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Electroporation enhances antimicrobial photodynamic therapy mediated by the hydrophobic photosensitizer, hypericin, Electroporation enhances antimicrobial photodynamic inactivation

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Abstract

The effective transport of photosensitizers (PS) across the membrane and the intracellular accumulation of PS are the most crucial elements in antimicrobial photodynamic therapy (aPDT). However, due to the morphological complexity of Gram-negative bacteria the penetration of PS is limited, especially hydrophobic PS. Electroporation (EP) could increase the effectiveness of aPDT, by promoting the formation of transient pores that enhance the permeability of the bacterial membrane to PS. In this study we evaluated the combination of aPDT mediated by the hydrophobic PS, hypericin and EP (aPDT/EP) against Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli*. These bacteria were exposed to light (590 nm) in the presence of hypericin (4μM), following electroporation. The results showed that aPDT/EP inactivated 3.67 logs more *E. coli* and 2.65 logs more *S. aureus* than aPDT alone. Based on these results we suggest that EP can potentiate the aPDT effect.

Keywords

antimicrobial photodynamic therapy; hypericin; electroporation; E. coli; S. aureus

1. Background

Antimicrobial photodynamic therapy (aPDT) is an emerging treatment for microbial infections, is a potential alternative to conventional antimicrobial agents [1]. aPDT can

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equally kill multi-drug resistant bacteria and no resistance mechanisms against it are yet known. aPDT is a multi-target process producing damage mainly to the cytoplasmic membrane leading to leakage of cellular contents and inactivation of membrane transport systems, and also to DNA [2] [3].

However, aPDT is limited against Gram-negative bacteria owing to the higher complexity of the cell wall that has a complex structure that consists of an inner cytoplasmic membrane and an outer membrane that are separated by the peptidoglycan-containing periplasm [4] forming an effective permeability barrier to many photosensitizers (PS) especially hydrophobic compounds [5]. Although cationic PS can effectively kill all classes of bacteria, it is still desirable to study methods to make non-cationic hydrophobic PS effective as well.

2. Aims

Electroporation (EP) [6] exposes the cell to short external electric pulses of high voltage, affecting the organization of cell wall and the plasma membrane forming transient permeable channels known as electropores that allow transport of various non-permeant molecules into the cell [7] (Fig. 1). An important advantage of EP is the enhanced selectivity of the treatment since the PS transportation rate is only elevated in the area of the electric field application [8, 9]. Several studies have shown that EP can potentiate the cytotoxicity of PDT, however those studies were applied against tumor cells [6–8, 10–13]. We here present the first study on electroporation combined with PDT applied to bacteria.

3. Methods

2.1. Bacterial growth

Escherichia coli (ATCC #25922) and *Staphylococcus aureus* (ATCC #35556) were grown overnight in brain heart infusion medium (BHI) at 37°C and 100 rpm, then 1 mL of them was transferred into fresh BHI medium and left to grow aerobically at 37°C until the mid-logarithmic phase. The cells were harvested by centrifugation (10min, 3000rpm) and resuspended in sterile PBS at pH 7.0. The final bacterial concentration was 10^8 colony-forming units (CFU) mL^{-1}.

2.2. Photosensitizer

Hypericin (Hy) was purchased from Sigma-Aldrich, (St Louis, MO). The stock solution of Hy (10 mM) was prepared in dimethylsulfoxide (DMSO) and stored at −20°C in the dark. All the experiments were performed by diluting the stock solution in PBS to 4μM (10μg/mL). The DMSO concentration did not have any anti-bacterial effect alone under these conditions.

2.3. Electroporation

Electroporation was performed by Easyject Optima– Equibio apparatus (St. Louis, MO) equipped with square-wave pulses, generating electrical pulses with the magnitude of 0–3000V, 10–600μs long, in the series of 1–99 pulses separated by the time interval of 100ms – 10s. The electroporation parameters were selected based on previously experiments (data not shown): 2 electric pulses at 1000 Vcm^{-1}, 50μs long, 1-Hz frequency. We used cuvettes fitted with electrodes with 0.1 cm between embedded aluminum plates (catalog number P41050, Invitrogen, Grand Island, NY).

2.4. aPDT procedure

50 μL of bacterial suspensions with 1×10^8 cells and 50 μL of hypericin solution (4μM) were mixed and added into electroporation cuvette and subjected to the electroporation procedure.

This suspension and a similar 100 μL of bacterial suspension with hypericin and no electroporation were incubated at 37°C in the dark for 10 minutes, and then irradiated with yellow light (590 nm) from a Lumacare LC122 lamp (fitted with a fiber optic filtered probe (570–630-nm) at different fluences (10–40 J/cm^2) delivered at 100mW/cm^2. Bacterial cells were serially 10-fold diluted with medium and the number of colonies counted after 24h incubation at 37°C. Control studies were performed (Table 1).

2.6. Statistics

Values are given as means and standard errors of four separate experiments. Differences between aPDT and EP/aPDT were tested for significance by one way ANOVA with Tukey post-hoc test. *p*-values of less than 0.05 were considered significant.

3. Results and discussion

Electroporation parameters were selected based on the viability of the microorganisms after EP (data not shown), considering an excessively high electric field can cause irreversible damage to the plasma membrane of the cells. So, the selected field energy was high enough to enhance the hypericin penetration and below the level to needed to kill the bacterial cells (Figure 2).

Hypericin is a plant derived phenanthroperylene quinone [14]. It possesses minimal dark toxicity, is not metabolized and is a photodynamically active molecule with red fluorescence [15]. The ability to generate singlet oxygen or reactive oxygen species upon irradiation also makes hypericin one of the most powerful natural PS [16, 17].

The control groups did not show significantly reduced bacterial viability (Fig 2), *S. aureus* (4.25 log reduction at 40J/cm^2) was more susceptible to hypericin-PDT than *E. coli* (0.93 log reduction at 40J/cm^2. A similar killing of these microorganisms was observed by Yow *et al* who evaluated the photodynamic antimicrobial effect of hypericin [16]. These results are consistent with the differences between Gram-positive bacteria with a relative porous layer of peptidoglycan that allows PS penetration and Gram-negative bacteria with a many-layered cell wall that acts as a permeability barrier [18]. Therefore EP was investigated to increase hypericin efficiency, especially against *E. coli*.

The combination of aPDT with EP potentiated the hypericin PDI effect on both bacteria more than aPDT alone (Fig 2). *S. aureus* showed significantly increased killing (2.5 log more than aPDT alone, $p<0.05$) at the lowest dose of 10J/cm^2 and as the fluence increased the difference between aPDT alone and aPDT/EP became even more pronounced with 20J/cm^2 giving 2.6 logs more killing ($p<0.01$) and 40J/cm^2 also giving 2.6 logs more killing ($p<0.01$). For *E. coli* there was also a significantly increased killing of 2.4 log more than aPDT alone ($p<0.05$) at the lowest dose of 10J/cm^2, and 20J/cm^2 giving 3.3 logs more killing ($p<0.01$) and 40J/cm^2 giving 3.5 logs more killing ($p<0.001$).

Importantly the relative effect of EP was greater in the case of the impermeable *E. coli* (at 40J/cm^2 there was 3.5 logs more killing than PDT alone) than it was in the case of the permeable *S. aureus* (2.6 logs more killing than PDT alone).

Caveats to this study are that: (i) host cells should also be tested as well as bacteria to demonstrate selectivity for microbial cells; and (ii) hypericin is almost certainly not the best antimicrobial photosensitizer to combine with electroporation, and methylene blue should also be tested.

4. Conclusions

Electroporation increased the phototoxic effect of hypericin against *E. coli* and *S. aureus* because EP increased the extent to which the PS penetrated and accumulated into the bacterial cells. This suggests that EP could be applied to improve the delivery of PS, especially hydrophobic ones such as hypericin, into microorganisms resulting in an enhancement of aPDT.

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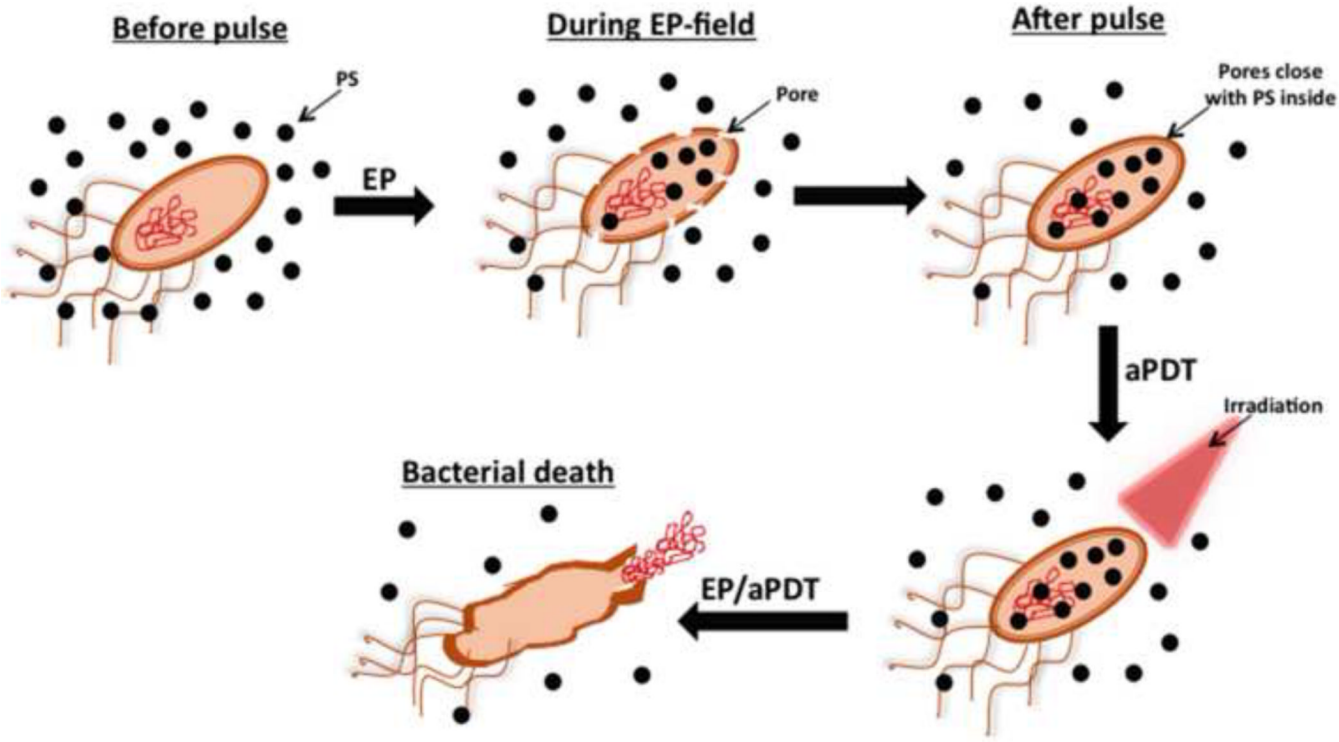

Figure 1. Electroporation in combination with aPDT

The PS in solution is allowed to gain intracellular access to the bacteria after temporary electropores are created by the pulse. The pores close up without killing the bacteria but when red light is delivered the bacteria are killed by aPDT more than simply incubating the bacteria with PS alone.

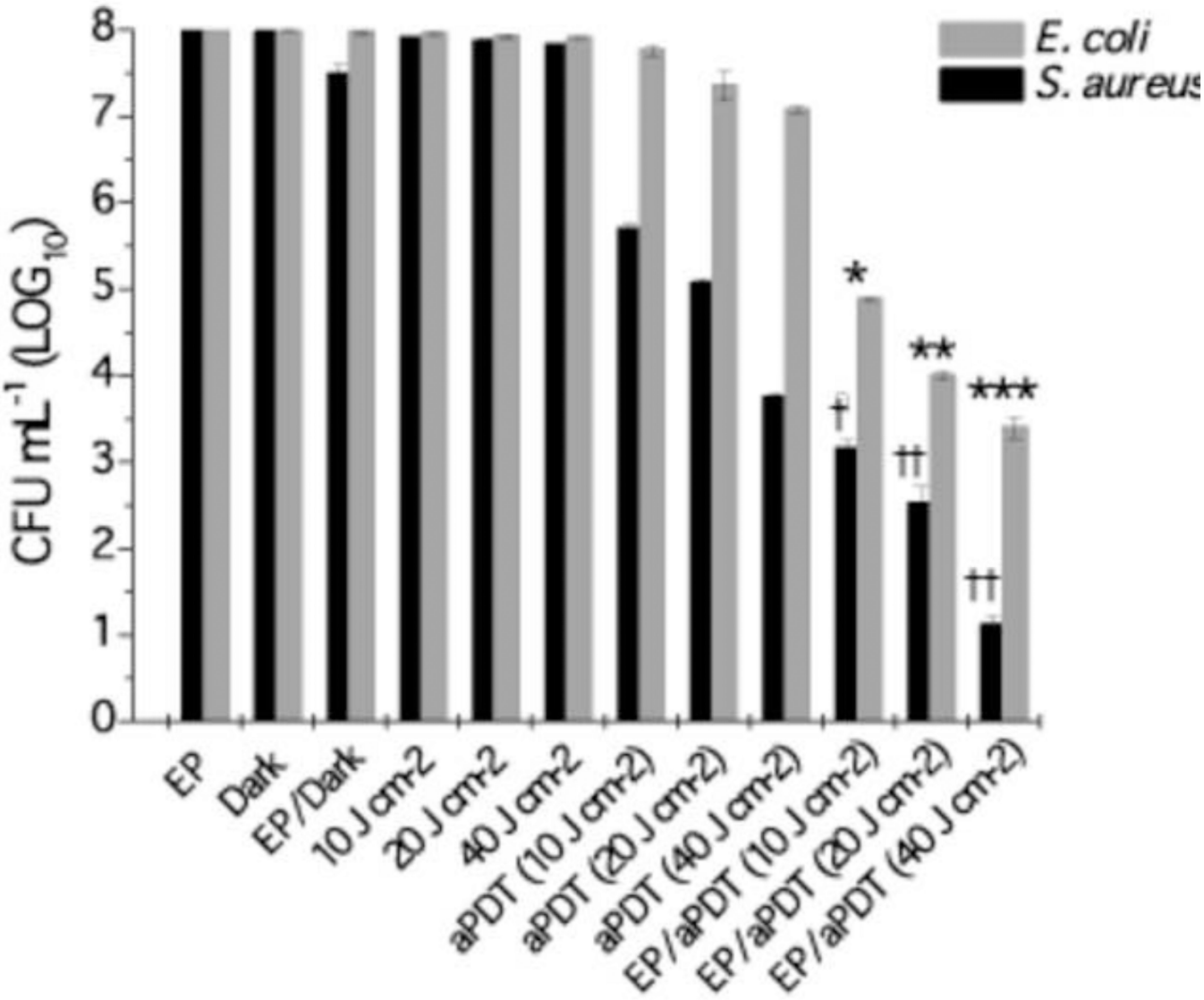

Figure 2.

Synergism of electroporation and aPDT mediated by hypericin. Against *S. aureus* and *E. coli*. Values are means of four separate experiments and bars are standard deviations. †,*$p < 0.05$ versus aPDT alone at 10 J cm^{-2}; ††, **$p < 0.01$ versus aPDT 20 J cm^{-2}; †† $p < 0.01$ and ***$p < 0.001$ versus aPDT 40 J cm^{-2}.

Table 1

Parameters of the electroporation study

Experiments	Electroporation (2 pulses / 1000 V cm^{-1})	Light (590 nm)	Hypericin (10 μg mL^{-1})
Control groups	X		
			X
	X		X
		10 J cm^{-2}	
		20 J cm^{-2}	
		40 J cm^{-2}	
aPDT groups		10 J cm^{-2}	X
		20 J cm^{-2}	X
		40 J cm^{-2}	X
EP/aPDT groups	X	10 J cm^{-2}	X
	X	20 J cm^{-2}	X
	X	40 J cm^{-2}	X

X: applied parameters