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Development of Sternum and Ribs in White New Zealand Rabbit (*Oryctolagus cuniculus)*

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ABSTRACT

The bone development and the assessment of the fetal skeletal improvement turn into a basic segment in the behavior of a prenatal toxicity study. The sternum is a unique bone in the distinctive shape and development. This study focuses on the development of the sternum and the ribs attached in the whit New Zealand rabbit, Specimens were collected prenatally (n=30) and postnatally (n=30). A group of specimens were double stained for bone and cartilage using Alizarin Red and Alcian blue. Another group was scanned by CT. The sternum was consisted of manubrium, four sternebrae and xiphoid cartilage. The sternum was in communication with 6 pairs of ribs. 7 ossification centers appeared in the sternum, one for each segment except the fourth one which has two. The first group centers appeared in the manubrium and the first two sternebrae on 22 days old embryos. All primary centers seen before birth. There are no secondary ossification centers for the sternum. Concerning ribs, there are four ossification centers for each except the last two ribs. The ossification centers of the body came from the extension of the transvers process of the thoracic vertebrae and it appears as early as two weeks of gestation. By the end of the third week of pregnancy, the ribs show primary center of ossification from the second to the last one. The second center was designed for the head and it appeared two weeks after birth. The third and fourth centers for the tubercle appeared a month after birth. Complete fusion between these centers takes place in three months old rabbit.

Key words: Rabbit, Sternum, Ribs, Development, Double staining, CT

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INTRODUCTION

The rabbit has been repeatedly used as a model to elucidate the typical and strange bone development since it takes after a fundamentally the same as example to the human infant, these factors are considered extremely valuable for the research in human models (Alberius and Selvik, 1986). The sternum is present in the mid ventral region of the thorax of the rabbit. The sternum is one of the skeleton parts with frequent variation in appearance (Jitender et al., 2015). It is made by five bony pieces called "Sternebrae". The most cranial sternebrae is called the presternum (manubrium). The sixth sternebra is the xiphisternum, which ends with xiphoid cartilage (Goodman et al., 1983; Gray et al., 1995; Graeber and Nazim, 2007; Restrepo et al., 2009). Seven ribs are articulated with sternum by their cartilage part

In human, the sternum developed over a long period of time which begins during the prenatal period and continues through the third and fourth decades of the postnatal period (Williams et al., 1989; Kozielec et al., 1973; O'Rahilly and Muller, 1992 and O'Neal et al., 1998). Moreover, the stages and pathways of appearance of the ossification centers in the skeleton during intrauterine in rodents and rabbit were considered a reliable indicators of fetal maturity in teratological experiments. In rats and mice, the ossification of the parietal and occipital bones, distal limb skeleton, sternum, and vertebral centra is particularly indicative (Strong, 1960).

The normal development of skeleton has been described for laboratory animals such as rat (Menegola et al., 2002) and hamster (Bruce and Hindle, 1994), mouse and rat (Fukuda and Matsuoka, 1980). However, there is no information available regarding normal development of ossification centers in the sternum and ribs of rabbit. This work is to determine the number and the time of the initial ossification centers for the sternum and ribs in the white New Zeeland rabbit. Moreover, the age of fusion of the resultant bony elements will be determined.

MATERIALS AND METHODS

The present study was conducted on white New Zealand rabbits (*Oryctolagus cuniculus)*, that were collected from different areas of Beheira and Cairo governorate, Egypt.

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Ethical approval

The use of the animal species was approved, before the study began, by the Institutional Animal Care and Use Committee (IACUC) of the faculty of veterinary medicine, university of Sadat city, Egypt.

Prenatal samples

Eight female rabbits were naturally mated at the same day and the day of coitus was considered as zero gestation day, then rabbits were sacrificed, the abdomen was opened the uterus to get the fetuses, which were collected on day 16th through 28th of pregnancy, n=30specimens (at least five specimens for every age). All specimens were counted and measured by metal caliber for the Crown-Rump Length (CRL) is the measurement of the length of embryos and fetuses from the top of the head (crown) to the bottom of the buttocks (rump). Fetuses were skinned, eviscerated and fixed in 95% ethyl alcohol for at least seven days.

Postnatal samples

A total of (30) rabbits were used at the ages of 1st day, 3rd day, 7th day, 15th day, 30th day, 45th day, 60th day, and 90th day. Animals were bled, skinned, eviscerated and stored in neutral buffer containing formalin 10%.

Double staining technique

The fetuses were held in 95% Ethyl alcohol for at least seven days, then placed in acetone for two days for fat removal. Maceration process was performed with different grades of potassium hydroxide(KOH) to remove muscle according to the age of the sample, in our laboratory 2% solution is used for the prenatal specimens and in the postnatal samples we used the concentration of 4% KOH solution.

For staining ossified bone, the Alizarin Red is enough. However, for staining the bone and cartilage, Alizarin Red and Alcian blue have been recommended by many researchers (Inouye, 1976; Kimmel and Trammel, 1981; Webb and Byrd, 1994 and Redfern et al., 2007). Immersion of the specimens in a mixture of Alcian blue for two days. After two days, the specimens were rehydrated in degrading series of alcohol. The specimens were immersed in this solution for 24 hours.

Transparency process

This process was performed in four successive steps:

1st step: stained fetuses were put in 1% KOH for one day. 2nd step: they were put in 80 cc 1% KOH and 20 cc 20% glycerin for five days. 3rd step: they were put in 50 cc 1% KOH and 50 cc 50% glycerin for five days. 4th step: they were put in 20 cc 1% KOH and 80 cc 80% glycerin for five days. Whole stained embryos were examined using stereoscopic microscope at a magnification of 30× and were photographed.

Computed tomography and 3D reconstruction

Settings for the Computed Tomography (CT) image technique in this study were as follows: 120 kV, 200MA and the image acquisition time was approximately 30 seconds at 2.00 mm thickness.

RESULTS

Sternum

The sternum was present as the cartilaginous bar in the ventral aspect of the chest wall at 16days old embryo. The sternum was formed of six sternebrae; the first one formed manubrium, four other sternebrae formed the mesosternum and the last one formed the xiphoid cartilage. Each sternebrae had a single ossification center. At the age of 22days old embryo, ossification centers appeared in the manubrium and the first two sternebrae. Within the next 24 hours, an ossification center appeared in the third sternebrae (Figure 1a). At the age of 25days old embryo, the fourth ossification centers appear in the fourth sternebrae and the xiphoid one. The whole sternum showed ossification centers before birth (Figures 1b, 2b).

The manubrium of the new born rabbit as well as the first three sternebrae had one primary ossification center. The fourth sternebrae had two ossification centers, primary and accessory ossification center. It was a small center distal to the primary center (Figure 1c). After one week, the manubrial ossification center was a large composite mass (Figure 2a) and the other three noteworthy essential centers of the sternebrae had lost all remnants of midline coalescence. Two weeks after birth, an increase in the size of ossified part and decrease in the cartilage between the sternebrae was observed (Figure 2c). In one-month old rabbit, ossification became increasingly evident as the ossification centers expand out toward the margins of the

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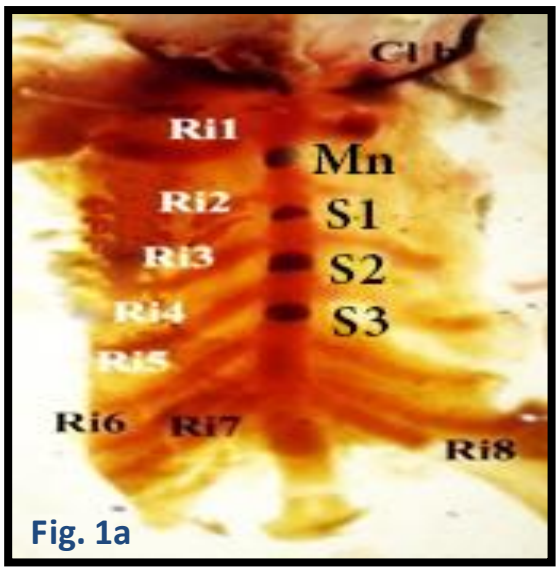
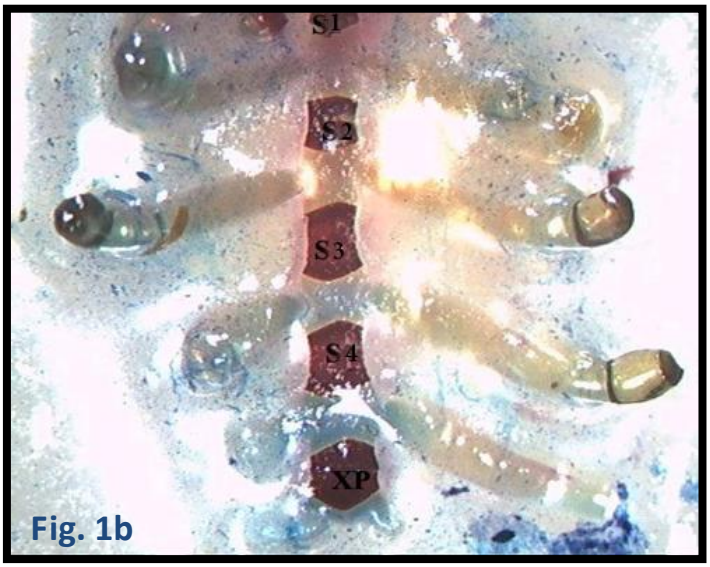
sternebrae and towards each other, they gradually developed small concavities at the junctions with the costochondral segments. 15 days later, the sternebrae developed a marginal lip at the junction of the costochondral segment with the sternum. Similarly, at the cranial end, the manubrium continued to develop. In two months old rabbit, the sternal segments two through four had undergone coalescence, although interestingly they still showed a longitudinal ossification response to the attachments of the rib costochondral segments. The original interosseous cartilage was being replaced by a bony plate. Three months after birth, complete ossification occurred in the sternum. It acquired the adult shape which formed the cartilaginous ends of the first pair of ribs articulate directly with the sides of the manubrium, the second through fifth pairs of ribs articulated with the inter sternebrae cartilages of the manubrium and the first to third sternebrae and the sixth and seventh pairs articulated with the cartilage between the fourth sternebrae and xiphoid process (Figure 2d).

Ribs

By the 15 days' post coitum, the transverse process of vertebrae in the thoracic part of the column were much longer than those of the other vertebral region. They extended ventrolaterally following the curve of the body wall forming the rib primordium. Is vertebral end formed a head, a neck, a tubercle and a shaft.

After three weeks of pregnancy in rabbit, the shaft of all ribs showed a primary center of ossification (Figure 1a 3a, 3b, 4a, 4c). All primary centers of ossification for ribs were present before birth (Figure 3c and Figure 4b).





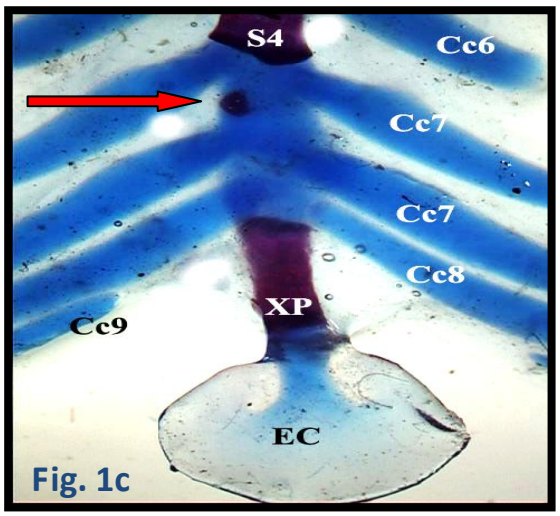


Figure 1.

A- Ventral view of rabbit sternum of 23 days' post coitum (dpc) Alcian blue and alizarin red S stained embryo show ossification centers of sternebrae 1-4 with the inter-sternal cartilage between the sternebrae attached to it the costal cartilage of ribs 1-8, the manubrium and xiphoid are still cartilaginous

B- Ventral view of the sternum of 28 days (dpc) double stained embryo of rabbit shows ossification of Sternebrae (S) 1-3 with the inter-sternal cartilage between the sternebrae attached to it the costal cartilage of ribs 1-4, the manubrium single ossification center Sternebrae (S) 1-4, Manubrium (Mn), Xiphoid Process (XP).

C- Ventral view of the sternum of new born rabbit double stained shows ossification of Xiphoid Process (XP), Ensiform Cartilage (EC), Ribs (Ri) from 6-9 costal cartilage. Red arrow; additional ossification center

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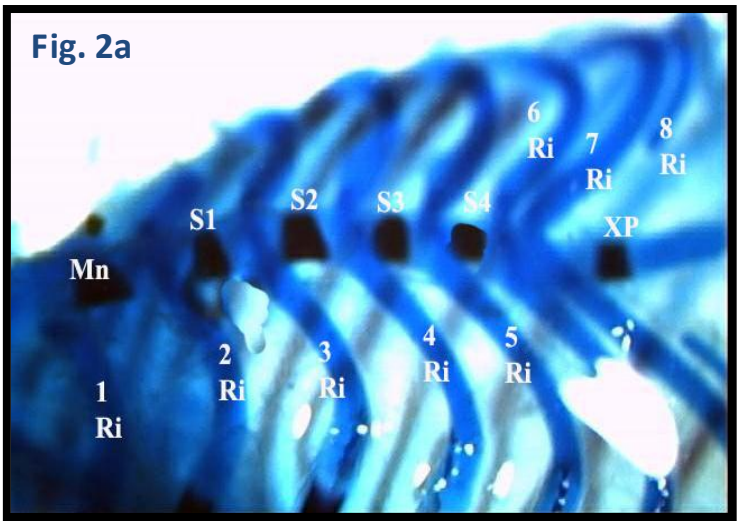

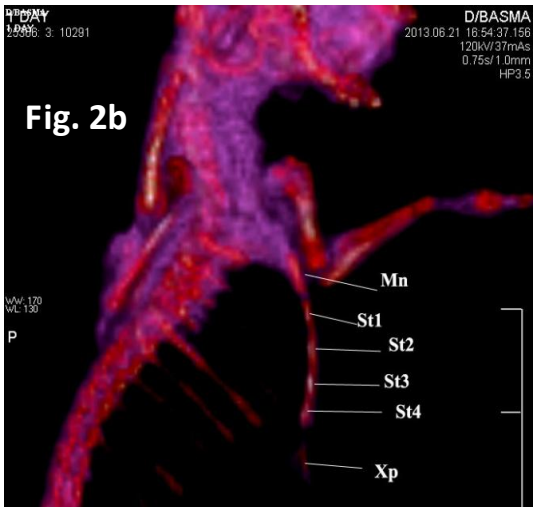

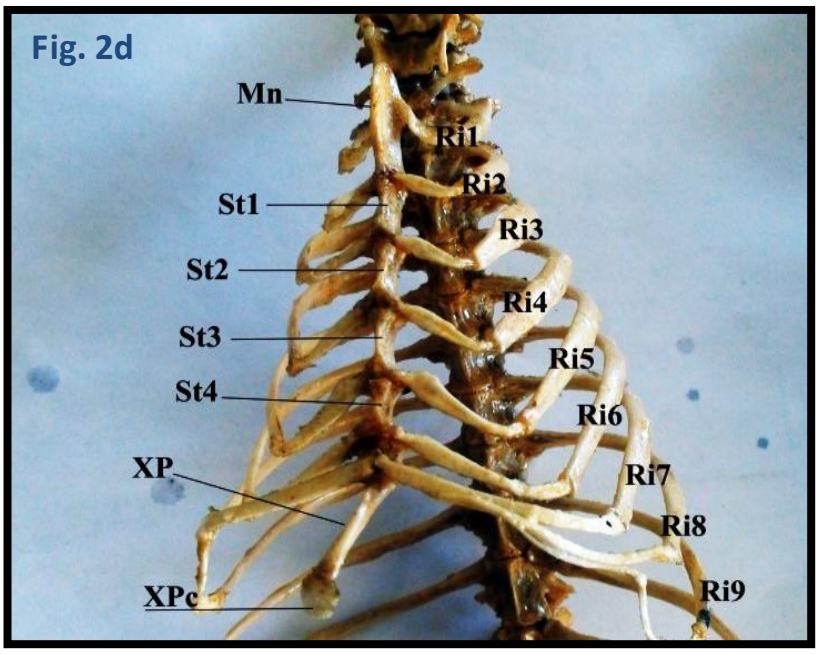

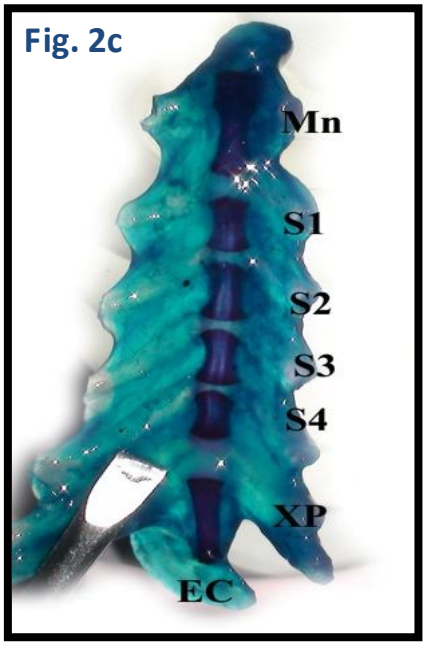

Figure 2.

A- Ventral view of the sternum of one-week old rabbit double stained shows ossification of sternebrae 2-4 attached to it Ribs (Ri) from 2-5 and in the inter-sternal cartilage between the 4th and xiphoid costal cartilage of ribs from 6-8.

B- Reconstruction of the axial skeleton lateral view of new born old rabbit showing the primary ossification center in the sternum manubrium (Mn), Sternbare1-4, (St. 1-4), and Xiphoid Process (XP).

C- Ventral view of sternum of two weeks old rabbit double stained showing ossification of Manubrium (Mn), Sternebrae (S) from 1 to 4 xiphoid Process (Xp), Ensiform Cartilage (EC).

D- Ventral view of sternum of 90-day old rabbit showing ossification of sternum parts manubrium (Mn), (St1-St4) sternebrae from 1 to 4, Xiphoid Processes (XP), Xiphoid cCrtilage (XC) attached to the sternum.

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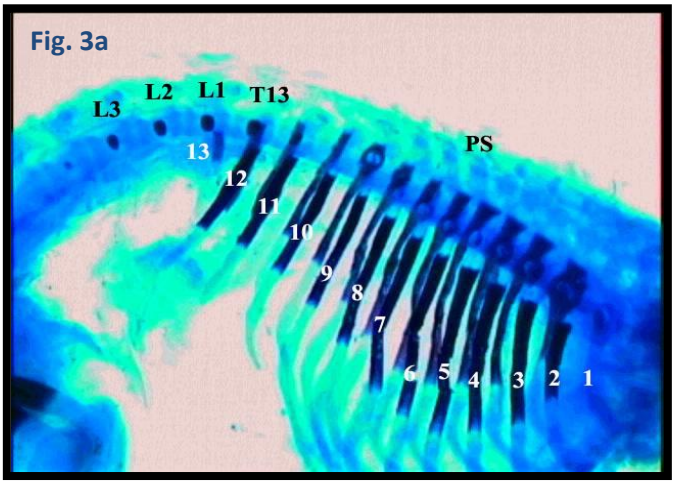


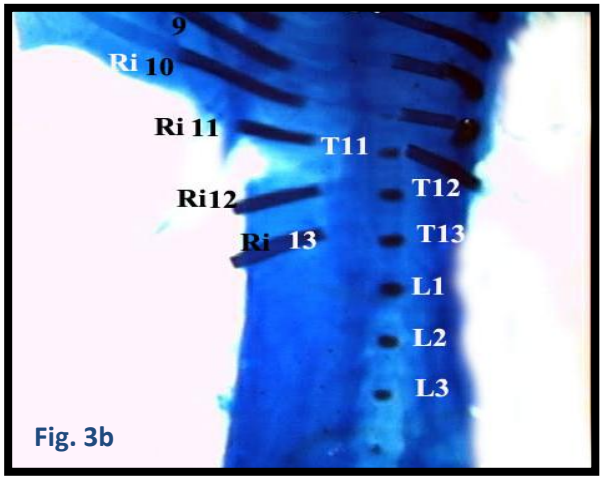
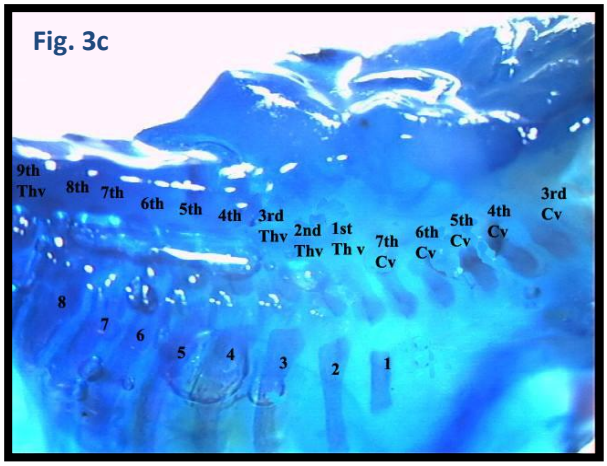


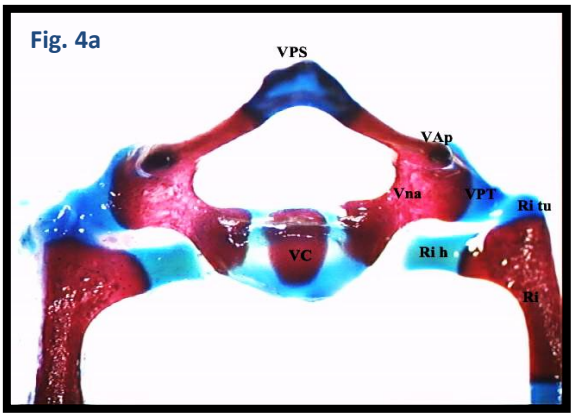
Figure 3.

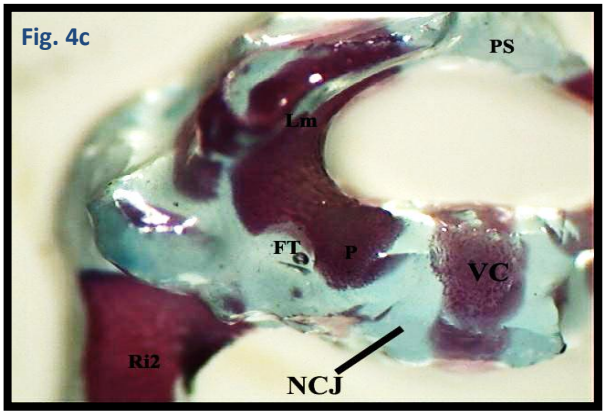
A- Lateral view of 21 days (dpc) double stained, in thoraco-lumber region of rabbit shows the ossification in the body of the ribs from (2-12) the first still cartilaginous and the last incompletely ossified, the vertebral centrum of the Thoracic (T) vertebrae 13 and first three Lumbar vertebrae, Processes Spinalis (PS) of the thoracic vertebrae are cartilaginous in nature.

B- Ventral view of 21 days (d.p.c) double stained, in thoraco-lumber region of rabbit shows the ossification in the body of the Ribs (Ri) from (9-13), the vertebral centrum of the Thoracic (T) vertebrae 11- 13 and first three lumber vertebrae.

C- Lateral view of cervico-thoracic region of 23 days (dpc) double stained of rabbit shows ossification in the body of the Ribs (Ri) from the 1-7, the cartilaginous nature of the Processes Spinalis (PS) and the ossification of the neural arch of the cervical vertebrae from the 1-7.







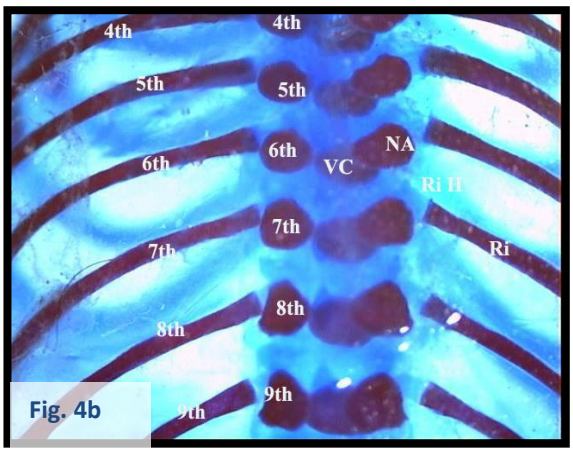


Figure 4.

A- Cranial view of typical thoracic vertebrae of 25 days (dpc) double stained of rabbit showing ossification of Vertebral Centrum (VC), Pedicle (P), Lamina (LM), Forman Transversus (FT), Processus Transversus (PT) is completely cartilaginous, Neurocentral Junction (NCJ), Processus Spinous (PS) is still cartilaginous, Rib (Ri) no.2.

B- Lateral view of typical thoracic vertebrae of 25 days (dpc) double stained of rabbit shows ossification of Vertebral Centrum (VC), Neural Arch (NA) and note that the 10th thoracic vertebrae anticlinalis (V Ac) with processus spinous is still cartilaginous.

C- Cranial view of first thoracic vertebrae of 28 days (dpc) double stained of rabbit shows ossification of Vertebral Centrum (VC), Pedicle (P), Lamina (LM), Processus Transversus (PT), Neurocentral Junction (NCJ), Processus Spinous (PS) is still cartilaginous, Rib (Ri), Rib head(Ri h), Rib tubercle (Ri tu).

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Fifteen days postnatal

An ossification center appeared in the head of the rib from the first one to the last one with cartilage collar separating the ossification center of the head from the body.

One month old rabbit

Another two ossification centers appeared in the tubercle one in the articular part and another one in the non-articular part. But in the last two ribs only single ossification center appeared as the head and tubercle was fused together.

Forty five days old rabbit

There was a marked increase in the total length of the ossified part of the ribs and appearance of ossification center at the tubercle of the rib with cartilage collar between the tubercle and the transverse processes.

Sixty days old rabbit

There was a marked decrease in the size of cartilage plate between the head and the tubercle and the body of the rib.

Ninety days old rabbit

A complete ossification occurred in the body of the ribs with bony fusion between the body, head and the tubercle (Figure 2d).

DISCUSSION

The normal development for laboratory animals, such as guinea pig (Gonzalez, 1932; Draper, 1920; Isben, 1928) and hamster (Bruce and Hindle, 1994) are depicted. In any case, there is no data accessible with respect to ordinary improvement of ossification centers in the sternum and ribs of rabbit.

The present study took after the ossification of the sternum and related ribs in the white New Zeeland rabbits prenatally and postnatally by the aide of the double recoloring procedure (Alcian blue and Alizarin red) and CT scanning.

Sternum

In human, the sternum begins to form in the lateral mesoderm plates during the sixth week of the prenatal period (Hanafi et al., 2014; Garriock et al., 2015). In orthograde (upright) Posture mammals, the sternum is typically made up of seven segments and differs from pronograde (quadrupedal) mammals in the shape of xiphoid process (Hill, 2016).

. In calf, the first focal ossification starts from the seventh sternal segment in 75 days old fetus (Lindsay, 1969). In the rabbit, the sternum begins to show up at 21 years old days old pre-birth that concurs with the consequences of Cozens (1965), who expressed that in the white New Zealand rabbit hardening begins on days 22 and 23 pre-birth. The human sternum was created from a couple of longitudinal mesenchymal buildups named sternal bars that structure in the ventrolateral body wall. The sternal bars fusion starts along the midline by the end of the 10th week of pregnancy (Hanafi et al., 2014). Fusion of the sternal bars will be finished with the formation of the xiphoid. In this study, the sternum was present as cartilaginous bar in the ventral aspect of the chest wall. Ossification of the sternal bars is an endochondral type. In human, it begins from the cranial to caudal part, producing the definitive bones of the sternum (Larsen, 1997). In this study the appearance of the ossification centers of the sternum runs in a Craniocaudal direction.

Ossification centers appear for the first two sternebrae at the age of 22 days old fetus then another ossification center appears in the third sternebrae. At the age of 25 days old fetus, an ossification centers appear in the fourth sternebrae. At the age of 28 days old fetus, the six segments of the sternum show ossification center. All previously mention data agree with the results in albino rat (Strong, 1961), in sheep (Harris, 1938) and in Sprague-Dawley rats (Alberius and Selvik, 1986) and in rabbit (Winkelmann and David, 2009).

At birth, in the human sternum, the calcified areas can only be observed in the manubrium and the mesosternum. Xiphoid process calcification appears at six years of age (O'Neal et al., 1998; Skandalakis el al., 1994; Hanafi et al., 2014). In this study, Xiphoid Processes (XP) ossification center appear before birth in rabbit. In human, three distinctive ossification models are portrayed by ossification of the manubrium and

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the body of the sternum during the postnatal period. Type one model is portrayed by one center in the manubrium and one center in the sternebrae of the body. Type two models are characterized by one center in the manubrium and in the first sternebrae of the body, two ossification centers in the other sternebrae. In the third model, the sternum has one center the manubrium and two centers in the sternebrae of the body. The type 2 ossification pattern was the most common pattern (Ashley, 956; Wong and Carter, 1988 and Hanafi et al., 2014 and Delgado et al., 2014). In rabbit the ossification pattern was similar to the type 1 ossification pattern; only two centers appeared in the fourth sternebrae of the body. At sternum of sixty days old rabbit, sternal segments two through four have undergone coalescence. At Ninety days old rabbit, complete ossification occurs in the sternum and the cartilaginous separation is replaced by bony part. In human the fusion of the manubrium and the body occurs between 6–10 years old, however as the xiphoid fusion with the body of sternum is attained between 20-25 years (Hanafi et al., 2014).

Ribs

In human, every rib, except for the last two, is ossified from four focuses. an essential focus for the body, and three epiphyseal focuses, one for the head and one each for the articular and non-articular parts of the tubercle (Louise and Sue, 2000).

In human, primary centers of ossification first appear in ribs 5-7 in the region of the posterior angle between 8-9 weeks of fetal life (Geddes, 1912; Noback and Robertson, 1951 and Ogden, 1979). Ossification centers then appear in a bidirectional manner, with the primary center for the first rib appearing before that of the last (Noback and Robertson, 1951). At the end of the third month of pregnancy, all ribs (except the last) have a single primary center of ossification (Flecker, 1932). The rib therefore commences ossification in advance of its corresponding vertebra, indicating that in terms of development it is divorced from the primitive mesenchymal vertebra, at a very early fetal age (Ogden, 1979). The epiphyses for the head and tubercle show up between the 16th and 20th years and are joined to the body during the 25 year (Gray, 1918).

In this study, there are four ossification centers for each except the last two ribs. The ossification center of the body of ribs comes from the extension of the transvers process of the thoracic vertebrae and it appears as early as two weeks of gestation. By the end of the third week of pregnancy, the ribs show primary center of ossification from the second to the last one. The second center was designed for the head and it appeared two weeks after birth. The third and fourth centers for the tubercle appeared a month after birth.

All in all, the formative pathways of the rabbit sternum and ribs were near the human model, and that could be useful in toxicological examinations as the rabbit give a decent case for the human advancement in brief time and more characteristic results.

Competing Interests

The authors declare 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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