THE EFFECT OF LOW AC ELECTRIC FIELD ON BACTERIAL CELL DEATH

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Abstract. Many bacterial species developed numerous mechanisms that render bacteria resistant to some and in certain few cases to nearly all antibiotics. Thus replacing or combining antibiotics with physical method becomes urgently needed. Effect of DC electric field on bacteria was studied decades before but DC usage has many drawbacks. Therefore some researches started to utilize very high AC electric field instead of DC but it is not very suitable for *in vivo* applications. In this paper we exposed *E. coli* to low electric AC fields with low voltage and high exposure time to make electric energy equivalent to those used in high AC field conditions. We tried frequencies ranging from 10 Hz to 1 MHz. We found significant reduction in the viable bacterial counts at 10 and 100 Hz while no significant changes were found at higher frequencies. We concluded that to replace high AC electric field with low one, equivalent electric energy should be maintained as well as low frequencies should be used.

Key words: Low AC electric field, frequency, time of exposure, *Escherichia coli*, bacterial cell death.

INTRODUCTION

Due to the widespread use of antibiotics, a variety of bacterial species have developed numerous resistance mechanisms to many well known antibiotics [4]. There are many important pathogens that are resistant to multiple antibiotic classes, and infections caused by multidrug resistant organisms are limiting treatment options and compromising effective therapy [14].

Due to continuous different resistances of bacteria to the antibiotics, different physical methods are used to kill the bacteria. The use of ultrasound [1], light sources [16] and electric fields [23] alone or in combination with antibiotics could represent an aid for modern medicine in the continuous battle against pathogenic microorganisms.

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The DC effects on bacterial cells have been studied for several decades [17, 18], and the studies have focused mainly on the viability, metabolism, and transport of the cells. In particular, viability studies have concentrated on the use of pulsed high voltage for inactivation [3] or moderate voltage for many hours or many days in some cases [23].

Even in bacterial biofilms; surface-adhering bacteria that form colonies characterized by the production of an exopolysaccharide matrix in which they reside [9], the bioelectric phenomenon is a synergy between a relatively weak DC and the antibiotic used to eradicate the biofilm bacteria [21].

There are limitations for the use of DC currents, including weak currents, for the killing of vegetative bacteria. The first is that such currents may stimulate nerves and muscles, causing pain and muscular contractions in the patient. The second relates to the spread of the currents in the body, which can be regarded as a volume conductor. Thus, unless the lesion is superficial or unless there is a conductor leading from the surface to a deeply situated lesion, a current density of sufficient intensity at the target can be obtained only when the density near the electrodes is of a damaging and stimulating magnitude. The third limitation is that DC currents cannot be generated by insulated electrodes and are therefore always associated with electrolysis, metal ions and free radicals [6]. The fourth reason in some applications is the requirement for long time DC exposure to achieve the required lethal effect which may extend to continuous seven days of exposure making its *in vivo* application impossible [2]. Although bioelectric technique in biofilms succeeds in some cases but not all the time because DC works as mechanical stress only that decreases the attachment of the bacteria on the solid surface and converts the situation to vegetative bacteria which may also resist the antibiotic [24].

Alternatively, new trials using AC instead of DC have been conducted. Very high electric field strength [22] or moderate electric field strength for longer time was used to exert heating effect [12]. Both conditions are still not optimum for *in vivo* application.

In this paper we investigated two equivalent energy low AC electric field protocols with the same energy derived from high fields protocol at different frequencies and time of exposures in order to test the possibility of achieving drug-free low-cost and low technology technique to kill vegetative bacteria surpassing the drawbacks of using DC and high AC electric field for *in vivo* applications.

MATERIALS AND METHODS

PREPARATION OF BACTERIA

E. coli isolate was obtained from microbiology department clinical laboratory samples, Medical Research Institute, Alexandria University, Egypt. Single colony was inoculated into Luria Bertani (LB) medium and incubated overnight at 37 °C

in a shaking water bath. The bacterial culture was diluted with sterile saline to achieve a turbidity equivalent to 0.5 McFarland standard (approximately 1.5×10^8 *CFU*/mL). Several serial 10 fold dilutions in sterile saline were performed to prepare reaction tubes containing 5 mL working bacterial suspension with approximate bacterial count of 1.5×10^3 *CFU*/mL.

EXPERIMENT DESIGN

According to Hamilton and Sale [10], who demonstrated that to affect microorganisms and produce permeabilization of a cell membrane, electric pulses must induce a membrane potential of more than 1.0 V. For a cell of radius a in an electric field, the transmembrane potential between the extracellular and the intracellular surfaces of the cell membrane, in the absence of pores, is given by equation (1) [11]:

$$\Delta V_{\text{memb}} = F \cdot a \cdot E \cdot \cos a \tag{1}$$

where F is a form factor determined by the shape of the cells; $F = 1.5$ for spherical cells; E is the electric field strength, and a is the orientation of the electric field at the poles of the cells which can be either 0° or 180°, so that $\cos a = \pm 1$, and only the sign of the transmembrane potential ΔV_{memb} is affected. For a rod shape cell, with three semiprincipal axes, and an axial ratio 1:1:3 (assimilated to *E. coli*), F is smaller (1.12) than for spherical cells if the long axes of the cell and field are parallel, and higher (1.8) if they are perpendicular [5]. In this paper we considered the average F of both orientations (1.46).

So to kill *E. coli* which have a diameter of about 1 μm, high AC pulses of 6849 V/cm that causes $\Delta V_{\text{memb}} = 1$ V/cm should be used. It was previously determined that the total exposure time required to cause 50% cell death of *E. coli* is 300 μs when high AC field of about 7 kV was used [11]. So we adopted electric field of 6849 V/cm for time exposure of 300 μs as the parameters required to induce *E. coli* irreversible cell membrane permeablization due to high AC field.

In this study we examined the using of train of low AC field pulses of 6.849 V/cm that causes only $\Delta V_{\text{memb}} = 0.001$ V/cm, but we make the final electric energy produced by the low field as same as generated by high electric field. We compensate the decrease in electric energy due to decrease in pulse voltage by increasing the exposure time to be 300 s to make equivalent energy. In order to be sure of the idea, we tried second protocol with the same equivalent energy by applying electric field of 10.274 V/cm ($\Delta V_{\text{memb}} = 0.0015$ V/cm) for exposure time of 133.3 s. In both protocols, frequencies of $10–10^6$ Hz were tested.

ELECTRIC FIELD EXPOSURE SYSTEM

The exposure of electric pulses was performed in a glass tube at 0 ºC (to counteract any heat that may be evolved) by placing 5 mL bacterial suspension between two stainless steel needle electrodes (width = 2 mm, thickness = 2 mm and length of 50 mm). Bipolar square pulses were applied to the cells through digital function generator (CALTEK, CA1640P-02 function generator/counter, serial number: 06mg0676, made in USA). The distance between the electrodes was kept at 1 cm.

BACTERIAL GROUPS

Groups 1–6 are exposed to electric field of 6.849 V/cm for 300 s for frequencies 10^1, 10^2, 10^3, 10^4, 10^5 and 10^6 Hz respectively. Groups 7–12 are exposed to electric field of 10.274 V/cm for 133.3 s for frequencies 10^1, 10^2, 10^3, 10^4, 10^5 and 10^6 Hz, respectively. Each group was tested in triplicate.

DETERMINATION OF PERCENTAGE OF VIABLE CELLS

Before and after exposure to electric field, 0.1 mL aliquot of each reaction tube was platted on LB agar plates. The viable count was determined by counting the grown colonies per plate after incubation at 37 °C over night. The lethal effect of each experimental condition was calculated using equation 2 as:

$$\textit{Percent of bacterial death} = [(CFU1 - CFU2)/CFU1] \times 100 \qquad (2)$$

$CFU1$ = viable bacterial count before exposure to electric field; $CFU2$ = viable bacterial count after exposure to electric field.

STATISTICAL ANALYSIS

The data analysis was performed using the SPSS-10 package (release 3, SPSS Inc., Chicago III) running on MCROVAX 3500. Paired t-test was used to compare between the means of the viable bacterial count of the samples of the same group before and after exposure to electric field. ANOVA test was used to compare between the different groups. A difference was considered significant at probability ($p < 0.05$).

RESULTS

Bacterial death percent of groups 1–6 after exposure to electric field is shown in Fig.1. Extremely high significant decrease of viable bacterial count in group 1 and 2 after the exposure to electric field of 6.849 V/cm for 300 s for

frequencies 10^1 and 10^2 Hz, respectively, compared to before exposure with bacterial death percent of 42.41 ± 4.28% and 30.76 ± 1.95% respectively. Exposure to higher frequencies in groups 3–6 showed insignificant lethal effect on bacteria. No significant difference could be observed between group 2 and group 1.

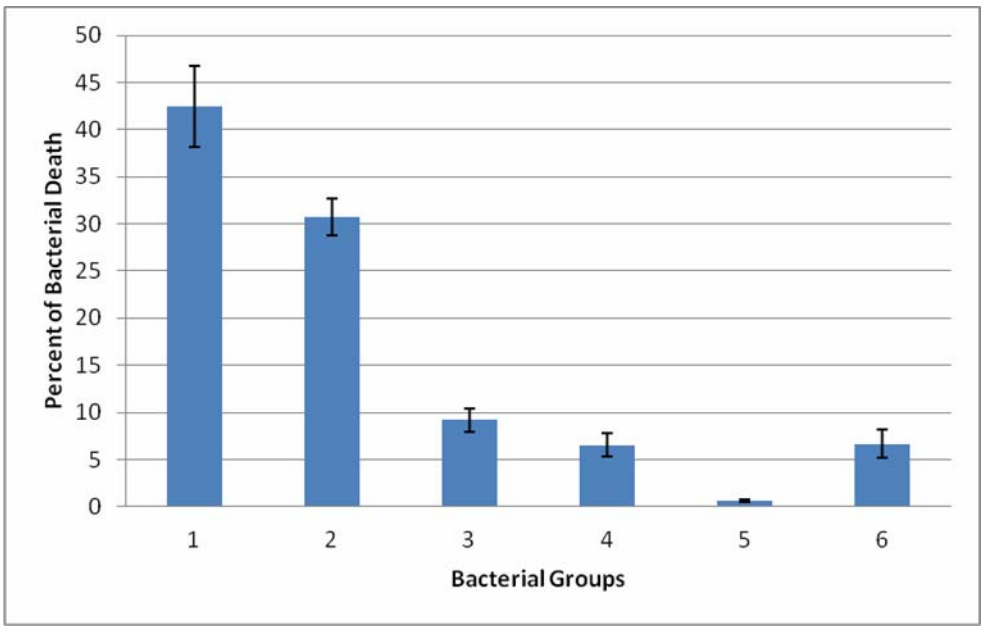

Fig. 1. Mean and standard deviation of the percent of bacterial death of groups 1–6 exposed to electric field of 6.849 V/cm for 300 s for frequencies 10^1, 10^2, 10^3, 10^4, 10^5 and 10^6 Hz respectively.

Figure 2 shows the bacterial death percent of groups 7–12 after the exposure to electric field. Extremely high significant decrease of viable bacterial count in group 7 and 8 after the exposure to electric field of 10.274 V/cm for 133.3 s for frequencies 10^1 and 10^2 Hz respectively compared to before exposure with bacterial death percent of 41.56 ± 3.59% and 30.42 ± 2.2% respectively. Exposure to higher frequencies in groups 9–12 showed insignificant lethal effect on bacteria. No significant difference could be observed between group 8 and group 7.

Comparing the results obtained from groups 7 and 8 to groups 1 and 2 respectively showed no significant difference indicating that both protocols are equally effective.

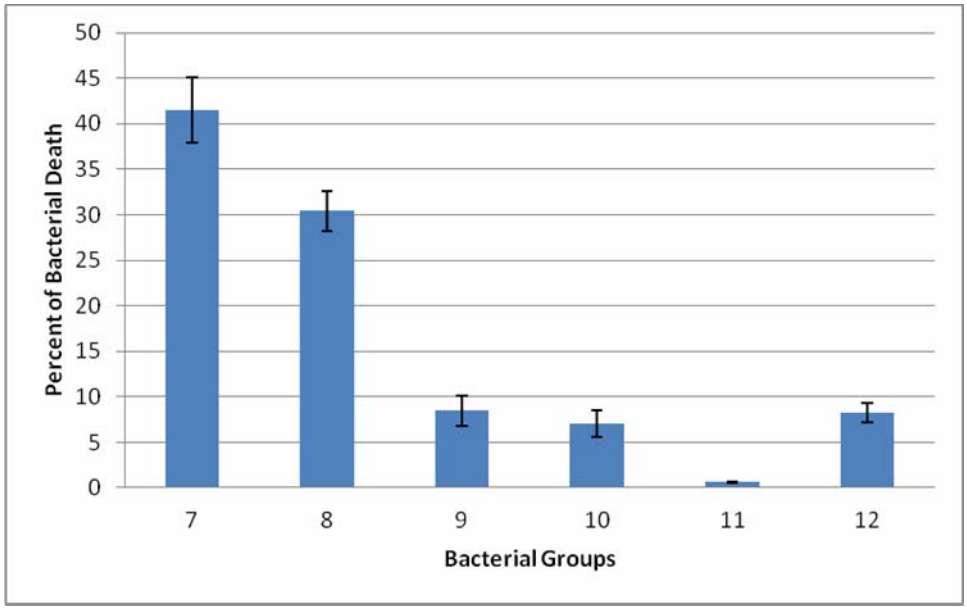

Fig. 2. Mean and standard deviation of the percent of bacterial death of groups 7–12 exposed to electric field of 10.274 V/cm for 133.3 s for frequencies 10^1, 10^2, 10^3, 10^4, 10^5 and 10^6 Hz respectively.

DISCUSSION

Problems of concern are the increased resistance of pathogens toward the host immune defense and antibiotic activity. Although the manner of acquisition of resistance may vary among bacterial species, resistance is created by only few mechanisms: (i) antibiotic inactivation; direct inactivation of the active antibiotic molecule, (ii) target modification; alteration of the sensitivity to the antibiotic by modification of the target, (iii) efflux pumps and outer membrane permeability changes; reduction of the concentration of drug without modification of the compound itself, or (iv) target bypass; some bacteria become refractory to specific antibiotics by bypassing the inactivation of a given enzyme [4].

Killing bacteria using DC was used from decades. The mechanism of the antibacterial activity of DC has been suggested to result from toxic substances (*e.g.*, H_2O_2, oxidizing radicals, and chlorine molecules) produced as a result of electrolysis, the oxidation of enzymes and coenzymes, membrane damage leading to the leakage of essential cytoplasmic constituents, and/or a decreased bacterial respiratory rate [2].

There were some researches of using AC on bacteria to avoid drawbacks of using DC [2, 6, 24] but most of them, according to the authors knowledge, were by using high pulsed electric fields as in case of food preservation [13].

Gross *et al.* [8] used moderate electric field (470 V/cm) involving the percentage cell lysis in SWLA-2 murine hybridomas produced by AC electric field pulses of varying amplitudes and pulse widths that produce membrane voltage of 0.58 V which is below the minimum 1.0 V deduced by Hamilton and Sale to produce permeabilization of a cell membrane. They got reduction in cell viability by 10% at pulse width of 1000 ms and frequency of 10 kHz. They put a question that more extensively evaluation of the effects of length of pulse duration as well as frequency on cell mortality at lower electric fields should be done.

The lethal AC high electric field effect mentioned in many researches is due to two factors; (i) the direct energy effect of high voltage pulses on the cell membranes causing electroporation and (ii) the release of toxic ions from the used electrodes due to the oxidation of the metal ions of the anode resulting in the dissolution of the anode [6]. In the case of the anode made from stainless steel, which is one of the most popular electrode materials, iron ions (Fe^{2+} and Fe^{3+}) are released from the anode which has toxic effect [19]. We achieved these two factors in this paper by using low AC electric field. As the direct energy effect of AC electric field on bacteria depends on the total electrical energy rather than the field strength, the difference in the field strength between high and low voltage electrical field was compensated by increasing the time of exposure resulting in equivalent total energy.

There are several works in the literature that present interesting results with the use of medium electric field. AC fields of weak intensity (e.g. 20–160 V/cm) have been shown to change membrane conductance and successfully achieve the transfection of *E. coli* by plasmid DNA [25]. Through the application of low voltage long duration pulses (100 ms pulses of 75 V/cm) with an interval of 100 ms to human cells, it was possible to achieve electroporation using electric fields as low as 75 V/cm [15], it was proposed that consecutive pulses of a voltage lower than the membrane breakdown threshold can accumulate the membrane potential, eventually until it reaches the threshold required to induce electroporation, disrupt the membrane integrity and resulting in the cell death.

The second factor can be explained as in case of low voltages, when voltage is applied to a pair of electrodes, the electrons in the electrolyte are transferred to the electrode surface, resulting in an increase in the thickness of ions, this layer is often called the electrical double layer capacitor since it behaves as a capacitor [20]. The current is utilized to charge the double layer until the threshold voltage equals that of the charging current, which does not produce any chemical reactions or charge transfer at this stage, once the capacitor is fully charged above the threshold voltage, faradaic current flows, and electrochemical reactions occur at the

electrode surface [7]. Under conditions such as low AC frequencies, a chain of chemical reactions involving mass transport of electroactive species to the electrode occur and accelerate electrode corrosion and release of toxic ions from the electrodes and hence the second factor is achieved as we found the significant lethal effect in groups 1, 2, 7 and 8.

On the other hand, with high alternating current frequencies (≥ 1 kHz) the rapid movement of electric charge periodically reverses direction supplied to the electrode. Consequently, the capacitor can no longer attain the threshold voltage because there was insufficient time to fully charge the double layer capacitor, only the charging current will flow at the electrode surface and electrochemical faradaic reactions will not start, based on this theory it has been shown that electrode corrosion can be limited by applying high frequencies [7] and due to the absence of the second factor, we could not achieve significant reduction in viable *CFU* in groups 3–6 and 9–12. Also we did not find significant differences between protocol 1 (groups 1–6) and protocol 2 (groups 7–12) because both have the same total electric energy and the same electrode type.

We think that in case of low electric field, both electric energy and release of toxic ions from the electrodes work synergistically. At the time that the low amplitude AC pulses accumulate the membrane potential producing cell pores, the toxic ions enter the cells causing cell death. The results of Xie and Tsong [25] indicated the production of reversible *E. coli* cell permeabilization due to low electric field AC without any lethal effect, according to our study these sub-lethal effects may be due to the absence of sufficient suitable toxic ions.

CONCLUSION

Low AC electric field can be used as a physical method to kill *E. coli*. There are two conditions to use low AC electric field as a lethal method. Firstly, the used field should have the same equivalent energy of the standard high AC electric field by increasing the time of exposure and the second condition is the use of low frequency from 10–100 Hz only and avoids using high frequency as 1000 Hz or above. In our bacterial groups, we achieved significant reduction in *E. coli* – viable *CFU* with the groups follow both conditions while a significant response was not achieved by using 1000 Hz or higher frequencies due to incomplete electrochemical faradaic reactions. We will try in the future work to optimize the method to reach higher death rates.

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