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Phototoxic effect of hypericin alone and in combination with acetylcysteine on *Staphylococcus aureus* biofilms

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Photodynamic inactivation;
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Summary
Background: Resistance of bacteria against antibiotics and antimicrobials is arising worldwide and there is an urgent need for strategies that are capable of inactivating biofilm-state pathogens with less potential of developing resistance in pathogens. A promising approach could be photodynamic inactivation (PDI) which uses light in combination with a photosensitizer to induce a phototoxic reaction. In this study, we evaluated the *in vitro* phototoxic effect of hypericin (HYP) alone and in combination with acetylcysteine (AC) on *Staphylococcus aureus* biofilms. AC, a mucolytic agent, reduces the production of extracellular polysaccharide matrix while promoting the disruption of mature biofilm.
Methods: In vitro phototoxic effect of HYP alone (0.5 μg/ml, light dose: 16 J/cm^2), and in combination with AC (10 mg/ml) on ten clinical *S. aureus* isolates and *S. aureus* (ATCC 25923) biofilms was studied. Effect of HYP concentration (0.5 μg/ml) and light dose (8 J/cm^2) on PDI of all eleven *S. aureus* strains in planktonic forms was also investigated.
Results: HYP-PDI did not result in a reduction in viable count for each of the strains when grown in biofilms. However, HYP-PDI applied on biofilms treated with AC was able to disrupt pre-formed biofilms (viable count reduction ranging from 5.2 to 6.3 $\log_{10}$-unit in comparison to controls in all tested strains). A 6.5 log killing was obtained for *S. aureus* (ATCC 25923) planktonic cells treated with 0.5 μg/ml at 8 J/cm^2. For this set of PDI parameters, ten clinical *S. aureus* isolates showed 5.5–6.7 log killing.
Conclusion: HYP-PDI in combination with AC had significant ability to eradicate the pre-formed mature biofilms of *S. aureus* strains.

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Introduction

In many environments bacteria exist as a complex, multispecies surface-associated community termed biofilm. Organisms within biofilms are embedded in a self-produced matrix of extracellular polymeric substance (EPS) composed of polysaccharides, proteins, lipids, and extracellular DNA. Several advantages exist for bacteria that live in a biofilm phenotype including structural stability, firm adherence to biotic or abiotic surfaces, increased virulence, and resistance to both antimicrobial therapy and the host immune response [1—3].

Staphylococcus aureus is an important human pathogen associated with numerous skin diseases including wound infections. The difficulty in eradicating *S. aureus* colonization with conventional antibiotic therapy may be due to the presence of biofilm [4]. EPS itself may slow drug-diffusion by its higher viscosity or can even act as a barrier [5]. Furthermore, development of a biofilm leads to an enormous genetic diversity of its cells, which provides insurance for the cells for better adaptation to rapid alteration of environment conditions [6]. Also, persister cells are being formed, when bacteria grow as a biofilm. These are in a dormant, non-dividing state and provide tolerance toward antimicrobial agents [7].

S. aureus biofilms have a negative effect on wound healing as evidenced by their ability to induce keratinocyte apoptosis *in vitro* and inhibit re-epithelialization in an *in vivo* animal wound model [8,9]. In addition, biofilms may also increase the inflammatory response characteristic of chronic wounds, thus promoting tissue damage and further contributing to the non-healing phenotype [10].

With increasing the resistance of pathogenic bacteria including *S. aureus* to antimicrobial agents, there is a need for the development of alternative antibacterial strategies; photodynamic inactivation (PDI) could be the method of choice. PDI involves killing of organisms by light in the presence of a photosensitizing agent [11]. The local combination of light and the photosensitive drug in the frame of PDI avoids systemic effects on bacterial flora and by this minimizes side effects. Reports of successful inactivation of multi-drug-resistant strains of *S. aureus* declare PDI to be the method of choice for treatment of such local infections [12,13].

Several studies have been reported PDI mediated by hypericin (HYP), a powerful photosensitizer [14—20]. HYP is a phenanthroperylene quinine pigment naturally occuring in *Hypericum* plants. It is characterized by a high quantum yield for formation of reactive oxygen species and very slow photobleaching [21].

In our pervious study, we evaluated the *in vitro* bactericidal effect of HYP-PDI on different bacterial species, assessing its photocytotoxicity to primary human fibroblasts to determine possible side effects [20]. Our results showed that HYP had a high phototoxicity to *S. aureus*, *Enterococcus faecalis* and *Escherichia coli* at extremely low drug concentrations and did not induce significant cytotoxic effects on human fibroblasts in culture. However, planktonic bacterial cultures as employed in that study represent the first step in the evaluation of a photoactive compound for its use in PDI. So, the purpose of the present study was to evaluate the *in vitro* phototoxic effect of HYP alone and in combination with acetylcysteine (AC) on *S. aureus* biofilms. AC, also known as N-acetylcysteine, is a mucolytic agent that disrupts disulfide bonds in mucus and, for this reason; it reduces the viscosity of secretions [22].

AC decreases biofilm formation by a variety of bacteria [22—24] and reduces the production of extracellular polysaccharide matrix [25] while promoting the disruption of mature biofilm [23,24].

Materials and methods

Bacterial species and culture conditions

A total of 10 clinical *S. aureus* recovered from acute and chronic wounds were used in this study. In addition, *S. aureus* (ATCC 25923) was also included. The *in vitro* antimicrobial susceptibility of clinical isolates was determined according to CLSI guidelines [26]. Antibiotic discs were purchased from Padtan Teb Company, Iran. Eight clinical isolates were resistant to the following antibiotics: amoxicillin—clavulanate (20/10 μg), oxacillin (1 μg), ciprofloxacin (5 μg), gentamicin (10 μg), and amikacin (30 μg). *S. aureus* (UTMC 1484) was resistant to ciprofloxacin, amoxicillin—clavulanate and gentamicin and sensitive to oxacillin and amikacin. *S. aureus* (UTMC 1474) was resistant to ciprofloxacin, amoxicillin—clavulanate and sensitive to oxacillin, gentamicin and amikacin.

Organisms were maintained by weekly subculture on nutrient agar (Merck, Germany). All organisms were grown aerobically in nutrient agar plates at 37 °C for 18—24 h. Then a suspension of each organism was prepared in sterile phosphate-buffered saline (PBS, pH 7.4) to reach the turbidity of McFarland standards. No. 2 (a concentration of 6×10^8 CFU/ml).

Photosensitizer (PS) and light source

HYP (Tocris Bioscience, UK, purity > 98%) was dissolved as a stock at 100 μg/ml in dimethyl sulfoxide (DMSO). Stock solution was kept at 4 °C in the dark and further diluted in sterile PBS (pH 7.4) when needed.

The light source consisted of a light-emitting diode (LED) array. The samples were exposed to LEDs (wavelength of 590 nm, measured power of 10 mW).

Photodynamic inactivation of *S. aureus* planktonic cells

Bacterial suspensions were incubated with 0.5 μg/ml HYP in the dark at 37 °C for 5 min. The HYP-treated cells were centrifuged (6000 rpm for 15 min), and washed twice with sterile PBS (pH 7.4). Aliquots of 200 μl treated cells were placed in a 96-well microtiter plate and irradiated with the light source (5 min, 8 J/cm^2). The plates were kept covered during the illumination in order to maintain the sterility of the culture. After that, 100 μl of cell suspension was spread on nutrient agar in 10-fold serial dilutions. Colonies were counted after incubation for 24 h at 37 °C. All experiments were repeated three times [20].

Every experiment was accompanied by three control samples: a negative control (no PS, no illumination), a light control (no PS, illumination same as PDI samples) and a dark control (with PS, no illumination).

Assessment of biofilm formation

S. aureus strains were grown overnight in tryptic soya broth (TSB, Merck, Germany) supplemented with 0.2% glucose at