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ARAŞTIRMA YAZISI / RESEARCH ARTICLE

TOTAL ANTİOKSİDAN SEVİYE VE TOTAL OKSİDAN SEVİYE ÖLÇÜMLERİNE HEMOLİZİN ETKİSİ

THE EFFECT OF HEMOLYSIS ON TOTAL ANTIOXIDANT STATUS AND TOTAL OXIDANT STATUS MEASUREMENTS

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ÖZET

AMAÇ: Total antioksidant seviye (TAS) ve total oksidan seviye (TOS) ölçümleri oksidatif stresin değerlendirilmesinde yaygın olarak kullanılan testlerdir. İn vitro hemoliz, preanalitik fazla ilişkili, biyokimyasal testler üzerine analitik süreçte etki eden önemli bir interferans nedenidir. Bu çalışmada in vitro hemolizin TAS ve TOS düzeyleri üzerine etkisini incelemeyi ve hemoliz indeksi (HI) eşik değerlerini belirlenmeyi amaçladık.

GEREÇ VE YÖNTEM: 22 sağlıklı gönüllü bireyden venöz kan örnekleri lityum heparinli tüplere alındı ve sonrasında 5 alikota ayrıldı. İlk alikota ek bir işlem uygulanmadı, diğer alikotlardaki numuneler sırasıyla ince uçlu iğneden 1, 3, 5 ve 7 kez aspire edilerek hafiften yükseğe doğru in vitro hemoliz yapıldı. Santrifüj sonrası elde edilen plazma örneklerinde hemoliz indeksi (HI), TAS ve TOS düzeyi ölçümleri Roche Cobas 6000 c501 otoanalizöründe spektrofotometrik yöntemle gerçekleştirildi.

BULGULAR: Bu çalışmanın sonucunda TAS ve TOS düzeylerinin her ikisinin de hemolizden etkilendiği görüldü. TAS düzeylerinde HI değeri 414 mg/dL'e kadar, TOS düzeylerinde ise HI değeri 29 mg/dL'ye kadar interferans olmadığı belirlendi.

SONUÇ: TAS ve TOS düzeyleri in vitro hemolizden etkilenmektedir. Özellikle TOS düzeyleri ciddi bir şekilde etkilenme göstermiştir. Bu testlerin güvenilirliği açısından hemoliz indeksi (HI) ölçümünün yapılması önerilir.

ANAHTAR KELİMELER: Total antioksidan seviye, Total oksidan seviye, İnterferans, İn vitro hemoliz, Hemoliz indeksi.

ABSTRACT

OBJECTIVE: Total antioxidant status (TAS) and total oxidant status (TOS) measurements are commonly used tests in assessing oxidative stress. In vitro hemolysis is a significant cause of interference in the analytical process of biochemical tests. This study aims to examine the impact of in vitro hemolysis on TAS and TOS levels and to determine the HI threshold values.

MATERIAL AND METHODS: Venous blood samples from 22 healthy volunteers were taken into lithium-heparin tubes and then separated into 5 aliquots. No additional manipulation was applied to the first aliquot, and the samples in the other aliquots were aspirated 1, 3, 5, and 7 passes through a small-gauge needle, respectively, and in vitro hemolysis from slight to high was performed in the aliquots. Hemolysis index (HI), TAS and TOS levels in plasma samples obtained after centrifugation were measured by the spectrophotometric method on Roche Cobas® 6000 c501 autoanalyzer.

RESULTS: As a result of this study, it was seen that both TAS and TOS levels were affected by hemolysis. No interference was observed at TAS levels up to an HI value of 414 mg/dL, and at TOS levels up to an HI value of 29 mg/dL.

CONCLUSIONS: TAS and TOS levels are affected by in vitro hemolysis. In particular, TOS levels were significantly affected. Measurement of the hemolysis index (HI) is recommended to ensure the reliability of these tests.

KEYWORDS: Total antioxidant status, Total oxidant status, Interference, In vitro hemolysis, Hemolysis index.

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INTRODUCTION

Hemolysis is characterized by the release of all intracellular components, especially hemoglobin, into the extracellular space and passing into serum or plasma when erythrocytes lose their structural integrity and break down (1). It is analyzed in two groups: in vivo and in vitro hemolysis. In vivo hemolysis results from the rupture of erythrocytes in the intravascular or extravascular space in various pathological conditions that cause a shortening of the life span of erythrocytes. In vitro hemolysis, on the other hand, occurs outside the body due to reasons such as improper sample collection, processing, transportation, or patient-dependent reasons. The most important one is in vitro hemolysis due to its much higher incidence in clinical laboratories (2). Hemolysis interferes with laboratory test results through various mechanisms. These are additive, spectral, chemical, and dilutional effects (3). Reactive oxygen species (ROS) play a crucial role in physiological and pathophysiological mechanisms in humans (4). Free radicals tend to interact with and damage all components of the cell, especially lipids, proteins and nucleic acids. These harmful effects are prevented by the body's enzymatic and non-enzymatic antioxidant defense systems. Biological damage occurs because of overproduction of ROS or insufficient antioxidant defense systems, and this is called oxidative stress (5). It has been demonstrated that oxidative stress contributes to the development of numerous diseases such as atherosclerosis, chronic obstructive pulmonary disease, Alzheimer's disease, and cancer (6). Various oxidants and antioxidants can be quantified individually to assess oxidative stress (7).

However, this is time-consuming, costly, workload-increasing and impractical. Instead, colorimetric kits are available that can measure both to total antioxidant status (TAS) and the total oxidant status (TOS) in various sample types. Owing to their ease of use and compatibility with diverse biological matrices, such kits remain commonly utilized in experimental and clinical research involving both human and animal subjects (8,9). This study aimed to assess the in vitro hemolysis interference on TAS and TOS levels and to determine the threshold HI values.

MATERIALS AND METHODS

Study design

In this study, 22 healthy volunteers who did not have a history of chronic disease and chronic drug use, were not pregnant or lactating, did not have recent surgery or trauma, and were able to continue their normal daily life were included. Venous blood samples from volunteers (9 women and 13 men aged 25-51 years) were collected into 4 mL vacuum tubes with dry lithium heparin (Vacuette; Greiner Bio-One GmbH, Kremsmünster, Austria) between 8:00 and 10:00 a.m. after at least 8 hours of fasting by the same phlebotomist. All blood samples were promptly separated into 5 aliquots. No additional procedure was applied to the first aliquot; the blood samples in the other aliquots were aspirated 1, 3, 5, and 7 times using a 22-gauge 0.70 x 32 mm needle (Ayset®, Turkey, Adana), respectively, and in vitro hemolysis was performed at increasing rates due to mechanical trauma (10). This study employed the shearing method to examine the impact of hemolysis (3). The aliquots were quickly centrifuged at 1000 g for 10 minutes, and the plasma samples were subsequently separated. The HI value provides a semi-quantitative representation of the hemolysis level (free hemoglobin) in serum or plasma. In this study, serum indexes were automatically studied on a Roche Cobas 6000 C501 autoanalyzer (Roche Diagnostic, Mannheim, Germany), and HI values were obtained. In this device, 1 HI corresponds to approximately 1 mg/dL of free hemoglobin. TAS and TOS levels of all samples were studied by the spectrophotometric method on Roche Cobas® 6000 c501 autoanalyzer (Roche Diagnostic, Mannheim, Germany). TAS and TOS levels were measured based on the manufacturer's protocols using Rel Assay brand kits (Rel Assay Diagnostics, Gaziantep, Turkey) developed by Erel (11,12) which can be applied to the autoanalyzer. Intra-assay coefficients of variation were reported as 3.3% -3.9% and inter-assay coefficients of variation 3.2%-2.8% for TAS and TOS, respectively. Different degrees of in vitro hemolysis were produced in aspiration aliquots. A total of 110 aliquots were divided into 5 groups according to their HI values. The 22 aliquots that did not undergo further manipulation cons-

tituted the hemolysis-free group (Group 1). Other aliquots (88 of them), except for the non-hemolysis group, were included in the groups according to the HI values measured independently of the number of aspirations.

Principle of TAS measurement

The antioxidants present in the sample convert the deep blue-green 2,2′-azino-di-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) radical into a colorless reduced ABTS form. The change in absorbance, measured spectrophotometrically, is inversely proportional to the total antioxidant content of the sample. The results were reported as Trolox equivalents (mmol Trolox Equiv./L), which is a vitamin E analogue.

Principle of TOS measurement

Oxidants in the sample oxidize the ferrous ion-o-dianisidine complex to a ferric ion, which then forms a colored complex with xylenol orange in an acidic environment. The absorbance of this colored complex, measured spectrophotometrically, correlates with the total oxidant content in the sample. The results were reported as micromolar hydrogen peroxide equivalents per liter (μmol H_2O_2 Equiv./L).

Ethical Committee

This study was carried out in alignment with the ethical guidelines established by the Declaration of Helsinki and received approval from the Clinical Research Ethics Committee of Yozgat Bozok University (Approval number: 2017-KAEK-189_2021.01.18_14). All participants in the study were provided with detailed information about the research, and their written consent was obtained.

Statistical Analysis

The percentage bias values for TAS and TOS results in the study were calculated using the formula: Bias% = 100 x (test result in hemolyzed sample – test result in non-hemolyzed sample) / test result in non-hemolyzed sample. Interference was considered significant if the percentage bias value exceeded 10% (13-15). The maximum HI value of the group preceding the one in which the bias exceeded 10% was designated as the threshold.

RESULTS

A total of 22 volunteers, 9 women (mean age 34.3 ± 5.8) and 13 men (mean age 35.4 ± 8.1), took part in this study. The levels of HI, TAS, and TOS were measured in the plasma obtained from the participants. Groups were categorized based on HI values. The HI ranges according to the groups, and the number of samples in the groups is shown in **Table 1**.

Table 1: Groups formed according to hemolysis index values.

Groups	Number of samples in groups	HI ranges
Group 1	22	0-22
Group 2	32	23-100
Group 3	37	101-300
Group 4	9	301-500
Group 5	10	501-970

* HI: hemolysis index

Since the degree of hemolysis varied among the aliquots due to mechanical trauma, and to accurately determine the percent bias, the non-hemolyzed group (Group 1) was paired individually with each hemolysis group. HI, TAS, TOS averages and bias percentages calculated for each paired group are shown in **Table 2**. In this study, the hemolysis interference threshold value for TAS was found to be 414 mg/dL. When we looked at the TOS, it was seen that the bias % value was very high even between group 1 and group 2. Therefore, to determine a lower threshold HI value for TOS, Group 2 was divided into subgroups and compared with the hemolysis-free group.

Table 2: Effects of hemolysis on TAS and TOS results.

Groups	Number of samples (N)	HI	TAS (mmol/L)	Bias %	TOS (μmol/L)	Bias %
roup 1		7±4	1.743±0.23	1.04	7.984±3.22	375.24
Group 2	N =32	63±24	1.761±0.22		37.943±27.22	
Group 1		7±5	1.737±0.25	5.80	7.140±3.58	1151.47
Group 3	N =37	199±59	1.838±0.25		89.351±39.33	
Group 1		8±6	1.781±0.26	9.40	6.927±4.08	1946.15
Group 4	N =9	367±32	1.948±0.24		141.739±46.83	
Group 1		2±1	1.378±0.15	14.82	3.498±1.67	5843.39
Group 5	N =10	665±138	1.582±0.14		207.894±64.29	

* HI, TAS, and TOS were shown as average ± SD. TAS: total antioxidant status, TOS: total oxidant status, HI: hemolysis index

Those with a HI value in the range of 23-30 were classified as Group 2a, those in the range of 31-40 were classified as Group 2b, those in the range of 41-50 were classified as

Group 2c, and those above 50 were classified as Group 2d. **Table 3** shows the results obtained by subgroups. The threshold HI value for TOS was calculated as 29 mg/dL.

Table 3: Determination of threshold HI value for TOS.

	HI range	Number of samples (N)	TOS (µmol/L)	Bias %
Group 1	5-11 (7.3±2.3)		9.25±2.98	105.81
Group 2a	23-29 (27.0±2.3)	4	19.03±4.45	
Group 1	3-7 (5.3±1.8)		8.73±1.92	191.94
Group 2b	38-40 (39.3±0.8)	4	25.50±3.23	
Group 1	3-11 (7.4±3.2)		9.00±2.53	209.53
Group 2c	41-50 (46.2±3.1)	5	27.85±6.01	
Group 1	3-22 (6.6±4.5)		7.29±3.46	547.18
Group 2d	51-98 (79.4±14.2)	19	47.20±31.80	

* HI was shown as min-max (mean ± SD), and TOS was shown as mean ± SD. TOS: total oxidant status, HI: hemolysis index

DISCUSSION

There are various sources of error in preanalytical, analytical, and postanalytical processes in clinical laboratories. The reliability of a test result depends on strict control of all phases. 70% of errors in the laboratory are caused by the preanalytical process, and the most common error is improperly taken samples. It has been reported that 40-70% of the inappropriately taken samples are hemolyzed samples. When the free hemoglobin concentration rises above 30 mg/dL, hemolysis becomes visible, and the color of the serum or plasma varies from pink to red depending on the degree of hemolysis (1). Hemolysis specimens are evaluated visually or with automated systems. Hemolysis, icterus, and lipemia indexes, called serum indexes, are integrated into some devices based on the wavelengths at which these substances are absorbed spectrophotometrically. Automated systems offer greater reliability in detecting hemolysis compared to visual assessment (16).

Nowadays, kit inserts usually contain information on interference studies conducted by the manufacturer. Different methods are used in hemolysis interference studies. These methods include osmotic shock, freezing/thawing, and shearing methods (3). In this study, the shearing method was used to investigate the effect of hemolysis on TAS and TOS levels. This approach more accurately simulates the true pathological processes of hemolysis (17). However, practice is required to achieve a wide range of hemolysis and may not produce gradual increases in hemoglobin concentration (3).

There is no information about hemolysis interference in the kit inserts used in this study. There are limited studies in the literature on hemolysis interference for TAS and TOS levels. In Erel's study, it was reported that hemolysis interferes with TOS measurement (12). However, no HI threshold value is specified. In addition, there is no information about which method was used in the study. In this study, the threshold HI value for TOS was found to be 29 mg/dL. This value is slightly below the visible hemolysis value, so there is a significant increase in TOS values even without visible hemolysis. For this reason, it would be appropriate to measure the HI and analyze the samples below 30 mg/dL before measuring the TOS level.

In another study conducted by Erel, it was reported that hemolysis did not cause interference in TAS measurement (11). However, in this study, TAS measurement was also affected by hemolysis. When the HI value exceeds 414 mg/dL, it causes a significant increase in TAS results. The reasons for this may include the presence of strong antioxidant enzymes (glutathione peroxidase-1, peroxiredoxin-2, and catalase), lower thiols and vitamins in erythrocytes, and the antioxidant properties of some amino acid residues in hemoglobin (18-20). Therefore, it would be appropriate to report the results by studying HI for TAS measurement.

The limitations of the study include a small sample size and a single-center design.

As a result, in this study, it was shown that TOS levels are seriously affected by hemolysis. TAS levels are also affected by hemolysis, but this interference occurs when the HI value rises above a certain level. To ensure reliable TAS and TOS results, measurement of the hemolysis index (HI) is recommended; accordingly, a decision should be made whether to reject or analyze the samples. However, further studies with larger sample sizes and multicenter designs are still recommended to support these findings.

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