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Changes in the Kinetics of Plasma Chemiluminescence as a Measure of Systemic Oxidative Stress in Humans

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Abstract—Oxidative stress acts as a pathogenetic factor in many diseases; estimating its level is important for early diagnosis and therapy adjustment. The antioxidant status was evaluated for the blood plasma. A set of chemiluminescence (CL) kinetic-curve parameters (latent period τ_{lat} and analytical signal increment ΔI_{CL}) in a 2,2'-azo-bis(2-amidinopropane)dihydrochloride–luminol system were proposed for estimating the oxidative stress level. Uric acid and albumin were identified as major components that are responsible for the changes in the plasma CL kinetic curve. UV light caused oxidative modification of serum albumin in a dose-dependent manner, thus enhancing its antioxidant properties. Changes in plasma CL kinetics were proposed as a means to measure oxidative stress in the human body.

Keywords: albumin, oxidative stress, oxidative modification of proteins, plasma, chemiluminescence
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INTRODUCTION

Oxidative stress (OS) manifests itself in the processes that occur as the balance between free radicals and their active metabolites, on the one hand, and the antioxidant system, on the other, shifts toward the former. Excess free-radical production causes damage to important biomolecules [1–4], thus impairing the total body function. Oxidative stress acts as a pathogenetic factor in many diseases. More than 200 diseases and pathological conditions have been shown to involve free radicals as an etiological and pathogenetic factor, including cardiovascular (cardiomyopathy, atherosclerosis, coronary heart disease, hemochromatosis, and Wegener's granulomatosis), gastrointestinal (liver injury induced by endotoxins, quinones, iron, and acetaminophen; hepatitis B; and gastric and duodenal ulcers), neurodegenerative (Parkinson, Alzheimer, and Werdnig–Hoffman diseases; epilepsy; and schizophrenia), and metabolic (amyloidosis, collagenopathy, and diabetes) disorders [5]. Monitoring oxidative stress, which is the excessive generation of free radicals that causes changes in the human body, is of both theoretical and applied interest, as it is significant for assessment of disease risk, early diagnosis, and evaluation of treatment efficacy.

Many methods and approximations to estimate the oxidative-stress level have been described in the literature, including volumetric [6, 7], electrochemical [8, 9], and photometric methods. The last group includes colorimetric measurement of the total antioxidant capacity (TAC) [9], use of an ABTS/H_2O_2/horseradish peroxidase system [10], and estimation of the Trolox equivalent antioxidant capacity (TEAC) [11]. Spectrophotometric techniques measure deoxyribose oxidation products [9], the production of colored iron–thiocyanate complexes [12], the radical oxidation rate [13], liposome oxidation products [14], and the iron–tripyridyl triazine complex (ferritic-reducing antioxidant power, FRAP) [9]. Light-sensitive chemiluminescence (CL) methods measure the total radical-trapping potential (TRAP) [15–18] and total antioxidant reactivity (TAR). Fluorimetric techniques include the oxygen radical absorption capacity (ORAC) [19] and Yang and Guo [20] methods.

Oxidative stress accompanies various disorders; it is possible to affect this complex process at various stages. On the one hand, antioxidant therapy is performed to alleviate oxidative stress. On the other hand, free-radical reactions are stimulated to achieve an opposite effect. Two biophysical approaches, laser and UV irradiation, are used to activate oxidative stress in blood irradiation therapy. UV irradiation (a physical model of oxidative stress [21]) is the most convenient (dosing is possible) and affordable method to model oxidative stress. The mechanism by which blood irradiation affects the human body is complex and diverse. There is still no unified theory on the manner by which UV irra-

Abbreviations: OS, oxidative stress; CL, chemiluminescence; ABAP, 2,2'-azo-bis(2-amidinopropane)dihydrochloride; HSA, human serum albumin.

diation affects the human body. Different spectral regions affect the physiological reactions of individual tissues and the total body differently. The main effects of UV irradiation include stimulation of antibody production, phagocytosis, and agglutinin accumulation in the blood at wavelengths of 280–400 nm; pigment production at 340 nm; erythema development at 297–302 nm; and vitamin D synthesis at 280–310 nm. UV radiation at wavelengths of 280 and 260–265 nm is maximally absorbed by proteins, leading to their denaturation. UV irradiation at 180–280 nm exert a bactericidal effect, which is at a maximum at 254 nm.

While the total body is affected by oxidative stress, the blood is the most convenient and available object for clinical testing; it possesses a great antioxidant potential. The CL method has received broad application to measure the total antioxidant capacity of the blood. CL curves make it possible to measure the activity for weak (TAR) or potent (TRAP) antioxidant systems [17, 22, 23].

However, certain difficulties arise when using the CL method to test the blood plasma because the kinetics of CL development (the time dependence of CL) is more complex than commonly believed. Several changes are seen in the CL curve. The CL intensity decreases at the first stage, which is seen as a latent period, and increases at the next one. Similar changes in CL kinetics have been observed in studies that focused on the antioxidant activity of the blood plasma in various pathological conditions [24]. However, the natures of these events are still not fully understood. The objectives of this work were to study CL kinetic curves and to identify the parameters that are potentially suitable for evaluating the oxidative stress level in the body for clinical purposes.

MATERIALS AND METHODS

CL measurements were performed with a Lum-5773 chemiluminometer (DISoft, Russia) in thermostated conditions at 37°C. The chemiluminometer was coupled with a computer using the original PowerGraph 3.3 software product (www.powergraph.ru). The antioxidant activity was estimated using a luminol-activated CL method [20] with minor modification. In brief, 50 μL of 0.05 M 2,2'-azo-bis(2-amidinopropane)dihydrochloride (ABAP; Fluka, Germany) (aqueous solution) and 20 μL of 0.1 mM luminol (5-amino-1,2,3,4-tetrahydro-1,4-phthalazinedione; Fluka, Germany) in 100 mM phosphate buffer (pH 7.4) were preincubated in the dark at room temperature for 20 min. Thermal decomposition of the radical source was triggered by adding the necessary amount of a preheated phosphate buffer (37°C). The main modification of the method is adding the test sample after the analytic signal has reached a plateau. CL of the ABAP–luminol system was recorded in a control experiment. A phosphate buffer that is added to the system in the place of the test sample has previously been shown to exert no effect on the curve shape. The system volume was 1.0 mL. Plasma samples were stored at –20° and were diluted with distilled water immediately before testing. The final plasma dilution factor was 1000.

Sample irradiation. To model oxidative stress, we used a Bio-Link UV irradiation system (Vilber Lourmat, France), which allowed dosed exposure at an effective wavelength of 254 nm for short-wave radiation. Samples (1 mL) that contained 6.6 μM protein in a phosphate buffer were irradiated in quartz cuvettes. By recording absorption spectra (data not shown), the concentration was selected so that the optical density did not exceed 0.2, especially in the region of 254 nm (the effective UV wavelength). This limitation was used to ensure that short-wave radiation acts homogeneously throughout the sample volume.

RESULTS AND DISCUSSION

Plasma samples were obtained from healthy subjects and patients with various disorders involving oxidative stress as a pathogenetic factor, such as Parkinson's disease, type 2 diabetes mellitus, and Wegener's granulomatosis (Fig. 1). The resulting CL curves differed in the duration of the latent period, the increment of CL intensity, and the ratio between the two values. To study the causes of the differences, basic parameters were introduced for quantifying the differences.

Analysis of the CL curve (Fig. 2) showed that the CL intensity increased and reached a plateau in the first part of the curve (prior to adding a sample). The pattern is due to the generation of free radicals and the steady-state character of the process, which results from thermal decomposition of the diazo compound ABAP. This part of the curve is determined exclusively by the system we employed. The second part of the curve is recorded after adding a plasma sample to the system and is biphasic (plasma part). Phase 1 corresponds to CL inhibition and is defined as the latent period (τ_{lat}). Phase 2 corresponds to further CL development with the CL intensity always increasing over the plateau level by a certain value (ΔI_{CL}). It is important to note that the two phases were seen in all cases, regardless of the patient's disease. The effect seen in phase 1 is known to reflect the antioxidant properties of the blood plasma, while the nature of the phase 2 effect is unclear.

To better understand the natures of phases 1 and 2, we studied the effects on the individual plasma components. The plasma antioxidant potential is determined by several major elements (Fig. 3a), including low-molecular-weight antioxidants, such as urea, ascorbic acid, and uric acid, and albumin, which may occur in several states. Hence, the next step was testing

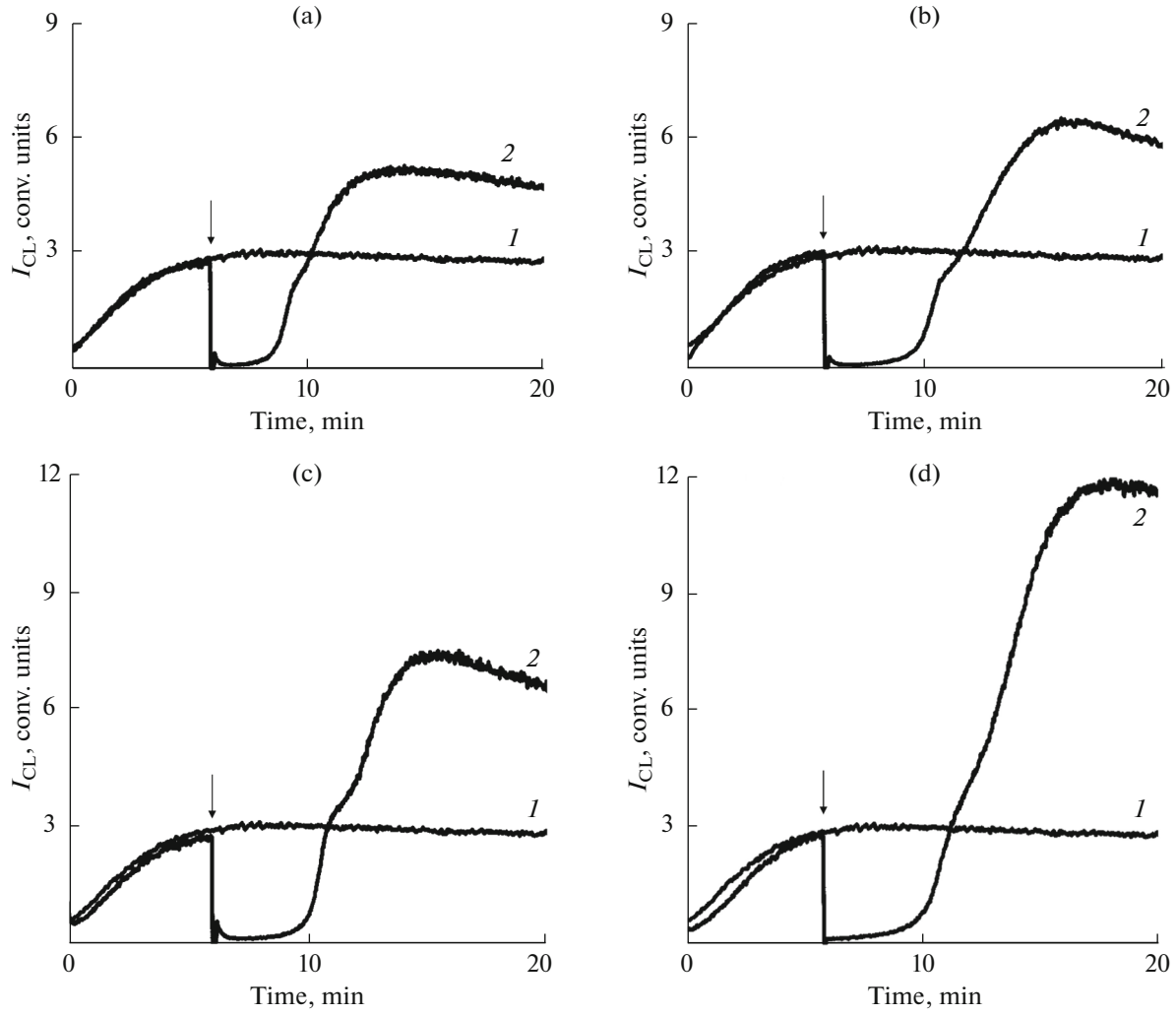

Fig. 1. The CL curves of plasma samples from (a) a healthy subject and patients with (b) Parkinson's disease, (c) type 2 diabetes mellitus, and (d) Wegener's granolomatosis. Curve *1*, control; *2*, test. The time of the addition of the plasma sample is indicated with an arrow.

the major components for their effect on CL development. The component contents were modeled based on the plasma chemistry tests that were performed for patient K with Wegener's granulomatosis.

Urea was not found to exert a considerable effect on the shape of the CL curve (Fig. 3b). Ascorbic acid is thought to be a potent antioxidant. However, its content is low; only a short latent period (less than 1 min) was observed in the model experiments (Fig. 3c), while the duration of phase 1 was 5 min in the experiments with plasma samples. Thus, ascorbic acid normally makes almost no contribution to the antioxidant properties of the blood plasma because of its low concentration. Uric acid is a product of purine catabolism and acts as an efficient acceptor of OH radicals and singlet oxygen [21]. When uric acid was added to the system in the model experiments, the latent period was substantial, viz., 5 min, owing to the relatively high uric acid concentration (0.2–0.45 mM in the plasma normally). As the antioxidant was consumed, the CL was restored to the control level (Fig. 3d). The phase 1 duration in the experiments with uric acid was much the same as in experiments with plasma samples, indicating that uric acid makes a major contribution to the antioxidant properties of the blood plasma. Albumin has a low antioxidant capacity. This is evident from the finding that the CL intensity decreased at first, while there was no latent period in its classical sense (Figs. 3e, 4). In addition, the CL subsequently developed to a level higher than the control, while such an increase was not observed with the other plasma components. Thus, uric acid and human serum albumin (HSA) were found to determine the shape of the plasma CL curve to a great extent.

It was of interest to study the effects of uric acid and HSA on the CL kinetics. Various uric acid concentrations were tested in a series of experiments; the latent period was found to increase with the increasing uric acid concentration (data not shown). The finding reflects the function of a classical potent antioxidant.

Next, HSA was tested (Fig. 4). It should be noted that the initial CL intensity was halved immediately after HSA was added at concentrations ranging from 0.015 to 0.06 mg/mL. The higher the HSA content in the system was, the greater the increment of ΔI_{CL} was. The concentration 0.015 mg/mL was identified as a definite threshold, because changes in CL kinetics were observed at lower HSA concentrations. First, the CL intensity decreased by only one-fourth of the initial level and, second, its steady-state level was lower than the control.

It is possible that the CL intensity initially decreases because HSA fully absorbs a component of the ABAP–luminol system. The subsequent increase might occur because a substance that acts as an additional CL activator accumulates to increase the quantum yield.

When the plasma component was added to the ABAP–luminol system free radicals were trapped and the CL intensity decreased. Further interaction with radicals yielded oxidation products that did not affect the CL development in the case of urea, ascorbic acid, and uric acid. Overt changes were seen in the case of albumin (Fig. 3e).

Although oxidative stress-inducing factors vary, all of them eventually cause oxidative modification of macromolecules, including proteins. Oxidative modification of proteins is the focus of intense research. Studies in the field are aimed at identifying the proteins that are affected by oxidative modification to the greatest extent, determining their targeted degradation pathways, especially those associated with a start of pathological changes in the body, and estimating the contributions of particular proteins to the progression of chronic diseases. Answering these questions will provide a better understanding of the mechanisms that underlie common human disorders and the development of new therapeutic approaches.

Albumin is one of the major blood proteins that face attack by free radicals according to the literature [22]. We believe that it is expedient to use albumin as a marker of oxidative stress because, first, its composition is invariable in pathological conditions of various origins and, second, its source (the blood plasma) is easy to obtain for clinical testing. In view of these facts, another series of experiments was carried out to identify the changes that arise in HSA upon exposure to free radicals that affect its CL properties.

Albumin was exposed to short-wave radiation as a physical model of oxidative stress (Fig. 5). Experimental CL curves were obtained for HSA; they showed two effects. First, the antioxidant properties of HSA increased already after low-dose UV irradiation (0.050 J/cm^2, curve *1*). This change is responsible for CL quenching (the occurrence of a latent period τ_{lat}). Second, a considerable increase in CL (ΔI_{CL}) was observed after a negative peak in the CL curve. As the UV radiation dose increased, the CL intensity change rapidly reached a plateau and remained relatively constant, while the area under the curve grew. The inset shows the radiation dose dependence for the antioxidant activity, which was measured as the latent period duration in minutes. The dependence clearly demonstrates that the antioxidant activity of HSA increased with increasing oxidative modification.

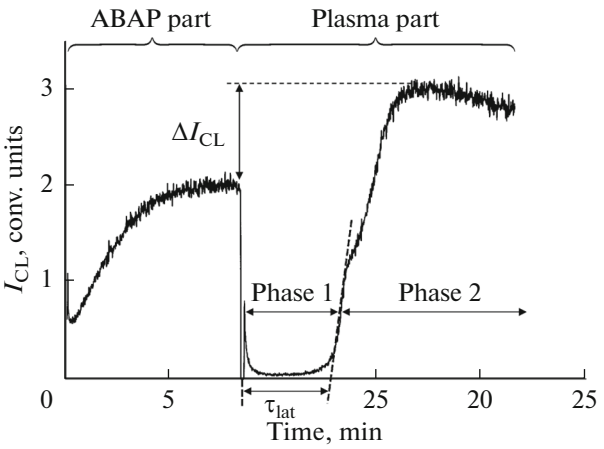

Fig. 2. Analysis of the CL kinetic curve in the ABAP–luminol system with the addition of a plasma sample. The ABAP and plasma parts are isolated. The plasma part is divided into two phases. Phase I corresponds to CL inhibition; phase 2 is further CL development after the inhibition. Two parameters, the latent period τ_{lat} and CL increment ΔI_{CL}, were introduced to characterize the CL curve.

The aromatic amino-acid residues tyrosine and tryptophan are among the major targets of free radicals in HSA. Oxidation products of these residues are probably responsible for the antioxidant properties of HSA and the increase in CL. Similar effects have been observed for tyrosine and tryptophan when tested individually [25]. The amino-acid derivative L-DOPA is a possible product of HSA oxidative modification. The above data make it possible to conclude that albumin experiences oxidative modification upon exposure to low-dose UV radiation. As the dose increases, the antioxidant properties (the latent period, which is a more sensitive parameter) grow stronger, while the CL change rapidly reaches a plateau and then remains relatively constant.

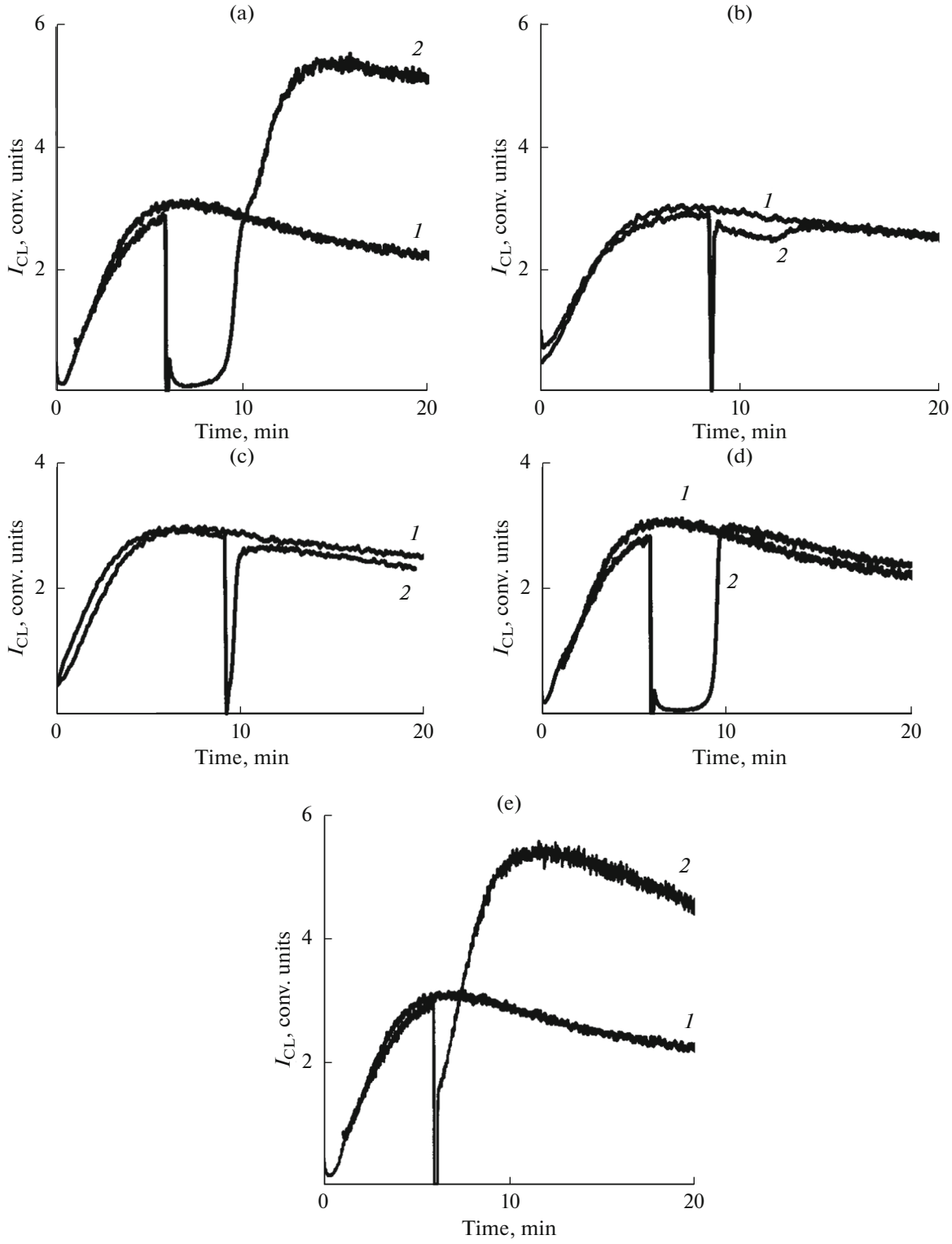

Fig. 3. The CL curves of the ABAP–luminol system in a phosphate buffer with the addition of (a) plasma or its individual components: (b) urea (4.7 μM), (c) ascorbic acid (0.01 mg/L), (d) uric acid (0.331 μM), or (d) albumin (0.038 g/L). Curve *1*, control; *2*, test. The final concentrations of the components correspond to their concentrations as obtained by blood chemistry for patient *K* with Wegener granulomatosis.

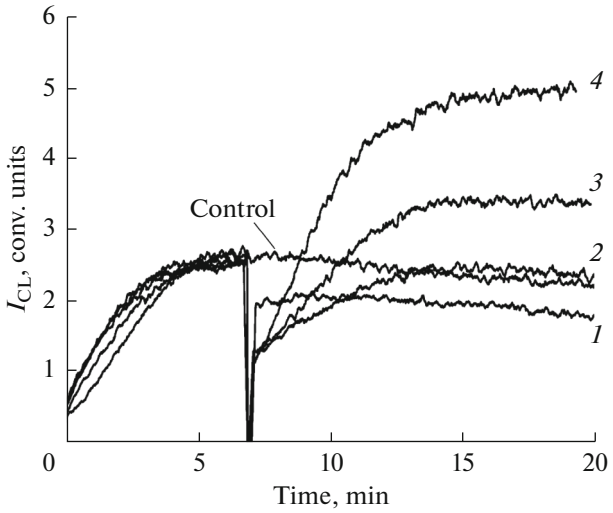

Fig. 4. The CL kinetics in the ABAP–luminol system at various HSA concentrations: (*1*) 0.006, (*2*) 0.015, (*3*) 0.03, and (*4*) 0.06 mg/mL.

CONCLUSIONS

The kinetics of plasma CL were analyzed qualitatively and semiquantitatively in this work. Major plasma components were tested for their effects on the CL curve shape and contribution to the total antioxidant capacity. Oxidative stress-related changes were found to occur in the plasma in several disorders of various origins. A biphasic character (CL quenching in phase 1 and an increase in CL in phase 2) was demonstrated for the CL curve and the proportion of the two phases changed depending on the pathological condition (Parkinson disease, type 2 diabetes mellitus, and Wegener's granulomatosis). Two parameters, viz., the latent period τ_{lat} and CL increment ΔI_{CL}, were introduced to characterize the two phases. Uric acid and, to a minor extent, oxidized albumin were identified as factors that are responsible for the negative peak of the CL curve. HSA alone was found to be responsible for the CL increase in phase 2. Urea did not exert a substantial effect on CL. The effect of ascorbic acid was similar in character to that of urea. The effect on the CL kinetics was studied for various HSA concentrations and threshold values at which the CL kinetics significantly changed were identified. Changes that occur in HSA in model oxidative stress and their effect on the CL properties of HSA were studied. Low-dose UV irradiation was shown to increase the intrinsic antioxidant activity of HSA. The CL intensity consequently increased, rapidly reached a plateau, and then remained relatively constant. The oxidation products of aromatic amino-acid residues form as a result of HSA modification in oxidative stress. The latent period and CL change provide measures of oxidative stress. Oxidative stress can be quantitatively characterized using these parameters.

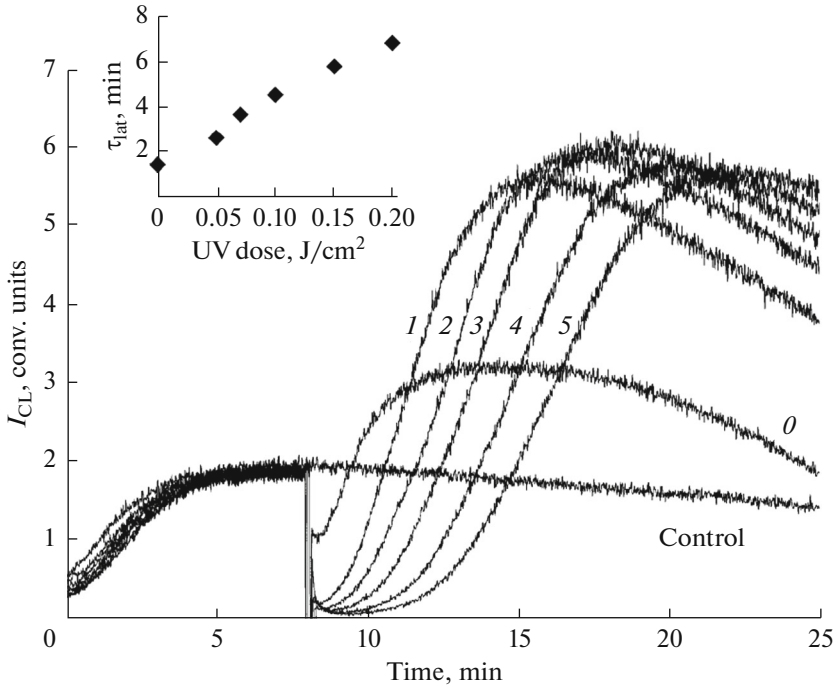

Fig. 5. The CL kinetics in the ABAP–luminol system with HSA (0.66 μM) that was preliminarily exposed to UV radiation at various doses: (*1*) 0.050, (*2*) 0.070, (*3*) 0.100, (*4*) 0.150, and (*5*) 0.200 J/cm^2. The inset shows the latent period τ_{lat} as a function of the irradiation dose.

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