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Improved metabolic control in tetrahydrobiopterin (BH4), responsive phenylketonuria with sapropterin administered in two divided doses vs. a single daily dose

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Abstract

Background: Phenylketonuria (PKU) often requires a lifelong phenylalanine (Phe)-restricted diet. Introduction of 6R-tetrahydrobiopterin (BH4) has made a huge difference in the diets of patients with PKU. BH4 is the co-factor of the enzyme phenylalanine hydroxylase (PAH) and improves PAH activity and, thus, Phe tolerance in the diet. A limited number of published studies suggest a pharmacodynamic profile of BH4 more suitable to be administered in divided daily doses.
Methods: After a 72-h BH4 loading test, sapropterin was initiated in 50 responsive patients. This case-control study was conducted by administering the same daily dose of sapropterin in group 1 (n = 24) as a customary single dose or in two divided doses in group 2 (n = 26) over 1 year.
Results: Mean daily consumption of Phe increased significantly after the first year of BH4 treatment in group 2 compared to group 1 ($p < 0.05$). At the end of the first year of treatment with BH4, another dramatic difference observed between the two groups was the ability to transition to a Phe-free diet. Eight patients from group 2 and two from group 1 could quit dietary restriction.
Conclusions: When given in two divided daily doses, BH4 was more efficacious than a single daily dose in increasing daily Phe consumption, Phe tolerance and the ability to transition to a Phe-unrestricted diet at the end of the first year of treatment.

Keywords: BH4; divided daily doses; phenylketonuria.

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Introduction

Phenylketonuria (PKU) is one of the most common inborn errors of metabolism. It is a recessively inherited disease caused by mutations in the gene encoding the enzyme phenylalanine hydroxylase (PAH; EC 1.14.16.1) [1]. Deficiency in PAH results in abnormally elevated concentrations of phenylalanine (Phe), which then can affect brain function, causing intellectual disability, microcephaly, delayed speech, seizures and behavioral abnormalities [1]. For patients responsive to 6R-tetrahydrobiopterin (BH4), a treatment for PKU that can prevent neurologic impairment and mental retardation, dietary restriction of Phe intake and/or administration of BH4 is imposed. The consensus is that an optimal outcome can be achieved if treatment starts as early as possible and that strict control of blood Phe is of primary importance, particularly during the early years of life [2, 3]. PAH activity is stimulated by BH4 in approximately 20% of the patients with PKU and in those patients, it serves as a useful adjunct to the Phe-restricted diet because it increases Phe tolerance and allows significant dietary relaxation [4]. Sapropterin dihydrochloride (Kuvan®, BioMarin, San Rafael, CA, USA), a pharmaceutical formulation of BH4, is an approved drug for the treatment of PKU. Since the approval of BH4 supplementation by the United States Food and Drug Administration (FDA) in 2007 and the Europe, Middle East and Africa countries (EMEA) in 2008, an increasing number of PKU patients have switched from the classic Phe-restricted diet to supplementation with BH4, allowing only moderate or no Phe restriction [5–7]. In BH4-responsive patients, this treatment allows natural protein consumption to be increased, improving therapy compliance and metabolic control [8].

To the best of our knowledge, to date, sapropterin dihydrochloride has been used only as a single daily dose in the treatment of PKU. Therefore, we investigated the efficacy of divided daily doses of BH4 in BH4-responsive patients with PKU compared to its common single daily dose. We observed a significant beneficial effect of divided

daily doses when compared to a single daily dose during the first year of BH4 treatment.

Patients and methods

This study was performed in 50 BH4-responsive PKU patients. All patients were diagnosed through the newborn screening program. A blood Phe level ≥ 360 μmol/L was considered the threshold for administering the Phe-restricted diet.

A 72-h BH4 loading test was performed using sapropterin dihydrochloride, which consisted of a single oral dose of 20 mg/kg. Responsiveness was defined as a reduction of more than 30% in blood Phe levels [9]. Those treated for a primary BH4 deficiency were excluded. Before intervention, a 7-day home dietary nutrition record was evaluated for calculating daily consumption of Phe and protein. To determine baseline Phe tolerance, serum Phe levels were measured three times a day for 5 days.

This was a single center case-control study. Patients were divided into two groups. Those in group 1 (n = 24) received the same daily dose (20 mg/kg/day) as a single dose at breakfast, while those in group 2 (n = 26) took the same daily dose in two equally divided doses: one at breakfast and the other at dinner. This study was conducted at Çukurova University Department of Pediatric Metabolism and Nutrition from January 2013 to December 2014. The Ethics Committee approval was obtained. Patients were followed for their ability to increase dietary Phe consumption (to reach the maximal Phe tolerance). For each participant, we determined patient characteristics, PKU genotyping when available, results of the 72-h BH4 loading test and plasma Phe levels at neonatal screening and during follow-up.

PAH gene sequence analysis was performed by using the MiSeq next generation sequencing (NGS) platform, an FDA-approved diagnostic system (Illumina, Inc., San Diego, CA, USA). All coding exons and their flanking splice site junctions were amplified using polymerase chain reaction (PCR) primers, designed with PRIMER© Version 2.0 (Sci-Ed software, Denver, USA). PCRs were validated by using agarose gel electrophoresis. After PCR amplification, the libraries were prepared with the NexteraXT kit (Illumina, Inc., San Diego, CA, USA), according to the manufacturer's instructions. Next-gene sequencing was carried out on MiSeq (Illumina, Inc., San Diego, CA, USA). Sequences were aligned to the *hg19* genome within MiSeq Reporter software (Illumina, Inc., San Diego, CA, USA). Visualization of the data was performed with IGV 2.3 (Broad Institute, Cambridge, MA, USA) software.

Statistical analyses

Data were analyzed using Statistical Package for Social Sciences (SPSS, Inc., Chicago, IL, USA) version 15.0. Frequency distribution was used to describe the sample and mean ± standard deviation was used to describe continuous data. Means of groups were compared using the Mann-Whitney U test. Significance was recognized when $p < 0.05$.

Results

We studied 518 PKU patients who were followed with the Phe-restricted diet in our clinic. The threshold for starting a Phe-restricted diet has been 360 μmol/L in our practice. Patients who had 360–600 μmol/L basal plasma Phe levels were considered to have mild hyperphenylalinemia (HPA; four in group 1, three in group 2) and those with 600–1200 μmol/L basal Phe levels were considered as having mild PKU (19 patients in group 1 and 22 in group 2). There was one classical PKU patient in each group.

After a 72-h BH4 loading test, BH4 treatments were initiated in 50 responsive patients; 24 patients using a single dose (group 1) and 26 using BH4 in divided daily doses (group 2). The age at diagnosis was 29.7 ± 24 days in group 1 and 44.2 ± 42.2 days in group 2 ($p = 0.294$). BH4 responses were 38.2 ± 6.6% and 41.8 ± 10.7% reductions in groups 1 and 2, respectively ($p = 0.335$). There were no differences between the mean Phe levels at diagnosis ($p = 0.786$), mean daily Phe consumption at the beginning of BH4 treatment ($p = 0.379$) and age of BH4 treatment initiation between the two groups ($p = 0.109$).

Mean daily consumption of Phe increased significantly after the first year of BH4 treatment in group 2 compared to group 1 ($p < 0.05$). At the end of the first year of treatment with BH4, another dramatic difference observed between the two groups was the ability to transition to a Phe-free diet. Eight patients from group 2 and two from group 1 were able to quit dietary restriction. Patient characteristics are summarized in Table 1.

Table 1: Characteristics of the PKU patients treated with BH4.

	Group 1 (n = 24)	Group 2 (n = 26)	p-Value
Age of diagnosis, day	29.7 ± 24 (20.5 [5–120])	44.2 ± 42.2 (30 [6–180])	0.294
Phenylalanine levels at diagnosis, μmol/L	888 ± 234 (900 [456–1440])	870 ± 216 (891 [504–1380])	0.786
BH4 responsiveness (mean ± SD), %	38.2 ± 6.6 (39 [30–49])	41.8 ± 10.7 (40 [30–68])	0.335
Age of BH4 treatment initiation, months	48.3 ± 29.7 (40.5 [11–140])	55.9 ± 18.9 (53 [9–100])	0.109
Phe consumption before BH4 therapy, mg/day	766 ± 170 (750 [500–1200])	732 ± 218 (725 [400–1300])	0.379
Phe consumption at the end of first year with BH4, mg/day	1025 ± 419.9 (900 [500–2000])	1400 ± 535.8 (1200 [650–2500])	**0.007**
Phe-free diet	2/24 (8.3%)	8/26 (30.8%)	
Secondary unresponsiveness	17/24 (70.8%)	7/26 (26.9%)	

Results are expressed mean ± SD (median [min–max]). $p < 0.05$ was considered significant.

A spectrum of 22 genotypes was determined in 48 patients. The most common *PAH* allele variants found were: IVS10 – 11G > A (c.1067–11G > A) (39.6%), p.R261Q (c.729G > A) (20.8%), IVS4 + 5G > T (c.441 + 5G > T) (6.3%), IVS2 + 5G > C (c.168 + 5G > C) (3.1%), p.P281L (c.842C > T) (3.1%), IVS11 + 1G > C (c.1999 + 1G > C) (2.1%), p.F55Lfs*6 (c.165delT) (2.1%), p.Y343C (c.1028A > G) (2.1%), and p.R176* (c.526C > T) (2.1%). Mutation results are shown in Tables 2 and 3.

The IVS10-11G > A mutation was homozygous in nine patients from group 2 and Phe tolerance was increased more than 50% with BH4 treatment in six of them. The IVS10-11G > A mutation also was homozygous in five patients from group 1 and Phe tolerance was increased more than 80% with BH4 treatment in two of them. Out of the five patients heterozygous for the IVS10-11G > A mutation in group 2, three did not respond to BH4 treatment and only one could quit the Phe-restricted diet. Out of the six patients heterozygous for the IVS10-11G > A mutation in group 1, four did not respond to BH4 treatment and only one could quit the Phe-restricted diet. When patients with the p.R261Q mutation in any allele from group 2 were evaluated for BH4 response, we observed transition to a Phe-unrestricted diet (six patients) and secondary non-responsiveness (four patients). Surprisingly, with the same mutation in group 1, secondary non-responsiveness was observed in three of the seven patients, but any patient could quit to a Phe-free diet. Among the two siblings who had a homozygous p.P281L mutation, one in group 1 had a 65% increase in Phe tolerance, while the other in group 2 did not respond. Genotypes and phenotypic features of the patients are shown in Tables 2 and 3 for groups 1 and 2, respectively.

Discussion

Response to pharmacologic doses of BH4 with reduced Phe levels in the blood of PKU patients was first documented in 1999 by Kure et al. [10]. At pharmacologic doses, BH4 was shown to act as a molecular chaperone by increasing the stability of partially misfolded PAH proteins as well as the effective intracellular concentration of the functional PAH enzyme [11–14]. Treatment with BH4 resulted in significant (at least 30%) and sustained

Table 2: Genotype and the results of BH4 treatment of the patients in group 1.

Patient	Allele 1	Allele 2	Phe level at diagnosis, µmol/L	Phe consumption before BH4 treatment, mg/day[a]	Phe consumption at the end of first year with BH4 treatment, mg/day[a]	Phe free diet	Secondary unresponsiveness to BH4
P1	IVS10-11G > A	IVS10-11G > A	882	900	800		(+)
P2	IVS10-11G > A	IVS10-11G > A	798	750	800		(+)
P3	IVS10-11G > A	IVS10-11G > A	1440	700	700		(+)
P4	IVS10-11G > A	IVS10-11G > A	1140	500	900		
P5	IVS10-11G > A	IVS10-11G > A	1080	500	1100		
P6	IVS10-11G > A	p.39delF	822	750	750		(+)
P7	IVS10-11G > A	p.F39L	954	750	1000		(+)
P8	IVS10-11G > A	p.R261Q	888	500	650		(+)
P9	IVS10-11G > A	p.R261Q	1074	625	1200		
P10	IVS10-11G > A	p.Y417C	648	850	1100		(+)
P11	IVS10-11G > A	p.D338Y	780	750	2000	(+)	
P12	IVS4 + 5G > T	IVS4 + 5G > T	1110	800	850		(+)
P13	IVS4 + 5G > T	IVS4 + 5G > T	936	750	500		(+)
P14	p.R261Q	p.P281L	1200	500	1300		
P15	p.R261Q	p.R243*	900	720	900		(+)
P16	p.R261Q	p.R261Q	594	850	1100		(+)
P17	p.R261Q	p.R261Q	900	900	1200		(+)
P18	p.R261Q	p.R261Q	630	1100	1700		
P19	p.F55Lfs*6	p.F55Lfs*6	594	1050	1100		(+)
P20	p.P281L	p.P281L	516	700	500		(+)
P21	p.R155H	p.P314H	456	900	2000	(+)	
P22	IVS2 + 5G > C	p.V388M	930	750	800		(+)
P23			960	800	700		(+)
P24			1056	900	1100		(+)

[a]While blood Phe level between 120 and 360 µmol/L.

Table 3: Genotypes and the results of BH4 treatment of the patients in group 2.

Patient	Allele 1	Allele 2	Phe level at diagnosis, µmol/L	Phe consumption before BH4 treatment, mg/day[a]	Phe consumption at the end of first year with BH4 treatment, mg/day[a]	Phe free diet	Secondary unresponsiveness
P1	IVS10-11G>A	IVS10-11G>A	708	800	850		(+)
P2	IVS10-11G>A	IVS10-11G>A	924	800	1200		
P3	IVS10-11G>A	IVS10-11G>A	876	700	800		(+)
P4	IVS10-11G>A	IVS10-11G>A	1002	500	1200		
P5	IVS10-11G>A	IVS10-11G>A	900	680	1100		
P6	IVS10-11G>A	IVS10-11G>A	1176	450	1000		
P7	IVS10-11G>A	IVS10-11G>A	1098	800	1000		(+)
P8	IVS10-11G>A	IVS10-11G>A	990	450	1000		
P9	IVS10-11G>A	IVS10-11G>A	774	400	1250		
P10	IVS11+1G>C	IVS11+1G>C	852	500	950		
P11	IVS10-11G>A	p.E390G	504	900	2250	(+)	
P12	IVS10-11G>A	p.F331C	930	1100	1500		(+)
P13	IVS10-11G>A	p.F331C	606	750	900		(+)
P14	IVS10-11G>A	p.R111*	966	550	650		(+)
P15	IVS10-11G>A	p.R176*	1380	900	1400		
P16	IVS2+5G>C	IVS2+5G>C	1128	750	1300		
P17	IVS4+5G>T	IVS4+5G>T	978	700	900		(+)
P18	p.R261Q	IVS7+5G>A	696	1000	1850	(+)	
P19	p.R261Q	p.R261Q	762	1000	2250	(+)	
P20	p.R261Q	p.R261Q	1140	600	1800	(+)	
P21	p.R261Q	p.R261Q	564	750	2500	(+)	
P22	p.R261Q	p.R261Q	582	500	2000	(+)	
P23	p.R261Q	p.L48S	882	1300	1900	(+)	
P24	p.P281L	p.P281L	648	850	1400		
P25	p.S16*	p.S16*	906	700	1200		
P26	p.Y343C	p.Y343C	630	600	2200	(+)	

[a]While blood Phe level between 120 and 360 µmol/L.

reductions in Phe concentrations in blood and dietary Phe tolerance was increased in responsive PKU patients.

Although sapropterin dihydrochloride has been used only as a single daily dose in the treatment of BH4-responsive patients with PKU, pharmacokinetic studies with BH4 indicate a peak blood concentration of BH4 in 2–4 h following oral intake and a half-life of 6 h [15]. In consideration of this short efficiency period and a relatively short half-life, to prevent the fluctuations in blood Phe levels throughout the day, a daily dose divided in two or three doses was suggested to be more beneficial [16]. The in vivo effect of BH4 is significantly shorter in the mouse Pah$^{enu1/2}$ [17]. Based on these pharmacodynamic facts, we performed a study comparing the same daily dose in one or two administrations in our BH4-responsive PKU patients. The outcome measures were daily Phe consumption, Phe tolerance and ability to transition to a Phe-unrestricted diet in two groups. At the end of the first year, we observed that mean daily Phe consumption increased from 766 to 1025 mg/day in group 1 and from 732 to 1400 mg/day in group 2 ($p<0.05$). Consistently, Phe tolerance was significantly higher in group 2 (91.26%) than in group 1 (33.81%) ($p<0.05$). While eight patients from group 2 could switch to a Phe-unrestricted diet, only two from group 1 were able to do so. In addition to these striking results, secondary non-responsiveness was higher in group 1 (70.8%) than in group 2 (26.9%). All these outcomes suggested beneficial effects of a divided daily dose of BH4 for the metabolic control of PKU.

To assess the well-known effect of genotype on BH4 responsiveness, we performed a genotype-phenotype correlation according to the predicted residual activity in vitro listed in the PAHvdb database (http://www.biopku.org/pah) and/or PAH database and phenotype association in these two groups of patients. According to the PAHvdb database, 5.68% of the patients who are homozygous for the IVS10-11G>A mutation respond to BH4, while 100% who are compound heterozygous for the IVS10-11G>A and p.E390G or IVS10-11G>A and p.D338Y mutations respond to BH4 and can switch to a Phe-free diet (http://www.biopku.org/pah). In six patients from group 2, with a homozygous IVS10-11G>A mutation, Phe tolerance was increased more than 50% and in two patients from

group 1 Phe tolerance was increased more than 80% with BH4 treatment. These differences between groups may be related to the low fluctuation of sapropterin blood level or other unknown factors. Out of the five patients heterozygous for the IVS10-11G > A mutation in group 2, three did not respond to BH4 treatment and only one could quit to a Phe-free diet. Out of the six patients heterozygous for the IVS10-11G > A mutation in group 1, four did not respond to BH4 treatment and only one could quit to a Phe-free diet. Among patients who are homozygous for the p.R261Q mutation, 78% were BH4-responsive. The rate of BH4 responsiveness was 75.86% for compound heterozygous patients with p.R261Q and p.L48S mutations (http://www.biopku.org/pah). When patients with a p.R261Q mutation in any allele from group 2 were evaluated for BH4 response, we observed the transmission to a Phe-free diet (six patients) and secondary non-responsiveness (four patients). Surprisingly, with the same mutation in group 1, secondary non-responsiveness was observed in three of the seven patients, but any patient could quit to a Phe-unrestricted diet. For the two siblings homozygous for the p.P281L mutation, the one who took two divided daily doses of BH4 had a 65% increase in Phe tolerance, whereas the other sibling who took a daily dose of BH4 did not respond to BH4 at the end of the first year. Despite their same genetic base and physical and social environments, non-responsiveness was observed in the sibling in group 1, while a significant increase was detected in the daily Phe consumption of the sibling in group 2. While we believe that this was the effect of the divided dose, we realize that the possible action of modifier genes cannot be excluded. Our results suggest a beneficial effect in the two divided daily doses group irrespective of the genotype.

Although the mechanisms of non-responsiveness resulting from the various mutations could not be determined, we have detected that a greater number of daily doses increases daily Phe tolerance and partially affects non-responsiveness. Where mutation responsiveness is known, the divided dose significantly increases Phe tolerance as well as the ratio that can switch to a free diet.

In conclusion, to our knowledge, this is the first reported study evaluating the efficiency of two divided daily doses of BH4 compared to a single daily dose. When given in two divided daily doses, BH4 was more efficacious than a single daily dose in increasing daily Phe consumption, Phe tolerance, and ability to transition to a Phe-unrestricted diet at the end of the first year of treatment. With two divided daily doses of BH4 treatment, the consumption of high protein content foods increased, so diet compliance and quality of life of the patient and the family increased. This study demonstrated the safety, efficacy, and good metabolic control of PAH experienced by PKU patients treated with 20 mg/kg divided into two doses, but further studies are needed to predict long-term effects.

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