Determination of Reference Intervals For Dihydrorhodamine 123 (DHR) Assay in Healthy Children

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Özet

Bu çalışmada, sağlıklı çocuklarda dihidrorodamin 123 (DHR) testi için referans aralıkların belirlenmesi amaçlanmıştır.

Selçuk Üniversitesi Tıp Fakültesi, Çocuk Sağlığı ve Hastalıkları Anabilim Dalı'na sağlam çocuk muayenesi için başvuran herhangi bir hastalığı olmayan veya minör travma ile gelen 0-18 yaş arası 100 sağlıklı çocuk ve 18 yaş üstü 10 yetişkin çalışmaya dahil edildi. Hastalar 0-1 ay, 1-3 ay, 4-6 ay, 7-12 ay, 13-24 ay, 25-36 ay, 3-5 yaş, 6-8 yaş, 9-11 yaş, 12-18 yaş ve yetişkin olmak üzere 11 gruba ayrıldı. DHR testi, EDTA'lı periferik kan numunelerinde çalışıldı ve akım sitometride ölçüldü. Sonuçlar belirlenen yaş gruplarına göre ortalama ve %95 güven aralığı olarak analiz edildi.

Sağlıklı kontrollerde stimülasyon indeksi değerinin 21 ile 451 arasında değiştiği (ort±SD; 105.9±77) saptandı. Gruplar arasındaki fark değerlendirildiğinde 1-3 ay arasındaki yaş grubunda, diğer yaş gruplarına göre stimülasyon indeksinin düşük olduğu bulundu ($p<0.05$).

DHR testi, reaktif oksijen radikallerinin özellikle hidrojen peroksitin azalmış seviyelerini indirek tespit eden bir yöntemdir. Bu çalışma ile sağlıklı çocuklarda DHR testi için referans değerler belirlenmiştir.

Anahtar Kelimeler: *Kronik granülomatöz hastalık, dihidrorhodamin testi, referans değerler*

Abstract

This study aimed to determine the reference intervals for dihydrorhodamine 123 (DHR) assay in healthy children.

A total of 100 healthy children, aged between 0 and 18 years and 10 adults, who admitted to Selcuk University Medical Faculty, Department of Pediatrics were included in this study. The DHR assay were evaluated in a total of 11 groups, each group consisting of 10 individuals (0-1 months, 1-3 months, 4-6 months, 7-12 months, 13-24 months, 25-36 months, 3-5 years, 6-8 years, 9-11 years, 12-18 years and adults). DHR assay was performed in peripheral blood samples with EDTA and the cells were immediately evaluated using flow cytometry. The 95% confidence interval was determined according to the mean, minimum, and maximum values obtained from this data.

The stimulation index was observed to vary ranging between 21 and 451 (mean ± SD, 105.9 ± 77). When the difference between the groups was evaluated, it was found that the stimulation index was found to be low in the age group of 1-3 months to compared with the other age groups ($p <0.05$).

The DHR assay is a reliable method to detect the levels of reactive oxygen radicals, especially hydrogen peroxide. The reference intervals of DHR assay in healthy children were determined in this study.

Key Words: *Chronic granulomatous disease, dihydrorhodamine assay, reference value*

Introduction

Chronic granulomatous disease (CGD) is a heterogeneous, hereditary primary immunodeficiency (1). The disease is characterized by a defect in nicotinamide dinucleotide

phosphate (NADPH) oxidase complex (2). Patients with CGD have severe and recurrent bacterial and fungal infections, formation of chronic granulomas, and poor wound healing (3). X-linked gp91phox defect was defined in approximately 70% all case of CGD (2). Studies in our country have been reported to constitute approximately 10% of primary immunodeficiencies (4).

Diagnostic tests in chronic granulomatous disease are based on methods of measurement superoxide production. The nitroblue tetrazolium (NBT) test, which is one of the commonly used methods to determine the neutrophil oxidative burst activity, is diagnosed by microscope, so the evaluation of this test is need to the experienced person (5). Dihydrorhodamine 123 (DHR) assay is a flow cytometry method that is a rapid, sensitive and the most widely used technique (6,7). Dihydrorhodamine settles in mitochondria in phagocyte cells and is reduced to strong fluorescent rhodamine with the effect of oxygen radicals and peroxynitrite after stimulation. Since Rhodamine emits light at 488 nm, it is analyzed according to the change in histogram in a flow cytometer. This method is much more sensitive and reliable than other methods such as NBT. It is also superior to other tests in determining that the mother in the X-CGD carrier status.

There is insufficient data on normal values for this test that used to in few centers in the Turkey. In our country, the first study with the DHR assay is Köker's thesis (8). In this study, they evaluated the DHR assay for the diagnosis of CGD and its subgroups. The DHR assay of patients with CGD and their family members was analyzed and the results were compared with 18 healthy control data. This test has been reported to be a practical method for the diagnosis of CHD and the determination of X-CGD carriers (8).

The second study was conducted in 2015 by Çiçeközü et al. using the DHR assay, and normal values were determined in 210 healthy controls. In that study, in addition to being a diagnostic test, have reported DHR assay can be used to determine the inheritance of this disease and its carriers. However, the distribution of age groups in healthy controls was not classified in that study (9).

Because of the limited number of studies (8,9), in this study aimed to determine reference intervals in healthy children for DHR assay.

Materials and Methods

Study population

100 healthy children between 0-18 years of age without any disease or with minor trauma who admitted to Selcuk University Medical Faculty, Department of Pediatrics for healthy child examination and 10 healthy adults were included in this study. Children with active infection, chronic disease and a history of recurrent infections were excluded from the study.

Healthy children were divided into 10 groups according to their age: 0-1 months newborn, 1-3 months, 4-6 months, 7-12 months, 13-24 months, 25-36 months, 3-5 years, 6-8 years, 9-11 years and 12-18 years.

DHR assay

DHR assay was performed to determine NAPDH oxidase activity of neutrophils. Peripheral blood samples of healthy children were stored in ethylene diamine tetra acetic acid (EDTA) tubes and studied on the same day. Two tubes were prepared as control and patient tubes for each individual. 10 µl of catalase was added to both tubes and then 100 µl of peripheral blood with EDTA was added. Catalase inactivates hydrogen peroxide by converting it into water and oxygen, thereby protecting the host tissue and cells by controlling the amount of reactive oxygen intermediates. 25 µl of PBS was added to the control tube and 25 µl of PMA was added to the patient tube and incubated at 37° C for 15 minutes in the water bath. PMA was used as an activating stimulus for neutrophils. After incubation, DHR was added to both tubes and incubated at 37° C for 5 minutes in the water bath. Then, lysing solution was added to lysis

erythrocytes. DHR is located into the mitochondria in phagocyte cells and is reduced to strong fluorescent rhodamine with the effect of oxygen radicals and peroxynitrite after stimulation. Rhodamine emitting light at 488 nm was analyzed by flow cytometry.

Flow cytometric analysis

BD FACS ARIA III flow cytometry and FACS Diva software program version 6.1.3 were used for analysis (BD Biosciences, Pharmingen, San Diego, USA). After the two tube samples were acquired with flow cytometry, neutrophils were gated on dot-blot graphics. Then geometric mean of Rhodamine-123 fluorescence intensity of neutrophils was determined on histograms.

Stimulation index (SI) was calculated by proportioning the geometric mean of the fluorescence intensity obtained from PMA-stimulated samples to the geometric mean of the fluorescence intensity obtained from non-stimulated samples. SI values were used to determine standard reference ranges in healthy subjects.

$$\text{Stimulation index (SI)} = \frac{\text{Geometric mean of fluorescence intensity of stimulated cells}}{\text{Geometric mean of fluorescence intensity of unstimulated cells}}$$

Statistical analysis

Statistical analysis of the data was performed using SPSS 11.0 program. Stimulation index values were examined for 0-1 months, 1-3 months, 4-6 months, 7-12 months, 13-24 months, 25-36 months, 3-5 years, 6-8 years, 9-11 years, 12-18 years and above 18 years-old age groups. Descriptive statistics such as number of children for each age group, geometric mean, arithmetic mean, minimum and maximum values as well as mean ± 2 standard deviation values are given. In addition, 95% confidence interval was established for each age group.

When examining the differences; in comparison of means between two groups, t-test was used in independent groups for data showing normal distribution, and Mann-Whitney U test was used for data without normal distribution. One-way analysis of variance (ANOVA) was used for normal distribution data and Kruskal Wallis test was used for non-normal distribution data. Significance level was accepted as $p<0.05$.

Results

Optimization of blood samples by comparison with patients:

DHR assay was performed in blood samples with EDTA according to the protocol specified in flow cytometry. Stimulation index was calculated. Two patients with chronic granulomatous disease were also studied. Stimulation index was below 10 in these patients. Flow cytometric analysis results of patient and control samples are shown in Figure 1.

Optimization of blood samples by run-time:

Blood samples with EDTA were optimized according to run-time for healthy control DHR assay. Blood samples were waited for 2 and 24 hours and then studied. The results showed a partial decrease in neutrophil functions. Therefore, all samples were studied within the first 6 hours on the day of arrival.

Dihydrorhodamine 123 assay results:

Stimulation index value ranged from 21 to 451 (mean ± SD; 105.9 ± 77) in healthy controls. The arithmetic mean, standard deviation, minimum-maximum values and 95% confidence interval of the stimulation index according to age groups obtained in the dihydrorhodamine assay are shown in table 1.

When the difference between the groups was evaluated, it was found that stimulation index was lower in the age group between 1-3 months compared to all age groups over 6 months ($p <0.05$).

Discussion

In this study, DHR assay, in which oxygen radicals formed after stimulation in neutrophils was detected was demonstrated to change with age, especially in children. These ROSs in PMA-induced neutrophils in healthy individuals provide a strong fluorescence reduction of DHR to rhodamine and form the basis of the DHR assay performed on flow cytometry. In the phagocyte oxidase defect, oxygen radicals (H2O2) cannot be synthesized and rhodamine does not occur with PMA stimulation. The "stimulation index'' calculated by the ratio of the mean fluorescence intensity obtained from unstimulated and stimulated neutrophils in flow cytometric analysis.

Chronic granulomatous disease was first described in 1957. It is a genetically heterogeneous disease characterized by recurrent, life-threatening bacterial and fungal infections and granuloma formation. Many patients are diagnosed before the age of five. This disease is caused by the inability of phagocytic leukocytes to produce reactive oxygen intermediates (ROIs). The source of these radicals is the superoxide produced by NADPH oxidase, an enzyme complex expressed in phagocytic leukocytes (neutrophils, monocytes, eosinophils and macrophages). This enzyme complex is responsible for the phagocyte respiratory burst (10,11).

Neutrophils constitute the largest portion of leukocytes in childhood and adulthood over four years of age and participate in the early phase of the inflammatory response. An adult person produces more than 100 billion neutrophils per day, and their half-life in the blood is only 6 hours. If circulating neutrophils do not settle at an infection site within this period, they are phagocytized by macrophages in the spleen and liver. In chronic granulomatous patients, both catalase-positive microorganisms and inflammation are present, as neutrophil functions are insufficient. Therefore, early diagnosis of patients is very important to prevent complications that may cause organ damage and death (12).

In Turkey Koker's thesis is the first study conducted in this regard, the SI value of healthy controls was found between 60-107 (mean ± SD. 79.6 ± 15.4). The control group consisted of 18 people, 14 of them were in the childhood and 4 of them were in the adult age group. There are no individuals under 3 years of age in the childhood (8). In our study, DHR assay normal values were evaluated in 110 individuals (100 children and 10 adults) in different age groups. For the first time, the potential of neutrophils to produce oxygen radicals in the 1-3 months age group was found to be insufficient. No difference in stimulation index between other age groups with the neonatal period suggests the presence of neutrophils passed on from the mother.

Another study was conducted in Turkey by Çiçekkökü et al., it was found that the stimulation index was between 20.1-125.2 (mean±SD; 36.8±18.3) in healthy control samples, although lower and upper values were similar to our study, mean and stardard deviation values were lower (our study; 21-451, mean±SD; 105.9±77) (9). Çiçekkökü et al. evaluated the stimulation index in healthy controls regardless of age, whereas healthy controls were divided into age groups in our study.

Köker et al reported that 55% of 89 patients with CGD from 73 Turkish families were autosomal recessive. It was reported that this disease was associated with residual NADPH activity in patients with mild clinical findings that appeared later in life. It has been reported that stimulation index can be increased up to 17 with DHR assay especially in subtype with p47 mutation (13). Therefore, reference values are important especially in the determination of mild clinical presentation in autosomal recessive cases with CGD.

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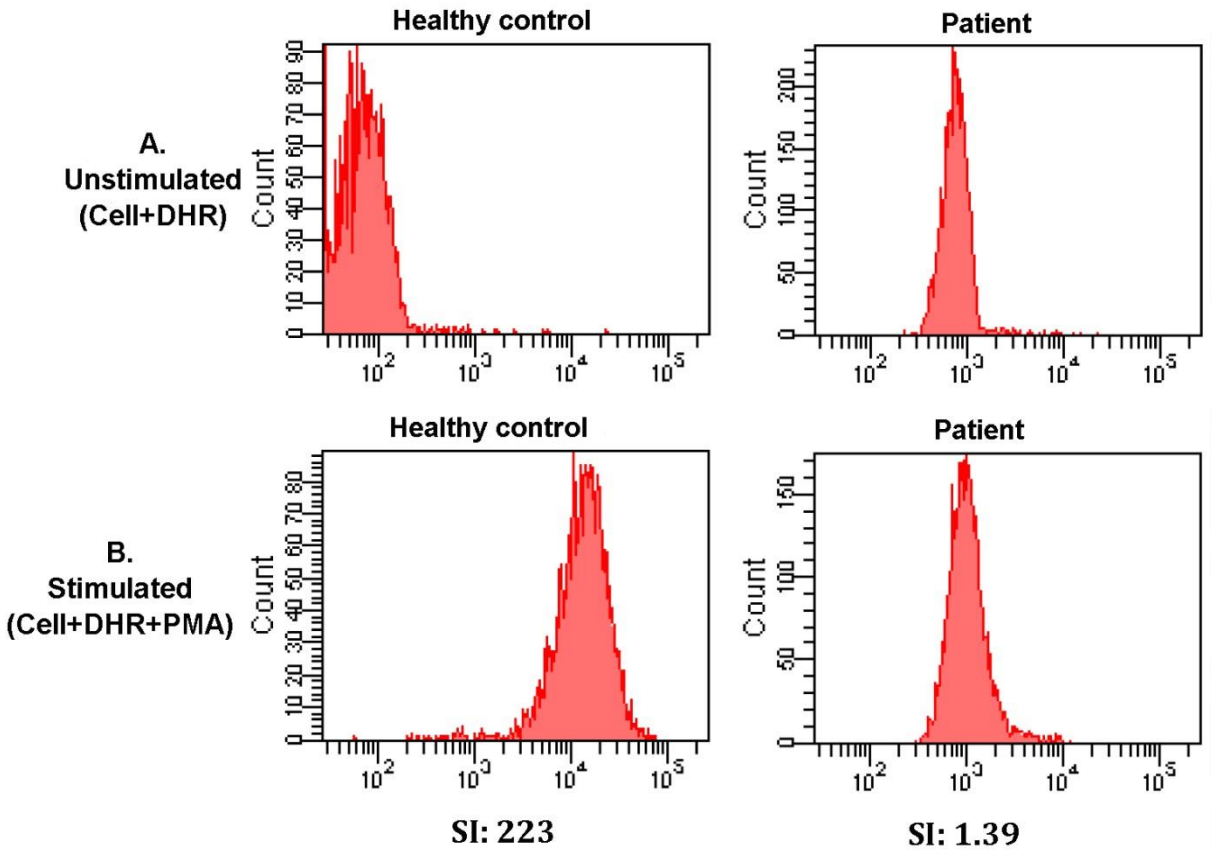

Figure 1: Histogram view and stimulation index observed in DHR test of healthy control and patients

Table 1: Age-related stimulation index in healthy children

Groups	n	mean ± SD	min - max	95% confidence intervals
0-1 months	10	67.2 ± 23.6	36 - 119	[54.08-81.87]
1-3 months	10	51.3 ± 22.9	24.2 – 81.9	[38.15-65.66]
4-6 months	10	76.5 ± 36.4	24.4 - 134	[56.21-97.47]
7-12 months	10	107.2 ± 49.3	41 - 186	[77.89-135.83]
13-24 months	10	138.5 ± 95.1	48 - 356	[88.40-198.81]
25-36 months	10	102.2 ± 43.4	40 - 182	[77.91-129.60]
3-5 years	10	120.5 ± 72.6	50 - 316	[86.32-169.97]
6-8 years	10	98.5 ± 47.2	32.2 - 205	[71.08-130.19]
9-11 years	10	99.5 ± 46.2	40 - 205	[75.02-126.87]
12-18 years	10	174.8 ± 153.4	21 - 451	[86.63-271.66]
Adults	10	129.9±97.7	23 - 366	[81.07-199.45]