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Original Article

Determination of Biotinidase Enzyme Levels in Umbilical Cord Blood and Comparisons with Dried Blood Spot Testing in Newborns

Gözde Uzunyayla[1], Tanyel Zübarioğlu[1], Esra İşat[1,2], Mehmet Şerif Cansever[2,3], Emre Dinçer[4], Adil Umut Zübarioğlu[5], Birol Topçu[6], Güner Karatekin[4], Ertuğrul Kıykım[1], Ayşe Çiğdem Aktuğlu Zeybek[1,2]

[1]İstanbul University-Cerrahpaşa, Cerrahpaşa Faculty of Medicine, Department of Pediatric Nutrition and Metabolism, İstanbul, Türkiye
[2]İstanbul University-Cerrahpaşa, Cerrahpaşa Faculty of Medicine, Research Laboratory of Metabolism, İstanbul, Türkiye
[3]İstanbul University-Cerrahpaşa, Vocational School of Health Services, Department of Medical Documentation and Techniques, Division of Medical Laboratory Techniques, İstanbul, Türkiye
[4]University of Health Sciences Türkiye, Zeynep Kamil Women and Children Training and Research Hospital, Clinic of Neonatology, İstanbul, Türkiye
[5]Yeni Yüzyıl University Gaziosmanpaşa Hospital, Department of Neonatology, İstanbul, Türkiye
[6]Tekirdağ Namık Kemal University Faculty of Medicine, Department of Biostatistics, Tekirdağ, Türkiye

ABSTRACT

Aim: Biotinidase deficiency (BD) is an autosomal recessive inherited metabolic disorder caused by enzymatic deficiency. Untreated patients may develop neurologic symptoms, hearing loss, optic atrophy, skin eruptions and/or alopecia. Although cord blood has been explored as a possible sampling type for various newborn screening tests, there is limited data on its use to measure biotinidase activity. In particular, there are no standard reference ranges or established cut-off values for BD detection in cord blood. In this prospective cohort study, we aimed to determine biotinidase activity levels in cord blood samples from newborns and to investigate their comparability with dried blood spot (DBS) measurements.

Materials and Methods: This prospective cohort study was conducted between October 2020 and December 2021. Biotinidase activity was measured in umbilical cord blood samples at birth and in DBS samples collected as part of the national screening program. In addition, venous blood samples were taken from 20 newborns who agreed to have their biotinidase activity remeasured from venous blood after six months.

Results: This study included 97 newborns, 53 girls (54.6%) and 44 boys (45.4%). Measurements of biotinidase activity in umbilical cord blood revealed an enzymatic deficiency in twenty patients (20%). These 20 patients had normal biotinidase enzyme activities according to their DBS samples. None of the 97 patients had a biotinidase enzyme activity of less than 65 U in their DBS samples. The sensitivity of the cord blood measurement, which was defined as the reference standard compared to the DBS test results, was calculated to be 79.38%.

Conclusion: The measurement of biotinidase activity in umbilical cord blood may not be suitable for routine screening due to its high rate of false-positive results and uncertain specificity.

Keywords: Biotinidase deficiency, newborn screening, cord blood

Corresponding Author
Assoc. Prof. Tanyel Zübarioğlu, İstanbul University-Cerrahpaşa, Cerrahpaşa Faculty of Medicine, Department of Pediatric Nutrition and Metabolism, İstanbul, Türkiye
E-mail: tanyel.zubarioglu@iuc.edu.tr **ORCID:** orcid.org/0000-0002-7159-4008
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Introduction

Biotinidase deficiency (BD) is an inherited metabolic disease caused by enzymatic deficiency in releasing and recycling endogenous biotin. Biotin is an essential water-soluble vitamin and a cofactor of carboxylase enzymes. In this context, progressive biotin depletion in BD leads to a deterioration in amino acid catabolism, fatty acid synthesis and gluconeogenesis as a consequence of the effects on carboxylase reactions in which biotin acts as a cofactor (1,2). Patients with a residual enzyme activity of less than 10% are classified as "severe BD" and patients with an activity between 10-30% as "partial BD" (2). The clinical severity of the disease depends mainly on the amount of free biotin in the diet and the residual activity of the enzyme. Untreated patients may develop refractory seizures, hypotonia, lethargy, ataxia, developmental delay, hearing loss, optic atrophy, skin eruptions and/or alopecia. Although the clinical phenotype of severe BD is often characterized by neurologic and cutaneous symptoms which can progress to coma and even death, other manifestations can also be observed. Myopathy, peripheral neuropathy and symptoms mimicking neuromyelitis optica can occur in older individuals with partial deficiency (3).

The incidence of BD is reported in the literature to be 1:40,000-60,000 live births. It is known that the incidence of this disease varies according to region and can increase up to 1:9,000 (4). Recent studies have found a higher incidence in some countries such as Brazil and Italy, which are similar to Türkiye (5-7). The incidence of BD in Türkiye, which has been investigated since 2008 as part of the "National Newborn Screening Program" in our country, is reported in the literature as being 1:7,116 (8-10).

There are various methods for measuring biotinidase activity, such as the fluorometric method, the spectrophotometric method and measurement by high-performance liquid chromatography (8-11). However, the most commonly used method is the colorimetric (spectrophotometric) measurement of enzyme activity in plasma or serum samples (12,13). After the development of a colorimetric method for the determination of biotinidase activity in dried blood spots (DBS'), BD has been included in the screening program of many countries around the world (2,14) and BD patients are diagnosed at an early stage and life without sequelae has become possible (15).

Newborn screening using umbilical cord blood sampling for various diseases has been discussed in the literature as a possible option, but it is not without controversy (16-18). There is a lack of information on the utility of measuring biotinidase activity in cord blood samples. Furthermore, there are currently no established reference ranges or cut-off values for biotinidase activity in cord blood. In this preliminary study, we aimed to present the biotinidase activity levels in cord blood samples from newborns and to investigate their comparability with DBS measurements.

Materials and Methods

Study Design and Participants

This prospective study was conducted with babies born between October 2020 and December 2021 in three tertiary hospitals in İstanbul.

The inclusion criteria were: those babies whose mothers were monitored regularly throughout their pregnancy, those whose mothers did not have any chronic/pregnancy-related diseases and/or drug treatments, and those whose birth history did not include perinatal asphyxia or difficult labor. Additionally, the babies included had to have reached at least 37 weeks gestation.

The exclusion criteria were: those babies who were born before 37 weeks gestation, those who were small for their gestational age, and those who had intrauterine growth retardation, or were treated in a neonatal intensive care unit.

Those babies who met the inclusion criteria and whose families had given their consent were enrolled into this study. Ethical approval was obtained from the İstanbul University-Cerrahpaşa, Cerrahpaşa Faculty of Medicine Clinical Research Ethics Committee (approval number: E-83045809-604.01.02-3183, date: 05.01.2021).

Sample Collection

Two milliliters of umbilical cord blood were collected after clamping the umbilical cord immediately after birth. The collected cord blood was centrifuged at 3,000 rpm for 5 minutes and then the serum was separated. It was stored at -80 °C. The following demographic and clinical data were collected from those patients from whom samples were taken as part of national newborn screening prior to discharge: the identity of the baby and mother, contact details, maternal age, maternal medical history, birth method, gestational age and birth weight, and time of DBS sampling.

Preparation of the Samples

The collected samples were transferred via a cold chain to the pediatric metabolism research laboratory. After transfer, biotinidase activity was measured immediately after thawing

the samples using a colorimetric spectrophotometric method. Those patients who experienced pre-analytical/analytical problems during the study period, such as inappropriate samples, incorrect transfers and/or missing data, were excluded from this study.

Spectrophotometric Enzyme Measurement

This study was carried out using the Secomam S.750 spectrophotometer. Biotinidase enzyme activity was measured spectrophotometrically using the method of Wolf et al. (19). In this method, the enzyme activity of biotinidase was determined by measuring the hydrolysis of the substrate N-biotinyl-p-aminobenzoate (20). Buffer A pH 6.0 solution was prepared with a total volume of 1 mL [0.0067 mol potassium dihydrogen phosphate, 0.0067 mol potassium phosphate, 0.0003 mol ethylenediaminotetraacetic acid (EDTA), 26.3 mg serum albumin, 1.64 µmol biotinidase substrate (N-biotinyl-p-aminobenzoate)]. Buffer B solution was prepared to contain a pH 6.0 mixture (0.0067 mol potassium dihydrogen phosphate, 0.0067 mol potassium phosphate, 0.0003 mol EDTA, 26.3 mg serum albumin) so that the total volume was 1 mL. To assay biotinidase enzyme activity, the samples were prepared as described in Table I and incubated at 37 °C for 30 minutes. Then, 100 µL of 30% trichloroacetic acid was added to each tube and the reaction was stopped. Centrifugation was carried out at 10,000 rpm for 5 minutes. The supernatant was removed and put into separate tubes. During the reaction, p-aminobenzoic acid (PABA), released by the activity of the biotinidase enzyme in the patient's serum, was diazotized with 100 µL of fresh sodium nitrite. The tubes were vortexed and left for 3 minutes. Following this, 100 µL of ammonium sulfate was added to remove excess nitrite. The tubes were vortexed and allowed to stand for 3 minutes. Finally, diazotized PABA was reacted with 100 µL of N-1-naphthyl ethylenediamine dihydrochloride to form a colored product. The resulting product was measured spectrophotometrically at a wavelength of 546 nm. Net absorbance was measured in relation to the amount of PABA released and thus the biotinidase enzyme activity in the sample was determined. Enzyme activity was expressed as µmol/min of PABA released into the serum (19). In accordance with the literature, BD is typically defined as enzyme activity below 30% of the normal reference range. Cut-off values should be set individually by each laboratory, taking into account their own reference values for enzyme activity (2). Based on our reference range, a value below 3.5 IU/L corresponds to an activity of less than 30% and was therefore considered as an indicator of BD in this study.

The national newborn screening program standards were used to define BD in the DBS samples. Accordingly, it was recommended that those babies with an enzyme activity of less than 65 U, as measured by a fluorometric method, be screened for BD (21).

Evaluation of the Measurements of the Biotinidase Activity of Umbilical Cord Blood in Comparison to DBS

After the cord blood sample measurements were completed, the results were compared with the DBS results obtained from the national newborn screening in the same participant. After this procedure, all patients in this study were called back for further sampling and the measurements were repeated in new peripheral blood samples. Peripheral blood samples were collected from those participants who agreed to provide new samples within six months of birth. As there may be a temporary decrease in enzyme activity in early infancy, repeated measurements are recommended for a more accurate diagnosis (22).

Statistical Analysis

Statistical analyses were performed using the Statistical Package for Social Sciences Version 25.0 (SPSS Inc., Chicago, IL, USA). Continuous variables are presented as arithmetic means ± standard deviations; categorical variables are presented as frequencies or percentages. The normal distribution of the data was assessed using the Kolmogorov-Smirnov test. The repeated measures analysis of variance test was used to determine the relationship between measurements. Normally distributed categorical variables were analyzed using the simple Paired Samples t-test. A p value ≤0.05 was set to determine statistical significance.

Table I. Measurement of biotinidase activity in serum samples using the spectrophotometric method

Tubes	Buffer A solution	Buffer A solution	PABA (standard)	Serum	Distilled water
Standard blind	-	950 µL	-		50 µL
Standard	950 µL	-	50 µL		-
Sample	950 µL	-	-	50 µL	-

PABA: P-aminobenzoic acid

Results

This study included 97 newborns, 53 girls (54.6%) and 44 boys (45.4%). The initial testing of the cord blood samples from all participants was performed at birth. DBS analyses were performed at different time points: in the first 24 hours of life in 77 (79.3%) and between 24-48 hours of life in 9 (19.5%) babies, in accordance with the national newborn screening protocol. All participants were called back to the hospitals six months after birth and invited to provide a new peripheral blood sample for the measurement of biotinidase enzyme activity. New peripheral blood samples were analyzed from 21 patients who accepted the invitation.

Comparison of Umbilical Cord Blood and DBS with Regard to the Measurement of Biotinidase Activity

The measurement of biotinidase activity in umbilical cord blood revealed an enzymatic deficiency in twenty patients (20%). These 20 patients had normal biotinidase enzyme activities according to their DBS samples. None of the 97 patients had a biotinidase enzyme activity of less than 65 U in their DBS samples. Based on these results, the sensitivity rate of cord blood measurement was estimated to be 79.38%. Due to the insufficient number of samples, the specificity rate could not be determined and was assumed to be uncertain. The accuracy rate of cord blood sampling was also determined to be 79.38%; however, the false positive rate of the test was 20.6%.

Based on the DBS results, which are considered the reference standard, the diagnostic performance of biotinidase activity in cord blood was further evaluated. Since no true positive cases were detected among the 97 participants, the positive predictive value was calculated as 0% (0/19). The negative predictive value was 99% (77/78), indicating a high rate of correctly identified unaffected individuals. The overall diagnostic accuracy, defined as the proportion of true results (true positive and true negative) out of the total number of cases, was 79% (77/97).

Comparison of Umbilical Cord Blood and Peripheral Blood when Measuring Biotinidase Activity Six Months Apart

Biotinidase activity was measured in the peripheral blood samples of 21 patients (21%) at six months of age. Three patients (14%) had BD when their cord blood was measured. However, all 21 patients had normal biotinidase activity on the second measurement in their peripheral blood samples. A statistically significant difference between the two measurements was found in all individuals ($p<0.001$) (Table II).

Discussion

In this study, we aimed to evaluate biotinidase activity in umbilical cord blood samples from newborns and to compare it with DBS measurements. We found that the sensitivity of the cord blood samples was 79.38%, but the specificity could not be determined due to the limited number of samples. The false positive rate of the test was 20.6%. The overall diagnostic accuracy was 79% (77/97). Based on these preliminary results of this study, the measurement of biotinidase activity in cord blood may not be suitable for routine screening due to its high rate of false-positive results, uncertain specificity and limited detection of true positives.

Several factors can influence the measurement of biotinidase enzyme activity and these must be carefully considered when interpreting results. Pre-analytical variables, such as sample collection and storage conditions, are particularly critical. Blood spots from newborn screening should be thoroughly dried before delivery to the laboratory in order to prevent the loss of enzyme activity due to moisture. For serum or plasma samples, freezing is essential in order to preserve enzyme activity. However, storage at -20 °C can lead to a decrease in enzyme activity over time. For quality control, long-term storage should therefore be at -80 °C (23). Apart from pre-analytical conditions, reduced enzyme activity in the first weeks of life should be taken into consideration, especially in preterm infants and those individuals with liver disease (24,25). In our

Table II. Comparison of umbilical cord blood and peripheral blood biotinidase activity measurements performed 6 months apart

	Mean ± SD	Median (min.-max.)	p value
Biotinidase activity in umbilical cord blood at birth*	4.67±1.57	4.70 (0.60-10.10)	**<0.001**
Biotinidase activity in peripheral blood at 6 months of age**	7.45±1.10	7.50 (5.70-9.50)	

*n: 97 patients **n: 21 patients
SD: Standard deviation, min.-max.: Minimum-maximum

study, prematurity was an exclusion criterion. Additionally, there was no evidence of liver disease in any of the babies. The storage procedures and transfer periods followed the guidelines appropriately. For these reasons, the results of the analyses can be considered to be reliable.

The use of umbilical cord blood for newborn screening for inherited metabolic diseases has been reported in a large-scale study involving 24,983 newborns. In that study, cord blood testing was limited to the analysis of acylcarnitine and amino acids and its effectiveness in detecting inherited metabolic diseases was found to be limited (26). To our knowledge, only one study has reported the measurement of biotinidase activity in cord blood samples. In 2022, in addition to congenital hypothyroidism, cystic fibrosis and glucose-6-phosphate dehydrogenase deficiency, BD was assessed in the cord blood samples from 26 newborns. None of the babies were found to have profound BD. As that study was performed on a very limited number of patients, the enzyme measurement was not repeated and the cord blood sample results were not compared with other samples, which was considered a limitation by its authors, Singh et al. (16). In contrast, our prospective study included a larger sample size and it directly compared the biotinidase activity measured in cord blood with DBS and peripheral venous samples, allowing for a more comprehensive assessment of diagnostic performance. Therefore, this study provides the first comparative data from Türkiye on biotinidase activity in cord blood and it can serve as a reference for future studies aiming to establish standardized cut-off values and screening protocols.

Various recommendations and examples of specific target values for the sensitivity and specificity of screening tests can be found in the literature. Although there are no defined target values, it is generally expected that screening tests should have a high sensitivity and specificity (27,28). In a study of Turkish patients in whom BD was confirmed by *BTD* gene analysis, spectrophotometric measurements of enzyme activity in serum samples showed a diagnostic sensitivity and specificity of 93.1% and 95.1%, respectively (29). In addition to these findings, Göksoy (30) evaluated the results of newborn screening for BD in 211 infants from southeastern Türkiye in a recent large-scale study. Their study reported that 48.3% of patients were ultimately diagnosed with BD, while 51.7% were classified as false-positive cases. Notably, molecular analysis confirmed BD in 26.8% of those patients who had normal quantitative enzyme activity, emphasizing the limitations of enzymatic testing alone and the additional value of genetic confirmation. These results highlight important diagnostic challenges, including increased false-positive rates and genetic diversity in different regions, and they support the need for region-specific screening strategies. Although our study is preliminary, it provides an initial insight into the use of cord blood for measuring biotinidase activity and it may contribute to future studies aimed at refining and optimizing BD screening approaches.

Study Limitations

Our study had some limitations. The most important limitation was the small cohort of the study. Another important limitation was the fact that not all individuals underwent follow-up at six months, which significantly limited the power of this study, especially for comparisons between the subgroups.

Conclusion

In conclusion, the measurement of biotinidase activity in umbilical cord blood may not be suitable for routine screening due to its high rate of false-positive results and uncertain specificity. Further studies with larger sample sizes are needed in order to determine the normal reference ranges of biotinidase activity in cord blood samples for future screening strategies.

Ethics

Ethics Committee Approval: Ethical approval was obtained from the İstanbul University-Cerrahpaşa, Cerrahpaşa Faculty of Medicine Clinical Research Ethics Committee (approval number: E-83045809-604.01.02-3183, date: 05.01.2021).

Informed Consent: Those babies who met the inclusion criteria and whose families had given their consent were enrolled into this study.

Authorship Contributions

Concept: G.U., T.Z., B.T., E.K., A.Ç.A.Z., Design: G.U., T.Z., E.İ., M.Ş.C., B.T., E.K., A.Ç.A.Z., Data Collection and/or Processing: G.U., E.İ., M.Ş.C., E.D., A.U.Z., G.K., Analysis and/or Interpretation: G.U., E.İ., M.Ş.C., E.D., A.U.Z., G.K., Literature Search: G.U., T.Z., A.Ç.A.Z., Writing: G.U., T.Z., B.T., E.K., A.Ç.A.Z.

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