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Determination of Bone Developments of Rat Anterior and Posterior Extremity Bones in Prenatal and Postnatal Period by Double Staining Method

Sıçan Ön ve Arka Ekstremite Kemiklerinin Prenatal ve Postnatal Dönemdeki Kemik Gelişimlerinin İkili Boyama Yöntemi ile Belirlenmesi

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ABSTRACT

Aim: In our study, we aimed to determine the morphological development of the bones of the anterior and posterior extremity by staining the rat fetus and offspring skeletons with the double staining method.

Methods: In the current study, seven groups three prenatal (16th, 18th, and 20th days) and four postnatal (0th, 3th, 7th and 12th days) were formed from the foetuses and offsprings obtained from 13 pregnant rats. Then, it was stained with double staining method. Anterior and posterior extremity images of the fetuses and offsprings were examined under a stereo microscope, and ossification findings were determined. Total bone and ossification lengths as well as ossification areas were measured using the ImageJ software.

Results: The first cartilage destruction in fetuses occurred on the 16th day of pregnancy in the clavicle, scapula, humerus, radius and ulna; It was seen in the femur, tibia and fibula on the 18th day of pregnancy. The first ossification centres were in the clavicle, scapula and humerus on the 18th day of pregnancy; It was seen in the radius, ulna, femur, tibia, fibula and 2-5 metatarsal bones on the 20th day of pregnancy. The secondary ossification centre was seen on the 0th day (birthday) in the scapula and humerus, on the 7th day after birth in the ulna and radius, and on the 12th day after birth in the femur and tibia. In the study, while the ossification rate in 20-day-old foetuses was 48.9% in the humerus, 53.2% in the radius, 55.7% in the ulna, 33.6% in the femur, 43.2% in the tibia, 44.3% in the fibula, it was determined that it reached 69.7% in the humerus, 78.4% in the radius, 73.3% in the ulna, 63.5% in the femur, 75.5% in the tibia, and 69.2% in the fibula on the 12th day after birth.

Conclusion: In this study, we revealed the morphological changes of the anterior and posterior extremity bones of fetuses and offsprings in the normal developmental course. We think that these results will shed light on the studies to be conducted on the detection of skeletal anomalies in teratological studies and contribute to a more comprehensive evaluation of the findings to be obtained from the studies to be conducted.

Key Words: Rat, Double Staining, Ossification, Bone Development, Image J, Toxicology

ÖZET

Amaç: Çalışmamızda sıçan fetus ve yenidoğan iskeletinin ikili boyama yöntemi ile boyanarak, ön ve arka ekstremiteye ait kemiklerin morfolojik gelişimlerinin tespitini amaçladık.

Yöntem: Bu çalışmada 13 adet gebe sıçandan elde edilen fetüs ve yavrulardan 3'ü prenatal (16.,18. ve 20. gün) ve 4'ü postnatal (0, 3.,7. ve 12.gün) olmak üzere 7 grup oluşturuldu. Daha sonra ikili boyama yöntemiyle boyandı. Fetüs ve yavruların ön ve arka ekstremite görüntüleri stereo mikroskop altında incelenerek ossifikasyon bulguları belirlendi. ImageJ yazılımı kullanılarak toplam kemik ve ossifikasyon uzunlukları ile ossifikasyon alanları ölçüldü.

Bulgular: Fetüslerdeki ilk kıkırdak yıkımı clavicula, scapula, humerus, radius ve ulna'da gebeliğin 16. gününde; femur, tibia ve fibula'da ise gebeliğin 18. gününde görüldü. İlk kemikleşme merkezi clavicula, scapula ve humerusta gebeliğin 18. gününde; radius, ulna, femur, tibia, fibula ve 2-5 metatarsal kemiklerde gebeliğin 20. gününde görüldü. İkincil kemikleşme merkezi scapula ve humerusta 0. günde (doğum günü), ulna ve radiusta doğumdan sonra 7. günde, femur ve tibiada doğumdan sonra 12. günde görüldü. Çalışmada 20 günlük fetuslerde kemikleşme oranı humerus'ta %48.9, radius'ta %53.2, ulna'da %55.7, femur'da %33.6, tibia'da %43.2, fibula'da %44.3'ken, doğumdan sonraki 12. günde humerus'ta %69.7, radius'ta %78.4, ulna'da %73.3, femur'da %63.5, tibia'da %75.5, fibula'da %69.2'e ulaştığı tespit edildi.

Sonuç: Bu çalışmada, fetüslere ve yenidoğan yavrulara ait ön ve arka ekstremite kemiklerinin normal gelişim seyrindeki morfolojik değişimlerini ortaya koyduk. Bu sonuçların teratolojik çalışmalarda iskelet anomalilerinin (malformasyon, varyasyon ve diğer anomaliler) tespitine yönelik yapılacak çalışmalara ışık tutacağını ve yapılacak çalışmalardan elde edilecek bulguların daha kapsamlı değerlendirilmesine katkı sunacağını düşünmekteyiz.

Anahtar Kelimeler: Sıçan, İkili Boyama, Kemikleşme, Kemik Gelişimi, Image J, Toksikoloji

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Introduction

Skeletal evaluations are one of the standard evaluations of developmental and toxicology studies [1]. Today, different techniques are used to evaluate the skeletal system [1,2]. One of these techniques is double skeleton staining. Double skeletal staining provides simultaneous staining of both bone and cartilage areas of the skeleton. This method of Inouye has now become a safe method for skeletal staining of developmental and toxicology studies [3].

Many parameters such as fetal or offspring weight, headstern length, bone lengths, the number of ossification centers, where and in which time period the ossification centers ossify and how long they are, and whether there is a delay in the development of bones, are examined in double skeleton staining studies for skeletal evaluation. In the light of the data obtained from these parameters, evaluations are made about the development of the fetal or offspring skeleton [3-7].

In developmental toxicity studies using the double skeletal staining method, the pregnancy is terminated shortly before birth (often on the 20th day of pregnancy) and skeletal examinations are performed on the fetuses to examine the effect of the toxic substance given to pregnant animals on the fetus [8,9].

However, these evaluations on fetuses collected close to birth do not allow an explanation for the outcome of the prenatal induced change in the postnatal period. In particular, it does not provide information about when the changes detected on fetuses collected by cesarean section in fetal skeletal evaluations begin, how they develop, how the process continues and the reversibility of these changes in the postnatal period [10].

Considering that the development of the skeletal system begins in fetal life with intramembranous and endochondral ossification and continues after birth, it is necessary to know well the normal development of the skeleton in both prenatal and postnatal periods. For this reason, in our study, we aimed to reveal the normal morphological development of the bones of the anterior and posterior extremities during the rat skeletal development, both in the prenatal and postnatal periods, by staining the fetuses and offspring obtained from healthy pregnant rats with the double skeleton staining method.

We think that this study will shed light on developmental and toxicology studies covering prenatal and postnatal periods and contribute to a more comprehensive evaluation of the findings to be obtained from these studies in the light of the findings we have presented.

Materials and Methods

This study was carried out in the laboratories of Erciyes University Experimental Research Application and Research Center (DEKAM). The entire procedure related to the study was designed according to the principles of the Erciyes University Local Animal Ethics Committee (Date:10.08.2011 No:11/86) and was supported by the Erciyes University Scientific Research Projects Unit (No: TSY-11-3723).

Analysis of data; The data obtained from the research were analyzed using the SPSS 24.00 package program. Descriptive statistics (arithmetic mean, standard deviation, percentage) were used to analyze the data.

Selection and Mating of Experimental Animals

In this study, a total of 70 fetuses and offsprings obtained from 13 adult female Wistar-albino rats weighing between 170 and 270 grams were equally seperated to seven groups as follows: Three prenatal (16th, 18th, and 20th days of pregnancy), and four postnatal (0th, 3th, 7th and 12th days).

One female and one male rat were taken into the cages at 17 pm to obtain the fetuses and offsprings. Vaginal smears were taken from the female rats at 08.00 the next morning and examined under a microscope. The female rats that had a sperm positive vaginal smear were accepted to be on day 0 of pregnancy. The rats were fed standard on a 12 hours of light-12 hours of darkness in DEKAM (Experimental Studies Research and Implementation Center).

Pregnant rats were anesthetized with 75 mg/kg ketamine and 10 mg/kg xylazine on the 16th, 18th and 20th days of pregnancy, and their fetuses were explanted by opening the anterior abdominal wall under anesthesia.

Those who were damaged during the explantation procedure of the fetuses were not included in the study. A study group for the 16th, 18th and 20th days was formed, with at least 10 fetuses in each group. The remaining pregnant rats were fed until birth. After the rats gave birth, offspring were sacrificed under anesthesia with 75 mg/kg ketamine and 10 mg/kg xylazine on the 0th, 3th, 7th and 12th days of birth, and four separate groups were formed with at least 10 offspring in each group.

Preparation Of The Rat Fetuses And Offsprings For Staining

Height and weight parameters were measured for whole fetuses and offsprings. After the sacrifization and removal process, fetal and offspring tissues were kept in 95% eth-

yl alcohol for seven days, and then they were fixed. Following this procedure, they kept in pure aceton for three days to clear their oil. Then, their skins were peeled and their internal organs and eyes were removed. The staining process of the skeletons of rat fetuses and offspring was carried out within the framework of the protocol in Table 1 [10,11].

Right and left anterior and posterior extremity bones of fetus and offspring rats, whose skeletal system became fully visible after the transparency phase, which lasted for approximately 16 days, were examined under a stereomicroscope and their photographs were taken. The obtained images were opened in ImageJ program and total bone length, ossification length and ossification areas were calculated. For long-term storage of finished fetuses and offspring, they were kept in pure glycerin in separate containers.

Calculation of the ratio of bone and cartilage areas: The obtained photo images were opened in the ImageJ program [12]. Then, the boundaries of the structures were determined with the help of mouse to obtain the superficial areas of the images by selecting the polygon selections in the ImageJ program. Bone and cartilage surface areas were measured by performing Ctrl-M (measure) on the keyboard. Bone and cartilage surface areas were recorded in pixels. As a result, how much area the bone and cartilage occupy in the extremity was calculated as a percentage. The following formula was used for this.

$$V_v(\text{cartilage, bone}) = \frac{\sum Pcartilage}{\sum Pbone} \times 100$$

Similarly, in the measurement of the lengths of the bones and ossification centers, the calibration was made on the set scale in the ImageJ program, and then the length measurements were made using a straight line [12].

Results

Effects on growth parameters:

Before starting the staining process in all groups, the heights and weights of the fetuses and offspring were determined. The findings of the height (Head-Stern lengths) and weight measurements obtained from all groups are shown in Table 2.

Effects on cartilage destruction:

In our study, the first cartilage destruction was in the anterior extremity bones the clavicula, scapula, humerus, radius, and ulna were seen on day 16, and in the posterior extremity bones, on the femur, tibia, and fibula, on day 18 (Figure 1).

Effects on the general growth morphology of the skeleton:

In the general morphological examination of the whole skeleton after staining, it was observed that the first ossification took place in the mandible, maxilla, scapula,

Table 1. Double skeleton staining protocol

Technical Stages	Solutions	Time
Fixation	70% ethyl alcohol	4-7 days
Degreasing	Pure acetone	1-3 days
Double Staining	preparing the double staining solution 1st solution: 300mg Alcian Blue + 100ml 70% ethyl alcohol 2nd solution: 100mg Alizarin Red S + 100ml 95% ethyl alcohol 3th solution: 1st solution + 2nd Solution + 100ml Glacial acetic acid 4th solution: Prepared by adding 1700 ml of 70% ethyl alcohol to the first three solutions	7 days 38-40 °C incubated
Transparency	1) 1% KOH 2) 1% KOH (80 ml) +%20'lik glycerin (20 ml) 3) 1% KOH (50 ml) +%50'lik glycerin (50 ml) 4) 1% KOH (20 ml) +%80'lik glycerin (80 ml)	1-3 days 5-7 days 5-7 days 5-7 days
Safekeeping	100% pure glycerine	

Acta Medica Alanya 2023;7(2): 190-199
Doi: 10.30565/medalanya.1262518

Table 2. Height (Head-Stern length) and weight (gr) measurements of fetuses and offspring groups

		PRENATAL GROUP			POSTNATAL GROUP			
	No	16-day old fetus	18-day old fetus	20-day old fetus	0-day-old offspring	3-day-old offspring	7-day-old offspring	12-day-old offspring
HEIGHT (mm)	1	11	17.4	31.1	41	45	57	61.2
	2	10.3	19.1	29.5	41.5	47.5	46.6	63.8
	3	13.3	20.1	32.9	43.0	48.3	52.2	61
	4	11.1	19.2	31.1	41.1	48.5	49.5	61
	5	11.2	19.5	30	41.5	46.1	48	73.0
	6	10.6	18.8	31.8	43	46.1	54.2	62.1
	7	10.4	18.4	30.5	40	43.5	52.2	53.2
	8	10.6	19	31.4	43.4	51	52.3	69.7
	9	11	17.9	31.7	40	44.2	43.2	56.5
	10	11	18	30.5	43.4	49.2	52.2	50
	11	-	18.7	32.4	39.8	45	51.5	54.2
	12	-	18	29.8	-	-	39.2	64.5
	13	-	19.2	33.7	-	-	-	-
$\bar{X}$±SD		**11.05±0.84**	**18.71±0.75**	**31.26±1.24**	**41.6±1.39**	**46.76±2.32**	**49.84±4.92**	**60.85±6.66**
WEIGHT (g)	1	0.24	1.98	3.5	5.11	7.46	10.67	14.53
	2	0.22	1.85	3.25	5.6	7.22	8.72	18.22
	3	0.24	2	3.07	4.94	6.11	10.84	11.4
	4	0.22	1.96	3.83	5	5.13	7.36	11.08
	5	0.23	2.4	3.39	4.82	6.6	7.56	15.59
	6	0.23	1.98	2.87	5.35	6.55	5.93	12.56
	7	0.24	1.74	3.36	5.59	7.36	10.08	20.34
	8	0.23	1.9	3.2	5.44	8	7.13	17.17
	9	0.22	2.1	3.03	5.21	7.88	10.37	10.68
	10	0.21	1.97	3.19	5.16	7.53	6.86	9.13
	11	-	1.98	3.2	5.59	7.29	10.65	18.31
	12	-	1.86	2.8	-	-	11.12	13.72
	13	-	1.80	2.9	-	-	-	-
$\bar{X}$±SD		**0.22±0.10**	**1.96±0.16**	**3.19±0.28**	**5.25±0.27**	**7±0.84**	**8.94±1.87**	**14.39±3.56**

clavicle and humerus bodies and extramitas vertebralis of the 1-8 ribs of the 18-day-old fetuses. It was observed that cartilage destruction continued in the bodies of other long bones and these regions had a spongy appearance (Figure 1).

Effects on the anterior extremity long bones:

The time of appearance of the first ossification centers of the anterior extremity skeleton bones is shown in Table 3, and the appearance of the first ossification centers of the anterior extremity skeleton is shown in Figure 2.

Effects on the posterior extremity long bones:

The first ossification center in the posterior extremity; femur, tibia, fibula, and 2-5 metatarsal bones at day 20 of gestation, 0-day-old offspring in 2-5 phalanges, and 3-day-old offspring in calcaneus, talus, 1st metatarsal and 1st phalanx (Figure 3).

Secondary ossification center in the anterior extremity was first seen in the scapula and humerus in 0-day-old offspring, while it was seen in the radius and ulna at the proximal end on the 7th day after birth and at the distal end on the 12th day after birth, and in the metetarcarpal bones on the 12th day after birth. Secondary ossification center in the posterior extremity was seen at the distal end of the femur, both the distal and proximal end of the tibia in the 12-dayold offspring (Figure 4).

In our study, bone length and ossification rate increased with age in all groups. Bone length, ossification length and ossification rates of anterior extremity and posterior extremity bones in prenatal and postnatal groups are given in Table 4.

Table 3. The time of first ossification center of the bones of the anterior extremity

	Bone Name	Prenatal period	Postnatal period
Anterior extremity	Clavicula	18th day before birth	-
	Scapula	18th day before birth	-
	Humerus	18th day before birth	-
	Radius	20th day before birth	-
	Ulna	20th day before birth	-
	Ossa carpi		12th day after birth
	Ossa metacarpi (2-4. Metacarpal bone)	20th day before birth	-
	Ossa metacarpi (5th metacarpal bone)		0 days old(birthday)
	Ossa metacarpi (1th metacarpal bone)	-	12th day after birth
	Phalanges (Both distal and proximal phalanges of the 2nd-4th finger)	20th day before birth	-
	Phalanges (Distal and proximal phalanx of the 5th finger)		0 days old(birthday)
	Phalanges (In the middle phalanx of the 2nd-5th finger and the proximal phalanx of the 1st finger)	-	3th day after birth
	Phalanges (distal phalanx of the 1st finger)	-	3th day after birth

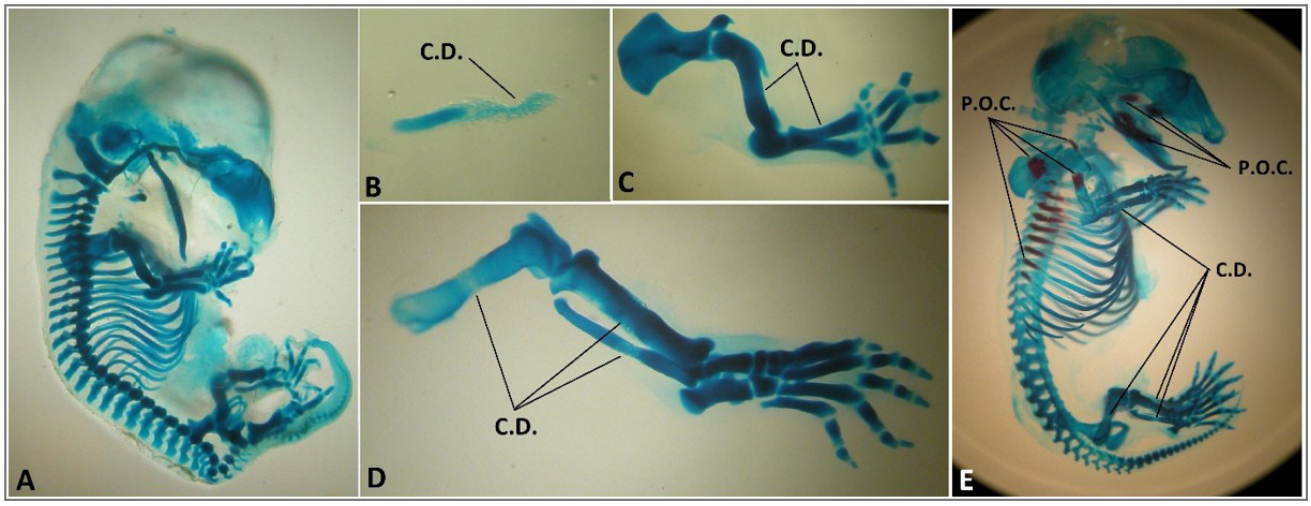

Figure 1. A: General view of the entire skeleton of the 16-day-old fetus. B: Clavicle (16-day-old fetus) C: Scapula, Humerus, Radius and Ulna (16 days old fetus) D: Posterior extremity (16-day-old fetus) E: General view of the entire skeleton of the 18-day-old fetus.
*C.D: Cartilage destruction, P.O.C: Primer ossification center

Discussion

In developmental toxicity studies, the degree of ossification is an indicator of skeletal maturity [11]. In the skeletal evaluations made using the double staining technique; parameters such as fetal or offspring weight, head-stern length, bone lengths, the number of ossification centers, where and when these centers appear, how long they are, whether there is a delay in bone development are examined [4,9,13-15]. When we scan the literature in the light of these parameters, we see that studies are generally carried out on 20-day-old fetuses and offspring. However, it is seen that the studies revealing the stages of skeletal development in the prenatal and postnatal groups are limited and the data in the findings of the control groups of the experimental studies are mostly shared.

Fetal weight and head-stern length are indicators of fetal skeletal development and are frequently evaluated in toxicology studies. In many studies, it has been reported that there is a decrease in weight and head-stern length in fetuses and offspring of pregnant mothers who have

Acta Medica Alanya 2023;7(2): 190-199
Doi: 10.30565/medalanya.1262518

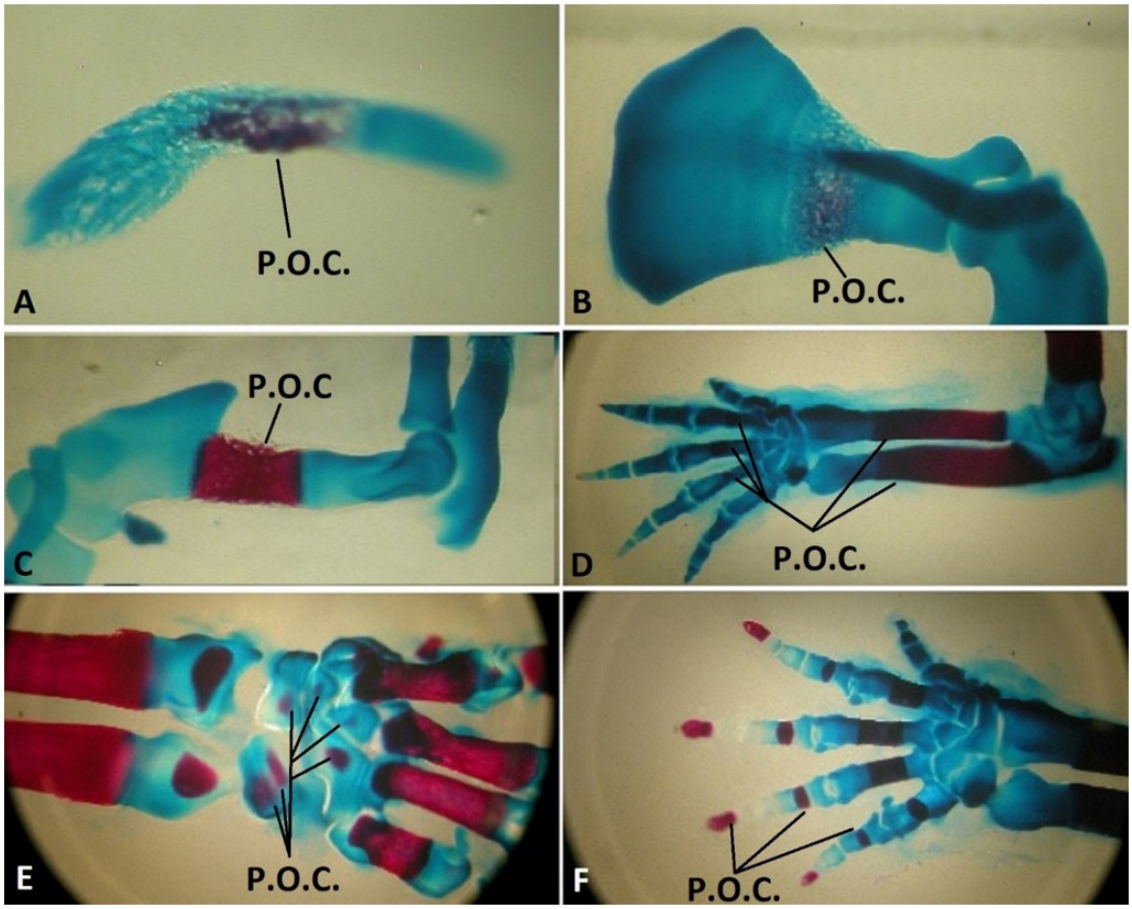

Figure 2. The appearance of primary ossification centers in the bones of the anterior extremity A: Clavicle (18-day-old fetus) B: Scapula (18-day-old fetus) C: Humerus (18-day-old fetus) D: Radius, ulna and ossa metacarpi (20-day-old fetus) E: Ossa carpi (12-day-old offspring) F: Ossa digitorum phalanges (0-day-old offspring).
P.O.C: Primer ossification center

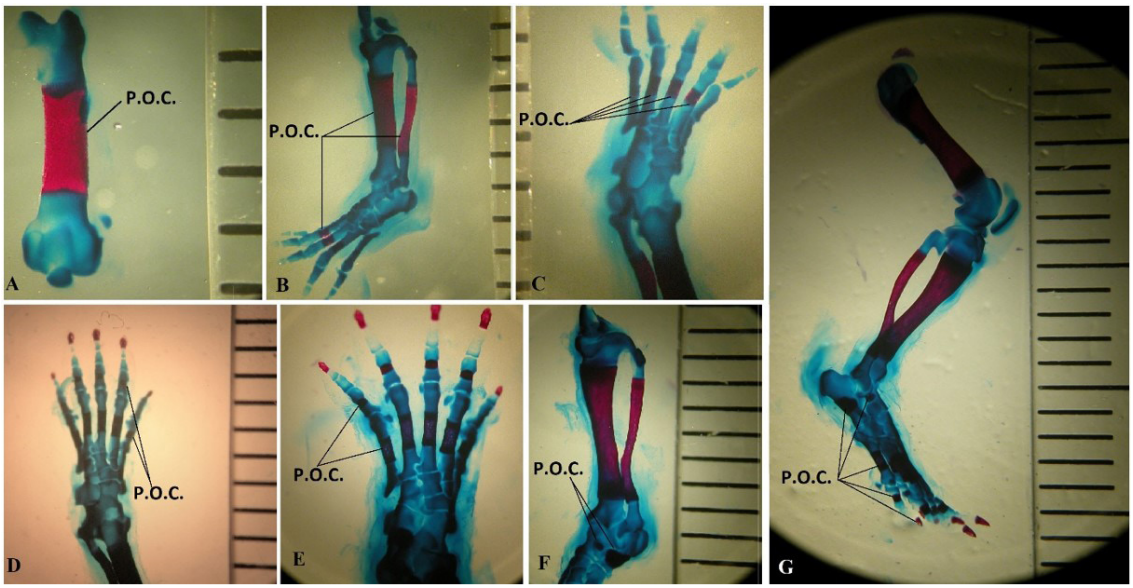

Figure 3. The appearance of the first ossification centers of the posterior extremity. A: Femur (20-day-old fetus) B: Tibia, fibula and metatarsal bones (20-day-old fetus). C: 2-5 metatarsal bones (0-day-old fetus) D-E: Ossa metatarsi and ossa digitorum phalanges (0-day-old offspring) F: Calcaneus and talus (3-day-old offspring) G: General view of the posterior extremity of the 3-day old offspring
P.O.C: Primer ossification center.

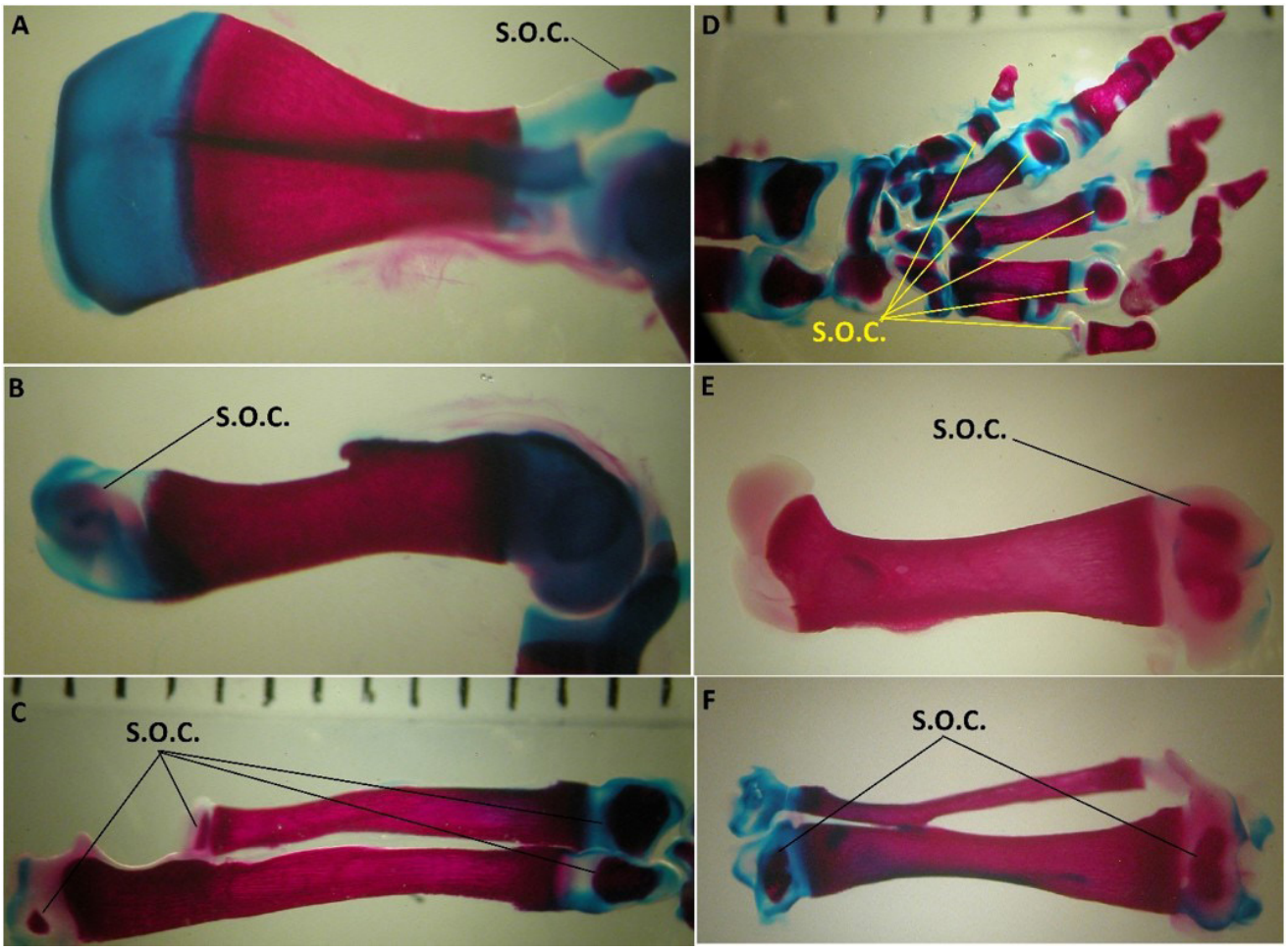

Figure 4. The appearance of secondary ossification centers in the bones of the anterior and posterior extremity. A: Scapula (0-day-old offspring). B: Humerus (0-day-old offspring). C: Radius and Ulna (12-day-old offspring). D: Ossa metacarpi (12-day-old offspring). E: Femur (12-day-old offspring). F: Tibia and fibula (12-day-old offspring).
S.O.C: Seconder ossification center

been exposed to toxic substances [13,15-19]. In our study, unlike other studies, we determined the course of ossification in the postnatal period by measuring the weight and head-stern lengths of fetuses and offspring of both prenatal and postnatal periods.

The formation of the primary ossification center, which is another indicator of bone development, is an indicator of the transition from cartilage tissue to bone tissue. In some studies, it has been reported that the primary ossification center in the clavicle, scapula and humerus is seen between the 15th and 17th days of pregnancy [15,20,21]. In our study, the first cartilage destruction in the clavicula, scapula and humerus was observed on the 16th day of pregnancy, and the first ossification center was observed on the 18th day of pregnancy.

In some studies, it has been reported that the primary ossification center in the radius and ulna is seen between the 15th and 17th days [15,20,21]. In our study, the first cartilage destruction in the radius and ulna started on the 16th day of pregnancy and the first ossification center was seen on the 20th day of pregnancy.

In studies conducted on the day of the first appearance of the primary ossification center in the femur, tibia and fibula from the posterior extremity bones, it has been reported that it occurs between 16 and 17 days of pregnancy [15,20,21]. In our study, the first cartilage destruction in the femur, tibia and fibula was observed on the 16th day of pregnancy, and the first ossification center was observed on the 20th day of pregnancy.

When the primary ossification center first appeared, it was observed that the center of the bone took a spongy appearance due to cartilage destruction and was stained blue with alcian blue, and ossification began in this area in the next period and stained red with alizarin red S for this reason, it was observed that there were differences between the day of cartilage destruction and the first day of ossification.

The secondary ossification center is an indicator of the maturation of the bone tissue and the final shape of the

Table 4. Findings of anterior and posterior extremity bones

	Total Bone Length (cm)	Length of Ossified Part (cm)	Ossification Rate	Total Bone Length (cm)	Length of Ossified Part (cm)	Ossification Rate	Total Bone Length (cm)	Length of Ossified Part (cm)	Ossification Rate
		HUMERUS			**RADIUS**			**ULNA**	
	X̄±SD	**X̄±D**	%	**X̄±SD**	**X̄±SD**	%	**X̄±SD**	**X̄±SD**	%
16 day old fetus	0,18±0,01	-	-	0,11±0,01	-	-	0,15±0,01	-	-
18 day old fetus	0,32±0,02	0,07±0,02	15,4	0,23±0,01	-	-	0,30±0,01	-	-
20 day old fetus	0,54±0,01	0,27±0,02	48,9	0,40±0,02	0,22±0,01	53.2	0,52±0,03	0,28±0,02	55.7
0 day old offspring	0,70±0,11	0,40±0,04	52,2	0,56±0,10	0,34±0,02	62.1	0,68±0,03	0,42±0,03	63.9
3 day old offspring	0,76±0,05	0,46±0,05	55,6	0,61±0,03	0,41±0,04	64.5	0,77±0,06	0,52±0,04	66.2
7 day old offspring	0,90±0,06	0,59±0,04	58,5	0,75±0,03	0,56±0,05	67.4	0,94±0,04	0,67±0,04	66.7
12 day old offspring	1,04±0,07	0,68±0,07	69,7	0,89±0,08	0,66±0,07	78.4	1,21±0,12	0,87±0,12	73.3
		FEMUR			**TIBIA**			**FIBULA**	
	XX̄±SD	**XX̄±SD**	%	**X̄±SD**	**X̄±SD**	%	**X̄±SD**	**X̄±SD**	%
16 day old fetus	0,16±0,03	-	-	0,13±0,01	-	-	0,11±0,01	-	-
18 day old fetus	0,35±0,05	0,10±0,16	-	0,29±0,01	0,04±0,01	-	0,26±0,02	0,04±0,01	-
20 day old fetus	0,60±0,07	0,24±0,03	33,6	0,47±0,06	0,22±0,04	43,2	0,44±0,06	0,22±0,04	44,3
0 day old offspring	0,74±0,13	0,41±0,06	44,6	0,70±0,16	0,44±0,11	56,6	0,65±0,16	0,41±0,10	60,1
3 day old offspring	0,83±0,07	0,47±0,04	46,6	0,83±0,11	0,55±0,06	63,6	0,74±0,06	0,52±0,05	67,2
7 day old offspring	1,09±0,08	0,66±0,06	51,3	1,07±0,08	0,73±0,07	69,5	0,96±0,08	0,67±0,06	68,3
12 day old offspring	1,18±0,09	0,81±0,08	63,5	1,39±0,08	0,91±0,07	75,5	1,18±0,08	0,82±0,08	69,2

X̄: Arithmetic Mean, SD: Standard Deviation

bone, so the time of appearance of the secondary ossification center is another indicator of skeletal development [22,23]. However, when we look at the literature, it is seen that the findings about the initial development of the secondary ossification center are limited and are obtained from studies on some specific bones.

In their study, Morini S and et al. [5] reported that ossification that started on the 4th day after birth in the humeral head became evident on the 11th day and the secondary ossification center developed.

Campion S.N. and et al. [24] reported that a secondary ossification center develops at the proximal end of the femur between 15 and 20 days after birth.

In their study on rats, Hedberg A and et al. [25] reported that on the 10th day after birth, secondary ossification center formation started at the distal femur and proximal end of the tibia, and the epiphyseal ossification reached a relatively mature state on the 15th day.

In our study, the secondary ossification center was first seen in the 0-day-old offspring (in offspring) of the scapula and humerus. 7-day-old offspring was seen at the proximal end of the radius and ulna, and at the distal end of the radius and ulna in 12-day-old offspring. In our study, a secondary ossification center was observed at the distal end of the 12-day-old offspring femur and both the distal and proximal ends of the tibia.

Other parameters used to evaluate the effect of the toxic substance are fetal bone length, ossification length and ossification rate. However, it is seen that measurements for these parameters are frequently made in 20-day-old fetus groups [6,7]. When the findings of these parameters belonging to our study are compared with some studies in the literature, it is seen that the results are close to each other. In our study, we measured these values in a total of 7 groups, including prenatal and postnatal, and revealed the course of ossification in a wider time interval.

Evaluation of fetuses after administration of the toxic substance to pregnant animals is common and current practice in developmental toxicology studies [18,19]. However, it is wondered how the postnatal outcome and developmental process of the fetus, which has been exposed to toxic effects in the prenatal period, which has been discussed for a long time, is affected. The postnatal bone formation process continues rapidly and the evaluation of whether the anomaly seen on fetuses collected during the fetal period is reversible and transient (variation) or permanent (malformation) cannot be made without more data in most cases. For this reason, it is necessary to include not only studies covering the fetal period, but also studies covering the postnatal period.

Hofmann T. and et al. [10] suggested that anomalies such as delayed ossification, presence of extra or rudimentary ribs, and twisted long bones can only be evaluated as malformations with more data, therefore the animal exposed to toxic substance should be evaluated in the postnatal period.

Saeidinezhad M. and et al. [26] reported that morphine had a toxic effect on 7-day-old offspring mice in their study examining the effect of morphine, and that morphine reduced the growth of longitudinal bone by reducing the growth of primary and secondary ossification centers.

Carney E.V. and Kimmel C.A. [27] suggested that both delayed ossification and wavy rib findings should be evaluated in a larger time frame in conjunction with other findings.

Hayasaka I and et al. [28] reported that mothers treated with 90 mg/kg/day azosemide had a significant increase in skeletal abnormalities in their fetuses, such as wavy ribs, twisted scapula, and twisted arm bone, in rats, but these observed anomalies resolved in adult offspring.

Limitations of the Study

This study has limitations. In order to follow the comprehensive developmental process, it is necessary to increase the number of postnatal groups and to follow them over a longer period of time.

Conclusion

Considering that the development of the skeletal system begins in fetal life with intramembranous and endochondral ossification and continues after birth, the normal development of the skeleton in both prenatal and postnatal periods should be well known. For this reason, in our study, we revealed the normal morphological developmental course of the bones of the anterior and posterior extremities during rat skeletal development in both prenatal and postnatal periods. We think that our study will be a source for developmental toxicology studies covering prenatal and postnatal periods and will contribute to a more comprehensive evaluation of the findings to be obtained from these studies in the light of the findings we have presented.

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