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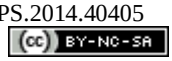
ISSN 2231-3354 

The stress of phenylalanine on rats to study the phenylketonuria at biochemical and molecular level

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ABSTRACT

The present study was aimed to study the stress of phenylalanine on rats to study the effect of Phenylketonuria at molecular and biochemical level. In our study, the rats' weight ranged from 132 to 190 gm. They were housed 25 day and the diet was prepared 5% phenylalanine and the weight is recorded every week. The rats divided into 2 groups, control group and phenylalanine group. After feeding with 5% phenylalanine diet, we take blood samples to measure biochemical markers as (ALT, AST, creatinine, Lipid profile and S100B) and tissues for PCR. Our biochemical results showed significant increase in S100B in phenylalanine group and reduction in total cholesterol, HDL, LDL and triglyceride in phenylalanine group. The molecular study which based on comparing the DNA obtained by RAPD-PCR showed a specific DNA bands which may be responsible for Phenylketonuria and may be used for identification of disease at earlier time of injury. The excess of phenylalanine in diet lead to neural tissue damage and may cause mutation combined with the induced PKU (Phenylketonuria).

INTRODUCTION

Phenylketonuria (PKU; OMIM 262600), one of the most common inborn errors of metabolism, is caused by recessively inherited deficiency of the enzyme phenylalanine hydroxylase (PAH) (Blau *et al.*, 2010). Also (Widaman, 2009) said, Phenylketonuria (PKU) is an inborn metabolic error in which metabolism of phenylalanine (Phe) into tyrosine (Tyr) is disrupted. The increased level of phenylalanine and its metabolites (i.e., phenylpyruvic acid) in PKU exert their toxicity exclusively on the central nervous system (CNS). This toxicity is believed to be a result from either: (a) direct neural damage by phenylalanine and its metabolites; or (b) indirect CNS damage by competing with neurotransmitter precursors such as tyrosine and tryptophan, resulting in lower dopamine, serotonin, epinephrine, and nor epinephrine needed for brain function (Martynyuk AE, 2005). Phenylalanine not needed for tissue synthesis, it is converted to tyrosine via phenylalanine hydroxylase, an oxygenase enzyme that requires the presence of oxygen and tetrahydrobiopterin (part of the folic acid molecule). The reaction is irreversible, making phenylalanine an essential amino acid and tyrosine a non-essential one. (Wurtman & Ritter-Walker, 1988). There are two routes by which the excess phenylalanine can be metabolized: oxidation to tyrosine (the normal and main route for degradation of phenylalanine, and the normal route for biosynthesis of Tyr), and transamination to phenylpyruvate and subsequent further metabolism (a minor route, which comes to the fore when the main route is blocked) (Felig,1975). There were many studies have detected Side Effects and Toxicity of phenylalanine, LD-50 of D-phenylalanine in mice is more than 10 g/kg. (Ehrenpreis and Sicuteri,1983). Short-term stimulant-type side effects have been reported, including elevation of blood pressure, headache, irritability, aggressiveness, and insomnia. Long-term side effects have not been studied. (Simonson, 1985) Phenylalanine hydroxylase (PAH) is an iron-dependent, tetrahydrobiopterin (BH4)-dependent mono-oxygenase that catalyses the rate limiting step in the catabolism of phenylalanine. Defects in PAH enzymatic activity caused by mutations in the *PAH* gene were associated with hyper-phenylalaninemia and phenylketonuria (Scriver and Kaufman, 2001). If there was inadequate PAH activity, with consequently little or no conversion of phenylalanine to tyrosine. The catabolism of phenylalanine was blocked and serum levels of phenylalanine raised

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(hyperphenylalaninemia). Side reactions that under normal conditions were undetectable then start to produce metabolites of phenylalanine Scriver *et al.,* (1995). There are two main reasons for a lack **of** phenylalanine hydroxylase activity.

1. The most common cause for a lack of phenylalanine hydroxylase activity is a genetic defect in the gene for phenylalanine hydroxylase. Most patients suffering from Phenylketonuria have one or another of several possible mutations in the gene for phenylalanine hydroxylase.
2. A secondary cause of lack of phenylalanine hydroxylase activity is a defect in the generation of adequate amounts of the cofactor tetrahydrobiopterin (BH4). Defects in biopterin metabolism account for 1%-3% of all cases of hyperphenylalaninemia.

Phenylketonuria is a recessive trait, so a person will exhibit symptoms of Phenylketonuria only if the person receives a defective phenylalanine hydroxylase gene from each parent. If a person receives a defective phenylalanine hydroxylase gene from only one parent, the person shows no symptoms of Phenylketonuria but is a carrier of the defect. (Robin *et al.*,2008).Fortunately, Savio Woo and his colleagues are obtaining information about the PAH gene. They isolated a human PAH cDNA clone and characterized the human gene. By restriction enzyme analysis of normal and Phenylketonuria -linked mutant chromosomes, they have found extensive polymorphism at the phenylalanine hydroxylase locus (Kwok *et al.*,1985).

There are three forms of polymorphisms

I) Seven biallelic restriction fragment length polymorphisms (RFLPs: BglII, PvuIIa, PvuIIb, EcoRI, MspI, XmnI, and EcoRV);

ii) Multiallelic polymorphisms (variable number of tandem repeats, VNTR, and short tandem repeats, STR)

iii) Silent single nucleotide polymorphisms, SNPs (e.g., Q232Q). RFLP, VNTR and STR alleles can be combined to generate PAH haplotypes. Although several thousands of different polymorphic phenylalanine hydroxylase haplotypes could be generated from combinations of these alleles, far fewer have actually been observed on human chromosomes. Particular phenylalanine hydroxylase haplotypes are associated with disease-causing mutations in European populations (Scriver and Kaufman, 2006). The human phenylalanine hydroxlase gene is located on chromosome 12q23.2, spans about 171 kb and contains 13 exons. The total cDNA length is about 2.4 kb; it encodes for a polypeptide of 452 amino acids of near identical sequence to the human phenylalanine hydroxylase protein, indicating little post translational modification. Hyperphenylalaninemia can be caused by either mutation at the phenylalanine hydroxylase locus, which results in Phenylketonuria, or from mutations in a number of loci which effect BH4 synthesis (Robin *et al.*, 2008). Initially the most prevalent mutations in the Western European population were identified and characterized with regard to the in vitro residual enzyme activity associated with the respective mutation (R.C. Eisensmith *et al.*, 1992). Database now lists a total of 564 PAH mutations discovered world wide as well as the knowledge available about the respective mutation including the residual enzyme activities of ~200 mutations. The phenylalanine hydroxylase mutations were missense mutations not preventing transcription or translation and that the majority of the patients are compound heterozygotes, meaning they carry a different mutation in each of their alleles. PAH deficiency thus most often results from complex interactions of mutant alleles or rapid intracellular destruction of mutant enzyme subunits making genotype/ phenotype correlations based on the knowledge about individual mutations challenging (Blau, 2011). Our study showed a specific DNA bands which may be responsible for Phenylketonuria and may be used as indicator for presence of mutation and identification of disease at earlier time of injury. The excess of phenylalanine lead to neural tissue damage and may cause mutation combined with the induced PKU (Phenylketonuria).

Subject and method

Subject

The rats weight ranged from 132 to 190 gm. Rats were ranked by body weight; they were housed individually in stainless steel cages with raised wire floors for 25 day**.** The diet was prepared 5% phenylalanine and the weight was recorded every week. The rats divided into 2 groups, control group and phenylalanine group. after feeding with 5% phenylalanine diet we take blood samples from all individual of group to measure biochemical markers as(ALT, AST, creatinine, lipid profile, s100B).and tissues from some individuals of each group for PCR.

Methods

The kinetic determination of creatinine concentration in serum and urine was done according to the method of Fabing & Ertinghausen (1971). Quantitative determination of serum cholesterol was carried out calorimetrically using method of (Richmond, 1973). Serum triacylglycerols concentration was determined according to the method of Fossati & Prencipe (1982). Quantitative determination of HDL-cholesterol concentration in serum was done according to method of (Assmann, 1979).

LDL-cholesterol was determined according to method of (Okada *et al.*, 1998). Determination of serum S100B (by ELISA method) this method based on the reports of (Lasn, 2006). Using ELISA Kit purchased from Bio Vendor-Laboratornimedicina a.sn USA. The PCR was performed for amplification of the genomic DNA. This reaction was carried out using the PCR kit purchased from SIGMA**.** Ten different primers used to optimize the genomic DNA. These primers Purchased from BIONEER with melting temp. 32° C and concentration 100 Pmol /µl.

Primer	The sequence (5' 3')	Primer	The sequence (5'3')
1	5'-TCGGCGATAG-3'	6	5'-TTCCGAACCC-3'
2	5'-AATCGGGCTG-3'	7	5´-TCTGTGCTGG-3´
3	5'-GAAACGGGTG-3'	8	5´-GACCGCTTGT-3´
4	5'-CAATCGCCGT-3'	9	5´-GAAACGGGTG-3´
5	5'-GTGATCGCAG-3'	10	5´-AGGGGTCTTG-3´

RESULT

The results indicated that the mean values ±SE of (AST andALT) in control group were 8.6±0.9 and 12.8±0.8 IU/L respectively with ranges 5-10.2 and 10-15. But the mean value of (AST and ALT) in phenylalanine group 37±3.8 and 34±3.5 IU/L respectively with ranges 28-47 and 23-40.

From the liver function comparisons between control and the phenylalanine group the result showed significant correlation (P<0.05). The results indicated that the mean values ±SE of Creatinine in control group were 0.6±0.17 with ranges 0.5-0.9. But the mean value of creatinine in phenylalanine group 0.8±0.25 with ranges 0.5-1.2. From the creatinine comparison between control and the phenylalanine group the result showed insignificant correlation (P<0.05).

The results indicated that the mean values ±SE of (cholesterol, triglyceride, HDL and LDL) in control group were 193±16.7 , 87±16.6 , 40±7.3 and 136±18.8 mg/dl respectively with ranges 170-214 , 75-113 , 30-50 and 105-154. But the mean value of (cholesterol, triglyceride, HDL and LDL) in phenylalanine group 98.8±8.8, 73.2±5.8, 33±5 and 51.16±10.5mg/dl respectively with ranges 90-113, 68-80, 29-41 and 40.2-67. From the lipid profile comparison between control and the phenylalanine group the result showed significant correlation in cholesterol, LDL (P<0.05) also the results showed insignificant correlation in triglyceride and HDL (P<0.05). The results indicated that the mean values ±SE of S100B in control group were 498±1 l with range 497-499 Pg/m. But the mean value of S100B in phenylalanine group 3539±0.83 Pg/ml with ranges 3539-3541Pg/m. From the S100B comparison between control and the phenylalanine group the result showed significant correlation (P< 0.05).

The molecular study showed that four primers only which react with the DNA of control and phenylalanine group (primers 1, 2, 4, 7). The amplified PCR products generated by primer 1 with molecular size from 1200 to 2790 bp. Two distinguishable bands were characteristic for control group. Three bands were characteristic for phenylalanine group. There were no common bands between the control and phenylalanine group The similarity index (SI) between the control and phenylalanine group was (0) zero. The higher genetic distance (GD) between them was 1. The molecular size (m.s) of PCR products generated by primer 2 ranges (1173–4773 bp). Three distinguishable bands with different molecular size (2204, 2790, and 4773) were characteristic for control group. Three bands with molecular size (m.s) (1973 to 3229) were characteristic for phenyl alanine group.

There was one common band was noticed between the control and phenylalanine group (1250 bp) with RF (35.906), and protein band density is equal 0.69.

The higher similarity index (SI) between the control and phenylalanine group was the higher similarity index (SI) between the control and phenylalanine group was (0.25). The higher genetic distance (GD) between them was (0.75). The molecular size (m.s) of PCR products generated by primer 4 ranges (1240-2794 bp). Two distinguishable bands with different molecular size (2200, 2794) were characteristic for control group. Three bands with RF (0.743 to 0.432) , m.s (1240 to 2450) were characteristic for phenylalanine group.

There were no common bands between the control and phenylalanine group The similarity index (SI) between the control and phenylalanine group was (0) zero. The higher genetic distance (GD) between them was 1.

The molecular size (m.s) of PCR products generated by primer 7 range (1200 – 4591 bp). Four distinguishable bands with different molecular size ranged from (210 to 4591 bp) were characteristic for control group. Three bands with RF (0.762 to 0.431), m.s (1200 to 2450) were characteristic for phenylalanine group. There were no common bands between the control and phenylalanine group. The similarity index (SI) between the control and phenylalanine group was (0) zero. The higher genetic distance (GD) between them was 1.

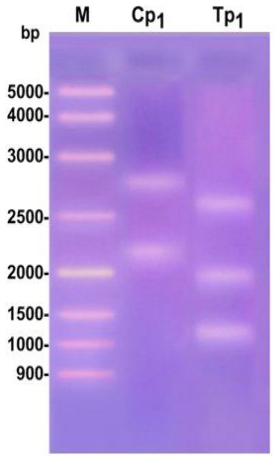

Fig. 1: Agrose gel electrophoresis analysis of PCR products represents the amplified fragments from genomic DNA of control and phenyl alanine treated using primer 1. Lane M: marker, Lane 1: control, Lane 2: phenyle alanine group.

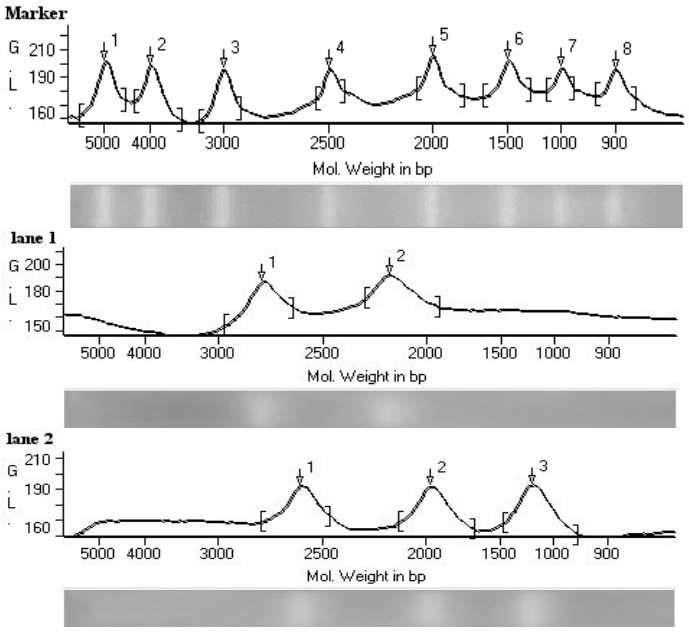

Fig. 2: Electropherogram of PCR Product using primer 1, corresponding to control and phenylalanine treated group. Lane M: marker, Lane 1: control Lane 2: phenyle alanine group.

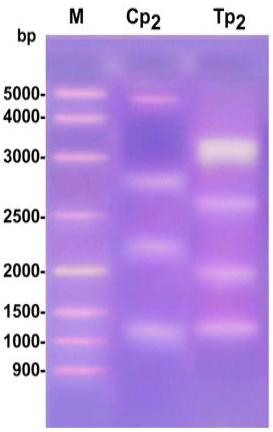

Fig. 3: Agrose gel electrophoresis analysis of PCR products represents the amplified fragments from genomic DNA of control and phenyl alanine treated using primer 2. Lane M : marker, Lane 1: control, Lane 2: phenylealanine group.

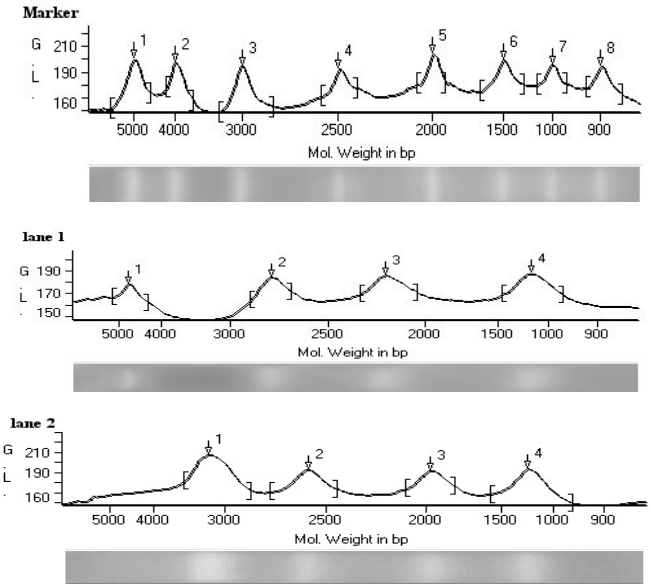

Fig. 4: Electropherogram of PCR Product using primer 2, corresponding to control and phenylalanine treated group. Lane M: marker, Lane 1: control, Lane 2: phenyle alanine group.

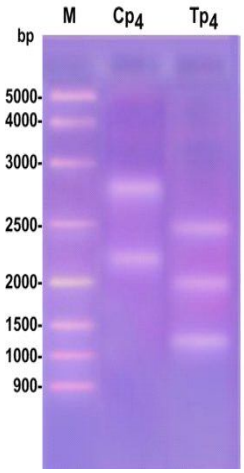

Fig. 5: Agrose gel electrophoresis analysis of PCR products represents the amplified fragments from genomic DNA of control and phenyl alanine treated using primer 4. Lane M: marker, Lane 1: control, Lane 2: phenyle alanine group.

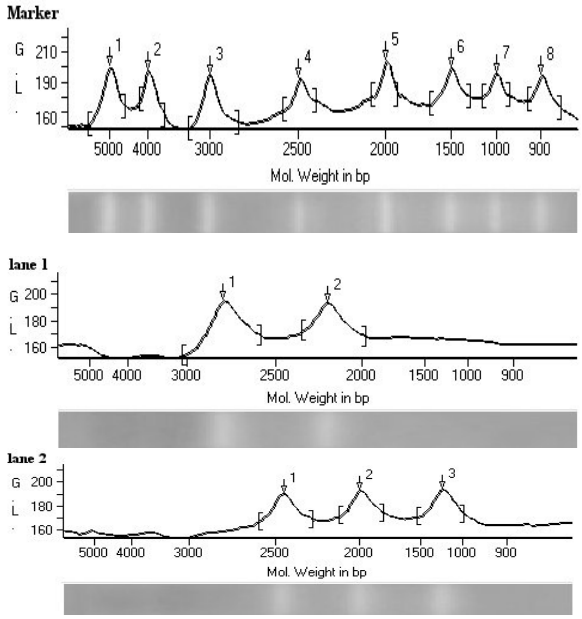

Fig. 6: Electropherogram of PCR Product using primer 4, corresponding to control and phenylalanine treated group. Lane M: marker, Lane 1: control, Lane 2: phenyle alanine group

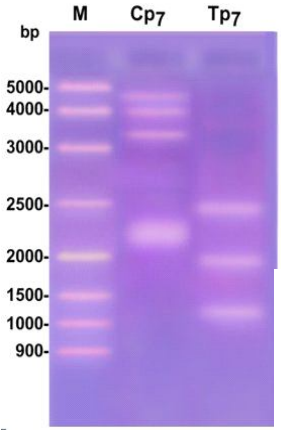

Fig. 7: Agrose gel electrophoresis analysis of PCR products represents the amplified fragments from genomic DNA of control and phenyl alanine treated using primer 7. Lane M: marker, Lane 1: control, Lane 2: phenyle alanine group.

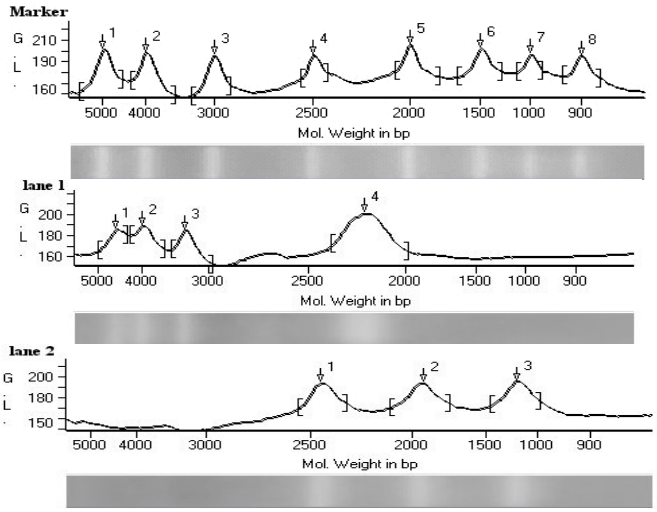

Fig. 8: Electropherogram of PCR Product using primer 7, corresponding to control and phenylalanine treated group. Lane M: marker, Lane 1: control, Lane 2: phenyle alanine group.

DISCUSSION

The objective of the current study was focused on the effect of stress of phenylalanine diet and detection of genetic mutation that may combined with the induced Phenylketonuria. Moreover to identify and determine the variances between the healthy individuals and Phenylketonuria individual on the molecular and biochemical levels. The molecular analysis was based on comparing the DNA obtained by RAPD-PCR. Consequently specific DNA band may be detected as responsible for Phenylketonuria and may be used for identification of the disease at earlier time of injury. On the other hand, the biochemical studies are based on determination serum neural protein (S-100B), lipid profile, kidney function and liver function and comparing the result between control and Phenylketonuria individual. In the current study, the evaluation of lipid profile showed a reduction in total cholesterol, triglyceride, HDL-C and LDL-C .These findings support the hypothesis of a relationship between high plasma phenylalanine level and the inhibition of cholesterogensis by inhibiting the two main regulatory enzymes (3-hydroxy-3-methylglutaryl-coA-reductase and mevalonate-5-pyrophosphate decarboxylase) of brain and liver cholesterogensis (Artuch *et al.*,2004) Some recent studies have showed a significant reduction in the total cholesterol level in Phenyl-ketonuria patient in comparison to the control group. However, HDL-c, LDL-c and triglycerides were not different from controls (Carolina *et al.,* 2010).our result showed a significant increase in S100B level in phenylalanine group. The measurement of biomarker of neural tissue damage such as S100B protein may offer an alternative and direct indicator of cell damage in the nervous system when (Steiner *et al.,* 2010). And these indicate that presence of brain damage due to the stress of phenylalanine. And this agree with previous authors who reported that higher concentrations of serum S100B protein reflect the ongoing central neuro-developmental processes occurring during different stages of life (Mori *et al.,* 2009).

Our study showed a specific DNA bands which may be responsible for Phenylketonuria and may be used for identification of disease at earlier time of injury. The excess of phenylalanine lead to neural tissue damage and may cause mutation combined with the induced PKU (Phenylketonuria).and these agree with a previous studies which showed that elevated phenylalanine concentrations (>907 µmol/I) had also a direct effect on DNA and protein synthesis in rat brain (Schulpis *et al.,* 2004) Our results indicated a genetic variation between control and phenylalanine group, this variation may be due to mutation or polymorphism in gene/s carry DNA sequence complementary to that of the used primers which may cause phenylketonuria, and predict persons at risk of Phenylketonuria.

CONCLUSION

The present investigation was carried out to study the stress of phenylalanine diet at biochemical and molecular level. The results revealed that excess of phenylalanine have effect on different body system and also may cause mutation. The above finding would open avenue to explore the mutation which occurs in the next generation and early detection for Phenylketonuria.

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