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Histological Observations of the Developing Human Fetal Humerus at Different Trimesters of Pregnancy

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ABSTRACT

Background: Although femur length is the preferred method for fetal age assessment, the humerus is sometimes the right choice, especially in the second half of pregnancy. Fetal bone development significantly impacts adult bone quality and senescent bone disorders, including osteoporosis.

Purpose of the study: The present study aimed to ascertain the histological changes in the human fetal humerus across the three trimesters. After the institutional ethical committee clearance and parent consent, the study was carried out on stillborn or medically terminated human fetuses from the 10th to the 32nd week of intrauterine life.

Results: First trimester: The primary bone collar appeared with the primary ossification center, marked by more vascular invasion and a pool of mesenchymal cells. Trabeculations begin from the bony collar and insignificant periosteum. Second trimester: The changes showed longitudinal growth of periosteal bone towards the proximal and distal ends of the growing bone. Growth plate with distinguished zones and gradual fusion of epiphysis with growth plate were observed. Third trimester: Trabeculation number and thickness increase with calcification. Towards the third trimester, the marrow cavity with increased and prominent trabeculations is consistent.

Conclusion: Understanding normal microstructural and cellular events chronologically is an ideal platform for future studies to develop cell-based or cell-targeted therapies for adult bone disorders, traumatic bone injuries, or bone engineering.

KEYWORDS: Histology, Developing Bone, Humerus, Fetuses, Embryology.

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INTRODUCTION

The humerus bone develops, grows, and mineralizes during development into a highly complex and designed to withstand various biomechanical stresses produced by upper limb usage [1].

A detailed report of the histological details of the humerus from its first appearance until birth is unavailable, especially for human fetuses.

Humeri are developed through endochondral ossification, where a simple cartilage model is turned into a complex and calcified bone before birth. The ossification starts close to the middle of the diaphyseal region of the growing bone and extends longitudinally in either direction, chiefly regulated by the growth plate at both ends. Generally, the growth plate region sequentially goes through processes such as chondrocyte proliferation and maturation, extracellular matrix synthesis, chondrocyte hypertrophy, mineralization of the matrix, and vascular ingrowth accompanied by osteoblasts. The rate of longitudinal bone growth is proportional to the size and organization of the growth plate, especially by the rate of proliferation of chondrocytes and the maturation rate of chondrocytes into hypertrophic cells. Simultaneously, as the mineralized zone develops in length, radial growth of the already mineralized diaphysis and rearrangement occur via intramembranous bone production and resorption, generating the definitive bone. [2, 3].

Cartilage canals are the spaces invaded by the vascular mesenchyme through the periosteum and differentiated to form a vascular network to recruit osteoprogenitors to form the appearance of a secondary center of ossification [4,5]. The canals nourish the chondrocytes and provide mesenchymal osteogenic cells to long bones [6]. The general shape of the humerus with distinct proximal and distal parts was quite similar to that of the adult from the first trimester. The humerus's exact shape and torsion appeared challenging to differentiate due to their fragile nature and adhesion of muscles and connective tissue. The characteristic features of the humerus became more sharply accentuated in the second half of the gestation, mainly due to the ossification, remodeling, and torsion [7].

Understanding the normal microstructural and cellular events chronologically is an ideal platform for future studies to develop cell-based or cell-targeted therapies to address adult bone disorders or traumatic bone injuries. Thus, the present study was done to determine the histological changes in the developing human humerus bone across the three trimesters.

METHODS

This study followed the ethical guidelines for biomedical research involving human subjects as outlined in the Declaration of Helsinki and was approved by the institutional ethics committee.

The present study was carried out on stillborn or medically terminated human fetuses from the 10th to the 32nd week of intrauterine life. Fetuses were procured from the Departments of Medical Genetics and Obstetrics for routine fetal autopsy. Only the fetuses without visible congenital musculoskeletal defects and genetic disorders affecting the skeletal systems were considered for the present study. Parent consent was taken to perform an autopsy, and relevant medical history was taken from hospital records as per the institutional protocol.

The humeri of the right side were carefully isolated from the shoulder girdle. The attached muscles and periosteum were removed with care to retain the proximal and distal ends of the bone and were fixed in a 10% formalin solution for 24 hours.

Decalcification was done with an acidic EDTA decalcifying solution (5.5 gms EDTA in 10% formalin) for 2-4 weeks till the satisfactory endpoint of decalcification. The longitudinal section was made through the bone to get epiphysis, metaphysis, and most diaphysis. Thin sections of 5 µm paraffin were taken and stained with hematoxylin and eosin stain. Also, the slides were subjected to Masson-Goldner trichrome staining protocols [8]. The slides were observed, photographs were taken under an Olympus BX53 research microscope, and selected sections were photographed using an Olympus DP 74 camera and cellSens imaging software.

RESULTS

First Trimester:

Epiphysis appeared covered with a highly vascularised connective tissue coat (Fig 1a). Chondrocytes were predominantly fusiform and oriented parallel on the developing epiphysis's outer aspect but irregular in their inner aspect. A thick perichondrium without

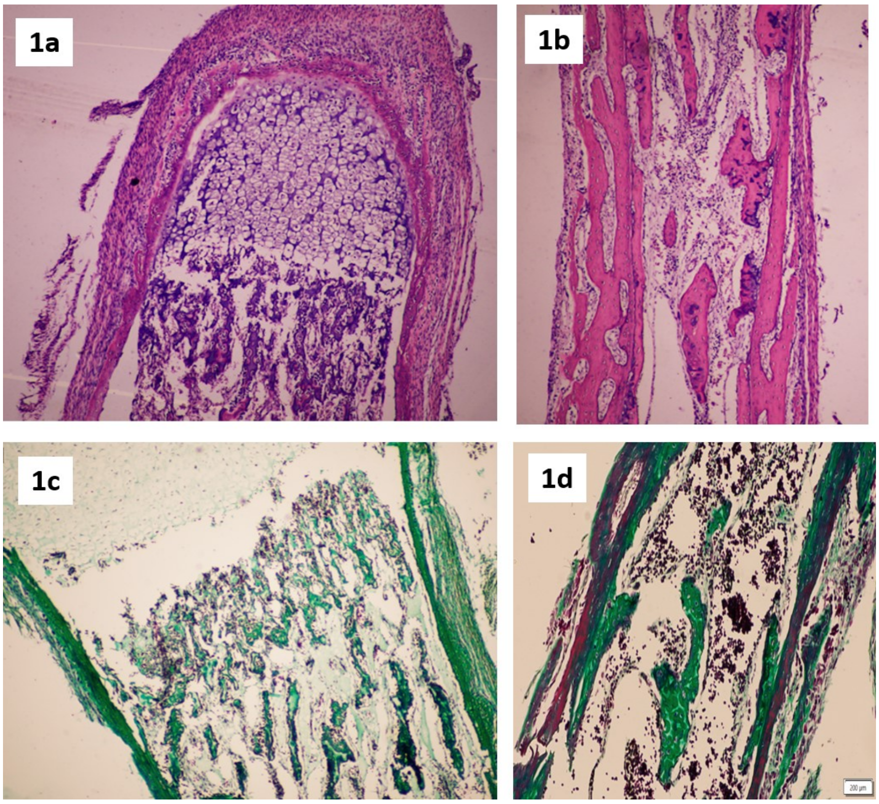

Fig. 1: First trimester photomicrographs with a scale 200µm in 4x magnification - Underdeveloped epiphysis and growth plates covered by a thick connective tissue coat (1a), Diaphyseal region showing developing trabeculations and ill-defined perichondrium and periosteal bony collor (1b), Metaphyseal region (1c) in Masson Trichrome stain, Diaphyseal region of trabeculation to identify calcification (red color) seen in 1d.

definite layers was visible. More proliferative and hypertrophic zones in the proximal epiphysis evidenced the developing growth plate (Fig 1c).

Cartilage canals begin to appear toward the later part of the first trimester with well-differentiated vascular mesenchyme within the proximal epiphysis. Clusters of mesenchymal cells within the developing epiphysis begin to arrange somewhat circularly at a few places and differentiate to form vessels and plexus of capillaries, probably recruiting the osteoprogenitor cells. Dense eosinophilic material was evident around the canals.

Diaphysis at early gestation shows more significant blood vessels, and trabeculations don't appear organized (Fig 1b). They are smaller and connected to the developing outer periosteal bone. The periosteum is delicate and without definite layers. The periosteal thickening was noted in some places, probably representing the muscle attachment site.

The developing humerus's medial side showed well-marked periosteal collar formation. Bony trabeculations, like the periosteal collar, were also predominant on the medial side. Masson trichrome stain shows the amount of bone calcification within the trabecula; the compact bone is not yet wholly seen (Fig 1d).

Second trimester:

The developing epiphyseal cartilage (Figure 2a) shows a progression of morphological changes from quiescent, proliferative, and hypertrophic cartilage to calcified cartilage, approaching the diaphysis. This change appears more anterior to the distal epiphysis than proximal epiphysis. The width of the metaphysis is larger at this stage of pregnancy, with greatly increased calcification.

The longitudinal section through the humerus shows calcified cartilage spicules on which the bone has deposited. The tiny spicules have already grown to create bony trabeculae in some places. The number of trabeculations decreases; however, the thickness of each increases. Many trabeculations join with each other and with the periosteal bone (Fig 2b & 2c). Thus, the formation of compact bone progresses in the diaphyseal region of the developing humerus till the growth plate. A

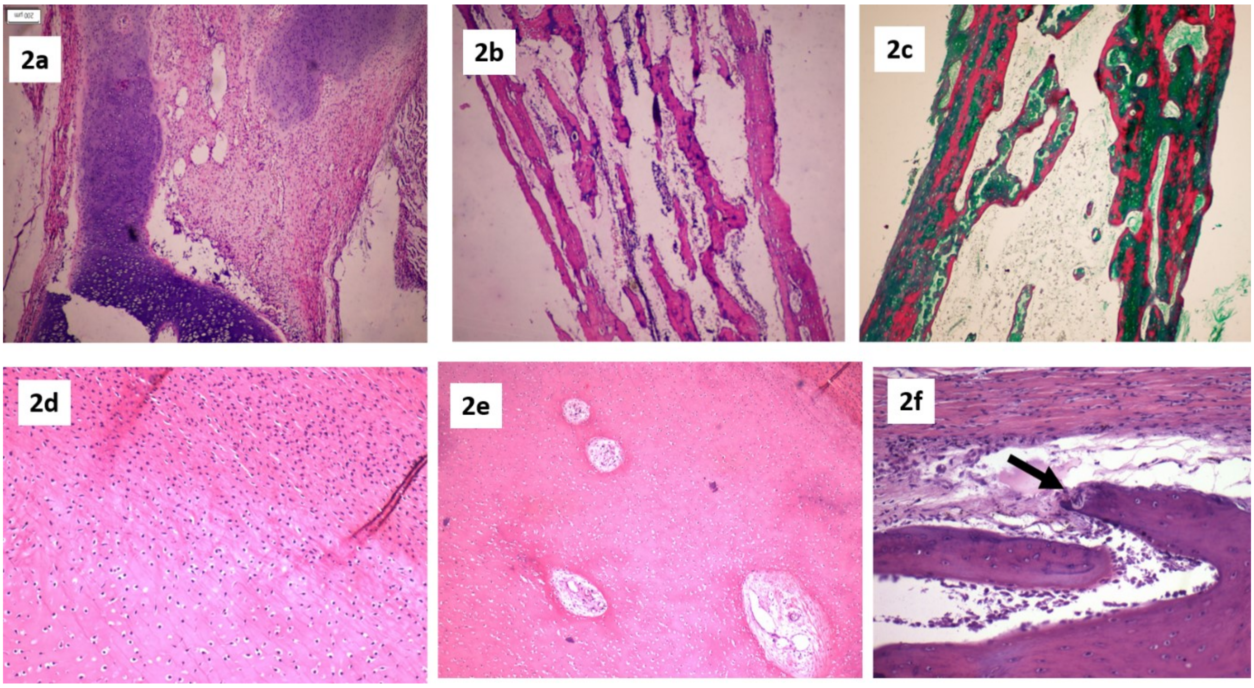

Fig. 2: Second trimester photomicrographs with a scale 200µm in 4x magnification-Growth plate region with epiphysis (2a), Diaphyseal region to show trabeculations in H & E stain (2b) and in Masson trichrome stain (2c), proximal epiphysis section in H & E stain to show the orientation of mesenchymal cells (2d), cartilage canals differentiating into vessels in 10 x magnification(2e), and trabeculation containing osteocytes, lining osteoblasts, and arrowed indicates osteocytes in 10 x magnification (2f)

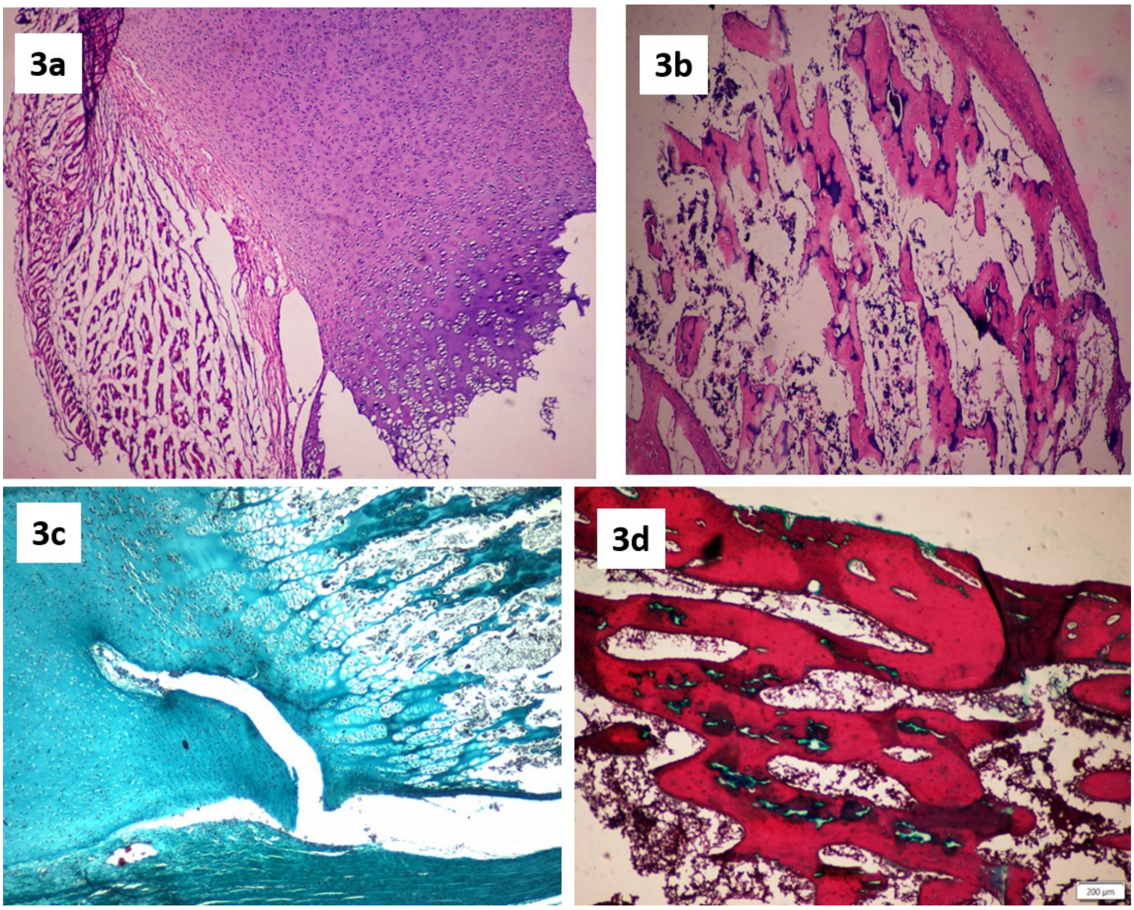

Fig. 3: Third trimester photomicrographs with a scale 200µm in 4x magnification –Zones of growth plate differentiation, metaphyseal region, and thickened perichondrium at the site of muscle attachment (3a), Diaphyseal region with bone marrow cavity, calcified trabeculations, and thick compact bone (2b), Masson trichrome stain to show cartilage canals carrying blood vessels (3c), and Masson trichrome stain of diaphysis to show dense calcification (3d) as indicated by red color.

thick periosteum with distinct layers covers the mid-diaphyseal region.

Cartilaginous canals were observed to dominate the epiphysis (Fig. 2d) and contain blood vessels, which differentiated into distinct arterioles and veins (Fig. 2e). It is clear that as gestation progresses, the epiphyseal plate becomes more developed and becomes more easily appreciated.

Third trimester:

The developing cartilaginous region showed well-differentiated areas of resting, proliferative, hypertrophic, calcified, and ossified cartilage adjacent to the metaphyseal region (Figure 3a). Some of these areas may overlap, which is often observed in areas of calcification and ossification. Cartilage cells begin to hypertrophy and deposit bone matrix, which then calcifies.

A vascular channel emerges from the perichondrium, thickening at the muscle attachment site (Fig 3c). The formation of bony trabeculae is expected to increase in the third trimester. Each of the trabeculations contains more calcified bone. The compact bone at the diaphysis achieves its maximum thickness (Fig. 3b & d). The marrow cavity enlarges withmore trabeculations and intertrabecular spaces. The intertrabecular spaces of the bone are richly vascularised and contain adequate adipose tissue and primitive stem cells from which all the cellular elements of bone are derived.

DISCUSSION

Long bone growth has been extensively studied on the femur, but no studies have been done on the humerus. The humerus is formed by endochondral ossification, in which the cartilaginous plane is gradually transformed into the mineralized bone in its future diaphyseal region [3,9]. Mineralization begins in the middle of the growing bone and continues longitudinally to both ends, dominated by growth cartilage. The growth plate is divided into resting, proliferative, hypertrophic, and mineralized zones. As the mineralized zone elongates, radial growth of the mineralized axis and its reorganization occur through intramembranous bone formation and resorption, forming the periosteal skeleton [3].

The humerus is ossified from eight centers in the diaphysis, head, greater and lesser tuberosities, lateral capitulum and process, medial process, and epiphysis. In 80% of the population, the diaphysis begins to ossify centrally at 8 weeks and the humerus within 6 months. Large and small nodules begin to form in the 2nd and 5th years in males and about a year earlier in females. Distally, during the first year after birth, ossification begins at the capitulum. The trochlear center appears in the 9th year in women and the 10th year in men. Medial and lateral epicondyle centers begin at years 4 and 12 [10].

Putz described the histological details of the developing epiphysis in 1996. Local and systemic factors have influenced these events, including mesenchymal aggregation, chondrocyte differentiation, and vascular invasion [11, 12, 13]. The present study documented the evidence of these cellular changes throughout gestation.

As suggested by Stump in 1925 and Blummer in 2004, the cartilage canals contribute to the development of the secondary ossification center. Appeared as a collection of mesenchymal cells from the adjacent perichondrium, converted as the vessels of the primitive bone arranged in a complex network of capillaries within the bony matrix [14]. The available literature on the cartilage canal was documented only from avian and other mammalian studies and rarely from human fetuses, even absent on humerus bone. Such canals contribute to the endochondral and intramembranous bone formation [7].

The Salle observed histological details of the developing metaphyseal region in 2002 on the proximal femur. They noted the growth of trabecula from the mature chondrocytes at the rate of 3ìm per day during the gestation. Kembler, in 1976, noted that the growth rate of a growth plate is approximately 35 µm per day from 5 to 8 years, whereas the cell cycle time is 20 days. He also quantified the metaphyseal region from birth till adolescence - Maturing and proliferating cells, Hypertrophic cells, and the height and width of each zone [15].

Gardner and Gray (1970) observed a primary bony collar covering two-thirds of the diaphysis and the ossification center. Numerous immature trabeculations in the diaphyseal region relate to the inner part of the periosteal bone. The periosteal bone is much thicker and less trabeculated in the mid-diaphyseal region, whereas the reverse is true for the proximal and distal parts of the humerus. The periosteal bone thickness gradually increases proximally and distally with the progression of the ossification [7].

The calcified cartilage was noted in the middle of the diaphyseal region, which marks the site of the primary ossification center in the first trimester. Bone progenitor cells in the inner periosteum continue to differentiate into osteoblasts, depositing bone material around the remaining calcified cartilage plates and forming the periosteal skeleton. New periosteal bone formation occurs at the rate of new endochondral bone formation. The bone collar increases in thickness and compactness as development progresses. The thickest part of the skeleton is found in the central part of the developing bone called the diaphysis. The main center of ossification is the diaphysis, where the initial periosteal skeleton forms [9].

Beneath the periosteum is growing bone. Periosteal cells differentiate into osteoblasts, forming anastomosing bone trabeculae surrounding the primitive marrow cavities. In the medullary cavities are cells and fibers of embryonic connective tissue, blood vessels, arterioles, and nerves. At the periphery, collagen fibers of the periosteum are continuous with fibers of the embryonic connective tissue of the adjacent marrow cavities and collagen fibers in the bone trabeculae.

Osteoblasts comprise the bone matrix and are arranged linearly along the growing bone trabeculae. A newly synthesized osteoid is visible at the edges of the trabeculae. Osteocytes are in the spaces of the trabeculae. Multinucleated osteoclasts resorb and remodel bone during formation [9]. With increasing gestational age, the area of the bone's body that approaches the metaphysis is characterized by a woven bone. On the other hand, moving away from the metaphysis, we observe the characteristics of a spongy bone with scattered trabeculae. In most specimens, the periosteal ring and trabeculae are more prominent on one side of the shaft.

In conclusion, homogeneity, even within a particular type of bone sample, is a much-debated topic. This is understandable because of the variations in the proportions of the constituent compartments in each bone segment and the dynamics of bone growth [16].

CONCLUSION

The primary ring of bone appears along with the primary ossification center, which is marked by more vascular invasion and a cluster of mesenchymal cells. Bony trabeculae originating from the neck of the bone and negligible periosteum are characteristic of femoral development during the first trimester. Changes during the second trimester show longitudinal growth of the periosteal bone toward the proximal and distal ends of the growing bone. A growth plate with prominent areas and gradual fusion of the epiphysis with the growth plate were observed. The number and thickness of trabeculae increase with calcification. Around the third trimester, the medullary cavity with bone trabeculae increases and becomes steadily prominent.

An understanding of endochondral ossification will bring about not only developmental bone biology but could also improve the treatment of several skeletal diseases, such as fracture non-unions, chondrodysplasias, and osteochondrosis. Furthermore, such developments may improve our understanding of osteoarthritis and the development of novel tissue engineering strategies for treating significant bone defects.

Author Contributions

LCP: Conceptualization, Formal analysis, and writing the original draft; **VM:** Data curation, Visualization, and writing the original draft preparation. **KMR:** Supervise, review, edit, and manage the project administration.

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Conflicts of Interests:

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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