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The Influence of Depleted Glutathione Levels on the Photodynamic Action of Zinc Phthalocyanine in CHO K1 Cells

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ABSTRACT

Objective: **The current study focuses on any influence that depletion of endogenous glutathione in CHO K1 cells may have on the photodynamic action of zinc phthalocyanine (ZnPc).** ***Materials and Methods:*** **Two lasers—a HeNe laser, 632.5 nm, maximum power output 3.5 mW, and a Toshiba semiconducting laser, 670 nm, maximum power of 7 mW—were used. Chinese Hamster Ovary cells (CHO K1) were exposed to light, 2–10 J. Cellular reduced glutathione levels [GSH] were depressed prior to exposure to ZnPc and laser light, using buthionine sulphoximine, a potent inhibitor of γ-glutamylcysteine synthetase. The influence of hypoxic intracellular conditions was studied by reduction of oxygen content of cells by 80% following purging of cell cultures with nitrogen.** ***Results:*** **In well-aerated cells, doubling times are reduced by the photodynamic action of ZnPc by 29 ± 6%, fig 2 ($p = 0.01$). Cells with lowered [GSH] do not show this effect ($p = 0.1$). When hypoxic cells are studied at normal [GSH], no photodynamic effect is observed ($p = 0.1$). When cell viability is studied, using the 670-nm laser, a photodynamic effect is observed, (80% fall from controls, $p < 0.001$), irrespective of the cellular [GSH] level for a single dose of 6 J. This effect is observed in cells with normal [GSH], for varied doses of 2 J and higher (63% fall at 2 J, $p < 0.001$).** ***Conclusions:*** **Lowered [GSH] was observed to depress the photodynamic effect of ZnPc when cell-doubling times were the endpoint. The photostimulating effect of ZnPc was similarly suppressed by hypoxic conditions. When cell viability was the endpoint, then a photodynamic effect of ZnPc was observed irrespective of the endogenous [GSH] values.**

INTRODUCTION

IN THE LAST FEW YEARS, noticeable progress in the field of photochemotherapy has been made through the introduction of photosensitizers with absorption bands in the 600–900-nm interval. The molecules, normally present in animal tissue, with the exception of melanin, exhibit no appreciable absorbency in this spectral region[34]; consequently, only upon administration of red-light absorbing dyes can biological systems be damaged by such wavelengths. At the same time, the decreased importance of light-scattering processes at longer wavelength allows deep penetration of red light into the tissues.[10] The dyes are characterized by a polycyclic chemical structure; the extensively delocalized electron cloud lowers the energy gap between excited state and ground state, so that electronic excitation may also be achieved by irradiation with low-energy light wavelengths.

Phthalocyanines and metallophthalocyanines are a class of porphyrin-like compounds (tetraazobenzopyrdic dyes) that can be exploited as photosensitizers in photodynamic treatment of cancer and other clinical conditions.[5,21,29,30,33] Zinc phthalocyanine (ZnPc) is a highly hydrophobic dye, which preferentially localizes in cellular membranes (plasma membrane, mitochondrial membrane, endoplasmatic reticulum, and nuclear envelope).

It is believed that ZnPc interacts with oxygen in the high-energy state brought about by excitation with laser light of a suitable wavelength. This interaction yields reactive oxygen

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species such as the superoxide radical $O_2^{\cdot-}$. Because of the short lifetime of the free radicals generated during PDT with ZnPc, the damage is accumulated in the close vicinity of the dye.

A number of studies have indicated the protective effect of sulphydryl (-SH) groups against free radical actions within the living cell.[4,7] Some of these studies have involved determining the amplitude and kinetics of the O_2 effect in cells exposed to ionizing radiation and pre-treated to enrich them in SH groups, mostly reduced glutathione, or deprived of SH groups (N-ethylmaleimide fixation of SH groups). These studies have demonstrated the important role of SH groups in the disappearance of oxygen species free radicals.

We report studies in this paper involving exposure of CHO K1 cells to ZnPc together with laser light at 632.5-nm wavelength from a helium-neon (HeNe) laser and 670-nm wavelength from a semiconducting laser. In particular, the studies have focused on a possible photodynamic effect in cells previously depleted in reduced glutathione by exposure to the buthionine sulphoximine, a potent inhibitor of γ-glutamylcysteine synthethase, using cell proliferation viability and growth rate as endpoints. BSO itself is not expected to influence the interaction of ZnPc with the reactive oxygen species produced by laser light. The hypothesis is that lowered GSH levels produced by BSO may give enhanced possibility of intracellular damage by removal of a potential scavenger of reactive oxygen species, GSH.

Some experiments have also been carried out in cells in which hypoxia has been induced. It has been reported[9] that the most significant protective effects of glutathione against reactive oxygen species have been observed in cells where the oxygen concentration has been reduced by incubation in oxygen-free conditions before exposure to radiation or, as in this case, a photosensitizing dye plus laser light. Hence, the hypothesis here is that changes in [GSH] may have more marked effects under hypoxic conditions when the overall concentration of reactive oxygen species will not be so high.

MATERIALS AND METHODS

Cell culture

Chinese hamster ovary cells (CHO K1) were routinely maintained in basal medium (Eagle) with 25 mM Hepes buffer, 10% newborn calf serum, 1% lactoalbumin hydrolysate, 2 mM of 200 mM L-glutamine, 0.075% of 7.5% (v/v) sodium carbonate, and 1% antibiotic/antimycotic solution. All constituents were purchased from Gibco-BRL.

Zinc phthalocyanine administration/laser irradiation conditions

ZnPc (Acros Organics) was stored in the dark at −10°C as stock solutions of 0.5, 1, 2, 3, and 12 × 10^{-4} μmol/L in 96% dimethyl sulphoxide (DMSO). ZnPc solution was added to give final concentrations of 0.5, 1, 2, 3, and 12 × 10^6 mol/L. Cells were incubated with the dye for 20 min in the dark as a monolayer, after which the dye suspension was discarded and cells were washed twice with HBSS. Laser irradiation studies were carried out on cells either in a monolayer in 25-cm^3 flasks or cell suspension (2 mL of 0.5 × 10^6cells/mL) at room temperature. The equipment used was a HeNe laser, 632 nm with a maximal power output of 3.5 mW, or a semiconducting Toshiba laser, 670 nm with the maximal power of 7 mW. The measured energy doses at the monolayer surface were 2, 4, 6, 8, and 10 J.

Growth rate experiments

Cell growth rates were determined using multiwell dishes. Each well was inoculated with 5 × 10^5 cells in 2 mL of medium. Cells were removed each day by trypsinization of individual wells, followed by counting. This procedure continued for 7 days, and the results were standardized on growth in cell numbers by the 5th day.

Later studies, involving aerated and hypoxic conditions, were carried out under slightly different conditions where the initial cell inoculate was decreased to 1–2 × 10^5 cells per well, and growth was estimated as increased cell number per well at 24 and 48 h after plating.

Reduced glutathione/buthionine sulphoximine

Reduced glutathione (GSH) levels in cells were determined by the Ellman method.[11] D-1,-Buthionine S-R-Sulphoximine (BSO) was prepared at an initial concentration of 1 mM in phosphate-buffered saline shortly before adding it to the cells. The final concentrations were 70, 100, and 200 μM, and incubation with BSO took place over 0.5–48 h. After incubation, the medium containing BSO was discarded and cells were washed twice with HBSS. Protein determinations were carried out using the Lowry method.[19]

MTT cytotoxicity assay

MTT is a yellow water-soluble tetrazolium dye that is reduced by live but not dead cells to a purple formazan product that is insoluble in aqueous solutions. The amount of MTT-formazan produced can be determined spectrophotometrically once the MTT-formazan has been dissolved in 96% DMSO.[22,24]

The cells were trypsinized, counted and seeded into 25-cm^3 flasks. The confluent cells were incubated with BSO (200 μM) for 18 h. Later, the medium was discarded and cells were washed two times with HBSS buffer; BME medium (without phenol red, serum, and lactoalbumin) for ZnPc incubation was added. The final concentration of ZnPc was 2 μM. The cells were left in the incubator for 20 min. After that, they were washed three times, trypsinized, and counted. For the irradiation, 3 mL of cell suspension (5 × 10^5 cells/mL) was used; the energy dose was 6 J. In the case of laser/ZnPc experiments, cells were irradiated with energy doses of 2, 4, 6, 8, and 10 J. Cells were constantly stirred during the irradiation.

After the irradiation, the cells were seeded into the 96-well plates (5000 cells per well) and left in the incubator. The reading was carried out using an ELISA reader (Stat Fax 2100, Awareness Technology Inc.) at the wavelengths of 545 and 630 nm. The final concentration of MTT was 0.5 mg/mL dissolved into the BME medium (without phenol red, serum, and lactoalbumin).

RESULTS

The time dependence of the depression of intracellular GSH content for cells incubated at three different concentrations of

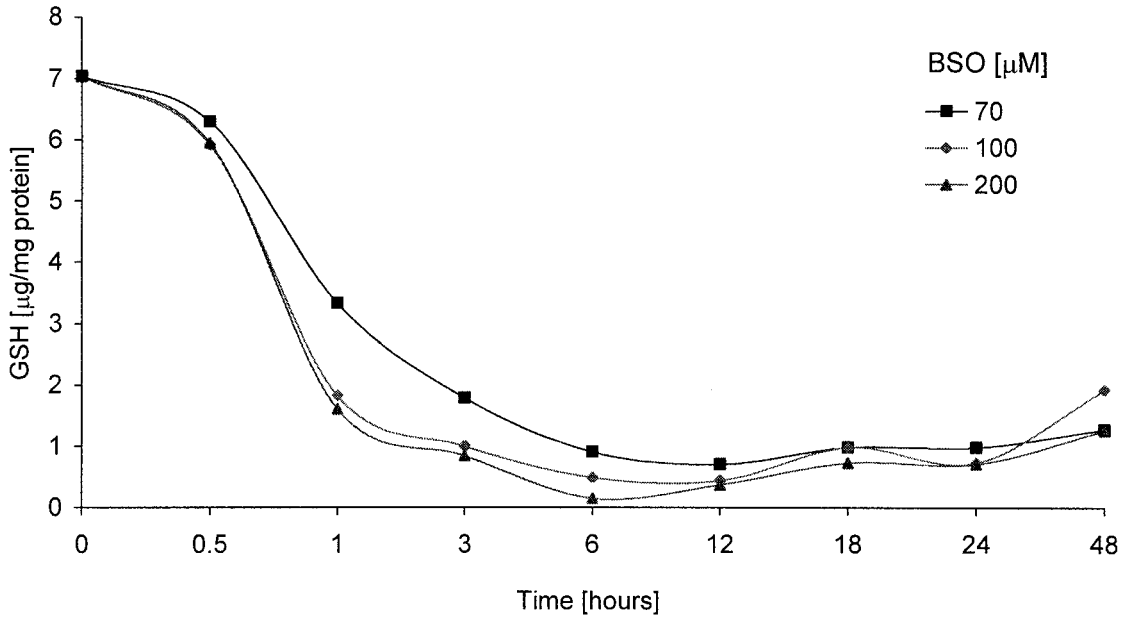

FIG. 1. The time dependence of the depression of intracellular GSH content for CHO K1 cells incubated with BSO (70, 100, and 200 μM).

BSO is given in Figure 1. A significant reduction in GSH levels was observed after 1 h of incubation. Subsequent experiments with zinc phthalocyanine and laser light were carried out on cells previously exposed to 200 μM BSO for a period of 18 h. At this time, the GSH levels were less than 15% of those found in untreated cells.

Growth rates were depressed in well-aerated cells exposed to BSO alone under dark conditions. A similar effect was observed when BSO-treated cells were exposed to ZnPc (2 μM) under dark conditions. In contrast, when cells were exposed to ZnPc alone under light conditions (632 nm, 3.5 mW, 6 J), a significant stimulation of growth rate was observed (Fig. 2, p =

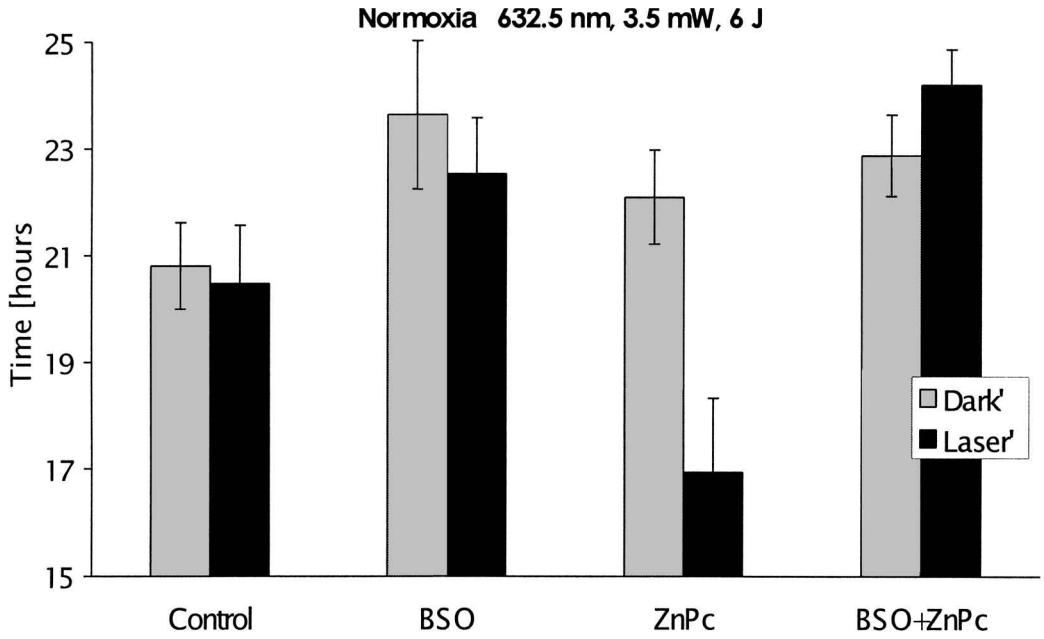

FIG. 2. Growth rates of CHO K1 cells in normoxia in the dark and after laser irradiation with a single dose (635.5 nm, 3.5 mW, 6 J). ZnPc concentration, 2 μM. After irradiation of the cells in the presence of ZnPc, a significant increase of cell growth was observed (doubling time was 16.9 ± 2.1 h compared to the control of 20.4 ± 1.8 h, significant for $p = 0.01$).

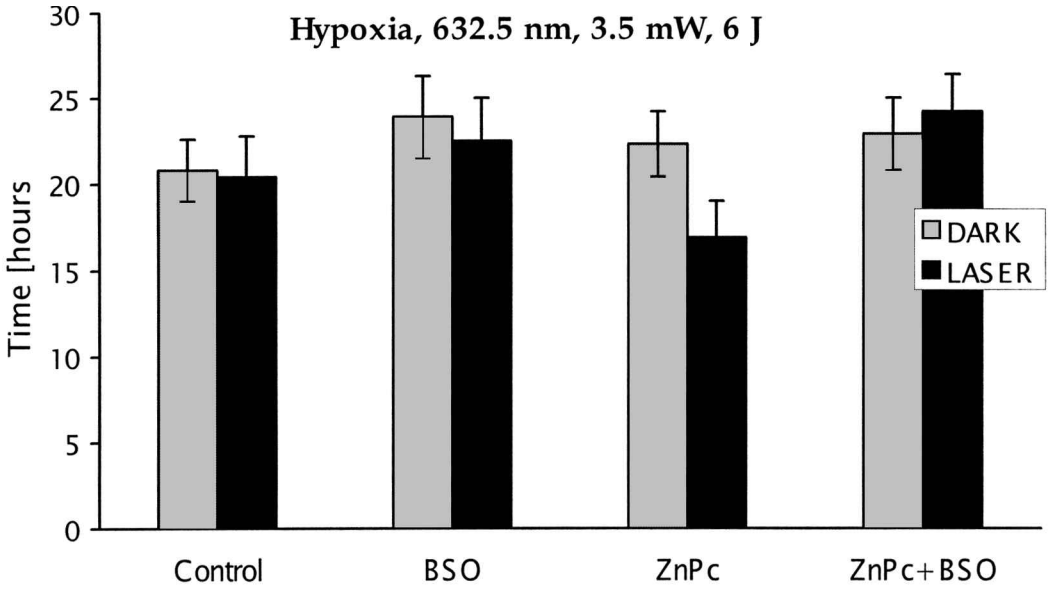

FIG. 3. Growth rates of CHO K1 cells in hypoxia in the dark and after laser irradiation with a single dose (635.5 nm, 3.5 mW, 6 J). ZnPc concentration, 2 μM. No significant changes of growth rates were observed ($p = 0.1$).

0.01), suggesting a photodynamic effect. This stimulation was not observed ($p = 0.1$) in cells treated with BSO and ZnPc under light conditions, suggesting that lowered GSH concentrations may suppress any photodynamic effect.

When oxygen levels in cells were suppressed by purging the system with nitrogen for a period (Fig. 3), the photostimulating effect of ZnPc (2 μM) and laser (632.5 nm, 3.5 mW, 6 J) on cell growth was lost, together with the growth depression reported in Figure 2 as a result of treatment with BSO ($p = 0.1$). This finding was at odds with the hypothesis that the influence of loss of endogenous GSH levels will be observed more easily under hypoxic conditions.

The cytotoxicity of the ZnPc treatment alone, laser treatment (670 nm, 7 mW) alone, and combined treatment with both modalities was studied, using the MTT dye test for cells exposed previously to BSO and cells with normal GSH intracellular concentrations. This test identified the viability and proliferative capacity of cells at 24 h after treatment. The results, using a single laser energy dose of 6 J, are displayed in Figure 4. ZnPc treatment alone (2 μM) reduced the viable cell number by

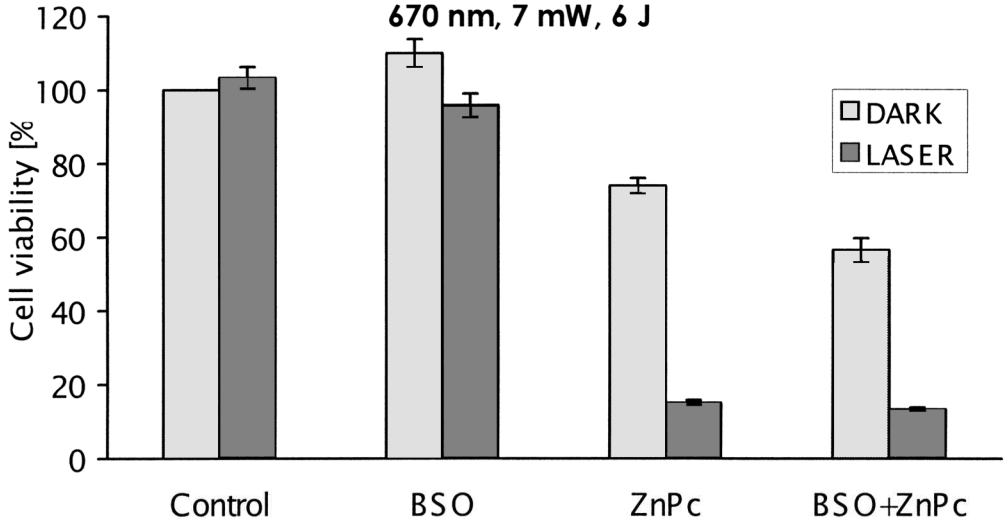

FIG. 4. Cell viability (MTT assay) at 24 h after the experiment in the dark and after laser irradiation with a single dose (670 nm, 7 mW, 6 J). Treatment of the cells with ZnPc combined with the light resulted in a dramatic reduction of cell viability of 80 ± 0.7%. A similar result was observed for the combined effect of ZnPc, light, and BSO (significant for $p < 0.001$).

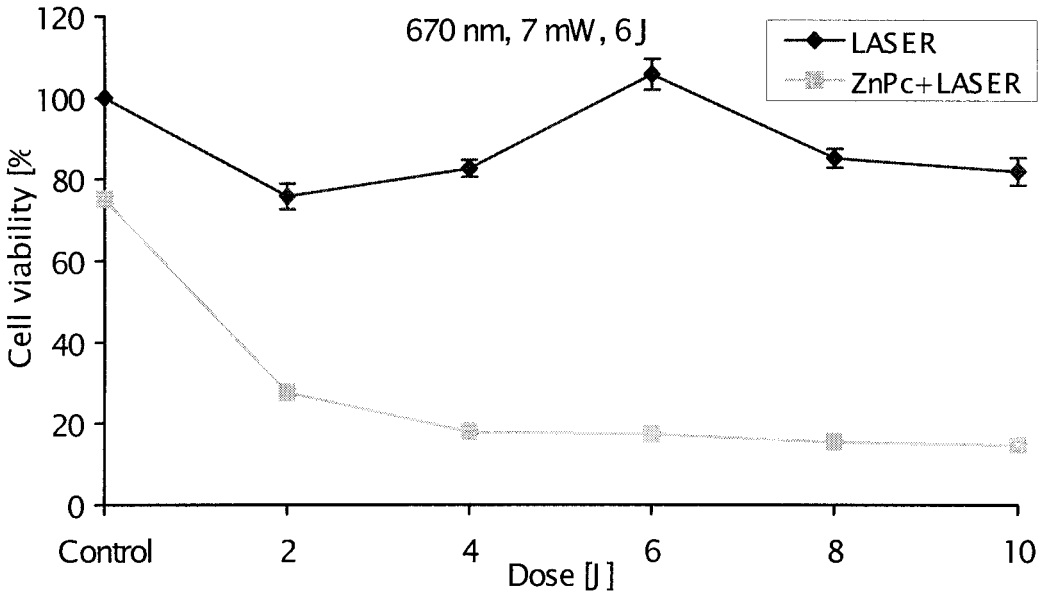

FIG. 5. Cell viability (MTT assay) at 24 h after laser irradiation with varied doses (2–10 J, 670 nm, 7 mW) with and without ZnPc (2 μM). Low-power laser irradiation alone slightly decreased cell viability (over 25 ± 3.1% for energy dose of 2 J, significant for $p = 0.01$, for dose of 6J cell viability was similar to this of control, not significant, and small decrease for higher doses). A dramatic decrease of 65 ± 0.5% (significant for $p < 0.001$) was observed for a dose of 4 J or above when the cells were irradiated with laser light in the presence of ZnPc.

26 ± 0.9% (significant for $p = 0.01$). When combined with laser treatment (670 nm, 7 mW), a dramatic reduction of over 80 ± 0.7% (significant for $p < 0.001$) in cell viability was observed, suggesting a significant photodynamic (PD) effect of a similar magnitude as observed in Figure 2, as measured by cell-doubling times. This PD effect is comparable in cells with normal intracellular GSH levels and in those whose glutathione levels have been previously depressed by BSO treatment (significant for $p < 0.001$). This finding is in contrast to the results in Figure 2, which suggest that lower GSH levels prevented the photodynamic effect.

A second set of experiments, using the MTT-cytotoxicity test as endpoint, involved giving a varied energy dose of laser light (670 nm) in the range 2–10 J for a fixed ZnPc concentration of 2 μM. These cells had not been treated with BSO and hence had normal GSH levels. The results are displayed in Figure 5 for cells assayed at 24 h post-exposure. A small but varied effect of laser light alone is observed for energy doses of 2, 4, 6, 8, and 10 J (significant for $p = 0.01$; not significant for dose of 6 J when cell viability was similar to that of control, $p = 0.1$). When light is combined with ZnPc, a dramatic decrease in viability is observed, with a fall of more than 65 ± 0.5% (significant for $p < 0.001$) recorded at light energy doses of 4 J or above. This finding can be attributed to a photodynamic mechanism.

DISCUSSION

In the present work, cell viability after laser irradiation alone (670 nm, 7 mW, 2–10 J) and after incubation with ZnPc (2 μM) was studied (Fig. 5), without prior incubation with BSO. For an energy dose of 2 J, a decrease of cell viability to 75% (±3.1, significant for $p < 0.001$) of control was observed. A decrease to 82% of control was observed for an energy dose of 4 J (82 ± 2.1%, significant for $p = 0.01$), and at 6 J it was slightly higher than control (105%, not significant). For higher energy doses, the cell viability decreased (81 ± 0.3% for 10 J, significant for $p = 0.01$). This kind of bell-like curve is very characteristic for laser light action. A similar shape was obtained after irradiation of human erythrocytes with the same kind of laser (for energy doses of 3–15 J), taking as an endpoint the activity of the membrane-bound enzyme, acetylcholinesterase.[31] The activity was significantly higher than in controls up to an energy dose of 9 J and subsequently decreased when energy dose increased. When cell survival, as measured by clonogenic ability, was chosen as the endpoint, the maximal enhancement of the endpoint was observed for an energy dose of 10 J.[26]

Fibroblasts are cells of paramount importance in the process of wound healing. Therefore, it is not surprising that a large amount of data exists on their behavior after light exposure. In the work of Lubart et al.,[20] a nonlinear dose and intensity dependence of the mitosis rate of human fibroblasts was reported after irradiation with two different wavelength ranges of noncoherent light. Webb et al.,[36] who used an 860-nm diode laser to irradiate human fibroblasts, obtained similar results. Their results suggested a relationship between fibroblast proliferation and succidic dehydrogenase (an enzyme located in the respiratory chain of cell's mitochondria). At the dose of 2 J/cm^2, both cell proliferation and enzyme activity were significantly increased, whereas at a dose of 16 J/cm^2, inhibition of both parameters was noted, once more pointing to the dose dependence of biological responses after light exposures. In a study performed by Yu et al.,[38] cell proliferation and release of basic

fibroblast growth factor (bFGF), a potent mitogen and chemotactic factor for fibroblasts cultures, was found to be maximal at a dose of 2.2 J/cm^2, using a 660-nm laser.

Loevshall and Arenhold-Bindslev[18] investigated the effect of 812-nm laser irradiation on DNA synthesis of human buccal fibroblasts, as measured by ^{3}H-thymidine uptake. In this study, relatively low-energy densities, between 0.0005 and 5 J/cm^2, were administered. With a constant intensity of 4.5 mW/cm^2, a maximal increase in DNA synthesis was found at the dose of 0.5 J/cm^2.

Pourreau-Schneider et al.[25] investigated the ability of a single HeNe-laser irradiation (dose of 1.2 J/cm^2) to transform fibroblasts into myofibroblasts, both *in vitro* and *in vivo*. These modified fibroblasts play a role in granulation tissue contraction during wound healing. The results obtained showed a direct and massive transformation of the cultured fibroblasts into myofibroblasts starting 24 h after light exposure. Moreover, a large number of myofibroblasts were found in the connective tissue of laser-treated gums, compared to unirradiated control sites. A dose-dependent enhancement of cell proliferation, resulting from low-intensity HeNe-laser irradiation, was observed by Atabey et al.[2] in normal human fibroblasts. An excellent overview of both inhibitory and stimulatory effects of laser irradiation on the control of connective tissue metabolism and their possible dermatological relevance is available from Abergel et al.[1] They also reported on significant procollagen production by cultured fibroblasts after 632.8- and 904-nm laser irradiation.[1]

Incubation of CHO cells with zinc phthalocyanine decreased cell viability by 26 ± 0.9%, significant for $p = 0.01$ (Fig. 4). According to many authors, phthalocyanines exhibit low cytotoxicity to mammalian cells. Dummin et al.[8] did not observe changes of cell viability (measured with the method of trypan blue exclusion), cell division, and respiratory activity (even for the concentration of ZnPc of 50 μM), or changes of mitochondria ultrastructure. It is supposed that phthalocyanines bind to the inner membrane of mitochondria, especially to hydrophobic proteins of the oxidation chain,[14] although some authors claim that the outer mitochondrial membrane is their final target (due to low amount of cardiolipin).[28]

Laser light irradiation with zinc phthalocyanine (Fig. 5) drastically decreased cell viability compared to controls (to 37 ± 0.8% of control for energy dose of 2 J and 24 ± 0.5% for 4 J, $p < 0.001$). Higher doses—up to 10 J—decreased cell viability still further, but not lower than 20% ($p < 0.001$) of control.

Many mitochondrial ultrastructural changes after photosensitization have been described.[6,16,17,35] Swelling of mitochondria is the most often reported morphological change, caused by destruction of mitochondrial membranes. Transmembrane potential (TMP) then breaks down, and the sensitizer enters the cytoplasm. In a second step, photosensitization catalyzes the destruction of the cytoplasm. Dummin et al.[8] suggest that the sensitizer molecules again bind to lipophylic cytoplasmic vehicles by hydrophobic interactions and destroy them on irradiation. Irregularly formed large vehicles are formed also. All structural changes are preceded by a decrease of activity of respiratory chain enzymes, especially cytochrome c oxidase and succinyl dehydrogenase.[13,32] Fluorimetric measurements showed destruction of ADP/ATP translocater, which affects oxidative phosphorylation.[3] The enzymes are situated between mitochondrial membranes (adenyl kinase) and are also located on the outer plasma membrane (monooxidase).[23] It is believed that the decrease of activity of these enzymes has a lower cytolethal effect than does activation of cytochrome c.

It was found in experiments reported in this paper that, shortly after adding BSO to growth medium (0.5 h), the GSH content decreased about 15% (Fig. 1). After 6 h of incubation, GSH levels had fallen below 1 μg/mg of protein (15% of initial concentration) and remained at this level. Such a depression in GSH levels seems to have no effect on cell viability (Fig. 4) in the presence or absence of laser light. However, it was observed that incubation of cells containing low GSH levels to zinc phthalocyanine in the dark produced a further decrease in cell viability compared to the effect on ZnPc on cells with normal GSH levels (Fig. 4). Exposure of BSO-treated cells to both laser light and ZnPc had no obvious effect on the photodynamic depression in cell viability observed with ZnPc alone.

When cell doubling times were considered (Fig. 2), a marked increase in growth rate, as evidenced by a decrease in doubling time, was observed after irradiation of CHO cells with zinc phthalocyanine ($p = 0.01$). This paradoxical result (compared to cell viability experiment, where viability was decreased) may possibly be explained by lower laser power (3.5 mW). Revazova et al.[27] found that low-power laser radiation can stimulate the growth of metastases in patients with a history of malignancy. Similar findings were reported by Yamada[37] who exposed clonal osteoblastic cells to continuous He-Ne laser beam a wavelength of 632.8 nm and found an increase of cell growth and DNA synthesis, but only when irradiated culture was in the growing phase. In our experiment, the energy dose was the same (6 J), but the irradiation time was double that used in viability experiments. For laser irradiation, wavelength, frequency, power output, spot diameter, irradiation time, intensity, dose, and treatment intervals are essential parameters. Changing the spot size, the exposure time, or both parameters automatically alters the intensity and the dose, thus leading to different cell responses.

It is said that mild oxidative stress (that is, the state when the balance between prooxidants and antioxidants still is not lost) may be beneficial. Examples include radiation hormesis (stimulating the effect of low doses of ionizing radiation, activating defense mechanisms, leading to an adaptation response of cells), hyperthermia-induced heat shock proteins (HSP), and chemical stimulation of the synthesis of the enzymes. Such a mechanism could explain why oxidative stress induced by PDT in these conditions (632 nm, 3.5 mW, 0.3 J/cm^2, concentration of zinc phthalocyanine at 2 μM) may stimulate cell proliferation. Similar experiments were performed under hypoxic conditions (Fig. 3), and no significant changes in growth rates were observed ($p = 0.1$). Presumably, reduced oxygen levels under hypoxic conditions will lead to a decreased potential for oxidative stress to exert its stimulating action.

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