Pediatric RESEARCH

BASIC SCIENCE ARTICLE

Assessment of antioxidant enzymes, total sialic acid, lipid bound sialic acid, vitamins and selected amino acids in children with phenylketonuria

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BACKGROUND: In this study, children with phenylketonuria and healthy control subjects were assessed for glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), catalase (CAT) activity, malondialdehyde (MDA), glutathione (GSH), retinol, cholecalciferol, α-tocopherol, phylloquinone, total sialic acid (TSA), lipid bound sialic acid (LSA), total antioxidant (TAS), total oxidation (TOS), and amino acid levels, and the relationships of these variables with phenylketonuria were evaluated.
METHODS: The study included 60 children with phenylketonuria and 30 control subjects. Children with phenylketonuria were divided into hyperphenylalaninemia (HPA) and amino acid mixture (AAM) groups.
RESULTS: The HPA group had significantly lower levels of GSH-Px, CAT, GSH, TAS, α-aminobutyric acid, and taurine levels ($p < 0.01$, $p < 0.05$, $p < 0.05$, $p < 0.001$, $p < 0.01$, $p < 0.05$, respectively) than the control group. Additionally, the AAM group had significantly lower levels of CAT, TAS, and phylloquinones ($p < 0.05$, $p < 0.05$, $p < 0.05$, respectively) than the control group. It was observed in our study that in the HPA group, a significantly strong positive linear correlation was observed between phenylalanine and α-aminoadipic acid ($r = 0.777$; $p = 0.002$).
CONCLUSIONS: It was concluded that the levels of α-aminoadipic acid and phylloquinone might be an appropriate choice for the determination of phenylketonuria in parallel with the levels of phenylalanine. α-aminobutyric acid and phylloquinone as a supplement can decrease HPA damage.

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INTRODUCTION

Phenylketonuria (PKU) is an autosomal recessive inborn error of metabolism that results from mutations in phenylalanine hydroxylase (PAH, EC 1.14.16.1).[1–4] This enzyme is a cytosolic homotetramer that catalyzes the hydroxylation of phenylalanine (Phe) to tyrosine (Tyr) using tetrahydrobiopterin (BH_4) as a cofactor.[2] The PAH enzyme activity is decreased in the presence of DNAJC12 (MIM: 606060) mutations. DNAJC12 molecular monitoring and treatment are important in preventing children with unresolved hyperphenylalaninemia.[5]

Enzyme deficiency causes the Phe levels to elevate in the blood and in other tissues such as the brain along with relevant neurotoxic effects.[1,6] Phe accumulates and is converted into phenylpyruvate, also known as phenylketone, which is detectable in urine.[3] Untreated PKU causes severe mental disability, microcephaly, epilepsy, growth retardation, delayed psychomotor development, microcephaly, seizures, and mental health issues.[7] Treatment for PKU involves a reduction in the dietary intake of natural protein and Phe and includes amino acid supplementation with manufactured protein substitutes other than Phe.[6] To ensure daily requirements, these dietary supplements are enriched with various micronutrients such as minerals and vitamins.[6–8]

Many harmful effects of oxidative stress on various biological functions can be measured, and higher exposure to the oxidative stress causes certain diseases such as cardiovascular and neurological diseases. In particular, a high lipid content in the brain, especially in the form of unsaturated fatty acids, is vulnerable to oxidants, as the antioxidant defense system in the brain is relatively weak.[9]

In PKU, oxidative stress during normal metabolism causes the cells to become unstable; the oxidative stress is caused by potentially harmful substances referred to as reactive oxygen species (ROS), which includes hydroxyl radicals ($OH^•$), superoxide anion (O_2^-) radicals, hydrogen peroxide (H_2O_2), and reactive nitrogen species (RNS) (e.g., nitric oxide (NO) and peroxinitrite ($ONOO^-$)).[9] The group of antioxidants includes enzymes that catalyze reactions and ultimately eliminate ROS; these antioxidants include SOD, CAT, and GSH-Px.[10,11] Non-enzymatic antioxidants include glutathione, metal ion sequestration proteins, thiols, some vitamins (E and C), minerals, carotenoids, and phytochemicals, such as flavonols and isoflavones.[10]

Sialic acids include acylated derivatives of neuraminic acid. These are commonly found in mammals and generally exist as terminal components at the non-reducing end of carbohydrate chains of glycolipids and glycoproteins.[12] TSA comprises LSA, protein-bound sialic acid and free sialic acid.[13] In humans, sialic acid is found in large quantities in inflammatory markers such as orosomucoid, $α_1$-antitrypsin, ceruloplasmin, fibrinogen, haptoglobulin, transferrin, and complement proteins.[14]

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The purpose of our study was to investigate whether altered antioxidant enzyme activity (CAT, GSH-Px, and SOD), MDA, GSH, vitamin A (retinol), vitamin D (cholecalciferol), vitamin E (α-tocopherol), vitamin K (phylloquinone), TAS, TOS, TSA, LSA, and amino acid (phenylalanine, tyrosine, α-aminobutyric acid, aminoadipic acid, β-alanine, ethanolamine, phosphoethanolamine, phosphoserine, gamma-amino butyric acid (GABA), carnosine, glycine, ornithine, cystathionine, citrulline, taurine) levels had an interactive association with phenylketonuria in children and whether these levels were correlated with phenylketonuria in children.

METHODS

Study population

The study included 60 children with phenylketonuria (36 girls and 24 boys) and 30 healthy subjects. Children with phenylketonuria were divided into hyperphenylalaninemic (HPA) and Phe-free amino acid mixture (AAM) groups based on the initial level of plasma Phe before and after the treatment as follows: PKU children before treatment (phenylalanine values >600 μmol/L) and PKU children after treatment (Phe values <600 μmol/L). Levels of Phe were categorized according to the following European PKU Guidelines:[1] mild hyperphenylalaninemia (120–600 μmol/L); mild-moderate PKU (600–1200 μmol/L); and classic PKU (>1200 μmol/L). A total of 30 children with a mean age of 6.92 ± 0.61 years (18 girls and 12 boys) were in the hyperphenylalaninemic (HPA) group (23 mild-moderate PKU, 7 classic PKU), and 30 children with a mean age of 7.54 ± 0.58 years (18 girls and 12 boys) were in the Phe-free amino acid mixture (AAM) group (25 mild hyperphenylalaninemia, 5 Phe values <120). Normal children ($n = 30$) with a mean age of 7.89 ± 0.74 years (17 girls and 13 boys) who had normal examination findings served as the healthy control group.

The study protocol was approved by the Van Yuzuncu Yil University (YYU) Faculty of Medicine Ethics Committee (YYU, 14.05.2014/Decision no: 2014/13) and conducted in accordance with the Helsinki Declaration. Parents were properly informed about the study and signed a written consent. Children with phenylketonuria were examined in the Division of Pediatric Endocrinology of YYU. Data and sample collection occurred between June 2014 and August 2015. The following variables were collected from each patient: age, sex, height, and weight.

Sample preparation and analytical methods

Five milliliters of venous blood were drawn after patients fasted overnight, and the blood was centrifuged for 10 min at 2500 rpm. The serum was separated and kept in covered polypropylene tubes and stored at −65 °C until TSA, LSA, TAS, TOS, α-tocopherol, retinol, phylloquinone, and cholecalciferol were measured.

Erythrocytes and plasma were obtained as whole blood samples from fasting individuals (PKU and controls) via venipuncture with heparinized vials. Blood was centrifuged at 1000 × g, and plasma was removed by aspiration and stored at −65 °C until plasma amino acids were measured. Plasma amino acid levels were determined using an ion exchange chromatographic method with the Aracus amino acid analyzer (membraPure-GmbH, Neuendorfstraße20a;16761 Hennigsdorf-Berlin, Germany).

Erythrocytes were washed three times with saline (0.153 mol/L NaCl) and centrifuged at 1000 rpm for 5 min. The erythrocytes were processed immediately to measure GSH and MDA. The erythrocytes were stored at −65 °C until the determination of CAT, GSH-Px, and SOD enzyme activities.

Measurements of biochemical parameters, namely, lactate dehydrogenase (LDH), alkaline phosphatase (ALP), phosphorus (P), sodium (Na), and chloride (Cl) were determined using standard procedures at the laboratory of the YYU Medical School using the Architect CI-16000 (Abbott Diagnostics, Abbott Park, IL). The dietary amino acid mixture used by the children with PKU was PKU2 (Milupa, Friedrichsdorf), which was Phe-free with vitamin and mineral supplementation.

Using the method of Beutler et al. and Rizzi et al., the erythrocyte GSH was analyzed. This method uses m-phosphoric acid to precipitate the protein and supernatant, which was obtained on reaction with 5–5′ dithiobis-2-nitrobenzoic acid (DTNB), resulting in a yellow compound. The optical density was measured at 412 nm and expressed as units of enzyme per gram of hemoglobin[15,16]. The erythrocyte MDA level was determined according to the method described by Gutteridge et al. and Sushil et al. based on the reaction of MDA with thiobarbituric acid (TBA). MDA was determined from the absorbance with a spectrophotometer at 532 and 600 nm and expressed as units of enzyme per gram of hemoglobin.[17, 18]

Erythrocyte antioxidant enzyme activities (GSH-Px, SOD, and CAT) were measured using a spectrophotometer (Shimadzu UV-1800 UV-VIS Spectrophotometer, Kyoto) and expressed as units of enzyme per gram of hemoglobin. The erythrocyte GSH-Px (E. C.1.11.1.9) activity was measured at a 340-nm wavelength by the method of Paglia et al.[19]. The SOD (EC 1.15.1.1) activity was determined at a 505-nm wavelength according to the method of Sun et al.[20]. The CAT (EC 1.11.1.6) activity was estimated at 240 nm by the decomposition of hydrogen peroxide, which is in accordance with the method described by Aebi.[21]

Vitamin (A, D, E, and K) analysis

α-tocopherol, retinol, phylloquinone, and cholecalciferol stock solutions were prepared at 500 μg/mL. To prepare a standard solution, the stock solutions were diluted suitably with methanol. To determine the proper calibration, linear regression analysis of the peak area was used to standardize the solution concentrations.

Extraction process

To minimize the sample degradation due to exposure to UV light, the samples, which were covered with plastic sleeves, were thawed at ambient temperature under fluorescent lights. α-tocopherol, retinol, phylloquinone, and cholecalciferol in serum were extracted as follows: 100 μL serum was deproteinized by adding 100 μL ethanol and adding antioxidants such as 0.025% BHT to the extraction solvent. The samples were mixed via vortex for 1 min.[22] The samples were extracted twice with 600 μL *n*-hexane. After mixing the samples via vortex, they were centrifuged at 8000 rev/min for 10 min. A total of 500 μL of hexane layer was extracted, and it was evaporated under a nitrogen stream of 37 °C to dryness. The residue was dissolved in 50 μL tetrahydrofuran and was added to 150 μL methanol. After vortexing the samples for 1 min, 100-μL samples were auto-sampled using amber glass vials.

Chromatographic conditions

The chromatographic system consisted of HP Agilent 1100 with a G-1328 Diode Array Detector (DAD) and G1329 ALS autosampler (−8 °C). Agilent Technologies HP software was used to process the data. A 5-μm Gl Science C_{18} reversed-phase column (250 × 4.6 mm ID) was used for separation. Then, the mobile phase of a methanol–tetrahydrofuran mixture (80:20, v/v) was modified.[23] The pump was arranged to a flow rate of 1.5 mL/min. Chromatographic analysis was performed at 40 °C using isocratic elution. The chromatogram was monitored with DAD array detection at 290, 325, 265, and 248 nm for the simultaneous measurement of α-tocopherol, retinol, cholecalciferol, and phylloquinone, respectively.

Using the method described by Sydow et al.[24], serum TSA levels were determined. The optical density measurement was made at 525 nm. Using the method specified by Katopodis et al.[25], serum LSA was detected from the absorbance with a spectrophotometer at 580 nm.

The total antioxidant status (TAS) is often used to evaluate the overall antioxidant state of the body and in the monitoring of antioxidant therapy. The total antioxidant status (TAS) of serum was determined with 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS). The results are expressed as mmol trolox equivalent/L.[26] The total oxidant status (TOS) was used to determine the patient's overall oxidative status. The serum total oxidation status (TOS) was measured using the method developed by Erel. The results are expressed in terms of millimoles of hydrogen peroxide (H_2O_2) equivalent per liter (mmol H_2O_2 equivalent/L).[27]

The oxidative stress index (OSI) is defined as the ratio of the TOS level to the TAS level. The OSI is an indicator of the degree of oxidative stress. The OSI is calculated using the following formula:

$$\mathrm{OSI} = \frac{\mathrm{TOS}\,(\mu\mathrm{mol\,H_2O_2\,Eq./L})}{\mathrm{TAS}\,(\mathrm{mmol\,trolox\,Eq./L})} \times 100$$

Statistical analysis

The results are expressed as the arithmetic means ± the standard error of the mean ($\bar{X}$ ± SEM). The analysis of variance (ANOVA) was used for statistical analysis, and Tukey's test was used for post hoc comparison of the means. The Pearson correlation coefficient was used for correlation studies in the hyperphenylalaninemia and amino acid mixture groups. The statistical analyses were performed using the SPSS®, version 22 statistical software (SPSS Inc. Chicago Illinois).

RESULTS

The characteristics of the hyperphenylalaninemia group, Phe-free amino acid mixture group, and healthy control subjects included in the study are shown in Table 1. As a result of the analysis, it was found that the values of body mass index (BMI), age (years) and sex in the HPA and AAM groups did not significantly change compared to those in the control group ($p > 0.05$). We determined the Phe concentrations (963.30 ± 85.37 μmol/L) and Tyr concentrations (40.03 ± 4.84 μmol/L) in the HPA group, the levels of Phe and Tyr (269.50 ± 49.75 μmol/L and 59.62 ± 10.58 μmol/L) in the AAM group, and the levels of Phe and Tyr (51.07 ± 3.52 μmol/L and 57.58 ± 4.45 μmol/L) in the control group.

Table 2 shows the concentration of erythrocytes, SOD, GSH-Px, CAT, MDA, GSH serum, TSA, LSA, TAS, TOS, OSI, α-tocopherol, retinol, cholecalciferol, phylloquinone, ALP, LDH, P, Na, and Cl in the HPA, AAM, and control groups.

The statistical analyses showed that the HPA group had significantly lower levels of CAT, GSH-Px, GSH, and TAS ($p < 0.05$, $p < 0.01$, $p < 0.05$, and $p < 0.001$, respectively) than the control group. Additionally, the AAM group had significantly lower levels of CAT, TAS, and phylloquinone than the control group ($p < 0.05$, $p < 0.05$, $p < 0.05$, respectively), whereas the HPA group had increased levels of MDA, TSA, LSA, TOS, OSI, and ALP compared to the control group ($p < 0.01$, $p < 0.05$, $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.01$, respectively). Similarly, the AAM group had significantly higher levels of MDA, TOS, OSI, and LDH than the control group ($p < 0.05$, $p < 0.01$, $p < 0.01$, and $p < 0.05$, respectively). However, the HPA group had a significantly higher level of ALP than the AAM group ($p < 0.01$). Moreover, no significant differences in the SOD, α-tocopherol, retinol, cholecalciferol, P, Na, or Cl levels were observed in the HPA and AAM groups compared to the control group ($p > 0.05$) (Table 2, Figs. 1–4).

The mean plasma amino acid levels of the HPA, AAM, and control groups are shown in Table 3. The statistical analyses showed that the HPA group had significantly lower levels of α-aminobutyric acid and taurine than the control group ($p < 0.01$ and $p < 0.05$, respectively). The HPA group had significantly higher phenylalanine levels than the control group ($p < 0.001$). Similarly, the AAM group had significantly higher phenylalanine and cystathionine levels than the control group ($p < 0.01$ and $p < 0.05$, respectively). However, the AAM group had significantly higher levels of α-aminobutyric acid and GABA than the HPA group ($p < 0.05$ and $p < 0.05$). Moreover, no significant differences in the tyrosine, α-aminoadipic acid, β-alanine, ethanolamine, phosphoethanolamine, phosphoserine, glycine, carnosine, ornithine, or citrulline levels were observed in the HPA and AAM groups compared to the control group ($p > 0.05$) (Table 3).

Table 1. Characteristics of phenylketonuric children and control subjects (mean ± SEM)

	Control ($\bar{X}$ ± SEM)	HPA ($\bar{X}$ ± SEM)	AAM ($\bar{X}$ ± SEM)
Gender (M/F)	13/17	12/18	12/18
Age (years)	7.89 ± 0.74	6.92 ± 0.61	7.54 ± 0.58
BMI (kg/m^2)	17.64 ± 0.53	15.79 ± 0.35	16.16 ± 0.54

HPA hyperphenylalaninemia, *AAM* phenylalanine-free amino acid mixture, *BMI* body mass index

Table 2. The mean antioxidant enzymes (SOD, GSH-Px, CAT), MDA, GSH, TSA, LSA, TAS, TOS, OSI, α-tocopherol, Retinol, Cholecalciferol, Phylloquinone and some biochemical parameters in phenylketonuric children and controls

Parameters	Control ($\bar{X}$ ± SEM)	HPA ($\bar{X}$ ± SEM)	AAM ($\bar{X}$ ± SEM)
SOD (IU/g Hb)	1962.29 ± 32.48	2040.95 ± 42.79	1978.88 ± 35.45
GSH-Px (IU/g Hb)	10.89 ± 0.35^{b}	9.231 ± 0.37^{b}	10.15 ± 0.41
CAT (IU/g Hb)	727.57 ± 29.21^{c,c1}	622.11 ± 25.05^{c}	629.60 ± 26.57^{c1}
MDA (nmol/g Hb)	5.16 ± 0.26^{b,c}	6.67 ± 0.36^{b}	6.31 ± 0.39^{c}
GSH (μmol/g Hb)	0.92 ± 0.029^{c}	0.81 ± 0.032^{c}	0.83 ± 0.03
TSA (mmol/L)	1.41 ± 0.070^{c}	1.67 ± 0.091^{c}	1.54 ± 0.072
LSA (mmol/L)	0.49 ± 0.012^{c}	0.54 ± 0.014^{c}	0.50 ± 0.012
TAS (mmol trolox equivalent/L)	1.52 ± 0.055^{a,c}	1.19 ± 0.063^{a}	1.31 ± 0.059^{c}
TOS (μmol H_2O_2 equivalent/L)	15.21 ± 0.54^{b,b1}	18.74 ± 0.64^{b}	17.98 ± 0.76^{b1}
OSI	1.04 ± 0.078^{a,b}	1.62 ± 0.083^{a}	1.41 ± 0.072^{b}
α-tocopherol (μmol/L)	2.39 ± 0.16	2.24 ± 0.16	2.26 ± 0.19
Retinol (μmol/L)	3.03 ± 0.14	2.87 ± 0.18	2.91 ± 0.21
Cholecalciferol (μmol/L)	0.38 ± 0.033	0.42 ± 0.026	0.43 ± 0.035
Phylloquinone (μmol/L)	1.01 ± 0.056^{c}	0.77 ± 0.088	0.73 ± 0.078^{c}
Alkaline phosphatase (U/L)	284.75 ± 27.41^{b}	429.13 ± 31.90^{b,b1}	305.74 ± 22.17^{b1}
LDH (U/L)	348.01 ± 27.83^{c}	488.82 ± 47.08	533.60 ± 63.74^{c}
P (mg/dL)	4.98 ± 0.14	5.11 ± 0.19	4.99 ± 0.29
Na (mmol/L)	137.45 ± 0.92	138.37 ± 0.73	137.88 ± 0.82
Cl (mmol/L)	104.35 ± 1.11	107.01 ± 0.82	107.08 ± 0.83

SOD superoxide dismutase, *GSH-Px* glutathione peroxidase, *CAT* catalase, *MDA* malondialdehyde, *GSH* glutathione, *TSA* total sialic acid, *LSA* lipid bound sialic acid, *TAS* total antioxidant status, *TOS* total oxidation status, *OSI* oxidative stress index, *LDH* lactate dehydrogenase, *P* phosphorus, *Na* sodium, *Cl* chloride

Different letters, significant differences between groups. a: $p < 0.001$; b,b1: $p < 0.01$; c,c1: $p < 0.05$

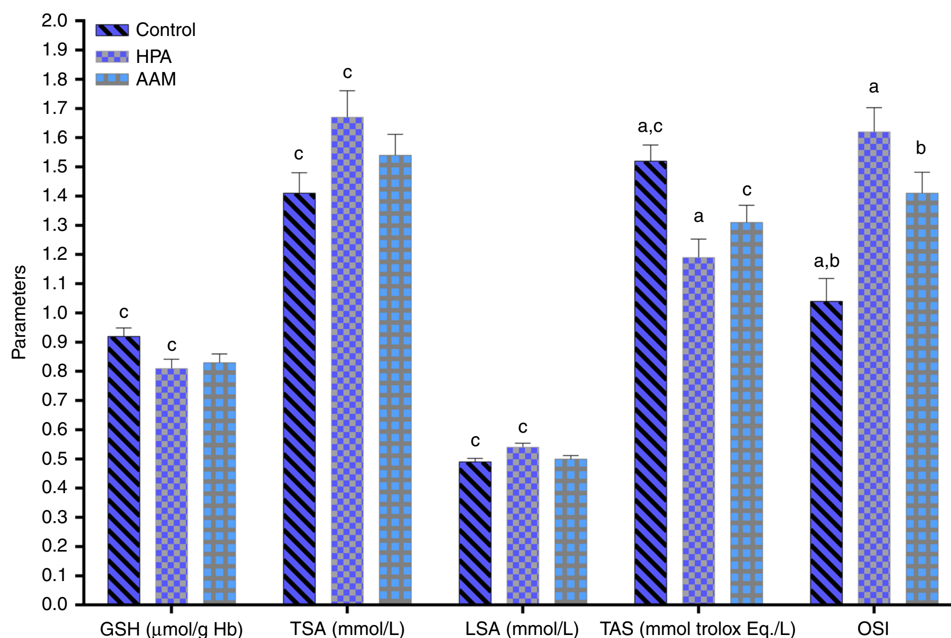

Fig. 1 GSH, TSA, LSA, TAS, and OSI levels of the control, HPA and AAM groups (a: $p < 0.001$, b: $p < 0.01$, c: $p < 0.05$)

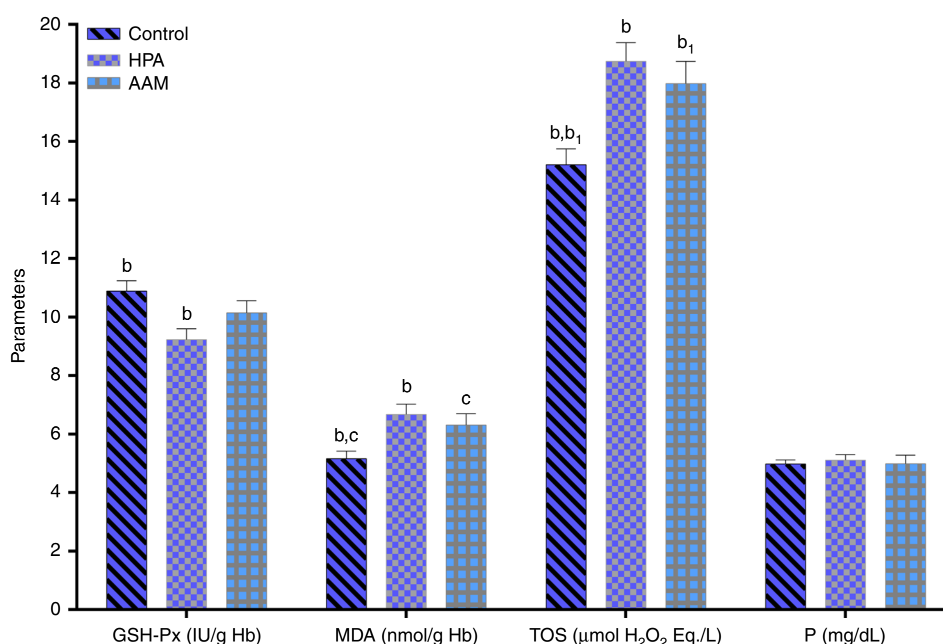

Fig. 2 GSH-Px, MDA, TOS, and P levels of the control, HPA and AAM groups (b, b_1: $p < 0.01$, c: $p < 0.05$)

Table 4 shows significant correlations between the parameters in the HPA group. Relationships between SOD activity, MDA, GSH, TSA, LSA, phylloquinone, cholecalciferol, α-tocopherol, and amino acids (i.e., phenylalanine, tyrosine, α-aminobutyric acid, α-aminoadipic acid, β-alanine, phosphoethanolamine, phosphoserine, GABA, glycine, ornithine, cystathionine, and taurine) in the HPA group were estimated by calculation of the correlation coefficient *r* and significance level, namely, the *p* value.

As a result of the analysis, a positive linear correlation was observed among GSH, SOD, and cystathionine levels in the HPA group ($r = 0.584$; $p = 0.046$, and $r = 0.809$; $p = 0.001$, respectively). Additionally, the following positive correlations were observed: SOD–GABA ($r = 0.642$; $p = 0.033$) and MDA–cystathionine ($r = 0.725$; $p = 0.005$). The following negative correlations were observed: MDA–β-alanine ($r = -0.666$; $p = 0.050$), TSA–phylloquinone ($r = -0.632$; $p = 0.020$), TSA–cholecalciferol ($r = -0.732$; $p = 0.004$), and TSA–α-tocopherol ($r = -0.704$; $p = 0.007$). In the children with HPA, significantly strong positive correlations were observed as follows: LSA–α-aminobutyric acid ($r = 0.782$; $p = 0.003$), tyrosine–ornithine ($r = 0.554$; $p = 0.050$), and phenylalanine–α-aminoadipic acid ($r = 0.777$; $p = 0.002$). Additionally, the following positive linear correlations were observed in subjects with HPA: phosphoserine–glycine concentrations ($r = 0.576$ $p = 0.039$), P–α-tocopherol concentrations ($r = 0.564$; $p = 0.045$), and α-tocopherol–cholecalciferol concentrations ($r = 0.568$; $p = 0.043$) (Table 4).

Table 5 shows significant correlations among the parameters in the AAM group. Our findings indicate the following negative linear

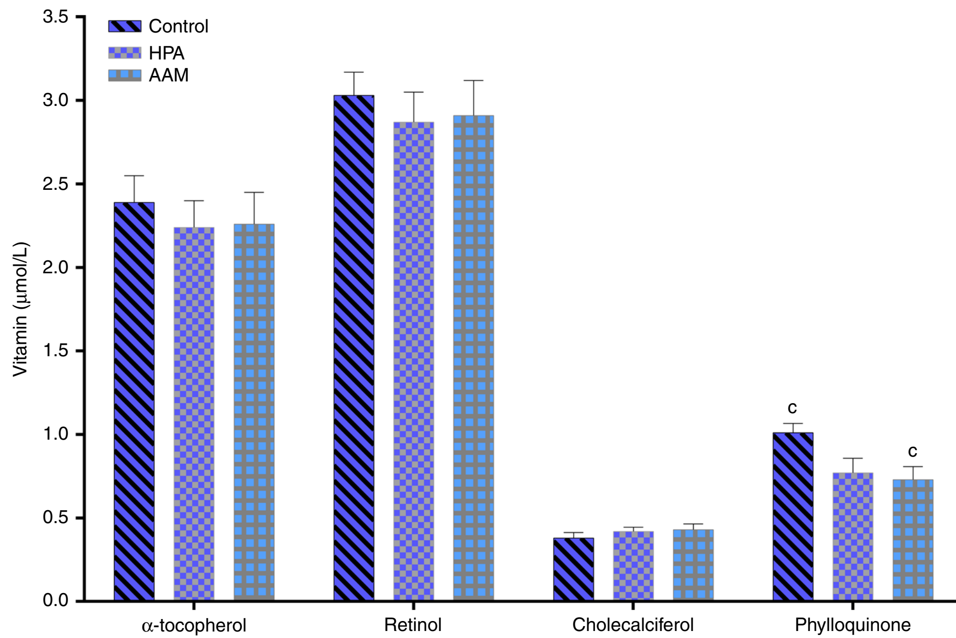

Fig. 3 α-tocopherol, retinol, cholecalciferol, and phylloquinone levels of the control, HPA and AAM groups (c: $p < 0.05$)

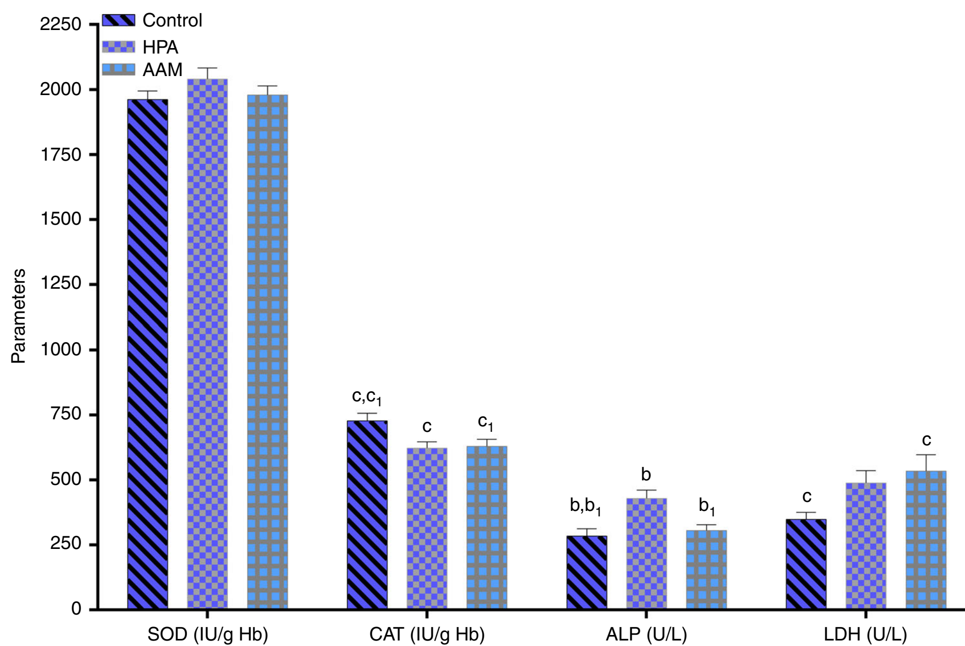

Fig. 4 SOD, CAT, ALP, and LDH levels of the control, HPA and AAM groups (b,b_1: $p < 0.01$, c,c_1: $p < 0.05$)

correlations: between the GSH and citrulline ($r = -0.542$; $p = 0.030$), CAT and LSA ($r = -0.503$; $p = 0.047$), and SOD and TOS concentrations ($r = -0.566$; $p = 0.022$). CAT levels were positively correlated with the β-alanine and cholecalciferol concentrations ($r = 0.499$; $p = 0.049$ and $r = 0.674$; $p = 0.008$). Negative correlations were observed between the following parameters: the TAS and GABA ($r = -0.513$; $p = 0.042$), phenylalanine and taurine ($r = -0.602$; $p = 0.014$), and α-aminobutyric and ALP concentrations ($r = -0.524$; $p = 0.045$). A positive correlation was observed between the MDA and α-tocopherol concentrations ($r = 0.618$; $p = 0.019$), between the TAS and P concentrations ($r = 0.790$; $p = 0.001$), and between α-aminoadipic acid and the ornithine and glycine concentrations ($r = 0.724$; $p = 0.003$ and $r = 0.628$; $p = 0.016$). Additionally, positive correlations were observed between the following parameters: the phosphoserine and phylloquinone concentrations ($r = -0.606$; $p = 0.022$), the GABA and α-tocopherol and P concentrations ($r = -0.658$; $p = 0.011$, $r = -0.688$; $p = 0.007$), the phylloquinone and glycine and ornithine concentrations ($r = -0.712$; $p = 0.004$, $r = -0.594$; $p = 0.025$), the carnosine and α-tocopherol concentrations ($r = -0.708$; $p = 0.049$), and the GABA, carnosine, and glycine ($r = 0.788$; $p = 0.020$, $r = 0.550$; $p = 0.027$) concentrations in subjects from the AAM group (Table 5).

DISCUSSION

The present study was conducted to evaluate antioxidant enzyme (GSH-Px, SOD, CAT) activity, MDA, GSH, TSA, LSA, α-tocopherol,

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Table 3. The mean selected amino acids in phenylketonuric children and controls

Parameters (μmol/L)	Control ($\bar{X}$ ± SEM)	HPA ($\bar{X}$ ± SEM)	AAM ($\bar{X}$ ± SEM)
α-aminobutyric acid	10.57 ± 0.89[b]	6.24 ± 0.91[,b,c]	9.91 ± 0.88[c]
α- aminoadipic acid	2.67 ± 0.69	2.25 ± 0.67	2.32 ± 0.72
β-alanine	21.64 ± 3.82	12.31 ± 2.12	25.35 ± 4.88
Ethanolamine	4.73 ± 0.70	4.72 ± 1.17	5.96 ± 0.98
Phosphoethanolamine	10.21 ± 2.40	17.19 ± 3.64	10.10 ± 3.35
Phosphoserine	11.95 ± 1.15	9.80 ± 1.32	11.01 ± 1.01
GABA	22.81 ± 3.96	12.08 ± 1.65[c]	25.65 ± 4.35[c]
Glycine	232.95 ± 15.07	209.83 ± 17.15	239.80 ± 24.58
Carnosine	3.03 ± 0.48	2.21 ± 0.47	2.46 ± 0.66
Ornithine	69.99 ± 7.45	47.18 ± 3.51	61.79 ± 7.52
Cystathionine	0.93 ± 0.22[c]	1.95 ± 0.52	2.43 ± 0.46[c]
Citrulline	21.81 ± 1.47	20.94 ± 1.70	19.34 ± 1.93
Taurine	35.64 ± 3.07[c]	22.76 ± 2.37[c]	33.91 ± 3.38

GABA gamma-amino butyric acid
[b] $p < 0.01$
[c] $p < 0.05$

Table 4. Correlations between the parameters in phenylketonuric children (HPA)

Parameters	*r*	*p*
HPA		
GSH–SOD	$r = 0.584$	$p = 0.046$
GSH–Cystathionine	$r = 0.809$	$p = 0.001$
SOD–GABA	$r = 0.642$	$p = 0.033$
MDA–Cystathionine	$r = 0.725$	$p = 0.005$
MDA–β-alanine	$r = -0.666$	$p = 0.050$
TSA–Phylloquinone	$r = -0.632$	$p = 0.020$
TSA–Cholecalciferol	$r = -0.732$	$p = 0.004$
TSA–α-tocopherol	$r = -0.704$	$p = 0.007$
LSA–α-aminobutyric acid	$r = 0.782$	$p = 0.003$
Phenylalanine–α- aminoadipic acid	$r = 0.777$	$p = 0.002$
Tyrosine–Ornithine	$r = 0.554$	$p = 0.050$
Phosphoethanolamine–Taurine	$r = -0.708$	$p = 0.010$
Phosphoserine–Glycine	$r = 0.576$	$p = 0.039$
P–α-tocopherol	$r = 0.564$	$p = 0.045$
α-tocopherol–Cholecalciferol	$r = 0.568$	$p = 0.043$

retinol, cholecalciferol, phylloquinone, TAS, TOS and amino acid (phenylalanine, tyrosine, α-aminobutyric acid, α-aminoadipic acid, β-alanine, ethanolamine, phosphoethanolamine, phosphoserine, GABA, carnosine, glycine, ornithine, cystathionine, citrulline, and taurine) contents and related levels in children with phenylketonuria.

To the best of our knowledge, this is the first study to examine the levels of some amino acids, TSA, LSA, and phylloquinone in children with phenylketonuria. The results obtained provide substantive evidence in favor of the protective potential of AAM mixture supplementation.

Table 5. Correlations between the parameters in phenylketonuric children (AAM)

Parameters	*r*	*p*
AAM		
GSH–Citrulline	$r = -0.542$	$p = 0.030$
CAT–LSA	$r = -0.503$	$p = 0.047$
CAT–β-alanine	$r = 0.499$	$p = 0.049$
CAT–Cholecalciferol	$r = 0.674$	$p = 0.008$
SOD–TOS	$r = -0.566$	$p = 0.022$
MDA–α-tocopherol	$r = 0.618$	$p = 0.019$
LSA–CAT	$r = -0.503$	$p = 0.047$
TAS–P	$r = 0.790$	$p = 0.001$
TAS–GABA	$r = -0.513$	$p = 0.042$
Phenylalanine–Taurine	$r = -0.602$	$p = 0.014$
α-aminobutyric acid–ALP	$r = -0.524$	$p = 0.045$
α-aminoadipic acid–Ornithine	$r = 0.724$	$p = 0.003$
α-aminoadipic acid–Glycine	$r = 0.628$	$p = 0.016$
Phosphoserin–Phylloquinone	$r = -0.606$	$p = 0.022$
GABA–α-tocopherol	$r = -0.658$	$p = 0.011$
GABA–P	$r = -0.688$	$p = 0.007$
GABA–Carnosin	$r = 0.788$	$p = 0.020$
GABA–Glycine	$r = 0.550$	$p = 0.027$
Glycine–Phylloquinone	$r = -0.712$	$p = 0.004$
Carnosin–α-tocopherol	$r = -0.708$	$p = 0.049$
Ornithine–Phylloquinone	$r = -0.594$	$p = 0.025$

In a study by Artuch et al., on the erythrocyte samples taken from children with phenylketonuria, erythrocyte GSH-Px, and SOD activities were not significantly different from those of the AAM and healthy groups. However, regarding CAT, it is reported that a significantly lower activity was present in children with PKU that in controls ($p = 0.001$).[28] In another study, Sitta et al. reported that erythrocyte GSH-Px activity was significantly decreased in the PKU group compared to the control groups ($p < 0.05$).[29] Sirtori et al. also showed a significant decrease in erythrocyte GSH-Px activity in the PKU group vs. the control group ($p < 0.05$);[30] however, Colomé et al. measured GSH-GPx activity in the plasma of children with PKU and reported that plasma GSH-Px activity did not differ significantly ($p > 0.05$), although it was higher than that in healthy subjects. The activities of CAT and SOD in PKU children showed no significant difference from those of the controls.[31] Conversely, Fisberg et al. found that a reduction in SOD activity was not significantly different between the assessed groups.[32]

In the study, statistical analysis showed that the HPA group had significantly lower levels of GSH-Px and CAT ($p < 0.01$ and $p < 0.05$, respectively) than the control group. Additionally, the AAM group had significantly lower CAT levels than the control group ($p < 0.05$). Moreover, no significant differences in SOD levels were observed in the HPA and AAM groups compared to the control group ($p > 0.05$) (Fig. 4). In this study, the findings of decreased GSH-Px and CAT levels in children with PKU were consistent with the findings of other studies.[28–31] The findings of elevated SOD values in children with PKU were not supported by other researchers.[32]

In the present study, significant decreases in CAT and GSH-Px activities were observed in children with PKU. Decreased enzyme activities (CAT and GSH-Px) in children with PKU may be a result of increased ROS generation, such as $OH^{\bullet}$, O_2^{-} radicals, and H_2O_2, which in turn leads to enzyme inhibition.

Sitta et al. reported that erythrocyte GSH was significantly reduced in children with PKU compared to the control group

$p < 0.01$.[29] Colomé et al. proved a significant increase in plasma MDA levels ($p < 0.05$) compared to the control group. However, no significant differences were observed in the α-tocopherol and retinol concentrations between the PKU and control groups ($p > 0.05$).[31]

The statistical analysis clearly indicates that the HPA group had significantly lower GSH levels than the control group ($p < 0.05$) (Fig. 1), whereas the HPA group had increased levels of MDA compared to those of the control group ($p < 0.01$). Similarly, the AAM group had significantly higher levels of MDA than the control group ($p < 0.05$) (Fig. 2). Furthermore, no significant differences in the α-tocopherol or retinol levels were observed in the HPA and AAM groups compared to the control group ($p > 0.05$) (Fig. 3). In this study, the significant decrease in GSH and elevated MDA, α-tocopherol and retinol values were consistent with those of other authors.[29, 31]

As a result of the analysis, it was found that the AAM group had significantly higher levels of GABA than the HPA group ($p < 0.05$). Moreover, no significant difference in GABA level was observed in the HPA group compared to the low GABA level in the control group ($p > 0.05$). Neurological symptoms may arise from deficiencies in specific compounds derived from amino acids. Patients with defects in the synthesis of GABA from glutamate may face developmental delays and seizures.[33] It was also demonstrated that taurine amino acid levels in PKU children were significantly ($p < 0.05$) lower than those in healthy children. Taurine deficiency is associated with epilepsy, anxiety, depression, and hyperactivity, while taurine supplementation can alleviate these symptoms.[34]

It was also demonstrated that α-aminobutyric acid levels in children with PKU were significantly ($p < 0.01$) lower than those in healthy children. However, the AAM group had significantly higher levels of α-aminobutyric acid than the HPA group ($p < 0.05$). As a result of the analysis, very strong positive linear correlations were observed between phenylalanine and α-aminoadipic acid ($r = 0.777$; $p = 0.002$) in the children with HPA. Kynurenic acid (KYNA) function in the brain can be modulated by α-aminoadipic acid; therefore, KYNA has a role in the pathogenesis of neurodegenerative and convulsive diseases.[35]

Statistical analysis showed that the AAM group had significantly lower phylloquinone levels than the control group ($p < 0.05$). Moreover, no significant differences in low α-tocopherol, retinol and high cholecalciferol levels were observed in the HPA and AAM groups compared to the control group ($p > 0.05$) (Fig. 3). Low values of phylloquinone, α-tocopherol, and retinol could be associated with insufficient phylloquinone, tocopherol, retinol supplementation, and increased plasma lipid peroxidation in PKU. We have recently demonstrated that despite sufficient calcium,[36] because of cholecalciferol (vitamin D) intake, children with PKU tended to have high cholecalciferol levels based on the results.

Our results show that the AAM changed the levels of some plasma amino acids. Moreover, it is suggested that the decreased levels of phenylalanine and ALP and the increased level of α-aminobutyric acid in the AAM group might be due to the phenylalanine-free amino acid mixture. Our findings indicate that treatment with the AAM could markedly decrease Phe, which is an essential amino acid. In this study, α-tocopherol, retinol, P, Na, phosphoserine, and citrulline levels were similar in both groups of untreated and treated children. This means that the AAM treatment does not change the serum levels of these parameters. Upon evaluation of the biochemical results, supplementation with the AAM mixture (phenylalanine-free amino acid mixture) was found to be effective in the AAM group compared to the HPA group. We suggested that amino acid mixture supplementation should be given with food that is fortified with vitamins, minerals, and antioxidants.

There was a positive correlation between phenylalanine and α-aminoadipic acid ($r = 0.777$; $p = 0.002$). Additionally, plasma tyrosine levels in the HPA group were positively correlated with plasma ornithine levels ($r = 0.554$; $p = 0.050$) in the AAM group. Correlations were observed between phenylalanine and taurine ($r = -0.602$; $p = 0.014$) and α-aminobutyric acid and ALP ($r = -0.524$; $p = 0.045$). Additionally, a significantly high positive correlation ($r = 0.790$; $p = 0.001$) was observed between the concentrations of TAS and P. Plasma phenylalanine levels were positively correlated with plasma α-aminoadipic acid levels, showing low levels in the plasma of children with PKU. We can conclude that low serum TAS, phylloquinone, α-aminobutyric acid, β-alanine, phosphoserine, GABA, glycine, carnosine, ornithine, and taurine levels and high levels of ALP were directly associated with PKU.

CONCLUSIONS

Serum levels of CAT, GSH-Px, GSH, TAS, and phylloquinone were significantly lower in children with PKU (HPA and AAM groups) than in the control subjects. Within this context, deficiencies in these parameters were correlated with a number of plasma amino acids such as GABA, cystathionine, β-alanine, phosphoserine, glycine, and phenylalanine, which are used in plasma testing to monitor PKU, and this was positively correlated with α-aminoadipic acid ($r = 0.777$; $p = 0.002$). At the same time, GSH was positively correlated with the plasma concentration levels of cystathionine ($r = 0.809$; $p = 0.001$) in the HPA group. There were negative correlations between TAS and GABA ($r = -0.513$; $p = 0.042$) and between glycine and phylloquinone ($r = -0.712$; $p = 0.004$) in the AAM group.

As a result of the regression analysis performed between the plasma levels of α-aminoadipic acid and phenylalanine, it was determined that the level of α-aminoadipic acid might be an appropriate choice for the determination of the deficiency in conjunction with the levels of Phe. Our findings indicate that treatment with the phenylalanine-free amino acid mixture had significant effects on α-aminobutyric acid, GABA, ALP, α-aminobutyric acid, and phylloquinone as a supplement and could reduce HPA damage. It was found that the results can be helpful in monitoring children with phenylketonuria in terms of an insufficiency or excess of amino acids. It is important that phylloquinone and α-aminoadipic acid be considered together with the values of ALP when evaluating Phe.

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ADDITIONAL INFORMATION

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