



Research Article

Effect of Experimental Phenylketonuria on the Bone of Pregnant Mothers and their young during Perinatal Life and after Delivered Newborn of Albino Rats**Hassan I. Elsayyad¹, Mahmoud E. Mohallal², Hany A. Hefny^{2,3*} & Hala M. Ebied²**¹*Zoology Department, Faculty of Science, EL Mansoura University, Egypt.*²*Zoology Department, Faculty of Science, Suez Canal University, Egypt.*³*Biology Department, University College, Umm Al Qura University, Al-Jomoum, KSA.*

Abstract: Phenylketonuria (PKU) is a genetic disorder that is characterized by an inability of the body to utilize the essential amino acid, phenylalanine. The disease results from a deficiency in phenylalanine hydroxylase, the enzyme catalyzing the conversion of phenylalanine to tyrosine. Although, this inborn error of metabolism was among the first in humans to be understood biochemically and genetically, little is known about the mechanisms involved in the pathology of PKU during neonatal development. Elevated concentrations of plasma phenylalanine were induced in pregnant rats by oral administration of 50mg/100g body weight alpha-methylphenylalanine plus phenylalanine supplementation at a dosage of 60mg/100g body weight two times daily after the 6th day of onset of gestation till 14 & 16 days of gestation as well as at parturition. Treatment with alpha-methylphenylalanine/phenylalanine resulted in a significant decrease of accumulated body weight gain during pregnancy as well as exhibited marked growth retardation of prenatal fetuses and delivered newborn. The growth retarded fetuses was manifested by decreased body weight, malformed both fore- & hindlimb, oedematous skin & superficial hematomas widely spread in different parts of the body. Ossification of bones was greatly altered. Skeletal abnormalities restricted mainly in skull, sternbrae, lumbar, caudal vertebrae and distal phalanx of both fore- & hindlimb. Histological examination of femoral bone revealed varieties of histopathological abnormalities which illustrated and discussed. These results suggested that exposure of the fetus to high plasma concentrations of phenylalanine cause deformities of bone.

Keywords: PKU, Bone, Pregnant, Rat.

1. Introduction

Phenylketonuria (PKU) disease was first described in 1934 by a Norwegian doctor named Asbjorn Folling [1]. Folling described that the disorder of phenylalanine metabolism was the cause of the children's mental retardation and gave the name Phenylketonuria to this condition. Subsequent examinations of PKU patients excreting phenylpyruvic acid proved that phenylalanine was accumulated in their bodies.

In Phenylketonuria, phenylalanine accumulates in the blood as the result of a deficiency or malfunction of the liver enzyme phenylalanine hydroxylase (PAH), which under normal conditions converts phenylalanine to L-tyrosine [2]. Consequently, individuals with PKU are low in L-tyrosine [3] which may contribute to behavior problems [4].

Phenylalanine abnormally accumulates in the blood. Inability to remove excess phenylalanine from

the blood during infancy and early childhood produces a variety of problems including mental retardation. Universal newborn screening can identify the genetic defect and these problems can be greatly reduced by placing the child on a special diet within the first few days of life [5]. The most common cause for a lack of PAH enzyme is a genetic defect in the gene for PAH, where most patients suffering from PKU have one or another of several possible mutations in this gene; Also the lack of PAH activity is a defect in the generation of adequate amounts of the cofactor tetrahydrobiopterin (BH₄). Tetrahydrobiopterin is an essential coenzyme not only for the hydroxylation of phenylalanine to tyrosine but also for the hydroxylation of tyrosine to L-dopa required for dopamine biosynthesis and for the hydroxylation of tryptophan to 5-hydroxytryptophan, the substrate for serotonin biosynthesis. This group appears to constitute 3% of all hyperphenylalaninemia patients [6].

*Corresponding author:
E-mail: hanyhefny56@yahoo.com.

Genetic analysis, using recombinant DNA techniques, has established that the genetic locus for PKU is on chromosome 12, DNA analysis has shown that the classical form of the disease is due not to deletion of the entire gene for PAH, but is instead due to mutations within the gene's sequence leading to amino acid substitutions; results in an enzyme that does not work properly and therefore the body cannot metabolize phenylalanine [2]. The gene for PAH was cloned by Robson *et al.*, 1982, and by now more than 400 different mutations in the PAH gene have been identified [7, 8]. Maternal PKU is of particular concern. Excessively high or low levels of phenylalanine may occur during pregnancy, both of which may adversely affect the fetus [9]. Maternal PKU can lead to fetal malformations, and mental retardation [10, 11]. Adverse effects on the offspring can be reduced by family planning and by careful dietary control both prior to and during pregnancy [12-14]. In addition, low L-tyrosine levels in pregnant women with PKU may contribute to fetal damage [15]. Untreated children with persistent severe hyperphenylalaninemia (Elevated plasma Phe concentration; HPA) show impaired brain development. The excretion of excessive phenylalanine and its metabolites can create a mousy body odor, sensitivity to sunlight and skin conditions such as eczema. The associated inhibition of tyrosinase is responsible for decreased skin and hair pigmentation, light skin. Affected individuals also have decreased myelin formation and dopamine, norepinephrine, and serotonin production. Further problems can emerge later in life and include exaggerated deep tendon reflexes and paraplegia or hemiplegia [16, 17]. The present work aims to illustrate the adverse effect of Phenylketonuria on mother's rat and its reflection on the development of the bone on the developmental stages 14th and 16th day of gestation and on delivered newborn through determination. The ossification of the skeletal system (the presence or absence of ossification centers) of prenatal fetuses at the 14th and 16th day of gestation as well as delivered newborn and the histogenesis of ossification of auditory regions, ribs, fore and hindlimb of 14 and 16 days fetuses.

2. Materials and Methods

2.1. Experimental animals

Eighty fertile virgin female and fertile males of albino rats with an average body weight of 100–110 grams (ratio of 1 male: 3 females) were obtained from Helwan Animal Breeding Farm, Ministry of Health, Cairo, Egypt and used for experimentation. Rats were housed in cages in the Animal House of Department of Zoology, Faculty of Science, Suez Canal University at a ratio of four/cage. They were maintained at a temperature of 20–25°C with 12h light- 12h dark cycle and stayed for acclimatization for one week before starting the experiments. They were fed on standard diet composed of 50% grinding barley, 10% grinding Yellow Maize, 20% milk and 10% vegetables was

supplied. Barley is a very useful grain source for growing, gestating, and lactating dairy cattle, providing more protein than the most other grains as well as showed a highly digestible starch and useful fiber. Barley is an economical nutrient source that should be strongly considered in formulating rations for dairy animals [18]. Water and food were available for consumption *ad libitum* throughout the experimental period. Rats were observed daily and only healthy animals were used in these experiments.

2.2. Experimental work

Mating was carried out by housing females' rat with fertile males in separate cages at a ratio of three females with one male for an overnight between 8 PM to 8 AM. The presence of vaginal bogs or the presence of sperms in the vaginal smear determined the zero date of gestation.

The pregnant mothers were divided into two main groups, thirty animals per each:

- i. **Control:** Thirty pregnant mothers were divided into three subgroups ten animals in each; sacrificed at 14th and 16th day of gestation as well as at parturition.
- ii. **Experimental Phenylketonuria group:** Each selected pregnant females on the 6th day of gestation was intragastrically administered 30mg DL- α -methylphenylalanine/100g body weight (to inhibit maternal phenylalanine hydroxylase) plus 60mg/100g body weight L-phenylalanine (to raise fetal plasma phenylalanine) dissolved in milk, at 12h intervals. The applied dose was selected according to Spero and Yu [19], and Rech *et al.*, [20]. The thirty pregnant mothers diseased with experimental PKU were divided into three subgroups ten animals in each; sacrificed at 14th and 16th day of gestation as well as at parturition.

2.3. Investigated parameters

2.3.1. Percentage of mean body weight gain during pregnancy

All of the control and experimental were weighted at during pregnancy till parturition. The percentage of body weight gain was calculated at the 8th, 10th, 12th, 14th, 16th, 18th and 20th days of gestation as follows:

$$\frac{(\text{Mean final body weight} - \text{Mean initial body weight})}{\text{Mean initial body weight}} \times 100$$

2.3.2. Effects on mothers during utero life as well as at parturition

The total numbers of prenatal fetuses at 14th and 16th day of gestation as well as delivered newborn for both control and experimental groups were recorded. The incidence rates of growth morphological abnormalities of the bone; were recorded. Photographs for both control and experimental groups were taken. Both fetuses and delivered newborn of both control and

experimental groups were fixed immediately after separating in 10% formal saline for 24 hours.

2.3.3. Ossification of the skeleton

The skeletal system was examined for the presence or absence as well as the rate of bone defects in skull, limbs, and girdles. For this purpose, fixed specimens of prenatal fetuses on the 14th and 16th day of gestation as well as delivered newborn of both control and experimental groups were freed of their skin, viscera, adipose tissue, and eyes are immersed in 2% aqueous KOH solution for five days till ossified areas were clearly visible through the soft tissue. The specimens were stained Alizarin red-s according to the method described by Bancroft *et al.*, [21]. The axial and appendicular bones were examined for the presence (+) or absence (-) of ossification centers. The stained preparations were carefully examined under the dissecting binocular microscope to study the various parts of the axial and appendicular skeleton. Photographs were carried out. The incidence of missing bones or delaying the formation of both axial and appendicular regions was recorded. Length measurements of mandibular bone as well as of ischium, ilium (girdles) and fore- (humerus and radius-ulna) and hind (femur and tibia-fibula) limbs were carried out in 15 specimens per each developmental stage of both control and experimental groups to assess the degree of bone growth defects of experimental groups compared with the control.

2.3.4. Light microscopic observations of maternal, fetuses at 14 and 16 days and newborn tissue

At parturition, ten mothers of both control and experimental PKU were sacrificed femur bone were removed and immediately fixed in formal saline for 24 hours. The femur bones were decalcified in 5% nitric acid for 3 days followed by careful washing in water

and returned to 10% formal saline. To assess retarding histogenesis of ossification, auditory regions, ribs, fore and hindlimb of 14 days fetuses of control and the experimental group as well as cervical vertebrae and cervical spinal cord of 14 and 16 days fetuses were separated and immediately fixed in 10% formal saline. The previously mentioned specimens were then washed several times in tap water, dehydrated in ascending grades of ethyl alcohol, cleared in terpineol for two days, then washed in benzene for 10 minutes and embedded in three changes of molten paraplast 58-62°C. Serial 6 μ thick histological sections were cut and stained in haematoxylin and eosin [22], examined carefully under bright field light microscopy and photographed.

2.3.5. Statistical analysis

Statistics were calculated with SPSS for Windows version 13.0; the mean values obtained in the different groups were compared by paired student's t-test. All results were expressed as mean values \pm SE and significance was defined as $p < 0.05$ and highly significant when < 0.01 [23].

3. Results

3.1. Skeletal abnormalities

3.1.1. Ossification of skull

In control 14 days' fetuses, ossification centers made their first appearance in nasal, frontal, premaxilla, maxilla, zygomatic arch, exoccipital and mandible (Plate 1: Fig. A). However, in those of experimentally induced PKU mothers, the nasal, premaxilla and exoccipital appeared missing. Meanwhile the other ossified bones showed a considerable reduction of mineralized bones as detected by faintly staining affinity (Plate 1: Figs. B and C).

14th day fetus control (A),(B)and(C)PKU fetus

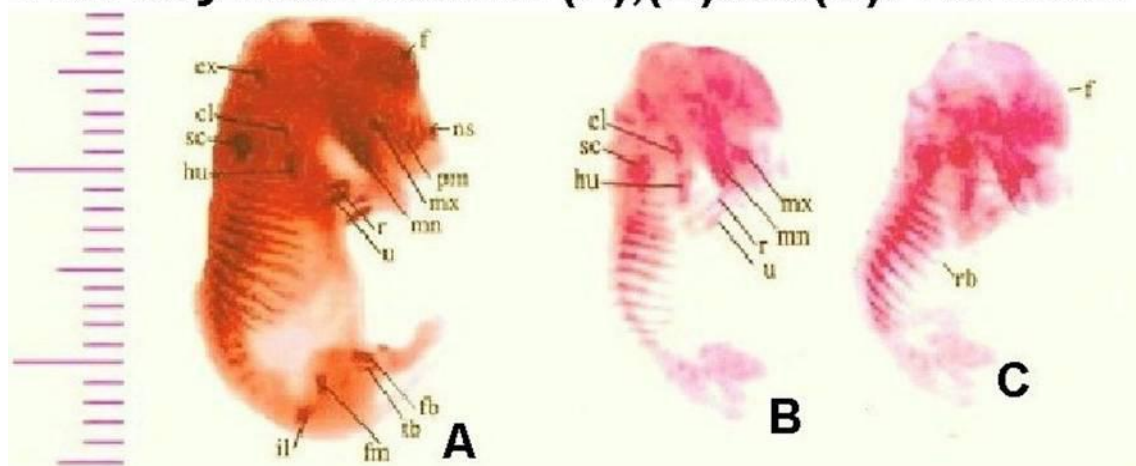


Plate (1). Photomicrograph of 14th day fetus: showing ossification of the skeleton of control and PKU (na - Nasal, f - Frontal, pm - Premaxilla, mx - Maxilla, mn - Mandible, ex - Exoccipital, sc - Scapula, cl - Clavicle, hu - Humerus, r - Radius, u - Ulna, il - Ileum, fm - Femur, tb - Tibia and fb - Fibula).

In control 16 days fetuses, extra ossification centers were detected in parietal, intraparietal, zygomatic arch, squamosal, malleus, basioccipital, exoccipital and supraoccipital (Plate 2: Fig. A). On the other hand, 16-day fetuses of experimentally induced PKU mother showed missing ossification centers in squamosal and basioccipital (Plate 2: Figs. B and C). Delivered newborn maternally diseased with PKU possessed a considerable delayment of ossification

centers, especially in intraparietal, hyoid arch, squamosal, incus, malleus, stapes, tympanic ring, basioccipital and supraoccipital (Plate 3 and Table 4). The incidence of missing bones was predominated in different skull bones, especially in nasal, ossicles and supraoccipital (Table 1). Length measurements of ossified mandibular bone revealed a considerable reduction in 14 and 16 days fetuses and delivered newborns of PKU mothers (Table 2, 3, 4 and Fig. 1).

16th day rat fetus control (A) ,(B)and(C). PKU fetus

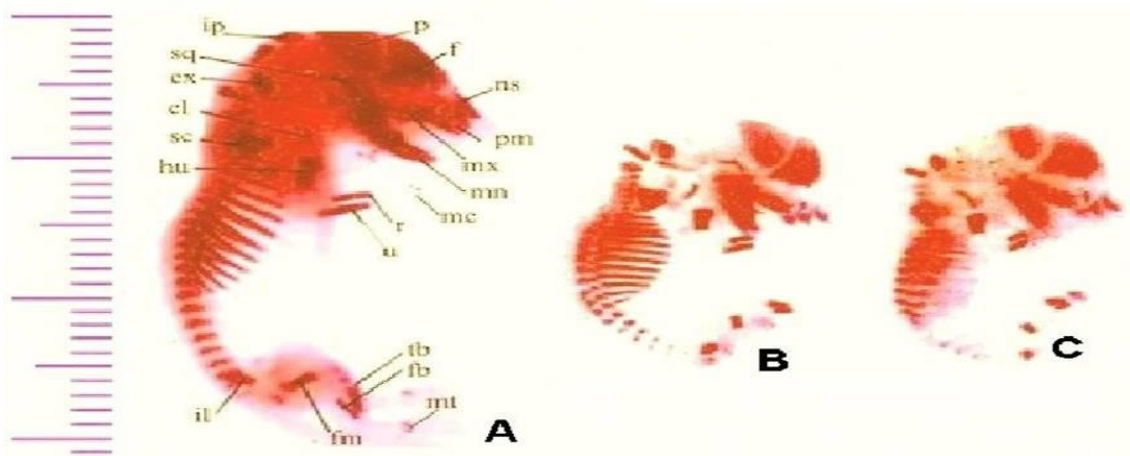


Plate 2. Photomicrograph of 16th fetus: showing ossification of the skeleton of control and PKU (na - Nasal, f - Frontal, pm - Premaxilla, mx - Maxilla, mn - Mandible, ex - Exoccipital, sc - Scapula, cl - Clavicle, hu - Humerus, r - Radius, u - Ulna, il - Ilium, fm - Femur, tb - Tibia and fb - Fibula).

DELIVERED NEWBORN CONTROL AND PKU FETUS

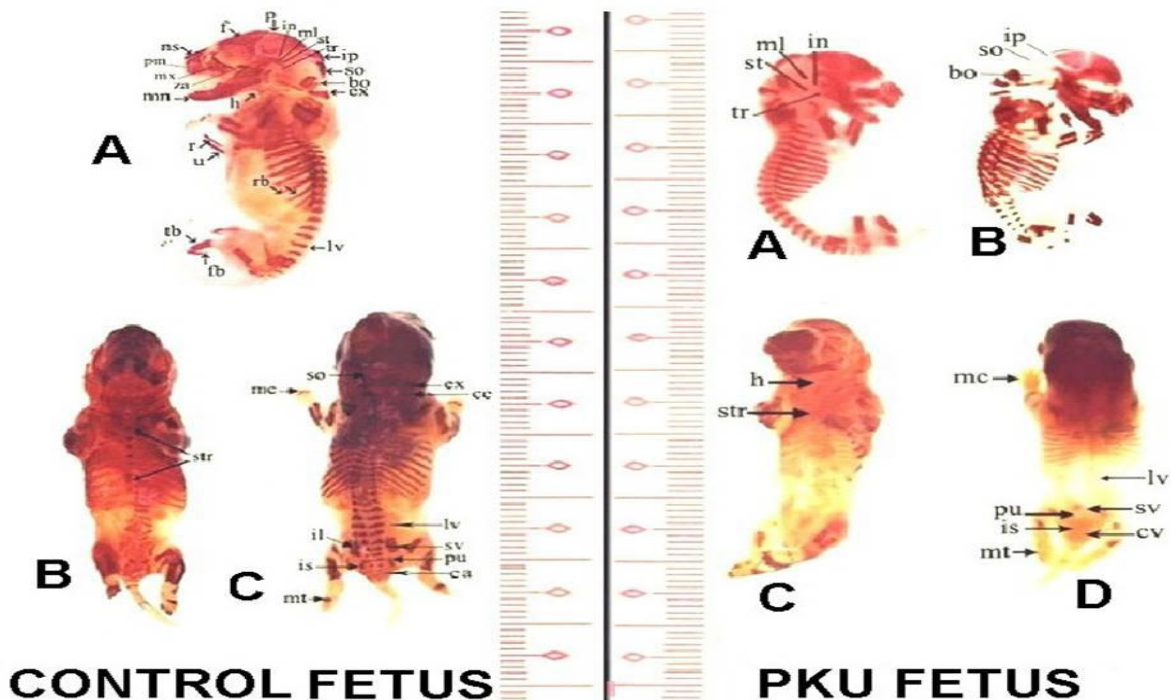


Plate 3. Photomicrograph of delivered fetus: showing ossification of nasal (na), frontal (f), premaxilla (pm), maxilla (mx), mandible (mn), exoccipital (ex), scapula (sc), clavicle (cl) the skeleton of control and PKU (cl), humerus (hu), radius (r), ulna (u), ilium (il), femur (fm), tibia (tb) and fibula (fb). IN control A - Lateral view, B- Ventral view and C - Dorsal view and in PKU A, B, C, D.

Table (1). Ossification centers in skull of prenatal fetuses at 14th and 16th day of gestation and delivered newborn of both control and experimentally induced PKU mothers.

	Prenatal at 14 Day of gestation		Prenatal at 16 Day of gestation		Delivered Newborn	
	c	PKU	c	PKU	c	PKU
Nasal	+	-	+	+	+	+
Frontal	+	+	+	+	+	+
Parietal	-	-	+	+	+	+
Interparietal	-	-	+	+	+	-
Premaxilla	+	-	+	+	+	+
Maxilla	+	+	+	+	+	+
Zygomatic arch	-	-	+	-	+	+
Hyoid arch	-	-	-	-	+	-
Mandible	+	+	+	+	+	+
Squamosal	-	-	+	-	+	-
Incus	-	-	-	-	+	-
Malleus	-	-	+	+	+	-
Stapes	-	-	-	-	+	-
Tympanic ring	-	-	-	-	+	-
Basioccipital	-	-	+	-	+	-
Exoccipital	-	-	+	+	+	+
Supraoccipital	-	-	+	+	+	-

(+ Presence; -) Absence

Table (2). Ossification centers in girdles of prenatal fetuses at 14th and 16th day of gestation and delivered newborn of both control and experimentally induced PKU mothers.

		Prenatal at 14th Day of gestation		Prenatal at 16th Day of gestation		Delivered Newborn	
		c	PKU	c	PKU	c	PKU
Pectoral Girdle	Scapula	+	+	+	+	+	+
	Clavicle	+	+	+	+	+	+
	Sternum	-	-	-	-	+	-
Pelvic Girdle	Ilium	+	-	+	+	+	+
	Ischium	-	-	+	-	+	-
	Pubis	-	-	-	-	-	-

(+ Presence; -) Absence

Table (3). Skeletal bone abnormalities of delivered newborn of PKU mothers.

Total number	Control	PKU
	(100%)	(30.10%)
Nasal	(0%)	(48.38%)
Interparietal	(0%)	(19.35%)
Maxilla	(0%)	(25.80%)
Zygomatic arch	(0%)	(22.58%)
Premaxilla	(0%)	(29.03%)
Squamosal	(0%)	(19.35%)
Ossicles (incus, malleus and stapes)	(0%)	(38.70%)
Tympanic ring	(0%)	(25.80%)
Basioccipital	(0%)	(22.58%)
Supraoccipital	(0%)	(45.16%)
Malformed fontanelles	(0%)	(19.35%)
Mandibular	(0%)	(16.12%)
Hyoid arch	(0%)	(29.03%)
Kyphotic lumbar vertebrae	(1%>)	(41.93%)
Forelimb distal phalanx	(0%)	(48.38%)
Hindlimb distal phalanx	(0%)	(64.51%)

Table (4). Ossified length (mm) of mandible of prenatal fetuses at 14th and 16th day of gestation and delivered newborn of both control and induced PKU mothers.

Statistical parameter	Prenatal at 14th Day of gestation		Prenatal at 16th Day of gestation		Delivered Newborn	
	c	PKU	c	PKU	c	PKU
Mean	3.56	2.50	6.32	5.04	8.18	5.76
± S.D.	0.16	0.15	0.05	0.16	0.02	0.34
± S.E.	0.07	0.07	0.02	0.07	0.01	0.15
T. Test	-	9.08	-	14.63	-	15.98
P. Value	-	.001	-	.000	-	.000
Significance	-	s	-	s	-	s

3.1.2. Ossification of girdles

In 14 days fetuses of treated mothers, scapula and clavicle made their first appearance; meanwhile, the ilium was completely missing compared with the control. Maternally treated prenatal fetuses at 16th day of gestation were still showing delayment of ossification mainly of the ischium. In delivered newborn of treated mothers, delayment of ossification was highly assessed in sternum, ischium, and pubis (Table 2). The Length of ossified scapula were markedly reduced in 14 and 16 days fetuses and delivered newborns of PKU mother. The Higher incidence of bone reduction was detected in the offspring of PKU mother (Table 2 and Fig. 1).

3.1.3. Ossification of fore and hindlimbs

Fourteen days fetuses of treated mothers possessed marked delayment of ossification of appendicular bones, especially radius and ulna of forelimb and hindlimb regions (Table 3). At 16 days of prenatal, the treated fetuses showed ossification centers of both fore- and hindlimb regions except metacarpal

and metatarsal bones which were still missing (Table 3). In delivered newborn of treated mothers, delayment of ossification was highly assessed in metacarpal, metatarsal and distal phalanx of both fore and hindlimbs (Table 3). The ossified bones of both fore- (humerus and radio-ulna) and hindlimb (femur and tibia-fibula) were markedly reduced in 14 and 16 days fetuses and delivered newborns of PKU mother. The High incidence of bone reduction was detected in 14 days fetuses of PKU mother (Table 5, 6, 7, 8, 9 and 10).

Table (5). Ossified length (mm) of forelimb of 14 days rat fetuses of both control and induced PKU mothers.

Statistical parameter	Humerus		Radius		Ulna	
	c	PKU	c	PKU	c	PKU
Mean	1.67	1.02	1.40	0.54	1.53	0.61
± S.D.	0.05	0.06	0.03	0.08	0.01	0.03
± S.E.	0.02	0.03	0.01	0.03	0.01	0.01
T. Test	-	29.36	-	18.84	-	49.65
P. Value	-	.000	-	.000	-	.000
Significance	-	S	-	S	-	S

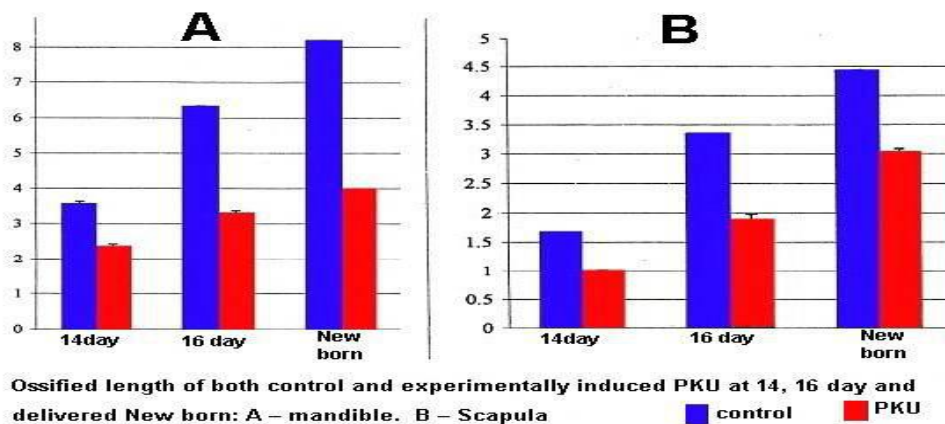


Fig. (1). Ossified length (mm) of mandible and scapula of prenatal fetuses at 14th and 16th day of gestation and delivered newborn of both control and experimentally induced PKU mothers.

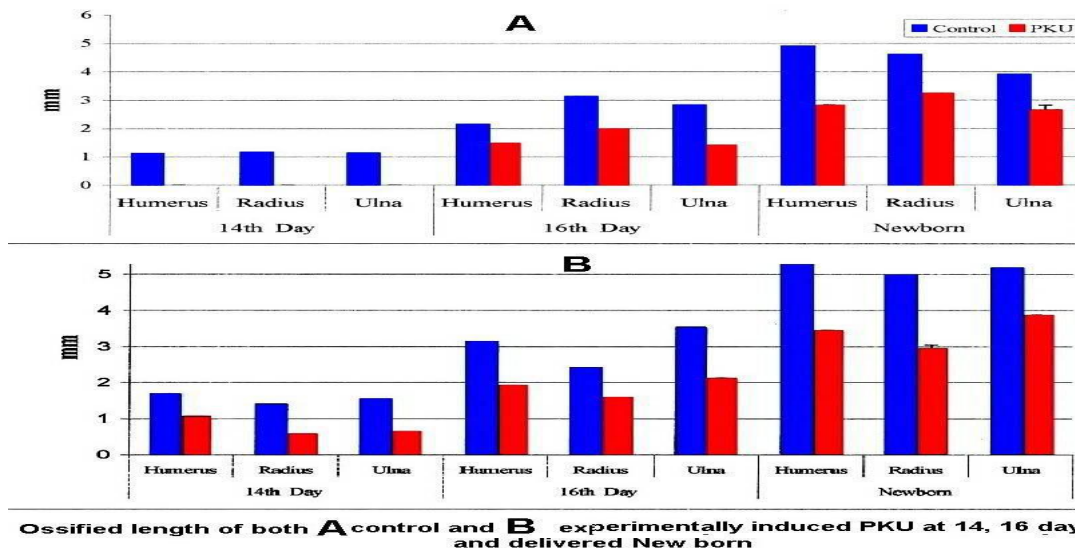


Fig. (2). Ossified length (mm) of A- forelimb, and B- hindlimb of prenatal fetuses at 14th and 16th day of gestation and delivered newborn of both control and experimentally induced PKU mothers.

Table (6). Ossified length (mm) of forelimb of 16 days prenatal fetuses of both control and induced PKU mothers.

Statistical parameter	Humerus		Radius		Ulna	
	c	PKU	c	PKU	c	PKU
Mean	3.10	1.88	2.39	1.56	3.01	2.09
± S.D.	0.06	0.08	0.03	0.06	0.02	0.08
± S.E.	0.02	0.03	0.01	0.02	0.01	0.04
T. Test	-	32.41	-	20.79	-	39.70
P. Value	-	.000	-	.000	-	.000
Significance	-	S	-	S	-	S

Table (7). Ossified length (mm) of forelimb of delivered newborn of both control and induced PKU mothers.

Statistical parameter	Humerus		Radius		Ulna	
	c	PKU	c	PKU	c	PKU
Mean	5.52	3.42	4.98	2.94	5.16	3.86
± S.D.	0.04	0.07	0.04	0.25	0.02	0.05
± S.E.	0.01	0.03	0.01	0.11	0.01	0.02
T. Test	-	56.31	-	19.73	-	56.32
P. Value	-	.000	-	.000	-	.000
Significance	-	S	-	S	-	S

S.D. =Standard; S.E. =Standard error; P =Probability; S =Highly significant (p<0.01).

Table (8). Ossified length (mm) of hindlimb of prenatal rat fetuses at 14th day of gestation of control and PKU mothers.

Statistical parameter	Femur		Tibia		Fibula	
	c	PKU	c	PKU	c	PKU
Mean	1.10	0.00	1.14	0.00	1.11	0.00
±S.D.	0.01	0.00	0.01	0.00	0.06	0.00
±S.E.	0.01	0.00	0.01	0.00	0.02	0.00
T. Test	-	131.47	-	224.74	-	37.20
P. Value	-	.000	-	.000	-	.000
Significance	-	S	-	S	-	S

Table (9). Ossified length (mm) of hindlimb of prenatal rat fetuses at 16th day of Gestation fetuses of control and PKU mothers.

Statistical parameter	Femur		Tibia		Fibula	
	c	PKU	c	PKU	c	PKU
Mean	2.14	1.47	3.12	1.95	2.81	1.40
±S. D.	0.02	0.01	0.01	0.02	0.02	0.03
± S. E.	0.01	0.01	0.01	0.01	0.01	0.01
T. Test	-	43.22	-	81.99	-	81.79
P. Value	-	.000	-	.000	-	.000
Significance	-	S	-	S	-	S

Table (10). Ossified length (mm) of hindlimb of delivered newborn of control and PKU mothers.

Statistical parameter	Femur		Tibia		Fibula	
	c	PKU	c	PKU	c	PKU
Mean	4.90	2.80	4.60	3.22	3.90	2.65
±S. D.	0.01	0.07	0.01	0.06	0.01	0.36
±S. E.	0.01	0.03	0.01	0.02	0.01	0.16
T. Test	-	55.72	-	44.67	-	7.76
P. Value	-	.000	-	.000	-	.001
Significance	-	S	-	S	-	S

S.D. =Standard deviation; P = Probability; S.E. =Standard error; S =Highly significant (p<0.01).

3.2. Histological abnormalities of prenatal fetuses at 14 and 16 days of gestation

3.2.1. Ossification of the ear

In control prenatal embryo at 14 days of gestation, the inner ear consists of varying sizes and structures of skeletal labyrinths. Each is divided into smaller compartments by the membranous labyrinth. The skeleton of labyrinths is formed by hypertrophied cartilage cells aligned adjacent to each other with a considerable reduction or missing of their surrounding matrix. The outer limiting membrane possessed marked

differentiation of osteoblast progenitor cells lying adjacent to sprouts of periosteal ossification (Plate 4: Figs. A and B).

In 14 days fetuses of experimentally induced PKU mothers, the inner ear formed of early entirely cartilaginous materials in the form of early developed chondroblasts widely separated from each other by hyaline matrix (Plate 4: Figs. A1 and B1).

3.2.2. Ribs

In the control 14 days fetuses, the body of the ribs formed by hypertrophied cartilage cells with

marked breakdown of cells at their periphery allowing periosteal ossification and increased deposition of mineralized calcium salts. The periosteum formed mainly of osteoblast cells and thin collagen fibers. Dark-brown mineralized early calcium deposits were detected along the periosteal sheath (Plate 4: Figs. C and C1). In fourteen days rat fetuses of PKU mothers, there were a considerable retardation of ribs growth. The ribs were formed of less developed cartilage cells separated widely from each other by hyaline matrix. The ribs were enclosed by a perichondrial sheath in the form of chondroblasts and collagen fibers (Plate 4: Figs. C and C1).

3.2.3. Cervical vertebrae

In the control 14 day's fetuses, the different regions of the vertebral body are formed of well-developed cartilage cells. The pedicle and transverse processes attained much more differentiation. Periosteal ossification begins to be detected on both sides of the pedicle (Plate 5: Fig. A). However, 14 days fetuses of experimentally induced PKU exhibited retarded skeletal differentiation of vertebral body which is formed of early developed chondroblasts. The cartilage cells appeared densely distributed in between wide matrix (Plate 5: Fig. A).

Besides, the cervical spinal cord of the control 14 days fetuses possessed marked differentiation of neuronal cells of both the dorsal and ventral horn. The dorsal horn appeared less developed from the ventral ones. In the dorsal horn, the neuronal cells appeared oval-shaped with basophilic nuclei and densely crowded. On the other hand, the neuronal cells of the ventral horn possessed marked development and take the spindle-shaped characteristic structures as well as possessed centrally located nuclei with prominent nucleoli. Nerve fibers appeared densely distributed in between the neuronal cells (Plate 5: Figs. B and C).

However, 14 days fetuses of experimentally induced PKU exhibited marked atrophy and degeneration of neuronal cells of both dorsal and ventral horn regions (Plate 5: Fig. D). In the control 16 day's fetuses, the different regions of the vertebral body are formed of well-developed cartilage cells. The pedicle and transverse processes attained much more differentiation. Periosteal ossification is detected in both sides of the pedicle (Plate 5: Figs. G and H). However, 16 days fetuses of experimentally induced PKU exhibited retarded skeletal differentiation of vertebral body which is formed of less differentiated chondrocytes. The cartilage cells appeared densely distributed between wide matrix (Plate 5: Figs. I and J).

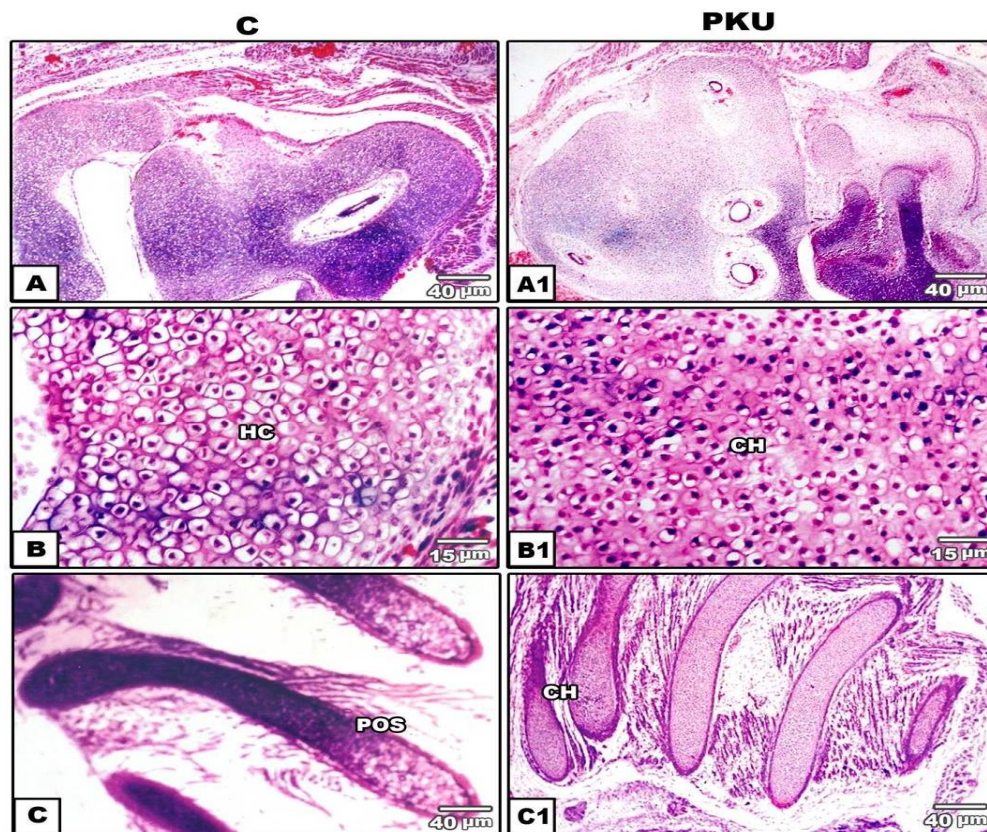


Plate 4. Histological abnormalities of prenatal fetuses at 14 and 16 days of gestation and delivered Newborn on the ear and ribs.

Photomicrographs of horizontal histological sections of ear and ribs of 14-days fetuses showing **A and B**: control fetuses showing hypertrophied cartilage cells (he) aligned adjacent to each other with a considerable reduction or missing of their surrounding matrix (AX40-BX250); **A1 and B1**: experimentally induced PKU mother showing retarded chondrocyte differentiation which widely separated from each other by hyaline matrix (arrow) (AX40-BX250); **C**: control fetuses showing hypertrophied cartilage cells with increased average of degenerated cells allowing periosteal ossification and deposition of mineralized calcium salts. Dark-brown mineralized calcium deposits were detected along the periosteal sheath (AX40- BX100); **C1**: fetuses of induced PKU mother showing considerable retardation of ribs growth. The ribs were formed of less developed cartilage cells widely separated from each other by hyaline matrix (AX40-BX100).

Cervical vertebra and spinal cord

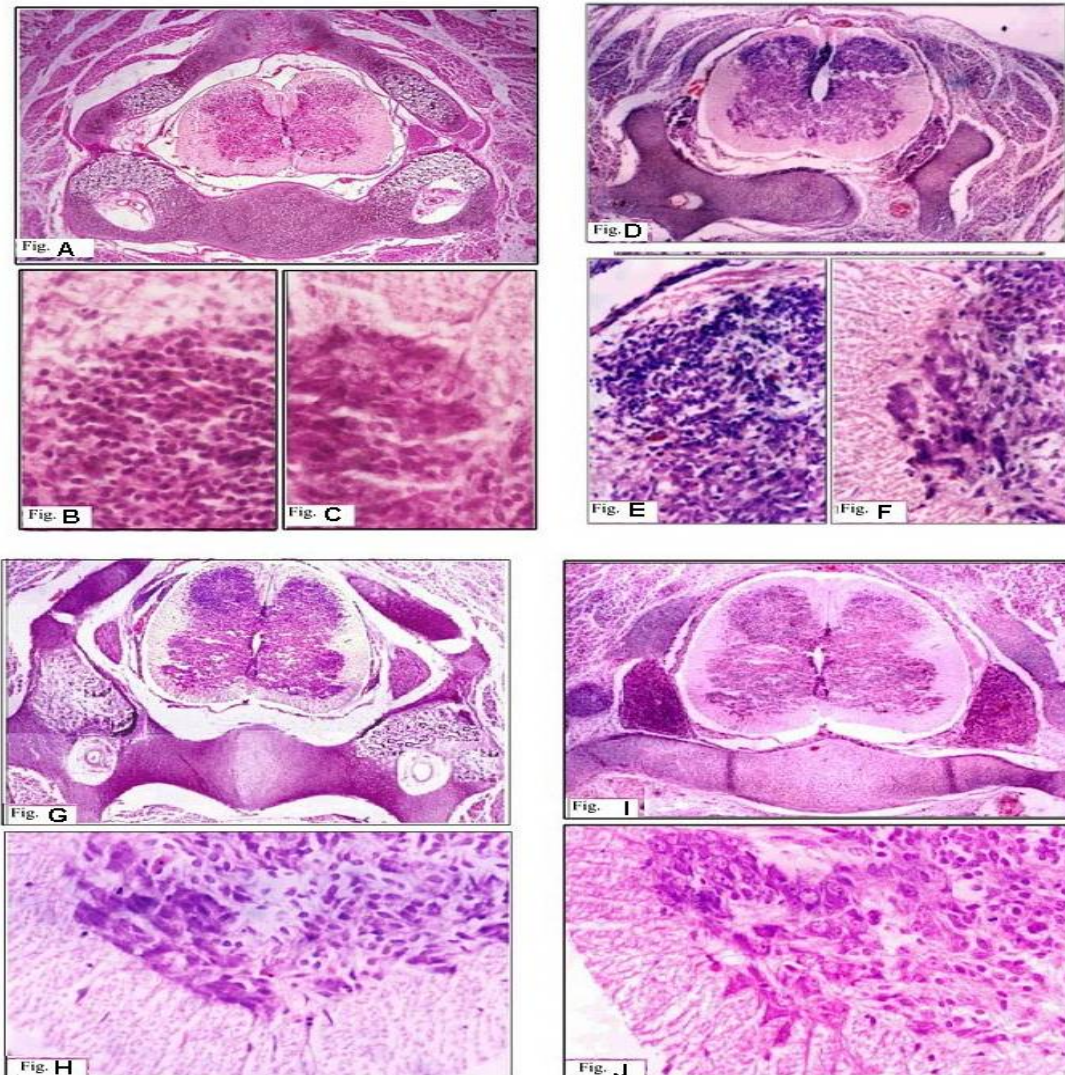


Plate 5: Photomicrographs of horizontal histological sections of 14 days fetuses Cervical Vertebra and spinal cord showing

A: Control 14 day's fetuses, the different regions of the vertebral body are formed of well-developed cartilage cells (X40); **D:** Induced 14 days fetuses PKU exhibited retarded skeletal differentiation of vertebral body which is formed of early developed Chondroblasts. The cartilage cells appeared densely distributed in between wide matrix (X40); **B and C:** Control 14 days fetuses 'cervical spinal cord of possessed marked differentiation of neuronal cells of both the dorsal and ventral horn. The neuronal cells of the ventral horn possessed marked development and take the spindle-shaped characteristic structures as well as possessed centrally located nuclei with prominent nucleoli (X40); **E and F:** cervical spinal cord of experimentally induced PKU exhibited marked atrophy and degeneration of neuronal cells of both dorsal and ventral horn regions (X40); **G and H:** control 16 days' fetuses, the different regions of the vertebral body are formed of well-developed cartilage cells. The pedicle and transverse processes attained much more differentiation. Periosteal ossifications are detected in both sides of the pedicle (X40); **I and J:** experimentally induced PKU 16 day's fetuses of exhibited retarded skeletal differentiation of vertebral body which is formed of less differentiated chondrocytes. The cartilage cells appeared densely distributed in between wide matrix (X40).

3.2.4 Forelimb

3.2.4.1. Humerus and radio-ulna

In the control fourteen-day fetuses, both humerus and radio-ulna exhibited marked rearrangement of remodeling of cartilage cells differentiation arranged respectively, including reserve cartilage cells, proliferating zone, zone of mature chondrocytes, zone of hypertrophy, zone of eroding cartilage cells and ossification and ossified bone. In the middle of the shaft of both humerus and radio-ulna, there was a marked eroding of cartilaginous tissue and active invasion of periosteal ossification including richly supplying of blood vessels, vascularization of the

centre of the shaft, flourishing of osteoblast cells leading to increase dark-brown deposits of mineralized calcium salts. There was no active osteogenesis and in the centre of the shaft hypertrophy of chondrocytes is well remarked (Plate 6: Figs. A, B and C). However, in treated fourteen days fetuses, both humerus and radio-ulna formed of hypertrophied cartilage cells and their perichondrial sheath become transformed into periosteal sheath. In the center of the shaft, moderate osteogenic activity was detected and characterized by increase osteoblast cells differentiation and decreased mineralized calcium salts deposition. In the center of the radio-ulna, there was a considerable reduction of the osteogenic activity (Plate 6: Figs. A1, B1 and C1).

3.2.4.2. The hand

The hand is formed of carpals, metacarpals and distal phalanx. In control fourteen days, the hand regions exhibited a mild osteogenic activity manifested by the presence of central zone hypertrophied chondrocytes, as well as both carpals, metacarpals and distal phalanx, become ensheathed with periosteal sheath composed of osteoblasts and collagen fibers (Plate 6: Figs. D and E). However, in 14-day rat fetuses of PKU mothers, the hand region appeared formed of chondrified tissue, but no osteogenic activity was detected (Plate 6: Figs. D1 and E1).

3.2.5. Hindlimb

The hindlimb is formed of femur, tibia-fibula, tarsus, metatarsus and distal phalanx. In control fourteen days, the femur and tibia-fibula exhibited similar remodeling of periosteal ossification as in forelimb regions. Both periosteal sheath and center of the shaft exhibited a marked increase of dark-brown mineralized calcium deposits, on the other hand, both femur and tibia-fibula of 14 days fetuses of PKU mothers appeared formed of cartilaginous tissues. The center of the shaft showed marked hypertrophy of chondrocytes.

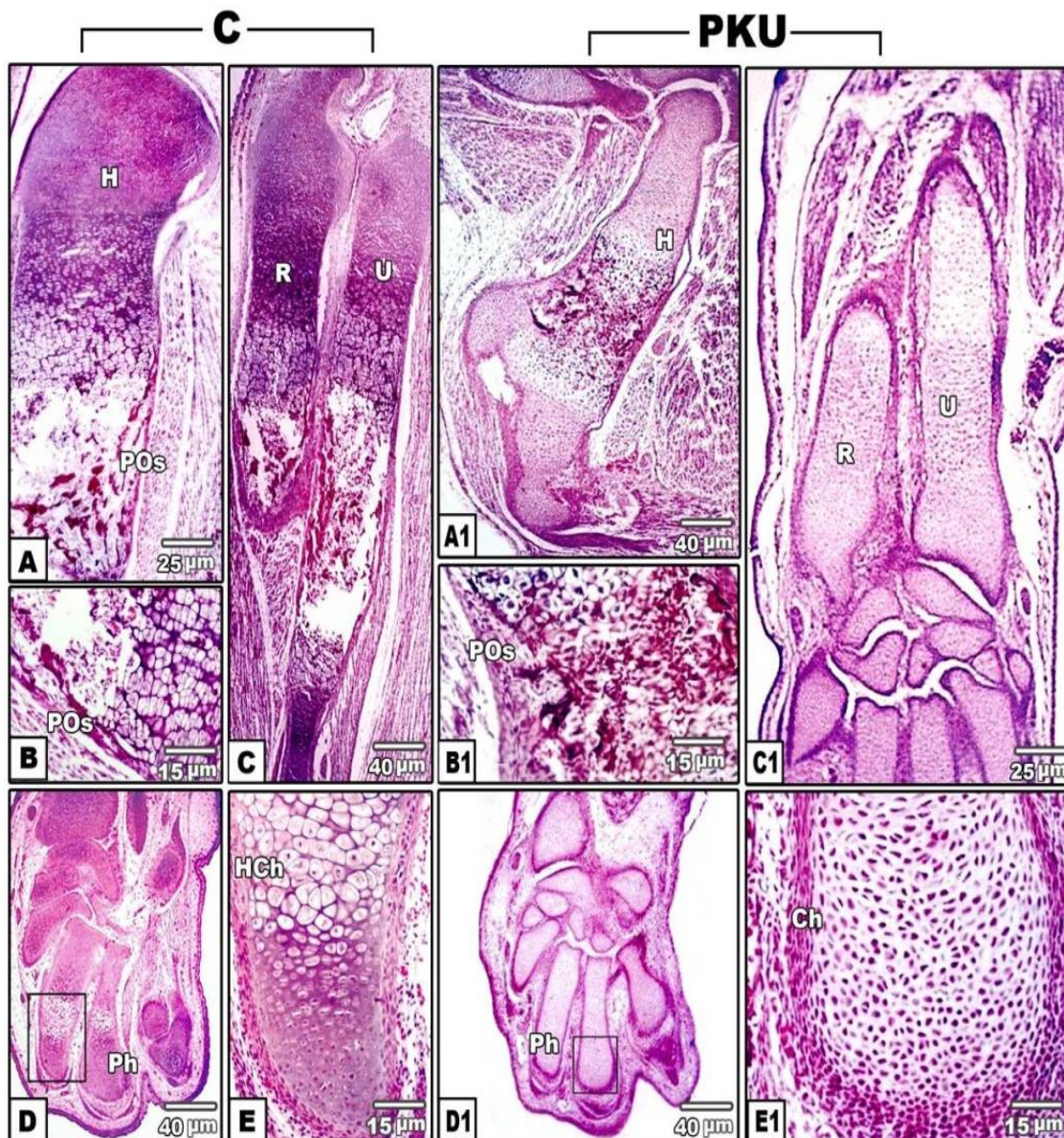


Plate 6. Photomicrographs of histological sections of humerus, radio-ulna, and hand of both controls and induced PKU, fetuses of 14th day.

A, B and C: Photomicrographs of histological sections of femur (A) and tibia-fibula (B-C) of control 14-days fetuses showing similar remodeling of periosteal ossification as in forelimb regions. Both periosteal sheath and center of the shaft exhibited a marked increase of dark-brown mineralized calcium deposits (AX40 - BX40 - CX100); **A1, B1, and C1:** Photomicrographs histological sections of femur (46A- 46B) and tibia-fibula (46C-46D) of 14-days rat fetus of experimentally induced PKU appeared formed of cartilaginous tissues. The center of the shaft showed marked hypertrophy of chondrocytes (AX40 - BX100 - CX40 - DX100); **D and E:** Photomicrographs of histological sections of the hand of control 14-days fetuses showing, carpals, metacarpals and distal phalanx, exhibited a mild osteogenic activity manifested by the presence of central zone of hypertrophied chondrocytes (he). Carpals, Metacarpals and distal phalanx become ensheathed with periosteal sheath (arrow) composed of osteoblasts and collagen fibers (AX40-BX200); **D1 and E1:** Photomicrographs histological sections of the hand of 14-days rat fetus of experimentally induced PKU mother showing the hand region appeared formed of chondrified tissue and no osteogenic activity was detected (AX40-BX200).

3.2.6. Femoral bone of control and induced PKU mothers

Morphometric observation of mother femur bones the depths of epiphyseal cartilage of femoral bone of control and induced PKU mothers Table (2). The results showed that PKU mothers exhibited a significant reduction of epiphyseal cartilage and bone trabecular (Fig. 7A). Histological investigation of the control showed endochondral proliferation of cells into cartilage columns of epiphyseal cartilage of epiphyseal growth plate. The columns of cartilage cells radially arranged in an aregular manner, the three exacting zones of the cartilage (zone of proliferation, maturation, and hypertrophy) are well demarcated. By the end of

the epiphyseal plate, the metaphysis is highly vascular and traversed by regular arrangement, primary bone trabeculae (Plate 7: Figs. A, B, and C).

In experimental PKU mother, there was a marked resorption of the epiphyseal cartilage with deranged epiphyseal line. The cartilage columns irregularly disrupted and atrophied to stunting groups of cartilage cells. Fewer numbers of cartilage cells were aggregated in clusters and lacked basophilic criteria of cartilage cells. The cartilage stroma becomes widened and widely separated the cartilage cells. The metaphysis bone trabeculae attained a considerable thinning with marked depletion of bone marrow cells in between them (Plate 7: Figs. D and E).

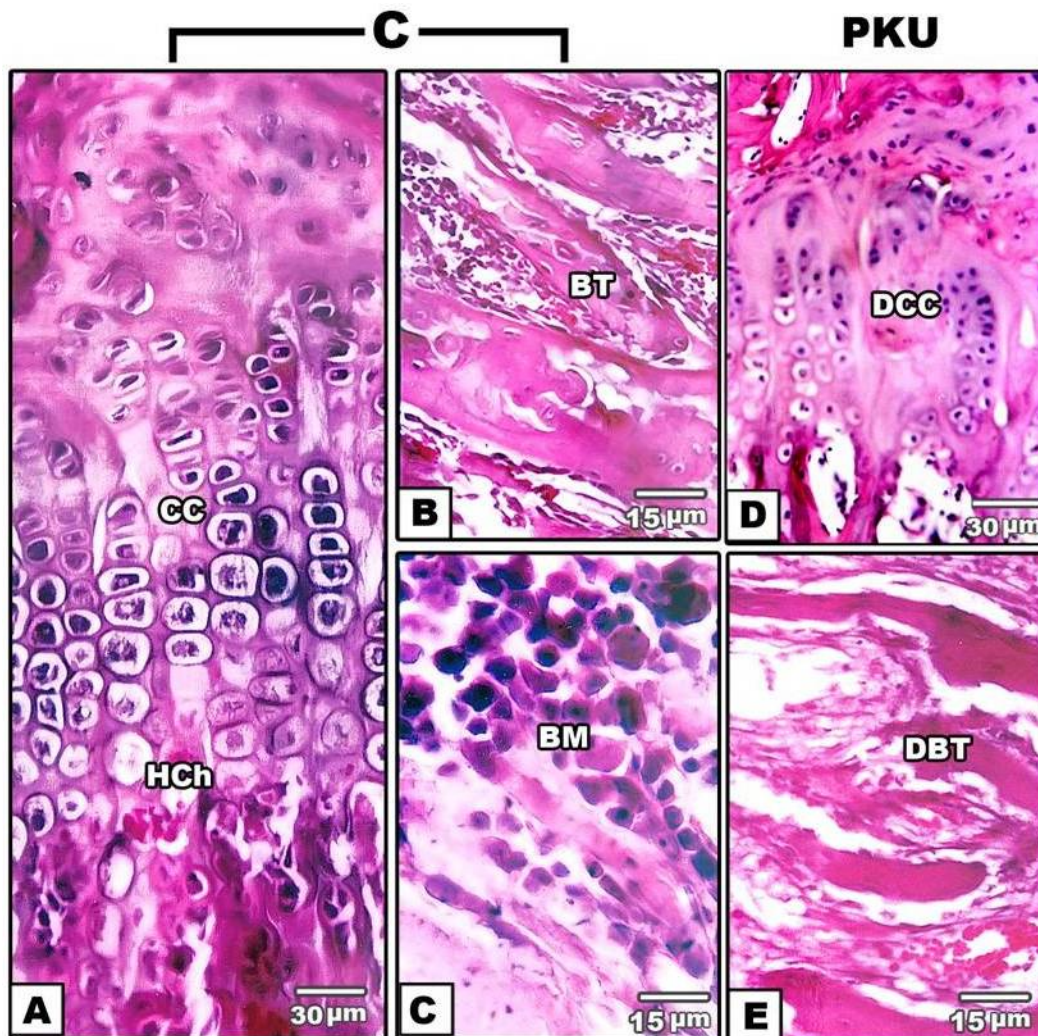


Plate 7: Photomicrographs of histological sections of mother femur bone of both controls and induced PKU.

A, B and C: In the controlling mother, endochondral proliferation of cells into cartilage columns of epiphyseal cartilage of epiphyseal growth plate. The columns of cartilage cells radially arranged in regular manner are the metaphysis is highly vascular and traversed by regular arrangement, primary bone trabeculae; **D and E:** In experimental PKU mother, there was a marked resorption of the epiphyseal cartilage with deranged epiphyseal line. The cartilage columns irregularly disrupted and atrophied to stunting groups of cartilage cells. Few numbers of cartilage cells were aggregated in clusters and lacked basophilic criteria of cartilage cells.

4. Discussion

Phenylketonuria (PKU) is an inherited metabolic disease, caused by mutations carried out through a "recessive" gene on chromosome 12 which intern caused marked partial or complete inhibition of the

liver enzyme phenylalanine hydroxylase (PAH) [24], and consequently, interrupt the conversion of amino acid phenylalanine to tyrosine. The disturbance of enzyme activity leads to excessive accumulation of the amino acid phenylalanine in the blood, to a concentration, sufficiently, high to activate an

alternative pathway of degeneration [25, 26]. Attempts to understand the underlying cause for the abnormalities associated with maternal PKU have led to the use of the animal model in which the morphological, histopathological and cytopathological investigations were encountered in different body organs to clarify the disease pattern. From the present work, intragastric administration of 30mg DL-a-methyl phenylalanine/kg body weight plus 60mg/kg body weight L-phenylalanine to pregnant rats exhibited various clinical manifestations of the disease. These symptoms are irritability, vomiting, mousy skin odor and abnormal skin appearance. These findings were closely similar to those described in human by Beloso and Lowitt [27]; Brass *et al.*, [28]. Fed pregnant rats on a diet supplemented with 0.5% alpha-methyl-phenylalanine and 3% phenylalanine from the 12th day of gestation to term and reported that the fetuses showed small reductions in the body. However, no differences were seen in crown-rump length, litter size or in postnatal survival. While, Sadava and Sutcliffe [29] used the same treatment in pregnant rats and found that the experimental litters did not differ from controls in a number of offspring, birth weight, or subsequent growth on an unsupplemented diet but there was a learning deficit in the lives of offspring.

4.1. Effects on maternal tissues and congenital abnormalities

In accordance with Gadallah [30]; MacDonald *et al.*, [31], both PKU patient and experimentally induced PKU animals failed to consume their protein substitute as a result of impairing conversion of phenylalanine to tyrosine impairing protein synthesis. Retarded body growth and increased incidence of malformations may be attributed to liver hepatitis, which led to disturbance of protein synthesis as a result of impairing; the important factor for supplying. The observed maternal histological defects may be attributed to the oxidative stress induced by hyperphenylalaninemia. The oxidative stress was defined as an increase in pro-oxidative damage to cells due to an increase in free radical production or impairment in oxidative defense. When glutathione is decreased, cells enter a low-antioxidant mode and are more prone to oxidative damage caused by free radicals [32]. Similar patterns of growth malformations were reported in fetuses of untreated PKU mothers. The detected intrauterine growth retardation was with microcephaly, facial dysmorphism, congenital heart disease, and abnormal intellectual development [33-35]. The pattern of foetal and neonatal defects may also closely similar to fetal alcohol syndrome [36-38] and congenital pyruvate dehydrogenase deficiency [39]. Both syndromes possess similar pathological manifestations concerning the transplacental passage of both Phe and alcohol penetrating to the fetus, such as Phenylpyruvate - a byproduct of Phe metabolism and acetaldehyde - a byproduct of alcohol metabolism which are potent inhibitors of pyruvate dehydrogenase. It is known that

phenylalanine and tyrosine compete for the same transporter across the blood barrier. The phenylalanine had more affinity for transporting more than tyrosine leading to increase phenylalanine level as well as their metabolites and consequently disturbed the metabolism [40, 41].

4.2. Bone defects

From the present findings, morphometric observations revealed that experimentally diseased PKU mothers developed a marked atrophy of both epiphyseal cartilage and bone trabeculae. The diseased epiphyseal cartilage was markedly resorbed with deranged epiphyseal line. The cartilage columns irregularly disrupted and stunting to few clusters of cartilage cells with marked decreasing of their basophilic criteria. The metaphysis bone trabeculae appear thinner with decreasing inclusion of bone marrow cells in between them. The observed disruption of maternal femoral epiphyseal cartilage agrees with the work carried out by [42] in the left hand and wrist in children and young adults with PKU and HPA. Although, the mean protein, vitamin D and mineral intakes met the recommended dietary allowance in PKU patients, [43-45] reported a severe osteopenia in these patients. The patients suffered from bone loss characterized by significantly higher serum calcium and magnesium, lower alkaline phosphatase, higher ratios of urinary Calcium-Creatinine ratio, lower urinary Phosphorus-Creatinine ratio and lower urinary Hydroxyproline-Creatinine ratio. The biochemical data indicate a metabolic state of low bone turnover in PKU patients. Similar defects of femur bone were reported in femur of experimentally induced PKU mice assessed by decrease weight and strength as well as of depletion of osteocalcin [46]. Osteopenia and osteoporosis developed in PKU patients were attributed to marked reduction of osteocalcin and collagen type I cross-linked C-telopeptide [47]. In addition, vitamin D2 (Ergocalciferol) and vitamin D3 (Cholecalciferol) are present in the normal diet and the latter is also synthesized in the skin by the action of ultraviolet on 7-dehydrocholesterol. Both vitamin D2 and D3 are transported to the liver and then in the kidney. Hydroxylation at the 25 positions of these molecules takes place in the liver and a second hydroxylation which occurs in the kidney involve either the one or 24 positions. The induce of both liver and renal disease as a result of experimental PKU may increase the catabolism of 25-hydroxy-vitamin D to biological inactive product which are rapidly excreted in the bile or urine and consequently impaired the action of 1,25-dihydroxy vitamin D on bone [48] causing bone defects [49], investigated the growth and skeleton of 82 children of untreated PKU mother and reported early infantile head circumference and growth appear to be predictors of cognitive development. The dramatic effects of organs of PKU mother on skeleton of fetuses and newborn. Ossification of different parts of the axial and appendicular regions was retarded with the

advancement of prenatal growth as well as of delivered newborn. Fourteen days of PKU mothers was highly susceptible to the effect of the disease and characterized by the presence of ossification centers in different regions of the skeleton. The incidence of ossified bones were determined in delivered newborn of PKU mothers and were restricted mainly in tympanic regions, sternbrae, mandibular, ischium and distal phalanx of fore and hindlimb. The growth of appendicular bones was markedly retarded. The observed growth retardation of skeletogenesis was confirmed by histogenesis of both elementary structures of the skeleton, including the chondroblast and osteoblast cells of tympanic regions, fore and hindlimb regions. The observed findings may be attributed to the active accumulation of phenylalanine in the placenta of PKU mothers and these interns reproduce its potential teratogenic effect [50, 51]. Disruption of placental structures as a result of Phenylalanine concentration or to thyroid dysfunction or to a decrease of both osteocalcin and collagen 1 [47] which play a great role during bone growth attributed the hazardous effects of PKU to the oxidative stress induced by hyperphenylalaninemia. These stresses resulted from liberation of free radicals [32] or to the decrease of glutathione peroxidase activity or to the blocking of normal metabolic pathway of phenylalanine, leading to increase of phenylalanine level and consequently cause the progress of increase of phenylalanine level and consequently cause the progress of oxidative damage of cells and consequently growth retardation [52]. Belloso and Lowitt [27] reported that a man with Phenylketonuria who was reported to have lichen sclerosus et atrophicus at age 16 years with large confluent areas of atrophy. Although sclerodermatous changes have been described in children with Phenylketonuria, this case offers a longitudinal view of the progression of skin lesions in a middle-aged man with Phenylketonuria. Finally, the present study led to the conclusion that adequate nutrition is very important to pregnant, but in women with PKU, two factors need to be controlled. In the first, the pregnant need careful supply of adequate diet for maintaining fetal growth. The second is to restrict phenylalanine supplementation before conception and throughout pregnancy to avoid drastic effects and protect the growing fetus. Although the biochemical analysis of PKU is of high cost and investigation and characterization of this disease need higher practice in medication, unknown anemic pregnant and offspring should run all of the available investigations to get answers about the unknown disease and nowadays we know about diseases not previously occurred such as celiac disease and so on. Women with PKU need early identification, education, regarding the importance of dietary compliance, careful monitoring, and emotional support to increase better outcomes. Exposure to the mother's metabolic abnormalities affects the fetus during the entire pregnancy. The abnormalities produced by the PKU mothers are not genetic, but "Intrauterinely

Environmental." Our results and those of others confirm this hypothesis. Jervis [53] stated that the damage to the children of PKU mothers is not a genetic consequence. This can be proven by the fact that PKU fathers produce normal offspring [54] and also that well-controlled pregnant PKU mothers can produce children without obvious handicap.

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