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Original Research

Growth of upper limb bone: A study by differential staining

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ABSTRACT:
The present study was conducted with an aim to study the total and ossified length of long bones of upper limb in the fetus and calculate the ratio of ossified vs. calcified component of a growing bone. Ten fetal upper limb bones of right side from the fetuses of 12th to 18th weeks were used in this method of differential staining of cartilage and bone. The double staining of cartilage and bone was done by using alcian blue and alizarin red in the present study. Results showed that the ossification start at the primary centre in the diaphysis and progresses above downwards keeping in pace with the total increase in the length of the long bones. The ossified component of these bones was in the range of 60- 70 % of the total length of the bone in the age group of 12 which increased to 70 -80 % in the age group 18 of weeks. At this stage the whole diaphysis of the bone appeared to be ossified with only the proximal and distal ends are cartilaginous.
Keywords: ossifications, long bones, growth, upper limbs.

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Introduction

Diaphysial lengths of dried material of fetal skeletons had already been investigated in forensicsciences1.Several studies [1-6] have considered the relationships between measurements and degree ofossification of long bones in human fetal limbs as well as their correlation to CR length.In the current study different techniques have been employed, measurement after cleaning andstaining is one of them. Direct radiological measurements (in vivo)of length of ossifying bones inhuman fetal limb bones has been debatable since they vary over wide range due to differences infetal position. [7,8,9]

Ossification begins in the long bones by eight week of embryonic development and initially occursin diaphysis of bones from primary centers of ossification. By twelve weeks primary ossification centres have appeared in all bones of the limbs.10Ossification centres of upper extremities form earlier than those of the lower extremity.[7]

The process of growth of cartilage and extension of ossification into its proceeds until, at birth, the result is a bony shaft with cartilaginous extremities. The most part of carilagenous extremeties ossified after birth by the appearance within them secondary centers of ossification.[11]

The problem of age estimation stays for osseous remains, both for entire bones and ossifed parts, since most of reference tables come from ultrasonographic measurements, which are not easily reproducible on fetal osseus remains. Furthermore, the ultrasonographic measurements contain slight errors in comparison to the realanatomical ones.12

The main advantage of measurement of the length of the long bones of extremities is that the age of even severely damaged fetuses can be readily determined with acceptable accuracy up to the 22nd weekof fertilisation.[1]

Many studies done on the assessment of growth patterns in human fetal limbs employed the method of gross staining [3,4,13-16] of bones to obtain a differential detection of the ossified part of the bone fromtotal.

Studies of osteogenesis in small vertebrates and mammalian foetuses differential staining of skeleton in whole. Differential staining of cartilage and bone with the use of alcian blue for cartilage staining andalizarin red S for staining the bone has been used for this purpose.It carries several applications including developmental toxicology (i.e.teratology) studies for new chemical candidates for pharmaceutical,industrial and environmental use.17
The present study was conducted with an aim to study the total and ossified length of long bones of upper limb in the fetus and calculate the ratio of ossified vs. calcified component of a growing bone.

MATERIAL AND METHODS
Gross staining: - 10 fetal upper limb bones of right side from the fetuses of 12th to 18th weeks were used in this method of differential staining of cartilage and bone. The double staining of cartilage and bone was done by using alcian blue and alizarin red in the present study.

Procedure-
1. PREPARING THE SPECIMEN : skin and fat of upper limb is removed.
2. FIXATION: fixation of skinned specimens in formalin(10%) for minimum 2 days was done immediately to prevent them from decaying.
3. WASHING: now the specimens were kept in distilled water to remove excess of formaldehyde for 2 days.
4. CARTILAGE STAINING- the specimens of the bones were placed in the following staining solution, shaken for a few second and left for almost 20-24 hours at room temperature:
Alcian blue – 20-40mg
Glacial acetic acid - 80 ml
95%Ethanol - 120 ml
5. REHYDRATION : the specimen was put in 95% ethanol for 2 days
Now the specimen was transfer to 70%ethanol for 2 days
Now it was kept in distilled water for one day
MACERATION-specimens of the bones were next placed in 2% KOH for 8 hours for maceration. This aided in removal of soft tissue attached to the bones.

7. BONE STAINING :the specimens of the bones were placed in the following staining solution, shaken for a few second and left for almost 20-24 hours at room temperature:

Alcian blue – 0.025gm

2%KOH - 5gm

Distilled water - 250 ml

8. CLEARING- it was followed by clearing by putting the specimens into following solution for one day

0.5%KOH :150ml

Glycerin :50ml

3%Hydrogen peroxide : 2ml

9. STORAGE of stained specimens-the specimens were further stored in pure glycerine.

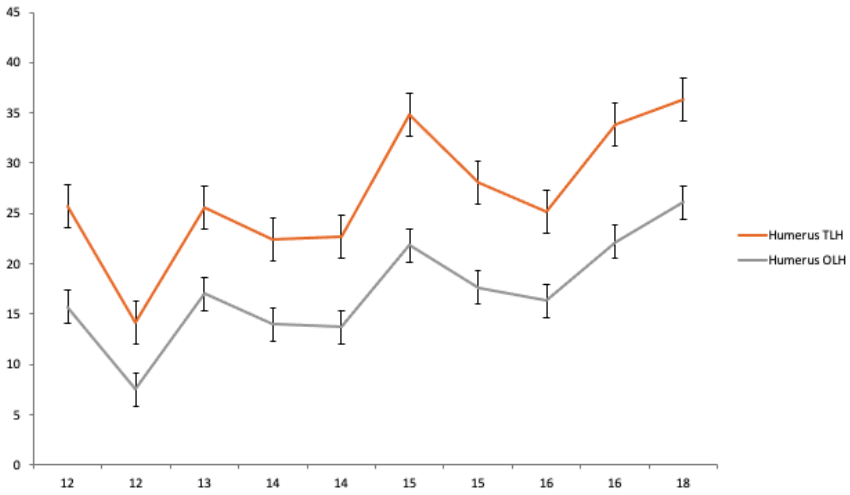
Results
This was done to obtain differential detection of the ossified part within the comprehensive outline between cartilaginous epiphysis. The ossified part of the bone was evident after staining. The osseous part was rendered red with alizarin red S and cartilaginous part blue with alcianblue in this procedure. The total length and the ossified segment was then measured.
The cartilaginous parts of the bones were stained blue with Alcian Blue, while the bony part was stained red with Alizarin RedS. After staining the total length(TL) and Ossified Length(OL) of the bones were measured

.
Table 1

Sr. No.	Fetus No.	GESTATIONAL AGE (in weeks)	HUMERUS		ULNA		RADIUS		OL_H/TL_H	OL_U/TL_U	OL_R/TL_R
			TL_H	OL_H	TL_U	OL_U	TL_R	OL_{Ra}	Ratio	Ratio	Ratio
1	1320	12	25.7	15.7	20.5	14	18.3	11.8	61.09	68.29	64.48
2	1830	12	14.2	7.5	13.9	7.5	9.3	6.5	52.82	53.96	69.89
3	1478	13	25.6	17	20.2	14.3	18.3	13.4	66.41	70.79	73.22
4	1313	14+5	22.4	14	17.6	10.8	15.8	10.3	62.50	61.36	65.19
5	1359	14	22.7	13.7	18	10.8	17.5	11.4	60.35	60.00	65.14
6	1565	15	34.8	21.8	28.9	20.9	23.5	16	62.64	72.32	68.09
7	1852	15+6	28.1	17.6	23.8	16.5	19.2	14.8	62.63	69.33	77.08
8	1389	16	25.2	16.3	20.6	13.6	18	13.1	64.68	66.02	72.78
9	1409	16	33.8	22.2	28.7	20.2	25	18.7	65.68	70.38	74.80
10	1408	18+2	36.3	26.1	32.6	24.2	28	21.9	71.90	74.23	78.21

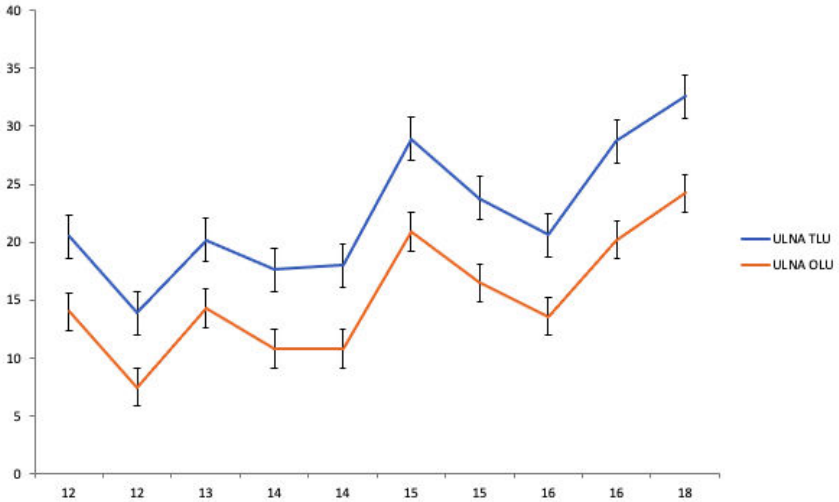
Fig1 a) Total Length andOssified Length of Humerus

FIG1 b). Increase in TLH and OLH of Humerus with respect to the increasing GA



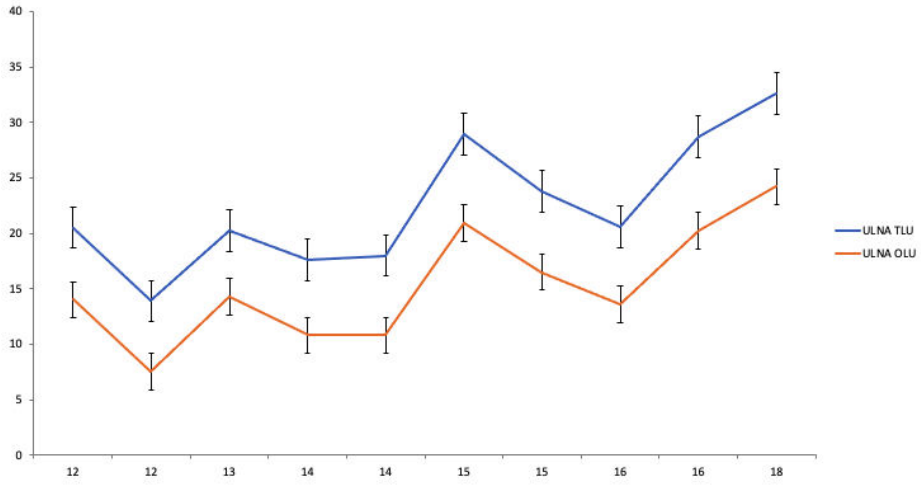
GA(in weeks)	Humerus	
	TL_H	OL_H
12	25.7	15.7
12	14.2	7.5
13	25.6	17
14	22.4	14
14	22.7	13.7
15	34.8	21.8
15	28.1	17.6
16	25.2	16.3
16	33.8	22.2
18	36.3	26.1

Figure 2 Increase in TLU and OLU of Ulna with respect to the increasing GA



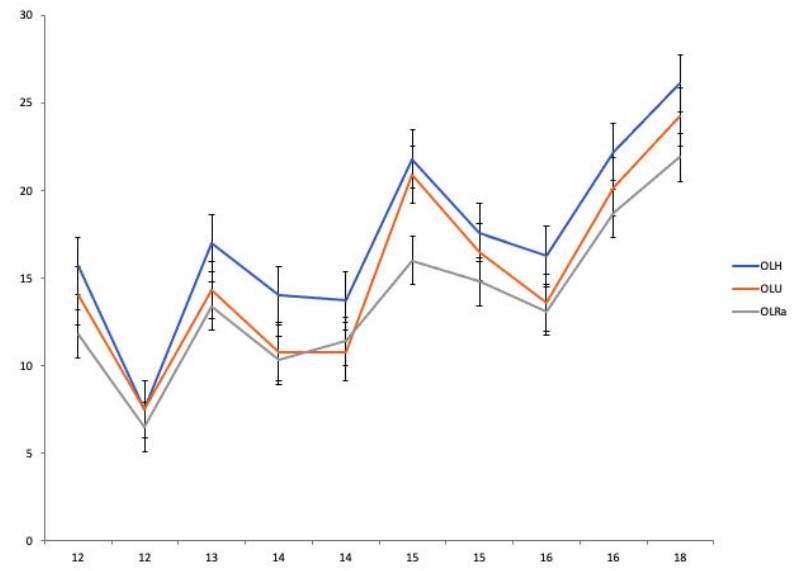
GESTATIONAL AGE(in weeks)	ULNA	
	TL_U	OL_U
12	20.5	14
12	13.9	7.5
13	20.2	14.3
14	17.6	10.8
14	18	10.8
15	28.9	20.9
15	23.8	16.5
16	20.6	13.6
16	28.7	20.2
18	32.6	24.2

Figure 3. Increase in TLR and OLR of Radius with respect to the increasing GA



GESTATIONAL AGE(in weeks)	RADIUS	
	TL_R	OL_{Ra}
12	18.3	11.8
12	9.3	6.5
13	18.3	13.4
14	15.8	10.3
14	17.5	11.4
15	23.5	16
15	19.2	14.8
16	18	13.1
16	25	18.7
18	28	21.9

Figure 4 Increase in OL of all the long bones of the upper limb with respect to the increasing GA



GA(in weeks)	Ossified length		
	OL_H	OL_U	OL_{Ra}
12	15.7	14	11.8
12	7.5	7.5	6.5
13	17	14.3	13.4
14	14	10.8	10.3
14	13.7	10.8	11.4
15	21.8	20.9	16
15	17.6	16.5	14.8
16	16.3	13.6	13.1
16	22.2	20.2	18.7
18	26.1	24.2	21.9

Figure 5 Ratio of ossified length vs total length

S. No.	GA	OLH/TLH %	OLU/TLU %	OLR/TLR %
1	12	61.09	68.29	64.48
2	12	60.54	77.39	57.40
3	13	66.40	70.79	73.22
4	14	62.5	61.36	65.18
5	14	60.35	60	65.14
6	15	62.64	72.31	68.08
7	15	62.63	69.32	77.08
8	16	64.68	66.01	72.77
9	16	65.68	70.38	74.8
10	18	69.10	79.36	75.89

Discussion

Foetal Humerus length is not currently used parameter for assessment of gestational age. There are very few studies on estimation of gestational age by humerus length because humerus is difficult to define accurately, because of its proximity to the chest wall and its apparent continuity with the scapula and clavicle

However, our study show the following parameters:-

- The ossified lengths of all bones were seen to show a gradual increase with the increasing GA (fig.4). A substantial difference was seen between the increase in OLR and OLU 15th week of gestation. On the contrary, the difference between the OLU and OLH was seen to be quite less leading to the overlapping of the line graph
- The line graph plotted between the ossified lengths of all the bones with the CRL at increasing gestational ages showed a gradual increase in OL (fig.4). A parallel increase was seen between the ossified lengths of all the three bones.
- Vivek Patre[18] study of for HL and GA, Pramila[19] study of association of GA with FL and HL revealed that HL would contribute to maximum accuracy next to FLamong all the other parameters.
- Mounika. V et al[20] study for HL and found that Humerus length can be an important additional parameter for estimating gestational age along with other parameters in certain conditions like hydrocephalous, anencephaly breech presentation, multiple gestations, and uterine anomalies .
- Wisniewski M et al[21] observed through their study that The ulna's shaft primary ossification center grows linearly with respect to its length. The obtained morphometric data of the ulna's shaft primary ossification center is considered normative for respective prenatal weeks and may be of relevance in both the estimation of fetal ages and the diagnostic process of congenital defects.
- Bareggi et al.[22] suggested that the evaluation of total length of bones may provide only debatable information relevant to the assessment of fetal age, whereas the ossification center may be considered an important parameter related to the degree of skeletal ossification.

Conclusion

Thus it is evident from the above observations that growth of all the long bones of the upper limb is proportional to increase in the gestational age of the fetus. The ossification start at the primary centre in the diaphysis and progresses above downwards keeping in pace with the total increase in the length of the long bones. The ossified component of these bones was in the range of 60- 70 % of the total length of the bone in the age group of 12 which increased to 70 -80 % in the age group 18 of weeks. At this stage the whole diaphysis of the bone appeared to be ossified with only the proximal and distal ends are cartilaginous.

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