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Tracking Appearance and Development of the Ossification Centers in Cranium and Skull Base Bones of Iraqi Sheep Fetuses (*Ovis aries*) Using Double Stain Method

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ABSTRACT

The skull is a group of flat and irregular bones that protect the brain and special sense organs. The shape of the head depends on the shape of the skull, which is closely related to certain structural and phenotypic features commonly used to identify and characterize different breeds of animals, their genetic profiles, and their relationship with the surrounding environment. 29 samples of indigenous Iraqi sheep fetuses were collected through daily visits to the slaughterhouses in Mosul city. The crown-rump length was measured to determine gestational ages in days. In the current study, the gestational ages of the fetuses ranged between 40 and 71 days, and a crown-rump length between 2.2 and 17 cm was used to accurately determine the locations of the ossification centers using the double stain (alizarin red S and alizarin blue stain) using an anatomical microscope. Histological examination of the head of the fetus was to determine the different stages of ossification. At 42 days of gestation, several primary ossification centers appeared in the right frontal bone near the right orbit. At 45 days of gestation, ossification centers appeared in the zygomatic processes of the temporal bone and the parietal bone. At 48 days of gestation, ossification centers appeared in the temporal bone. At 50 days of gestation, ossification centers appeared in the sphenoid bone. At 57 days of gestation, ossification centers appeared in the squamous and basilar parts of the occipital bone. At 61 days of gestation, the ossification of the inter-parietal bone and the occipital condyles were observed. Histological examination showed that the ossification center consisted of separate groups of osteoblasts in the membranous neurocranium, which ossified intramembranous. In contrast, the base of the skull was cartilaginous in origin, which ossified the endochondral. The very significant result of this study is that the appearance of ossification centers and their development happen within the seventh to ninth weeks of pregnancy, which makes this period the most dangerous time for bone growth, particularly cranium and skull base bones. Thus, it's important to prevent giving any medicine or treatment during that period, which might interrupt or delay this vigorous progression.

Keywords: Cranium and skull base, Double stain, Fetuses, Ossification centers, Sheep.

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INTRODUCTION

Iraqi sheep are considered to have rough wool and a wide tail filled with fatty material. In recent years, the average number of sheep in Iraq has reached 8 million. Iraqi sheep have included three main breeds: Awassi, Kurdish, and Arabi. Over the years and for long periods, they have been exposed to harsh environmental conditions such as drought, food shortages, and diseases, and this adaptation to such conditions was at the expense of important economic qualities **(Mustafa *et al.*, 2022).**

The skull is a group of flat bones that are relatively thin and protect the brain and special sense organs (eyes, nose, ears, and tongue). The shape of the head depends on the skull and is closely related to certain structural features **(Künzel *et al.*, 2003)**. These structural and phenotypic features were commonly used to identify and characterize different breeds of animals, their genetic profiles, and their relationship with the surrounding environment **(FAO, 2007; Shawulu *et al.*, 2011).**

The skull had four surfaces: the dorsal, the two lateral and the ventral. The skull bones are divided into

cranial bones (neurocranium) and facial bones (visceral cranium). The cranial bones (neurocranium) have two sets of bones: the bones of the cranial vault and the basilar bones (**Dyce *et al.*, 2009).** The bones of the cranial vault are: occipital, inter-parietal, parietal, frontal, temporal, and ethmoid bones. As for the basilar bones of the skull, they include the basal part of the occipital bone and the sphenoid bone with its two parts: the basi-sphenoid bone and the pre-sphenoid bone (**Popoola and Oseni, 2018; Gündemir *et al.*, 2020; de La Barra *et al.*, 2020).**

Congenital anomalies of the skull require that the normal morphology of the skull be understood **(Fahrioglu et al., 2023**). Skull development has been extensively studied in humans, with numerous studies recording the time and pattern of ossification in humans **(Susan, 2015)** and mice **(Marghoub *et al.*, 2019).** There is little research on cranial development in animals, such as studying cranial development in the prenatal stages of camel fetus skulls using radiography and magnetic resonance imaging **(Arencibia *et al.*, 2005; Hena *et al.*, 2012; Teja and Rajendranath, 2017)** who used alizarin red S stain to study the primary ossification centers in the buffalo fetuses' skulls.

This study aims to understand the formation of cranial bones in Iraqi sheep fetuses during the fetal period and provide a database related to their growth by specifying the dates of the appearance of primary and secondary ossification centers and their locations in each cranial bone, as well as double staining techniques to show bone and cartilage, in addition to histological study at early developmental stages. This study provides data on the normal growth of the cranial bones so that researchers in other medical fields can discover changes in bone development or deformities that affect these bones when studying the effect of a particular substance, food, or medicine on the growth of fetal bones.

MATERIALS AND METHODS

Twenty-nine (29) samples of indigenous Iraqi sheep fetuses were collected through daily visits to the Al-Saadoun Slaughterhouse and nearby slaughterhouses in Mosul city for five months and a half, starting from September 2023 to mid-February 2024. Physical and clinical examinations were conducted to select healthy fetuses and exclude those suffering from structural deformities and infections. Animal management and section gathering were achieved following the institutional Animal Care and Use Committee rules of the College of Veterinary Medicine, University of Mosul, Mosul, Iraq (UM.VET.2023.082). The crown-rump length was measured in centimeters using a measuring tape to determine gestational ages in days as described by **Arthur (1975)** using Richardson's formula: Estimated age (in days) = 2.1 (crown-rump length (cm) + 17). This length represents the distance extending from the top of the head (represented by the coronal edge of the frontal bone) and along the vertebral column to the tail root **(Fig.1).**

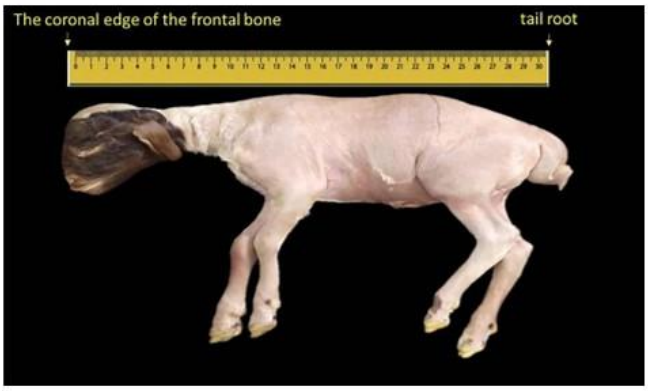

Fig.1: A macroscopic photo showing the method for calculating the estimated age of the fetus by measuring the crown-rump length.

In the current study, the ages of the fetuses ranged between 40 and 71 days, and their crown-rump length ranged between 2.2 and 17 cm. This was used to accurately determine the locations of the ossification centers using an anatomical microscope (Humanscope Stereo 110-250v, 50-60Hz, Germany).

To prepare fetus samples, the head was separated from the body (in the area of the atlantic-occipital joint) **(Vimini *et al.*, 1983).** To ensure accuracy and efficiency and to speed up the fixation with alcohol, staining with double stain, and maceration with sodium hydroxide (NaOH), soft tissue was removed, including the skin, eyes, tongue, and most of the muscles.

The double staining method described **by Salih and Ahmed (2022)** was used. The procedure began by moving the fetuses from ethyl alcohol to the double staining solution and keeping them in this solution for 24 hours. At the end of the staining procedure, the head samples were moved to the maceration solution, an aqueous solution prepared from 2%–4% sodium hydroxide solution, with monitoring every two hours. After that, the samples were passed with gradual concentrations of glycerin, which was diluted with distilled water, starting from a concentration of 25% (for 12 hours), 50% (for 24 hours), and 75% (for three days) until the tissues became transparent and the bones and cartilage could be seen through them. The samples were stored in 87% glycerin supplemented with a few thymol crystals to avoid fungal contamination **(Aliesfehani, 2015; Atabo *et al.*, 2020).**

Histological Examination

A histological examination was done to determine the different stages of ossification. For fixation, 10% neutral formalin was used for 3 days. The decalcification of the samples was done using a diluted formic acid solution (5%) for two weeks. After that, the samples were moved to increasing concentrations of ethyl alcohol (70%, 80%, 90%, and 100%) for dehydration. Then the samples were placed in xylene to remove the remaining alcohol. To prepare the tissue slides, the samples were immersed in liquid paraffin at 58–60 °C so that the paraffin penetrated the tissue in three passages, one hour for each passage, then cast to obtain wax molds and cut using a rotary microtome to a thickness of 6 micrometers. The slides were stained using Hematoxylin & Eosin (H&E) and Masson's trichrome stain **(Suvarna *et al.*, 2018; Dey, 2023).**

RESULTS

The smallest samples of indigenous sheep fetuses stained using the double staining technique were at an estimated age of 40-41 days with a crown-rump length of 2.2-2.5 cm, no ossification center appeared, the cranial bones were still membranous and the basilar bones were still cartilaginous and did not contain any ossification centers **(Fig.2A, B&C and table 1**).

Table 1: Times of appearance of ossification centers in the cranium and skull base of indigenous sheep fetuses.

Crown-rump length/cm	Age of fetus/day	Age of fetus/week	Name of bone	Time of appearance of ossification centers
2.2	40	6	Membranous cranium/ Cartilaginous skull base	----------
2.5	41	6	Membranous cranium/ Cartilaginous skull base	----------
3	42	6	Frontal bone	several primary ossification centers
4.5	45	7	Zygomatic process of the temporal bone	One primary ossification center
4.5	45	7	Parietal bone	Two primary ossification centers
6.3	48	7	Temporal bone	several primary ossification centers
6.3	48	7	Eye orbit	the ventral edge of the orbit beginning to appear ossified
7	50	8	Sphenoid bone	One primary ossification center
7.5	51	8	Eye orbit	completion of the growth of the frontal edge of the orbit
9	55	8	Zygomatic process of the temporal bone	the zygomatic process of the temporal bone continued to grow above the zygomatic process of the zygomatic bone
10	57	9	Squamous part and basilar part of the occipital bone	several primary and secondary ossification centers
12	61	9	inter-parietal bone	several primary and secondary ossification centers
12	61	9	Occipital condyles	several primary and secondary ossification centers
16	69	10	Fontanel area	the appearance of the fontanel area between the frontal and parietal bones

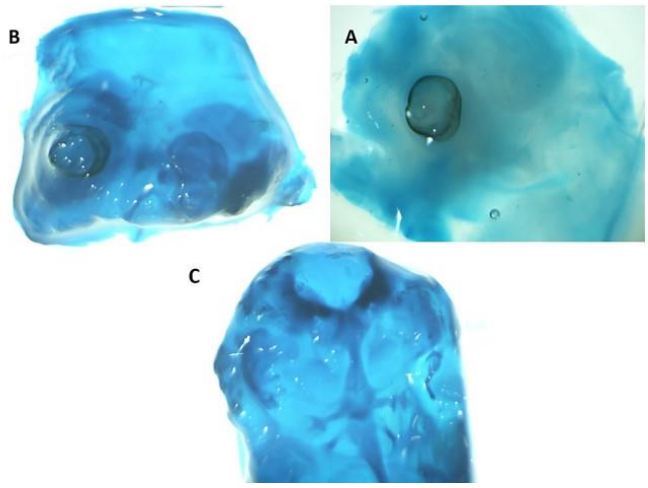

Fig. 2: An anatomical micrograph, (**A**) lateral view of the head of an indigenous sheep fetus, aged 40 days of gestation with a crown-rump length of 2.2 cm (20x). It shows that the skull bones did not show any ossification center. (**B**) lateral view of the head of an indigenous sheep fetus aged 41 days of gestation and with a crown-rump length of 2.5 cm, showing membranous skull bones and with no ossification center, (20x). (**C**) ventral view of the head of an indigenous sheep fetus aged 41 days of gestation with a crown-rump length of 2.5 cm, the basilar bones of the skull appear cartilaginous with no ossification center (15x).

At 42 days of gestation with a crown-rump length of 3 cm, where several primary ossification centers appeared in the right frontal bone near the right orbit **(Fig.3).**

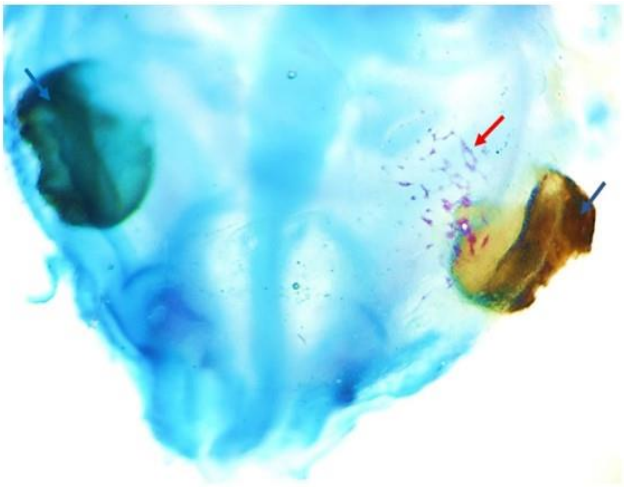

Fig.3: An anatomical micrograph (dorsal view) of the head of an indigenous sheep fetus aged 42 days of gestation with a crown-rump length of 3 cm, showing the ossification centers in the right frontal bone (red arrow). The blue arrow shows the right and left eyes, (20x).

At 45 days of gestation with a crown-rump length of4.5 cm, an ossification center appeared in the region of the zygomatic process of the temporal bone **(Fig. 4)** and two ossification centers in the parietal bone **(Fig.4**). The stage of appearance of ossification centers is followed by the stage of extension of the ossification centers; the extension of bone material begins from the lateral edge of the frontal bone and the parietal bone on the right and left sides, towards the top dorsally and towards the median suture of the frontal bones. In the region of the zygomatic process of the temporal bone, the extension of bone material begins from the backward to forward.

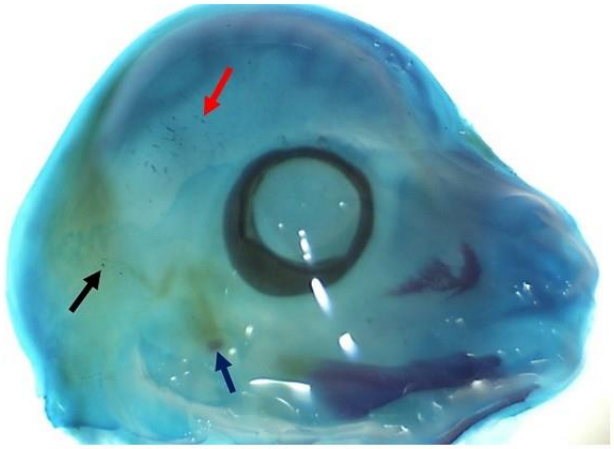

Fig.4: An anatomical micrograph (lateral view) of the head of an indigenous sheep fetus, aged 45 days of gestation with a crown-rump length of 4.5 cm, showing the ossification centers in the right frontal bone (red arrow). The blue arrow shows the ossification center of the zygomatic process of the temporal bone, and the black arrow shows the ossification center of the parietal bone (10x).

At 48 day of gestation with a crown-rump length of 6.3 cm, the ossification centers of the frontal bone and the parietal bone extended in the form of a network of bony spicules with a suture line separating the two halves of the bones; also the zygomatic process of the temporal bone appeared as a finger-like protrusion extending anteriorly towards the zygomatic process of the zygomatic bone, and the ventral edge of the orbit beginning to appear ossified. At this age, also noticed the appearance of ossification centers in the temporal bone. The bones of the base of the skull were cartilaginous and no ossification center appeared in them yet **(Fig. 5A&B).**

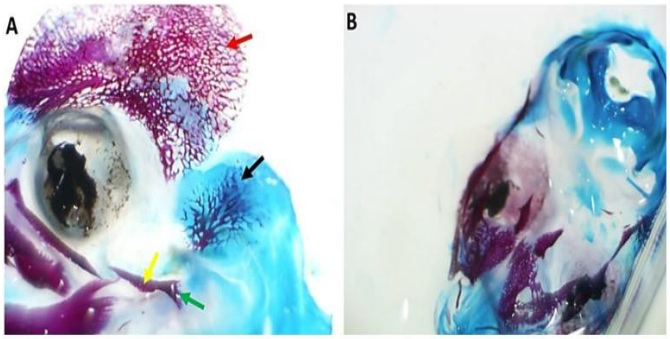

Fig.5: An anatomical micrograph of the head of an indigenous sheep fetus aged 48 days of gestation with a crown-rump length of 6.3 cm. (**A**) lateral view showing the extension of the ossification centers of the frontal bone (red arrow), the parietal bone (black arrow), the zygomatic process of the temporal bone (yellow arrow), and the temporal bone (green arrow) (7x). (**B**) ventral view showing the basilar bones of the skull (7x).

At 50 days of gestation with a crown-rump length of 7 cm, ossification centers appeared in the sphenoid bone **(Fig.6A)**. At 51 days of gestation with a crown-rump length of 7.5 cm, an increase in the thickness of the bone material in the frontal bone was observed; while in the parietal bone, the bone spicules extended to the median suture, with the completion of the growth of the frontal edge of the orbit **(Fig.6 B&C).** At the same age, the extension of the bony spicules in the temporal bone increased caudally, and the extension of the zygomatic process of the temporal bone increased rostrally **(Fig. 6B&C).** While the squamous part of the occipital bone was still cartilaginous, as it appeared in blue color **(Fig. 6D)**, and the extension of the ossification centers of the pre-sphenoid bone and the outer edge of the auditory bulla was also observed **(Fig.6E).**

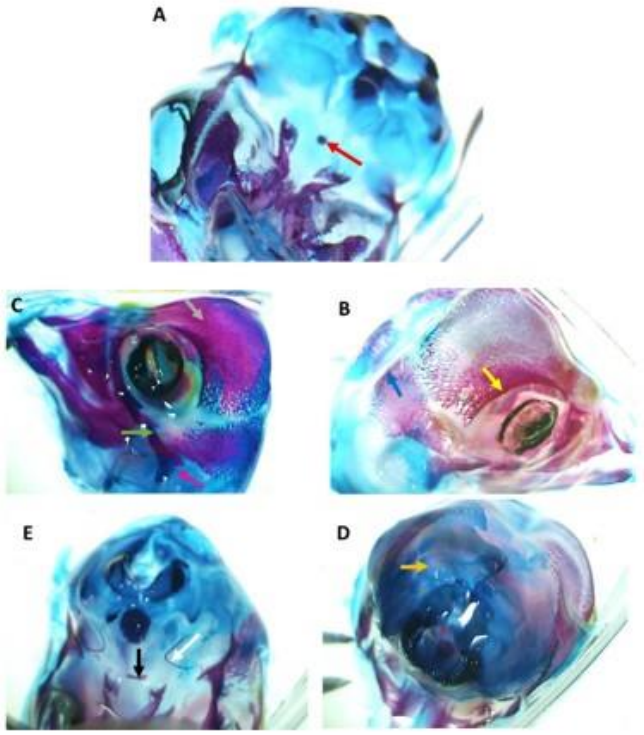

Fig. 6: An anatomical micrograph of the head of an indigenous sheep fetus at 50-51 days of gestation with a crown-rump length of 7-7.5 cm. (**A**) ventral view shows the ossification center of the sphenoid bone (red arrow) (7x). (**B**) dorsal view the extension of the bony spicules in the parietal bone (blue arrow) and the ventral edge of the orbit (yellow arrow) (7x). (**C**) lateral view shows an increase in the thickness of the bone material in the right frontal bone (gray arrow), the temporal bone (pink arrow), and the zygomatic process of the temporal bone green arrow (7x). (**D**) caudal view shows the squamous part of the occipital bone (yellow arrow) (7x). (**E**) ventral view shows the extension of the ossification centers of the pre-sphenoid bone (black arrow) and the outer edge of the auditory bulla (white arrow) (7x).

At 55 days of gestation with a crown-rump length of 9 cm, the zygomatic process of the temporal bone continued to grow above the zygomatic process of the zygomatic bone **(Fig. 7 A).** The bony spicules continue to extend in the sphenoid bone from the middle towards the lateral side of the bone, with the increasing thickness of the auditory bulla **(Fig.7 B).**

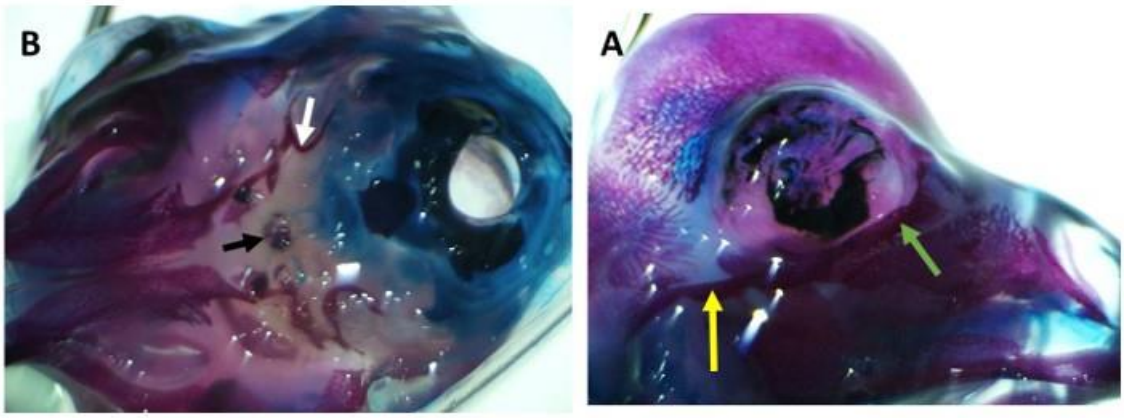

Fig. 7: An anatomical micrograph of the head of an indigenous sheep fetus at 55 days of gestation with a crown-rump length of 9 cm. (**A**) lateral view shows the zygomatic process of the temporal bone (yellow arrow) and the zygomatic process of the zygomatic bone (green arrow) (7x). (**B**) Ventral view shows the increase of ossification centers of the sphenoid bone (black arrow) and increasing the thickness of the auditory bulla (white arrow) (7x).

At 57 days of gestation with a crown-rump length of 10 cm, the ossification centers appeared in the squamous part of the occipital bone **(Fig.8A**); and in the basilar part of the occipital bone, with the continued extension of bone spicules into the sphenoid bone **(Fig.8 B).**

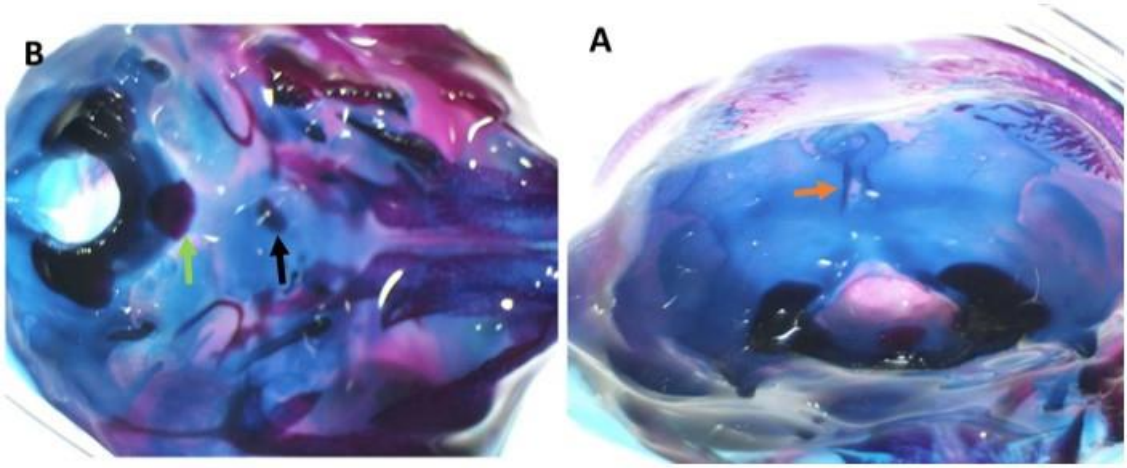

Fig. 8: An anatomical micrograph of the head of an indigenous sheep fetus at 57 days of gestation with a crown-rump length of 10 cm. (**A**) caudal view shows the ossification centers of the squamous part of the occipital bone (orange arrow) (7x). (**B**) ventral view) shows the ossification centers of the basilar part of the occipital bone (green arrow) and the sphenoid bone (black arrow) (7x).

At 59-60 days of gestation with a crown-rump length of 11-11.5 cm, increasing the thickness of the bony spicules of the frontal bone **(Fig.9A)** and parietal bone **(Fig.9B)**; and the continued extension of bone spicules in the temporal bone (laterally and caudally) **(Fig.9B**), and in the sphenoid bone (from the top (basi-sphenoid bone) and the bottom (pre-sphenoid bone)) **(Fig.9C).**

At 61 days of gestation with a crown-rump length of 12 cm, the ossification of the inter-parietal bone, the occipital condyles **(Fig.10A),** and the basilar part of the occipital bone were observed **(Fig.10B).**

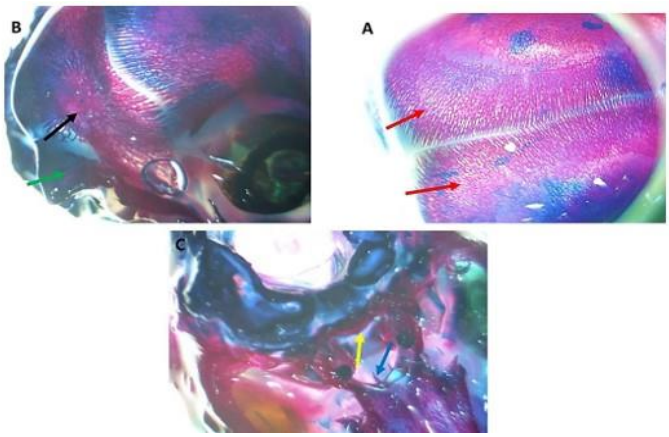

Fig.9: An anatomical micrograph of the head of an indigenous sheep fetus at 60 days of gestation with a crown-rump length of 11.5 cm. (**A**) dorsal view shows the continued increase in thickness of the bone spicules in the frontal bone and their extension along the length and width of the bone (red arrows) (7x). (**B**) lateral view shows the continued increase in thickness of the bone spicules in the parietal bone (black arrow) and the continued extension of bone spicules into the temporal bone laterally and caudally (green arrow) (7x). (**C**) ventral view shows the continued extension of bone spicules into the basi-sphenoid bone (yellow arrows) and pre-sphenoid bone (blue arrow) (7x).

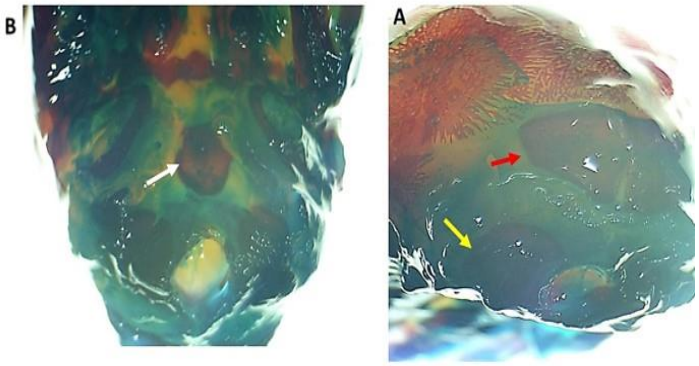

Fig.10: An anatomical micrograph of the head of an indigenous sheep fetus at 61 days of gestation with a crown-rump length of 12 cm. (**A**) caudal view shows ossification of the inter-parietal bone (red arrow) and the occipital condyles (yellow arrow) (7x). (**B**) ventral view shows ossification of the basilar part of the occipital bone (white arrow) (7x).

At 62 days of gestation with a crown-rump length of 12.5 cm, the completion of the upper edge of the orbit **(Fig.11A)**, and an increase in the growth and thickness of the basilar part of the occipital bone were observed **(Fig.11B).**

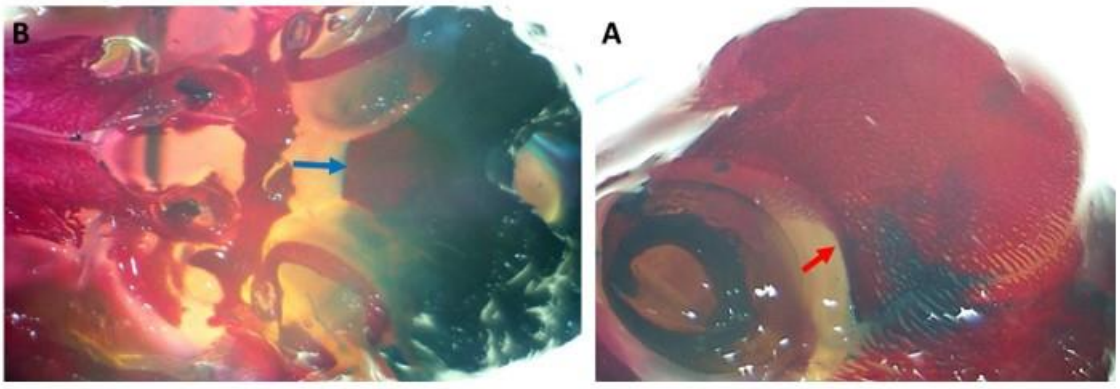

Fig. 11: An anatomical micrograph of the head of an indigenous sheep fetus at 62 days of gestation with a crown-rump length of 12.5 cm. (**A**) lateral view: shows the completion of the upper edge of the orbit (red arrow) (7x). (**B**) ventral view: shows the increasing growth and thickness of the basilar part of the occipital bone (blue arrow) (7x).

At 64-67 days of gestation with a crown-rump length of 13.5-15 cm, the extension of the bony spicules in the temporal bone continued to increase caudally **(Fig.12 A),** with the continued ossification of the sphenoid bone was observed **(Fig.12B).**

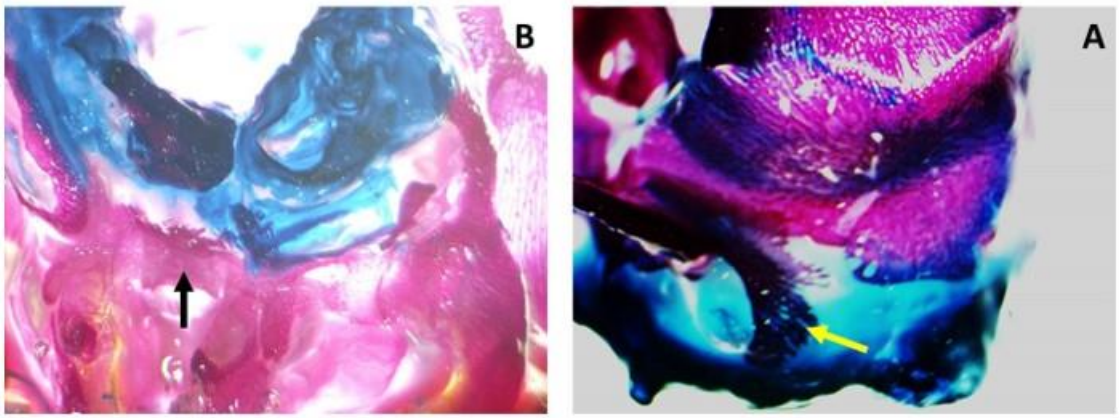

Fig. 12: An anatomical micrograph of the head of an indigenous sheep fetus at 64&67 days of gestation with a crown-rump length of 13.5&15 cm. (**A**) lateral view shows the continued extension of the bony spicules of the temporal bone caudally (yellow arrow) (7x). (**B**) ventral view shows the continued ossification in the sphenoid bone (black arrow) (7x).

At 69 days of gestation with a crown-rump length of 16 cm, there was an increase in the thickness of the bone spicules in the frontal bone **(Fig.13A)**, the parietal bone, and the inter-parietal bone **(Fig.13C),** with the appearance of the fontanel area between the frontal and parietal bones **(Fig.13B)**, the extension of ossification in the squamous part of the occipital bone **(Fig.13D)**, the basilar part of the occipital bone and the sphenoid bone **(Fig.13E).**

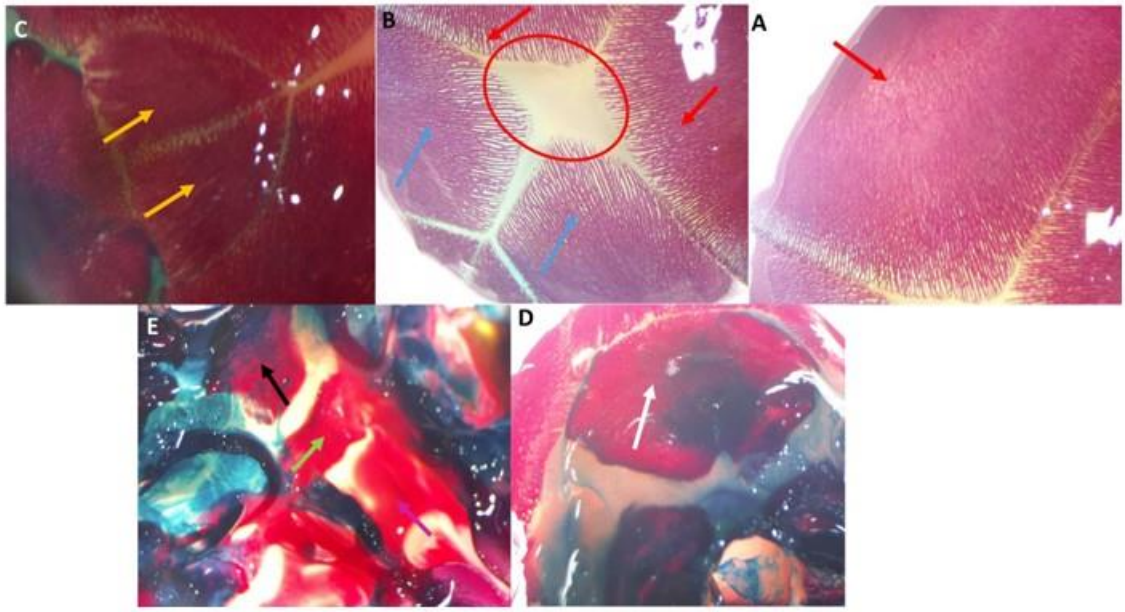

Fig. 13: An anatomical micrograph of the head of an indigenous sheep fetus at 69 day of gestation with a crown-rump length of 16 cm. (**A)** dorsal view) shows the increasing in thickness of the bony spicules of the frontal bone (red arrow) (7x), (**B**) parietal bone (blue arrows, and the appearance of the fontanel area between the frontal and parietal bones (red circle) (7x). (**C**) dorsal view shows the continued extension of bony spicules into the inter-parietal bone (yellow arrow) ((7x). (**D**) dorsal view shows continued ossification in the squamous part of the occipital bone (white arrows) (7x). (**E**) ventral view shows the continued ossification of the basilar part of the occipital bone (black arrow), the basi-sphenoid bone (green arrow), and the pre-sphenoid bone (purple arrow) (7x).

At 71 day of gestation with a crown-rump length of 17 cm, the continuation of ossification of the squamous part and the basilar part of the occipital bone **(Fig.14A&B),** and the sphenoid bone **(Fig.14B).**

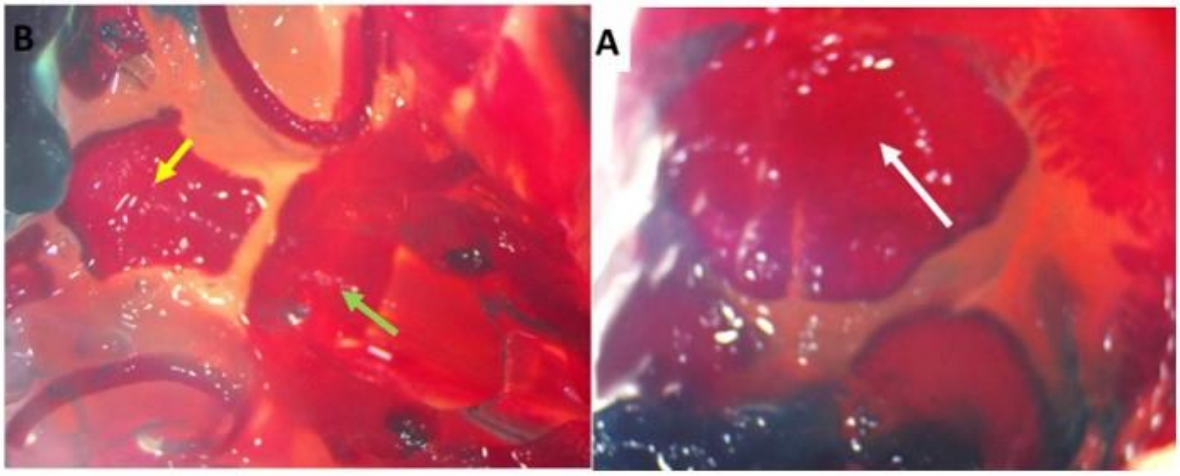

Fig.14: An anatomical micrograph of the head of an indigenous sheep fetus at 71 days of gestation with a crown-rump length of 17 cm. (**A**) dorsal view shows ossification of the squamous part of the occipital bone (white arrow) (7x). (**B**) ventral view showing continued ossification of the basilar part of the occipital bone (yellow arrow) and the completing the ossification of the sphenoid bone (green arrow) (7x).

Histological examination

The histological examination of the tissue sections taken from the skull of an indigenous sheep fetus at 48 days of gestation (7^{th} week), the crown-rump length of 6 cm showed the presence of an ossification center in the frontal bone **(Fig.15A),** then the ossification centers and bone spicules had begun to extend in the frontal bone, where the bone matrix appeared in green color when stained with Masson's Trichrome stain **(Fig.15B),** with the presence of undifferentiated mesenchymal cells. The ossification center consisted of separate groups of osteoblasts, which are spherical cells containing a basic cytoplasm with a single nucleus located on the side of the cell and surrounded by collagen fibers within mesenchymal tissue and blood vessels **(Fig.15A).** Histological examination of the frontal bone using Masson's trichrome stain showed the type of ossification in this bone was intramembranous ossification, where the mesenchymal cells differentiated into osteoblast cells, without a cartilaginous matrix where the bone appears as a membranous structure.

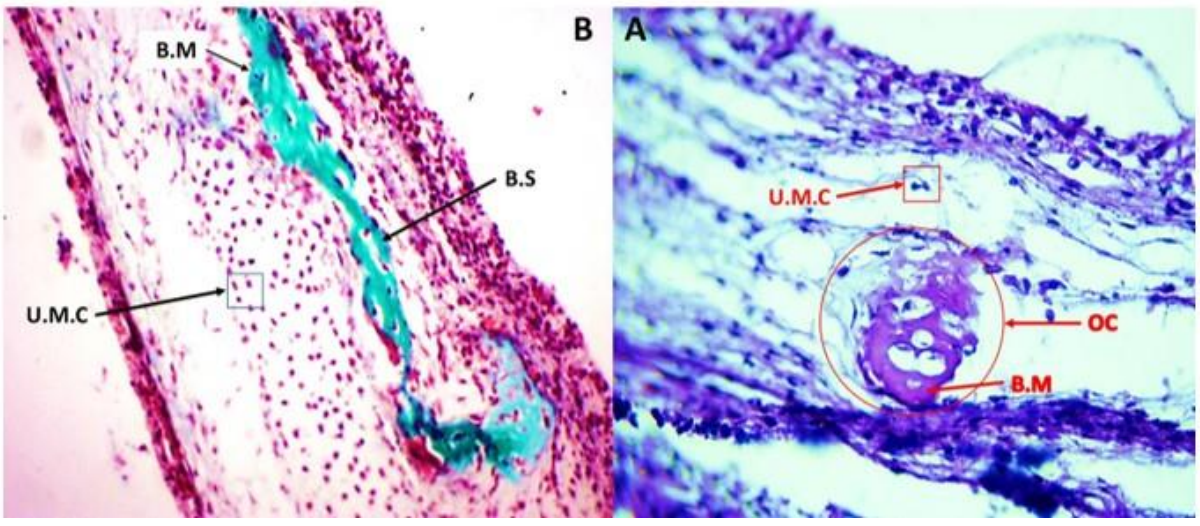

Fig. 15: Micrographs of the skull at 48 days of gestation with a crown-rump length of 6 cm. (**A**) shows the ossification center in the frontal bone: O.C (red circle), B.M: Bone Matrix with red color (H&E) (100x). (**B**) shows the extension of ossification centers in the frontal bone. B.M.: Bone Matrix (green color), U.M.C.: Undifferentiated Mesenchymal Cells, B.S.: Bone Spicules, Masson's Trichrome stain (100x).

At 50 days of gestation (crown-rump length of 7 cm), the presence of an ossification center within the cartilage of the sphenoid bone (endochondral ossification) was observed **(Fig16A & B).**

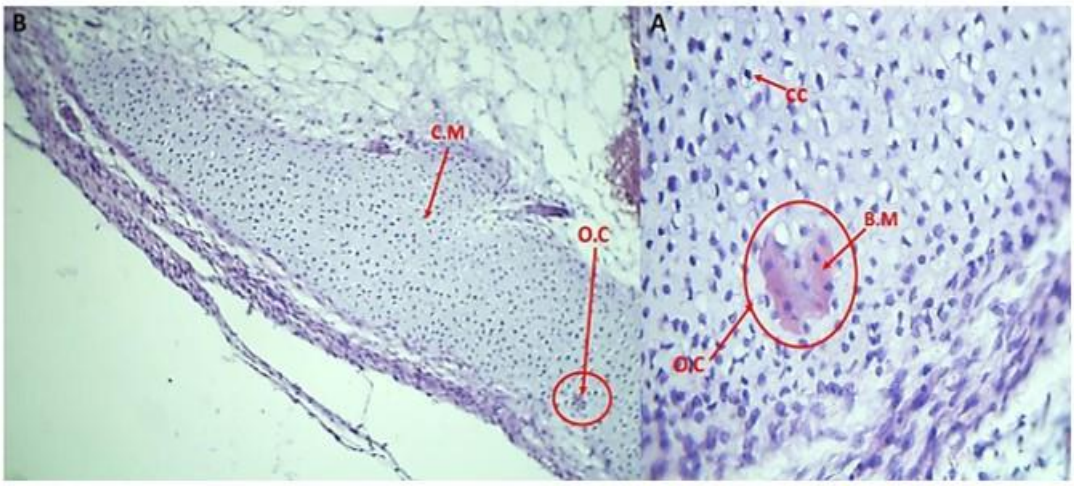

Fig. 16: Micrographs of the skull at 50 days of gestation with a crown-rump length of 7 cm. (**A**) shows C.M.: Cartilage Matrix, OC: Ossification Center (H&E) (100x). (**B**) C.C: Chondrocyte, BM: Bone matrix, OC: Ossification Center (H&E) (400x).

At 55 days of gestation (eighth week) a crown-rump length of 9.5 cm, an increase in the number, length, and extension of bone spicules was observed, and the green color completely changed to red color when the histological section stained with Masson's Trichrome stain **(Fig. 17).**

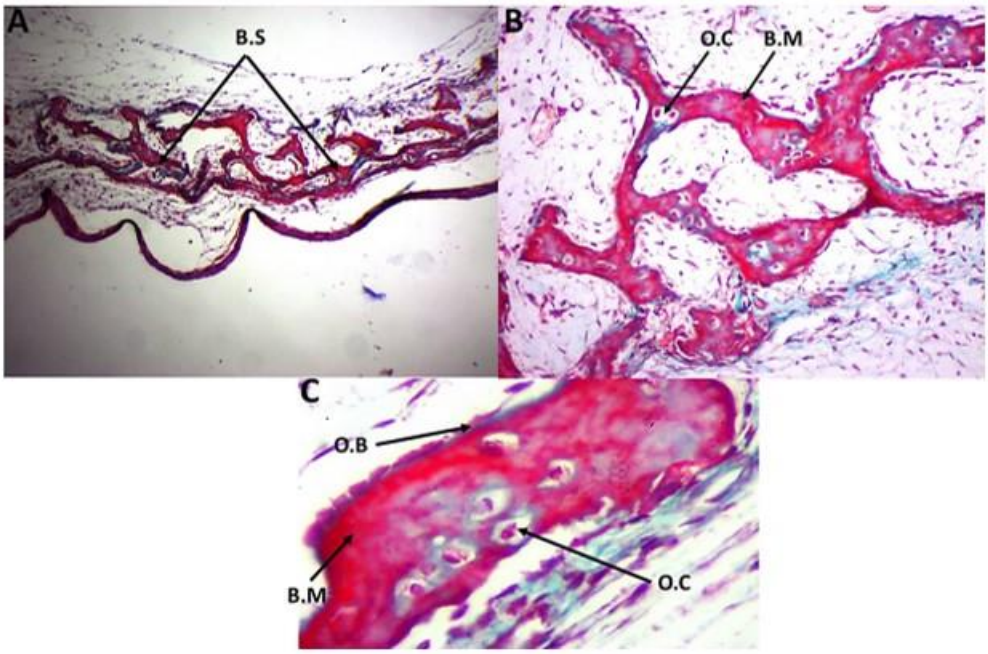

Fig. 17: Micrographs of the skull at 55 days of gestation with a crown-rump length of 9.5 cm. (**A**) shows an increase in the bone spicules numbers B.S.: Bone Spicules, B.M.: Bone Matrix (40x). (**B**) O.C: Osteocyte, B.M: Bone Matrix (100x), (**C**) O.B: Osteoblast: O.C: Osteocyte, B.M: Bone Matrix (400x). Masson's Trichrome stain.

At 58 days of gestation (ninth week), the crown-rump length of 11 cm, the size of the bony spicules in the frontal and parietal bones was increased and extended in several directions. The bone at this stage is called the septal bone **(Fig.18A&B)),** where the presence of bone cells (osteocytes) inside this bone was observed, in addition to the presence of osteoblasts around it. Osteocytes are stellate-shaped cells that contain acidic cytoplasm; their nucleus is located in the center of the cell, and they are classified as long-living cells **(Fig.18C)).**

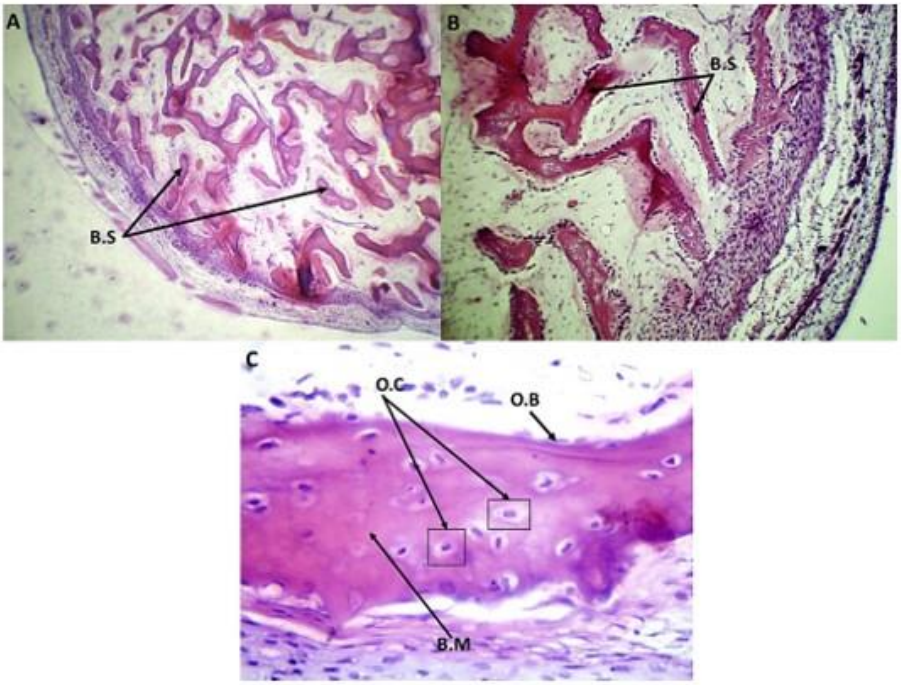

Fig 18: Micrographs of the skull at 58 days of gestation with a crown-rump length of 11 cm. (**A**) shows the bony spicules in the frontal bone B.S: Bone Spicules, (H&E) (40x). (**B**) shows the bone spicules in the parietal bone. B.S: Bone Spicules, (H&E) (100X). (**C**) O.B: Osteoblast, O.C: Osteocyte, B.M: Bone Matrix (red color) (H&E) (400X).

DISCUSSION

The general pattern of the extension of ossification centers in the cranial bones varies from one bone to another. In the frontal and parietal bones, the appearance of the ossification centers was at the lateral edge of the bone, and their extension was upward dorsally towards the median suture between the right and left bones. As for the temporal bone, the ossification centers appeared from the caudal edge of the zygomatic process of the temporal bone and their extension was to the back of the skull. In the inter-parietal bone, ossification centers appeared in its central part and extended in forward and backward directions. As for the squamous part of the occipital bone, the ossification centers appeared in the central part, and their extension was in the lateral direction of the bone. In the sphenoid bone, the ossification centers appeared in the middle of the basi-sphenoid bone, and then the ossification centers began to extend forward, backward and laterally. As for the pre-sphenoid bone, the ossification centers appeared in the apex of the bone, then the ossification centers began to extend backward and laterally.

In the current study, the beginning of the appearance of ossification centers in the cranial bones was in the frontal bone at an estimated age (42 days), this result was in agreement with **Succu *et al.,* (2023)** in their work on the development of the skeleton of the Sarda sheep fetus using ultrasound and staining techniques that allow observing the first ossification processes during the first trimester of gestation and the growth rate of skeletal components, such that the ossification in the skull began in the day (40) of gestation and was completed between the day (65) to the day (70) of gestation; with **Mogheiseh *et al.,* (2023)** who found in their study to evaluate the skeletal ossification of sheep fetuses at various stages of gestation (20-95 days) using radiography and staining of the skeleton that ossification of the skull occurred in the day (40) of gestation; and **(Vernunft *et al.,* 2022)** stated in their study of the growth of the local pig fetus by ultrasound that the beginning of ossification in the skull was on the day (37) of gestation. The bony spicules observed during the ossification of the frontal bone, the parietal bone, the inter-parietal bone, the temporal bone, and the zygomatic process of the temporal bone in this

study indicate that they were all ossified via intramembranous ossification. This result was consistent with the study conducted by **Mahmood (2007)** in her study of the development of the facial bones in the Awassi sheep fetuses.

Frontal bone

The current study showed that the frontal bone was the first cranial bone in which ossification centers appeared at day 42 of gestation, and several ossification centers appeared in the right frontal bone near the right orbit. This result was consistent with the results of **Teja and Rajendranath (2017)** who identified the primary ossification centers in the skull of the buffalo fetus using alizarin red stain, where they found that the ossification centers of the frontal bone appeared on the lateral border of the frontal bone.

In their study to compare ossification of the skull in three breeds of Nigerian sheep using alizarin red stain, Atabo et al. (2020) mentioned that the appearance of ossification centers in the frontal bone began in the first trimester of gestation, days (45–47) of gestation in the Yankasa breed, and days (48–50) of gestation in the Uda and Balami breeds. The results of the current study agreed with the results of these researchers in the timing and method of the extension of the bone matrix, where the appearance of the ossification centers was at the lateral border of the frontal bone and its extension was upward dorsally toward the median suture of both the frontal bones. The early appearance of the ossification centers in the current study could be clarified by the importance of the mother's nutrition and health status on the embryo's development and the difference in the environment between the two countries.

In their study on the development of prenatal membranous cranial bones in buffaloes, **Lakshmi *et al.*, (2012)** noted that the development of the frontal bone arises from a single ossification center in the body of the bone near the supraorbital groove on the day 49% of gestation. The extension of ossification occurred on the day (55) of gestation, and the orbital part extended on the day (75) of gestation and was completed during the day (83) of gestation. **Soana *et al.*, (1996)** studied bone formation in the skulls of cow fetuses and found that the frontal bone ossifies in cows on the 52nd day of gestation. The results of the current study agreed with the results of both works mentioned above in buffaloes and cows in terms of the timing of the appearance of the frontal bone in the first trimester of gestation. The delayed appearance of ossification centers in the current study can be explained by the two previous studies in buffaloes and cows due to the difference between the animal species.

Parietal bone

The appearance of ossification centers in the parietal bone was at day 45 of gestation as two ossification centers. This result was consistent with the results of **Atabo *et al.*, (2020),** who studied ossification in the skull of three breeds of Nigerian sheep, in the timing of the appearance, where they mentioned that the beginning of the appearance of ossification in the parietal bone was between 45 and 47 days of gestation in the Yankasa breed and differed from the Uda and Balami breeds, which appeared between 48 and 50 days of gestation; this difference could be due to the difference between the animal breeds. Also, the results of the current work agreed with the results of this work in terms of the extension method of the ossification centers, where the appearance of the ossification centers was at the lateral edge of the parietal bone and their extension was upward dorsally towards the median suture of the bone.

In their study on the development of prenatal membranous cranial bones in buffaloes**, Lakshmi *et al.*, (2012**) stated that the parietal bone developed from a single ossification center, and the first sign of ossification was evident at day 60 of gestation. Most of the parietal bone was completely ossified by day 141 of gestation. The results of the current study agreed with the results of these researchers in buffaloes in terms of the timing of the appearance of the parietal bone in the first trimester of gestation. The delayed appearance of ossification centers in the current study compared to the previous study in buffaloes can be explained by the difference between the animal species.

Soana *et al.*, (1996) who studied bone formation in the skull of cow fetuses, found that the parietal bone ossifies in cows on day 97 of gestation. The results of the current study differed from the results of these researchers in cows in terms of the timing of the appearance of the parietal bone at the beginning of the second trimester of gestation. The early appearance of the ossification centers in the current study could be clarified by the difference in the type of nutrition between the two species.

The zygomatic process of the temporal bone

Ossification centers appeared in the zygomatic process of the temporal bone on the 45th day of gestation as a single ossification center on the left and right lateral surfaces of the skull. The ossification centers continued to extend rostrally as a finger-like protrusion towards the zygomatic process of the zygomatic bone, where they passed over it. This result was consistent with **Atabo *et al.*, (2020)** who studied ossification in three breeds of Nigerian sheep, in the timing of the appearance, where they mentioned that

the beginning of the appearance of ossification in the parietal bone was between 45 and 47 days of gestation in the Yankasa breed and differed from the Uda and Balami breeds, which appeared between 48 and 50 days of gestation; this difference could be due to the difference between the animal breeds.

Temporal bone

Ossification centers appeared in the temporal bone on the 48th day of gestation. One ossification center began to appear on both sides, caudally to the end of the zygomatic process of the temporal bone, and then ossification centers continued to extend in a in a backward direction. These results agreed with **Atabo *et al.*, (2020)** in terms of the location and method of extension of the ossification centers in the heads of fetuses of three breeds of Nigerian sheep. They mentioned that it may share the same ossification center with the zygomatic process of the temporal bone, but they differed in terms of the date of appearance of the ossification centers in the temporal bone, as they stated that the temporal bone had not yet ossified in the age groups studied, which ranged from 42 to 67 days of gestation. This could be due to the maternal malnutrition of Nigerian sheep, which affects the health status of the embryo's development.

Laxshmi *et al.*, (2012) mentioned in their study about the time and order of the appearance of ossification in the skull of buffalo before birth that ossification in the squamous temporal bone occurred on the day (55) of gestation and in the petrous part of the temporal bone on the day (111) of gestation, and Soana et al. (1996) who studied bone formation in the skull of cow fetuses, found that the temporal bone ossifies in cows on the day (52) of gestation. The results of the current study agreed with the results of both works mentioned above in buffaloes and cows in terms of the timing of the appearance of the squamous temporal bone in the first trimester of gestation. The delayed appearance of ossification centers in the current study can be explained by the two previous studies in buffaloes and cows due to the difference between the animal species.

Sphenoid bone

The appearance of ossification centers in the sphenoid bone occurred on the 50th day of gestation. They appeared in the middle of the basi-sphenoid bone, and then the ossification centers began to extend backward, laterally, and forward in the pre-sphenoid bone. As for the pre-sphenoid bone, the ossification centers appeared in the apex of the bone, and then the ossification centers began to extend laterally and backward in the basi-sphenoid bone. These results agreed with **Atabo *et al.*, (2020**) in terms of the timing of the appearance of ossification centers in the three breeds of Nigerian sheep in the first trimester of gestation because they are from the same species.

Laxshmi ***et al.*, (2012)** mentioned in their study about the time and order of the appearance of ossification in the skull of buffaloes before birth that ossification in the body of the pre-sphenoid bone occurred on the 89th day of gestation. The results of the current study agreed with the results of these researchers in buffaloes in terms of the timing of the appearance of the sphenoid bone; and with **Soana *et al.*, (1996)** who studied bone formation in the skull of cow fetuses, found that the sphenoid bone ossifies in the day (97) of gestation which meaning at the ending of the first trimester of gestation; and in the body of the basi-sphenoid bone in the day (62) of gestation in buffalo fetuses which meaning early of the first trimester of gestation; this differed from the current study results could be clarified from two opinions, initially, the difference is due to they are from different species, furthermore, these differences in the time of growth of ossification centers of basi-sphenoid bone that were recognized in current data could be as a result of the genetic related that regulator each cell action through endochondral ossification **(Ibrahim *et al.*, 2020).**

Inter-parietal bone

The current study showed that this bone began to appear on day 53 of gestation, where the bony spicules of the inter-parietal bone appear and extend caudally in a separate manner on the right and left sides, while at day 61 of gestation, the bony spicules on both sides united to form a single bone. This result differed from **Atabo *et al.*, (2020)** in terms of the timing of the appearance of ossification centers, which appeared between 48 and 49 days of gestation in the Yankasa breed and between 49 and 50 days of gestation in the Uda and Balami breeds; while they reached their full development in the day 61 of gestation in both breeds, these findings are consistent with current study results. These variations could be due to the differences between the animal breeds and the environment between the two countries.

Laxshmi *et al.*, (2012) mentioned in their study about the time and order of the appearance of ossification in the skull of a buffalo before birth that the ossification of the inter-parietal bone occurred on day 64 of gestation. The results of the current study agreed with the results of these researchers in buffaloes in terms of the timing of the appearance of the inter-parietal bone in the first trimester of gestation. **Soana *et al.*, (1996**) studied bone formation in the skull of cow fetuses, found that the inter-parietal bone ossifies on the day (117) of gestation. The results of the current study differed from the results of these researchers in terms of the timing of the appearance of the inter-

parietal bone in the second trimester of gestation. This variation is due to the differences between the animal species.

Occipital bone

Ossification centers appeared in the basilar and squamous parts of the occipital bone on day 57 of gestation. These results were consistent with the results of **Atabo *et al.*, (2020)** in terms of timing and the method by which ossification centers appear and extend, as ossification appeared in the squamous part of the occipital bone between 54 and 56 days of gestation in Uda and Balami sheep breeds and differed from the Yankasa breed, which appeared between 51 and 53 days of gestation. These variations could be due to the differences between the animal breeds and the environment between the two countries.

In their study on the development of prenatal membranous cranial bones in buffaloes, **Lakshmi *et al.*, (2012)** stated that the squamous part of the occipital bone develops by intramembranous ossification, while the basilar part develops by intracartilaginous ossification. Ossification was observed in the lower part of the squamous part of the occipital bone for the first time in buffalo fetuses on the day (64) of gestation, while the upper part of the squamous part of the occipital bone was observed on the day (65) of gestation; ossification of the squamous part of the occipital bone was completed on the day (132) of gestation. While they mentioned that ossification of the exoccipital condyles, tympanic bulla, and basi-occipital appeared on the day (62) of gestation, this differed from the results of the current study, where ossification of the occipital condyles appeared on the day (61) of gestation, which was at the beginning of the second trimester of gestation. **Soana *et al.*, (1996)** found that the squamous part of the occipital bone, the occipital condyles, and the basilar part of the occipital bone ossify on the day (97) of gestation. The results of the current study differed from the results of these researchers regarding the timing of the appearance of the occipital bone at the beginning of the second trimester of gestation. This difference could be clarified from numerous opinions; initially, the difference is due to the fact that the fact that they are from different species; furthermore, the mechanism for bone development of cranium bones is through intramembranous ossification, which is different from the skull base bones, which are developed through endochondral ossification; their cartilages aren't replaced by bone as flat bones immediately **(Caplan, 2010)**. An additional explanation for such dissimilarities in the appearance and development of ossification centers between the cranium and skull base bones is that the cranium bones are membranous in structure, which cover the brain dorsally and laterally and need to be protected during gestation periods.

The results of the histological examination revealed that ossification centers began to appear in the cranial bones in the seventh week (48 days of gestation), as the mesenchymal cells in the periosteum layer developed into osteoblast cells. These cells aggregated and formed colonies, which were referred to as ossification centers. These colonies of osteoblast cells secrete collagen matrixes, surrounded by osteoblast cells, after which pieces of primitive bone spicules are formed. These results were consistent with **Atabo *et al.*, (2022**) in their study on the formation of calvaria histogenesis of the skull before birth in the Uda and Yankasa breeds of sheep, where they stated that mesenchymal cells develop into osteoblast cells and form bone spicules in the late first trimester of gestation (48–50 days) in Yankasa and the early second trimester of Oda fetuses (51–53 days of gestation), which differed from the current study results. In the eighth week (55 days of gestation), bone cells (osteocytes) appeared, produced by osteoblasts and immersed within the bone matrix. This bone matrix is known as bone spicules. In the ninth week (58 days of gestation), it was observed that there were many large pieces of bone known as bony septa, and this differed from **Atabo *et al.*, (2022**) in their study on the formation of calvaria histogenesis of the skull before birth in the Uda and Yankasa sheep breed, where they mentioned that in the third trimester of gestation, the bony septa fused, increased in size, and ossified more. These variations could be due to the differences between the animal breeds and the environment between the two countries.

Histological examinations in the current study confirmed the development of the membranous neurocranium through intramembranous ossification, where the transformation of mesenchymal cells into osteoblasts was followed by osteocytes, then bony septa, and finally cancellous bone **(Moore *et al.*, 2018). Lakshmi *et al.*, (2012)** studied the development of the membranous neurocranium in prenatal buffalo, histologically showed that the frontal bone develops from a single ossification center within the membranous neurocranium near the region of the supraorbital foramen on day (49) of gestation, and the parietal bone also develops from a single ossification center inside the membranous neurocranium, and they stated that the first sign of ossification in the parietal bone was clear on day (60) of gestation, while the squamous part of the occipital bone ossified in the manner of endochondral ossification on day (64) of gestation and was completed on day (132) of gestation, the results of the current study were in agreement with their results in terms of the number of ossification centers and the method of ossification (Intramembranous and endochondral ossification). The results of histological examination of a study conducted by **Hena *et al.*, (2012)** revealed that in the

first trimester of gestation (35–130 days), the membranous cranium contains an abundance of mesenchymal cells with a light-colored, ill-defined bony septa with a space between the septa, while in the second trimester of gestation (131–260 days), there were many osteoblast cells scattered within the immature cranium with a light-colored, ill-defined bony septa with a space between the septa. As the fetus grows older in the third trimester of gestation (261-390 days), there are prominent bone spicules or septa with a small number of osteoblast cells surrounding them, and the bone cells are arranged regularly within the bone matrix with fewer spaces between the bone septa. The results of the current study were in agreement with the results of these researchers, especially in the first trimester of gestation. These variations could be clarified from numerous opinions; initially, the difference is due to the fact that the fact that they are from different species; furthermore, these differences in the time of growth of ossification centers that were recognized in current data could be a result of the genetic factors that regulate each cell action **(Ibrahim *et al.*, 2020)**; and the mechanism for bone development of cranium bones is through intramembranous ossification, which is different from the skull base bones, which are developed through endochondral ossification **(Caplan, 2010).**

CONCLUSION

The ossification centers were observed in the first trimester in the frontal bone, the zygomatic process of the temporal bone, the parietal bone, and the temporal bone, respectively. But, in the second trimester, the ossification centers began in the sphenoid bone, the squamous part, the basilar part of the occipital bone, the interparietal bone, and the occipital condyles, respectively. The ossification center consisted of separate groups of osteoblasts in the membranous neurocranium, which ossified intramembranous. In contrast, the base of the skull was cartilaginous in origin, which ossified the endochondral. The very significant result of this study is that the appearance of the ossification centers and their development happen within the seventh to ninth weeks of pregnancy, which makes this period the most dangerous time for bone growth, particularly cranium and skull base bones. Thus, it is important to prevent giving any medicine or treatment during that period, which might interrupt or even delay this vigorous progression.

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Conflict of interests

The authors declare no potential conflict of interest.

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