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INACTIVATION OF FUNGI *IN VITRO* BY PHOTOSENSITIZATION: PRELIMINARY RESULTS

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Abstract: Photosensitization is based on the interaction of 2 completely non-toxic agents - a photosensitizer, accumulated in microorganisms, and visible light. This interaction induces radical-based cytotoxic reactions in the presence of oxygen. The photosensitization phenomenon is widely involved in the treatment of tumors in oncology, in curing arthritis and atherosclerosis. In this work, the possibility to inactivate pathogenic and harmful fungi by photosensitization is shown. A new treatment methodology is proposed on the basis of effective inactivation of the several micromycetes, such as *Aspergillus flavus, Trichothecium roseum, Fusarium avenaceum, Rhizopus oryzae*, by photosensitization.

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INTRODUCTION

In the early 1900s, Raab [6] was the first to observe the death of *Paramecium caudatum* after light exposure in the presence of acridine orange. At that time it was impossible to understand the mechanism of the microorganism's death. Now, it is accepted world-wide that this phenomenon might be named "photosensitization". In general, this treatment involves the organic dye (for instance, acridine orange, methylene blue or hematoporphyrin that usually accumulates in the target microorganism and is photoactive) and subsequent irradiation with visible light. This combination of 2 completely non-toxic elements – dye and light – in an oxygenated environment induces damage and total destruction of target cell in which it accumulates. In 1924, this phenomenon was applied to cure skin cancer. Afterwards, in 1978, Daugherty [5] started successful application of this novel technique for treatment of different cancers. Moreover, numerous investigators demonstrated possible practical usefulness of photosensitization in the broad field of different sciences: virology, microbiology, immunology, and dermathology [16, 18, 22, 31, 34, 39].

Pathogenic and harmful microorganisms can be present everywhere: in the air, buildings, on different surfaces, plants and food [20, 33, 35]. Moreover, the methods used for inactivation of these microorganisms are not always efficient and ecologically inert. For instance, novel non-thermal technologies increasing food microbial control can alter the structure of proteins and polysaccharides, causing changes in texture, physical appearance and nutritious value of the food. High-intensity ultrasound can denature proteins and produce free radicals adversely affecting the flavor of fruit-based or high-fat food [26]. Proteins, fats and carbohydrates are not notably altered by irradiation, although certain doses may cause slight colour changes in beef, pork and poultry [37].

Natural compounds, such as essential oils, chitosan, nisin or lysozyme, are being investigated to replace chemical preservatives and to obtain "green label"

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products. Their application is mainly hampered due to the interaction of the natural compounds with food ingredients, and due to changes in the organoleptic properties after introduction into food [3].

In addition, the viability of bacterial spores and an existence of highly resistant microbial subpopulations currently limit the efficacy of emerging non-thermal technologies [9]. The viral contamination of food can occur anywhere along the way "from farm to fork", but most food-borne viral infections can be traced back to infected persons who handle food that is not heated, or otherwise treated afterwards [8].

The last decade has been characterized by a significant interest in the microbiological quality of the indoor environment [7, 11, 33, 38]. The point is that the microbiological quality of air, contamination of buildings by airborne bacteria or fungi, could be potential causes of inhabitants health complains [33]. Lugauskas and Stakeniene [12] have isolated fungal strains from the air, vegetables and fruits. They detected that intensive secondary metabolite production was characteristic of 51% of the investigated strains; among them, 39% were lethal to mice within a period of 2 weeks. Most known mycotoxins are produced by species belonging to the genera *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* [28, 38]. Consequently, work in modern agricultural and industrial environments exposes the respiratory system to chemical agents derived from bacteria and fungi, which often cause asthma-like syndrome, extrinsic allergic alveolitis, mycosis, etc. [25, 33, 35], or simply induce some health complications without clear origin (such as bronchitis, sore throat, concentration difficulties, backaches, irritation of eyes and mouth cavity, weakness, etc.) [21, 23]. In spite of tremendous scientific progress, knowledge about microbiological indoor air pollution, biocontamination of buildings (Sick Building Syndrome), and ways to solve these problems seems to be still insufficient [7].

Thus, it is easy to understand that today's existing methods for inactivating harmful and pathogenic microorganisms in different fields, including food manufacturing and safety, occupational environment, or in some cases, conservation of cultural objects and archives, are not always effective. Based on that, a new approach to inactivation of harmful microorganisms in a cost-effective and environmentally inert way is still a problem.

Taking all the above into account, the pilot study was focused on the possibility to inactivate several potentially pathogenic fungi of different morphology using biophotonic technology – photosensitization.

MATERIALS AND METHODS

Objects. We selected micromycetes of several strains, which are harmful to the food industry, plant substrates, grains of corn, induce corresponding diseases, and belong to a different morphological type.

For instance, *Rhizopus oryzae* Went et Prinsen Geerl. is distributed in food, indoor and isolated from soil, grain, vegetables, fruits and nuts. *R. oryzae* is the most frequent agent of the human mucormycosis.

The occurrence of other representative of micromycetes *Aspergillus flavus Link* is mostly restricted to fermented food products or industrial environments, common in (ground) nuts, spices, oil seeds, cereals, and occasionally in dried fruits (e.g. figs). Invasive aspergillosis is found most commonly among patients with leukemia, lymphoma, and other malignant diseases, renal or bone marrow transplantation [29].

Trichothecium roseum (Pers.) *Link* ex Gray is a world-wide distributed micromycete, mostly detected on different substrates: decaying plant, soil, ears of corn, foodstuffs, outdoor air. *T. roseum* has been considered a primary pathogen of stored apples and tomatoes in greenhouses, but it is also regarded as a bioagent against other pathogens [4].

The last one selected for experiments was *Fusarium avenaceum* (Fr.) Sacc. Fungi of the *Fusarium Link* genus are widely distributed on plants and in soil, being the usual components of fungal flora of rice, bean, soybean, and other crops [30]. The grains contaminated with these toxins may give rise to allergic symptoms or be carcinogenic in long-term consumption [24]. It is of importance to note that *Fusarium* fungi are among the most drug-resistant [1].

Photosensitizer. The stock solution of hematoporphyrin dimethyl ether (HPde) (a gift from Prof. G.V. Ponomarev, Russia) was prepared in physiological saline ($2.5\ 10^{-3}$M) and stored in the dark below 10ºC [14].

Irradiation. The light source used for irradiation of microorganisms was constructed at the Laser centre of Vilnius University [15]. It consisted of a tungsten lamp (500 W), an optical system for light focusing and an optical filter for UV and infrared light elimination (370 nm$<\lambda>$680 nm). Light intensity at the position of the cells was 30 mW/cm^3. Irradiation time was 15 min [14].

Experimental setup and evaluation of treatment efficiency. Stock cultures of fungi were stored at 4ºC on malt extract agar (MEA, Sigma). MEA containing Petri dishes were inoculated with agar plugs obtained from stock cultures. The fungi were cultivated at 25ºC in the dark to achieve typical growth and sporulation. Conidia (or spores – *Rhizopus oryzae*) were harvested by flooding grown Petri dishes with 10 ml of distilled water and scraping the surface of colonies with glass rods. The resulting suspension was agitated and filtered though double-layered sterile cheesecloth to remove hypal fragments; the obtained suspension was decanted, replaced with PBS, and diluted up to 10^6 spore ml^{-1}. Stock solutions of HPde in phosphate buffer (PBS) or control solution (to obtain 2.5, 5.0, 7.5, 10.0, 25.0, 51.0 and 71.0 μM HPde concentration in spore suspension) was added to spore suspensions and incubated at 25ºC for 20 min.

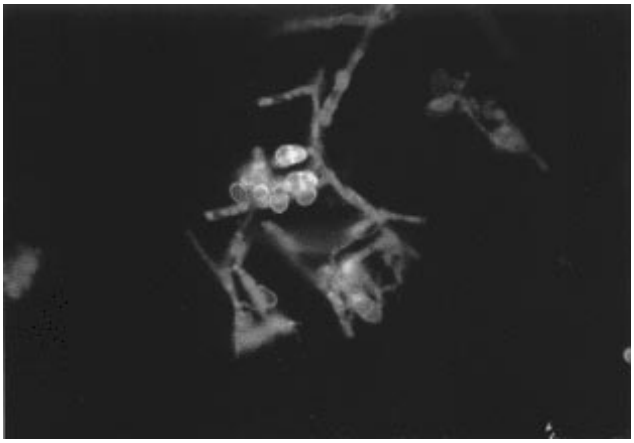

Figure 1. Fluorescent microphotograph of *Rhizopus oryzae* (×400). Red fluorescence reflects the loci of HPde accumulation.

After the exposure, the solutions were decanted and replaced with PBS (1 ml). One sample was irradiated with visible light in the cell culture dishes, while the other sample was left in the dark. At least 3 separate experiments were conducted for each fungus investigated.

After irradiation, the buffer was removed and replaced with 1 ml of fresh medium. Suspensions were spread onto water agar (WA, Oxoid) on the glass slides, placed in Petri dishes on the glass rods and incubated in the dark at 25ºC. Plates were incubated for 24-48-72 h prior to the assay number of germinated spore (or conidia) for the detection of their survival. Control dishes were prepared to evaluate the spore germination (%). Five microscope fields were observed on every glass slide of the control, just HPde treated, and irradiated with light variants [10]. Germinated and non-germinated conidia were counted in random fields at ×40 with a light microscope (Motic microscope B1-setries biological microscopes). A total of 250–300 conidia were counted.

Assessment of conidia germination. After each exposure session the fungal suspension was pipetted on cover slips and placed conidia-side down on water agar medium in 9-cm Petri dishes. The plates were incubated at 25ºC for 24 h to induce conidial germination; at the time, the conidia germination was assessed [10]. Germinated and non-germinated conidia were counted with a light microscope (magnification: ×100). A total of 300–500 conidia were examined on each cover slip, with a higher number of conidia in the case of low germination. The conidia were considered germinated if their length was ≈20 μm. The percent germination was calculated as follows:

$$\frac{\text{No. of germinated conidia}}{\text{Total No. of conidia}} \times 100.$$

Fluorescence measurements. Photosensitizer accumulated in the microorganisms was detected by fluorescence microscope (Nikon Eclipse E-400). Excitation of red porphyrin fluorescence was performed using excitation at λ = 330–380 nm.

RESULTS

In the first phase, we tried to prove that when was used it to accumulate in the fungi selected for investigation because of their sensitivity to this treatment. For this purpose, all investigated fungi were incubated with HPde (10^{-4}M). Due to the fact that accumulated photosensitizer can produce fluorescence, visualization of every fungus was monitored and photographed using a fluorescence microscope. The microphotograph (Fig. 1) clearly indicates that living *Rhizopus oryzae* is able to accumulate the HPde. Indeed, the red fluorescence of HPde shows the loci of compound accumulation and localization. It is worth mentioning that all investigated strains of micro-fungi show rather high fluorescence of HPde located in the conidia (*Rhizopus*, *Aspergillus*, *Fusarium*).

The data presented (Fig. 2) clearly indicate that *Aspergillus* is very sensitive to HPde treatment alone (dark toxicity). Increasing the concentration of this photosensitizer acts drastically on conidia germination, inhibiting it by up to 100% (5.1×10^{-4} M). It is interesting to note that following irradiation of this microorganism by visible light increases the inhibition by about 10%, if

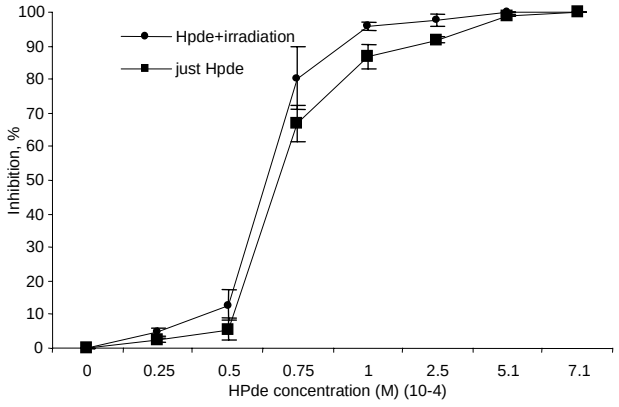

Figure 2. Inhibition of *Aspergillus flavus* conidia germination as function of photosensitizer (HPde) concentration. Incubation time - 18 hours; irradiation with a visible light - 15 min. The error bars represent the standard deviation of 3-5 repeats.

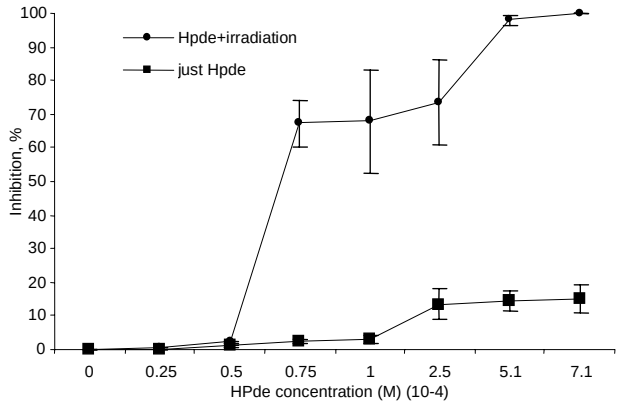

Figure 3. Inhibition of *Rhizopus oryzae* conidia germination as function of photosensitizer (HPde) concentration. Incubation time - 18 hours; irradiation with a visible light - 15 min. The error bars represent the standard deviation of 3-5 repeats.

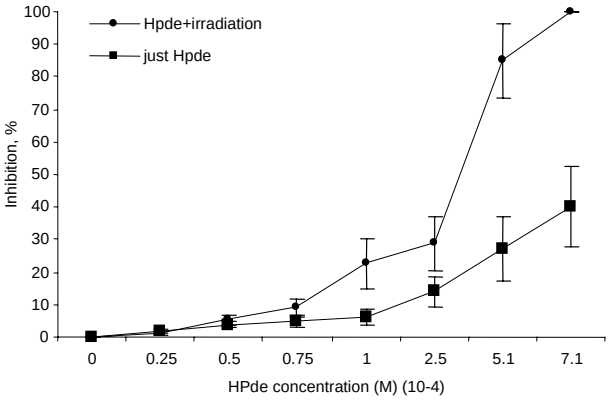

Figure 4. Inhibition of *Fusarium avenaceum* conidia germination as function of photosensitizer (HPde) concentration. Incubation time - 18 hours; irradiation with a visible light - 15 min. The error bars represent the standard deviation of 3-5 repeats.

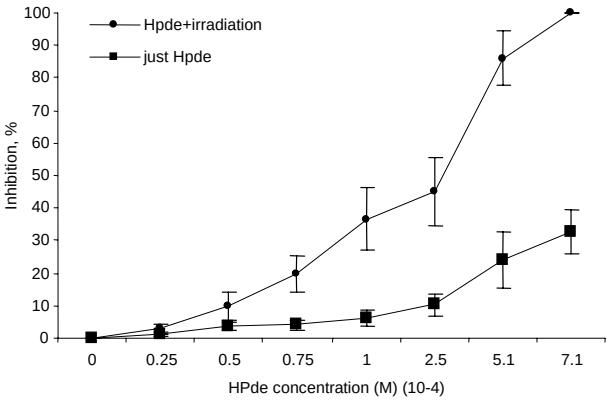

Figure 5. Inhibition of *Trichothecium roseum* conidia germination as function of photosensitizer (HPde) concentration. Incubation time - 18 hours; irradiation with a visible light - 15 min. The error bars represent the standard deviation of 3-5 repeats.

compared with the action of HPde alone. It is obvious that HPde action on the inhibition of *Aspergillus* is extremely high and practically important, and therefore needs further investigation for deeper understanding of the mechanism of this inhibition.

Therefore, other experiments were carried out with *Rhizopus*. Data (Fig. 3) revealed that this microorganism is much more resistant to HPde treatment: it could induce *per se* just 15% inhibition of spore germination. On the contrary, following irradiation with visible light increased the inhibition up to 100% when higher concentrations of HPde were used (7.1×10^{-4} M). At first sight, both objects examined have a concentration dependent inhibition response to photosensitization (0.75-7.1×10^{-4} M HPde concentration range).

It was therefore of interest to investigate the sensitivity of *Fusarium avenaceum* to this treatment. Results indicate that under analogous conditions employed in this study, resistance of this microorganism to photosensitization-induced destruction is much higher: even high concentration of HPde (5.1×10^{-4} M) was inhibiting conidia germination of this fungus. As a matter of fact, the dark toxicity of HPde (Hpde only) observed in the case of *Fusarium avenaceum*, was rather high and reached 40% (at concentration of 7.1×10^{-4} M) (Fig. 4). Nevertheless, the inhibiting action of the highest HPde concentration (7.1×10^{-4} M), and following irradiation, did not reach that observed in the case of *Rhizopus* or *Aspergillus*.

Eventually, the last one under investigation was *Trichothecium roseum*. As previously described, first of all the HPde action on inhibition of conidia germination was examined. The data (Fig. 5) reflect a rather high resistance of *T. roseum* to the action of HPde – only the highest concentrations of this compound (7.1×10^{-4} M) might reach 33% inhibition. Understandably, a subsequent experiment was performed to evaluate the inhibition induced by photosensitization. As a rule, fairly significant concentration-dependent inhibition of conidia germination was observed, and eventually at concentration 7.1×10^{-4} M HPde reached 100%.

DISCUSSION

The struggle against harmful and pathogenic microorganisms has continued due particularly to the wide variety of encountered pathogens and existing disadvantages of methods applied to inactivate them. Thus, new approaches towards this problem seem to be imperative. In this context, novel, cost-effective, environmentally inert biophotonic technology – photosensitization – is available to supplement the armoury of existing tools.

So far, only a few reports have been published on the possibility to inactivate several yeasts by photosensitization [2, 13].

Our previous data clearly indicate that yeast *Saccharomyces cerevissiae*, as well as micromycetes *Ulocladium oudemansii*, might be inactivated by this treatment [13, 17]. Moreover, inhibition of growth under other experimental conditions was further observed in *Aureobasidium* sp., *Rhodotorula* sp., *Penicillium staloniferum* (unpublished data). Intrinsically, all data obtained in our laboratory support the idea that the plethora of harmful micromycetes destroying food, buildings, and items belonging to cultural heritage might be inactivated by the photosensitization method, which is completely safe, reproducible, not-mutagenic, environmentally and human inert. Actually, the combination of 2 completely non-toxic constituents, i.e., organic dye and visible light, might actually contribute to inactivation of several fungi as the most hazardous enemies in this context. Moreover, from the data obtained, it is easy to draw the conclusion that different microfungi have individual sensitivity to HPde dark toxicity as well as photosensitization. For instance, *Aspergillus* can be described as most sensitive to HPde treatment (100% inhibition). Photosensitization of this fungus by HPde and light was just 10% increasing the inhibition of conidia germination. With regard to the sensitivity of *Rhizopus oryzae* to HPde treatment, this was much less (15% at concentration 5.1×10^{-4} M HPde). Nevertheless, the

photosensitization of this fungus was very fast and significant. On the contrary, *Fusarium* and *Trichothecium* exhibited certain resistance to this treatment up to 1×10^{-4} M HPde. Nevertheless, a further increase of HPde concentration and following irradiation drastically inhibited all investigated fungi up to 90-100%.

What is the mechanism of inhibition of investigated fungal conidia germination after HPde treatment? The point is that spores of many pathogens (plants or human) are ready to germinate almost immediately upon arrival on a surface, although germination is often poor or absent in other situations. Spore germination on "a suitable" surfaces can be stimulated by several factors, of which the most important are attachment, physical or chemical induction and loss of autoinhibitors [32]. The surface may provide physical features that induce germination. The spores of many phytopathogenic fungi contain potent autoinhibitors what prevent germination until they have been washed or diluted out of the surface. It seems that HPde as photosensitizer in some cases may serve also as a spore germination inhibitor. This statement is in agreement with the conclusions of other authors; for instance, Wainwright and Crossley [36] postulate the capacity of several photosensitizers to have an inherent or "dark" toxicity against microbial species.

It has been shown convincingly that photosensitization by HPde and light might totally inhibit conidia germination of all investigated fungi. The question arises, what might be the mechanism of this inhibition? It is more or less accepted that the loci, where photosensitizer is preferentially accumulated, will be destroyed after photosensitization [36]. The point is that reactive $^1O_2$, generated during physico-chemical stages of photosensitization has limited migration, and the sites of initial damage after photosensitization are closely related to the localization of the sensitizer [19]. Consequently, if fungus is accumulating photosensitizer, it will be sensitive to this treatment. So far, promising and effective methodology for the inhibition of fungal conidia germination and perhaps, total destruction and elimination of these potentially pathogenic microorganisms seems possible.

CONCLUSIONS

In conclusion, the presented data support the idea that photosensitization might be an effective tool for inactivation of several microfungi. The general antifungal action of photosensitization, observed *in vitro*, offers great potential for inhibiting the development of the fungi, and might be used to sterilize or decontaminate various surfaces in a cost-effective, environmentally and human friendly way. It is important to note, that HPde *per se* in some cases may serve also as a spore germination inhibitor.

While it is not suggested that photosensitization will solve all antimicrobial problems in special cases, improvements may be obtained using this new approach or combining photosensitization with accepted methods for microbial control.

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