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Original Article *Özgün Araştırma*

A NOVEL AND SEMIAUTOMATED ASSAY FOR THIOL OXIDATIVE STRESS INDEX: TOSI

Tiyol Oksidatif Stres İndeksi için Yeni ve Yarı-Otomatik Bir Test: TOSİ

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ABSTRACT

Objective: The aim of the study is to develop a new and automated method determining irreversible and reversible thiol oxidation products which are formed by the exposure of the plasma to oxidation and to measure the resistance of thiol groups to oxidation.

Material and Methods: The method is based on thiol-disulphide homeostasis measurement of plasma thiol groups before and after exposure to oxidative stress. According to the measurements, the levels of reversible thiol oxidation products (RTOP), irreversible thiol oxidation products (ITOP), and thiol oxidative stress index (TOSI=ITOP/RTOP) were determined.

Results: Plasma levels of ITOP were 24.02±12.1 µmol/L, levels of RTOP were 26.68±14.96 µmol/L and TOSI was 0.67±0.60 in the healthy control group. The level of ITOP of patients with lung and colon cancer were found to be significantly higher than the control group, while the level of RTOP was found to be significantly lower. When the parameter of TOSI which was used to determine the direction of the balance was examined, it was observed that the formation of ITOP was more dominant in both colon and lung cancer patients when compared to the control group.

Conclusion: Practical, inexpensive and semi-automatic spectrophotometric RTOP, ITOP and TOSI tests, developed by us for the first time, can be used to investigate many diseases associated with oxidative stress.

***Keywords**: Irreversible thiol oxidation products, reversible thiol oxidation products, thiol*

ÖZ

Amaç: Çalışmanın amacı, plazmanın oksidasyona maruz kalmasıyla oluşan geri dönüşümsüz ve geri dönüşümlü tiyol oksidasyon ürünlerini belirleyen yeni ve otomatik bir yöntem geliştirmek ve tiyol gruplarının oksidasyona direncini ölçmektir.

Gereç ve Yöntemler: Yöntem, oksidatif strese maruz kalmadan önce ve sonra plazma tiyol gruplarının tiyol-disülfid homeostaz ölçümüne dayanmaktadır. Ölçümlere göre, geri dönüşümlü tiyol oksidasyon ürünleri (RTOP), geri dönüşümsüz tiyol oksidasyon ürünleri (ITOP) ve tiyol oksidatif stres indeksi (TOSİ=ITOP/RTOP) seviyeleri belirlenmiştir.

Bulgular: Sağlıklı kontrol grubunda ITOP plazma düzeyleri 24.02±12.1 µmol/L, RTOP düzeyleri 26.68±14.96 µmol/L ve TOSI düzeyleri 0.67±0.60 olarak bulunmuştur. Akciğer ve kolon kanseri olan hastaların ITOP düzeyleri kontrol grubuna göre anlamlı olarak yüksek iken RTOP düzeyi anlamlı olarak düşük bulunmuştur. Dengenin yönünü belirlemek için kullanılan TOSI parametresi incelendiğinde, ITOP oluşumunun hem kolon hem de akciğer kanserinde kontrol grubuna göre daha baskın olduğu görülmüştür.

Sonuç: İlk defa geliştirdiğimiz pratik, ucuz ve yarı otomatik spektrofotometrik RTOP, ITOP ve TOSI testleri; oksidatif stres ile ilişkili birçok hastalıkta araştırılabilir.

***Anahtar Kelimeler**: Geri dönüşümlü tiyol oksidasyon ürünleri, geri dönüşümsüz tiyol oksidasyon ürünleri, tiyol*

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INTRODUCTION

Albumin and other plasma proteins represent a large part of plasma thiol pool while low molecular weight thiols including cysteine, cysteinyl-glycine, glutathione, homocysteine, and γ–glutamylcysteine form a small part of thiol pool in human plasma (1) . Thiol groups (RSH) can undergo oxidation reaction via oxidants and form both reversible thiol oxidation products (RTOP) and irreversible thiol oxidation products (ITOP) such as disulfide, sulfenic acid, sulfinic acid and sulfonic acid. RTOP is a dynamic oxidation product (e.g. disulphide) and can be reduced again to thiol groups via antioxidants in the body, thereby the thiol-disulfide balance is maintained (2-4). The dynamic thiol-disulfide homeostasis plays a critical role in the mechanisms of transcription and cellular signal transduction in the regulation of antioxidant defense, detoxification, apoptosis, and regulation of enzyme activities (5). Abnormal thiol-disulfide homeostasis play a significant role in pathogenesis of diabetes mellitus, cardiovascular diseases, cancer, ankylosing spondylitis, chronic renal failure, AIDS, Parkinson's disease, Alzheimer's disease, multiple sclerosis and liver diseases (6-15). On the other hand, it is remarkable that irreversible oxidation of protein cysteine that contains thiol groups has remained relatively unstudied (2). ITOP represents permanent oxidative damage of thiol groups. For instance, sulfinic acids are produced when the sulfur atom of a sulfenic acid is oxidised by taking two-electrons (16). Furthermore, the ratio of the reversible (RTOP) and irreversible (ITOP) oxidation products of the thiol groups (RTOP / ITOP) is named as thiol oxidative stress index (TOSI). TOSI is defined to determine the direction of oxidation balance.

Plasma thiol levels are most commonly measured by the conventional Ellman Reactive (DTNB). This compound is stoichiometrically reduced with free thiol groups. The resulting 5-thionitrobenzoic acid is measured at 412 nm (17, 18). Several recent studies have shown that native thiol and disulfide levels of plasma low molecular weight thiol compounds are determined using HPLC, capillary electrophoresis and bioluminescence systems. But these sophisticated systems can measure structural disulphide bonds, while it cannot measure the level of functional and dynamic thiol-disulfide homeostasis in plasma (19-22).

The aim of the study is to develop a new and automated method determining irreversible and reversible thiol oxidation products (ITOP, RTOP) which are formed by the exposure of the plasma to oxidation and to measure the resistance of thiol groups to oxidation. These oxidation products were measured using the thiol-disulfide homeostasis measurement method that was previously published by Erel&Neselioglu (18).

MATERIALS AND METHODS

Chemicals

Hydrogen peroxide (H_2O_2), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), ethylenediaminetetraacetic acid (EDTA), sodium borohydride, Tris (hydroxymethyl) aminomethane (Trizma), methanol and formaldehyde manifactured by Sigma-Aldrich Chemical Co. (Milwaukee, WI) and Merck Co. (Darmstadt, Germany) were used. All chemicals were ultrapure grade, and type I reagent-grade deionized water was used.

Plasma Samples

Venous blood samples were collected and plasma samples were separated from cells by centrifugation at 1500×g for 10 min. The samples were immediately stored at −80 °C. The study was approved by the local ethics committee (*Yıldırım Beyazıt University Ethics Committe of Clinical Research, 03.09.2014-138).*

Apparatus

A Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) with a temperature

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controlled cuvette holder and an automated analyzer (Cobas c501, Roche Diagnostics, Mannheim, Germany) were used.

Assay

The newly developed method is based on thiol-disulphide homeostasis measurement of plasma thiol groups before and after exposure to oxidative stress (18). Determination of thiol-disulfide homeostasis is performed by direct measurement of native thiol (−SH) and total thiol (−SH + −S−S) levels. Also, the level of disulfide, another component of homeostasis, is obtained by calculating the half of the difference between the total thiol and the native thiol levels.

Total Thiol and Native Thiol Measurement

Reversible disulphide bonds in the sample are reduced to functional thiol groups by $NaBH_4$. The unused $NaBH_4$ remnants are completely removed by formaldehyde. Formaldehyde prevents the extra reduction of the DTNB by $NaBH_4$. Thus, the total thiol content of the sample is measured using modified Ellman reagent. Simultaneously-measured native thiol content is subtracted from the total thiol content and half of the obtained difference gives the disulphide bond amount which is a reversible thiol oxidation product (Table1)(18).

Table 1: Automated measurements of total thiol and native thiol concentrations

	Volume	Reagent content
Sample	10 µL	Blood plasma
Reagent 1' (R1')	10 µL	NaBH4, 10 mM
Reagent 1 (R1)	10 µL	NaCl, 10 mM
Reagent 2 (R2)	110 µL	6.7 mM formaldehyde and 10 mM EDTA in tris buffer, 0.1 M, pH: 8.2
Reagent 3 (R3)	10 µL	10 mM DTNB in methanol
Wavelength		415 nm
Reading point		End-point
Calibration type		Linear (Mercaptoethanol)

R1 is reagent 1 of native thiol; R1' is reagent 1 of total thiol; R2 and R3 are common reagents of both native thiol and total thiol.

The New Assay

In the new method, the sample is divided into two portions. In the first portion, native and total thiol levels are measured according to thiol-disulphide homeostasis measurement method. In the second portion, after exposed to oxidative stress, native and total thiol levels are measured again. Then, the value of the oxidized sample is subtracted from the value of the unoxidized sample. Thus, RTOP, ITOP and TOSI levels could be calculated (Figure 1, Table 2).

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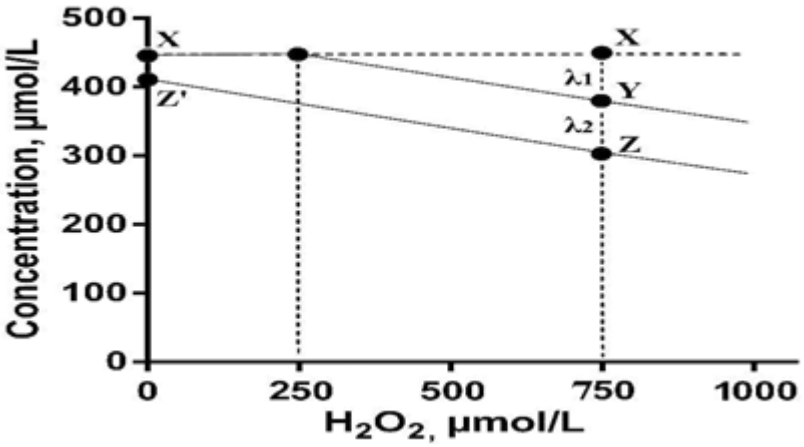

Figure 1: Design of measurements made before and after exposure of the sample to oxidative stress

Table 2: Identification of new parameters reflecting resistance to oxidative stress of thiol groups

	Explanation
X point	Total thiol [-SH+Reversible thiol oxidation products] (0 µM H2O2, baseline)
Y point	Total thiol [-SH+Reversible thiol oxidation products] (750 µM H2O2)
Z point	Native thiol [-SH] (750 µM H2O2)
Z' point	Native thiol [-SH] (0 µM H2O2, baseline)
[X]-[Y]=λ1	Irreversible thiol oxidation products (ITOP)
[Y]-[Z]=λ2	Reversible thiol oxidation products (RTOP)
λ1/ λ2	Thiol oxidative stress index (TOSI)

Measurement Steps

Measurements: Before the application of oxidative stress to the plasma

Native thiol (Z' point in Figure 1) and total thiol (X point in Figure 1) concentrations of plasma are detected before exposure to oxidative stres (18).

Measurements: After the application of oxidative stress to the plasma

The sample was exposed to oxidative stress for 15 minutes by adding 750 µM H_2O_2 (final concentration in the sample). After that, native thiol (Z point in Figure 1) and total thiol (Y point in Figure 1) concentrations were measured.

Identification and Graphical Display of Rtop, Itop and Tosı Levels

The measured values were plotted as oxidant (H_2O_2) concentration on the x-axis and thiol concentration on the y-axis (Figure 1). Thus, it was determined both RTOP and ITOP levels and the thiol oxidative stress index (TOSI) obtained by the ratio of these two values (Table2).

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Table 3: The level of ITOP, RTOP, and TOSI of the healthy control group and patients with lung cancer and colon cancer

	Control group	Lung cancer	p^1 value	Colon cancer	P^2 value
ITOP	24.02±12.1	44.31±27.77	0.036*	75.51±45.87	<0.001*
RTOP	26.68±14.96	16.84±10.90	0.022*	9.07±7.10	<0.001*
TOSI	0.67±0.60	3.21±2.77	0.029*	4.95±2.90	0.043*

p^1 value: Control and lung cancer were compared; p^2 value: Control and colon cancer were compared

* Indicates a significant statistical difference with p< 0.05

Statistical Analyses: The data were evaluated using visual (histograms) and statistical methods (Shapiro-Wilktest) to determine whether the data were normally distributed. Descriptive analyses were presented using mean and standard deviation (mean ± SD) for the normally distributed variables. As the data were normally distributed, independent sample t-tests and one-way ANOVA were conducted to compare the parameters among groups. In all analyses, a p-value of less than 0.05 was considered to be statistically significant. SPSS software Ver. 22.0 was used for statistical calculations (SPSS Inc., Chicago, IL, USA)

RESULTS

We identified a new test cluster containing X, Y, Z, Z', λ1, λ2 and λ1/ λ2 parameters in the method. This test cluster was obtained by using measured native thiol, total thiol, and reversible thiol oxidation product (disulphide) levels before and after exposure to oxidative stress.

The expose of samples to oxidation

Each of plasma samples was aliquoted into two tubes. Native and total thiol levels were measured as baseline values in the first aliquot. Simultaneously, the sample in the second aliquot was exposed to oxidative stress, and the levels of native and total thiol were measured again. The values of the oxidized sample were subtracted from baseline values of the sample. Thus, new parameters have been determined (Table 2, Figure 1).

Optimization of concentration and application time of H_2O_2

The optimization study was performed to determine optimum oxidant agent (H_2O_2) concentration and duration of application. Native thiol and total thiol levels of the plasma pool were monitored by adding hydrogen peroxide at increasing concentrations. It was determined that the optimal concentration of oxidant is 750 µM and the optimal duration of the application is 15 minutes (Figure 2).

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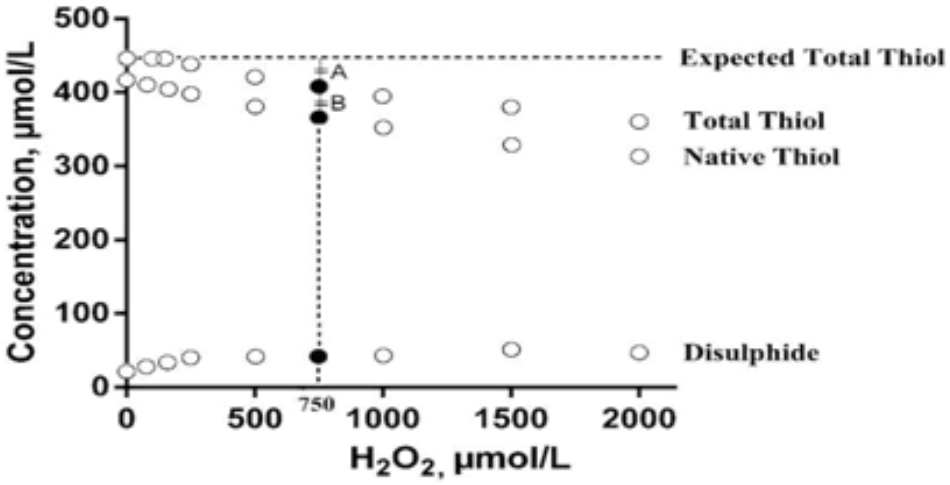

Figure 2: The changes in native thiol, total thiol and reversible thiol oxidation product (Disulphide) concentrations of plasma pool by increasing the oxidant, hydrogen peroxide

Identification of New Test Cluster

The parameters reflecting resistance to applied oxidative stress of thiol groups were identified by measuring native thiol and total thiol as follows (Table 2, Figure 1).

X Point: It is described as measured total thiol level of the sample before exposing oxidation.

Z' Point: It is described as measured native thiol level of the sample before exposing oxidation

Y Point: It is described as measured total thiol level of the sample after exposing oxidation. This point was determined by measuring the total thiol levels after the sample was exposed to 750 µM H_2O_2 for 15 min.

Z Point: It is described as measured native thiol level of the sample after exposing oxidation. This point was determined by measuring the native thiol levels after the sample was exposed to 750 µM H_2O_2 for 15 min.

[X]-[Y]=λ1=ITOP: It was determined by subtracting level of total thiol after exposure from the level of total thiol before exposure to hydrogen peroxide.

[Y]-[Z]=λ2=RTOP: It was determined by subtracting level of native thiol level from the level of total thiol of the sample exposed to hydrogen peroxide.

[X]-[Y] / [Y]-[Z] = λ1/ λ2: This ratio was calculated by dividing level of ITOP by level of RTOP. It was termed as Thiol Oxidative Stress Index (TOSI) reflecting resistance to applied oxidative stress of plasma thiol groups.

The first clinical preliminary study experiments of the new method were performed using serum samples from patients with colon and lung cancer. Thirty-two healthy controls, 30 patients with lung cancer and 30 patients with colon cancer were included in this study. Gender, body mass index (BMI), and age of the healthy and cancer groups were not different significantly ($p > 0.05$ for all groups). According to our first preliminary results; ITOP levels of the patients with lung and colon cancer were found to be significantly higher than the control group, while RTOP levels were found to be significantly lower. When the parameter of TOSI which is used to determine the direction of the balance was examined, it was observed that the formation of ITOP was more dominant in both colon and lung cancer when compared to the control group (Table 3).

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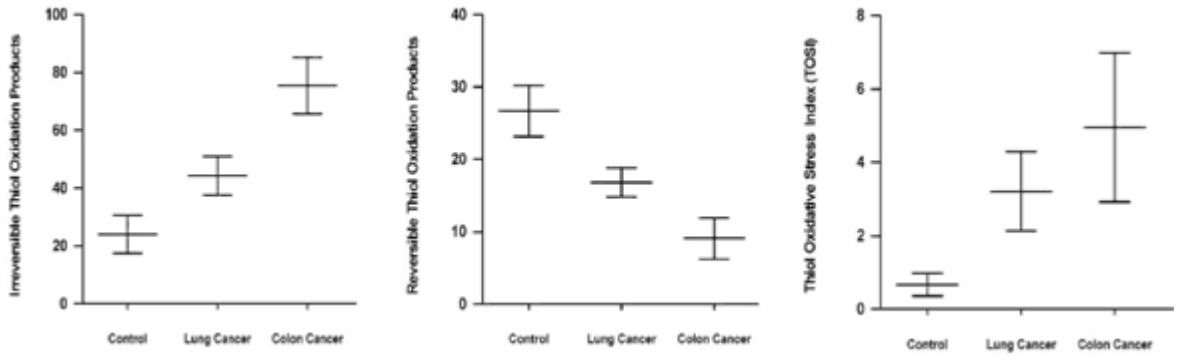

A B C

Figure 3: Plasma ITOP (A), RTOP (B), and TOSI (C) levels (±σM) of patients with colon and lung cancer and healthy subjects

DISCUSSION

Thiol groups can undergo oxidation reaction via oxidants and form both ITOP and RTOP such as disulfide, sulfenic acid, sulfinic acid, and sulfonic acid (4). Thiol-disulphide homeostasis tests were accomplished according to a recently developed method known as native thiol, total thiol and the parameters calculated using these two values. Simply, thiol levels and its reversible oxidation products can be measured in serum using this method. The results can be presented both separately and totally (18). Using the newly developed method, the level of ITOP can also be determined in addition to the parameters obtained by using thiol-disulphide homeostasis method. Thus, RTOP, ITOP and TOSI levels which constitute the plasma thiol profile were determined. While in the new method the first part of the sample was exposed to oxidative stress with H_2O_2, a natural oxidant that can oxidize protein thiols, the second part was not exposed (23). Native thiol and total thiol levels were measured simultaneously in both parts of the sample. As shown in Figure 1 and Table 2; RTOP and ITOP levels of the sample were determined by subtracting values of the oxidized sample from values of the non-oxidized sample measured at baseline. Also, the TOSI ratio was calculated by dividing level of ITOP by level of RTOP. Optimal concentration and duration of the application according to optimization studies were established as 750 µM H_2O_2 and 15 min. The values obtained at the end of the optimization studies were determined according to a point in which they are approximately equal to each other ($\lambda1 \approx \lambda2$) (Figure 2). Besides this, TOSI is a ratio obtained using ITOP and RTOP levels ($TOSI=\lambda1/\lambda2$) and is an index showing the resistance to the applied oxidative stress of the plasma thiol groups (Figure 1). Before hydrogen peroxide application, the native thiol (SH) level of the sample pool is the Z' point while the total thiol (SH + reversible thiol oxidation products) level is the X point. The difference between these two parameters is due to the amount of reversible thiol oxidation products. When the concentration of H_2O_2 in the sample is increased, the native thiol concentration decreases linearly as expected (from Z' to Z). The total thiol level is linearly decreased at higher H_2O_2 concentrations (from 250 µM H_2O_2 to Y), although it does not change up to the application of 250 µM H_2O_2 (from X to 250 µM H_2O_2). Therefore, H_2O_2 administration at concentrations lower than 250 µM causes RTOP to be formed, and sodium borohydride, a reductant chemical

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used in total thiol test, can convert these RTOP into thiol groups. In other words, ITOP does not occur up to the application of 250 μM H_2O_2 (Table 2, Figure 1). However, as ITOP begin to form at higher H_2O_2 concentrations (> 250 μM), these irreversible products cannot be converted to thiol groups with sodium borohydride. Therefore, the total thiol level also decreases (from X to Y). Subtraction of the total thiol level (Y point) measured after 750 μM H_2O_2 administration from initially measured total thiol level (X point) of the serum pool gives the level of ITOP. After 750 μM H_2O_2 administration, subtraction of native thiol (Z point) level from the total thiol (Y point) level gives the level of RTOP. Thus, the level of ITOP, identified as λ1, the level of RTOP, identified as λ2, and the TOSI (λ1/λ2) parameters were found. Namely, the levels of parameters reflecting resistance to applied oxidative stress of thiol groups were identified (Table 2, Figure 1). In many diseases, especially cancer diseases, the thiol homeostasis is affected (8, 24-27) . According to our first preliminary results; ITOP levels of the patients with lung and colon cancer were found to be significantly higher than the control group, while RTOP levels were found to be significantly lower. When the parameter of TOSI which is used to determine the direction of the balance was examined, it was observed that the formation of ITOP was more dominant in both colon and lung cancer when compared to the control group (Table 3). Compared with the control group, ITOP in both diseases were significantly higher due to the oxidation of the thiol groups. This may be due to a decrease in the resistance to oxidation of thiol groups in cancer patients. The mechanisms underlying the resistance to an oxidative attack of particular cancer cells were highlighted in some studies. Also, it is shown that the effects of oxidative stress-based therapies go beyond local cytotoxicity, being propagated in the close vicinity and having even a systemic echo mediated by the immune response (28-30). This test cluster (TOSI, RTOP, and ITOP) could make a contribution to clinical assessment according to our first preliminary findings in lung and colon cancers.

In conclusion, this new method demonstrates levels of reversible thiol oxidation products formed under mild oxidative stress conditions and irreversible thiol oxidation products formed under advanced oxidative stress conditions. In this way, the level of resistance to oxidation is determined in patients via thiol groups, the major antioxidant molecule in the plasma. Also, we foresee that this new method may be useful in assessing the clinical progression of the disease.

Conflict of Interests: Authors have no conflict of interest.

Financial Disclosure: There are no organizations financing the research.

Ethics Committe Aproval: Yıldırım Beyazıt University Ethics Committe of Clinical Research, 03.09.2014-138.

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