DOI 10.1007/s10517-016-3362-x
Bulletin of Experimental Biology and Medicine, Vol. 161, No. 1, May, 2016

EXPERIMENTAL METHODS FOR CLINICAL PRACTICE

Kinetic Chemiluminescence as a Method for Oxidative Stress Evaluation in Examinations of Patients with Type 2 Diabetes Mellitus

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 161, No. 1, pp. 149-152, January, 2016
Original article submitted February 13, 2015

We propose a new approach to evaluation of oxidative stress based on kinetic chemiluminescence: measurement of antioxidant and pro-oxidant activities of the plasma. The study included 50 patients with type 2 diabetes mellitus receiving peroral hypoglycemic therapy. In addition to the above parameters, the levels of TBA-reactive products, inflammation markers, clotting parameters, and biochemical values were studied. The new method provides information on oxidative stress in patients with type 2 diabetes mellitus irrespective of the clinical and laboratory values. The use of this method in complex with the clinical, laboratory, and instrumental studies allows comprehensive evaluation of patient's status for the diagnosis and choice of therapy.

Key Words: *oxidative stress; blood plasma; kinetic chemiluminescence; type 2 diabetes mellitus*

Recent data indicate that oxidative stress is involved in the development of diabetes mellitus, specifically, in the pathogenesis of insulin resistance and cell dysfunction – the main mechanisms of development of type 2 diabetes mellitus (DM2) and its vascular complications [2,4,7,8]. High glucose level activates various enzyme cascades in mitochondria, activates NADPH-oxidase, uncouples NO synthases, and stimulates xanthine oxidases [5]. Lipid peroxidation and protein glycation in fact unite the carbonyl and oxidative stress in a common metabolic circle [6].

Oxidative stress consists in imbalance between the oxidant and antioxidant systems, and hence, its evaluation involves quantitative description of these two constituents. The chemiluminescent (CL) method, due to its advantages (it is simple, cheap, sensitive, and informative), can be effectively used for this purpose. Numerous CL methods for measurements of ROS and evaluation of antioxidant characteristics of substances are available [9]. We suggest a new approach for simultaneous evaluation of the parameters of plasma oxidant and antioxidant systems in the same experiment.

We study the possibility of using this approach in clinical practice in patients with DM2.

MATERIALS AND METHODS

The study was carried out in 50 patients (22 women and 28 men) with DM2 receiving oral sugar-reducing therapy. The mean age of patients was 60.80±7.0 years, mean duration of diabetes 5.4±3.6 years. Com-

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prehensive clinical laboratory studies were carried out with evaluation of lipid metabolism values, glycemic control, blood clotting, parameters of chronic inflammation and oxidative stress (TBA-reactive products). Twenty normal subjects (men aged 55.5±4.0 years) previously examined by the new method within the framework of prophylactic checkup at the Central Clinical Hospital of Civil Aircraft served as control group.

The patients were enrolled into the study group according to the following criteria: age above 18 years, DM2, fasting glycemia 6-10 mmol/liter, glycated hemoglobin <10%; ALT, AST, alkaline phosphatase levels surpassing the upper threshold normal level no more than 2-fold.

Patients with hepatic and/or renal failure, severe heart failure, using estrogens or oral contraceptives, with alcohol or narcotic dependence, malignant tumors detected less than 5 years before, lung rale, pregnancy, and lactation were excluded. All patients signed consent to voluntary participation in the study after they were informed about the conditions and targets of the study.

CL analysis of the plasma was carried out on a single-cuvette chemiluminometer SmartLum 5773 (DISoft) with original PowerGraph 3.0 software. The following reagents were used: luminol, 2,2'-azo-bis(2-amidinopropane dehydrochloride (ABAP), sodium ascorbate (Fluka), KH_2PO_4 (Sigma), H_2O_2 (Sigma-Aldrich), and horseradish peroxidase (HP, 124 U/mg; Sigma).

Solution (50 mM) of ABAP (50 μ) and luminol (0.1 mM, 20 μl) were put into a microtube (1.5 ml). The mixture was processed (2 min) on a Yellow Line TTS2 vortex at 1400 rpm and incubated (20 min) in darkness at ambient temperature. An appropriate volume of buffer solution, pre-warmed (37°C) in a thermostat, and 70 μl of ABAP+luminol mixture were put into the cuvette and the fluorescence was recorded till the stationary level was attained at 37°C, after which 10-fold diluted plasma (10 μl) was added. Registration was discontinued after the signal repeatedly reached the plateau. The total volume of the sample in cuvette was 1 ml.

The signal calibration in measurements of antioxidant activity was carried out with ascorbic acid; in evaluation of the pro-oxidant activity the stationary plateau signal in system: HP+H_2O_2(0.01 mM)+luminol (80 μM)+PBS (pH 7.4) (100 mM). The Δ/CL was converted to peroxidase activity units.

Analytical values – plasma antioxidant and pro-oxidant activities – were studied in the main group and the data were compared with the values in the control group. The data were statistically processed by Statistica software (StatSoft).

RESULTS

Chemiluminescent analysis of plasma antioxidant activity is based on registration of CL kinetics in ABAP+luminol system [1]. Modified method – with a new method for calculation of analytical signal – was used in clinical studies.

The kinetic CL method consists in registration and processing of the complete CL curve reflecting its time course. Organic azocompound ABAP serves as free radical generator; during warming (37°C) it degraded to form two radicals. Fluorescence reaching the plateau I_0 (Fig. 1, phase 1) was registered in the presence of luminol. The fluorescence ceased after addition of blood plasma due to neutralization of the radicals by plasma antioxidants, primarily by uric acid (phase 2).

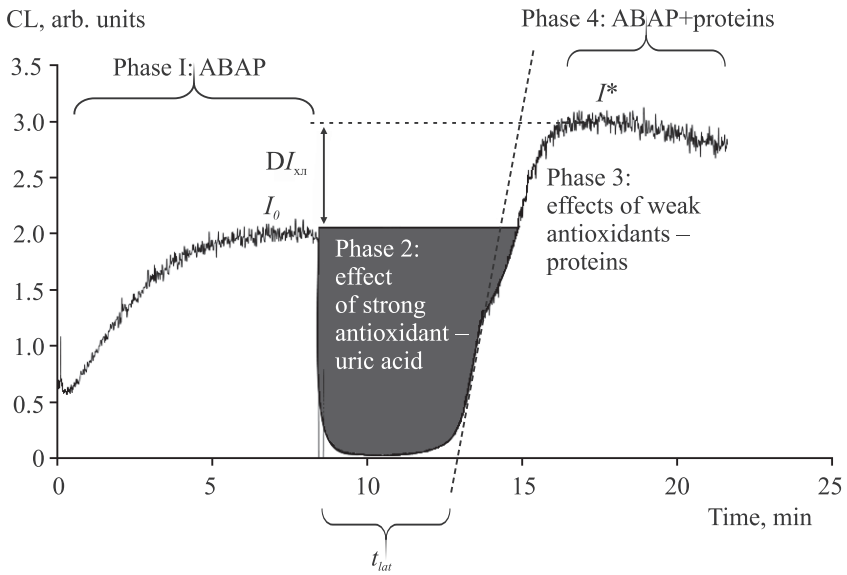

Fig. 1. Chemiluminescence curve in ABAP+luminol+plasma system. The area of the dark region is proportional to plasma antioxidant activity. Dotted line: latent period area t_{lat}.

The fluorescence restarted after the antioxidants were over (phase 3) and reached a new plateau I^* (phase 4), which could serve as the indicator of pro-oxidant activity of plasma proteins if CL level was surpassed before plasma addition.

Commonly the antioxidant activity is evaluated by the latent period – time between the beginning of phase 2 and time shown in the abscissa by the tangent (dotted line) to the rising part of CL curve (Fig. 1). This approach is not free from flaws, *e.g.*, the latent period depends on the initial stationary fluorescence I_0, while the probable effects on the weak antioxidants' CL are neglected. Estimation of the antioxidant activity by the area under the CL curve "gap" (dark area in Fig. 1) is more accurate, as the contributions of all antioxidants to the activity are taken into consideration and the result does not depend on the initial level of fluorescence [3].

Theoretically the analytical signal can be calibrated by any radical capturer, as the "gap" area is a universal measure of antioxidant activity irrespective of the antioxidant strength. Practically it is convenient to calibrate the system by a strong plasma antioxidant – ascorbic acid. The graduation curve equation is approximated by the number line:

$$y=21.3x+0.32,\ r^2=0.993$$

The data on antioxidant activity (AOA) of patients (group B) and healthy subjects (group A) differ from the normal (Shapiro–Wilk test): n(B)=50, p(B)=0.15, n(A)=20, p(A)=0.031. Description of the data massive: median (AOA-B) 1.76 mM (in conversion to sodium ascorbate), interquartile range (AOA-A) 1.64 mM, interquartile range (AOA-A) 1.32-2.12 mM.

The following data were obtained for the two independent groups by Mann–Whitney test: U=430, z=-0.78, p=0.43, which confirmed the hypothesis on the absence of a significant difference between the samples. Hence, the level of antioxidant defense in compensated patients with DM2 corresponded to the norm.

In a previous study, we have shown that an increase in the stationary fluorescence level Δ/CL (Fig. 1) is determined by changes in the blood proteins (mainly albumin) and can be used as an index for oxidative stress evaluation.

Distribution of the sign differs from the normal in group A (p=0.001) and in group B (p=0.025). In arbitrary units of HP activity: median (A) 1.34, interquartile range 1.02-2.02; median (B) 2.49, interquartile range 2.10-3.63. The hypothesis on a significant difference is confirmed by Mann–Whitney test: U=236, z=5.7, p<0.001.

Hence, the pro-oxidant activity of plasma proteins in patients with DM2 is significantly higher than in healthy subjects, this indicating a chronic oxidative stress in patients with this diabetes type.

We have shown with the use of a new precise method for estimation of plasma antioxidant activity that the antioxidant activity in patients with satisfactory glycemic control virtually does not differ from the control. A new index is suggested, which can serve as a measure of plasma protein pro-oxidant activity. The new approach provides independent information about oxidative stress in patients with DM2. The use of this method in complex with clinical, laboratory, and instrumental studies leads to more accurate objective evaluation of the patient's status and prediction of the disease course.

The study was supported by the Russian Science Foundation (project No. 14-15-00375).

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