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DNA Damage after Ozone-Photodynamic Therapy in Cancer Animals: Experimental Research

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Abstract: Objectives: To assess the genotoxic effect of a new antitumor ozone-photodynamic therapy using the improved modification of the COMET assay. Methods: Xenograft cancer models on 58 rats were used. The sarcoma RA was transplanted subcutaneously, and after increasing of tumor volume from 0.5 to 4.2 cm^3, rats were divided into the four groups: "Intact"—healthy, "Control"—with xenografted tumors and no treatment, "PDT"—the rats treated with the photodynamic therapy, "PDT + ozone"—the rats were treated with both photodynamic therapy and injections of ozonated saline solution. The toxicity of treatment was assessed by DNA damage in leukocytes using the new modification of the COMET assay. The analysis of the "COMETs" was performed following the percentage of DNA in the tail of the "COMET" (% TDNA). Results: A combination of PDT and ozone makes the strongest negative impact on tumor growth. The tumor growth inhibition is associated with low genotoxic exposure of ozone-photodynamic therapy on whole blood leukocytes of cancer rats. Conclusions: A new modification of the COMET assay can provide the assessment of the genotoxic effect of the antitumor therapy in experimental neoplasia.

Key words: COMET assay, DNA damage, ozonetherapy, photodynamic therapy, experimental neoplasia.

1. Introduction

PDT (photodynamic therapy) is used in the treatment of different types of cancer: skin [1-3], esophageal [4, 5], lungs [6-8], nervous system [9, 10], female genital organs [11-13], biliary tract [14], head and neck [15-17]. However, the widespread usage of PDT in clinics is limited by a number of factors such as inefficient sources of singlet oxygen [18], low depth of penetration into human tissue [19], phototoxicity [20] and genotoxicity caused by DNA single strand breaks of leukocytes of blood [21, 22]. Therefore, there is a real necessity to find new approaches for improving the therapeutic effects of PDT.

One way to improve PDT is to combine with other photosensitizers [23-26]. However, the authors of these works pointed out the possibility of long-term side effects caused by accumulation of mutations due to the incomplete repair of DNA damage. It was proposed [27-29] that it is single oxygen, the product of the combined PDT with photosensitizers, that causes cytotoxic and mutagenic effects by damaging DNA and cell organelles. However, there are only a limited number of works studying DNA damage and repair after PDT [21, 29, 30].

A level of DNA damage and repair in single non-dividing nucleated cells can be determined by a single cell gel electrophoresis—the COMET assay [31]. Currently, there are two versions of the COMET assay: alkaline and neutral. The alkaline version is used to detect a broad spectrum of induced DNA damage, such as DNA single strand breaks, nucleotide base modifications, and alkali-labile sites [32-38]. The neutral version of COMET assay is typically used for detecting DNA double-strand breaks [39].

However, the standard implementation of COMET test is not used in medical practice. In practice, it is difficult to standardize an impact of radiation. Therefore, we used a new version of the COMET

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assay that makes it possible to introduce this test to clinical medicine [40].

In this study, we proposed and explored a new approach for enhancing efficiency of PDT by increasing the oxidative stress in a tumor tissue. To this end, we used medical ozone as a source of reactive oxygen. It is known that medical ozone has antihypoxic [41] and antineoplastic [42] effects. At certain concentration, ozone can normalize the oxidation-reduction processes [43] and stimulate the immune defense of the organism [44, 45].

We proposed that a combination of PTD with ozone will enhance an exposure of tumor tissue to free radicals increasing the therapeutic impact of PDT. This hypothesis was tested in experimental models of neoplasia in rats. We found that: (1) a combination of PDT and ozone makes the strongest negative impact on tumor growth; (2) an inhibition of tumor growth is associated with low genotoxic impact of ozone -photodynamic therapy on whole blood leukocytes in rat models of cancer. We also showed that a new modification of the COMET assay can be used to assess the genotoxic effect of the antitumor therapy in experimental neoplasia.

2. Materials and Methods

The experiments were performed on 58 white non-linear male-rats weighing 250 ± 25 g (Fig. 1).

The animals were removed from the experiment by decapitation under anesthesia. All manipulations have been carried out in compliance with the international principles of the Helsinki Declaration 2000 of the humane attitude of the animals.

The tumor strain of carcinoma RA was selected as a

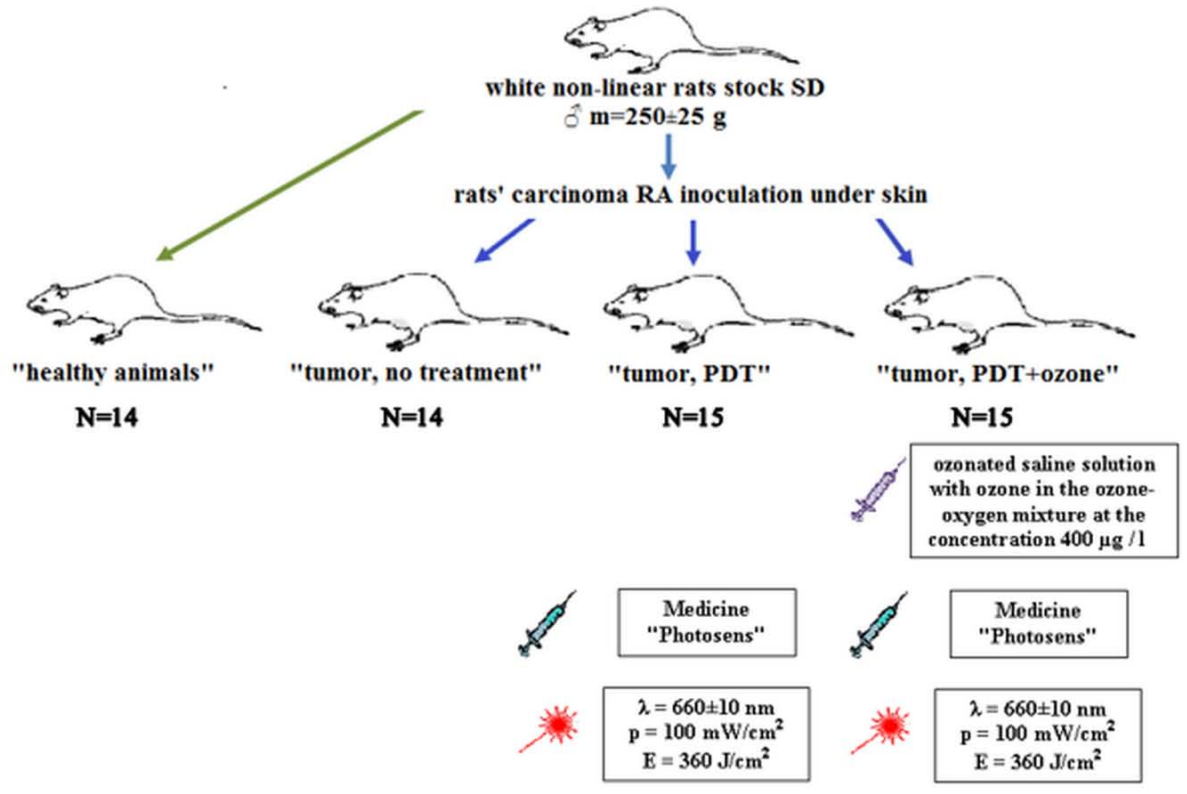


Fig. 1 General schema of the experiment. Rates of tumor growth, survival differences and levels of remaining DNA damage in leukocytes were assessed four groups of animals: (1) "No tumor" (*n* = 14)—healthy animals; (2) "Tumor, no treatment" (*n* = 14)—the rats with tumor but without treatment; (3) "Tumor, PDT" (*n* = 15)—the rats with tumor after photodynamic therapy (PDT); (4) "Tumor, PDT+Ozone" (*n* = 15)—the rats with tumor after the course of injections of ozonated saline solution and photodynamic therapy.

model of neoplasia. The tumor strain was made by under skin inoculation to non-linear rats. The RA carcinoma strain was obtained from the Bank of tumor strains of the Blokhin Russian Cancer Research Center of Russian Academy of Medical Science. The incubation period of this tumor strain is short (7-10 days). The development of the tumor is typically completed on 26th day from the time of inoculation. This tumor strain is characterized by a large biomass and a high rate of successful inoculation [46].

The animals were divided into four experimental groups (Fig. 1): (1) "healthy animals" (n = 14); (2) "Tumor, no treatment", control (n = 14)—the rats with tumor but no treatment; (3) "Tumor, PDT" (n = 15)—the rats with tumor after photodynamic therapy; (4) "Tumor, PDT + ozone" (n = 15)—the rats with tumor after the course of injections of ozonated saline solution and photodynamic therapy. Only rats with a tumors volume ranging from 0.5 to 4.2 cm^3 were selected into groups 2-4.

The synthetic second-generation photosensitizer—gidroksialyuminiya trisulfoftalotsianin ("Photosens") (FSUE "SRC" NIOPIK, Russia) was used for photodynamic therapy. It has the maximum of the absorption (676 nm) in the region of spectrum which is transparent in biological tissues. This makes it possible to expose the deeper layers of the tumor tissue. However, "Photosens" has some disadvantages. The main of them is a high value of the dose (0.5-0.8 mg per kg of body weight). As a result, it has adverse dermatological toxicity. The local (intratissual) method of injection of the photosensitizer in tumor tissue was used to solve this problem [47, 48]. The 0.3% solution of the drug "Photosens" was injected in the three points of tumor based on 30% of the tumor volume. Then each of the three points of the tumor was exposed to LED radiation ($\lambda = 660 \pm 10$ nm, p = 100 mW/cm^2) for 10 minutes in 6-12 hours after the "Photosens" injections. The physiotherapy LED device APS ("Polironik", Russia) was used as light source. The two PDT were performed on the 15th and 19th day after inoculation. This is conformed to the period of active growth of the carcinoma RA.

Starting on the 10th day after inoculation of the tumor strain, injection of ozonated saline solution was performed for 10 days (the 5 exposures every other day). To this end, the 0.5 mL of ozonated saline solution with ozone in the ozone-oxygen mixture at the concentration 400 μg /L were injected abdominally. This scheme was chosen based on the published results [42]. The selection of the ozone concentration was made based on the experimental data of the potentiating of the antitumoral effect of ionizing radiation, 5-fluorouracil and doxorubicin [49] as the most appropriate for the systemic exposure on tumor. The ozonation was carried out with using the medical ozonator "TEOZONE" (the Russian Federal Nuclear Center, All-Russian Scientific Research Institute of Experimental Physics, RFNC-VNIIEF, Sarov, Russia).

The antitumoral effect of the therapy was evaluated using the coefficient of the absolute tumor growth (C). It was calculated using the following Eq. (1):

$$C = \frac{V_t - V_o}{V_o};\ V = \frac{\pi}{6} \cdot \left(\frac{d_1 + d_2}{2}\right)^3 \quad (1)$$

where: d_1 and d_2—the two mutually perpendicular cross-section of tumor, cm;

V_o—the tumors volume before exposure, cm^3;

V_t—the tumors volume at the period of observation, cm^3 [50, 51]. $C \geq 0$—the extended tumors growth, $-1 \leq C < 0$—the tumors regression.

The level of DNA damage in leukocytes of whole blood of rats was determined with the new version of the COMET assay protocol [40].

DNA reparation was evaluated after exposure to ozone in the ozone-oxygen mixture at the concentration of 900 μg/L for 10 minutes on the cells in the slides and incubation in phosphate buffer saline (pH 7.4-7.5) for 1 hour at 37 °C. The characteristics of the DNA reparation were derived based on the level of

remaining DNA damage after incubation.

The analysis of the "COMETs" was based on assessment of the percentage of DNA in the tail of the "COMET" (% TDNA). The 100 COMETs from 2 slides in each glass have been observed and analyzed with specialized software "COMET.exe". Totally, 162 slides on 81 glasses were analyzed for evaluation of the level of DNA damage and repair.

These data were analyzed using the methods of nonparametric statistics. The uniformity of tumor volume distribution in the experimental groups was evaluated using Kruskal-Wallis test at the confidence level of 0.95. Significance of the observed survival differences between experimental groups was assessed by Mann-Whitney test.

3. Results and Discussion

The results of the dynamics of tumor growth, the average values of the levels of spontaneous (%TDNA of "healthy animals" = 4.3 ± 0.3; %TDNA of "tumor, no treatment" = 5.8 ± 0.6; %TDNA of "tumor, PDT = 4.2 ± 0.7; %TDNA of "tumor, PDT + ozone" = 7.6 ± 1.0) and remaining DNA damage in leukocytes of whole blood of rats after the therapy are shown in the Figs. 2 and 3. The details of COMET tails morphology for spontaneous and remaining DNA damage in whole blood leukocytes after the therapy are shown in Table 1.

The analysis revealed statistically significant difference between tumors growth rate in the group of combined exposure of photodynamic therapy with ozone (p = 0.045) as compared to the tumor growth rate in the group without ozone exposure (Fig. 3).

The photosensitizers are mainly localized in the cytoplasm of cells or in the cells membrane [34]. Therefore, it would be reasonable to expect that the ozonated saline solution with ozone in the ozone-oxygen mixture at the concentration 400 μg/L will improves the penetration and uniform distribution of the photosensitizer in the tumor cells because the main effects of ozone are associated with the impact on the cell membrane. It is known that ozone does not penetrate into the cell at the low concentrations. Ozone reacts with the cell surface components forming

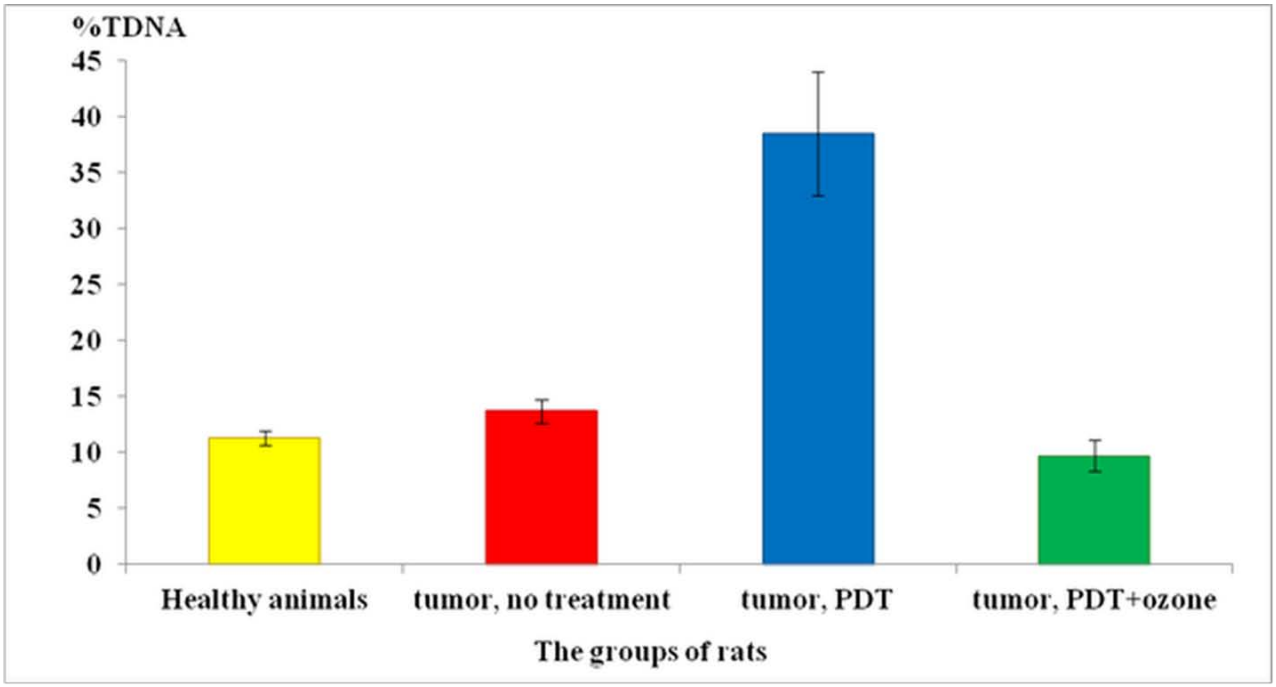


Fig. 2 The average values of the levels of remaining DNA damage in leukocytes of whole blood of rats after the therapy. The levels of remaining DNA damage are measured by a percentage of DNA in the tail of the "COMET" (%TDNA). Adding ozone to organism drastically reduces the genotoxic effect of photodynamic therapy.

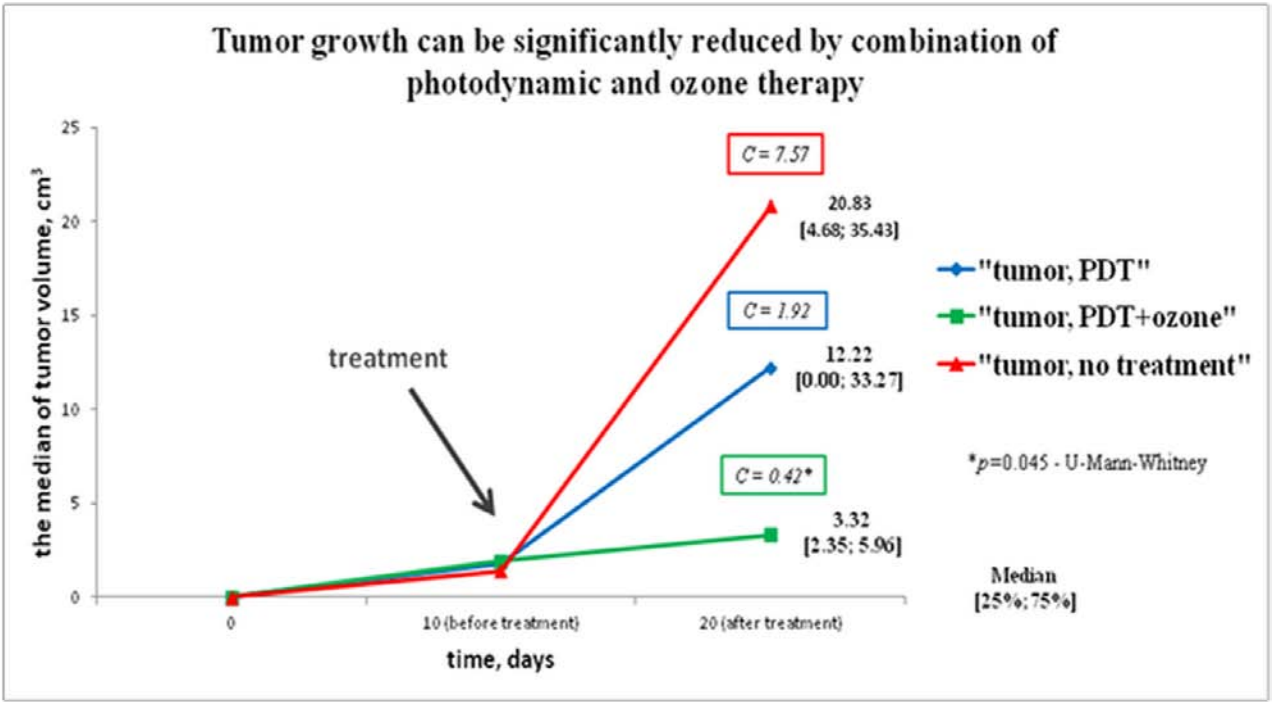
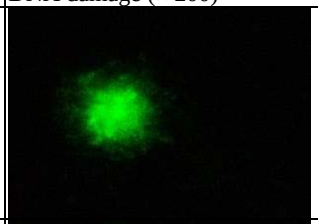
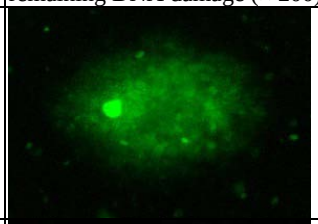
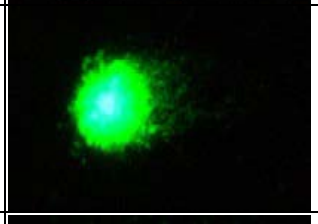
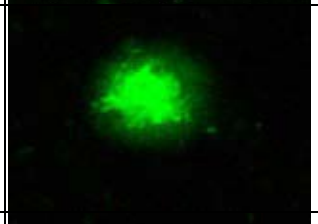
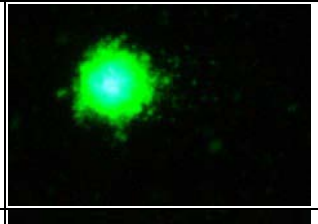
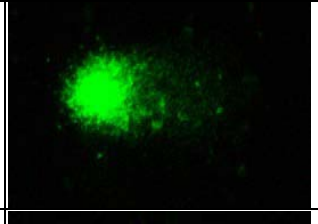
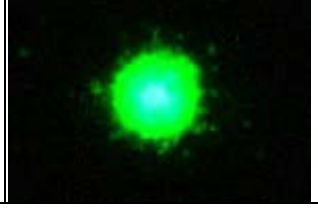
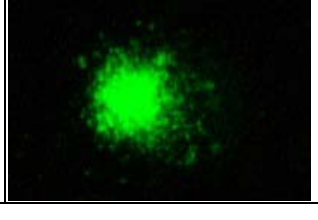


Fig. 3 The dynamics of tumor growth in vivo (red), after photodynamic therapy (blue) and combined ozone-photodynamic therapy (green). The tumor growth coefficient C was calculated by Eq. (1).

Table 1 COMET assay images of DNA from blood leukocytes made for different types of therapy.

Types of therapy:	The microphotography of the COMETs for spontaneous DNA damage (× 200)	The microphotography of the COMETs for remaining DNA damage (× 200)
Tumor, PDT (15 rats)		
Tumor, PDT + Ozone (15 rats)		
Tumor, no treatment (14 rats)		
Healthy animals (14 rats)		

free radicals, and peroxides, and then—ozonides [52]. These products cause the functional changes such as the oxidative modification of proteins and lipids of cells membrane [53, 54]. As a result, the membrane structure is broken, the stability of the lipid bilayer is reduced and the membrane permeability is increased [55]. These effects facilitate diffusion of the photosensitizer in tumor cells and make possible more uniform distribution of photosensitizer molecules in tumor tissue. Therefore, the impact of PDT would result in breaks of nuclear cell membranes at the early stages of treatment that is been detected by DNA damage [34]. As a result, the tumor cells are killed and the tumor growth is stopped [51].

Images of DNA were taken with using SYBR GREEN I staining. Note the biggest COMET tail for PDT therapy and no essential difference between COMET tails for “PDT + Ozone”, “No treatment” and “Healthy animals” treatment groups.

However, our results obtained from the new modification of COMET assay protocol showed that, the average level of spontaneous DNA damage in whole blood leukocytes of rats after PDT with ozone exposure was not significantly higher than the values obtained without the ozone exposure. The average level of spontaneous DNA damage in whole blood leukocytes of rats after PDT with ozone went up only by 2%. However, the average level of remaining DNA damage of whole blood leukocytes after incubation and photodynamic therapy with “Photosens” was significantly increased (38.5 ± 5.5) as compared to the average level of remaining DNA damage without ozone exposure (13.7 ± 1.0) (Fig. 2).

Thus, the ozone-photodynamic therapy enhanced antitumor activity with a low genotoxic effect. Obviously, more works are needed to warn the effect on cancer cells and normal cells of the organism.

It is, on the one hand, the positive effect of the combinational therapy can be explained by the therapeutic effects of the systemic action of ozone low concentrations. These include normalization of redox system as a result of neutralization of ROS (reactive oxygen species) by antioxidants. Consequently, ozone low concentrations activate antioxidant system and stimulate oxygen metabolism [42, 56]. ROS and ozonides in ozone-photodynamic therapy oxidize hypoxic tumor cells enhancing oxidative stress in the tumor. This results in tumor destruction.

On the other hand, ozone low concentrations cause detox and immunomodulatory effects [57, 58]. Possibly, induction of antitumor immunity by ozone is due to increased synthesis of cytokines [58] reduces of genotoxic effect after ozone-photodynamic therapy as we have shown in this paper.

Consequently, ozone-photodynamic therapy is both inhibition of tumor growth and a low genotoxic effect on the organism.

4. Conclusions

We proposed and studied antitumor effects of photodynamic therapy in combination with ozonated saline solution. We showed that the proposed therapy significantly reduces tumor growth in models of cancer in rats. Using our newly developed version of COMET assay, we discovered a new effect of low genotoxic impact of photodynamic therapy with ozone on whole blood leukocytes of rats in experimental neoplasia.

The results of our investigations may be used in clinical practice both for a new version of Comet assay in personal diagnostics and new ozone-photodynamic treatment to enhance the efficiency of PDT.

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