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Formation of long-lived reactive products in blood serum under heat treatment and low-intensity laser irradiation, their role in hydrogen peroxide generation and DNA damage

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Long-lived reactive protein products were shown to be evolved under heat treatment and low-intensity laser irradiation in blood serum in presence of dissolved oxygen from the air. These reactive protein products generate hydrogen peroxide for a long time, which results from conjugated electron-radical chain reactions. Long-lived reactive protein species play an important role in the adaptation of living systems to stress factors. Apparently, the formation of visible light- and heat-induced reactive protein species is not specific to just blood serum proteins, rather than it could also be a feature of other proteins.

Keywords: Action of heat, Laser radiation, Long-lived reactive protein species, Oxidative stress, Reactive oxygen species

Reactive oxygen species (ROS) are permanently formed in living cells both in normal metabolic processes and under the action of external factors (ionizing, ultraviolet, microwave and shortwave radiation, xenobiotics, heat treatment, visible and infrared radiation in oxygen absorbance bands, *etc*.)[1-7]. Elevation of intracellular ROS concentration above the antioxidant protection system capacity causes oxidative stress. Oxidative stress is commonly accompanied by such dangerous processes as peroxidation of lipids, oxidative modification of nucleic acids and proteins. It was established that ROS do not only damage biomolecules but also play signaling and regulatory roles in the organism[8]. The main signaling ROS molecule among all the ROS in a mammalian organism is hydrogen peroxide. Several reviews[9-11] summarize modern data on the role of hydrogen peroxide in intracellular and intercellular signaling. Alterations of hydrogen peroxide level in the organism caused by different physical actions could be important factors of therapeutic effect and adaptation of the organism to dangerous environmental factors[12]. A number of physiotherapeutic procedures based on both hyperthermia and light sources are widely used in medical practice, but biological mechanisms of their therapeutic effect are not studied well.

We have earlier established in our studies that electromagnetic irradiation of water solutions saturated by atmospheric oxygen in visible and near infrared range leads to the observable formation of ROS (singlet oxygen, superoxide anion, hydroxyl radical and hydrogen peroxide)[4,5]. ROS generation was shown to occur only during treatment by irradiation in a range corresponding to the absorption band of molecular oxygen dissolved in water. Meanwhile, a part of oxygen dimoles is converted from triplet to singlet state. The analogous data were obtained under heat treatment. Physico-chemical mechanism of the process was established. Singlet oxygen formation was shown to be the key element of ROS generation during both heat treatment and irradiation by a set of optical wavelength ranges. There is cause to believe that the formation of hydrogen peroxide is one of the reasons for the therapeutic effects of heat and optical radiation[13]. Being abundant and reactive, proteins are the main and the most vulnerable targets for ROS[14]. Proteins

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comprise 15% of cell mass (~70% of dry cell weight). Oxidation by ROS leads to loss of specific protein functions in normal cells, thus being the cause of development of pathological processes[15].

Formation of long-lived reactive protein species (LRPS), which include long-lived protein radicals and protein hydroperoxides, is observed during the interaction of ROS with proteins[16-18]. LRPS formation in model systems was shown after treatment with gamma-, X-ray, UV-radiation, hyperthermia, peroxynitrite, uranyl ions, *etc*. for many proteins, including blood serum proteins[17-22]. Blood serum is a liquid of complex composition contacting with all the tissues in the body, which makes it one of the first targets of harmful actions during oxidative stress progression[23,24]. LRPS obtained in model conditions were shown to be capable of causing mutations and cell transformations[18]. It was earlier shown in model systems, that LRPS are the source of secondary free radicals which cause further oxidation of biomolecules including DNA[25-28]. Development and intensity of oxidative stress are usually corrected with antioxidants[29-31]. Some natural antioxidants including those present in blood serum are capable of neutralizing LRPS and leveling the harmful consequences of their effectiveness on living systems[18-20]. Presence of antioxidants in living systems including blood serum significantly alleviates the negative effect of LRPS in native conditions, but the degree of alleviation is not exactly known yet.

In the present work, we have studied LRPS formation in blood serum. We investigated the ability of blood serum proteins to generate LRPS under treatment with visible light corresponding to absorbance lines of dimoles of molecular oxygen and under hyperthermia. Both visible light irradiation and hyperthermia were shown to cause LRPS formation in the serum. Such LRPS can generate ROS, particularly hydrogen peroxide, for a long time, and lead to DNA damage. Comparison of the results obtained for native blood serum and model systems was carried out.

Materials and methods

Materials

The following reagents and materials were used: bovine serum (Innova, Australia), 4-iodophenol, coumarin-3-carboxylic acid (CCA), 7-OH-coumarin-3-carboxylic acid (7-OH-CCA), horseradish peroxidase, superoxide dismutase (SOD), tiron (Sigma-Aldrich, USA), sodium chloride (NaCl), (Solvey, France), sodium phosphate, mono, and dibasic ($NaH_2PO_4 \times 2H_2O$ and $Na_2HPO_4 \times 12H_2O$) (Amresco, USA), luminol (Appli Chem, Germany), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Sigma-Aldrich, USA), hydrogen peroxide and sodium azide (NaN_3) (Khim Med, Russia); and 99.9% D_2O (Isotope, Russia). The oxygen concentration in the solution was altered using 99.5% oxygen and 99% argon (Gas Com, Russia). Gas bubbling was carried out using an aqueous trap to remove particulate impurities. Phosphate buffer (20 mM, pH=7.4) was used to dilute the serum.

Physical effects

Helium-neon laser LHN 208A (Med Apparatura, Russia) was used as a source of light with radiation at 632.8 nm (power 1.7 mW, flux density 0.7 mW/mm^2). The spectral characteristics of light fluxes were determined with the help of an automated spectrometer complex MDR-41 (OKB Spectr, Russia) in the range from 200 nm to 1000 nm. Dosimetry of optical radiation was performed using a Field Master device (Coherent, USA). Hyperthermia was modeled using a U-10 liquid ultra thermostat (Prufgerate-Werk Medingen, Germany) with an accuracy of 0.1 degrees. Water solutions and animals were irradiated at room temperature using an X-ray therapeutic device RUT-15 (Mosrentgen, Russia) at doses ranging from 1 to 15 Gy at a dose rate of 1 Gy/min (focal length of 37.5 cm, current 20 mA, voltage 200 kV). X-ray irradiation was performed at the Center for Shared Use “Group of Irradiation Sources” Institute of Cell Biophysics RAS.

Induced chemiluminescence

LRPS were studied by measuring the light-induced chemiluminescence of blood serum using a specially developed highly sensitive chemiluminometer Biotoks-7 ultra (Econ, Russia). After heating, all samples were kept in the dark at room temperature for 30 minutes. Then measurements were carried out in dark at room temperature in 20 mL plastic polypropylene vials for liquid scintillation counting (Beckman, USA)[26]. The measured values of chemiluminescence were background-corrected.

Measurement of hydrogen peroxide

The concentration of hydrogen peroxide produced in serum solutions after exposure to He-Ne laser radiation and hyperthermia was measured by the method of enhanced chemiluminescence in the luminol-4-iodophenol-peroxidase system. The liquid

scintillation counter Beta-1 (Med Apparatura, Ukraine) for measuring β-radiation, which operates in the regime of counting single photons (without the scheme of coincidence), served as a chemiluminometer. Due to the high sensitivity of the method, it was possible to detect H_2O_2 at concentrations of less than 1 nM[32]. The H_2O_2 content was determined using calibration curves of chemiluminescence dependence on its known concentration in the solution. The initial concentration of hydrogen peroxide used for calibration was determined spectrophotometrically at 240 nm using a molar absorption coefficient of 43.6 M^{-1} cm^{-1} [33].

Measurement of hydroxyl radicals

The concentration of hydroxyl radicals in the solution was determined using coumarin-3-carboxylic acid, a highly specific to OH-radicals fluorescent probe[34]. The final concentration of CCA in working solutions was 0.5 mM. The fluorescence intensity was measured on a Cary Eclipse spectrofluorometer (Varian, Australia) with λ_{ex} = 400 nm and λ_{em} = 450 nm. Calibration for the formation of hydroxyl radicals was done using a commercial preparation of 7-OH-CCA[35].

Measurement of molecular oxygen concentration

The blood serum solutions were additionally saturated with argon or oxygen by gas bubbling for 15 min[26]. The oxygen concentration in solution was measured with the aid of an oxymetric electrode DKTP 02.4 on an Ekspert-001 device (Ekoniks, Russia).

Measurement of the concentration of 8-oxoguanine

For the quantitative determination of 8-oxoguanine in DNA, an enzyme immunoassay using monoclonal antibodies specific for 8-oxoguanine was used. The experimental procedures are described in detail earlier[36]. DNA samples were denatured in advance in a boiling water bath for 5 min and cooled on ice for 15 min. DNA solutions (43 μL, 355 μg/mL) were applied onto the bottom of the immunoenzyme plate wells (Costar, USA). DNA was immobilized by adsorption during drying for 2 h at 80°C till complete dryness. Nonspecific adsorption sites were blocked using 300 μL of 1% casein solution in 0.15 M Tris-HCl buffer, pH 8.7, 0.15 M NaCl. The plates were incubated at room temperature overnight (14-18 h). Formation of antigen complexes with 8-oxoguanine specific antibodies was carried out in the blocking solution by incubation with stirring for 2 h at 30°C. The samples were washed (300 μL per well) with a washing solution (50 mM Tris-HCl, pH 8.7, 0.15 M NaCl, 0.1% Triton X-100). Then secondary complex of antibodies with IgG–horseradish peroxidase conjugate was formed for 2 h at 30°C in blocking solution. This complex was washed three times by the above-described method after which a chromogenic substrate (18.2 mM ABTS) and 2.6 mM hydrogen peroxide in 75 mM citrate buffer, pH 4.2, were added. After the achievement of green staining, the reaction was stopped by addition of an equal volume of 1.5 mM NaN_3 in 0.1 M citrate buffer, pH 4.3. The sample absorption was measured on a multiplate scanning photometer (Thermo Scientific, USA) at λ = 405 nm. The content of 8-oxoguanine in DNA was determined on the basis of the known value of radiation–chemical yield[36].

Animals

Males of random-bred white Kv:SHK mice aged 5-6 weeks and weighing 18-22 g were used in the experiments. The animals were kept in a vivarium and fed a standard diet with free access to pelleted commercial mouse chow (Arno, Russia) and tap water. The institutional guide for the care and use of laboratory animals was carefully followed. All the experimental protocols received approval from the Bioethics Committee of the Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences.

Micronucleus test

The magnitude of cytogenetic damage in the bone marrow cells of mice was determined by the percentage of polychromatophilic erythrocytes (PCE) with micronuclei (MN). Mice were sacrificed by the method of cervical dislocation 28 h after irradiation. Histological preparations were produced and stained by the standard method. The calculation of PCE with MN was performed using a light microscope with an immersion objective at magnification × 1000[21].

Statistical analysis

Results are presented as mean values and their standard errors (SEM). The data were subjected to one-way analysis of variance (ANOVA), followed by the Fisher's post hoc test. Differences were considered significant at $P < 0.05$.

Results

Figure 1 shows the dependence of the intensity of luminescence of blood serum solutions treated with 632.8 nm irradiation and hyperthermia on dilution degree of blood serum. The dependence of luminescence intensity has a biphasic shape. It was

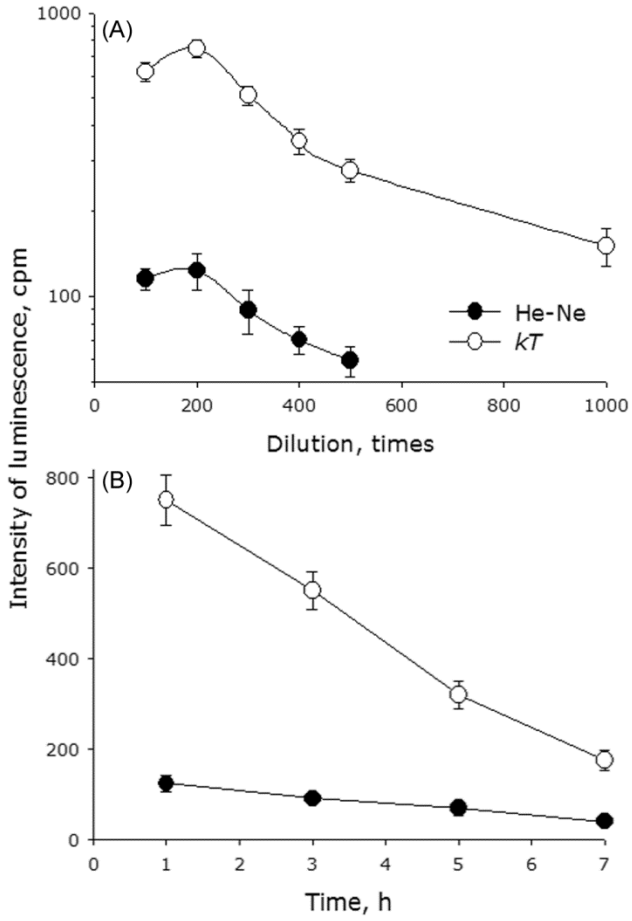

Fig. 1 — (A) Dependence of luminescence intensity of blood serum after the action of laser radiation (He-Ne, 632.8 nm, 30 min) or hyperthermia (kT, 45°C, 2 h) on dilution degree; (B) Change of luminescence intensity at different incubation times after physical treatment of blood serum solutions. He-Ne is 30 min treatment with He-Ne laser. kT is 2 h hyperthermia at 45°C. The measurement was carried out in 1 h after physical treatment. Background luminescence values were subtracted from the obtained data. Mean values of three independent experiments and their standard errors are shown

maximal at dilution degree around 200 times. This dilution was used for further experiments. Irradiation of blood serum with He-Ne laser (wavelength 632 nm) for 10-30 min at 1:200 dilution degree corresponding to maximal luminescence level led to LRPS formation. Extending the time of treatment of blood serum solution with optical radiation was accompanied with a proportional increase of chemiluminescence intensity. Changing of time of hyperthermic treatment in the range from 30 min to 2 h led only to increase of chemiluminescence intensity.

The decrease of chemiluminescence with time after treatment allows to determining the half-life period of LRPS (Fig. 1). As the presented dependences are close to linear, the average half-life period corresponded to double decrease of luminescence intensity. As it can be seen from plot 2, the half-life of radicals formed from blood serum protein was around 4-5 h after the treatment. Thus, it was shown that both hyperthermia and irradiation by light at a wavelength equal to molecular oxygen absorbance line result in observable LRPS formation.

Hydrogen peroxide formation after the action of He-Ne laser radiation and hyperthermia in blood serum solutions was studied. Dependence of hydrogen peroxide generation in 30 min after laser irradiation and 2 h after heat treatment is presented in (Fig. 2). For both optical irradiation by light in molecular oxygen absorbance range and hyperthermia, the decrease of hydrogen peroxide formation rate with blood serum dilution is observed.

As it was shown above, the effect of laser irradiation and hyperthermia leads to hydrogen peroxide formation. Time dependence of hydrogen peroxide formation by 632.8 nm laser irradiation was shown to be linear in the range from 5 to 30 min. The rate of hydrogen peroxide formation after different periods of heat treatment did not change monotonously. Hydrogen peroxide formation in the solution of blood serum affected by the action of physical factors in different time after the treatment is shown in (Fig. 2). It is shown that a long time after the influence of physical factors there is a tendency to increase the concentration of hydrogen peroxide. Meanwhile, in the case of hyperthermia, the elevation of hydrogen peroxide concentration is on the level of 5% per h. After laser irradiation of blood serum solution, a 50% elevation of hydrogen peroxide concentration is observed each h.

A fluorescent probe, coumarin-3-carboxylic acid (CCA), which is converted into hydroxylation product, 7-OH-coumarin-3-carboxylic acid, an intensive fluorophore, served for measurement of hydroxyl radicals in blood serum solutions after 30 min treatment with 632.8 nm laser and 2 h treatment with 45°C temperature. 7-OH-CCA content in blood serum solution (1:200) after laser treatment comprised 1.4 ± 0.2 nM, whereas after heat treatment it was four times lower.

To reveal the mechanism of hydrogen peroxide formation in blood serum solution under the action of laser radiation and hyperthermia, the inhibitory analysis was carried out. A number of compounds applied in inhibitory analysis significantly decrease hydrogen peroxide generation. This makes certain

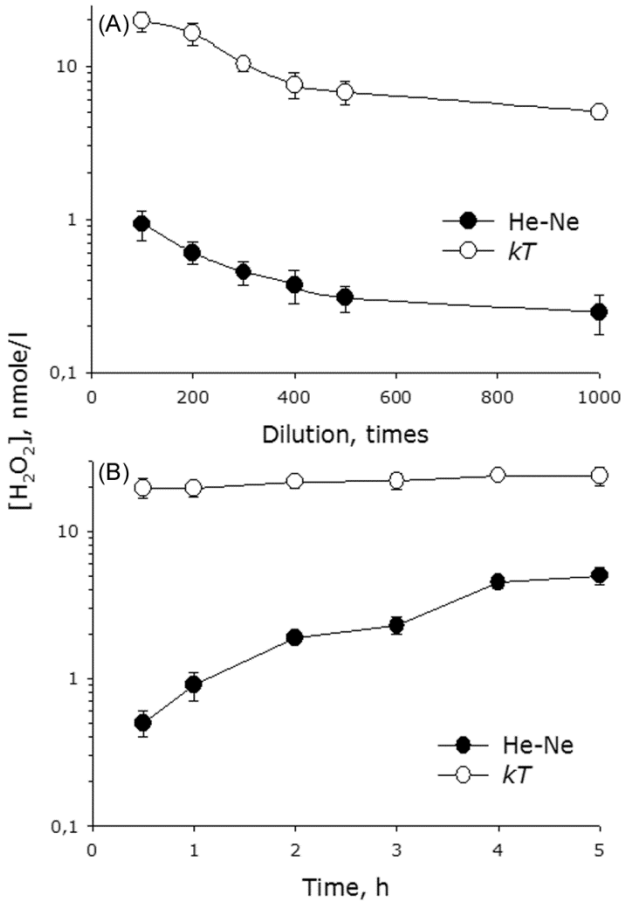

Fig. 2 — (A) Effect of blood serum dilution degree on hydrogen peroxide production by LRPS induced by physical treatment. He-Ne – 30 min treatment with helium-neon laser. kT – 2 h of heat treatment (45°C); (B) Hydrogen peroxide formation in blood serum solution treated by physical factors in different time after the treatment. He-Ne – 30 min treatment with helium-neon laser. kT – 2 h of heat treatment (45°C). The measurement was carried out in 1 h after physical treatment. The background values were subtracted from the obtained data. Mean values of three independent experiments and their standard errors are shown. Y axis is in the logarithmic form

measurements during laser irradiation of blood serum solutions impossible. Thus, to reveal the mechanism of hydrogen peroxide formation in blood serum solution, we used only one physical factor, hyperthermia (Table 1). Oxygen effect, *i.e.* influence of dissolved oxygen concentration on hydrogen peroxide formation by LRPS of blood serum, was established. The concentration of hydrogen peroxide generated by LRPS in the solution saturated with extra oxygen during 15 min before irradiation was 1.6 times higher. Under saturation of blood serum solution with argon, laser irradiation led to 20% lower hydrogen peroxide formation. It is known that the presence of D_2O increases the lifetime of singlet

Table 1 — Influence of different substances on hydrogen peroxide formation in blood serum solutions treated with 2 h of hyperthermia (45°C)

Treatment	$[O_2]$, μM	$\Delta[H_2O_2]$, nM	K
Control	270	19.6 ± 2.4	1
Saturated with O_2 **	420	30.4 ± 1.4 *	1.6
Saturated with Ar **	130	16.1 ± 1.4 *	0.8
D_2O (25% *v/v*)	270	23.6± 1.2 *	1.2
NaN_3 (0.1 μM) ***	270	15.7 ± 0.8 *	0.8
SOD (10^{-3} U/mL)	270	23.1 ± 1.8 *	1.2
Tiron (100 nM)	270	13.8 ± 0.9*	0.7
Prx2 (10^{-2} U/mL)	270	0.0 ± 0.0*	0.0

Background values of hydrogen peroxide concentration were subtracted from the obtained results. Mean values of three independent experiments and their standard errors are shown. The samples contained serum diluted 200 times. Measurement was carried out in 1 h after physical treatment. K - changes in H_2O_2 concentration by the action of the analyzed agent relative to the control value.

* - $P < 0.05$ *vs* control.

** - the serum was saturated with gas for 15 min by bubbling prior to hyperthermia (45°C, 2 h).

*** - sodium azide at this concentration did not markedly inhibit the activity of peroxidase.

oxygen. The concentration of hydrogen peroxide after hyperthermia was elevated by 20% in blood serum solution containing 25% D_2O. Sodium azide, a quencher of singlet oxygen, decreased LRPS-dependent hydrogen peroxide generation by 20%. These data give evidence on the participation of singlet oxygen in hydrogen peroxide formation. Application of superoxide dismutase (SOD) showed that superoxide anion radicals participate in hydrogen peroxide generation in water solution under the action of LRPS. SOD increased hydrogen peroxide concentration by 20% *via* additional dismutation of these radicals. Addition of tiron, a spin trap of superoxide radicals, led to decrease of hydrogen peroxide concentration by 30%. Prx2, an antioxidant protein which expectably degraded all the hydrogen peroxide formed, was used as absolute control.

LRPS are known to be capable of damaging other biomolecules. The most significant damage to living systems is DNA damage. Among all kinds of DNA damage, 8-oxoguanine formation is the most widespread, and it is is rightly considered a key biomarker of oxidative DNA damage[37]. The ability of blood serum treated with He-Ne laser or hyperthermia to lead to 8-oxoguanine formation in DNA in a

model system is shown in (Fig. 3). Incubation of laser-treated blood serum with DNA was shown to lead to the formation of around 31 molecules of 8-oxoguanine per million of guanine bases in DNA (Fig. 3, histogram 2). In the case of hyperthermia, around 14 molecules of 8-oxoguanine per million of guanine molecules were formed (Fig. 3, histogram 3). It should be noted that native blood serum taken as a control did not lead to the emergence of DNA damage. DNA treated with ionizing radiation was also used for comparison (Fig. 3, histogram 1), and in this case, around 78 molecules of 8-oxoguanine per million of guanine molecules in DNA were formed per 1 Gy. This dependence was observed in an absorbed dose range from 5 to 20 Gy (Fig. 3).

As we showed above, LRPS can damage DNA molecules in model conditions. However, it remains unclear whether LRPS of blood serum can directly damage DNA in living systems. Hematopoiesis system is one of the most sensitive systems for oxidative stress. One of the most studied and applied parameters of damage level of hematopoiesis system is the formation of micronuclei in erythrocyte progenitor cells. Figure 4 shows the effect of blood serum treated with He-Ne laser action or hyperthermia on micronuclei formation in polychromatophilic murine erythrocytes *in vivo.* Blood sera possess immunogenicity, thus we used intact blood serum as a control. Both intact serum and one treated with physical factors did not lead to an increase of micronuclei concentration in polychromatophilic erythrocytes of mice. Living organisms have the capability of repair, and, as we could not detect the effect of blood sera, we used the challenging dose method. The method allows study of weak effects on biological systems indirectly. The sense is in studying weak action on the background of stronger action. Usually, a living system is first treated with a weak action and then, in 5-8 h, with a strong one. If the weak action influences the studied factor, it leads to the development of tolerance to the damaging effect of strong action. In other words, this phenomenon is called adaptive effect. Figure 4 shows that irradiation of mice with 1 Gy dose significantly increases the number of polychromatophilic erythrocytes containing micronuclei. Injection of native blood serum does not change the situation significantly, whereas injection of blood sera treated with laser irradiation and hyperthermia leads to progression of adaptive effect, decreasing the number of polychromatophilic erythrocytes with micronuclei by 15% on average.

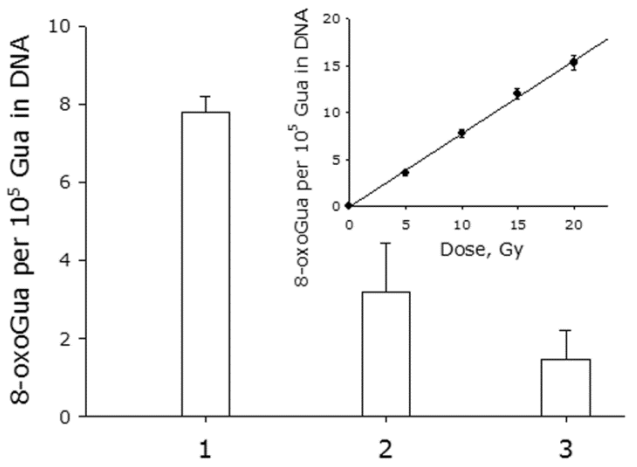

Fig. 3 — (A) Influence of blood serum treated with He-Ne laser (30 min) and hyperthermia (45°C, 2 h) on 8-oxoguanine (8-oxoGua) formation in DNA *in vitro*. DNA/protein ratio was around 10:1. Background values were subtracted from the obtained results. Mean values of three independent experiments and their standard errors are shown. (1) Effect of ionizing radiation on DNA (dose = 10 Gy); (2) 2 h DNA incubation with blood serum treated with He-Ne laser; and (3) 2 h DNA incubation with blood serum treated with hyperthermia. The insert shows the dependence of 8-oxoGua formation in DNA *in vitro* on the absorbed dose of ionizing radiation

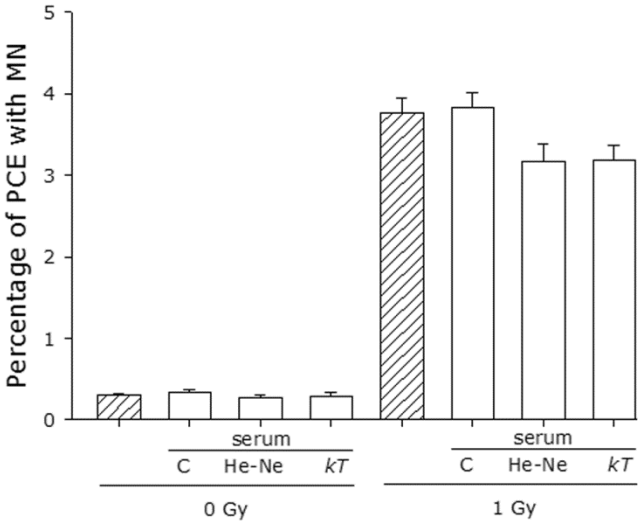

Fig. 4 — Effect of blood serum treated with He-Ne laser (30 min) and hyperthermia (45°C, 2 h) on the formation of micronuclei in polychromatophilic murine erythrocytes *in vivo*. Native serum or serum treated with physical factors was injected into mice intravenously in a final concentration of 5 mg/g. Mean values of five independent experiments and their standard errors are shown. C — injection of native serum not treated with physical factors. He-Ne – injection of serum treated with He-Ne laser for 30 min. kT – injection of serum treated with heat (45°C) during 2 h. 0 Gy – mice are not irradiated. 1 Gy – mice irradiated with a dose of 1 gray. Hatching shows the control group of mice which did not receive exogenous serum

Discussion

Normally, blood serum contains a large number of antioxidants. These can be high molecular weight compounds, *i.e.* such enzymes as extracellular SOD, catalase and glutathione peroxidase, *etc*., and low molecular weight compounds, such as glutathione, tocopherol, some free nucleosides, sulfur-containing amino acids, *etc*.[8]. Presence of such a large number of antioxidants in blood serum was supposed to prevent LRPS formation in native conditions. However, it was shown to be quite different (Fig. 1). LRPS were shown to be generated in blood serum, and their half-life was comparable to the LRPS half-life registered on purified proteins[21,25,26,28].

It was earlier established that prolonged irradiation of immunoglobulin solutions with low-intensity ultraviolet radiation results in the formation of hydrogen peroxide from molecular oxygen dissolved in the protein solution[38]. The authors proposed the transition of molecular oxygen from triplet to singlet state to be the key step of this process[38]. In these experiments, intensive hydrogen peroxide formation under the action of ultraviolet light was observed only in gamma-globulin solutions and not in solutions of other proteins. In our experiments, we activated blood serum solutions with laser radiation with a wavelength of 632.8 nm corresponding to absorbance line of molecular oxygen and with elevated temperatures. In these cases, the formation of hydrogen peroxide did also occur (Fig. 2).

It should be noted that kinetics of hydrogen peroxide generation under laser irradiation, hyperthermia, and ionizing radiation differs significantly[20,26,39]. In radiation-induced LRPS, the decrease of hydrogen peroxide generation rate after the end of treatment was close to exponential. In cases of LRPS induced by laser irradiation and heat, the rate of hydrogen peroxide generation after the end of the treatment did not decrease significantly in several h, and in some cases it even slightly increased (Fig. 2) This could possibly be related to different ratio of certain reactive oxygen species generated, on one hand, by water radiolysis[40], and on the other hand, by laser irradiation at wavelength of molecular oxygen absorption band[44,41] and hyperthermia[5]. As a result, impacts of two possible ways of hydrogen peroxide formation, one *via* recombination of OH-radicals and another one *via* dismutation of hydroperoxide radicals, could be different. As it follows from Table 1 and data on the generation of OH-radicals, $^1O_2$, $HO_2^{\bullet}$ and $^{\bullet}OH$ take part in LRPS-induced hydrogen peroxide generation.

It is supposed that the process of ROS generation by low-intensity physical factors can be an essential way related to the release of extra free energy *via* surface tension in air nanobubbles, or bubstons (bubbles stabilized by ions)[42,43]. This energy can be released in a form of standard high-energy processes as a result of bubston collapse under the action of visible light, a series of laser irradiations, in a way analogous to that taking place during ultrasonication[44,45]. There are proposals on the possibility of analogous processes under the action of heat[36,46]. Apparently, the triggering mechanism of cavitation collapse of bubstons is resonance excitation of molecular oxygen by wavelengths corresponding to its transition to singlet state leading to local electromagnetic disturbance[41,47].

Taking into account reactive oxygen species formation in the water under hyperthermia[4] and optical irradiation by light in molecular oxygen absorption lines[41], the following sequence of conjugated reactions leading to LRPS and hydrogen peroxide formation in protein solutions can be suggested:

$$O_2 + h\nu \rightarrow {}^1O_2 \,/\, O_2 + kT \rightarrow {}^1O_2 \quad \ldots (1)$$

where $h\nu$ is an energy quantum of the electromagnetic radiation corresponding to the transition of oxygen from triplet to singlet state[47], kT is thermal energy[5].

$$OH^- \rightarrow {}^{\bullet}OH + e + {}^1O_2 + H^+ \rightarrow HO_2^{\bullet} \quad \ldots (2)$$

where e is a hydrated electron. The mechanism of dissociation of hydroxyl anion was analyzed earlier both theoretically[48] and experimentally[49].

The reaction of $^{\bullet}OH$ with hydrogen atom attached to C_α-carbon of polypeptide chain leads to its release and formation of C_α-carbon radical[50,51]:

$$–NH–RC_\alpha H–CO– + {}^{\bullet}OH \rightarrow –NH–RC_\alpha{}^{\bullet}–CO– + H_2O \quad \ldots (3)$$

Reaction with oxygen leads to the formation of peroxyl radical in proteins[51]:

$$–NH–RC_\alpha{}^{\bullet}–CO– + O_2 \rightarrow –NH–RC_\alpha OO^{\bullet}–CO– \quad \ldots (4)$$

Then, hydroperoxide elimination occurs:

$$–NH–RC_\alpha OO^{\bullet}–CO– \rightarrow –N{=}RC_\alpha–CO– + HO_2^{\bullet} \quad \ldots (5)$$

and hydrogen peroxide is formed *via* dismutation of hydroperoxide radicals:

$HO_2^{\bullet} + HO_2^{\bullet} \rightarrow H_2O_2 + {}^1O_2$ … (6)

To prove the correctness of the proposed mechanism, further studies are required. It is known that α-carbon radical is also formed during proton release from side chains (β-, γ- and δ-carbons) of amino acids as a result of the transfer of radical product to Cα-carbon in proteins due to elevated stability of Cα-carbon radical resulted from electron delocalization[51].

Thus, a novel fundamental property of serum proteins has been revealed: they can be converted under the action of laser radiation and heat in presence of dissolved oxygen into LRPS, which could further generate hydrogen peroxide for long periods of time as a result of conjugated electron-radical chain autocatalytic reactions[52]. Apparently, the formation of LRPS induced by optical radiation and heat is not restricted to only serum proteins but should rather be a common property of at least the majority of proteins, as it is in the case of ionizing radiation[26].

One could have an impression that hydrogen peroxide concentration formed by active species of blood serum proteins in our *in vivo* experiments is too low to have a significant biological effect. But it is definitely not like that. Hydrogen peroxide is known to play a role of secondary messenger in mammals at concentration from several to hundreds of nanomoles per litre[53]. Comparing it with hydrogen peroxide concentrations after the action of ionizing radiation-induced LRPS, 20 nM concentration (a common level observed in our experiments) is formed at a dose of 0.25 Gy. This dose, for example, causes temporary male sterility[54]. It should be emphasized that in our experiments we measure average hydrogen peroxide concentration in very diluted solutions, whereas its local concentration close to protein surface could probably be much higher. Moreover, we directly estimate LRPS damaging potential both *in vitro* (Fig. 3) and *in vivo* (Fig. 4). Thus, we cannot exclude that optical irradiation by light at a wavelength equal to that of molecular oxygen absorbance line during physiotherapeutic procedures in medical practice is accompanied by LRPS formation[55]. Hydrogen peroxide generated under the action of LRPS can participate in signaling pathways and cause an adaptive reaction in humans[8-10,56] in a manner analogous to the scheme drawn in (Fig. 4).

The proteins are supposed to be the sensors warning the cells about the beginning of oxidative stress development. They are related to different intracellular signaling cascades participating in the regulation of pro- and antioxidant gene expression and serve the role of a link between oxidative damage of the cells and antioxidant protection systems[57-60]. Despite that, it should be noted that ROS formation induced by optical radiation or heat treatment at levels overwhelming the inactivating capacity of cellular antioxidant protection system can lead to the same consequences as ionizing radiation.

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