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Application of constant-current coulometry for estimation of plasma total antioxidant capacity and its relationship with transition metal contents

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Abstract

Simple and express coulometric method for the evaluation of the total antioxidant capacity (TAC) of human plasma based on the reaction with electrogenerated bromine is applied. TAC of plasma from patients with different ethiology of chronic renal failure was observed. The levels of antioxidant capacity for venous and arterial plasma are authentically different (15 ± 1 kCl/L versus 11.7 ± 0.7 kCl/L, $p < 0.01$). The application of Vitamin E and ximedon as an antioxidant treatment significantly increase TAC level of plasma. Free liposoluble antioxidants in plasma in α-tocopherol units was determined. Redox potential of plasma is measured and its correlation with lg(TAC) is obtained. Transition metal contents of Fe, Cu, Mn, Ni, and Cr in plasma of patients with chronic renal failure is significantly higher than that for a control group. Correlation analysis has shown negative linear regression between TAC value and transition metals concentration in plasma. This confirms interrelation of processes with participation of free radicals, antioxidants and transition metals as donors of electrons in chain radical processes. Moreover, it shows utility of common parameters, TAC for example, for estimation of efficiency of antioxidant defense system in living organism, in particular its antioxidant status.

Keywords: Total antioxidant capacity; Coulometric titration; Chronic renal failure; Human plasma; Antioxidant therapy; Transition metals; Atomic absorbtion spectroscopy; Redox potential

1. Introduction

Oxygen free radicals are the chemical species formed in all tissues and cells during normal aerobic cellular metabolism that can damage the various intracellular components on which correct cell functioning depends [1].

The free radicals are molecules with an unpaired electron on the outer orbit [2]. They are generally unstable and highly reactive. Examples of reactive oxygen species (ROS) are superoxide anion radical ($O_2^{\bullet -}$), hydroxyl, peroxyl ($RO_2^{\bullet}$), alkoxyl ($RO^{\bullet}$) and hydroperoxyl ($HO_2^{\bullet}$) radicals. Nitric oxide ($NO^{\bullet}$) and nitrogen dioxide ($^{\bullet}NO_2$) are the two nitrogen free radicals. Oxygen and nitrogen free radicals can be converted to other non-radical reactive species, such as hydrogen peroxide, hypochlorous acid (HOCl), hypobromous acid (HOBr) and peroxynitrite ($ONOO^-$). ROS, reactive nitrogen and chlorine species are produced in animals and humans under physiological and pathological conditions [3].

Free radicals can play an important role in the origin of life and biological evolution, implicating their beneficial effects on the living organisms [4]. For example, oxygen radicals exert critical actions such as signal transduction, gene transcription and regulation of soluble guanylate cyclase activity in cells [5,6]. Also, $NO^{\bullet}$ is one of the most widespread signaling molecules that participates in virtually every cellular and organ function in the body. Physiologic levels of $NO^{\bullet}$ produced by endothelial cells are essential for regulating the relaxation and proliferation of vascular smooth muscle cells, leukocyte adhesion, platelet aggregation, angiogenesis, thrombosis, vascular tone, and hemodynamics [7]. In addition, $NO^{\bullet}$ produced by neurons serves as a neurotransmitter, and $NO^{\bullet}$ generated by activated macrophages is an important mediator of the immune response

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[8]. However, as oxidants and inhibitors of enzymes containing an iron–sulfur center, free radicals and other reactive species cause the oxidation of biomolecules (e.g., protein, amino acids, lipid, and DNA), which leads to cell injury and death [4,8]. For example, radiation-induced ROS markedly alter the physical, chemical, and immunological properties of superoxide dismutase [9], which further exacerbates oxidative damage in cells. The cytotoxic effect of free radicals is eleterious to mammalian cells and mediates the pathogenesis of many chronic diseases, but is responsible for the killing of pathogens by activated macrophages and other phagocytes in the immune system [4]. Thus, there are "two faces" of free radicals in biology in that they serve as signaling and regulatory molecules at physiologic levels but as highly deleterious and cytotoxic oxidants at pathologic levels [8].

High level of generation of ROS and other radical species leads to oxidative stress. The last may be defined as a disruption of the balance between the level of oxidants to reductants in the living organism. Thus, oxidative stress can result from increased exposure to oxidants or from decreased protection against them, or even from both problems occuring simultaneously [10].

Transition metals such as iron and copper are ubiquitous metals in the cells, present in the structure of many enzymes and proteins. As transition elements, their ionic forms are prone to participate in one-electron transfer reactions, and this is one important attribute for their use as prosthetic groups in enzymes that catalyze redox reactions. However, this capacity enables iron and copper to generate radical species as well. For example, copper and iron participate in the Fenton reaction cycle in which the very reactive hydroxyl radical is generated [11]:

$$H_2O_2 + Fe^{2+} \rightarrow HO^{\bullet} + HO^{-} + Fe^{3+}$$

The rate constant for the Fenton reaction is higher for copper than for iron [12]. However, the abundance of iron in biological systems, as compared to copper, makes it a more likely source of $^{\bullet}$OH radicals [13,14]. A combination of iron accessibility and increased production of intermediates of oxygen reduction, like hydrogen peroxide and superoxide anion-radical, generates a prooxidant status in the cell. Furthermore, transition metals may also behave as non-enzymatic (pseudo-) peroxidases that bio-activate benign catechol-containing compounds (such as dopamine) into toxic *ortho*-semiquinone radicals [15].

Living cells are not defenseless against the oxygen radicals and other activated species to which they are constantly exposed. All aerobic organisms, including human beings, have various adaptive mechanisms of protection from oxidant damage. The antioxidant defense system is of major importance [16,17]. The term "system" is used here deliberately, since we believe the many different antioxidants work together in concert for the homeostatic regulation of the body's redox status.

All biological antioxidants can be classified into two principal groups: the enzymes and the low-molecular weight antioxidants [18]. The antioxidant enzymes consist of superoxide dismutase, catalase, peroxidase, glutathione reductase and contain a limited number of proteins (transferrin, ferritin, ceruloplasmin). The low-molecular weight antioxidants group contains a large number of compound capable of preventing oxidative damage by direct and indirect interaction with ROS [19].

Generally, an antioxidant may act in one of the three possible ways:

- preventing the production ab initio of ROS and their destruction with subsequent reactions (e.g., chelating agents: proteins; and catalase);
- chain-breaking/scavenging the free radicals (e.g., α-tocopherol, ascorbic acid);
- enzyme system to repair the damage to macromolecules caused by ROS and their derivatives [20].

Total antioxidant capacity (TAC) is the parameter summarizing overall activity of antioxidants and antioxidant enzymes.

Various methodologies have been proposed for the TAC determination. One of the main approach is two-part system based on the generation of different free radicals such as 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), crocin, and their detection (end-point), which is affected by the addition of an antioxidant to the system [21–25].

The chemilumiscence and fluorescence methods of TAC determination in living cells and tissues have been used [26,27].

High-performance liquid chromatography (HPLC) coupled with a coulometric multi-electrode array system provides specificity and sensitivity for determination of multiple redox-active low-molecular weight compounds based on differences in oxidation–reduction properties of analytes [28,29].

Another method based on HPLC coupled with electrochemical (coulometric) detection applied for determination the physiological levels of epicatechin, catechin and epicatechin dimmers which contents reflect antioxidant status of human organism [30].

A new interesting method has been developed that is capable of providing a complete profile of the most common monothiols and disulfides present in plasma or tissue extracts. The method utilizes reversed phase ion-pairing HPLC coupled with coulometric electrochemical detection to simultaneously quantify free oxidized and reduced aminothiols or total aminothiols after chemical reduction [31].

However, these different methodologies give different assessments of the relative importance of the antioxidant species and TAC and may lead to misleading conclusions.

At last electrochemical methods were successfully applied for TAC estimation of biological fluids, tissues [32–34].

Therefore, development of techniques for the TAC determination is still important. As shown earlier, coulometry is characterized by simplicity, sensitivity, cost-effeciency, precision, accuracy, and speed and may be useful in analysis of complicated biological objects.

2. Materials and methods

2.1. Patients

Thirty healthy volunteers, at the age of 21–54 years, were selected to form a control group for the determination of

the reference interval of the TAC. They were on a normal diet.

One hundred patients (44 males and 56 females) with chronic renal failure who have undergone hemodialysis treatment at the Center of an-of Kidney Organism Clearing (Department of Hemodialysis, Kazan) were included in the study. All patients were dialyzed three times a week for 3 h each session. Patients with higher proteinuria (over 300 mg/L) were excluded from the study. Patients were examined before standard session of hemodialysis using a dialyzer with polysulfon membrane (Fresenius) with an area of 1.3 m^2. Patients were on the odered diet and regimen.

The etiologies of the patients renal disease were chronic glomerulonephritis, pyelonephritis, polycystic disease and diabet mellitus.

Two groups of patients (22 and 9 persons) treated with antioxidant therapy by Vitamin E (0.2 g/24 h) and ximedon (2 g/24 h) per os. within month.

2.2. Preparation of plasma

Venous and arterial blood was collected before standard session of dialysis in glass tubes containing a small amount of heparin as an anticoagulant.

Plasma was obtained by centrifugation at 3000 rpm for 5 min and immediately was analysed for TAC and other parameters.

2.3. Determination of TAC

Determination of TAC is based on the coulometric titration of plasma by electrogenerated bromine [35–37].

2.4. Coulometric determination of free liposoluble antioxidants in human plasma

The procedure is based on the extraction of liposoluble antioxidants from human plasma by the 1:1 ligroin–alcohol mixture with further coulometric titration by electrochemically generated Br_2.

Bromine was electrochemically generated by a P-5827 M potentiostat at a constant current of 5.0 mA from 0.2 M $(C_2H_5)_4NBr$ in 0.1 M $HClO_4$ acetonitrile solution with 100% current yield. The titration end-point was detected amperometrically using two polarized platinum electrodes ($\Delta E = 300$ mV). The working electrode was a plain platinum plate of 1 cm^2; the auxiliary electrode was a platinum coil separated from the anode compartment of the cell by a semipermeable diaphragm.

A supporting electrolyte (20.0 mL) was placed in a 50 mL cell, the electrodes were dipped, and the generation circuit was turned on. After a certain value of the indicator current was achieved, a portion (0.5 mL) of the test extract was added into the cell and a stopwatch was started simultaneously. The titration end-point was detected by the achievement of the initial value of the indicator current. At that moment, the stopwatch was stopped and the generation circuit was turned off.

Content of free liposoluble antioxidants in plasma was expressed in α-tocopherol units.

2.5. Determination of plasma redox potential

Five milliliters plasma were added into the 50 mL electrochemical cell. The working (platinum microelectrode), auxiliary, and saturated calomel electrodes were immersed. A platinum coil separated from the anodic compartment with a semipermeable diaphragm served as the auxiliary electrode.

The redox potential of plasma was measured after 1 min.

2.6. Determination of transition metals in plasma by atomic absorbtion spectroscopy

The measurement of transition metals content was carried out on SIMAA 6000 (Perkin-Elmer, USA) multielement atomic absorbtion spectrometer equipped with a transversely heated electrothermal atomizer, THGA, autosampler AS-72 and longitudinal Zeeman-effect background correction system. A Perkin-Elmer Fe, Cu, Mn, Cr, and Ni hollow-cathode lamps operated at 15 mA were used as primary sources. Fe (305.9 nm), Cu (324.8 nm), Mn (279.5 nm), Cr (357.9 nm) and Ni (232.0 nm) resonance lines were selected for the determinations. Pd (5 μg) + $Mg(NO_3)_2$ (3 μg) modifier was used for the transition metals assay.

All the reagents used were of analytical-reagent grade. Multielement standard solutions for calibration were prepared from single-element stock solutions (Merck, Darmstadt, Germany) in 0.2% (w/v) nitric acid.

The human plasma samples were analysed directly after 1 + 2 dilution with ultra-pure water for Cr and Ni and 1 + 4—for Fe, Cu, and Mn. 0.2% HNO_3 could not be used for sample dilution because this would lead to relatively high blank values.

2.7. Statistical analysis

Statistical analysis of the results was performed using SPSS for Windows. All data are expressed as the mean value ± S.D. The difference of parameters were tested by Student's *t*-test. A $p < 0.05$ was considered as statistically significant.

The correlation analysis of data was performed using parametric methods with the aid of the Origin v 6.1 (OriginLab Corporation).

3. Results

3.1. TAC of plasma from patients with chronic renal failure

Venous plasma TAC levels were estimated in the control and patient's groups and it was found that they differ significantly (27.1 ± 0.9 kCl/L versus 15 ± 1 kCl/L, $p < 0.01$).

Fig. 1 shows the plasma's TAC for different types of chronic renal failure ethiologies and healthy controls. As can be seen, there are significant decrease of plasma's TAC level ($p < 0.01$) in hemodialyzed patients compared to the control group independently from ethiology of chronic renal failure. But a difference

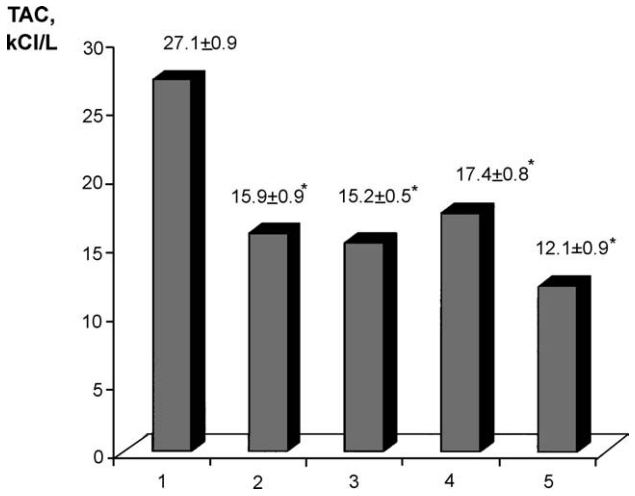

Fig. 1. Total antioxidant capacity of plasma for healthy group and patients with chronic renal failure: (1) control group; (2) chronic glomerulonephritis; (3) chronic pyelonephritis; (4) polycystic disease; (5) diabet mellitus.

in the TAC in the patients group is obtained. No significant differences were found in the TAC levels for patients with chronic glomerulonephritis and pyelonephritis (15.9 ± 0.9 kCl/L versus 15.2 ± 0.5 kCl/L). The highest value of TAC is observed for the polycystic disease (17.4 ± 0.8 kCl/L) and this difference is statistically significant. Diabet mellitus is characterized by the most decreased TAC level and it is significantly lower than in the other cases of chronic renal failure ($p < 0.01$).

The levels of antioxidant capacity for venous and arterial plasma are authentically different (15 ± 1 kCl/L versus 11.7 ± 0.7 kCl/L, $p < 0.01$).

The effect of antioxidant therapy was also investigated. The application of Vitamin E and ximedon increases TAC level of plasma (Table 1).

The TAC value is significantly increased after antioxidant therapy, but does not reach the level for the control group.

Free liposoluble antioxidants in plasma in α-tocopherol units were determined (Table 2). There is statistically significant difference in the free liposoluble antioxidants level before and after treatment by Vitamin E (1.1 ± 0.4 g/L versus 2.0 ± 0.3 g/L, $p < 0.01$).

Redox potential of plasma is determined and its correlation with lg(TAC) is obtained. The reverse linear regression is described by following equation $Y = a + bX$, where $a = 1.300 \pm 0.006$, $b = -(45 \pm 2) \times 10^{-4}$, $R = 0.9958$.

The content of transition metals Fe, Cu, Mn, Ni, and Cr in plasma of patients with chronic renal failure is higher than for the control group (Table 3) and this difference is statistically significant.

Correlation analysis has shown a linear regression between TAC value and transition metals concentration in plasma (Table 4) with the exception of nickel.

Table 1
Effect of antioxidant therapy by Vitamin E and ximedon on plasma's TAC level after 1 month of treatment

Antioxidant	Total antioxidant capacity (kCl/L)			
	Venous plasma		Arterial plasma	
	Before treatment	After treatment	Before treatment	After treatment
Vitamin E	15 ± 1	24 ± 1[a]	11.7 ± 0.7	19.9 ± 0.6[a]
Ximedon	15 ± 1	20 ± 2[b]	11.7 ± 0.7	17 ± 1[a]

[a] $p < 0.01$ vs. before treatment.
[b] $p < 0.05$ vs. before treatment.

Table 2
Plasma's free liposoluble antioxidants level in α-tocopherol units for patients with chronic renal failure

No.	Free liposoluble antioxidants level in α-tocopherol units (g/L)	
	Before treatment	After treatment
1	1.0 ± 0.1	1.9 ± 0.1
2	1.5 ± 0.1	1.65 ± 0.08
3	1.27 ± 0.09	1.8 ± 0.1
4	0.61 ± 0.08	2.4 ± 0.3
5	0.78 ± 0.07	2.0 ± 0.2
6	1.27 ± 0.09	2.02 ± 0.09
7	1.5 ± 0.2	2.0 ± 0.1
8	1.4 ± 0.1	1.95 ± 0.08
9	1.45 ± 0.09	2.1 ± 0.2
10	0.51 ± 0.06	2.3 ± 0.2

Table 3
Comparison of plasma iron, copper, manganese, nickel, and chromium concentration of patients and healthy subjects ($n = 13$)

Parameters (μg/L)	Patients	Healthy subjects	p
Fe	2504 ± 620	1111 ± 161	<0.05
Cu	3230 ± 490	1002 ± 147	<0.01
Mn	76 ± 20	5 ± 2	<0.05
Ni	90 ± 25	4 ± 1	<0.05
Cr	60 ± 20	30 ± 5	<0.05

Table 4
Correlation parameters between plasma's TAC and transition metals

Metal	Regression equation $Y = a + bX$		R
	a	$b \times 10^4$	
Fe	15.8 ± 0.3	-4.1 ± 0.8	0.8735
Cu	16.6 ± 0.4	-5 ± 1	0.8362
Mn	14.9 ± 0.2	-199 ± 43	0.8923
Cr	17.3 ± 0.7	-288 ± 63	0.9158

4. Discussion

Our data indicate that chronic renal failure is characterized by intensive decrease of antioxidant defence in organism and prooxidant state in plasma. This is in accordance with previous studies [38–40].

Significant changes in TAC of plasma for different types of chronic renal failure ethiologies is found. TAC value not significantly differs in glomerulonephritis and pyelonephritis cases. This is caused by the similar processes in kidney accompanied by injury of parenchyma. The lower TAC is obtained for diabet

mellitus, which is the most serious and dangerous disease and complicates significantly the renal failure status.

TAC for arterial and venous plasma is significantly differ. As known, arterial blood is saturated by oxygen. The first product of oxygen molecular activation is superoxide anion-radical ($O_2^{\bullet -}$). It is the source of all active forms of oxygen in vivo which intensively decrease the antioxidant capacity [41]. So, arterial plasma is characterized with the smaller antioxidant capacity.

So far as patients with chronic renal failure exhibit the decreased TAC, the efficiency of supplementary antioxidant therapy by Vitamin E and ximedon was investigated. The data obtained allow us to conclude that antioxidant therapy significantly increases TAC of plasma that is also confirmed in [42,43]. At first, it was shown that ximedon is effective as antioxidant that permits to enlarge the application area of this pharmaceutical.

Moreover, the authentical increase of free liposoluble antioxidants level after treatment by Vitamin E was observed. So, the intensity of lipid peroxidation in cells could be decreased by antioxidants which act as scavenger of free radicals in living systems.

The redox potential (E_h) of plasma has been determined and it correlates with lg(TAC).

It should be noted that redox potential is a measure of ability of a chemical system to exchange electrons with its environment. The situation concerning redox poise or balance may be likened to that of pH. The latter deals with the transfer of protons while the former is concerned with the transfer of electrons. The two equations defining the relationships show interesting similarities:

Henderson–Hasselbach equation for pH:

$$\mathrm{pH} = \mathrm{p}K + \lg\frac{[\mathrm{acid}]}{[\mathrm{base}]}$$

Nernst equation for redox balance:

$$E_h = E_0 + \frac{RT}{nF} \cdot \lg\frac{[\mathrm{oxidant}]}{[\mathrm{reductant}]}$$

Theoretically, the redox potential exists only in a system at equilibrium; therefore, the redox state is at best a steady-state approximation. An oxidant is the oxidized form of a redox system which serves as an electron acceptor in the couple. If a pH measurement is the sum effect of the different acid–base systems, then the redox state may be considered the sum of the different oxidant/reductant pairs.

$$E_{\mathrm{total}} = E_{\mathrm{system}(1)} + E_{\mathrm{system}(2)} + E_{\mathrm{system}(3)} + \ldots + E_{\mathrm{system}(n)}$$

The relationship between the pair is oxidant + electron(s) < = > reductant, which implies that depending on the physico-chemical circumstances an oxidant may become an antioxidant and vice versa. The clinical implication of maintaining a "normal" redox balance is to keep proteins and enzymes in their functional state and to maintain reducing equivalents ($NADH/NAD^+$, for example) for metabolic regulation [20].

So, correlation between redox potential and TAC shows that TAC determination based on the reaction with electrogenerated bromine correctly reflects antioxidant state of plasma.

As known, transition metals play one of the key roles in the ROS generation and the subsequent oxidative stress [44–47]. Our investigation has shown that patients with chronic renal failure are characterized by significantly high value of transition metals in plasma. This is indirect illustration of predisposition to oxidative stress development in organism particularly to lipids peroxidation.

TAC correlation with transition metals contents confirms interrelation of processes with participation of free radicals, antioxidants and transition metals as donors of electrons in chain radical processes. Moreover, it shows efficiency of common parameters, TAC for example, for estimation of efficiency of antioxidant defense system in living organism, in particular its antioxidant status.

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