb bud; H: hind limb bud; Eye: eye primordium, Bar=10mm. C-D: H & E, A. 4x; B. 40x.

Fig.2: Gross and microscopical features of the forelimb bud of E14 rabbit embryos. **A.** The bud shows an evidence of differentiation into two distinct parts by a well-defined constriction. The proximal part corresponds to the primitive shoulder girdle region and arm region while the distal part is more peripheral and circular forming a paddle shaped hand plate. F: forelimb bud; H: hind limb bud; Eye: eye primordium, Bar=10mm. **B.** Transverse section showing the cartilage primordium of the scapula of left limb; scapular blade (SB). H & E, 4x. **C.** Mediolateral section showing the cartilage primordium of the scapula. H & E,10x. **D.** Section of the scapula showing the transformation of mesenchymal cells (ME) within the limb bud into chondrocytes (Ch). Humerus (H). Safranin O, 10x. **E.** Transverse section stained with PAS showing positive reaction of the cells of right scapula. 4x. **F.** Photograph of double stained whole embryo, dorsal and lateral views, respectively showing chondrification of the scapula (S) and vertebral column (VC). 4x.

Fig.3: Sections of the forelimb bud of E17 rabbit embryos. **A.** Longitudinal section showing the beginning of primary ossification center (POC) at the center of scapular primordium following the invasion of blood vessel. **B.** Longitudinal section showing the invasion of the blood vessels through supraspinous fossa inducing ossification. Arrow referred to perichondrium. Arrow head represent blood vessel invasion. **C.** Longitudinal section showing the beginning of ossification within the centre of scapular primordium and the formation of three layers of chondrocytes (HCh, PCh, RCh). **D.** Lateral view showing the elongation of chondrified scapula with a distal hock shaped spine, which ends with acromion and metaacromion process but the proximal end of the scapular spine not developed yet. No signs of ossification were observed. GhJ: primordium of glenohumeral joint; H: primordium of humerus head; HCh: hypertrophic chondrocytes; PCh: Proliferative chondrocytes; RCh: resting chondrocytes; S: scapula; SP: scapular spine; ST: Supraglenoid tubercle. A: H & E, 4x; B: Masson's Trichrome, 10x; C-D: Safranin O and Fast Green Food, 10x.

Fig.4: Medio-lateral sections of the scapula of E21 rabbit embryos. **A.** A section showing the beginning of primary ossification centre in the middle of the scapula and spread bidirectionally toward the vertebral border and the distal end of scapula. **B.** Magnification of the boxed area in A. showing the five zones of endochondral ossification. Asterisks referred to the bone trabeculae, arrow heads referred to bone marrow, and the arrow referred to periosteum, which appears as a definite line between the subperiosteal bone collar and the surrounding connective tissue. A-B: H and E, 4x.

Fig.5: Photomicrographs of mediolateral sections of the scapula of E21 rabbit embryos with special stains. **A.** Photograph of the scapula showing the epiphyseal cartilage toward both ends of the scapula but the rest of

scapula is ossified. **B.** Photograph showing the calcified medullary bone trabeculae and calcified extracellular matrix. **C.** Photograph showing the the calcified bone trabeculae in the scapula. **D.** Photograph showing the ossification of the scapula with changing in the shape of spine from horizontal direction extending along the entire length and extended longer beyond the vertebral border but the acromion and metaacromion process were still cartilaginous. A: Safranin O stain and Fast Green Food, 4x. B. Van Kossa and Haematoxylin, 4x. C-D: Alizarin Red and Fast Green Food, 4x. C: clavicle; G. Glenoid; S: spine of scapula; V. vertebral border.

Fig.6. Photomicrographs of mediolateral sections of the scapula of E28 rabbit embryos. **A.** Photograph of the distal half of scapula showing woven hypercellular bone trabeculae (T) with lacunae arranged in a haphazard pattern. The Periosteum (Po) separate between the collar bone and the surrounding connective tissue. Arrowheads indicate the bony trabeculae covered by osteoblasts. **B.** Higher magnification photograph of the scapular blade showing plenty number of osteoclasts (arrowheads) and a small resorption pit; Howship's lacunae (Hs) containing osteoclasts. Observe osteocytes (asterisks) within the bony spicules of the bony trabeculae (T). **C.** Photograph of the lateral aspect of the scapula showing the ossification of acromion process (AC) and slight inclination of the spine while the metaacromion process, glenoid articular cavity and the vertebral border are still cartilaginous. A-B: H and E, 4x. C: Alizarin Red and Fast Green Food, 4x. H. Humerus; HT: hematopoietic tissue; M: Forming marrow spaces; S. scapula.

DISCUSSION

Understanding the basic pattern of scapular growth is not easy, since initially it appears somewhat different from a long tubular bone.[21] According to the available literatures, researches on prenatal development of the appendicular skeleton in rabbit were concerned mainly with the long bone[22-23] but there was scarcity of information about the flat bone of the appendicular skeleton.

Prenatal development of the forelimb: The first visible sign of limb primordium appears in early stages of development as a small bulge called limb buds, which grows out of either side of the lateral body wall at appropriate levels. In mouse and rabbit, the primordia of limbs appear at E9.5.[24, 25-26] At E11, we found the forelimb bud appeared as a protrusion from the ventrolateral body wall filled with undifferentiated mesenchymal cells. This limb bud was covered by a single layer of cuboidal ectoderm, which become more thickened at the distal border of the limb forming the AER. This ridge is a key signaling center, which controls the outgrowth and patterning of the proximal-distal axis of the limb through the secretion of various molecules.[27] It exerts an inductive influence on the adjacent mesenchyme causing it to remain as a population of undifferentiated, rapidly proliferating cells.

Chondrification of the scapula: At E14, the mesenchymal cells of the proximal part of the limb bud transformed into chondrogenic cells forming the cartilage template of the scapula. These chondrogenic cells secret glycosaminoglycans rich matrix, which were detected by Safranin-O positive stain and PAS stain. This is in agreement with previous reports in rabbit embryos mentioned that chondrification of the forelimb bud begins at the 14th day of gestation.[22-23]

Ossification of scapula: In our study, pre-ossification phase of the scapula started at E17 where two principal primary centers of ossification appeared in the body of the scapular blade and the scapular spine. Similarly, the primitive scapula of dogs at E40 has two primary ossification centers one for the body and one for the spine.[28] Meanwhile, the E15.5 albino mouse has only one primary ossification center started in the body of the scapula and spread thereafter until it reaches the spine.[29] In our study, the invasion of blood vessels into the scapular blade occurred in the infraspinous fossa where the nutrient foramen is present, while in human scapula the initial area of primary scapular ossification is located in the supraspinous fossa where nutrient artery arises from the suprascapular artery initiating the endochondral ossification.[30] Our results are coincided with previous findings in the human scapula[13] in that both of the ossification sites are located closer to the glenoid region than to the vertebral border. We found the scapular spine joined with the acromion and metaacromion processes forming a hock shaped like structure but the proximal extremity of the spine was still ill developed, which is coincided with the scapula of marsupial mammals.[9] At E21, we found the primary ossification centers extended bidirectionally in both sides and the elongation of scapula continues until it gained almost the definitive shape, which is similar to the findings in human.[19] We detected the formation of five layers within the developing scapula: zone of resting cartilage, zone of proliferating cartilage, zone of hypertrophic cartilage, zone of cartilage degeneration with cartilage matrix mineralization and the zone of woven bone formation on mineralized cartilage matrix. The manner of developmental sequences of endochondral ossification was similar to those in other mammals.[31] The growth rates are accelerated in the vertebral cone, which results in a greater expansion of the vertebral end compared to that for the caudal mass as found in human[13] and albino mouse.[29] The spaces between the two endochondral cones are then filled by perichondral ossification so that much of the blade of the scapula was formed by perichondral ossification where the chondroblasts directly differentiated into osteoblasts. We did not find mesenchymal cells within the scapula during and after the chondrification process, which contrasts that the space between the two cones of human scapula was filled by intramembranous.[13]

At E28, the scapula showed hypercellular woven bone covered by osteoblasts that produce and arrange the

organic matrix of the bone. Within the bony trabeculae, the osteoblast produces the matrix, surrounds itself with matrix, and inhabits a small lacunar space within the matrix becoming an osteocyte. Osteocytes are plumper and their orientation is more random and they are important for the maintenance of bone. Osteoclasts appear as multinucleated, large cells with foamy cytoplasm on the surface of the bone. They are responsible for the process of remodelling of bone that occur during this stage of development by release of their lysosomal contents in the resorption pit.[32]

The endochondral bone retained its longitudinal orientation with constant addition to each elongating column once it was formed. This retention and constant addition to each endochondral column and the creation of new columns peripherally (latitudinal growth) established a radiating pattern to the vertebral border cone.[13] Such retention thereby allowed a better understanding of basic growth in this flat bone. Longitudinal growth rates are rapid, depending upon the size of the scapular plate. These rates certainly are greater for the vertebral than the glenoid cone. More importantly, the scapula exhibits significant latitudinal growth along the vertebral border. This was more dominant in the infraspinous than in the supraspinous fossa. Our result confirmed that ossification of most parts of the scapula was a prenatal event meanwhile in dog,[27] mouse[24] and in human,[13, 33] the acromion process, glenohumeral cavity and the vertebral border remained cartilaginous until birth.

REFERENCES

1. H. Hristov, D. Kostov, D. Vladova. Topographical Anatomy of some abdominal organs in rabbits, Trakia Journal of Sciences, 2006; 4(3): 7-10.
2. Makkaway NH, EL - Mezian A, Abdel – Tawab M. A radiological appearance of prenatal ossification centers and epiphyseal lines of the thoracic limb in Egyptian buffaloes. Zag Vet J, 1988; 16(4): 310-322.
3. Percival CJ, Richtsmeier JT. Angiogenesis and Intramembranous Osteogenesis. Dev Dyn, 2013; 242: 909–922.
4. Mackie, E. J., L. Tatarczuch, M. Mirams. The skeleton: a multi-functional complex organ: the growth plate chondrocyte and endochondral ossification. J Endocrinol, 2011; 211(2): 109-21.
5. Ashton EH, Oxnard CE. The musculature of the primate shoulder. Trans Zool Soc Lond, 1963; 29: 554–650.
6. Schulter-Ellis, F.P. Evidence of handedness on documented skeletons. J Forensic Sci., 1980; 25(3): 624-630.
7. Shulin, P., Fangwu, Z. Estimation of stature from skull, clavicle, scapula and os coxa of male adult of Southern China. Acta Anthropologica Sinica, 1983; (3): 253-259.
8. Nathan M. Young. Function, ontogeny and canalization of shape variance in the primate scapula. J. Anat, 2006; 209: 623–636.
9. Marcelo R. Sa´ nchez-Villagra, Wolfgang Maier. Ontogenesis of the Scapula in Marsupial Mammals, With Special Emphasis on Perinatal Stages of Didelphids and Remarks on the Origin of the Therian Scapula. Journal of morphology, 2003; 258: 115–129.
10. Gardner, E. and O'Rahilly, R. The early development of the hip joint in staged human embryos. Anatomical Record, 1972; 172: 451-452.
11. Uhthoff, H.K. The development of the shoulder. In: The Embryology of the Human Locomotor System (H.K. Uhthoff, Ed.), pp. Berlin: Springer, 1990; 73-81.
12. Corrigan, G.E. The neonatal scapula. Biologia Neonatorum, 2: 159-167.
13. Ogden, J.A., Phillips, S.B. Radiology of postnatal skeletal development. VII. The scapula. Skeletal Radiology, 1983; 9: 157-169.
14. Ehehalt F, Wang B, Christ B, Patel K, Huang R. Intrinsic cartilage forming potential of dermomyotomal cells requires ectodermal signals for the development of the scapula blade. Anat Embryol (Berl), 2004; 208: 431–437.
15. Kuijper, S., Beverdam, A., Kroon, C., Brouwer, A., Candille, S., Barsh, G., Meijlink, F. U. Genetics of shoulder girdle formation: roles of Tbx15 and aristaless-like genes. Development, 2005; 132: 1601-1610.
16. Prols, F., Ehehalt, F., Rodriguez-Niedenfuhr, M., He, L., Huang, R., Christ, B. U.
17. Natalia Cortés-Delgado; Jairo Pérez-Torres, Julio Mario Hoyos. Staining Procedure of Cartilage and Skeleton in Adult Bats and Rodents Int. J. Morphol, 2009; 27(4): 1163-1167.
18. Bancroft, J., Gamble, M. Theory and Practice of Histological Techniques. Churchill Livingstone, 2008; 6th edition.
19. Menegola, E.; Broccia, M. L., Giavini. E. Atlas of rat fetal skeleton double stained for bone and cartilage. Teratology, 2002; 64(3): 125-133.
20. Soysal, H. , Erdogan ,U., Ayhan, D., Omur ,K., Nihat, E. Whole mouse fetuses by alcian blue and alizarin red S. Teratology, 2011; 22(3): 299-301.
21. Terence D. Capellini1, Giulia Vaccari, Elisabetta Ferretti, Sebastian Fantini, Mu He, Massimo Pellegrini, Laura Quintana, Giuseppina Di Giacomo, James Sharpe, Licia Selleri, Vincenzo Zappavigna. Scapula development is governed by genetic interactions of Pbx1 with its family members and with Emx2 via their cooperative control of Alx1. Development, 2010; 137: 2559-2.
22. Rivas, R., F. Shapiro. Structural stages in the development of the long bones and epiphyses: A study in the New Zealand white rabbit. J. Bone Joint Surg, 2002; Am., 84-A: 85-100.
23. Ahmed, Y.A., Eman A. Abdelrahim, Fatma Khalil. Histological Sequences of Long Bone Development in the New Zealand White Rabbits. Journal of Biological Sciences, 2015; 15(4): 177-186.

24. Kauffmann M. H. The Atlas of Mouse Development. Elsevier Ltd., 2008; Revised Edition pp 496.
25. Beaudoin S, Barbet P, Bargy F. Developmental stages in the rabbit embryo: guidelines to choose an appropriate experimental model. Fetal Diagn Ther, 2003; 18: 422–427.
26. Cameron, J., Mccredie, J. Innervation of the undifferentiated limb bud in rabbit embryo. J. Anat, 1982; 134, 4, 795 808.
27. Benazet JD, Zeller R. Vertebrate limb development: moving from classical morphogen gradients to an integrated 4-dimensional patterning system. Cold Spring Harb Perspect Biol, 2009; 1(4):a001339.
28. Melania Moioli. Appearance of ossification centers of the limbs and skeletal development in newborn toy-dog breeds: radiographic, morphometric and histological analysis. PhD in Veterinary Clinical Sciences. Università degli Studi di Milano, 2013. https://doi.org/10.13130/moioli-melania_phd, 2014-03-26.
29. Myra L Johnson. The time and order of appearance of ossification centers in the Albino mouse Am. J. Anatomy, 2005; 52(2): 241–271.
30. Scheuer L, Black S., Christie A. Development juvenile osteology, 2000; Chapter 8, 244-271.
31. Mackie, E.J., Y.A. Ahmed, L. Tatarczuch, K.S. Chen, M. Mirams. Endochondral ossification: How cartilage is converted into bone in the developing skeleton. Int. J. Biochem. Cell. Biol, 2008; 40: 46-62.
32. Gilbert-Barness E: Osteochondrodysplasia-constitutional diseases of bone. In Potter's Pathology of the Fetus, Infant, and Child, Second edition. Philadelphia, PA: Mosby Elsevier; 2007; 1836–1897.
33. Last, R.J. Anatomy, Regional and Applied. Edinburgh: Churchill Livingstone, 1973; 5th edition.