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Peroxynitrite and Peroxynitrous Acid Production of Gas-Discharge Spark Plasma Radiation.

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ABSTRACT

Peroxynitrite production in distilled water ($pH_0 = 5.9$) by gas-discharge (gas-air) spark plasma radiation was investigated. Active species were produced in water, which contained dissolved oxygen and nitrogen. The discharge pulse energy was 0.059 J. The reaction of DPPH[•] (2,2-diphenyl-1-picrylhydrazyl radical) ethanol solution with a sample of water treated with plasma radiation was investigated. The water sample was mixed with 1:1 ethanol DPPH[•] solution. Identification of peroxynitrite in the liquid sample and the measurement of DPPH[•] concentration in the mixture was made by means of spectrophotometry. Absorbed spectra of samples treated with plasma radiation and its reaction with DPPH[•] were measured immediately after treatment and for the next 14 days. Peroxynitrite is produced as a complex, which decays over 14 days. The steady state concentration of the complex is reached in 20 min treatment with plasma radiation and equals about 10^{-3} mol/l. The instantaneous concentration of peroxynitrite and peroxynitrous acid arising by complex decay is about 10^{-5} mol/l. Production of peroxynitrite and peroxynitrous acid after treatment by UV mercury lamp radiation (253.7 nm) was not observed. The generation of long-lived active species under spark plasma radiation can be a cause of the strong sporicidal effect that was observed earlier.

Keywords: Peroxynitrite and Peroxynitrous Acid, plasma

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INTRODUCTION

The factors determining the effect of cytotoxic plasma radiation on biological objects (prokaryotic and eucaryotic cells) are plasma-generating active species, which affect the sample on immediate contact of plasma with objects. Plasma radiation plays a specific role [1, 2]. It interacts with objects on its surface; active species cannot enter into the object to substantial depths. However, the UV radiation of plasma in contrast to active plasma species can enter in to a substantial depth (up to several cm). The inactivation of E.coli by non-thermal dielectric barrier discharge atmospheric pressure plasma was investigated in [3]. To see the effect of biomass on the plasma's antimicrobial efficacy, E.coli cell density-dependent experiments were conducted. These showed that 30 s of plasma treatment inactivated ~95% of 10^7 E.coli cells in the sample and caused complete inactivation when 10^6 cells were treated. But the inactivation was only several percent when 10^9 cells were in the sample.

When the same bacteria were treated with the UV radiation of a mercury lamp ($\lambda = 253.7$ nm), the value describing the inactivation process is the irradiation dose (treatment time) when the number of bacteria N is diminished after treatment, $N_t/N_0 = 0.1$. The inactivation law is: $dN/dt = -kI$, where $k = \text{constant}$, $t = \text{time of treatment}$ and $I = \text{intensity of UV-photons}$ [4]. The ratio $N_t/N_0 = e^{-kIt}$ is slightly dependent on initial bacteria concentration N_0 as radiation enters at almost full sample depth, and every bacterium has the probability of absorbing a quantum of radiation. Experimentally, the observed dependence of surviving cell numbers (in log scale) from UV radiation time exposure for E.coli is close to linear; that is, the ratio N_t/N_0 for a given irradiation dose can be equal to 10, both for $N_0 = 10^9$ and $N_0 = 10^7$. Plasma radiation is usually less intensive than mercury lamp UV radiation [1], but plasma radiation can have a disinfecting effect compared to a mercury lamp, and is stronger.

A strong sporicidal effect of spark discharge plasma radiation (IR) was found in [5]. The effect of UV-lamp radiation on the inactivation of E.coli was much greater than infrared plasma radiation (IR), but the sporicidal effect on micromycete A.Niger, A.Alternata, Ch.Globosum and P.Chrysogenum was many times more than UV-lamp radiation. Also, the flux of photons from the UV lamp exceeded by 430 times the radiation in the UVC range from IR. Therefore, it would be interesting to find the factor for plasma radiation, which determines the biocide effect. We used a pulse radiation source in which the parameters of spark discharge were chosen to provide maximal chemical effects in liquid (diminished pH, maximal concentration of reductants and oxidants after treatment) [6, 7]. The main active species generated in water with dissolved gases (oxygen and nitrogen) under plasma radiation that have the greatest concentration (including primary and secondary species) are the radicals $\text{HO}_2^{\bullet}/\text{O}_2^{\bullet-}$ ($\text{pK}_a = 4.8$) and peroxyntirite (peroxyntirous acid) $\text{pK}_a = 6.8$. Yields of these species have been calculated and determined by previous experiments [8, 9]. The more interesting chemical is peroxyntirite, as it has high level of chemical and biological activity. In neutral and acid conditions peroxyntirite itself decays to NO_3^- ions and exists as peroxyntirous acid ($\text{pK}_a = 6.8$) [10]. Therefore, it is of interest to study in detail the peroxyntirite production under spark gas-discharge plasma radiation and compare the outcome with the peroxyntirite yield under mercury UV lamp radiation. We undertook this study to investigate the kinetics of peroxyntirite and peroxyntirous acid production in water under spark gas-discharge plasma radiation and post-radiation storage time effects. Active species, having a long lifetime, such as peroxyntirite and peroxyntirous acid, can play a decisive role in the sporicidal effect.

MATERIALS AND METHODS

Experimental setup

A sketch of the setup and sample is shown in Fig. 1. Two types of radiation source were used: (1) a generator of spark gas-discharge plasma radiation IR-10 and (2) a low-pressure mercury UV lamp DBK-9. Glass vessel 3 was covered by Teflon plate 2 with a hole for radiation. Petri dish 5 with a water sample can be covered in some experiments by a simple glass or quartz glass filter (place 4). There was a directional light source – the sample could shut by means of light stopper, which is placed in position 6.

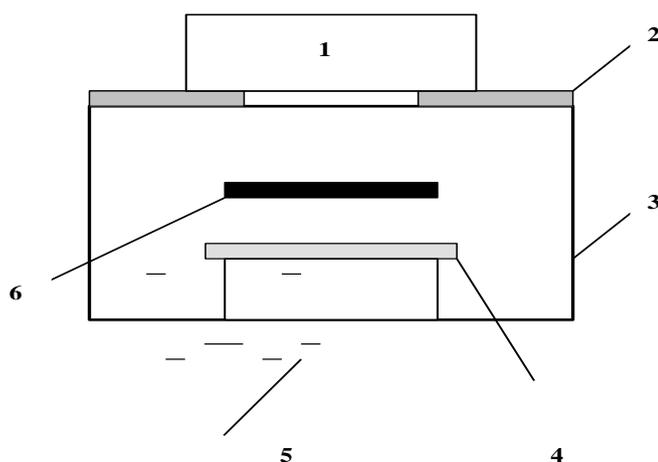


Figure 1: Sketch of the setup and sample position: 1 – radiation source; 2 – Teflon covering; 3 – glass vessel; 4 – place for filter; 5 – Petri dish with sample, 6 – place for light stopper.

Sources of radiation

The first source used was a generator of spark gas-discharge plasma radiation IR-10 [6]. The pulse energy was $5.9 \cdot 10^{-2}$ J, the pulse front duration was 50 ns, full pulse duration was 100 μ s and pulse repetition frequency was 10 Hz. The high voltage was 11 kV and the discharge capacitor was 3.3 nF. The density of energy flux for the UVC range at the time of pulse (100 μ s, 10Hz) at a distance of 1 cm from the electrodes (pulse power) was $2 \text{ J/cm}^2 \text{ s}^{-1}$; the average radiation power for the same distance was equal to $(2 \pm 0.3) \cdot 10^{-3} \text{ J cm}^{-2} \text{ s}^{-1}$ and the flux of the photons UVC range was $(1.26 \pm 0.3) \cdot 10^{-10} \text{ mol cm}^{-2} \text{ s}^{-1}$. Luminous emittance (in full wavelength range) was 230 lux, and the maximum of the radiation spectrum was at $\lambda = 220$ nm. When the spark cord is cooling, the maximum of the radiation spectrum dislocates to long wavelengths from UVC to the visible range at up to 800 nm. The power of the discharge (discharge capacitor value) was chosen to provide the maximum chemical effect in water (diminished pH and maximum concentration of oxidant and reductant after treatment) [6].

The second source was a low-pressure mercury UV lamp DBK-9 (9 W, $\lambda = 253.7$ nm). According to technical manual the density of the energy flux was $2.6 \cdot 10^{-2} \text{ J cm}^{-2} \text{ s}^{-1}$ at 3 cm from the lamp, and the photon flux was $5.4 \cdot 10^{-8} \text{ mol cm}^{-2} \text{ s}^{-1}$. Luminous emittance was 2400 lux. The photon flux of the UV lamp DBK-9 ($\lambda = 253.7$ nm) exceeded by 430 times the photon flux of the UVC range from the plasma generator IR-10 radiation [9].

Intensity of UVC radiation for both sources was determined by a ferrous oxalate dosimeter and solution of KI. The measured value of intensity for the UV lamp DBK-9 is in agreement with the technical manual of this lamp.

To test the role of light wavelength and active species production in the gas phase, filters (position 4, Fig. 1) were used that covered the water surface and had direct contact with the liquid [6, 7]. When the filter was ordinary glass, no chemical effects in the water were observed. With a quartz glass filter the yields of active species were the same as without the filter. This means that active species are generated in water which contains dissolved gases (oxygen and nitrogen). The depth of UV light penetration in liquid was about 40 mm [7]. Ordinary glass is not transparent for UVC light, and the absence of chemical effects in water with the ordinary glass filter means that active species production is caused by UVC light [6, 7].

In addition, in the experiment the direction from discharge region and water sample was shut by a dark Teflon plate (position 6, Fig. 1), but the contact of the liquid with the gas phase was free. The distance from sample–discharge was the same, as for all experiments. In this case no active species was found in water (no pH change), which means that the role of active species diffusion through air is negligible.

Solution of DPPH[•]

A solution of stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH[•]) in 96% ethanol, 5 mg DPPH[•] on 200 ml ethanol was used. To study the reactions of species produced in water under plasma radiation, treated water was mixed with ethanol DPPH[•] solution 1:1. The absorption spectrum was measured 3 min after mixing. The control was ethanol solution DPPH[•] mixed with non-treated distilled water 1:1. In the control sample the concentration was [DPPH[•]] = $3.15 \cdot 10^{-5}$ mol/l.

Sample treatment, absorption spectra and pH measurements, and chemicals

The treatment of water by spark gas-discharge plasma radiation (IR) was realized in sterile Petri dishes 40 mm diameter. The water volume was 4 ml and the time of treatment was 1–60 minutes. The distance from the water surface to electrodes (plasma cord) was 10 mm. The pH values were measured for untreated water immediately after treatment and during the next 14 days. Absorption spectra in visible and UV-range of treated water and treated water mixed with DPPH[•] were studied immediately after treatment and over the next 14 days.

The treatment of water samples by UV lamp radiation was realized in the same Petri dishes. The distance from the UV lamp to water surface was 30 mm. The values of pH, absorption spectra for non-treated and treated water, water mixed with DPPH[•] were measured as well as in the case of IR. All experiments were repeated 3–5 times.

The measurement of the absorption spectra of samples was made using a Fluorat-02 Panorama spectrophotometer (Lumex FIRm, St. Petersburg, Russia). The thickness of the cuvette was 10 mm. The optical density of the line, which can be discovered in our experiment in limit of experimental error was equal to $D \sim 0.01$. Values of pH and the oxygen concentration were measured using the Expert 001 device (Econics FIRm, Russia). Bovine serum albumin (fraction V modified) was used, as well as chemically pure grade reagents (NaNO_2 , HNO_3), and distilled water ($\text{pH}_0 = 5.9$). Distilled water was kept for one day before measurement to achieve steady state concentration of dissolved gases (oxygen, nitrogen, CO_2). The oxygen concentration measured with a Clark's electrode was 7 mg/l, and the water temperature was 20°C.

RESULTS

Extinction coefficients measurement for ions NO_2^- and NO_3^- :

Aqueous solutions of 10^{-2} mol/l of nitric acid and NaNO_2 were prepared. Absorption spectra in the range 250–400 nm were measured. Extinction coefficients were calculated on the basis of the optical densities of peaks and concentrations of ions NO_2^- and NO_3^- .

Absorption spectra of 10^{-2} mol/l solutions HNO_3 and NaNO_2 are shown in Fig. 2 For nitric acid there is a peak of $\lambda \sim 300$ nm, and the optical density related to the baseline is $D = 0.07 \pm 0.01$; for sodium nitrite there is peak at $\lambda \sim 360$ nm, and the optical density related to base line is $D = 0.18 \pm 0.01$. For the cuvette thickness of 10 mm the extinction coefficients are equal to $\epsilon(\text{NO}_3^-) = 0.07/0.01 = 7 \pm 1 \text{ l}(\text{mol cm})^{-1}$, and $\epsilon(\text{NO}_2^-) = 0.18/0.01 = 18 \pm 1 \text{ l}(\text{mol cm})^{-1}$. It was supposed in both cases that the degree of dissociation equals 1.

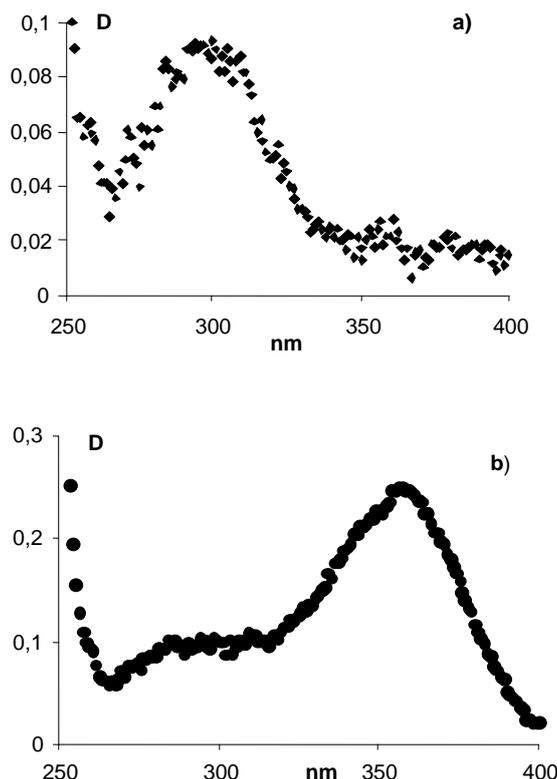


Figure 2: Absorption spectra for 10^{-2} mol/l solutions: (a) – HNO_3 , (b) – NaNO_2 .

Measurement of absorption spectra for treated water:

Water treated for 20 min by plasma radiation was kept afterwards for 14 days at room temperature. Absorbed spectra were measured daily at wavelength 250–400 nm. In the absorbed spectra peaks at $\lambda \sim 300$ and 360 nm were identified (see Fig. 3). Peak ~ 300 nm can be attributed to NO_3^- ions $\epsilon = 7 \text{ l}(\text{mol cm})^{-1}$ and peroxyntirite $\lambda = 301 \text{ nm}$, $\epsilon = 1670 \text{ l}(\text{mol cm})^{-1}$ [11]. The peak of ~ 360 nm can be attributed to NO_2^- ions, $\epsilon = 18 \text{ l}(\text{mol cm})^{-1}$ (see previous section).

The protonated form of peroxyntirite, peroxyntirous acid ONOOH ($\text{pK}_a = 6.8$), has a lifetime of $\sim 1.3 \text{ s}$ [13]. The absorption spectrum of peroxyntirous acid has a weak maximum at $\lambda = 355 - 360 \text{ nm}$ ($\epsilon = 100 \text{ l mol}^{-1} \text{ cm}^{-1}$) and an optical density that smoothly increases with decreasing wavelength [11]. As the lifetime of peroxyntirous acid is small, observation of this maximum in our experiment is unlikely. The lifetime of peroxyntirite ONOO^- is essentially longer, therefore a peak of 301 nm can be observed.

The measurement results for pH-values and optical densities for lines 300 and 360 nm, before treatment, immediately after 20 min plasma radiation treatment and for a post-treatment storage time of up to 14 days are shown in Table 1. The concentrations of NO_3^- ions in Table 1 were calculated on the basis of pH-values: $[\text{NO}_3^-] = [\text{H}^+] = 10^{-\text{pH}}$.

So, at $\lambda \sim 300 \text{ nm}$ we can observe two lines: nitric acid ($\epsilon = 7 \text{ l}(\text{mol cm})^{-1}$) and peroxyntirite ($\epsilon = 1670 \text{ l}(\text{mol cm})^{-1}$) [14]. The optical density of the line, which can be discovered in our experiment within the limit of errors, equals $D \sim 0.01$. The nitric acid concentration, which can be discovered spectrophotometrically, was $[\text{NO}_3^-] = 0.01/7 = 1.4 \cdot 10^{-3} \text{ mol/l}$ or $\text{pH} = 2.84$ or less. The peroxyntirite concentration at $D = 0.01$ was $[\text{ONOO}^-] \sim 6 \cdot 10^{-6} \text{ mol/l}$.

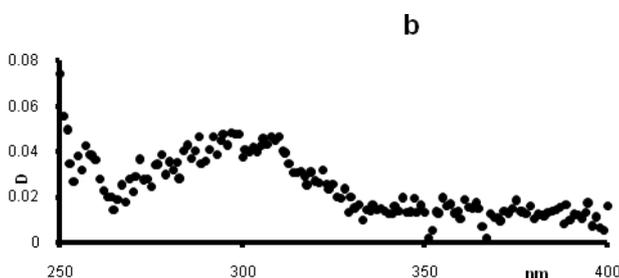
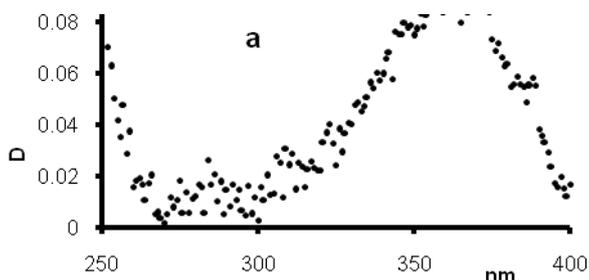
In our experiment distilled water with an initial value of $\text{pH}_0 = 5.9$ was treated to plasma radiation. After 20 min of treatment the pH value was 3.1. It has been shown previously [8] that the mechanism of pH

decrease is peroxy nitrite production and its decay: $\text{ONOO}^- \rightarrow \text{NO}_3^-$. Therefore, a decrease of pH $5.9 \rightarrow 3.1$ immediately after treatment means the production and decay of peroxy nitrite $[\text{ONOO}^-] = [\text{NO}_3^-] = (8 \pm 0.05) 10^{-4} \text{ mol/l}$ (see Table 1). A decrease of pH was observed during all the storage time for up to 14 days. The final concentration of nitric acid was $[\text{NO}_3^-] = (2 \pm 0.05) 10^{-3} \text{ mol/l}$.

Table 1: Stable reaction products in distilled water before and immediately after 20 min treatment (day 0) and for post-treatment storage time of 3, 10 and 14 days.

Product	Before treatment	Post treatment storage time, days			
		0	3	10	14
Water, pH	5.9 ± 0.1	3.1 ± 0.05	3.0 ± 0.05	2.9 ± 0.05	2.7 ± 0.05
$[\text{NO}_3^-]$ mol/l	0	$(8 \pm 0.05) 10^{-4}$	$(1 \pm 0.05) 10^{-3}$	$(1.25 \pm 0.05) 10^{-3}$	$(2 \pm 0.05) 10^{-3}$
D (300 nm)	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.04 ± 0.01	0.02 ± 0.01
$[\text{ONOO}^-]$ mol/l	0	$<6 10^{-6}$	$(1.2 \pm 0.6) 10^{-5}$	$(1.8 \pm 0.5) 10^{-5}$	$<6 10^{-6}$
D (360 nm)	0.01 ± 0.01	0.09 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
$[\text{NO}_2^-]$ mol/l	0	0.005 ± 0.0015	$<6 10^{-4}$	$<6 10^{-4}$	$<6 10^{-4}$

Line $\lambda \sim 300 \text{ nm}$ immediately after IRradiation was not observed (see Table 1 and Fig. 3a), and it appeared 3 days after treatment; the maximum optical density was reached in 10 days and its optical density had considerably diminished by 14 days after treatment. Immediately after 20 min treatment with plasma radiation there is line $\lambda \sim 360 \text{ nm}$ (nitrous acid, ions NO_2^-), which disappears in 3 days (Table 1, Fig. 3a,b).



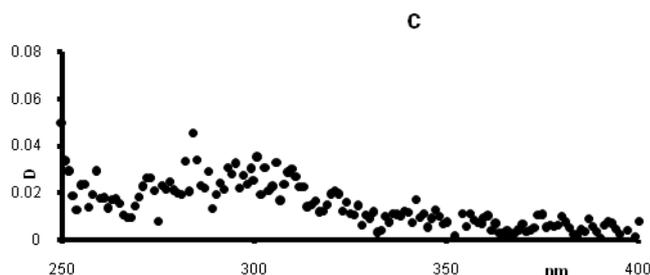


Figure 3: Absorption spectra for samples of water, treated for 20 min to gas-discharge plasma radiation: (a) immediately after treatment; (b) 10 days after treatment; (c) 14 days after treatment.

At 10 days the pH-value of treated water was 2.9, and the absorption spectrum is shown in Fig. 3b. Now, the line $\lambda = 300$ nm is seen, which has an optical density (related to baseline) of $D = 0.04 \pm 0.01$. The optical density for line 300 nm is the sum of optical densities $D(300 \text{ nm}) = D(\text{NO}_3^-) + D(\text{ONOO}^-)$. The optical density of the nitric acid according to its concentration (pH = 2.9) is $D(\text{NO}_3^-) = 10^{-\text{pH}} = 0.01$. The optical density of peroxyntirite is $D(\text{ONOO}^-) = 0.04 - 0.01 = 0.03 \pm 0.01$. Hence, the peroxyntirite concentration is $[\text{ONOO}^-] = (1.8 \pm 0.5) \cdot 10^{-5} \text{ mol/l}$.

At 14 days after treatment the pH-value of the treated water was 2.7 (Table 1), the optical density of line 300 nm was diminished and equal to 0.02 ± 0.01 (see Fig. 3c). For pH = 2.7 $[\text{NO}_3^-] = (2 \pm 0.05) \cdot 10^{-3} \text{ mol/l}$ and hence the optical density of nitric acid is $D(\text{NO}_3^-) = 0.014 \pm 0.005$. This means at 14 days after treatment the peroxyntirite was not observed, as all the value of optical density of the 301 nm line is within the limit of error can be attributed to NO_3^- ions.

So, immediately after plasma IRradiation, the pH value of water was reduced from 5.9 \rightarrow 3.1, which corresponds to the formation and decay of peroxyntirite with nitric acid production $[\text{ONOO}^-] = [\text{NO}_3^-] = (8 \pm 0.05) \cdot 10^{-4} \text{ mol/l}$. During the 14 days after exposure the pH-value was reduced from 3.1 \rightarrow 2.7, which corresponds to the formation and decay of peroxyntirite $[\text{ONOO}^-] = (1.2 \pm 0.05) \cdot 10^{-3} \text{ mol/l}$. The total concentration of peroxyntirite, which forms and decays at the time of IRradiation and during 14 days after treatment, determined on the basis of pH-value is $[\text{ONOO}^-] = (2 \pm 0.05) \cdot 10^{-3} \text{ mol/l}$. The concentration of peroxyntirite, which is seen in the optical spectrum, we will name as "instantaneous". The maximal instantaneous concentration of peroxyntirite, observed 10 days after treatment, was $(1.8 \pm 0.5) \cdot 10^{-5} \text{ mol/l}$, and diminishes to zero in 14 days.

Changes of pH values after treatment by UV mercury lamp radiation during 20 min within the limit of error was not observed. So, whereas the intensity of UV mercury lamp radiation at $\lambda = 253.7 \text{ nm}$ is 430 times more than that of spark discharge radiation in the UVC range, the peroxyntirite under mercury lamp radiation does not produce.

Reaction with stable free radical DPPH[•]

Concentration of peroxyntirous acid

It is known that free stable radical DPPH[•] has an absorption line of 520 nm [12]. Its compounds (non-radicals) have absorption lines as follows: DPPH-H ($\lambda = 330 \text{ nm}$), OH-DPPH-H ($\lambda = 302 \text{ nm}$), NO_2 -DPPH-H ($\lambda = 355 \text{ nm}$) [12], and ion DPPH^- ($\lambda = 428 \text{ nm}$) [13]. The spectra of the control samples (mix of distilled non-treated water and ethanol DPPH[•] solution 1:1) are in Fig. 4a,b,c (curve 1), and the spectra of treated to plasma radiation water and ethanol DPPH[•] solution (mix 1:1) are in Fig. 4a,b,c (curve 2). Optical densities of mixed solutions in the region of the observed lines are presented in Table 2, and the concentrations of DPPH[•] in mixed solution and ONOOH in water are in Table 3.

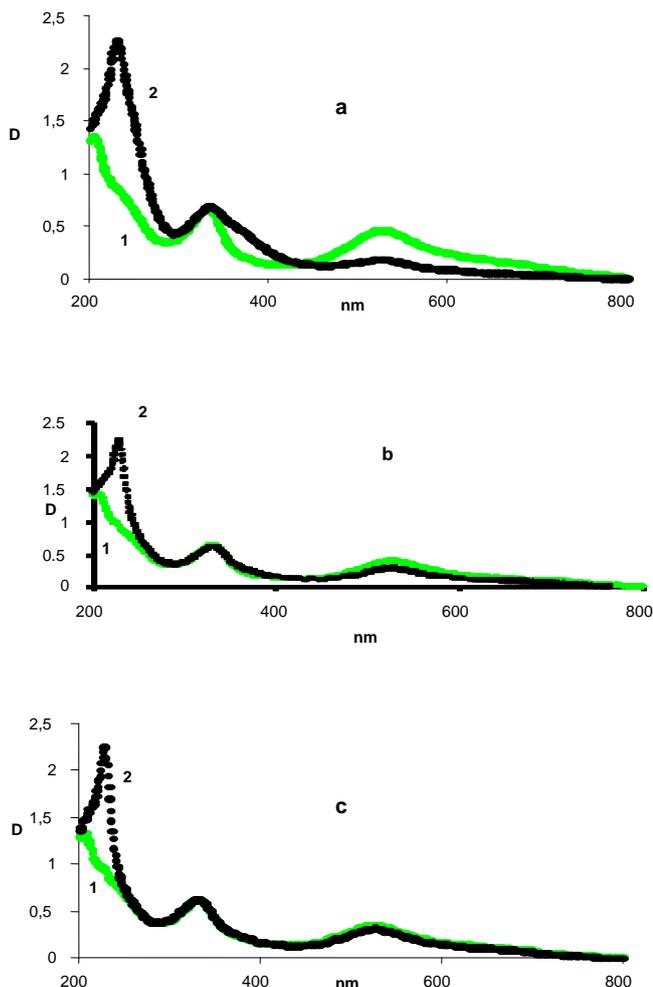


Figure 4: Absorption spectra for ethanol DPPH* solution, mixed with distilled water 1:1, curves 1, and for the same ethanol DPPH* solution, mixed with treated water 1:1, curves 2: (a) immediately after treatment, day 0; (b) day 3 after treatment; and (c) day 13 after treatment. Spectra were measured at 3 min after mixing.

Table 2: Optical density for region of absorption lines for DPPH* (520 nm), DPPH-H (330 nm) and NO₂-DPPH-H (355 nm) in mixture of ethanol DPPH* solution + water (1:1): untreated water, water immediately after 20 min plasma radiation treatment (day 0) and water after post-treatment storage time of 3 and 13 days.

DPPH lines	Optical density before treatment and after post treatment storage time, days			
	Untreated water	0	3	13
330 nm	0.67 ± 0.02	0.67 ± 0.02	0.67 ± 0.02	0.65 ± 0.02
355 nm	0.23 ± 0.02	0.43 ± 0.02	0.25 ± 0.02	0.24 ± 0.02
520 nm	0.46 ± 0.02	0.18 ± 0.02	0.29 ± 0.02	0.43 ± 0.02

Table 3: Concentration of [DPPH*] in mixture of ethanol DPPH* solution + water (1:1) and [ONOOH] for untreated water, water immediately after 20 min plasma radiation treatment (day 0) and water after post-treatment storage time of 3 and 13 days.

Products	Concentration, mol/l, before treatment and after post-treatment storage time. days			
	Untreated water	0	3	13
[DPPH*]	(3.5 ± 0.5) 10 ⁻⁵	(1.4 ± 0.5) 10 ⁻⁵	2.2 ± 0.5) 10 ⁻⁵	(3.3 ± 0.5) 10 ⁻⁵
[ONOOH]	0	(2.1 ± 0.7) 10 ⁻⁵	(1.3 ± 0.7) 10 ⁻⁵	< 0.5 10 ⁻⁵

Table 4: Initial yields of active species (experiment).

Species	Spark discharge plasma radiation	Low pressure mercury UV lamp
$\text{HO}_2^*/\text{O}_2^{*-}$	$(1.2 \pm 0.3) 10^{-6} \text{ mol(l s)}^{-1}$	$(1.1 \pm 0.5) 10^{-6} \text{ mol(l s)}^{-1}$
$\text{NO}_3^- + \text{NO}_2^-$	$(5.8 \pm 1.6) 10^{-7} \text{ mol(l s)}^{-1}$	$(3.4 \pm 1) 10^{-9} \text{ mol(l s)}^{-1}$
NH_4^+	$(1.7 \pm 0.5) 10^{-10} \text{ mol(l s)}^{-1}$	$(2.5 \pm 1.5) 10^{-8} \text{ mol(l s)}^{-1}$

In all spectra of the mix for treated water and DPPH^{*} solution there is line $\lambda = 225 \text{ nm}$ (curve 2, Fig. 4a,b,c). Its optical density does not change under different conditions. The peak at the same wavelength and with the same optical density is seen in the spectrum of treated water mixed with distilled water (1:1). The assignment of this peak may be as NO_3^- ions, which are produced under plasma radiation [8]. The peak at 225 nm is not essential for the given experiment.

In the control sample, there are two lines: $\lambda = 330$ and 520 nm (see Fig. 4a, curve 1). The presence of two lines may be explained as two compounds in the solution: DPPH^{*} and DPPH-H. In the sample immediately after treatment (Fig. 4a, curve 2) the 520 nm line is strongly decreased. The optical density at $\lambda = 330 \text{ nm}$ does not vary, but on the right side of this line there is a not resolved (not distinguished) line in the region $\lambda \sim 355 \text{ nm}$ (see Table 2).

The decrease of DPPH^{*} ($\lambda = 520 \text{ nm}$) concentration and the line appearing at $\sim 355 \text{ nm}$ could be caused by reaction with nitrous acid, which is produced under plasma radiation [8]:



Reaction (1) could play a decisive role during first 2 days after IRadiation, when nitrous acid exists before its decay. The donor of protons for DPPH^{*} could be peroxyntrous acid:



Peroxyntrous acid could be a reductant in this process as the formal oxidation state of the nitrogen atom in a peroxyntrous acid is +3, but for ion NO_3^- the oxidation state of the nitrogen atom is +5. The reaction (2) could play decisive role on the third day after radiation treatment, when nitrous acid is decomposed. Peroxyntrous acid can be part of a complex, produced under a radiation pulse (see section on mechanisms of peroxyntrite and peroxyntrous acid production). Then, in reaction (2) another chemical (not DPPH-H) will form. In fact, this takes place as the optical density of the 330 nm lines does not change (see Table 2). The reagent DPPH-H is not consumed, essentially as reagent has less chemical activity than DPPH^{*}.

In 3 days after treatment, line $\sim 355 \text{ nm}$ has disappeared (see Table 2 and Fig. 4b), line 330 nm is the same as initially and line 520 nm increased compared to in water immediately after treatment (see Table 2). The next day's optical density of 520 nm lines slowly increased, and in 13 days it was almost equal to initial value (for non-treated water; see Fig. 4c and Table 2).

So, the decrease of optical density of the 520 nm line in the 3 days after treatment compared to control sample is about 30% ($0.46 \rightarrow 0.29$; see Table 2). In the control sample, according to the spectral measurements $[\text{DPPH}^*] = (3.5 \pm 0.5) 10^{-5} \text{ mol/l}$ (see Fig. 4a, curve 1; for $\lambda = 520 \text{ nm}$, $\epsilon = 10^4 \text{ l mol}^{-1} \text{ cm}^{-1}$ [16]). This is in agreement with the chemical concentration of the initially dissolved reagent, $[\text{DPPH}^*] = 3.15 \text{ mol/l}$.

Let us evaluate the amount of peroxyntrous acid which is consumed in the reaction with DPPH^{*}. Both reactions (1) and (2) for water immediately after treatment (day 0) took place. By the third day nitrous acid in treated water fully decayed, and the only reduction agent is peroxyntrous acid. Therefore, it is possible to evaluate the concentration of peroxyntrous acid which is consumed by DPPH^{*} reduction. The decrease of $[\text{DPPH}^*]$ concentration equals the $[\text{ONOOH}]$ concentration if in the reduction of one molecule of DPPH^{*} one molecule of peroxyntrous acid is spent (according to reaction 2). On the third day the decrease of $[\text{DPPH}^*]$ after mixing with treated water was $\Delta[\text{DPPH}^*] = 1.3 \cdot 10^{-5} \text{ mol/l}$ (see Table 2). Then, the concentration of consumed peroxyntrous acid in treated water was $[\text{ONOOH}] = (1.3 \pm 0.7) 10^{-5} \text{ mol/l}$. This value is the

instantaneous concentration of ONOOH. With increasing post-treatment storage time, the concentration of peroxyntrous acid decreased, and it became undetectable in 13 days. The instantaneous concentration of peroxyntrous acid $(1.3 \pm 0.7) \cdot 10^{-5}$ mol/l was close to instantaneous peroxyntrite concentration, $[\text{ONOO}^-] = (1.8 \pm 0.5) \cdot 10^{-5}$ mol/l, found in absorption spectra.

Time dependence of D/D₀ (520 nm)

The optical density ($\lambda = 520$ nm) dependence of ethanol DPPH[•] solution mixed with treated water (1:1) versus the time treatment of distilled water by plasma radiation of an IR generator and mercury UV radiation lamp DBK-9 were investigated (for results see Fig. 5). In the case of UV-lamp IRradiation, the optical density D(520 nm) decreased after 60 min treatment, decrease did not exceed 2%, and within the limit of error the optical density did not change (Fig. 5, curve 1). This means the active species under mercury lamp radiation in water is not produced within the limit of experimental errors, while photon flux from mercury UV-lamp is 430 times more than that from plasma radiation. In the case of plasma radiation, the optical density of line 520 nm decreases and after 60 min treatment its value is 60% of the initial value (Fig. 5, curve 2). After 20 min treatment by plasma radiation the optical density of line 520 nm very slowly diminishes. This means the active species concentration of treated water reached a steady state concentration in ~20 min, and did not increase with IRradiation time.

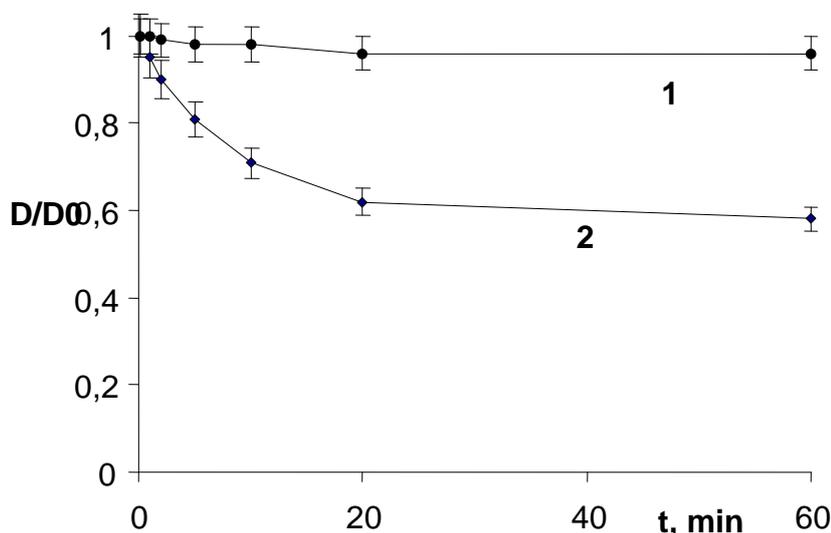


Figure 5: The time dependence of ratio D/D₀, where D is the optical density of line 520 nm for the mixture (ethanol solution of DPPH[•] + treated water, 1:1) after treatment at time t(min); D₀ is the optical density of the same line for solution (DPPH[•] + distilled water, 1:1): 1 – treatment by UV radiation of mercury lamp DBK-9; 2 – treatment by gas-discharge plasma radiation.

The high efficiency of plasma radiation compared to UV lamp radiation is due first to the pulse character of radiation and shorter wave length of radiation. The results of this experiment mean that under the mercury lamp radiation, the peroxyntrous acid and peroxyntrite are not produced.

Value pH of treated solutions

The kinetics of the pH-value variation for pure water and the aqueous solution of albumin depending on time treatment to spark gas-discharge plasma radiation is presented in Fig. 6. For pure water, the pH-value after 1–2 min treatment is diminished by up to 3.5–4 (curve 3), and after 30 min reached pH = 2.56. For bovine serum albumin solution (1 g/l) the diminution of the pH-value is weaker (2), and for the albumin concentration at 25 g/l during first minutes pH does not change within the limit of error and after that it slow diminishes up to pH = 5.05 at t = 30 min (1). The results of this experiment indicated that the presence in the solution of

dissolved organic compound prevents peroxyxynitrite and peroxyxynitrous acid production, decay of which leads the pH to diminish.

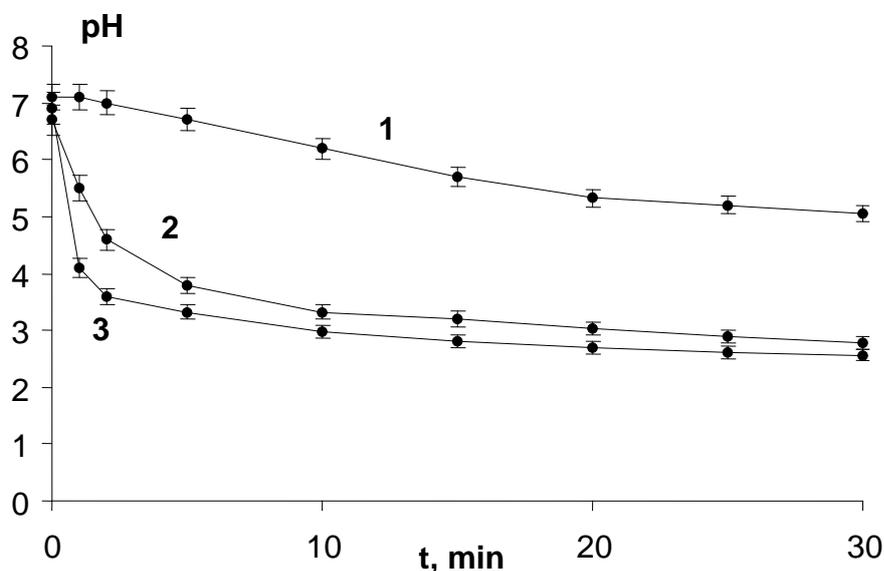


Figure 6: Value of pH of samples exposed to IR during time t (min): 1, 2 – solution of bovine albumin (1 – 25 g/l, 2 – 1 g/l); 3 – distilled water.

DISCUSSION

Mechanisms of active species generation in liquid under UV radiation

Photosensitizer

The most biological activity involved UVC (100–280 nm, in air really 180–280 nm) radiation. In pure water the direct absorption of UVC-photons and active particle (radicals) production plays the main role. The UVB (280–315 nm) and UVA (315–400 nm) radiation can produce a biological effect by means of the production of radicals if there is photosensitizer (chromophore) in the sample. Direct damage of the sample induced by UVB and UVA radiation was limited. There are two types of processes that govern the actions of the sensitizers, divided into Type 1 and Type 2 reactions. The review of photo destruction processes under UVA and UVB light is described in study [14]. In Type 1 reactions, the sensitizer in the triplet state interacts with the substrate by hydrogen atoms or electron transfer to form radicals. In the Type 2 reaction, the triplet sensitizer interacts with O₂ by energy transfer to generate singlet oxygen, which further reacts with the substratum. Both types of reaction lead to protein damage [4]. Nitrite can be a photosensitizer because the N-O bond of the nitrite ion in aqueous solution will be disrupted by the energy of the light within the UVA and UVB spectrum: $\text{NO}_2^- + h\nu \rightarrow \text{NO}^\bullet + \text{O}^{\bullet-}$ [14].

So, with photosensitizers (chromophores) it is possible for radical creation (Type 1 reactions) and singlet oxygen formation (Type 2 reactions) to take place [15]. In proteins, such chromophores are tryptophan, tyrosine and phenylalanine. Tryptophan is a stronger chromophore, but its concentration in proteins is relatively small (ratio Trp:Tyr is 1:18). The main energy part absorbed by these chromophores is emitted with photons, and only a small part is spent in Type 1 and 2 reactions. The initiator of tryptophan oxidation under UVA and UVB IR radiation is tryptophan itself. In lipids, there is no strong chromophore; therefore, this mechanism for does not work for them.

Photodestruction via Type 1 and 2 reactions has been experimentally observed in many investigations, but it should be emphasized that the light intensity (luminous emittance) must be large (about 30000 lux), the power of the lamp should be about 300 W and the treatment time should be 10–20 hours to

make the mechanism of photodestruction via chromophores appreciable [16]. Treatment time can be reduced if the chromophore is not tryptophan, but chemicals with a big extinction coefficient. In one study [17], clay is used as the chromophore, with $\varepsilon = 10^5 \text{ l mol}^{-1} \text{ cm}^{-1}$. The power of the light source was 300 W, and appreciable oxidation over 1 hour was observed. Thus, Type 1 and 2 reactions demand a large energy expense.

The maximum treatment time in our experiments was 60 minutes, and in many cases it was 20 minutes. The role of chromophores in our experiment was small, as radiation intensity and treatment time are small compared to conditions where Type 1 and 2 reactions take place. In addition, we used only pure distilled water. Therefore, the main photo oxidation mechanism in our work is direct active particles (radicals) produced in water under UVC radiation.

Power of radiation source

It should be emphasized that the active particles (radicals R^\bullet) produced by physical action (discharge) in solution can interact with dissolved molecules B at the rate $w_1 = k_1[R^\bullet][B]$ and terminate at the rate $w_2 = k_2[R^\bullet][R^\bullet]$; that is, they uselessly disappear. As we are interested first in chemical effects, the rate of reaction with molecules B must essentially be more than the rate of termination of radicals and must be $w_2 \ll w_1$. Hence, the condition for achieving maximum chemical effect is:

$$[R^\bullet] \ll \frac{k_1}{k_2} [B] \quad (3)$$

That is, the concentration of generated radicals must be small enough [18]. When the optimal condition was found, both a decrease and an increase in the power of the physical action (in our case, for spark discharge plasma radiation, the power of electric discharge and the discharge capacitor value) equally led to a decrease in the chemical effect. This case is realized in our work, as it was found that the optimal chemical effect in the liquid under the action of plasma radiation (decreasing pH, accumulation of oxidants and reductants) is achieved at a discharge capacitor value of 3.3 nF [7]. The chemical effect reduces with both decreases and increases of the discharge capacitor value.

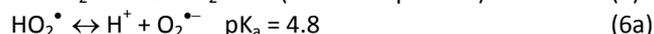
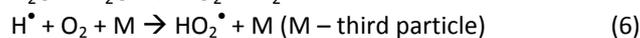
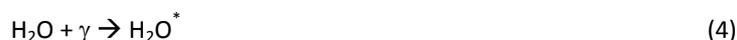
Direct active species production

Another mechanism of UV-induced reaction is the creation of direct active particles (radicals) without reference to the substratum, only in pure water containing dissolved oxygen and nitrogen (air). This mechanism is realized for UVC plasma radiation sources used in this work.

The possibility of creating active species under external physical impact is determined first of all by the energy conservation law – the process must be energetically possible. In case of plasma, which consists of ionized particles, the energy concentration is high and at plasma contact with the treated target many active species are produced, as in plasma itself.

For UV radiation, the energy concentration is essentially less (with the device of the same power as the plasma generator). The energy concentration is enhanced if pulse radiation is used; therefore, pulsed radiation provides stronger effects in the treated target.

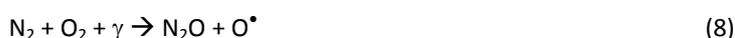
The investigation of the plasma radiation effects of the same electric discharge, which is used in our work and described in [7, 9], showed that under the action of plasma radiation in water the pH-value was decreased (produced NO_3^- ions) and oxidants and reductants were accumulated. The mechanism for active particle production in water under UV radiation of pulsed plasma gas-discharge through excited states of water molecules was proposed and analyzed in [8].





In the act of the production of one active species (HO_2^{\bullet} radicals), two photons are needed (two excited water molecules, reaction 5). Primary products (excited water molecules) created at the time of the photon flash, in view of their high instantaneous concentration, interact with one another (reaction 5). In a continuous photon beam this condition is not realized, and the probability of creation of active species is essentially lower. The production of HO_2^{\bullet} radicals was determined in reaction with oxalic acid and Fe^{2+} [6]: HO_2^{\bullet} radicals cannot oxidize oxalic acid but oxidize Fe^{2+} . The pH value is diminished during first minute of treatment with plasma radiation up to $pH < 5$; therefore, the main role in the reactions is played by HO_2^{\bullet} radicals, not ion-radicals $O_2^{\bullet-}$. Oxalic acid is not oxidized under spark discharge plasma radiation, as it can be oxidized only with OH^{\bullet} radicals and not with HO_2^{\bullet} radicals. Hence, in [6] it was stated that OH^{\bullet} radicals in course of treatment by plasma radiation of the UVC range are not produced as primary species.

The dissolved gases present in water are nitrogen and oxygen (air). The energy of UV photons with $\lambda = 200\text{--}250$ nm is enough to realize the following processes:



The probability of reactions 8 and 9 occurring in the course of treatment of 253.7 nm radiation is small, as the energy of photons is small, but they can have appreciable probability under spark plasma radiation for 200–220 nm wavelengths. Final products of all transformations for nitrogen compounds are nitric and nitrous acids. Therefore, the yield of reaction 8 was evaluated in [8] from pH diminution of water after treatment.

Thus, it has been stated [8] that the primary active species produced in water exposed to UV radiation in the case of spark discharge plasma are $HO_2^{\bullet}/O_2^{\bullet-}$ radicals, O^{\bullet} atoms and N_2O molecules. The final observed products are radicals $HO_2^{\bullet}/O_2^{\bullet-}$, ions $NO_3^- + NO_2^-$ and NH_4^+ . Initial yields of these products under spark discharge and mercury lamp radiation are presented in Table 4 [9]. The yield of ions NH_4^+ is small, they do not play an appreciable role. It should be emphasized that yields of $HO_2^{\bullet}/O_2^{\bullet-}$ at spark discharge and UV mercury lamp radiation used in this work from radiation sources are the same within limits of experimental error, but yields of $NO_3^- + NO_2^-$ ions are essentially different. Therefore, the comparison of effects under spark discharge and mercury lamp radiation may be a useful tool for scientific research. The mechanisms of nitric and nitrous acid production through peroxyxynitrite and peroxyxynitrous acid decay are discussed below.

Mechanisms of peroxyxynitrite and peroxyxynitrous acid production

The primary species produced immediately under UVC radiation activate a chemical transformation [8]. In one study [8], 25 reactions were considered in which secondary active species are produced: OH^{\bullet} , H_2O_2 , $O_2^{\bullet-}$, O_3 , NO^{\bullet} , NO_2^{\bullet} , N_2O_3 , N_2O_4 , HNO_2 , HNO_3 and $ONOO^-$.

The decrease of pH value is caused by peroxyxynitrite production and its decay:



According to energy and impulse conservation law, reaction 10 is possible only with the participation of third body M. This reaction can take place in liquid, but in practice is impossible in the gas phase, where the density of the surrounding particles is small. Ion-radicals $O_2^{\bullet-}$, which participate in reaction 10, are produced in reaction 6a and NO^{\bullet} radicals are produced in reaction 12 [8]:



In study [8] it was stated that an explanation of nitric acid yield, which is observed in the experiment, is impossible without reaction 10.

At a high instantaneous radiation flow at the time of the radiation pulse it is possible to form a complex including ONOOH and ONOO⁻. The possibility of producing such a complex has been discussed in [11]. Then, the process can be realized as follows. At the time of radiation burst a chemical compound X is produced, which continues to interact with chemicals accumulated in water treated with plasma radiation, in particular with NO₂⁻ and products of NO₂⁻ decay, transforming it into chemical compound X₁ (X → X₁), which in the course of time (up to 14 days) decays with peroxyxynitrite production: X₁ → ONOO⁻. Chemical compounds X and X₁ do not absorb in the UV and visible ranges [11]. The specific peroxyxynitrite spectrum appeared 3 days after treatment (after full NO₂⁻ ions decay), reaching the maximum of optical density in 10 days, and decreasing to the background level (optical density of NO₃⁻ ions) in 14 days. The peroxyxynitrous acid, which is a protonated form of peroxyxynitrite that exists in acid and neutral solutions, is found in reaction with DPPH[•]. So the post-radiation effects, which are found in our work, can be attributed to the formation and decay of the peroxyxynitrite-peroxyxynitrous acid complex.

Interaction of active species with biological sample

If there are dissolved chemicals in solution, the primary active species will interact first of all with these chemicals. The mechanism for active species transformation, which arises under spark gas-discharge plasma radiation, can be pictured as follows (see Figure 7).

Plasma radiation enters into the liquid through the liquid's surface. In water, primary active species are produced (HO₂[•]/O₂^{•-}, O[•], N₂O; level 1, Figure 7). First, these species are consumed in interactions with the substratum, as the substratum concentration is usually much greater than that of active species. After the substratum concentration is diminished in reactions with primary active species, the primary species will interact with one another and secondary species will be produced (level 2). The main secondary species are ONOO⁻ and ONOOH [8]. Peroxyxynitrite and peroxyxynitrous acid will interact with the substratum and with one another. As pulse radiation generates active species with a high instantaneous concentration, the ...ONOOH, ONOO⁻... complex can be produced (level 3). The complex itself has a low activity level; its lifetime is up to 14 days. Complex decay products (ONOOH, ONOO⁻, NO₂⁻, NO₃⁻) have chemical activity and can be reductants and oxidants. If there is no substratum, levels 1, 2 and 3 are connected, and the products of level 3 (NO₂⁻ and NO₃⁻) appear immediately.

Active species also are produced in region of electric discharge. Reactions in that region can be divided into several stages. The first stage is at the time of breakdown at the front of the pulse, when voltage applied to the discharge gap is maximal (in our case 11 kV) and electric field strength has a high value. The duration of this stage is 50–100 ns. At this stage of the discharge, active species are effectively generated. The second stage is started when the formation of the discharge channel is completed. The voltage of discharge gap falls by up to a 100 volts. The temperature of the heated plasma cord is high and active species can be generated. The concentration of active species is high and they will interact firstly with one another [18]. As a result, a less active compound appears, among which nitrosamine and hydrocarbons were found [7] (as in the gas phase, there are oxygen, nitrogen, water vapour and carbon dioxide). Nitrogen compounds (such as N₂O) can be generated, but are consumed in further reactions with products in the discharge region. At the third stage after discharge the products in the spark region are cooling and settling on the walls of vessel (and on sample surface). Among these products N₂O were not found. The proportion of the energy absorbed in the air compared to that in the water is negligibly small. Therefore, the processes in air under spark gas-discharge plasma radiation are not essential. Active species, produced in plasma itself, terminate in the place of their production and cannot reach the surface of the sample, as the distance of the sample–discharge region is 1 cm. So, in our case, active species, which are generated in region of discharge and in the gas phase do not play an essential role. All processes are determined by the radiation absorbed in the sample.

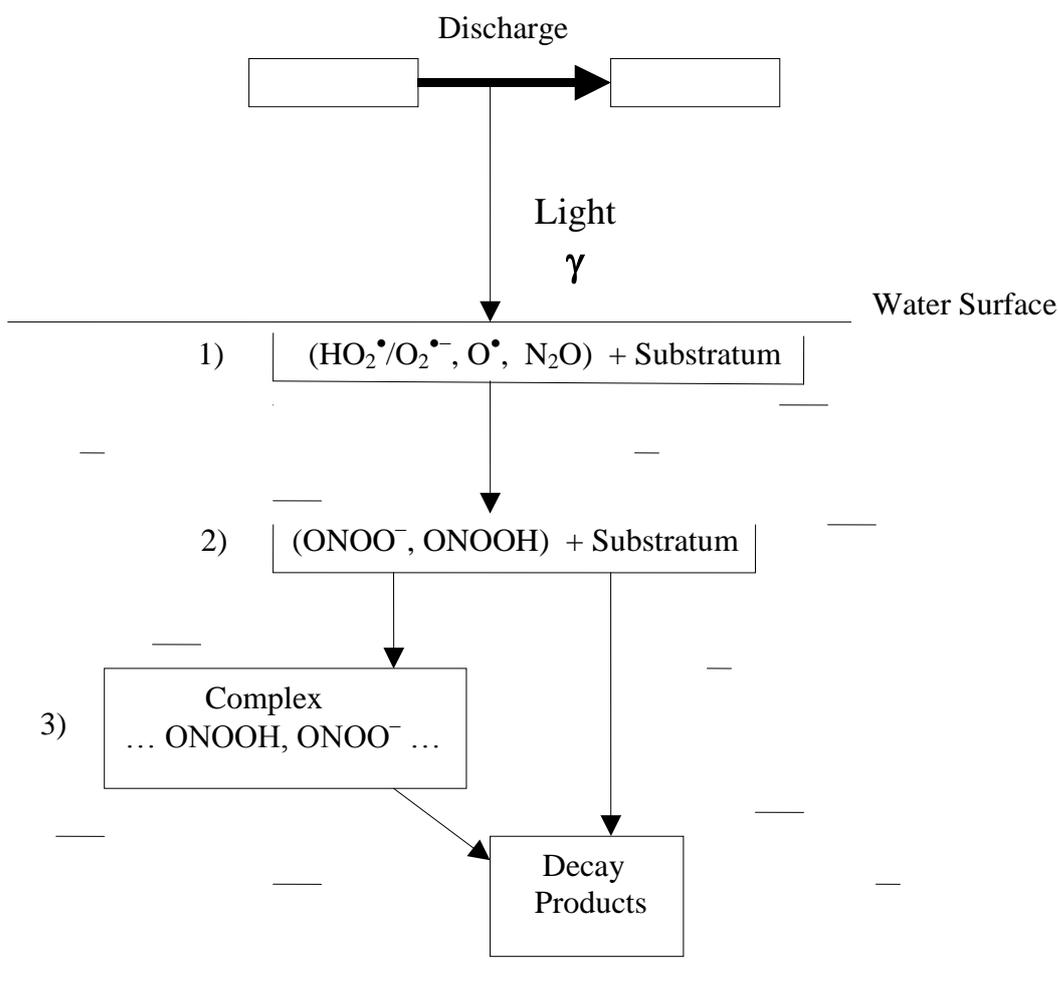
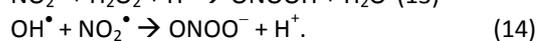
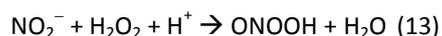


Figure 7: The proposed scheme of processes in aqueous solution under spark gas-discharge plasma radiation.

When the plasma is in direct contact with a liquid surface, as investigated in studies [19–21], there are other processes. Active species are generated on the liquid's surface and enter into the liquid, and diffusion takes place. In contrast, under spark gas-discharge plasma radiation the active species is generated directly in the liquid where the radiation has penetrated (up to 40 mm for water). The mechanism for the pH decreasing in both cases is the same: peroxyntirite production and its decay.

In the case of gas-phase discharge in contact with the water surface, the mechanisms of peroxyntirous acid and peroxyntirite production are [21]:



Reaction (10) in study [21] is also included in the mechanism of peroxyntirite formation, but this reaction can be realized only with a third body M (according to the energy and momentum conservation law). In the gas phase the concentration of circumambient bodies is essentially less than in water. Therefore, the role of this reaction in the case of peroxyntirite production in the gas phase must be small.

The production of a long-lived chemical compound, which decays on peroxyxynitrite, can be the cause of a strong sporicidal effect after treatment of the micromycets spore by spark gas-discharge plasma radiation (generator IR-10), as in [5, 22]. Micromycets spores are covered with an opaque peptidoglycan layer, through which UV radiation cannot penetrate the spore cell as short-lived species (radicals) that are terminated before entering through the cell envelope. The species living up to 14 days can enter into spore, they are decayed on peroxyxynitrite, which causes irreversible damage to DNA molecules [23]. As result the spores does not grow out and 100% sporicidal effect is achieved. Evaluation of the cytotoxic effect mechanisms of gas-discharge plasma radiation has been made in [24].

CONCLUSION

1. The primary active species produced in the course of treatment by gas-discharge pulsed plasma radiation in the sample are: $\text{HO}_2^\bullet/\text{O}_2^{\bullet-}$, O^\bullet and N_2O .
2. The main secondary active species, decay of which led to a pH decrease, are peroxyxynitrite and peroxyxynitrous acid.
3. Peroxyxynitrite and peroxyxynitrous acid are formed complexes, the lifetime of which is up to 14 days. After the complex decayed, peroxyxynitrite and peroxyxynitrous acid appeared; instantaneous concentrations are $[\text{ONOO}^-] = (1.8 \pm 0.5) 10^{-5} \text{ mol/l}$ and $[\text{ONOOH}] = (1.3 \pm 0.7) 10^{-5} \text{ mol/l}$.
4. Peroxyxynitrite and peroxyxynitrous acid are not produced under the radiation of the mercury lamp, although the intensity of lamp radiation is 430 times more than the intensity of the plasma discharge radiation in the UVC range.
5. The sporicidal effect, which is observed under plasma radiation, can be caused by peroxyxynitrite.
6. Comparative investigation of effects of spark discharge under IR and UV mercury lamp radiation may find applications in biomedical investigations, as the yield of $\text{HO}_2^\bullet/\text{O}_2^{\bullet-}$ is the same, but yields of NO_2^- and NO_3^- essentially differ according to the various types of radiation sources used.
7. The simplicity of the construction of the discussed IR generator and low power supply compared to the appreciable chemical effect produced in liquid could be a stimulus for application of the generator in the practice of biomedical investigations.

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