PHOTODYNAMIC TREATMENT CAN INDUCE ENHANCED GENERATION OF HYDROGEN PEROXIDE IN CELLS OUTSIDE THE IRRADIATED AREA: PROOF OF THE PHENOMENON ON CELL CULTURE IN VITRO

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Abstract. Photodynamic therapy is a minimally invasive cancer treatment modality based on the production of the reactive oxygen species by photoactive dye under light irradiation in the presence of molecular oxygen. During the development of the photodynamic reaction, various types of reactive oxygen species are formed, among which hydrogen peroxide is of the greatest interest since it can act as an extracellular and intracellular signaling molecule. Using a genetically encoded sensor of hydrogen peroxide, we have registered the development of oxidative stress in non-irradiated cells in response to local photodynamic exposure of a single cell using Photosens as a photosensitizer. The effect manifested when the cells were closely contacted to each other; if the irradiated cell was at some distance from the bulk of the population, the response of non-target cells was not observed. The oxidative stress in the irradiated cell is assumed to be the initiator of the signal transmission and triggering the response of non-target cells. That this response is more likely mediated by gap junction intercellular signaling. However, the mechanisms involved in the propagation of damaging effects to cells outside the area of photodynamic exposure have to be further investigated.

Keywords: photodynamic therapy, hydrogen peroxide, protein sensor HyPer, photosens, bystander effect.

List of Abbreviations

PDT – photodynamic therapy
PS – photosensitizers
ROS – reactive oxygen species
H_2O_2 – hydrogen peroxide
BE – bystander effect
IC50 – half-maximal inhibitory concentration

Introduction

Photodynamic therapy (PDT) is one of the most common treatments for cancer and some other diseases, known for over 100 years (Hamblin, 2019). The PDT mechanism is based on the relatively selective accumulation of a photosensitizing dye (PS) in the tumor, which is capable of generating cytotoxic agents when exposed to light with a certain wavelength in the obligatory presence of molecular oxygen (Castano *et al*., 2004; Mansoori *et al*., 2019). PDT has several advantages including minimal invasiveness, local selective action on tumor tissues and the possibility of combination with other clinical treatment modes, e.g., surgery or chemotherapy. PDT also has multiple cellular targets and, therefore, this method is not associated with formation of drug resistance (Agostinis *et al*., 2011; Bacellar et al., 2015).

The cytotoxic action of PS is based on the initiation of a series of photochemical reactions with massive production of reactive oxygen species (ROS), inducing free radical peroxidation of cellular lipids and proteins ultimately leading to death of the cell (Agostinis *et al*., 2011; Mansoori *et al*., 2019). Two different types of photodynamic reactions can occur: photochemical reactions of the 1st type are characterized by redox reactions between PS and substrate molecules with transfer of electron or proton; during the reaction of the 2nd type, the energy of the excited triplet state of PS is transferred to molecular oxygen thus generating extremely reactive singlet oxygen $^1O_2$. The contribution of particular types of reactions depends on oxygen concentration, the type of PS, and its intracellular localization (Bacellar *et al*., 2015; Hamblin, 2019). In should be noted, however, that independently of a predominant

type of primary photochemical reactions, various types of ROS are formed in cells, among which hydrogen peroxide is of the greatest interest (Garcia-Diaz *et al*., 2016).

Hydrogen peroxide (H_2O_2) has a rather long lifetime of about 1 ms, compared to lifetime of other types of ROS of <1 μs (Yang *et al*., 2020), which allows it to diffuse at a fairly long distance from the place of formation, comparable to the cell size, and also to accumulate in the extracellular environment (Nardin *et al*., 2019). In addition to its oxidizing properties, H_2O_2 can act as an extracellular and intracellular signaling molecule that mediates multiple effects in biological systems. For example, H_2O_2 can affect the cellular signaling cascades that are activated by external stimuli such as growth factors and cytokines (Castano *et al*., 2005; Rhee, 2006; Yang et al., 2020). H_2O_2 is mostly formed in mitochondria in the reaction of dismutation of superoxide anion radicals. Compared to normal cells, cancer cells show increased generation rate of H2O2 resulting in a higher level of H2O2 in the tumor than in normal tissues (Yang *et al*., 2020).

It is now hypothesized by many researchers that the death of tumor cells occurs not only as a result of direct photodynamic exposure, but also as a result of signal transmission from damaged cells to intact ones. This process is called the «bystander effect» (BE) (Dahle *et al*., 2000; Olivier *et al*., 2009; Bazak *et al*., 2019). It is a phenomenon when cells exposed to some physical or chemical stress can transmit signals to neighboring unaffected cells which results in a manifested response of the latter (Prise & O'Sullivan, 2009; Marín et al., 2015). BE is extensively studied for radiation therapy; however, it is much less known about similar effects in the case of photodynamic therapy (PDT) (Olivier et al., 2009; Bazak et al., 2019). In general, oxidative stress resulting from the excitation of the photosensitizer during photodynamic exposure and subsequent damage of cellular structures spread from the irradiated cells to the surrounding non-irradiated cells (Bazak et al., 2017; Nardin et al., 2019), but detailed mechanisms need to be further studied.

There are two possible ways of signal transmission to bystander cells from irradiated target cells: intercellular communication by virtue of gap junctions, and signal transmission through the surrounding extracellular medium (Verma & Tiku, 2017; Bazak *et al*., 2017). In both cases, the signal molecules are formed during PDT-induced oxidative stress and are able to diffuse within the cell and between neighboring cells. Therefore, the stress of the target cell is important for triggering the bystander effect, but not the cell death (Chakraborty *et al*., 2009).

It was previously assumed that H_2O_2 itself can be the signaling molecule causing the bystander effect under PDT treatment (Rubio *et al.*, 2009). Previously, we demonstrated that PDT treatment induce the long-term secondary production of H2O2 in cells lasting for more than an hour after light irradiation (Peskova *et al*., 2021). The characteristic times of the generation of this molecule and its physical-chemical properties are in good agreement with its possible role as a transmitted signal for bystander effect triggering.

In the present work, we analyze the generation of hydrogen peroxide in cells outside the irradiation area after a local photodynamic effect on a single cell. Using the genetically encoded H_2O_2-sensitive sensor allowed us to monitor the responses of individual cells depending on their relative positions from the target cell and to reveal the role of intercellular contacts.

Materials and methods

Cell line. The experiments were performed on cell line on human epidermoid carcinoma A431-HyPer-cyto. This cell line was created by stable transfection of the parental line A431 (Peskova *et al.*, 2021) and is characterized by expression of H_2O_2-sensitive protein sensor HyPer (Belousov *et al*., 2006).

Cells were cultured in Dulbecco's modified Eagle's Medium (DMEM, PanEco, Russia) supplemented with 2 mM glutamine and 10% fetal bovine serum (HyClone, USA) in 5% CO_2 atmosphere at 37 °C. Trypsin: EDTA (1:1) (PanEko, Russia) was used to detach cells from the culture flask when passaging.

Photosensitizer. The PDT treatment was performed using Photosens®, a synthetic photosensitizer of the second generation (Organic Intermediates and Dyes Institute, Russia), which is a mixture of di-, tri- and tetrasubstituted fractions of aluminum phthalocyanine with the number of sulfo groups 3.4. Photosens has an absorption peak at 676 nm and a fluorescence maximum at 685 nm (Lukyanets, 1999). Accumulation of Photosens is revealed in vesicular cell structures, including endosomes and lysosomes (Sobolev *et al*., 2004; Brilkina *et al*., 2019).

PDT treatment and monitoring of H_2O_2 intracellular concentration. Registration of dynamic changes in hydrogen peroxide intracellular concentration was performed using an Axio Observer Z1 LSM 710 NLO DUO laser scanning confocal microscopy system (Carl Zeiss, Germany) with a C-Apochromat 40x/1.20 W objective lens. All experiments were carried out in a chamber maintaining cell culture conditions (5% CO_2 and 37 °C), which was mounted around the microscope stage. The images were acquired and processed using the ZEN 2012 software (Carl Zeiss, Germany).

During the experiments, cells were seeded in 35 mm glass bottom Petri dishes (Eppendorf, Germany) at a concentration of 2×105 cells per dish and grown overnight. The next day, the culture medium was replaced with serum-free medium containing 5 μM Photosens and cells were incubated for 4 hours. Then the medium was replaced again with fresh complete culture medium and the dish was placed on the stage of the microscope.

For photodynamic treatment, the cells were irradiated through the objective lens using a laser with a wavelength of 633 nm. The irradiation dose was calculated taking into account the laser power on the objective, the pixel dwell time, the image resolution and the number of scans. Two doses were applied in the experiments, 50 or 100 J/cm^2. The power density in both cases remained constant, and the exposure time was no more than 10 seconds.

HyPer protein fluorescence was excited sequentially at two wavelengths: 405 nm and 488 nm. The chosen laser wavelengths are close to the absorption maxima of the reduced (420 nm) and oxidized forms (500 nm) of the HyPer protein. The lasers' power on the objective was the same and equal to 0.02 mW. The fluorescence at both excitation conditions was registered at the same gain settings in the range of 500–550 nm. The relative level of hydrogen peroxide was estimated by calculation the ratiometric index I488/I405 which is a ratio of fluorescence signals at the corresponding excitation wavelengths.

In the course of experiment, the photodynamic treatment was locally applied to a single cell in the field of view. The HyPer response was registered before and after irradiation in the irradiated target cell, cells in tight contact with the target one, as well as cells located far from it. Images were acquired every 4 minutes to avoid strong additional influence on sensitized cell culture; total monitoring time was 80–88 minutes after photodynamic exposure, until the cells retain their viability.

Results

To visualize the production of hydrogen peroxide in cells after PDT treatment, we used the cell line expressing genetically encoded H_2O_2 sensor HyPer (Peskova *et al*., 2021). HyPer is a chimeric protein created from the bacterial transcription factor OxyR by inserting a circularly permuted yellow fluorescent protein (cpYFP) into the OxyR regulatory domain. When HyPer interacts with H_2O_2, a disulfide bond is formed in the OxyR regulatory domain (OxyR-RD), thereby inducing the conformational changes in the cpYFP fluorescent protein. Oxidized HyPer can be reduced by glutaredoxin system of the cell, allowing long-term measurements within the same cell or cellular structure. In the presence of H_2O_2, a change in the ratio of the reduced and oxidized forms is observed (Belousov et al., 2006). In our experiments the dynamics of the H_2O_2 content was estimated by the ratio of the fluorescence intensities at excitation in the absorption band of the oxidized (488 nm) and reduced (405 nm) forms of the sensor, the ratiometric index I488/I405.

To sensitize the cells to light irradiation, they were incubated with Photosens at a concentra-

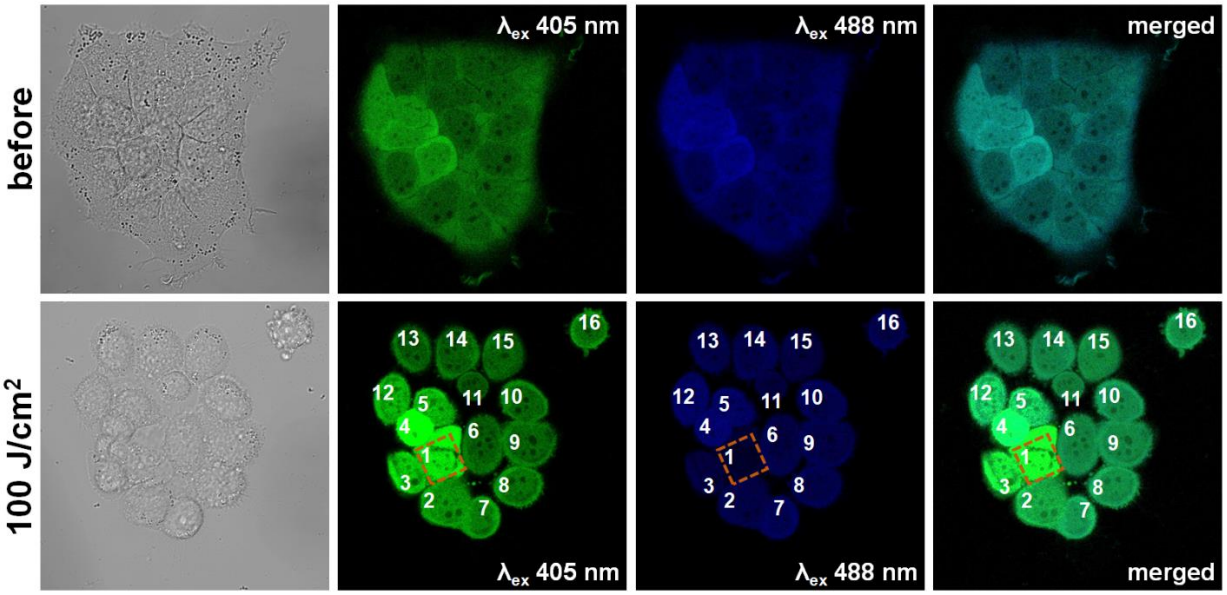

Fig. 1. An example of the response of A431-HyPer-cyto cells located in close group to photodynamic exposure of a single cell. Confocal images of cells in transmitted light, fluorescent images (λem 500–550 nm) with excitation at λex 405 nm, at λex 488 nm, and images with merged fluorescent channels are shown. The culture was treated with Photosens (5 μM for 4 hours); the images were obtained before and 80 minutes after irradiation at a dose of 100 J/cm^2. Images size 150 μm × 150 μm. The irradiated cell is marked as 1; cells contacting the irradiated one are marked as 2–6; the distant located cells are marked as 7–16

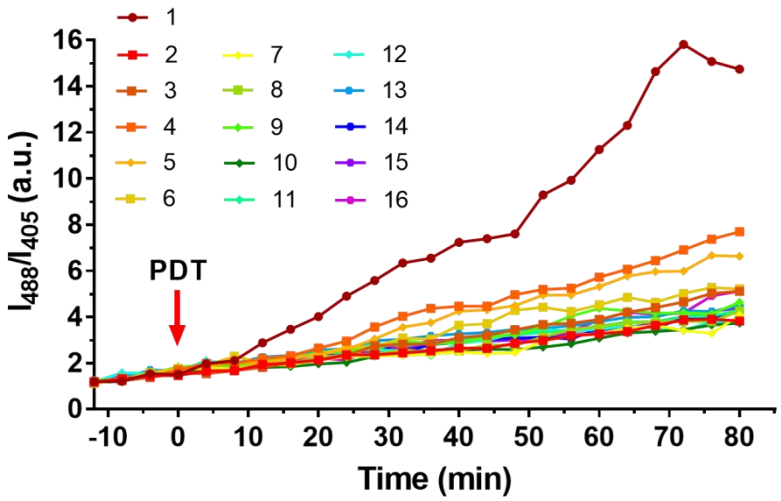

Fig. 2. Dynamics of ratiometric index I488/I405 before and after photodynamic exposure of a single cell (marked as 1). The number marks of other cells correspond to Fig. 1: cells contacting the irradiated one are marked as 2–6; the distant located cells are marked as 7–16

tion of 5 μM for 4 hours. The choice of the photosensitizer concentration was based on our previous studieds (Peskova *et al.*, 2021) and corresponded to the half-maximum inhibitory concentration (IC50) under irradiation at light doses in the range 2–5 J/cm^2. To induce strong cellular damage and stimulate the transmission of stress signal to neighboring cells, we applied

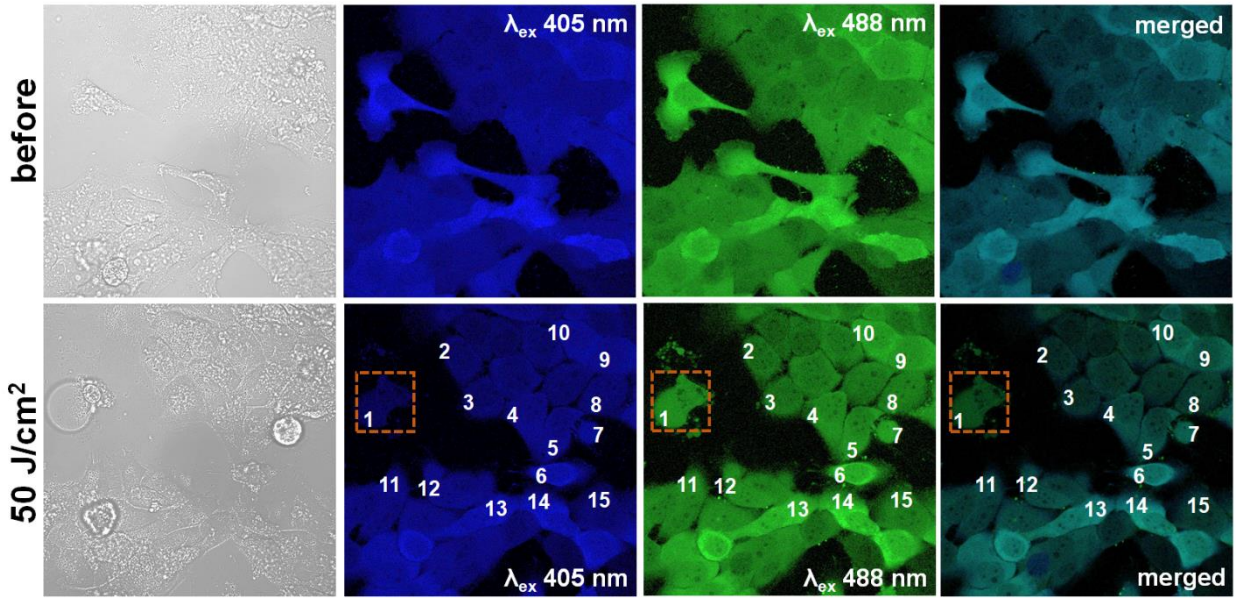

Fig. 3. An example of the response of A431-HyPer-cyto cells to photodynamic exposure of a single cell distant located from the rest of the cell population. Confocal images of cells in transmitted light, fluorescent images (λem 500–550 nm) with excitation at λex 405 nm, at λex 488 nm, and images with merged fluorescent channels are shown. The culture was treated with Photosens (5 μM for 4 hours); the images were obtained before and 88 minutes after irradiation at a dose of 50 J/cm^2. Image size 212 μm × 212 μm. The irradiated cell is marked as 1, cells neighboring the irradiated one is marked as 2, 3, 11, 12 the distant located cells are marked as 4–10 and 13, 14, 15

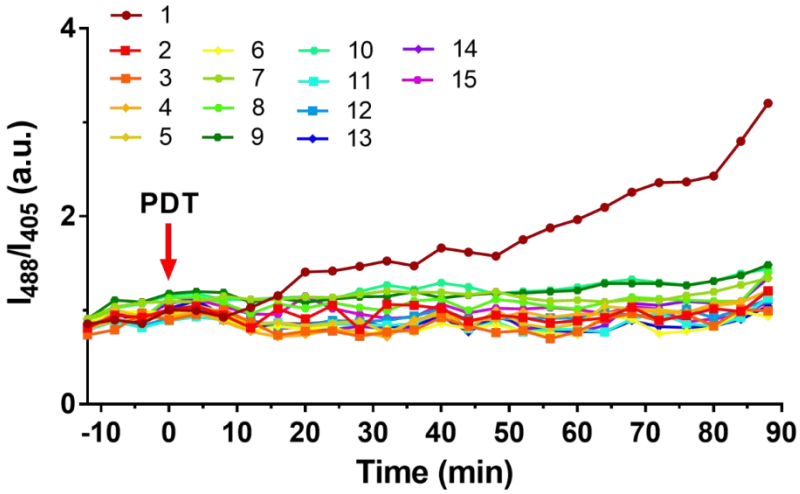

Fig. 4. Dynamics of ratiometric index I488/I405 before and after photodynamic exposure of a single cell (marked as 1). The number marks of other cells correspond to Fig. 3: the cells neighboring the irradiated one are marked as 2, 3, 11, 12; the distant located cells are marked as 4–10 and 13, 14, 15

light doses at least ten-fold higher than IC50-, 50 J/cm^2 or 100 J/cm^2. Only a single cell in the microscopic field of view was treated.

Two series of experiments with different relative cell layout in the field of view were carried out. In the first case, the irradiated target cell was located in close contact and was surrounded by a group of bystander cells (Fig. 1). Intensive photodynamic treatment of a single cell led to a strong deterioration in the morphological state of the cell. During the monitoring period (80 minutes), the irradiated cell demon-

strated the membrane bubbling, and later on the detachment from the bottom of the Petri dish, which can be considered as signs of photoinduced damage. Such a reaction of cells to photodynamic treatment with Photosens is typical and has been reported earlier in a number of works (Brilkina *et al*., 2018; Turubanova *et al*., 2019). Both nearby cells and more distant ones also underwent some morphological changes, however, less pronounced. All non-irradiated cells in a tight cell groups shrinks with a transition from a densely packed monolayer to a rather rarefied population.

Morphological changes were accompanied by an increase in the intensity of HyPer fluorescence upon excitation at λex 488 nm ('green' channel) and a decrease in intensity upon excitation at λex 405 nm ('blue' channel), which indicates an increase in the concentration of H_2O_2 in cells.

Dynamics of the peroxide content in the irradiated cell, as well as in contacting and distant cells was analyzed (Fig. 2). As can be seen from the plot, the answer in the the presented typical example did differ in terms of severity and timing of the response. In the irradiated cell (cell marked as 1), a statistically significant difference from the initial state was noted as early as 12 minutes after irradiation. Also, the response of contacting cells (especially, cells 4, 5, and 6) was registered in about 24–26 minutes after the irradiation. It is worth to highlight that the concentration of hydrogen peroxide have also increased in distant cells (cells 7–16) by about 4 times compared to the initial level during the observation period.

In the second series of experiments, the fields of view were chosen so that the irradiated cell was located at some distance from the rest of the cell population. After PDT treatment, the irradiated cell also shows a subsequent impairment of the morphological state accompanied by an increase in the intensity of HyPer fluorescence in the 'green' channel (λex 488 nm) and a decrease in the intensity in the 'blue' channel (λex 405 nm) (Fig. 3). The rise in the I488/I405 index, and accordingly the increase in H_2O_2 intracellular concentration depended on the irradiation dose. However, no statistically significant response of neighboring cells, either being in contact with the irradiated cell or distant, was detected (Fig. 4). We assume that this may be due to location of the irradiated cell somewhat apart from the majority of the cell population; and the results obtained are in line with the hypothesis that direct cell-cell contacts are necessary to transmit the signal from target cell to trigger the bystander effect.

Discussion

It is now recognized, that photoinduced cell death combines direct cellular damage with the subsequent spread of pernicious influence to non-target cells (Dahle *et al*., 1997; Castano *et al*., 2005; Poyer *et al*., 2012). 'Bystander effect' is the term attributed to response of neighboring cells triggered by the transmitted stress signals from treated cells. The effect has long been discussed by researchers and clinicians specialized in cancer therapy. It was demonstrated for the first time for ionizing radiation treatment by H. Nagasawa and J.B. Little as early as in 1992 (Nagasawa & Little, 1992); several years later it was shown that the bystander effect can also take place in cellular response to photodynamic treatment (Dahle *et al*., 1997; Dahle *et al*., 2000). Since then, a large amount of experimental data has been accumulated on the relevant topic in the field of radiation therapy (Mothersill & Seymour, 2004; Prise & O'Sullivan, 2009; Marín *et al*., 2015; Lara *et al*., 2015; Verma & Tiku, 2017), however, there are much less data on the bystander effect in response to PDT (Dąbrowska *et al*., 2005; Olivier *et al.*, 2009; Girotti *et al.*, 2021).

Using genetically encoded H2O2 sensor, we registered the development of the oxidative stress in non-irradiated cells in response to local photodynamic treatment of a single cell. Thus, our results are in line with the reports of other research groups on possibility of PDT-triggered bystander effect (Dahle *et al.*, 2000; Dąbrowska *et al.*, 2005; Olivier *et al.*, 2009; Bazak *et al.*, 2019).

Signal transmission to cells that have not been exposed to irradiation can occur through the surrounding media with the help of diffusing molecules and/or through gap junctions. We should underline that the pronounced response

of bystander cells was observed only when the cells in the population have closely contacted each other. Our results are in line with previous works, which show that the signal propagation process directly depends on the initial packaging of tumor cells (Dahle *et al.*, 2000; Lupu, 2009; Poyer *et al.*, 2012). The gap junctions-dependent mechanism seems to prevail in our experimental system, but we cannot exclude the role of diffusion either.

We assume that the PDT-induced accumulation of peroxide in the irradiated cell could be the reason for the signal transmission and initiation of the bystander effect we have recorded. Moreover, hydrogen peroxide can be the mediator molecule responsible for the signaling from target cells to non-irradiated cells both by means of gap junctions and diffusion through medium due to its long lifetime and ability to easily penetrate cell membranes.

Gap junctions between two cells are formed by transmembrane proteins, connexins (Cxs), which assemble to hexameric hemichannel, interacting with hemichannel of the neighboring cell and forming the gap junction channel that connects the cytoplasms of cells with each other. Gap junctions provide the passage of molecules and ions up to 1-1.5 kDa in size, for example, Ca^{2+} ions, inositol-3-phosphate (IP3), cGMP, cAMP, which are considered as candidate molecules responsible for signaling to neighboring cells (Verma & Tiku, 2017; Hoorelbeke *et al.*, 2018). In addition, ROS (e.g., H2O2, singlet oxygen, nitric oxide) can affect the tyrosine balance in cells by inhibiting the protein-tyrosine phosphatases and thus promoting the phosphorylated state of the tyrosine residues of connexin, which can influence the signaling cascades (Dahle *et al.*, 2000; Rubio, 2009; Verma & Tiku, 2017; Hoorelbeke *et al.*, 2018).

The second way of triggering the bystander effect is signal transmission by molecules capable of leaving the cell, for example, hydrogen peroxide, nitric oxide, calcium ions, etc. Thus, H2O2 released from the cell and accumulated in the extracellular medium is able to launch intercellular signaling pathways, in particular, to activate inositol-3-phosphate receptors (IP3R), which are involved in the intracellular release of calcium ions from its stores. The overload of the cytoplasm and mitochondria with calcium ions, possibly, leads to the release of apoptotic proteins cytochrome C and caspases, and, consequently, to triggering of apoptosis (Verma & Tiku, 2017; Nardin *et al.*, 2019). The direct activation of IP3R by hydrogen peroxide apparently results from modification of thiol groups of the receptor by H_2O_2 (Bansaghi *et al.*, 2014; Verma & Tiku, 2017).

To conclude, we registered the bystander effect in cell culture in vitro in response to a local photodynamic exposure on a single cell with Photosens used as a photosensitizer. The oxidative stress in the irradiated cell is assumed to be the initiator of the response of non-target cells. The effect manifested when the cells were closely contacted to each other; if the irradiated cell was at some distance from the bulk of the population, the response of non-target cells was not observed. In total, it allowed hypothesizing the prevail of intercellular signaling through gap junctions, however, the mechanisms involved in the propagation of damaging effects on cells outside the area of photodynamic exposure have to be further investigated.

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