Histopathological effects of experimental phenylketonuria on 15 days albino rat placenta

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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 of the mechanisms involved in the pathology of PKU during neonatal development. Hyperphenylalaninaemia (Elevated concentrations of plasma phenylalanine) were induced in pregnant rats by oral administration of 30 mg. DL–α-methylphenylalanine/100 g (to inhibit maternal liver phenylalanine hydroxylase) plus phenylalanine supplementation at a dosage of 60 mg/100 g body weight two times daily (to increase maternal and fetal plasma phenylalanine) after 6th day of onset of gestation till 15 days of gestation. Treatment with alpha-methylphenylalanine/phenylalanine affect placentation through reduction in placental weight and histopathologically, through increase in apoptotic cells in the labyrinth zone and basal zone, and hypoplasia of the labyrinth zone and basal zone, dilatation of the blood vessels and inducing haemorrhagic and degenerative changes in the layers of placenta. Conclusions: PKU affects placentation that may be reflected on the growth and development of fetuses.

1.Introduction

Placenta is the organ, which provides oxygen and nutrients to the developing conceptus as well as facilitating the exchange of metabolic products. The placenta functions are like a lung, liver, kidney and other endocrine organs in order to guide the exchange of all factors necessary for successful embryonic and later, fetal development and growth (Dodgson, 1991).

Phenyl ketonuria (PKU) disease was first described in 1934 by a Norwegian doctor named Asbjorn Folling. 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 (Pietz, 1998). Consequently, individuals with PKU are low in L-tyrosine (Roberts *et al.*, 2001) which may contribute to behavior problems (Tam and Roth, 1997).

Phenylalanine is one of the building blocks of protein. It is found in protein foods like milk, meats, eggs, and cheese. However, when the phenylalanine hydroxylase enzyme is absent or deficient, 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 (Medical Research Council Working Party on PKU 1993).

There are two main reasons for a lack of PAH activity. The most common cause for a lack of this 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. A secondary cause of 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-doba 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 hyperphenylalaninemic patients (Kaufman *et al.*, 1983).

Genetic analysis, using recombinant DNA techniques, has established that the genetic locus for PKU is on chromosome 12. The PAH gene contains 13 exons of various sizes, spread over approximately 90 kilo bases of DNA. 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 (Pietz, 1998). The gene for PAH was cloned in 1982 Robson *et al*. and by now more than 400 different mutations in the PAH gene have been identified, Zschocke (2003).

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 (Brenton and Lilburn, 1996). Maternal PKU can lead to fetal malformations, including small head size (microcephaly), cardiac abnormalities, intrauterine growth retardation and mental retardation (Levy and Ghavami, 1996; Koch *et al*., 2000). Adverse effects on the offspring can be reduced by family planning and by careful dietary control both prior to and during pregnancy (Cechak *et al*., 1996; Riva *et al*., 1996 and Rouse *et al*., 1997). In addition, low L-tyrosine levels in pregnant women with PKU may contribute to fetal damage (Rohr *et al*., 1998). Untreated children with persistent severe hyperphenylalaninemia (Elevated plasma Phe concentration; HPA) show impaired brain development. Signs and symptoms include microcephaly, epilepsy, severe mental retardation, and behavior problems. 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 (Pietz *et al.*, 1998).

A tremendous research has been performed on the teratogenic mechanism on the embryo and the other associated tissue, since it is generally believed that the site of action of a chemical teratogen is an embryo itself and in the tissue that are directly or indirectly involved in the fetal malformations, Singh (2005). Chemical induced alteration on the placenta could by itself cause adverse effect in the embryo. Inadequate embryonic nutrients may influence the morphogenesis either on its own or by modulating the homeobox genes and protooncogenes involved in the prenatal development, (Khera, 1992).

In order to evaluate whether the detrimental effects caused by Hyperphenylalaninaemia are due to its action directly on the fetus by crossing the materno-fetal barrier or indirectly by altering the placental architecture, the present experiment was undertaken.

2. Materials and methods
Experimental animals:

Twenty four fertile virgin female and 8 fertile males of albino rats with an average body weight of 100 –110 grams (ratio of 1 male : 3 females) were obtained from Hellwan 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 ratio of four / cage. They were maintained in a temperature of 20 –25 ^{0}C with 12 hours light – 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 ratios for dairy animals (**Christen *et al*., 1996**). All the rats had access to water and animal diet *ad libitum.* They were paired overnight. The morning of finding sperm positive vaginal smear was defined as day 0 of gestation

Experimental work:

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

I- Control:

Twelve pregnant mothers were sacrificed at 15th day of gestation.

II- Experimental Phenylketonuria group:

Each selected pregnant females at the 6th day of gestation was intragastrically administered 30 mg. DL–α-methylphenylalanine/100 g. body weight (to inhibit maternal phenylalanine hydroxylase) plus 60 mg/g body weight L-phenylalanine (to raise fetal plasma phenyalanine) dissolved in milk, at 12 hrs intervals. The applied dose was selected according to Spero and Yu (1983) and Rech *et al*., (2002). The twelve pregnant mothers diseased with experimental PKU were sacrificed at 15th day of gestation.

Each placenta was weighted. A total of 36 placentas were obtained randomly from the live fetuses in the dams of control and experimental groups (3placentas/dam) and immediately fixed in 10% formalin saline for 24 hrs. 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. For histopathological examination sections stained in haematoxylin and eosine (Harris HX, Drury and Wallington, 1980). For immunohistochemistry examinations were used capase-3 (apoptotic marker) positive cells were determined with streptavidin-biotin-peroxidase staining method (Karakusa *et al.,* 2011). Caspase-3 positive reactions were visualized and evaluated by high-power bright field light microscopic of approximately X20 objective.

Statistics were calculated with SPSS for windows version 13.0, the means value obtained in the different groups were compared by independent student's t-test. All results were expressed as mean values ± SE and significance was defined as $p < 0.05$ and highly significant when $p < 0.01$ (Field, 2000).

3. Results

Control gestation day 15 (GD 15) rat Placenta.

Twenty four pregnant albino rats were used throughout this study. They were classified into two groups (twelve dams for each) control, and PKU induced groups. The total number of GD 15 placentae were collected throughout this study were 141 placentae (82 and 59) from control and PKU induced groups respectively. The mean weight of placenta was 427.4mg (Fig. 1).
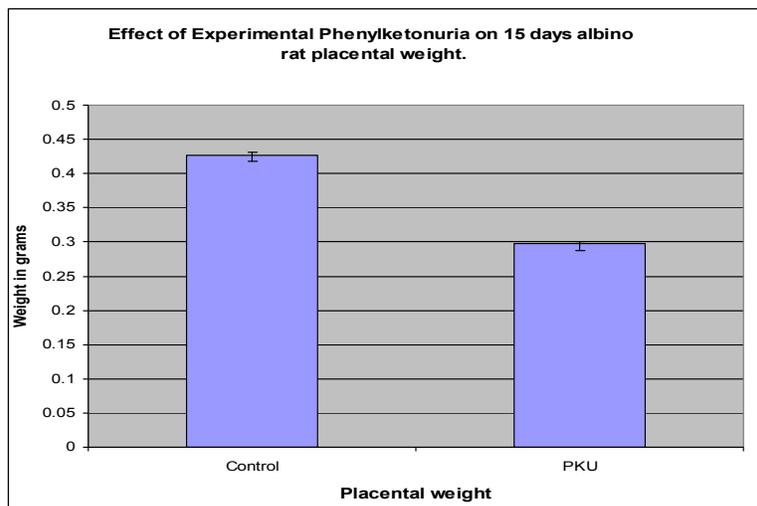

Fig. (1): Effect of Experimental Phenylketonuria on 15 days albino rat placental weight.

The rat chorioallantoic placenta morphologically has a discoid shape and is classified into the hemochorial type. Histologically, the chorioallantoic placenta is divided into a fetal part and a maternal part (Furukawa *et al.*, 2011). The maternal part of the placenta differentiates from uterine stromal cells after decidualization, and histologically consists of the decidua and metrial gland. The decidua basalis of placenta was composed of cellular and fibrous elements. It was separated from the basal zone by single layer of giant cells. The fetal part of the placenta originates from the trophectoderm of the embryo, and consists of the basal zone and labyrinth zone. According to Fonseca *et al.* (2012) the basal zone is composed of giant trophoblast cells, which represent the first trophoblast layer of the placenta, glycogenic trophoblasts and a highly packed basophilic spongiotrophoblast cells. The labyrinth zone contains giant trophoblast cells and syncytiotrophoblasts.

Treated GD 15 placenta.

The treated placenta revealed no abnormality on gross inspection, nevertheless, multiple foci of hemorrhagic area were observed. While the mean weight of the treated placenta was 297.4 mg ($P < 0.01$) showing a significant reduction when compared to the controls.

Histological changes in the GD 15 placenta

In the labyrinth zone, apoptotic cells, characterized by pyknosis or karyorrhexis, phagocytosis and cell debris and positively showed caspase-3 activity, were scattered in the trophoblastic septa in treated group (Figs.3 and 4), resulting in labyrinth zone hypoplasia. In addition, irregular dilatation of maternal sinusoids with hemorrhage was observed.

In the basal zone, apoptotic cells were scattered in the treated group (Figs.3 and 4), and a marked decrease in glycogen cell-islands was detected in GD 15 treated group. Spongiotrophoblasts were decreased resulting in basal zone hypoplasia.

Cystic degeneration of glycogen cells was also observed. In which abnormal retention of extensive cytoplasmic vacuolation within glycogen cells. The vacuoles contain eosinophilic fibrinous material and polymorphs. The degenerated cells undergo cytolysis and subsequently coalesce into multiple large cysts that are filled with a homogeneous acidophilic mass and multiple clusters of residual glycogen cells, macrophages, erythrocytes and cell debris (Fig.3).
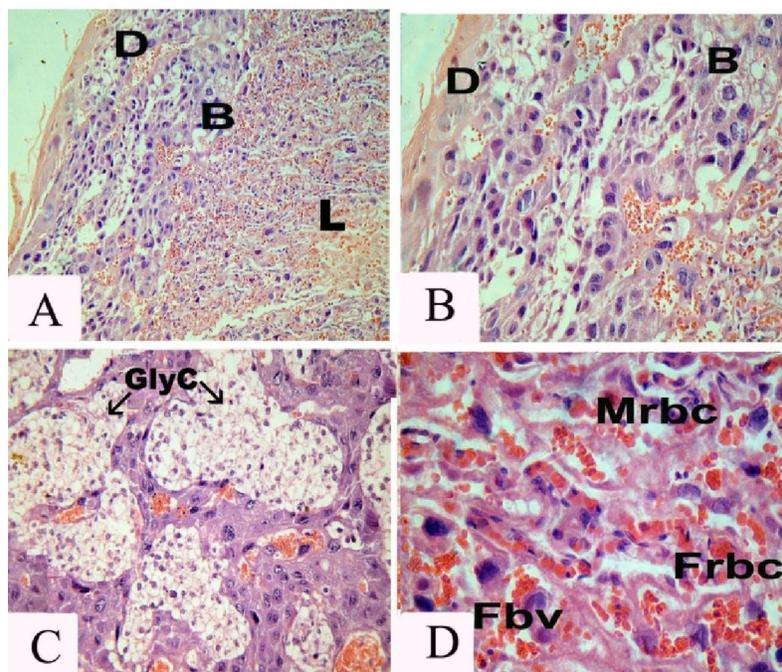
Fig. 2 : Control Rat placenta GD 15, HE stain. A, low magnification, 100 X; (D, decidua basalis; B, basal zone; L, labyrinth) . B, higher magnification 250 X; C, basal zone; GlyC, glycogen cell; D (400 X), labyrinth zone; showing trophoblastic trabeculae consisting of trophoblasts and syncitiotrophoblast, fetal capillaries lined by endothelial cells (FBV) containing fetal erythroblast (Frbc) and maternal lacunae containing maternal erythrocytes (Mrbc)
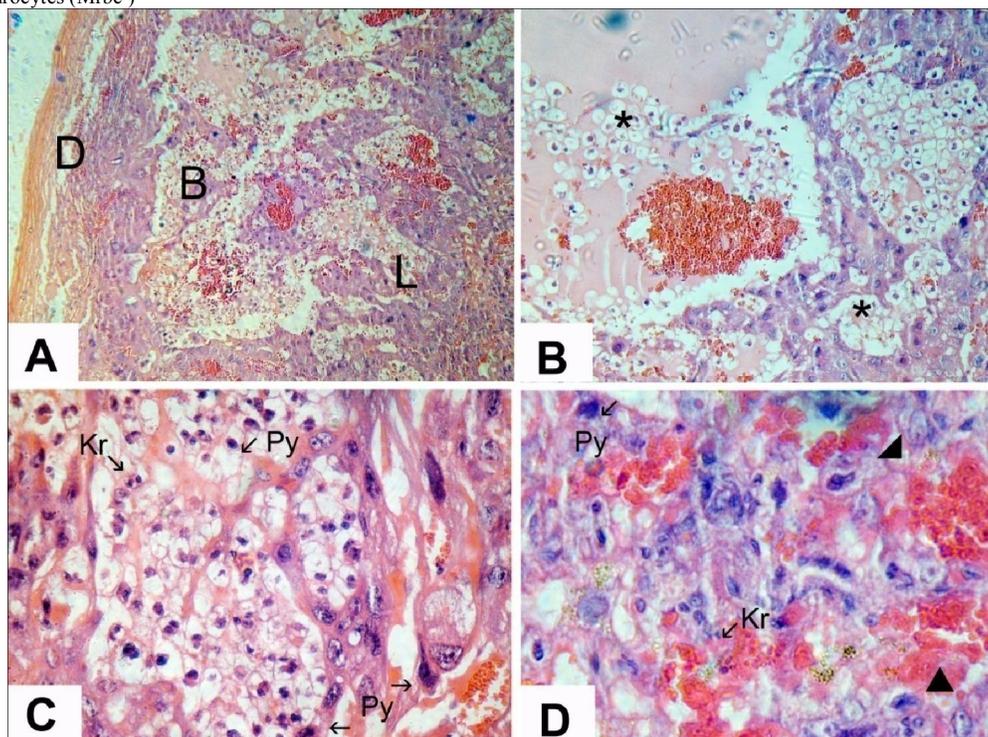

Fig. 3: Experimentally induced PKU Rat placenta GD 15, HE stain. A, low magnification, 100 X; (D, decidua basalis; B, basal zone; L, labyrinth). B, higher magnification 200 X; C, basal zone showing Cystic degeneration of glycogen cells (star). C, (400 X), apoptotic cells, characterized by pyknosis (py) or karyorrhexis (Kr) in glycogen cells and spongiotrophoblasts. D, (400 X), Labyrinth zone; showing disruption of trophoblastic septa, dilated fetal capillaries and maternal lacunae inducing haemorrhagic and degenerative changes (arrow head)
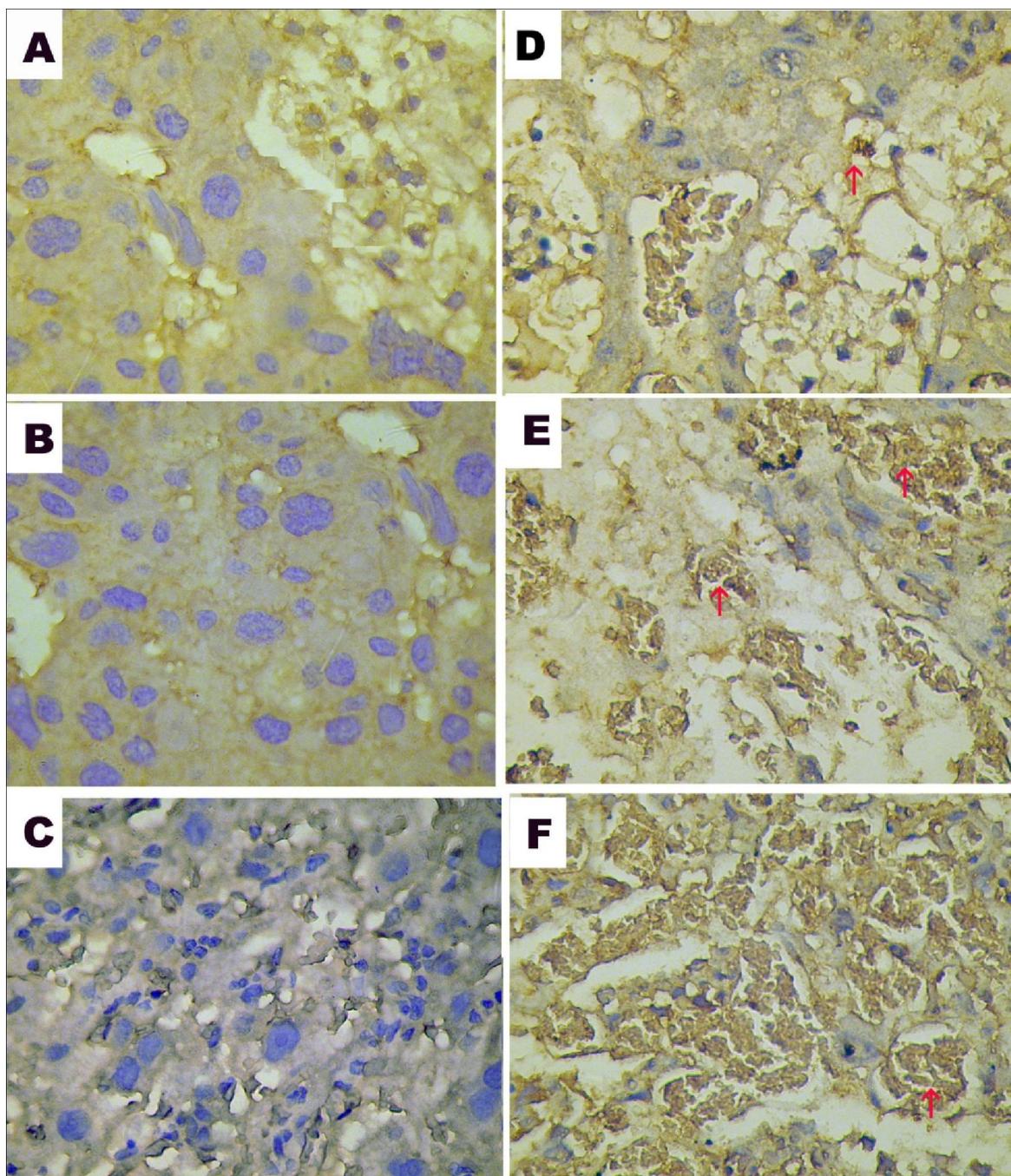
Fig. 4: Caspase-3 positive reactions in rat placenta GD 15. (A, B & C) Control, (D, f & E) Experimentally induced PKU. Arrow: Caspase-3 positive apoptotic cells (streptavidin-biotin peroxidas staining) A & B showing apoptosis in glycogen cells and trophoblasts in the basal zone. C showing apoptosis of trophoblasts in the labyrinth zone associated with remarkable decrease in cellularity (400 X)

4. Discussion

Phenylketonuria (PKU) is an inherited metabolic disease, carried through a "recessive" gene. Phenylalanine hydroxylase deficiency is caused by mutations in the PAH gene resulting in a primary deficiency of the liver enzyme phenylalanine hydroxylase (PAH), (Scriver and Kaufman, 2001), and consequently interrupt the conversion of amino acid phenylalanine to another amino acid, tyrosine that results in excessive accumulation of the amino acid phenylalanine and reduced levels of the amino acid L-tyrosine in the blood (Diamond, 1996). Thus phenylalanine accumulates in the blood to concentration sufficiently high to activate an

alternatively pathway of degeneration (Gazit *et al*., 2003). The placenta is an interface between dam and developing embryo/fetus, and is a specialized organ of O_2/CO_2 exchange and nutrient/metabolite requirements during embryonic development. It also secure the embryo/fetus to the endometrium as a protective barrier of xenobiotics and releases a variety of steroids, hormones and cytokines. Although the placenta is a temporary organ, its growth and function play important roles in the maintenance of pregnancy, and the influence on fetal growth and development. Therefore, placental dysfunction and injury have adverse effects on the maintenance of pregnancy, and fetal growth and development Furukawa *et al.* (2011)

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." (Fisch and Stassart, 2004). Drug- or chemical- induced histopathological changes of the placenta in rats are important in safety evaluation to understand the mechanism of teratogenicity and developmental toxicity. However, the placenta has not received proper consideration as a target organ in safety evaluation of the risks for dams and embryos/fetuses. Morphological or histopathological evaluation of placental development and abnormalities has been scarce and incomplete in experimental animals Furukawa *et al.* (2011)

Jervis, 1939 stated that the damage to the children of PKU mothers is not a genetic consequence (although the children will be at least heterozygous, as PKU is an autosomal recessive disorder). This can be proven by the fact that PKU fathers produce normal offspring (Fisch *et al.*, 1991) and also that well controlled pregnant PKU mothers can produce children without obvious handicap. these symptoms result from high Phe concentrations in the blood of the fetus. It is a consequence of intrauterine Phe excess resulting from the positive transplacental Phe gradient. To keep fetal blood Phe below 500 µmol/l maternal concentration should be below 300 µmol/l (Schoonheyt *et al.*, 1994).

The fetal part of the placenta originates from the trophectoderm of the embryo, and consists of the basal zone and labyrinth zone. These constitutive cells of the placenta proliferate rapidly, differentiate and undergo morphological changes in close relation to each other according to the development sequence in a short pregnancy period. Particularly, the labyrinth zone has high proliferative activity and becomes a major part of the placenta with pregnancy progression (Davies and Glasser, 1968).

Maternal and fetal bloods come very close together in the trophoblast septa and most of the maternofetal exchange of substances is carried out in the labyrinth zone (Takata *et al.*, 1997). Thus, dysfunction of the labyrinth zone relates closely to fetal toxicity.

The present study intragastric administration of 30 mg. DL–α-methylphenylalanine/kg body weight plus 60 mg/kg body weight L-phenylalanine to pregnant rats starting at the 6th day of , at 12 hrs intervals showed reduction in gestation day 15 placental weight. Histopathologically, an increase in apoptotic cells was detected in the labyrinth zone and basal zone, and hypoplasia of the labyrinth zone and basal zone was induced. This result was in agreement with Furukawa *et al.* (2013) in rat placenta due to administration of cisplatin at 2mg/kg/day during GDs 11–12.

The trophoblast cell lineage originates from the ectoplacental cone and differentiates into five differentiated trophoblast types in the labyrinth and basal zone; trophoblastic giant cells, glycogen cells, spongiotrophoblasts, syncytiotrophoblasts and cytotrophoblasts (Cross, 2006). Some of these trophoblasts exhibit high proliferative activity in early placental development.

From the previously mentioned dramatic histopathological alterations may be attributed to the disturbed metabolism of phenylalanine as a result of disturbed protein synthesis may contribute to the reduction in placental weight. Andersen (1976) reported that fetal plasma phenylalanine levels were several times higher than maternal plasma phenylalanine levels, indicating that the placenta actively concentrates maternal phenylalanine.

Intrauterine growth retardation is a feature of maternal PKU syndrome, due to fetus exposition to high Phe levels. Therefore it is conceivable that moderate phe high levels, to which the PKU affected fetus is probably, exposed during fetal life, may negatively affect the intrauterine growth and lower the body weight (Ebaid, 2006).

When Phenylalanine is extremely high and tyrosine is very low, the less descimiminative tyrosine hydoxylase acts on phenylalanine, making dihyroxyphenylanines. Oxidation of these catecholamines can produce superoxide and hydrogen peroxide in a very complicated series of reactions (Halliwell and Gutteridge, 1989). The oxidative stress was defined as an increase in proxidative damage to cells due to an increase in free radical production or impairment in oxidative defense . When glutathione is decreased , cells enter alow-antioxidant mode and are more prone to oxidative damage caused by free radicals This is especially true for cells which are innately more vulnerable to oxidative attacks , such as RBCs (Halliwell and Chirico, 1993).

The placenta serves as a major life support organ for the fetus. Its ability to move oxygen and nutrients from maternal to fetal blood is necessary for the normal growth and development of the fetus. It also serves as a protective barrier for the fetus against harmful compounds by preventing their passage from maternal blood (Deanna., 1998). So any changes in its structure and function may affect fetal growth and development.

Finally the present study strongly suggest the transplacental passage and accumulation of phenylalanine causing pathological effects in placenta. In addition, penetrating to fetus of PKU mothers. In conclusion, adequate nutrition is very important to pregnant but in women with PKU, two factors needs 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 to avoid drastic effects and protect the growing fetus.

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