

DETERMINATION OF TOTAL ANTIOXIDANT STATUS (TAS) AS A BIOCHEMICAL PARAMETER IN CONTROL OF WORKERS' HEALTH

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Abstract — Despite its importance for human health, the capacity of the antioxidative defense system (AOS) is still not included among regular biochemical analyses. In this study, we estimated the blood plasma level of the total antioxidant status (TAS) as an appropriate parameter for AOS monitoring with the aim of including it among other biochemical parameters in the regular control of health of the working population. We have applied a method developed by Randox Laboratories (England) that can be routinely performed on automatic chemical analyzers. The TAS level, in correlation with corresponding biochemical parameters, could contribute to better evaluation of the health of individuals and the working population.

Key words: Antioxidant status, oxidative damage, antioxidative defense, health control, Daytona automatic chemical analyzer

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INTRODUCTION

Production of reactive species, mostly of oxygen (ROS), is an integral part of every aerobic metabolism. Because of the high potential of ROS, especially very reactive free radicals (FR), to damage vital biological systems by forming oxidatively modified biomolecules (proteins, lipids, nuclei acids) that disturb their relationships and organization (Slater, 1987; Halliwell and Gutteridge, 1989; Floyd and Carney, 1992), living organisms have been forced to develop an antioxidative defense system (AOS). The human AOS has a complex and multilevel organization with the role of counteraction this reactivity by preventing formation or propagation of reactive oxygen and nitrogen species and reducing or repairing damage already done (Halliwell et al., 1992; Halliwell, 1996; Frei, 1999). As a first level of defense, the AOS includes enzymes, mostly intracellularly located, such as several forms of superoxide dismutase (SOD), glutathione peroxidases (GSHPx), glutathione-S-transferases (GST), and catalase (CAT); a group of macromolecules including albumin, ceruloplasmin, and ferritin; several small-molecule anti-

oxidants (present extra and intracellularly); including reduced glutathione (GSH), ubiquinol-10, uric acid, bilirubin, lipoic acid, methionine, and vitamins (ascorbic acid, α -tocopherol, β -carotene); and several micro or trace elements (Pryor, 1994; Jacob, 1995). Several hundred small-molecule compounds with antioxidant properties are currently recognized (Chapple, 1997). Most of them are derived from dietary sources (Djujić et al., 2000), but the cell itself synthesizes a minority of these molecules, ones such as glutathione and NADPH. The AOS has its own redox scavenging (injury-repairing) capacity that very much depends on the individual's antioxidative status (from the genetic base to current environmental influence). Under normal physiological conditions, a balance exists between production of ROS and AOS activity. Many factors disturb this equilibrium, thereby reducing the organism's defense capacity and allowing uncontrolled oxidation of biomolecules. Such factors include exposure of the organism to environmental toxicants, hazardous chemicals, and heavy metals; ionizing radiation; miscellaneous xenobiotics; and endogenous pathological metabolic changes. Generally,

depletion or incapability of the AOS for defense leads to a condition in the organism known as "oxidative stress". Oxidative stress has now been recognized to entail serious pathological changes in the organism that are related to more than a hundred disease states (Halliwell et al., 1992; Braganza et al., 1995) such as atherosclerosis (Witztum, 1994; Heinecke, 2003); rheumatoid arthritis (Heliovarra et al., 1994); cardiovascular diseases and primary heart attacks (Oliver, 1995; Miller, 1997); amyotrophic lateral sclerosis (Glabinski et al., 1993); cataract; neurodegenerative disorders like Parkinson's disease (Olanow and Tatton, 1999); Alzheimer's dementia and strokes (Ikeda and Long, 1990); cancers (Block, 1992; Afrasyap et al., 1998); and autoimmune diseases and factors underlying the aging process itself (Harman, 1995; Reiter, 1995). Oxidative stress, as a state of imbalance between prooxidative and antioxidative processes, can thus be the cause and also the consequence of most illnesses. In the light of these facts, estimation of the level of oxidative stress and the capacity of the AOS for defense is important for health of both the population at large and individuals.

The best way to monitor ROS activity and levels of the main AOS components in particular situations and obtain a complex picture contributing to health control would be by direct measurement. In routine practice, most of these methods are still time-consuming, labor-intensive, and costly. Moreover, they require complicated techniques and suffer from a lack of standardization and referent levels.

For practical reasons and because of the need for such data, biochemists have tried to develop a one-step method to quantify a body's antioxidant capacity or status. Using bodily fluids, mostly blood, different analytical methods for assessing total antioxidant capacity have appeared. These methods differ, depending on what technology and which FR generator or oxidant has been used. They include determination of the total peroxyl radical-trapping parameter (TPRAP), the most widely used assay of antioxidant capacity during the last decade (Wayner et al., 1985); fluorescence-based total antioxidant capacity (TAC) (Miller et al., 1993; Ghiselli et al., 1994); total antioxidant activity (TAA) in a phycoer-

ythrin fluorescence-based assay (Glazer, 1990); total antioxidant power (TAOP) or the ferric-reducing ability of plasma (FRAP) (Benzie and Strain, 1999; Benzie et al., 1993; Cao et al., 1995); total antioxidant status (TAS) or Trolox equivalent antioxidant capacity (TEAC) by a colorimetric method (Miller et al., 1993; Rice-Evans and Miller, 1994; Re et al., 1999); and other parameters. In the course of time, most of those methods have been improved or modified (Johnstone et al., 2006) and have additionally been used in the food and cosmetics industries (Wang et al., 1996; Arendt et al., 2001) to analyze the antioxidant potential of products for commercial uses.

The main aim of this study was to present a way of monitoring the state of the AOS by estimating antioxidant capacity as TAS (e.g., Trolox equivalent antioxidant capacity of TEAC) in blood plasma using the colorimetric method of Rice-Evans and Miller (Rice-Evans and Miller, 1994). This technique measures the ability of antioxidants in physiological fluids to quench absorbance of the FR cation formed by the reaction of 2,2-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS), a specific chromogene, with peroxidase (metmyoglobin) and hydrogen peroxide (Miller et al., 1993). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), an analog of vitamin E, is used as an antioxidant standard. This method was adopted by Randox Laboratories (Crumlin, England) for a commercial kit, which is also suitable for automatic biochemical analyzers. We advocate the addition of TAS analysis to the usual biochemical parameters in health control, especially for people working in a potentially hazardous environment, such analysis being performed by an automatic biochemical analyzer (in our study, one of the Daytona type). In addition, we compare our results of TAS measurement with results from other laboratories applying the same techniques. We also attempt to establish correlations between TAS and levels of biochemical blood compounds related to AOS capacity (serum total proteins, bilirubin, urea, creatinine, and fasting glucose).

MATERIALS AND METHODS

This study was conducted within the framework of periodical regular control of the health of

98 males working in the metallurgical industry, education, and nuclear research at the Department of Radiological Health Protection, Vinča Institute of Nuclear Sciences, Belgrade, Serbia. All blood sample donors were informed about the objective of the study and were asked for their permission before taking the blood sample. Personal data were considered confidential and were used only for statistical analysis, such data including gender, age, and physicians' conclusions for statistical evaluations. To establish the referent TAS value for the healthy working male population, individuals with a diagnosis of some acute or chronic disease, for example diabetes, malignancy, or high blood pressure (systolic blood pressure – SBP > 140 mm Hg and diastolic blood pressure – DBP > 90 mm Hg), were excluded from the group, leaving 72 relevant samples.

Chemicals for the TAS assay were ones supplied with the Randox TAS kit (metmyoglobin, hydrogen peroxide, ABTS chromogen, diluting agent, sample of control plasma, and Trolox as an equivalent antioxidant capacity standard). Additional chemicals and reagents for blood plasma sampling and measurement not supplied by Randox were obtained from Sigma-Aldrich (USA).

Samples for plasma TAS were prepared by separation of blood elements from heparinized and centrifuged (10 min, 4°C, 2500 x g) venous blood taken for biochemical analyses during regular periodic health control. Plasma samples were stored without further preparation at -20°C until use for TAS measurement.

The TAS assay was conducted applying Randox TAS kit components with Trolox as an equivalent standard (Rice-Evans and Miller, 1994). The assay is based on generation of a stable radical cation (ABTS•+) from a reaction where metmyoglobin, which acts as a peroxidase, in the presence of hydrogen peroxide form a radical that interacts with the chromogen ABTS. Quenching of absorbance of this species at 600 nm by plasma or individual antioxidants can be quantified and then compared to that from Trolox as a standard under the same conditions. The results are expressed as mmol Eq Trolox/L of plasma (Pl).

The TAS analyses were performed on a new-generation Daytona (RX) automatic chemical analyzer according to instructions provided by the Randox Co. The protocol quantified absorbance of plasma samples at 600 nm. The Trolox standard and the control plasma sample were run at the same time. From the same blood samples, blood chemistry, i.e., serum concentrations of total protein, cholesterol, triglycerides, urea, total bilirubin, creatinine, and glucose, as well as the activities of aspartate aminotransferase (AST), γ -glutamyltransferase (GGT), and alkaline phosphatase (AP), were evaluated on the same Daytona automatic analyzer according to routine protocols and standards.

For statistical analysis, the obtained values of biochemical blood parameters for the analyzed group of 86 working males and a referent group of 72 subjects at the end were expressed as means (AV) \pm standard deviation (SD) with the standard error of the mean (SE). The parametric coefficient of correlation was used to cross-compare TAS values [mmol/L Pl] of 72 plasma samples with selected biochemical parameters of compounds related to AOS capacity: total protein (TP), urea, bilirubin (TBil), creatinine (Cre), and glucose, with the confidence level estimated by regression analysis (using Microcal Origin statistics). The difference between two parameters or levels was considered statistically significant at $p < 0.05$.

RESULTS

Measuring of blood plasma samples for TAS was performed on a Daytona (RX) automatic chemical analyzer using the Randox TAS kit assay with Trolox as a standard. Variations were shown to be less than 3%. The mean TAS level for our group of 86 individuals (after excluding persons with a diagnosis of some acute or chronic disease, for example diabetes, malignancy, or unregulated blood pressure) with mean age of 47.5 ± 7.5 (31-60) was 1.779 ± 0.103 (0.011) mmol Trolox Eq/L Pl (min 1.40 and max 2.06 mmol/L). This mean TAS level was within the established normal range of 1.4-1.9 mmol/L given by the Randox protocol. Only six values exceeded our normal range, and that represents 7% of the analyzed samples.

Table 1. Blood plasma or serum mean levels (AV) of TAS and relevant biochemical parameters with correlations to TAS for healthy working men. Plasma TAS, serum total protein, urea, bilirubin, creatinine, and glucose were expressed as mean levels of 72 samples, AV \pm SD (SE). Differences at the level of $P < 0.05$ are considered to be statistically significant. N.S. - non significant correlation.

	AV \pm SD (SE)	Correlation (R) with TAS	$P <$
Age (years)	47.8 \pm 7.5		
TAS [mmol/L Pl]	1.768 \pm 0.102 (0.012)		
Bilirubin total [μ mol/L]	17.65 \pm 7.66 (0.91)	0.243	0.01
Urea [mmol/L]	5.84 \pm 1.35 (0.15)	- 0.085	N.S.
Proteins, total [g/L]	78.0 \pm 7.6 (0.9)	0.458	0.001
Creatinine [μ mol/L]	106.8 \pm 12.5 (1.5)	0.325	0.002
Glucose [mmol/L]	5.66 \pm 0.93 (0.52)	- 0.209	0.01
Blood pressure [mm Hg]	Sys 127.3 \pm 4.4 (0.53)	- 0.286	0.005
	Dia 79.5 \pm 4.5 (0.53)	- 0.303	0.005

Other relevant biochemical parameters measured from the same blood sample on the same Daytona automatic chemical analyzer showed the following AV \pm SD (SE) values: TBil, 18.52 \pm 7.79 (0.85) μ mol/L; urea, 5.86 \pm 1.40 (0.15) mmol/L; TP, 79.1 \pm 8.5 (0.9) g/L; Cre, 108.9 \pm 14.9 (1.6) μ mol/L; and glucose, 5.57 \pm 0.965 (0.106) mmol/L. The group of working males also had SBP 127.9 \pm 11 (1.25) and DBP 80.8 \pm 5.5 (0.69). Among them, seven men (8%) were considered to have mild hypertension (SBP > 140 mm Hg and DBP > 90 mm Hg).

To establish the referent TAS value for healthy working males coming in for regular health control, we excluded from the analyzed group individuals with higher blood pressure (leaving only normotensive subjects) and with extreme levels of more than two biochemical parameters, which left us with 72 relevant samples. Estimated TAS values, levels of some biochemical factors, and correlations of TAS with them are given in Table 1. The obtained average TAS value of 1.768 \pm 0.102 (0.012) mmol/L Pl for this referent normotensive group was not significantly different from a previous larger group, which had the same range of values (1.40-2.06 mmol/L). The other TAS-relevant biochemical parameters

also exhibited no significant difference from previous data, but had better homogeneity within the group, as was expected. The largest variation was found in the case of TBil (range 6.2-39.5 mmol/L), where 19 (26%) samples were within the overall referent range of 0-20.5 mmol/L. In the case of glucose, 16 samples (22%) were within the overall normal range of 4.2-6.4 mmol/L.

Calculated correlations between TAS and the other biochemical parameters are also presented in Table 1. Positive correlations, statistically significant as determined by regression analysis, were found between TAS and TBil ($R = 0.243$, $p < 0.01$), TP ($R = 0.458$, $p < 0.001$), and Cre ($R = 0.325$, $p < 0.002$). Between TAS and glucose, DBP, and SBP, we found negative or inverse correlations of $R = - 0.209$, $p < 0.05$; $R = - 0.286$, $p < 0.005$; and $R = - 0.303$, $p < 0.005$, respectively. There was no significant correlation between TAS and urea, only a positive trend without significance.

DISCUSSION

As it has been already mentioned, there is a constantly increasing need for new ways of monitoring human health to achieve better and faster control

and assist in diagnosing alterations. Blood TAS analysis as a measure of antioxidative defense system capacity is a relatively noninvasive method which we believe can contribute to that aim. We agree that quantitatively measuring the antioxidant capacity or antioxidant power of each relevant compound separately (or the cumulative effect of several) could provide more complete information about the antioxidant state of individuals. However, in routine health care, one-way brief analysis of TAS and other biochemical parameters can be more useful for individuals and populations, especially if estimated by automatic analyzers (Lantos et al., 1997; Ching et al., 2002; Erel, 2004; Johnstone et al., 2006).

After comparing several methods of assessing the total antioxidant capacity of human serum or plasma, we chose a method based on estimation of blood TAS with Trolox as an equivalent standard (Miller et al., 1993; Rice-Evans and Miller, 1994) because it can be used with an automatic chemical analyzer as a micromethod (if utilizes only a few μL of sample material) and has standardization established by a world-renowned laboratory (Randox). The critical reviews of this method pointed out a few problems related to technical performance. Schofield and Braganza (1996) demonstrated variations in serum TAS levels with reading time. Our results obtained using the manual variant of this method are in agreement with this observation (unpublished data). The fact is that TAS represents the total contribution from a wide range of plasma components with antioxidant properties. The combined antioxidant capacity of uric acid and albumin accounts for 51% of the total, the remaining activity being a measure of the combined power of other plasma antioxidants like creatinine, tocopherols, and ascorbate. The antioxidative rates of plasma proteins are not as high as that of Trolox as a pure substance. However, Trolox has been used as a traditional standard in measuring TAS. We agree with Lamont et al. (1997) in asserting that if estimation of serum TAS with an automatic analyzer employs standardized 3-min reading time in the same highly controlled conditions applied to all samples, variations depending on reading time are not relevant. Also, the observation that dilution of samples results

in an increase in the TAS level is correct, but in practice this is a negligible disadvantage (a serum or plasma TAS value > 2.5 mmol/L is the linear limit of method). Some critical comments have called attention to the fact that the plasma sample itself can influence the reliability of TAS measurement. The presence of lipids in the blood and in blood plasma (> 10.0 g/L), because of their turbidity, leads to increased absorbance of samples. Also, because of their color, icteric serum and haemoglobin (hemolysis causes the release of constituents from red blood cells) show a weak increase of absorbance in TAS measurement. The application of a micromethod employing an automatic analyzer reduces those errors to a great extent.

In our study, all extreme conditions pertaining to plasma samples or other conditions were avoided because we tried to establish the referent range of TAS in plasma of the normal working male. In automatic sampling, the plasma's relative dilution is high already, and the concentration of anticoagulants therefore is very low and does not affect incubation medium components. Thus, the anticoagulants did not act as either prooxidants or antioxidants. Analyses of the Trolox standard and referent plasma from the Randox kit were performed in triplicate with good agreement. With the employed method, there was no statistically significant difference of TAS values between serum and plasma (despite the difference in protein concentration).

The Randox TAS kit that we used and the automatic micromethod of estimation (by a Daytona chemical analyzer) allow various laboratories to perform TAS analyses under the same analytical conditions, reducing errors stemming from analytical procedure and permitting valid comparison of results. The obtained mean TAS value (1.768 ± 0.102 mmol/L of PI) was in the range of data obtained by other authors employing the same method, viz., 1.41 ± 0.12 mmol/L (Cao and Prior, 1998) and 1.48 to 1.63 mmol/L (Lamont et al., 1997), and in the upper part of the Randox reference range of 1.30-1.77 mmol/L of PI, but was 39% higher than that obtained by Koracevic et al. (2001) (1.08 ± 0.28 mmol/L of PI). The differences in mean TAS levels may result from the choice of groups in establishing

the control range (sex, mean age, environmental factors, life style). The applied method is precise enough to distinguish various populations and their plasma TAS values.

The correlations of mean TAS values to levels of relevant biochemical parameters (TP, creatinine, glucose, SBP, and DBP) for our working population were also in good agreement with results obtained by other authors (Lamont et al., 1997), with significance demonstrated by regression analysis. Unexpectedly, we found no correlation between TAS and mean urea concentrations in our male working group, although urea can be considered one of the main antioxidant compounds in blood plasma, along with albumin and ascorbate. The explanation could lie in the applied method of uric acid estimation or in the kind of working group we chose for TAS analysis. Further epidemiological investigation and TAS estimation of other populations are needed to establish more TAS referent ranges.

CONCLUSIONS

Our estimation of blood plasma TAS levels in a group of working people using established technical and chemical procedure (Randox TAS assay) and an automated chemical analyzer (Daytona RX) showed that TAS as a biochemical parameter can be easily included in regular health control of our working population and is additionally useful in medical diagnostics. This method of TAS analysis is easily performed, rapid, and reliable, and only small samples of biological material are needed for the analyses. Its low-cost makes it practical for routine measurement in our conditions. The results are comparable with standards from other countries and are correlated with related biochemical parameters already included in health control.

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УКУПНИ АНТИОКСИДАНТНИ СТАТУС (TAS) КАО БИОХЕМИЈСКИ ПАРАМЕТАР У ЗДРАВСТВЕНОЈ КОНТРОЛИ ЗАПОСЛЕНИХ

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Нова сазнања о улози слободнорадикалске равнотеже у успостављању и одржавању хомеостазе у организму условила су потребу да се одређивање параметара система заштите од оксидативних оштећења уведе међу обавезне биохемијске анализе при периодичном здравственом прегледу запослених, нарочито оних који раде у потенцијално штетним условима. У овом раду желели смо да укажемо на потребу укључења анализе укупног антиоксидантног статуса (Total Antioxidant Status, TAS) из крвне плазме међу биохемијске параметре при здравственом прегледу популације запослених. Са циљем да се установе референтне вредности TAS, издвојена је група здравих, нормотензивних особа мушког пола. Мерења нивоа

TAS у плазми рађена су спектрофотометријском методом користећи дијагностички комплет фирме Randox Lab. England, на аутоматском клиничком хемијском анализатору новије генерације Daytona RX, који омогућава добру прецизност и репродукбилност коришћене микрометоде. Одређена је и корелација са другим за TAS релевантним биохемијским параметрима, који су се стандардно одређивали из истих узорака крви при прегледу запослених (билирубин, уреа, укупни протеини, креатинин, глукоза, крвни притисак). Вредности TAS параметра доприносе комплетнијем мониторингу здравља како појединца, тако и одређене популације, нарочито у вези утицаја услова рада и животне средине.