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RESEARCH ARTICLE

Production of Low Protein Products Used in Treatment of Congenital Metabolic Diseases: A Pilot Study in the Stage of Experimental Animals

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Summary

Congenital metabolic diseases are not rare in Turkey. For instance, phenylketonuria frequency is 1/4500. Classic phenylketonuria occurs as a result of deficiency of phenylalanine hydroxylase which is a liver enzyme. If it is not treated, the most serious clinical finding of it is mental retardation. Signs of disease can be prevented with early and effective treatment. Basic treatment is nutrition therapy in which low protein and low phenylalanine medical foods are used. Methods such as protein precipitation from whey, amino acid disposal and adding free amino acids, vitamins and minerals to isolated soy protein or whey protein can be used in order to produce low protein product. We preferred the last method in the present study. We indicated via food analysis that product contains adequate nutrients. We demonstrated in experimental animals that consumption is at safe limits in terms of phenylalanine. It is necessary to continue the study in order develop and make the product suitable for use among human beings.

Keywords: *Congenital metabolic diseases, Nutrition therapy, Low protein products, Phenylketonuria*

Doğumsal Metabolik Hastalıkların Tedavisinde Kullanılan Düşük Proteinli Ürünlerin Üretimi: Deney Hayvanları Aşamasında Bir Pilot Çalışma

Özet

Ülkemizde doğumsal metabolik hastalık sıklığı nadir değildir. Örneğin fenilketonüri sıklığı 1/4500'dir. Klasik fenilketonüri bir karaciğer enzimi olan fenilalanin hidroksilaz enzimi eksikliği sonucu oluşur. Tedavi edilmezse en ciddi klinik bulgusu zekâ geriliğidir. Erken ve etkin tedavi ile hastalık bulguları önlenebilir. Temel tedavi düşük proteinli ve düşük fenilalaninli tıbbi besinler kullanılan beslenme tedavisidir. Düşük proteinli ürünü oluşturmak için whey'den protein çöktürme veya aminoasit uzaklaştırma yöntemleri, izole soya proteinine veya whey proteinine serbest aminoasitler, vitaminler ve mineraller ekleme yöntemi kullanılabilir. Çalışmamızda son yöntemi tercih ettik. Ürünün yeterli besin öğeleri içerdiğini besin analizi ile gösterdik. Fenilalanin açısından tüketiminin güvenli sınırlarda olduğunu deney hayvanlarında gösterdik. Ürünü geliştirip insanlarda kullanıma uygun hale getirmek için çalışmaya devam edilmesi gerekmektedir.

Anahtar sözcükler: *Doğumsal metabolik hastalıklar, Beslenme tedavisi, Düşük proteinli ürünler, Fenilketonüri*

INTRODUCTION

Congenital metabolic diseases (CMD) are notable among the diseases requiring food restriction. Genetic disorders underlie these diseases. Product of the enzyme, which cannot occur or function because of defective genetic information, either cannot be synthesized or it is produced inadequately. As a consequence, the product before the enzyme accumulates and/or it is turned into different products via alternative ways [1-4].

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Treatment of congenital metabolic diseases is mostly through diet. It is necessary to know the metabolic disorder well while planning the diet. Improper diet can lead to nutritional, metabolic and neurological problems [5]. Diet plan should be made at the level of amino acid in congenital metabolic diseases requiring protein restriction. Patients should controlledly be provided with essential amino acids. This cannot be completely achieved with natural foods. Special products are needed for a successful nutrition practice.

Phenylketonuria (PKU) is a CMD caused by deficiency of phenylalanine hydroxylase enzyme (PAH) [2-9]. Mental retardation, fetor, light-colored hair, eyes and skin, postural disorders, eczema, scleroderma, epilepsy, cataracts, and cerebral calcification develop in patients who are not treated [2].

Treatment of phenylketonuria requires a multidisciplinary approach. Diet is the most important part of the treatment. Enzyme replacement [6], liver transplantation [8] and gene therapy [9] are currently in the research phase.

Different approaches were developed in PKU treatment and follow-up [5,9-12]. Common idea is that treatment should be launched in the first three weeks following the birth. It is necessary to add fruits and vegetables to the diet following the period of transition to additional nutrients. To this end, natural foods were grouped according to the content of phenylalanine. Foods containing equal phenylalanine can be used interchangeably. Besides natural foods, drinks and flour-based foods with low phenylalanine are used in the diet for intake of allowed quantity of daily phenylalanine and lowest protein ensuring normal development. Content of low protein foods should both provide the building stones necessary for normal growth and development and keep the blood phenylalanine level at optimum level. Such a diet cannot be realized in practice just with natural foods. Different methods are used while producing low protein foods in order to decrease phenylalanine content [13,14].

Commercial formulas can be collected in two groups according to basic protein content: milk based and soy based [15-17]. Milk proteins are the proteins which remain at precipitating part of the milk after being processed with acids and appear in the liquid part. Casein is the phosphoprotein precipitating in acid environment. Whey is the name given to the proteins in the liquid following the precipitation of casein. Whey and casein are rich in different amino acids. Cow's milk is used in milk protein based formulas. Main whey protein in cow's milk is beta-laktoglobulin, and main whey protein in human milk is alpha-lactalbumin [18-21]. Whey protein lactalbumins contain more sulfur amino acid compared to the casein. Formulas are mixtures of whey and casein at particular proportions. Whey/casein rate may be 18:82 or 60:40 [15-21]. Even though whey/casein rate of the formulas differ according to fat content, nucleotide, and the status of having fiber and probiotic additionally, general rules of the components were arranged firstly by suggestion of American Academy of Pediatrics Committee on Nutrition for nutrient content of infant formulas [15], and then European Commission decisions [17]. Formulas prepared for congenital metabolic diseases follow these rules in general; but they include some changes according to the restrictions to be made in the disease for which they are recommended [14].

Special processes are needed in order to decrease the phenylalanine and total protein quantity of cow's milk. These processes are generally chemical and mechanical. Chemical processes refer to disposal of proteins through precipitation with chemical substances while mechanical processes are methods for disposal of proteins by breaking up a part of the proteins with high speed ultrafiltration [18-21]. Technology is needed in order to dispose auxiliary products used in chemical processes and to deal with the problems in mechanical processes.

Soy protein can be obtained in great quantities and cheaply. Cow's milk started to be used as protein source considering that it is advantageous as it does not contain allergens in the protein [17]. Fat content is just like milk based formula. It is lactose-free. It contains sucrose and/or corn starch hydrolysate, tapioca hydrolysate. American Academy of Pediatrics Committee on Nutrition suggests soy protein formula to the infants born mature having galactosemia, hereditary lactose intolerance, temporary lactase deficiency after diarrhea, cow's milk allergy [15-17]. However, allergic responses were identified also for soy proteins. It is necessary to add methionine, sulfur amino acids (particularly cystine) to the soy protein. Phytates prevent vitamin and mineral absorption. Taurine, carnitine and iron should be added. The phosphor is much. Soy formula is not recommended to the preterm infant with birth weight less than 1.800 g who is quiet weak considering the gestational age and those having enterocolitis and enteropathy triggered by cow's milk proteins in order to prevent colic and allergies [15].

Nutrients other than protein sources are also added to the formulas. They comprise of nucleotides, sugar (ribose, deoxyribose), nitrogenous base (purine, adenine, guanine, and pyrimidine; cytosine, thymine) and phosphoric acid. Nucleotides are added to the formula to be source of energy and to be used in cell repair. Taurine (amino ethane sulfonic acid) is sulfonic acid containing amino group. It is used for forming compounds with bile salts. Carnitine comprises of lysine in the organism. It is used for carrying fatty acids into mitochondria [15-17]. Through these things added, content of cow's milk and soy protein based formulas is tried to be likened to the human milk.

MATERIAL and METHODS

Composition of the product prepared by us and planned

to be used in patients with PKU is showed in *Table 1*. It was aimed at reducing the amount of phenylalanine, adding tyrosine and reaching the amounts suggested by European Society of Pediatric Gastroenterology, Hepatology, and Nutrition (ESPGAN) for baby foods in other elements [15].

Table 1. *Fat, carbonhydrate, protein, amino acid, vitamin content of the formed product*

Tablo 1. *Oluşturulan ürünün yağ, karbonhidrat, protein, aminoasidler, vitamin, mineral içeriği*

Nutrient		Content of 100 g Powder Food
Fat (g)		31.97
Carbonhydrate (g)		52.72
Energy (kcal)		517
Protein (g)		4.6
Essential Amino Acid	Arginine (g)	0.52
	Cystine (g)	0.18
	Histidine (g)	0.35
	Isoleucine (g)	0.20
	Leucine (g)	0.36
	Lysine (g)	0.95
	Methionine (g)	0.08
	Phenylalanine (g)	0.13
	Threonine (g)	0.28
	Tryptophan (g)	0.23
	Tyrosine (g)	0.07
	Valine (g)	0.20
Minerals	Sodium (mg)	966.3
	Potassium (mg)	921.11
	Calcium (mg)	144.6
	Magnesium (mg)	19.43
	Phosphor (mg)	244.97
	Chlorine (mg)	468
	Iron (mg)	3.65
Vitamins	A µg	550.22
	D µg	7.8
	E µg	6.05
	K µg	31.2
	B_1 mg	0.69
	B_2 µg	3.27
	B_6 µg	273
	B_{12} µg	0.78
	Niacin mg	1.64
	Folic acid µg	32.0
	Biotin µg	11.7
	C µg	12.51
	Carnitine mg	0.058
Trace Elements	Zinc mg	1.75
	Iodine µg	39
	Selenium µg	23.4

Amino acid profile contained by isolated soy protein was found out via high-pressure liquid chromatography (HPLC) method by Dupont Protein Technologies International Inc. Company. Isolated soy protein was provided by Dupont Protein Technologies International Inc.; amino acids Merck KGaA (Germany); vitamin and minerals Roch Türkiye AŞ (Istanbul), Sigma AŞ (Istanbul), Solgar AŞ (Istanbul), Tunçkaya Kimyevi Maddeler Tic. ve San. Ltd. Şti. (Istanbul), Yılmaz Kimya Tic. ve San. Ltd. Şti. (Istanbul).

Weighing operations were performed with 0.01 g precision digital scale and 0.0001 gram precision digital scale. Acidity measurements were made with Sentix 21 pH meter.

Nutrient analyses of the formed product were made in TUBITAK Marmara Research Center Institute of Food Science and Technology Research Laboratory.

Analysis methods and tools used are mentioned below [22-30]. Energy was calculated via Atwater method [22]; fat was analyzed via Soxtec manual method; protein was analyzed via "Termal Conductivity" method, Leco was analyzed via manual method; fiber was analyzed via AOAC method [23,30]; Vitamin A was analyzed via HPLC [24]; Vitamin C was analyzed via Titrimetric method [25]; Vitamin B_1 was analyzed via HPLC [26]; Vitamin B_2 was analyzed via HPLC [26]; Vitamin E was analyzed via HPLC [27]; niacin was analyzed via spectrophotometric method [28]; folic acid was analyzed via HPLC [29]; sodium, potassium, zinc, iron, calcium, magnesium were analyzed via Hitachi 180-50 atomic absorption spectrometry [25]; phosphore was analyzed via spectrophotometric method [25].

Five mongrel puppies born in the same abdomen, which were 60 days old and which weighed 5.000±240 g, were used in the animal experiment. Healthy puppies were not given any food for 8 h during the night; then in the morning, on an empty stomach, 2 ml venous blood was taken into sterile tube containing 3.2% sodium citrate. After special dry powder food whose protein was reduced 7.8 g was watered with 100 ml water and was given to the puppies with orogastric probe, 2 ml venous blood was taken again with same method 2 h later. The experiment was repeated with same animals with 100 ml pasteurized full fat cow's milk and low protein commercial drink at one day intervals. Puppies were kept under surveillance for 1 day in terms of health problems. They did not have any health problem. Animals were healthily sent to their natural environments.

Phenylalanine and tyrosine values were studied via HPLC method for plasma (Shimadzu Class-VP V6.12 SP1). Statistical studies were made via 11.0 SPSS statistical program. Intra-group comparisons of values *(Table 2)* obtained after nourishment with milk, product prepared for the study and commercial formula (LPD) whose phenylalanine was decreased were evaluated via Friedman Test.

RESULTS

Among nutrients used as energy source of the product, pH of olive oil making up the fat was found 2.4-2.9; pH of sunflower oil was found 2.9-3.5; pH of mixture of both of them was found 2.9-3.2; and pH of the whole mixture was found as 3.8-4.1. Acidity measurements were made at 25°C temperature. Fiber found in 100 grams of the product is 1.2 gram. Vitamin, mineral, fiber, amino acid content of the product was presented in *Table 1*.

Blood amino acid values of subjects were presented in *Table 2*. Intra-group comparisons were found insignificant for phenylalanine in the comparisons made between blood values taken after hunger-milk, hunger-study product and hunger-commercial formula via Friedman Test in SPSS 11.0 statistical program ($\chi^2 = 3.514$, P = 0.621). Intra-group comparisons were found insignificant for tyrosine ($\chi^2 = 6.600$, P = 0.252).

Table 2. *Serum phenylalanine and tyrosine levels after hunger of eighth and 2 h later following the nourishment with milk, study product, low protein commercial formula*

Tablo 2. *Sekiz saatlik açlıktan sonra ve süt, çalışma ürünü, ticari düşük proteinli formüla ile beslenmeden 2 saat sonra serum fenilalanin ve tirozin düzeyleri*

Aminoacids in Blood	Hungry	Milk	Hungry	Study Product	Hungry	Low Protein Commercial Formula
Phenylalanine (µmol/L)	44.3	57.6	48.0	52.0	43.2	42.3
	47.4	67.1	55.9	42.9	51.2	49.4
	43.5	45.1	53.4	39.4	48.5	57.0
	48.3	47.0	46.4	38.2	47.4	49.1
	50.9	48.0	63.5	51.3	54.9	49.4
Friedman Test Result	$\chi^2 = 3.514$, p=0.621					
Tyrosine (µmol/L)	23.8	37.2	23.3	24.7	23.5	28.0
	25.0	49.8	28.5	22.4	25.5	25.9
	31.9	44.2	29.4	18.5	30.6	24.0
	33.6	40.1	28.5	23.4	32.3	25.7
	29.6	29.4	37.3	31.3	30.3	24.9
Friedman Test Result	$\chi^2 = 6.600$, P = 0.252					

DISCUSSION

The most frequently used method in PKU treatment is the diet restricted of phenylalanine. Almost all of the products used in this diet are imported. It is important to produce alternative foods which can be used by patients with PKU through local facilities.

There are various ways for producing low protein milk products. Precipitation and ultrafiltration methods are used in order to reduce milk proteins. Phenylalanine amount in the milk is reduced by reducing whey protein amount, by precipitating casein from the milk by means of cation exchange resin, and by changing protein amino acid content via proteolytic enzyme. Advanced technology and expensive investments are needed for all these methods [18-21]. It is cheaper to provide the product with desired feature by supporting vegetable proteins (isolated soy protein) with essential amino acids and vitamins. On other hand, the fact that soybean production started to be based on genetically modified products and to be more foreign-depended gives rise to thought that it is more reasonable to use whey proteins which we can reach more easily in our country through our own resources. In the present study, soy protein isolate was used because of technical and financial reasons. However, whey proteins can also be used for the same purpose with the method used in the present study.

During the present study, we indicated that full fat milk, low protein commercial formula and study product did not create significant difference in plasma phenylalanine value. It was started to be thought that product will not create high level of phenylalanine and low level of tyrosine in the long-term use. This preliminary study gave rise to thought that constituted product can replace the low protein milk which is an important component of diet for patients with PKU.

REFERENCES

1. Burton BK: Inborn errors of metabolism in infancy: A guide to diagnosis. *Pediatrics,* 102 (6): e69, 1998.

2. Kniffin CL: Phenylketonuria. McKusick VA: www3.ncbi.nlm.nih.gov/omim/ PHENYLKETONURIA261600. *Accesed*: 20.07.2010.

3. Cockburn F, Barwel BE, Brenton DP, Chapple J, Clarck B, Curzon G, Davidson DC, Heeley AF, Laing SC, Lister-Cheese AF, Mc Donald I, Malcolm S, Pollitt RJ, Quinn D, Rylance G, Smith I, Stewart A, Surtees R, Thompson AJ, Tyfield L, Whittle MJ, Scriver JR, Frew J: Phenylketonuria, Report of the Medical Research Council Working Party on PKU. *Arch Dis Child,* 68, 426-427, 1993.

4. National Institutes of Health: Phenylketonuria: Screening and

management. *National Institutes of Health Consensus Development Conference Statement Online*, 17 (3): 1-27, 2000.

5. Acosta PB, Yannicelli S: The Ross Metabolic System Nutrition Support Protocols. Ross Laboratories Publication, Ohio, 1993.

6. Sarkissian CN, Shao Z, Blain F, Peevers R, Su H, Heft R, Chang TMS, Scriver CR: A different approach to treatment of phenylketonuria: Phenylalanine degradation with recombinant phenylalanine ammonia lyase. *Proc Nat Acad Sci,* 96, 2339-2344, 1999.

7. Vajro P, Strisciuglio P: Correction of phenylketonuria after liver transplantation in a child with cirrhosis. (Letter) *New Eng J Med*, 329, 363, 1993.

8. Eisensmith RC, Woo SLC: Gene therapy for phenylketonuria. *Eur J Ped,* 155 (1): S16-S19, 1996.

9. Report of Medical Research Council Working Party on PKU and Recommendations of the European Society for PKU on Dietary Management of PKU: British Guidelines for PKU Management: *National PKU News*, www.pkunews.org, 2000.

10. Singh RH, Kable JA, Guerrero NV, Sullivan KM, Elsas LJ: Impact of experience on phenylalanine levels, knowledge, attitudes, and healt biliefs relevant to nutrition management of phenylketonuria. *J Am Diet Assoc* 100, 797-803, 2000.

11. Van Spronsen FJ, Van Rijin M, Bekof J, Koch R, Smit PGA: Phenylketonuria: Tyrosine supplementation in phenylalanine-restricted diets. *Am J Clin Nutr*, 73, 153-157, 2001.

12. Greeves LG, Patterson CC, Carson DJ, Thom R, Wolfenden MC, Zschocke J, Graham CA, Nevin NC, Trimble ER: Effect of genotype on changes in intelligence quotient after dietary relaxation in phenylketonuria and hyperphenylalaninaemia. *Arch Dis Child,* 82, 216-221, 2000.

13. Lopez-Bajonero LJ, Lara-Calderon P, Galvez-Mariscal A, Velazques-Arellano A, Lopez-Munguia A: Enzimatic production of a low-phenylalanine product from skim milk powder and caseinate. *J Food Sci*, 56 (4): 938-942, 1991.

14. Acosta PJB, Grondalski RA, Liebrecht JW, Reynolds PA: Medical foods for the nutritional support of infant/toddler metabolic diseases. *United States Patent* 5, 550, 146, 1996.

15. Aggett PJ: Research priorities in complementary feeding: International Paediatric Association (IPA) and European Society of Peadiatric Gastroenterology, Hepatology, and Nutrition (ESPGAN) WORKSHOP: *Pediatrics,* 106 (5): 1271-1305, 2000.

16. Subcommittee on the Tenth Edition of the RDAs Food and Nutrition Board Commission on Life Sciences National Research Council: Recommended Dietary Allowances, 10th ed., National Academy Press. Wasthington DC, 1989.

17. European Commission-Scientific Committee on Food: Report of the Scientific Commission on Food on the Revision of Essential Requirements of Infant Formulae and Follow-on Formulae. Brussels: SCF/CS/NUT/IF/65Final, 2003.

18. Gruetzmacher TJ, Bradley RL: Acid whey as a replacement for sodium caseinate in spray dried coffee whiteners. *J Dairy Sci,* 74, 2838-2849, 1991.

19. Hollar CM, Law ARJ, Dalglleish DG, Brown RJ: Separation of major casein fractions using cation-exchange fast protein liquid chromatography. *J Dairy Sci*, 74, 2403-2409, 1993.

20. Imafidon GI, Farkye NY, Spanier AM: Isolation, purification, and alteration of some functional groups of major milk proteins: A review. *Crit Rew Food Sci Nutr*, 37 (7): 663-689, 1997.

21. Morr CV, Ha YW: Whey protein concentrates and isolates: Processing and functional properties. *Crit Rew Food Sci Nutr,* 33 (6): 431-476, 1993.

22. Watt BK, Merrill AL: Composition of Foods. Agriculture Handbook, 8th ed., pp. 159-161, Washington, DC, 1975.

23. Kenneth Herrich: Official Methods of Analysis of the Association of Official Analytical Chemists. Fifteenth ed., Arlington, Virginia, 1990.

24. Manz U, Philipp K: A method for the routine determination of vitamin A. Department of Vitamin Research and Development, Roche, Basel, Switzerland, 1988.

25. Kenneth Herrich: Official Methods of Analysis of the Association of Official Analytical Chemists, 16th ed., Arlington, Virginia, 1995.

26. Finglasa PM, Faulks RM: The HPLC analysis of thiamin and riboflavin in potatoes. *Food Chemi,* 15 (1): 37-44, 1984.

27. Manz U, Philipp K: A method for the routine determination of atocopherol in complete feeds, premixes and vitamin concentrates with the aid of high performance liquid chromatography. *Int J Vitam Nutr Res,* 51, 342-348, 1981**.**

28. Vulleumier JP, Probst HT, Brubacher G: Handbook der Lebensmittelchemic, Berlin, Heidelberg, 2nd ed., 669-876, New York, Springer, 1967.

29. Rizzolo A, Polesellob S: General review chromatographic determination of vitamins in foods. *J Chrom A*, 624, 103-152, 1992.

30. Guler GO, Çakmak YS, Zengin G, Aktumsek A, Akyıldız K: Fatty acid composition and conjugated linoleic acid (CLA) content of some commercial milk in Turkey. *Kafkas Univ Vet Fak Derg,* 16 (Suppl-A): S37-S40, 2010.