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***Corresponding Author:**
Basma Mohamed Kamal

Email:
omhabiba_kamal@yahoo.com

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Sequential Pattern of Prenatal Ossification in the Fore Limb Bones in White New Zealand Rabbits by Double Stained Techniques and Computed Tomography

Basma Mohamed Kamal*

Anatomy and Embryology Department, Faculty of Veterinary Medicine, Sadat-City University, Sadat City, Egypt.

Abstract:
The study was conducted to detect the sites of primary ossification centers and their sequence of appearance in the forelimb bones of white New Zealand rabbit's fetuses (*Oryctolagus cuniculus*) by using double staining method and computed tomography. The results showed that the complete chondrification of the primitive bone of the forelimb scapula, humerus, radius and ulna, carpus, metacarpus and phalanx at 16th days postcoitum. The primary ossification centers of the forelimb firstly appeared in the diaphyses of radius and ulna, humerus and at two centers of scapula at 18th days, the ossification centers at metacarpus from I to III appeared at 22nd days, but in metacarpus of IV and V at 30th days postcoitum. The appearance of ungual phalanx at 22nd days postcoitum, but the ossification centers of the first three phalanges begin to appear at 30th postcoitum. By the end of 30th days, the ossification occurred at the most parts of scapula except the glenoid cavity, also the ossification of humerus, radius and ulna begin to appear at epiphysis. This study can provide useful baseline information on the normal sequential pattern of chondrification and ossification in the forelimb in rabbit's fetuses by the computed tomography CT, and double stained technique.

Keywords: Ossification, forelimb, rabbits, double staining method, computed tomography.

INTRODUCTION

Rabbits are considered a popular house pet animal, and they are used on large scale as a laboratory model for researches due to its small size and descent in nature (Hristov *et al*., 2006), the pregnancy period in white New Zealand rabbits ranged from 31.71 ± 0.29 and 31.86 ± 0.33 days (Marai and Rashwan, 2003), the morphological studies of the development of bone and joints is necessary for early diagnosis of skeletal malformation in young age (Makkaway *et al*., 1988). The detection of limb ossification centers and chondrification provide a useful guide in the prenatal age estimation, and assessment of fetal bone maturation, and early diagnosis of congenital abnormalities of skeletal system of limbs (Oishi *et al.,* 1996).

The bones development in mammals is completed through two types of ossification; Intramembranous and endochondral, the endochondral type begin from proliferation, maturation and hypertrophy of chondrocytes, organized in ossification centers, to mineralization of cartilaginous matrix to form an osseous tissue. In case of long bones, endochondral bone development and bone elongation are associated with calcium deposition in the areas involved in architectural changes during the morphogenesis (Wongdee *et al.,* 2012). In a study done on the detection of ossification center of rabbit scapula by Elgendy et al. (2018) using histological and microscopic examination, it was found that; only two ossification centers appear at scapula of rabbit; one within the body of scapular blade and the second one in the scapular spines . Secondary ossification centers of humerus of rabbit appeared in the epiphyses of the humerus at first day postnatal (Fukuda and Matsuoka, 1981).

Micro-computed tomography (micro-CT) is an accurate imaging technology which has widely used since 2004. This advanced technique technology include precise analysis of boney structure and softy tissues, also it is widely practiced in the field of developmental embryology and toxicology. Now days, it is applied in the field of genetic embryology to evaluate the embryo that subjected to chemicals or any genetic deformities, and this technique will provide the scientist with a powerful and efficient means of scanning, reconstruction, visualization, segmentation, and analysis of micro-CT generated images of embryo development at prenatal stages (David *et al.,* 2013; Aoyagi *et al.,* 2012).

Micro-CT evaluations of rodent embryos have all used fixed specimens, and almost exclusively have included the use of various contrast stains to visualize soft tissues. Most interest has been with identification and quantitation of soft tissue structures, since skeletogenesis is just starting in the embryonic period. One of the first detailed studies of mouse embryos used micro-CT to examine fixed and OT-stained specimens between GD 9.5 and 12.5 (Aoyagi *et al.,* 2012; Johnson *et al.,* 2006).

The Combination between the histologic or microscopic view with the computed tomography for elucidating the sequential development of ossification centers of rabbits forelimbs and hence they give an accurate detection, and there is a little knowledge about the morphogenesis of fetal skeletal system and the prenatal development of the fore limb bones of rabbits fetuses such as; scapula, humerus, radius, ulna, carpus, metacarpus, and digits, and the detailed sequence of appearance of ossification centers of each and the times at which they develops. The purpose of this study was to determine the sequential progress involved in the chondrification and formation of ossification centers of forelimb bone in New Zealand rabbits in the prenatal stage from 16th day till birth by the double stained technique and computed tomography CT.

MATERIALS AND METHODS

Ethical consideration for laboratory animals

This study followed the guidelines for the care and use of laboratory animals and the animal welfare and ethics committee of the Faculty of Veterinary Medicine, Sadat City University, Egypt. The local animal ethics review panel approved this protocol.

Embryos collection and preparation

In this study, ten female white New Zealand rabbits collected from the distributed area of Behera governorate were naturally mated at the same day, then they were individually housed in suspended stainless-steel cages in environmentally controlled rooms with an approximately 12-hour light/dark cycle, and fed ad libitum, embryos were obtained from pregnant rabbits by cesarean section after anesthesia using ketamine (25 mg/kg) and sacrificed, only 5 fetuses were obtained from each female, and collection was as follow; (16^{th}, 18^{th}, 19^{th}, 20^{th}, 21^{th}, 22^{th}, 23^{th}, 25^{th}, 28^{th}, 30^{th} day post coitum), CVR length of fetuses was measured.

Double- Staining technique

The method was followed after (Cortés-Delgado *et al.,* 2009), in which the fetuses were skinned, then abdomen was opened to eviscerate the internal organs and the subcutaneous fat between the scapulae and along the back of the neck and then fixed 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 2% to remove muscle, and few drops of H_2O_2 were used for good results, exposure time was taken according to the age as follows; at 16^{th} days (15 min), 18th days (20 min), 19th days (25 min), 20th days (120 min), 21th days (180 min), 22th days (180 min), 23th days (360 min), 25th days (720 min), 28th days (1080 min), 30^{th} days day post coitum days (1440 min), for Cartilage staining the specimen was completely immersed in a mixture of alcian blue 10 mg 8GN, with 80 ml of 95 % ethyl alcohol, and 20 ml of glacial acetic acid for 2 days, for Rehydration, Place the specimen in a bath of 95 % ethyl alcohol for 2 hours, and repeat for 2 hours in a new bath. Place the specimen in baths of successively decreasing concentrations of 75 %, 40 %, and 15 % ethyl alcohol (2 hours per bath). Store the specimen in distilled water for about 8 hours, this step also helps to reinforce the fixing of the alcian blue to the cartilage. For Bone staining, transfer the specimen to 1.25 % aqueous KOH, then add alizarin red S in powder form until the solution becomes deep purple in color. Put the specimen in this solution for 24 hours. (If the specimens are overstrained, placed in a solution of 2 parts of 70% ethanol, 2 parts glycerin, and 1 part benzyl alcohol for 1 day). Transparency process was performed in four successive steps, according to (Soysal *et al*., 2011) as follows; 1st step: stained fetuses were put in 1% KOH for 1 day. 2nd step: they were put in 80 cc. of 1% KOH and 20 cc 20% glycerin for 5 days. 3rd step: they were put in 50 cc 1% KOH and 50 cc 50% glycerin for 5 days. 4th step: they were put in 20 cc 1% KOH and 80 cc 80% glycerin for 5 days. Then samples were examined in a glass dish by submerging in ethanol: glycerin to minimize glare during the examination using stereoscopic microscope at a magnification of 30X and were photographed. The stained samples transfer to glycerin (with a few crystals of phenol to prevent mold growth for storage (Kamal *et al.,* 2016).

Computed tomography and 3D reconstruction

In this study, all micro-CT imaging were visualized and analyzed using the Amira® 4.1 software platform (Mercury Computer Systems, Inc., Chelmsford, MA) running on a Windows XP (Microsoft Corp., Redmond, WA) workstation with 4 Gb of RAM and an Nvidia Quadro FX 5500 graphics card (Nvidia Corp., Santa Clara, CA). Individual fetuses were loaded into the Amira® image analysis software and visualized using the Voltex module with the Volrenglow color map. This module applies a color map to the image volume based on the individual voxel values. The colored 3D volume rendering of the fetus manipulated and rotated to visualize the

entire fetus at any angle (David *et al.,* 2013; Aoyagi *et al.,* 2012).

RESULTS

Results of the present study revealed the CVR length of recovered fetuses at different post coitum days as follow; it was 9-11 mm at 16 day postcoitum, 10-12 mm at 18 day postcoitum, 12-13 mm at 19 day postcoitum, 30-35 mm at 20 day postcoitum, 40-42 mm at 21 day postcoitum, 42-45 mm at 22 day postcoitum, 55-65 mm at 23day postcoitum, 67-70 mm at 25 day postcoitum, 71-73 mm at 28 day postcoitum, 74-75 mm at 30 day postcoitum, and the following results is ordered according the part of forelimb as follow;

Scapula

The double staining techniques clarified the complete chondrification of the scapula occurred at 16th days postcoitum (Figure 1), the appearance of two main ossification centers; one at the body or the center of scapula and the second at scapular spine, at 18th days postcoitum (Figure 2), Ossification extended bidirectionally in both sides and elongation of the scapula continuous until taken mostly the shape of the future scapula. Appearance of the scapular spine ossification extended to acromion process and begin of the ossification of metacromion process (Figures 3, 4, and 5). At 25th days postcoitum, ossification of supraspinous (SU)and infra spinous fossi (IN), scapular spine(SS) , ossification of acromion(AC) and appear of metacromin process (Figure 6), meanwhile the glenoid articular surface, coracoid process still cartilage 28th days postcoitum (Figure 7), finally; ossification of matacropmine (MC) and Coracoid process occured at 30th days postcoitum (Figure 8). For Computed Tomography; 3D reconstruction C.T has been elucidated the sequences of development of scapula; at 21st day old embryo, the scapula begin to appears, and increased the size gradually at 28th day old embryo till reach the 30th day old and most of the scapula structure has been completed (Figures 4, 7, and 8).

Humeuus

In the double staining techniques for humerus, it showed the complete chondrification of the humerus at 16th days postcoitum (Figure 1). The ossification center at the middle of diaphysis of humerus occurred at 18th days postcoitum (Figure 2), the ossification area increase toward the extremities it reach two third of shaft at 19th days postcoitum (Figure 3), the growth of ossification center involve the whole length of shaft of humerus and the extremities still completely cartilage at 25 days postcoitum (Figure 6), ossification center involve the whole length of shaft of humerus and begin of epiphysis ossification at 30 days postcoitum (Figure 8). For Computed Tomography; 3D reconstruction to follow the sequences of prenatal progress of the ossification center of humerus, at 21st day embryo the appearance of the part of the shaft of humerus begin to appeared (Figure 4), at 28th day embryo the appearance of the whole length of shaft of humerus only (Figure 7), finally at 30th day embryo the appearance of the whole length of shaft of humerus and parts of epiphysis (Figure 8).

Radius and ulna

The complete chondrification of the radius and ulna occurred at 16th days postcoitum by the use of double staining techniques (Figure 1), the ossification center at the middle of diaphysis of radius and ulna at 18th days postcoitum (Figure 2), the ossification area increase toward the extremities it represent the two third of shaft at 19th days postcoitum (Figure 3), the ossification at all diaphysis of radius and ulna and appearance of proximal epiphysis of radius and ulna has completed at 25th days postcoitum (Figure 6). The ossification at all diaphysis of radius and ulna and appearance of proximal epiphysis, and the semilunar notch appear partially ossified olecranon still cartilaginous at

28th days postcoitum (Figure 7), appearance Ossification of olecranon at 30 days postcoitum. For CT, at 28 day, the appearance of the whole length of shaft of radius and ulna only has done (Figure 7), at 30 day embryo the appearance of the whole length of shaft of humerus and parts of epiphysis and olecranon of ulna (Figure 8)

Carpus

The carpal bones still cartilaginous till 30 days postcoitum by double stained and CT. ***metacarpaus;*** Double staining techniques clarified the chondrification of metacarpus still at 21 days postcoitum (Figure 4), appearance of the ossification centers at metacarpus from I to III at 22 days postcoitum (Figure 5), appearance of the ossification centers at metacarpus of IV and, V at 30 days postcoitum by double stained and C .T (Figure 8).

Digit

The appearance of ungual phalanx at 22 days postcoitum (Figure 5), and appear of the phalanx ossification of fist three phalanges by double stained and C. T at 30 days postcoitum (Figure 8).

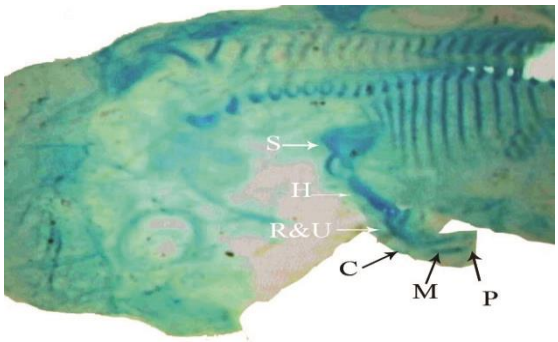

Fig. 1. Lateral view of 16 days postcoitum double stained (d.p.c) embryo show the complete chondrification of the primitive bone of the forelimb (S) scapula, (H) humerus, (R&U) radius and ulna , (C) carpus, (M) metacarpus and (P) phalanx

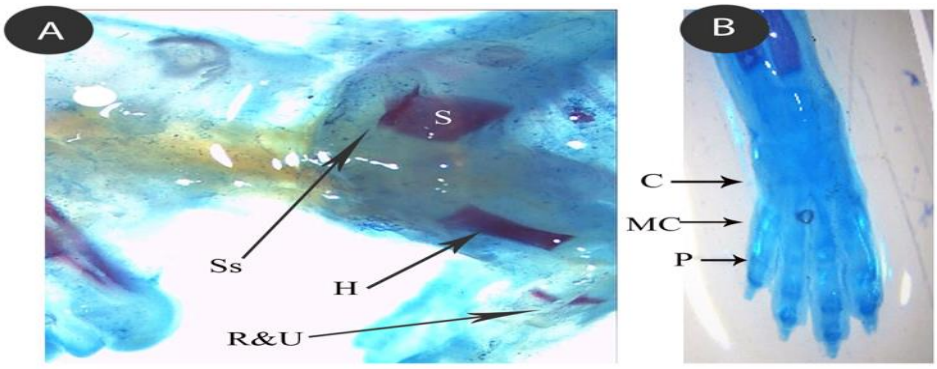

Fig. 2. (A) Lateral view of the chest of 18 days postcoitum double stained (d.p.c) embryo show that two ossification center at scapula one at the (S) center of scapula and at (sc) scapular spine, (H)ossification center at the middle of diaphysis of humerus ,(R&H) ossification center at the middle of diaphysis of radius and ulna. **(B)** Dorsal view of of manus region of forelimb of 18 days postcoitum double stained (d.p.c) embryo show that chondrification is still presents at , (C)carpus ,(M) metacarpus and (P)phalanx

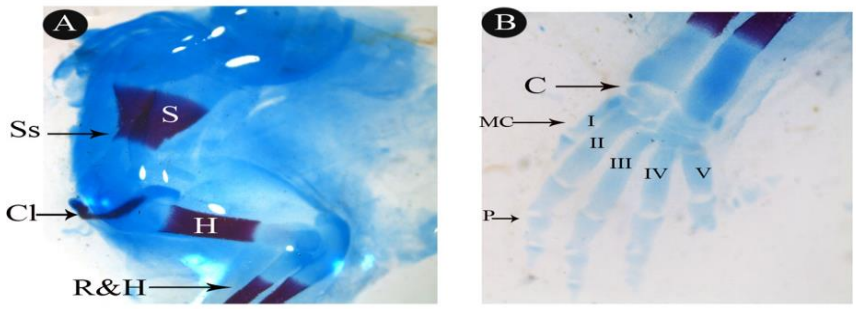

Fig. 3. (A) Lateral view of the chest (forelimb) of 19 days postcoitum double stained (d.p.c) embryo show that two ossification center at scapula one at the (S) center of scapula and at (Ss) scapular spine, (H)ossification center at the middle of diaphysis of humerus ,(R&U) ossification center at the middle of diaphysis of radius and ulna and appear of ossification center of clavicle. **(B)** Dorsal view of of manus region of forelimb of 19 days postcoitum double stained (d.p.c) embryo show that chondrification is still presents at , (C)carpus ,(M) metacarpus from I to V and (P) phalanx

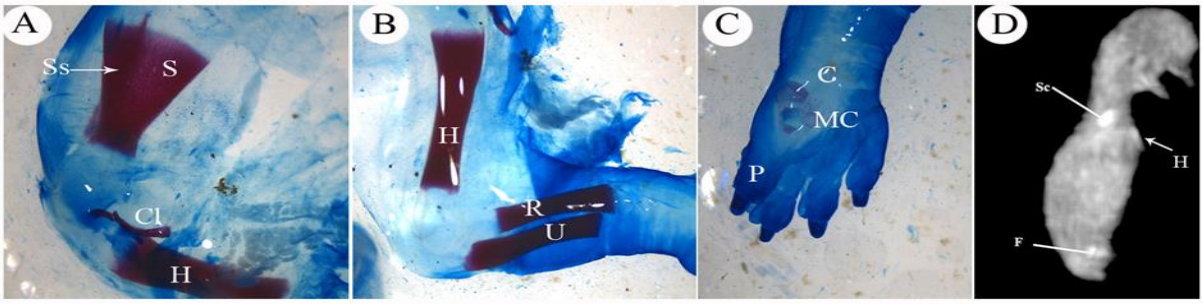

Fig. 4. (A) Lateral view of the chest **(B)** lateral view of arm and forearm **(C)** dorsal view of Manus of 21 days postcoitum double stained, (d.p.c) and **(D)** 3D reconstruction CT of 21 embryo Explained that, two ossification center at scapula one at the (S) center of scapula and at (Ss) scapular spine, (H) ossification center at the middle of diaphysis of humerus, (R&U) ossification center at the middle of diaphysis of radius and ulna and (CL) appear of ossification center of clavicle, the chondrification is still presents at (C) carpus, (M) metacarpus from I to V and (P) phalanx

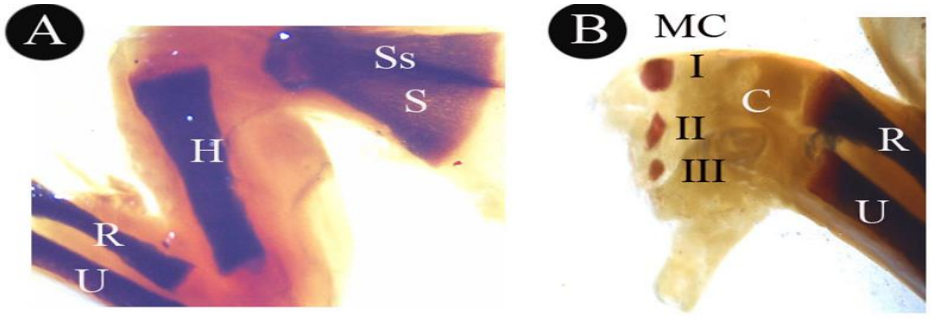

Fig. 5. (A) Lateral view of the the shoulder , arm and for arm **(B)** dorsal view of Manus of 22 days postcoitum double that stained showing that; two ossification center at scapula one at the (S) center of scapula and at (Ss) scapular spine, (H)ossification center of humerus Increases in size Involve nearly the most length of diaphysis,(R&U) ossification center of radius and ulna Increase in the length toward the two extremities, (C)carpus ,(M)appearance of the ossification centers at metacarpus from I to III

Fig. 6. (A) Lateral view of the shoulder region l. **(B)** lateral view of arm and for arm. **(C)** dorsal view of Manus of 25 days postcoitum double stained, (d.p.c) Explained that, appear of scapular spine ossification of the acromion process and begin of the ossification of metacromin process, (H) ossification center humerus growth involve the whole length of shaft and the extremities still completely cart, (R&U) ossification at all diaphysis of radius and ulna and appearance of proximal epiphysis of radius and ulna, (C) carpus, (M) appearance of the ossification centers at metacarpus from I to III and (P)phalanx

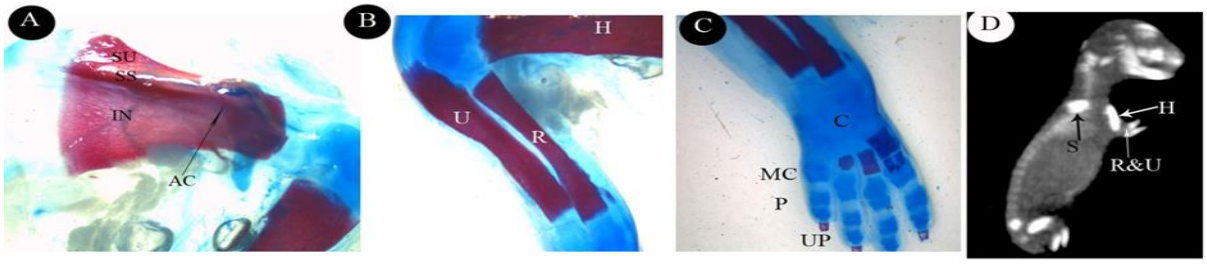

Fig. 7. (A) Lateral view of the shoulder region l. **(B)** lateral view of arm and for arm. **(C)** dorsal view of Manus of 28 days postcoitum double stained, (d.p.c). **(D)** 3D reconstruction CT of 28 embryo showing that; Ossification of supraspinous (SU)and infraspinous fossi(IN), scapular spine(SS) , ossification of acromion(AC) and appear of metacromin process meanwhile the glenoid articular surface, coracoid process still cartilage . (H)Growth of ossification center involve the whole length of shaft of humerus and the extremities still completely cartilage (R&U) ossification at all diaphysis of radius and ulna and appearance of proximal epiphysis, Semilunar notch appear partially ossified olecranon still cart. ,(C)carpus ,(M)appearance of the ossification centers at metacarpus from I to III and (P)phalanx (UP) ungual phalanx. Complete ossification of acromion and metacromin meanwhile the glenoid articular surface, coracoid process still cartilage.

Fig. 8. (A) Lateral view of the shoulder arm and forarme regions. **(B)** dorsal view of Manus of 30 days postcoitum double stained, (d.p.c) **(C)** 3D reconstruction CT of 30 days postcoitum embryo showing that; Ossification matacropmine (MC) and Coracoid process (C) (H) Growth of ossification center involve the whole length of shaft of humerus and Begin of epiphysis ossification (R&H) ossification at all diaphysis of radius and ulna and appearance Ossification of olecranon, (C)carpus still cartilagionous, (M)appearance of the ossification centers at metacarpus from I to IV and (P)phalanx ossification of fist three phalanges (UP) ungual phalanx.

DISCUSSION

Several authors have been studied the in-utero development of skeletal system, one of them was Maruyama (2011) who reviewed the skeletal development in prenatal stage and he stated that, the skeleton of vertebrates is divided into four components which are the skull, limbs (appendicular skeleton), vertebral column, and ribs with sternum, that is originated from the mesodermal germ layer and the neural crest, the limbs are firstly formed from cartilage then replaced with bone at end of gestation period, that is called endochondral ossification, but in flat bones as skull, is formed directly from mesenchymal cells without formation of cartilage and this type is called intramembranous ossification. One of the obstacle for embryology researchers is to completely clarify and understand the basic pattern of appendicular skeleton especially the scapular growth in utero which differ from long bones of limbs, as mentioned in previous studies (Terence *et al.,* 2010; Rivas and Shapiro, 2002; Ahmed *et al.,* 2015). Many researches on in-utero development of the appendicular skeleton in rabbit were concerned mainly with the long bone not scapula, only. Elgendy et al. (2018) in Egypt had worked on the prenatal development of scapula and forelimb of rabbits fetuses from day 11^{th} till birth, and they found that; during the prenatal stage the ossification of scapula passed by many steps, and there were only two centers for ossification in scapula, one at the body or the center of scapula and the second at scapular spine, and it appeared at 18^{th} days postcoitum, finally the ossification occurred at the most parts of scapula except the glenoid cavity at day 30^{th}, and this was agreed with the results of the present study. Our results are coincided with previous findings in the human scapula by Ogden and Phillips (1983) in that both of the ossification sites are found near the glenoid cavity than to the vertebral side.

The present study clarified that; the scapular spine joined with the acromion and metaacromion processes forming a structure like a hock, but the proximal extremity of the spine was still ill developed, which is coincided with the scapula of marsupial mammals as stated by Marcelo et al. (2003), also the finding of the present study was coinceded with Menegola et al. (2002) who conducted a similar study in rats and they mentioned that at day 21 prenatal, the ossification center in scapula extended in both sides and the prolongation of scapula is continued till it take the definitive shape. Benazet and Zeller (2009) have been clarified that; the longitudinal growth rates of scapula 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 infra-spinous than in the supra-spinous fossa. Result of the present study is confirmed this issues, that ossification of most parts of the scapula was a prenatal event meanwhile in dog, mouse, and in human, in which the acromion process, gleno humeral cavity and the vertebral border remained cartilaginous until birth. David et al. (2013) stated that, the use of computed tomography technology has gone beyond evaluation of bone anatomy and density to include now analysis of soft tissues imaged by contrast agents.

In our study, we have used some of image 3D reconstruction techniques for clarifying the bony structures of forelimb in fetuses of white rabbit CT images. The main aim of this work is to provide the reference evaluation of the appendicular skeleton during the chemical or toxicological studies in rabbits during prenatal stage till birth. Collins et al. (2007) had conducted a study and proved that micro-CT showed the feasibility of embryos phenotype in preparation for the International Knockout Mouse Consortium, and they stated that, resolutions of 8 and 27 mm were achieved; however, scan times were 12 and 2 hr, respectively. Segmentation of the 27mm images revealed the complex 3D neural tube malformations of Pax3: Fkhr transgenic

embryos, although quantitative measures were not collected. Micro-CT was projected to be a significant improvement in resolution, time, and expense over other potential tools (e.g., MRI and histology) for quantitative analysis of developmental abnormalities induced by genetically engineered mutations or chemical/drug-induced embryo toxicity (Boone *et al.,* 2004). Nagase et al. (2008) questioned the need for the long scan times and use of OT, so investigated a more rapid protocol that used hexamethyldisilazane as a contrast stain. Images of normal and abnormal GD 10–11 mouse embryos were presented. The results of present study was in agreement of all above mentioned data and it showed that; Computed Tomography; 3D reconstruction has been elucidated the sequences of development of scapula; at 21th day old embryo, the scapula begin to appears, and increased the size gradually at 28th day old embryo till reach the 30th day old and most of the scapula structure has been completed and clearly viewed. Several authors had worked on the prenatal development of the appendicular skeleton ossification centers such as; Fukuda and Matsuoka (1981), who had mentioned that, the ossification centers occurred along the whole length of the shaft of humerus, in which the primary ossification center start from the epiphysis at day 30 prenatal, and the secondary ossification center begin at first day postnatal that present in the proximal and distal epiphysis of the humerus, at the same time, Ahmed et al. (2015) found that; the secondary ossification center was first seen at 3rd day postnatal within the epiphysis by the histological methods, these studies was agreed to the present study that begin from day 16 till birth only and it revealed that; The ossification center at the middle of diaphysis of humerus occurred at 18th days postcoitum, and the ossification area increase toward the extremities till reach two third of shaft at 19th days postcoitum, the growth of ossification center involve the whole length of shaft of humerus and the extremities still completely cartilage at 25 days postcoitum, ossification center involve the whole length of shaft of humerus and begin of epiphysis ossification at 30 days postcoitum. On the other hand, DeSesso and Scialli (2018), had proved that, the metacarpals (bones of the palm of the hand) was ossified before the phalanges (bones of the fingers), because the phalanges undergo ossification near the time of birth in rodents and rabbits, and some sort of delay in the onset of ossification, also some time the early collection of fetuses may lead to reducing the numbers of ossification centers in the forepaws. There are a few papers that describe the ossification of the thumb beginning during the week before pregnancy termination (Danielson and Kihlstr€om, 1986), and this study provides an indication of the variability in the schedule of ossification in control rabbits. Understanding the schedule of development for the bones of the digits is important because evaluation of near-term rodent and rabbit fetuses for potential developmental toxicity involves inspection of the skeletal system, including the bones of the paws.

The results of the present study were in agreement with previous authors, in that the complete ossification centers occurred at all diaphysis of radius and ulna and appearance of proximal epiphysis of radius and ulna has completed at 25th days postcoitum and the semilunar notch appear partially ossified olecranon still cartilaginous at 28thdays postcoitum, and at 30 day embryo the appearance of the whole length of shaft of humerus and parts of epiphysis and olecranon of ulna which is completed postnatal. At the same time, the carpal bones still cartilaginous till 30 days postcoitum. The ossification centers of metacarpus I to III start at 22 days postcoitum, but metacarpus IV and, V at 30 days postcoitum. Finally for digit; the ossification centers of ungual phalanx at 22 days postcoitum, ossification of fist three phalanges at 30 days postcoitum. Previous had reviewed the micro-CT and mentioned that, there is a significant progress in the application of this imaging toward problems in developmental biology and toxicology studies (Guldberg *et al.,* 2004; Boone *et al.,* 2004;

Degenhardt *et al.,* 2010). As others have pointed out, micro-CT holds the possibility of directly relating quantitative assessments of morphogenesis with assays of structural or regulatory molecules, and thus providing new approaches to the mechanistic basis of development and dysmorphogenesis, and they have seen more refined analyses of bone growth and development, as well as of soft tissues with the use of contrast agents and stains. At the same time, other researchers had stated that, the tools for analysis have advanced such that new images can be automatically mapped to an atlas of a normal organism and thus aid in the detection of changes to growth and morphology (Dogdas *et al.,* 2007; Ford *et al.,* 2003; French *et al.,* 2012; Gregg *et al.,* 2012; Guldberg *et al.,* 2004).

Such automated analyses have provided a tool for screening the phenotype of knockout mice generated from the IKMC, and will soon provide the tool for identifying skeletal abnormalities in fetuses from EFD toxicity studies. French et al. (2012) stated that, to date, we are not aware of any micro-CT-based studies that have examined the prenatal fate and sequential development of normal appendicular skeletal variations in rabbit fetuses, and hence the data provided by this study will be very important to the researchers that deals with chemical, drugs or toxicant that exert a teratogenic or congenital deformities in prenatal stages till birth.

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CONFLICT OF INTEREST

The author declares that there are not significant personnel, professional or financial competing interest that might have influenced the presentation of the results of the study described in this manuscript.

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