POTENTIATION OF PHOTODYNAMIC INACTIVATION OF STAPHYLOCOCCUS AUREUS USING INORGANIC SALTS

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ACADEMIC ETHICS AND INTEGRITY STATEMENT

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ABSTRACT

POTENTIATION OF PHOTODYNAMIC INACTIVATION OF STAPHYLOCOCCUS AUREUS USING INORGANIC SALTS

Antibiotics are used in the prevention and treatment of bacterial infections. However, unnecessary, misuse or overuse of antibiotics may cause drug-resistant bacteria, which pose a significant threat to human health. Photodynamic therapy (PDT) is researched as a bacterial infection treatment modality. In PDT, photosensitive molecules (photosensitizer, PS) are excited by light to create reactive oxygen species. PDT has advantages over antibiotics, including selectivity and low risk of resistance. PDT can also treat infections, including localized bacterial infections and viral infections. Besides the advantages, some situations limit the effectiveness of PDT. For example, the wavelength of the applied light that is used to excite the PS molecules may not penetrate deep tissues effectively, which makes it hard to treat deeper or internal infections. Another limitation of aPDT is that the effectiveness of aPDT may vary depending on the specific bacterium and the photosensitizing agent used. Therefore, strategies to increase the efficacy of antimicrobial photodynamic inactivation protocols are researched for the increased application of this method in clinics. A widely researched strategy for improving PDT efficacy is using drug delivery systems to increase the solubility and accumulation of PSs in the tumor tissue. Active targeting strategies using monoclonal antibodies are also researched to increase the selectivity of the treatment method. Another strategy shown to increase the aPDT efficacy is to use inorganic salts as potentiators. Building on the literature, this study aimed to increase the efficacy of aminolevulinic acid (ALA)- and chlorin e6 (Ce6)-mediated aPDT using potassium iodide (KI) and potassium chloride (KCl). Although the efficacy of ALA-mediated PDT did not increase with the studied concentrations of the inorganic salts, this study showed for the first time that Ce6-mediated PDT can be potentiated using KCl.

Keywords: Photodynamic Therapy, Antibiotic-Resistance, Potassium Iodide, Potassium Chloride.

ÖZET

İNORGANİK TUZLAR KULLANILARAK STAPHYLOCOCCUS AUREUS'UN FOTODİNAMİK İNAKTİVASYONUNUN GÜÇLENDİRİLMESİ

Antibiyotikler bakteriyel enfeksiyonların önlenmesinde ve tedavisinde kullanılmaktadır ancak antibiyotiklerin yanlış veya aşırı kullanımı ilaca dirençli bakterilerin oluşmasına neden olabilir ve bu da insan sağlığını tehdit eder. Fotodinamik terapi (FDT), alternatif bir bakteriyel enfeksiyon tedavi yöntemi olarak araştırılmaktadır. FDT'de ışığa duyarlı moleküller (ışığa duyarlılaştırıcı, FS) bakteriler için toksik olan, reaktif oksijen türlerini oluşturmak için uyarılır. FDT'nin antibiyotiklere göre seçicilik ve düşük direnç riski gibi avantajları vardır. FDT ayrıca lokalize bakteriyel enfeksiyonlar ve viral enfeksiyonlar dahil olmak üzere çok çeşitli enfeksiyonları tedavi edebilir. Avantajlarının yanı sıra bazı durumlar FDT'nin etkinliğini sınırlamaktadır. Örneğin, FS moleküllerini uyarmak için uygulanan ışığın dalga boyu, derin dokulara etkili bir şekilde nüfuz edemeyebilir, bu da daha derindeki enfeksiyonların tedavisini zorlaştırır. aFDT'nin diğer bir dezavantajı, aFDT'nin etkinliğinin, kullanılan spesifik bakteriye ve fotosensitizana bağlı olarak değişebilmesidir. Bu nedenle, antimikrobiyal fotodinamik inaktivasyon protokollerinin etkinliğini artırmaya yönelik stratejiler araştırılmaktadır. FDT etkinliğini arttırmaya yönelik yaygın olarak araştırılan bir strateji, tümör dokusunda FS'lerin çözünürlüğünü ve birikimini arttırmak için ilaç dağıtım sistemlerini kullanmaktır. Tedavi yönteminin seçiciliğini artırmak için monoklonal antikorların kullanıldığı aktif hedefleme stratejileri de araştırılmaktadır. aFDT etkinliğini arttırdığı gösterilen başka bir strateji, güçlendirici madde olarak inorganik tuzların kullanılmasıdır. Bu çalışma, potasyum iyodür (KI) ve potasyum klorür (KCl) kullanılarak aminolevulinik asit (ALA) ve klor e6 (Ce6) aracılı aFDT'nin etkinliğini arttırmayı amaçlamıştır. Her ne kadar ALA aracılı FDT'nin etkinliği, inorganik tuzların çalışılan konsantrasyonlarıyla artmasa da, bu çalışma ilk kez Ce6 aracılı FDT'nin KCl kullanılarak güçlendirilebileceğini gösterdi.

Anahtar Sözcükler: Fotodinamik Tedavi, Antibiyotik Direnci, Potasyum iyodür, Potasyum klorür

TABLE OF CONTENTS

ACKNOWLEDGMENTS . . . iii
ACADEMIC ETHICS AND INTEGRITY STATEMENT . . . iv
ABSTRACT . . . v
ÖZET . . . vi
LIST OF FIGURES . . . ix
LIST OF SYMBOLS . . . x
LIST OF ABBREVIATIONS . . . xi
1. INTRODUCTION . . . 1
1.1 Motivation . . . 1
1.2 Objectives . . . 2
1.3 Outline . . . 2
2. BACKGROUND . . . 3
2.1 Antibiotics Resistant Bacteria . . . 3
2.2 Light Tissue Interactions . . . 3
2.3 Photodynamic Therapy and Its Applications on Antibiotics-Resistant Bacteria . . . 5
2.4 Potentiation of Antimicrobial Photodynamic Therapy by Inorganic Salts 8
3. MATERIALS AND METHODS . . . 10
3.1 Determination of the Number of Live Bacteria as a Result of Treatment and Bacteria Culture . . . 10
3.2 Investigation of the Light-free Toxicity of the Chemicals Used on Fibroblast Cells . . . 11
3.3 Absorption Spectra of Ce6 and KCl . . . 12
3.4 aPDT Applications and Use of Inorganic Salt . . . 12
3.5 Statistical Analysis . . . 14
4. RESULTS . . . 15
4.1 Cytotoxicity of KI on Fibroblast Cells . . . 15
4.2 Effect of KI Addition on ALA-PDT Activity . . . 16
4.3 Cytotoxicity of KCl on Fibroblast Cells . . . 16

4.4 Effect of KCl Addition on ALA-PDT Activity 17
4.5 Ce6-mediated PDT Results and the Effect of KCl Addition on Ce6-Mediated PDT Activity 18
4.6 Absorption Spectra of Ce6-KCl 18
5. DISCUSSION 20
6. CONCLUSION 23
6.1 APPENDIX A. Publication Produced From the Thesis 24
REFERENCES 29

LIST OF FIGURES

Figure 2.1	Light-Tissue interactions.	4
Figure 2.2	PDT Mechanism.	4
Figure 2.3	Jablonski diagram [1].	5
Figure 2.4	Biosynthesis pathway [2–4].	8
Figure 3.1	Incubation of PDT bacteria with inorganic salts, after 24 hours.	10
Figure 3.2	After 24 hours incubation, counting of bacteria colonies.	11
Figure 3.3	Experimental process of light-free toxicity assays on L929 fibroblast cells.	12
Figure 3.4	PDT applications setup, 660 nm LED.	13
Figure 3.5	PDT experimental setup and experimental process.	13
Figure 4.1	KI toxicity on fibroblast cells (ns: not significant, *: $p<0.05$).	15
Figure 4.2	Effect of KI addition on ALA-PDT activity (ns:not significant, NT: NonTreated group).	16
Figure 4.3	KCl toxicity on fibroblast cells (ns: Not Significant, *: $p<0.05$).	17
Figure 4.4	Effect of KCl addition on ALA-PDT activity (NT: NonTreated, *: $p<0.05$).	18
Figure 4.5	Efficacy of Ce6-mediated PDT (25 μM Ce6 and 30 J/cm^2 energy density) on *S. aureus* and the effect of KCl addition on Ce6-mediated PDT activity (NT: NonTreated group, *: $p<0.05$).	19
Figure 4.6	Absorption spectra of KCl-Ce6.	19

LIST OF SYMBOLS

H_2O_2	Hydrogen peroxide
$OH^{\cdot}$	Hydroxyl radical
$^1O_2$	Singlet oxygen

LIST OF ABBREVIATIONS

PDT	Photodynamic Therapy
aPDT	Antimicrobial Photodynamic Therapy
PS	Photosensitizer
S. aureus	Staphylococcus aureus
ROS	Reactive oxygen species
ALA	Aminolevulinic Acid
MB	Methylene Blue
PBS	Phosphate Buffered Saline
Ce6	Chlorin e6
CFU	Colony Forming Unit
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
RB	Rose Bengal
KCl	Potassium Chloride
KI	Potassium Iodide
JPIAMR	The Joint Programming Initiative on Antimicrobial Resistance
ANOVA	Analysis of Variance
S_0	Singlet ground energy level
S_1	Excited singlet state
T_1	Excited triplet state
min	minute
nm	nanometer
μM	micromolar
mM	millimolar
rpm	revolutions per minute
J	Joule
W	Watt
mW	milliwatt

1. INTRODUCTION

1.1 Motivation

Antibiotic-resistant bacteria, like Methicillin-resistant *Staphylococcus aureus* (MRSA), is becoming a growing threat to human health. Overuse and misuse of antibiotics, along with their application in the food industry, have contributed to the rise of these resistant strains [5], [6]. Antibiotic-resistant bacteria are responsible for approximately 700,000 deaths globally each year, with that number projected to reach 10 million by 2050 [5]. Despite efforts by researchers to develop new drugs, bacteria continue to acquire resistance, underscoring the urgent need for alternative treatment methods, especially for conditions like diabetic foot, where antibiotics may fail.

Photodynamic therapy (PDT) is a treatment method that induces cytotoxicity using light-activated photosensitizers (PSs), which are molecules that become excited when exposed to light [7]. Upon excitation, these photosensitizers undergo electronic and vibrational transitions. During an electronic transition from the triplet excited state to the singlet ground state, these molecules may interact with other molecules in the environment to produce reactive species [8], [9]. PDT is an important alternative treatment method for antibiotic-resistant bacteria because reactive species produced by PDT are highly toxic to microorganisms such as gram-positive and gram-negative bacteria [10], [11]. In the literature, there are successful antimicrobial PDT (aPDT) studies that use exogenous PSs. However, some studies also reported low aPDT efficacy due to factors including the type of bacteria, the PS used or the depth of infection. For example, the studies that used the endogenous PS, aminolevulinic acid (ALA)-induced protoporphyrin IX (PpIX) reported contradictory results [12–16].

One way to increase the efficiency of aPDT is by using inorganic salts such as potassium iodide (KI) or sodium azide [17–20]. The motivation of this study was to show for the first time that the use of inorganic salts, specifically KI, can increase the efficacy of aminolevulinic acid (ALA)-mediated aPDT.

1.2 Objectives

1. To assess the non-toxic and toxic concentration of KI on mouse fibroblast cells.

2. To investigate the potentiation effect of the addition of two different concentrations of KI on ALA-PDT activity.

3. To assess the non-toxic and toxic concentration of potassium chloride (KCl) on mouse fibroblast cells.

4. To investigate the potentiation effect of the addition of KCl on ALA-PDT activity.

5. (Optional) To include other PSs, such as chlorin e6, into the study to broaden the study.

1.3 Outline

This thesis consists of six chapters, Introduction, Background, Materials and Methods, Results, Discussion, and Conclusion.

The motivation and objectives of the study were explained in the first chapter. The second chapter presents a literature review of antibiotic-resistant bacteria, bacterial infections, photodynamic therapy, and inorganic salts-mediated potentiation of photodynamic therapy.

In the third chapter, the materials used, and experimental approaches were explained. The fourth chapter shows the results obtained at the end of the experiments. The interpretation of the results and their comparison with previous work, interpreting takes place in the fifth section. Finally, a conclusion section summarizes the study and provides future perspectives.

2. BACKGROUND

2.1 Antibiotics Resistant Bacteria

The discovery of penicillin in 1928 was an important steps in the fight against bacterial infections [21]. Later, Staphylococcus aureus (*S. aureus*) became resistant to penicillin and Methicillin was introduced to the market in 1960. Immediately afterward, a methicillin-resistant *S. aureus* variant emerged [22]. *S. aureus* is a type of bacteria that can cause skin and wound infections, which is one of the main causes of infections known today as "hospital infections" [23]. *S. aureus* is a methicillin-resistant gram positive bacterium [24]. As in the case of *S. aureus*, due to the overuse/misuse of antibiotics, as well as the use of antibiotics in the food industry, antibiotic-resistant bacterial variants may emerge, and these variants pose a threat to human health. According to the World Health Organization, diseases caused by antibiotic-resistant bacteria cause around 700,000 deaths per year in the world, and this number is expected to reach 10 million in 2050. In Europe, around 25,000 patients die annually due to antibiotic resistance. Considering the healthcare costs and production loss, the total cost of these diseases in Europe exceeds 1.5 billion Euros per year [5]. In addition to the European Union, organizations including the U.S. Centers for Disease Control and Prevention (CDC), and The Joint Programming Initiative on Antimicrobial Resistance (JPIAMR), provide large amounts of financial support for developing new drugs to fight antibiotic-resistant bacteria every year. However, as a result of the mutations they have undergone, bacteria can also become resistant to new antibiotics, so new treatment approaches are urgently needed [6]. For example, in the treatment of infections known as "diabetic foot", where antibiotics may be ineffective, developing alternative methods to antibiotics can prevent limb loss and even death [25].

2.2 Light Tissue Interactions

The interaction of light with a substance lead to four primary phenomena: reflection, refraction, absorption, and scattering (Figure 2.1). Absorption is crucial to achieve an effect on tissue and the extent of absorption varies depending on the properties of

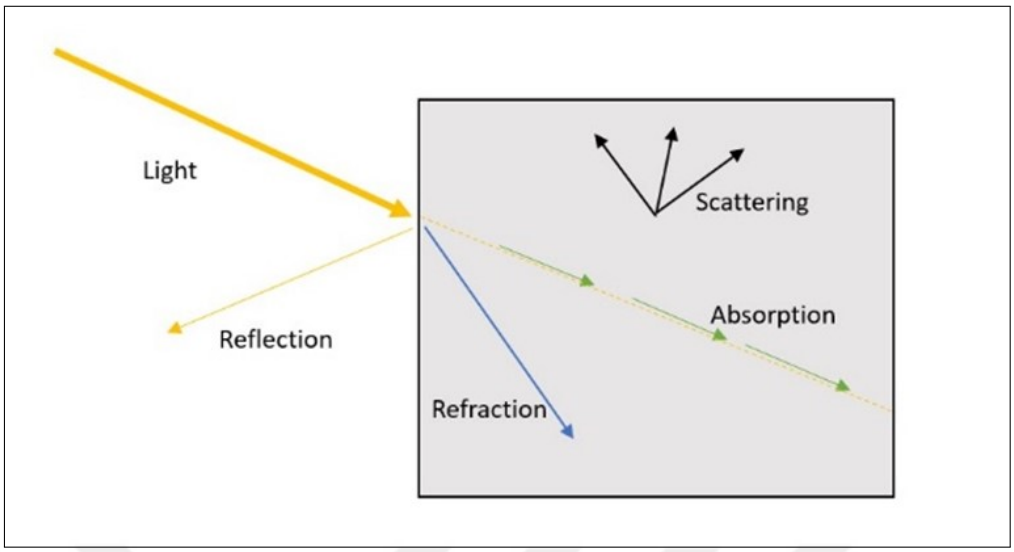


Figure 2.1 Light-Tissue interactions.

the the light and tissue. Upon absorption, the light can induce various interactions within the tissue, including photochemical interactions, photothermal interactions and photoablation. In this application, we aimed to induce a photochemical effect, which involves the interaction of light with tissue, leading to chemical changes. One of the most common methods for generating a photochemical effect in tissue is photodynamic therapy (PDT), which is a method that creates cytotoxicity using photosensitive molecules, photosensitizers, that are excited by light (Figure 2.2).

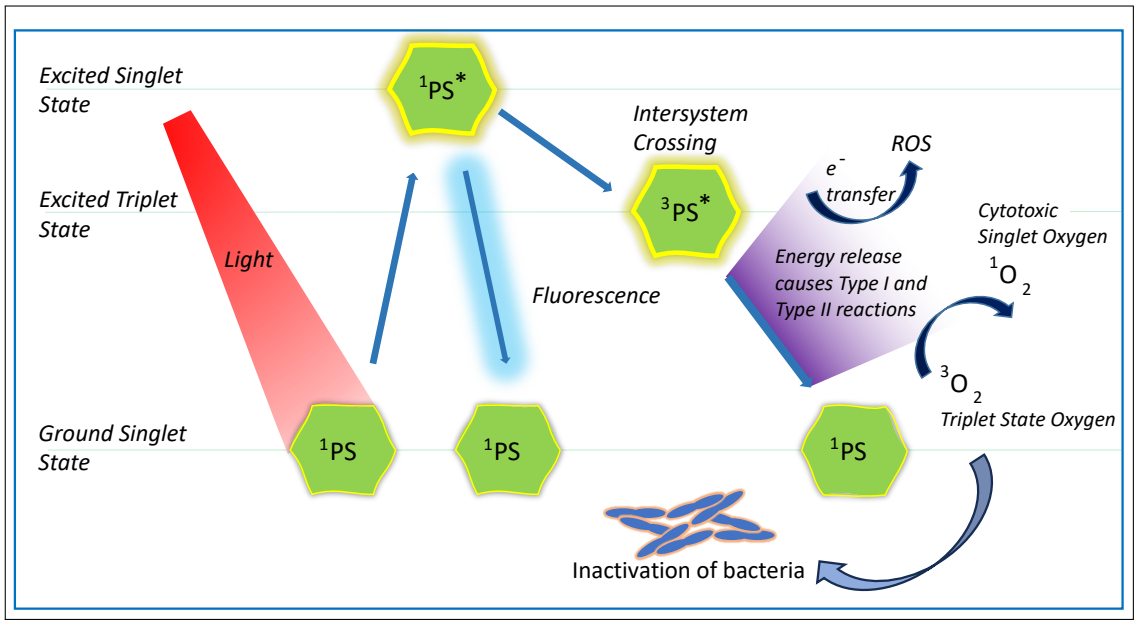


Figure 2.2 PDT Mechanism.

Upon excitation, photosensitizer electronic and vibrational transitions occur as shown in Jablonski Diagram (Figure 2.3). The terms S_0, $S1$ and $T1$ represent the multiplicity of the energy states and multiplicity can be calculated from the formula M=2S+1, where S is the magnetic quantum number. In the Jablonski diagram, it is shown that

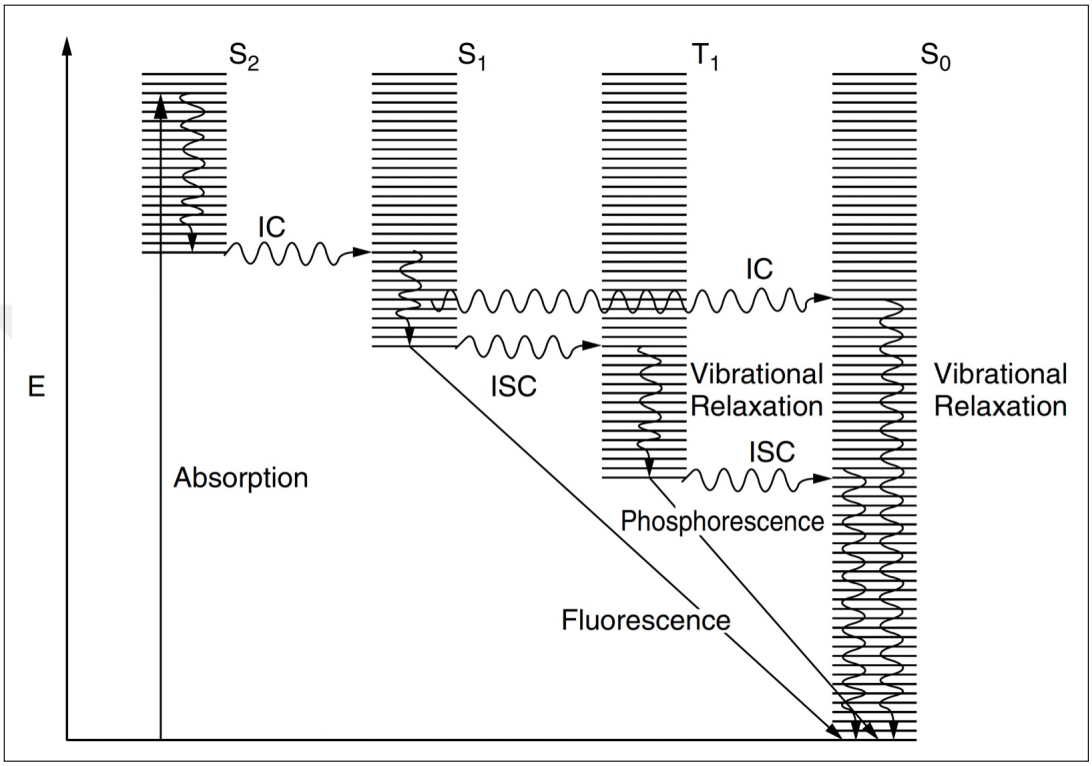


Figure 2.3 Jablonski diagram [1].

the absorbance of a photon excites an electron to a vibrational level of S_1. From that energy level, the electron loses energy by vibrational relaxation. Then, the electron can either return to the singlet ground energy level (S_0) by emitting a photon, called fluorescence, or it can make an intersystem crossing to the triplet excited (T_1) level. The relaxation of the photosensitizer molecule from T_1 to the ground energy level may lead to energy transfer to the molecular oxygen or other substrates to form radicals, which induces formation of reactive oxygen species that are toxic for cells [1], [26].

2.3 Photodynamic Therapy and Its Applications on Antibiotics-Resistant Bacteria

PDT causes cancer cell death or bacterial inactivation by the combination of three components: Photosensitizer, light, and molecular oxygen [7]. None of these compo-

nents are individually toxic but the excitation of the photosensitizer by an appropriate light starts the photochemical reaction that causes the ground state molecular oxygen to be excited [10], [27], [28]. Photosensitizer is a crucial parameter in PDT applications. The ideal photosensitizer should have high singlet oxygen quantum yield, low dark toxicity, rapid clearnce from normal tissues. Also, the molecules should have high absorption peak at wavelengths between 600-800 nm to be able to be excited by light at this range of wavelength [7], [29]. The excited photosensitizer returns to its ground state either by emitting fluorescent light and/or generating heat. The photosensitizer molecule that is electronically excited by light transitions back to its original state through two possible pathways: emitting fluorescent light (radiative relaxation) or producing heat (non-radiative relaxation). Alternatively, the molecule can undergo intersystem crossing to reach the triplet energy state. When the molecules return to the ground state, the molecule may interact with a substrate through electron transfer, leading to the formation of radicals. Reactive oxygen species (ROS) are generated if these radicals interact with molecular oxygen, constituting a Type I reaction. On the other hand, a Type II reaction occurs when there's a direct energy transfer between the photosensitizer in its triplet state and oxygen molecules. In this case, cytotoxic singlet oxygens are formed and damage cancer cells or bacteria [7], [8], [28], [30], [31].

One of the important advantages of PDT over conventional cancer treatment methods is that clinically approved photosensitizers have very low side effects. Furthermore, because this treatment method does not induce any resistance mechanism, multiple applications are possible [30]. PDT is clinically approved in many countries including the United States for the treatment of Actinic Keratosis, Lung and esophagus cancer. There are also numerous ongoing studies and clinical trials on other cancer types [29], [32–34].

PDT is an important alternative treatment method for antibiotic-resistant bacteria because ROS produced by PDT is also highly toxic to microorganisms such as gram-positive and gram-negative bacteria [10], [11], [35], [36]. As an example, osteomyelitis, a bone infection, can arise from local injury or conditions such as diabetes. The traditional approach to treating osteomyelitis involves surgically removing the infected tissue and administering potent antibiotics [37], [38]. Photo inactivation of various

Gram-positive bacteria has been demonstrated by several studies using PDT with exogenous sensitizers. A photosensitizer that shows promise in antibacterial photodynamic therapy, is chlorin e6. Chlorin e6 has been extensively investigated in cancer research because of its advantageous characteristics, such as strong absorption in the near-infrared range of the electromagnetic spectrum and specific accumulation in tumor tissues. Moreover, chlorin e6 is recognized for its ability to generate reactive oxygen species when activated by near-infrared light [39], [40]. In a comparative study examining the effectiveness of methylene blue and chlorin e6 in treating streptococcus mutans biofilms through photodynamic therapy, Nie *et al.* found that both chlorin e6 and methylene blue were effective in biofilm removal [41]. However, chlorin e6-based photodynamic therapy showed greater efficacy than methylene blue, particularly at higher photosensitizer concentrations.

Studies also showed that both gram-positive and gram-negative bacteria produce their porphyrins when incubated with ALA which is not a photosensitizer itself but is a biochemical precursor of natural photosensitizer protoporphyrin in the biosynthesis pathway (Figure 2.4) [42–44]. Although there are reports that reveal the potential of ALA-mediated aPDT (ALA-aPDT) on the inactivation of bacteria, these studies generally used relatively long irradiation times and a wide range of (0.5-40 mM) ALA concentrations, which can explain the reason why this method could not be translated into clinics. For example, Wardlaw *et al.* study aimed to inactivate four different bacterial species with ALA-aPDT [13]. Among these bacteria, *E.coli* did not respond to the treatment, while other bacteria responded to the treatment only after 2-3 hours of irradiation. In the *in vitro* study of Hsieh *et al.*, *S. aureus* and *P. aeruginosa* bacteria were aimed to be inactivated using ALA-aPDT. Complete bacterial inactivation was possible after at least one hour of laser irradiation (162 J/cm^2 energy density) [12]. In a similar *in vitro* study from Huang *et al.*, the irradiation time to inactivate bacteria was even higher with higher laser power density (0.4 W/cm^2) compared to other studies [14]. These reports show that potentiation by inorganic salts can be considered to achieve an efficient antimicrobial ALA-aPDT at shorter irradiation times.

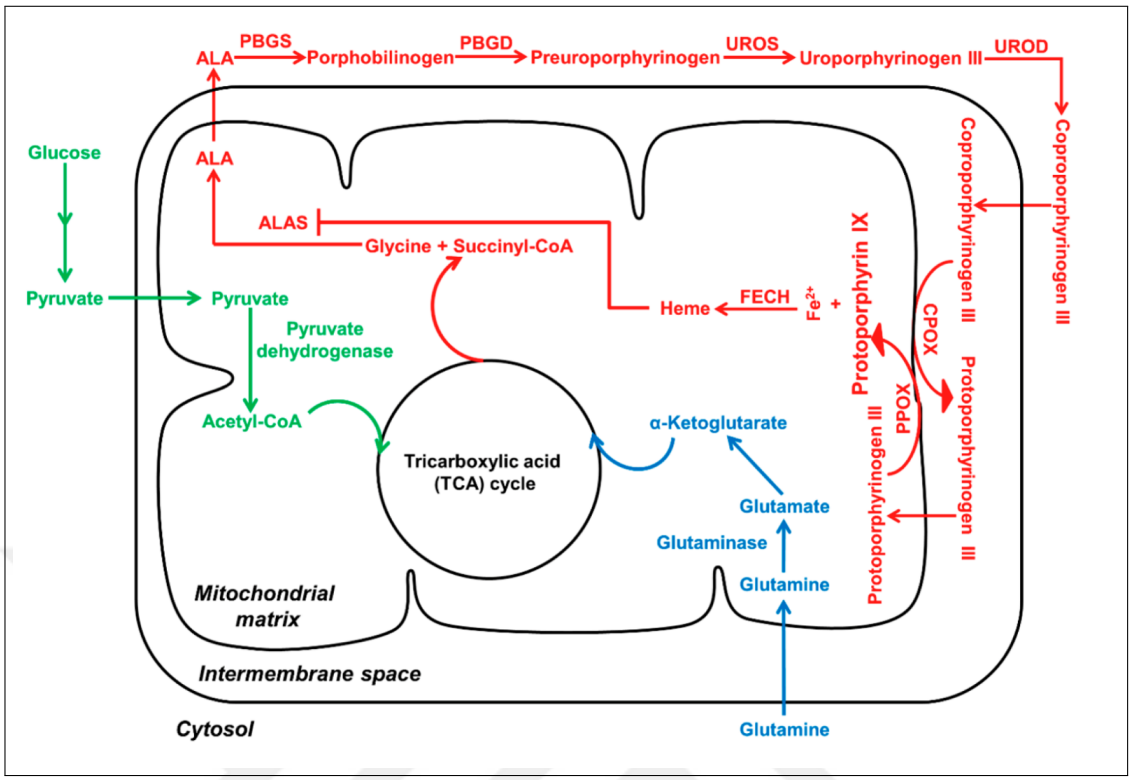


Figure 2.4 Biosynthesis pathway [2–4].

2.4 Potentiation of Antimicrobial Photodynamic Therapy by Inorganic Salts

The potentiation of antimicrobial photodynamic inactivation by inorganic salts has been researched by multiple studies. For example, Wen *et al.* showed that the addition of 100 mM potassium iodide potentiates rose bengal-mediated PDT on Gram-negative and Gram-positive bacteria by initiating iodide-mediated chemical reactions that lead to the formation of ROS [20]. In another aPDT study on Gram-positive and Gram-negative bacteria, Kasimova *et al.* determined that the efficacy of methylene blue (MB)- mediated aPDT can be increased by the addition of sodium azide due to electron transfer to the MB triplet state from azide anion, which leads to the production of azidyl radical [45]. One of the studies from our group on *E. faecalis* suspensions and biofilms also showed that toluidine blue-mediated aPDT can be potentiated using potassium iodide [46]. Another recent study by Topaloğlu *et al.* showed that photoinactivation using Ce6, which was also preferred in this current study, did not provide a high mortality rate on *P.aeruginosa*, yet, combining this protocol with potassium iodide could significantly increase its efficacy [47]. Based on the literature reviewed above, in

thi study, we hypothesized that the use of inorganic salts, specifically KI, can increase the efficacy of aminolevulinic acid (ALA)-mediated aPDT.

3. MATERIALS AND METHODS

This study was conducted under *in vitro* conditions by applying PDT on gram (+) bacteria *S. aureus* (ATCC 43300). Light sources used were 633 nm diode laser, 660 nm LED (M660L4-C1); photosensitizers used were ALA and Ce6; inorganic substances used were KI and KCl.

3.1 Determination of the Number of Live Bacteria as a Result of Treatment and Bacteria Culture

A colony was picked from the bacterial stock using a loop and incubated in tryptic soy medium overnight in a shaking incubator (rpm=225) at $37°C$. The bacterial suspension was then centrifuged (for 10 minutes at 3000rpm) before resuspending the pallets in phosphate. Finally, dilution was made using PBS so that the final bacterial concentration was $10^7 - 10^8$ CFU (colony forming unit)/ml. The number of bacteria was confirmed by measuring optical density on a UV-VIS spectrometer. After the

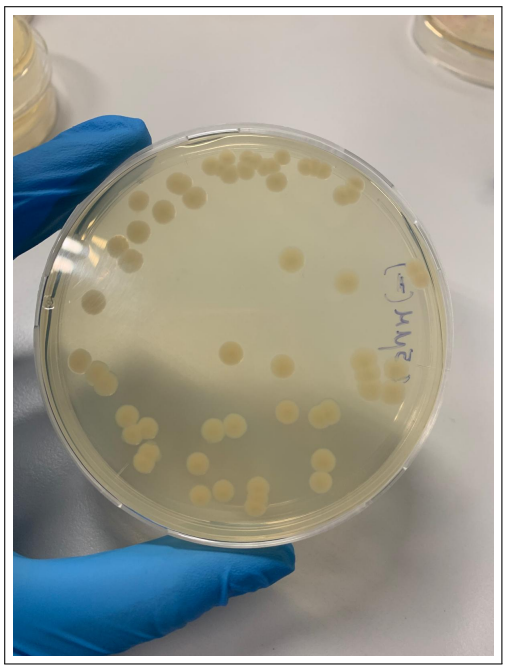


Figure 3.1 Incubation of PDT bacteria with inorganic salts, after 24 hours.

experiments, bacterial suspensions were serially diluted to determine the proportion of live bacteria. 30 μL of the diluted samples were taken and planted on tryptic soy

agar plates and incubated at $37°C$ for 24 hours (Figure 3.1). The number of CFU was counted and viable bacteria was calculated by multipluying CFU with the dilution factor.(Figure 3.2).

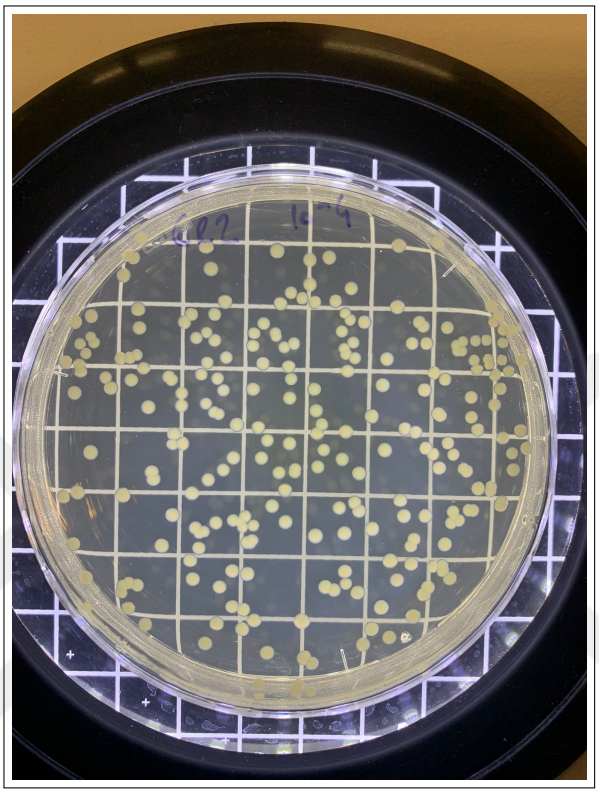


Figure 3.2 After 24 hours incubation, counting of bacteria colonies.

3.2 Investigation of the Light-free Toxicity of the Chemicals Used on Fibroblast Cells

It is a frequently applied method to analyze the toxicity of chemicals to be used on mouse fibroblast cells before planned experiments on bacterial killing. Similarly, the toxicity of all chemicals used in this project was analyzed on L929 mouse fibroblast cells. However, since ALA has a widely accepted optimum concentration in the literature (1 mM), this chemical was not tested on mouse fibroblast cells. In order to determine at what concentrations Ce6, MB, KI and KCl alone (without light application) cause toxicity, L929 cells were grown in RPMI-1640 medium (10% fetal bovine serum and 1% Penicillin-Streptomycin Solution were added). The flasks were kept in a cell culture incubator and the medium was refreshed twice a week. Before the experiments, L929

cells were seeded in a 96-well plate at a density of 10000 cells/well,and incubated in the incubator for 24 hours. At the end of this period, the culture medium of the cells was replaced with medium only (control group) or medium containing chemicals at different concentrations, and the plates were incubated for another 15-30 minutes. Incubation times of the chemicals were determined by taking into account the maximum incubation periods planned to be used in the following stages. At the end of the experiment, cell viability was determined by the MTT method, and cell viability in all groups was normalized compared to the control group of that experiment. The experimental process is shown in Figure 3.3.

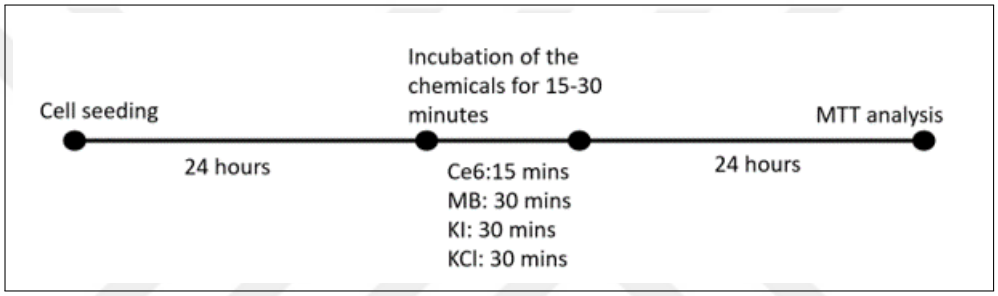


Figure 3.3 Experimental process of light-free toxicity assays on L929 fibroblast cells.

3.3 Absorption Spectra of Ce6 and KCl

The absorption spectra of Ce6 and KCl at concentrations 25 μM and 400 mM, respectively, were recorded between 300-800 nm using a Nanodrop 2000c UV-vis spectrophotometer.

3.4 aPDT Applications and Use of Inorganic Salt

In this experiment, three different photosensitizers, two different light sources and two different inorganic salts were used. Photosensitizers: ALA (precursor of PpIX, an endogenous photosensitizer), Chlorine e6 (Ce6) and Methylene blue (MB) Light sources: 633 nm diode laser and 660 nm LED light source. Inorganic salts: Potassium iodide (KI) and potassium chloride (KCl). In ALA-mediated PDT experiments, bacteria were incubated with 1 mM ALA for 4 hours and then irradiated at 200 J/cm^2 laser energy density (633 nm, 100 mW/cm^2, 33.3 mins) (Figure 3.4).

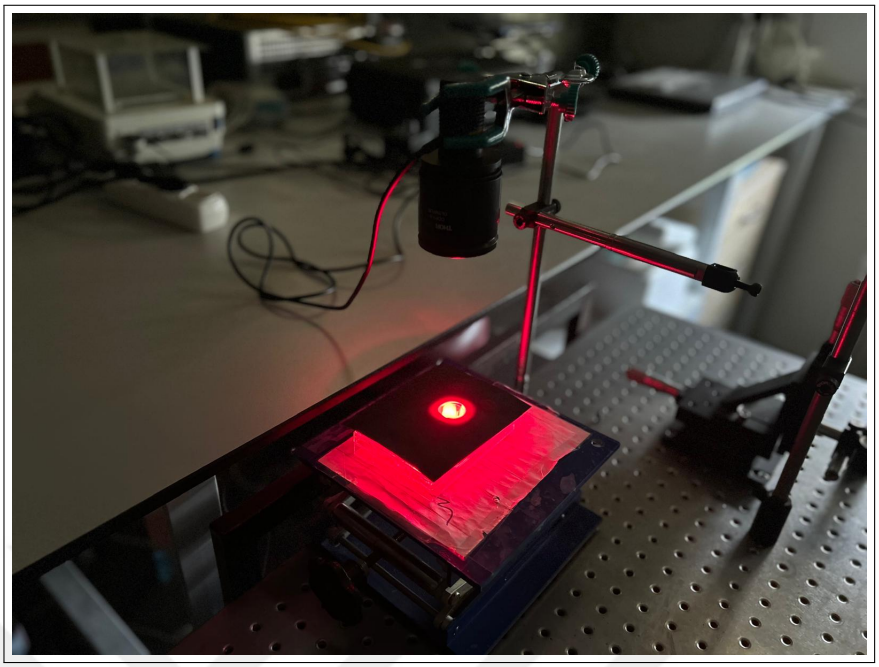


Figure 3.4 PDT applications setup, 660 nm LED.

These parameters were determined based on studies in the literature. In experiments using KI, KI was also incubated with ALA for 4 hours. In PDT applications, irradiation after chemical incubation was performed using the mechanism shown below. Before each application, the power of the light source was measured using a power meter and fixed to the desired power density. The experimental process and setup are shown in Figure 3.5.

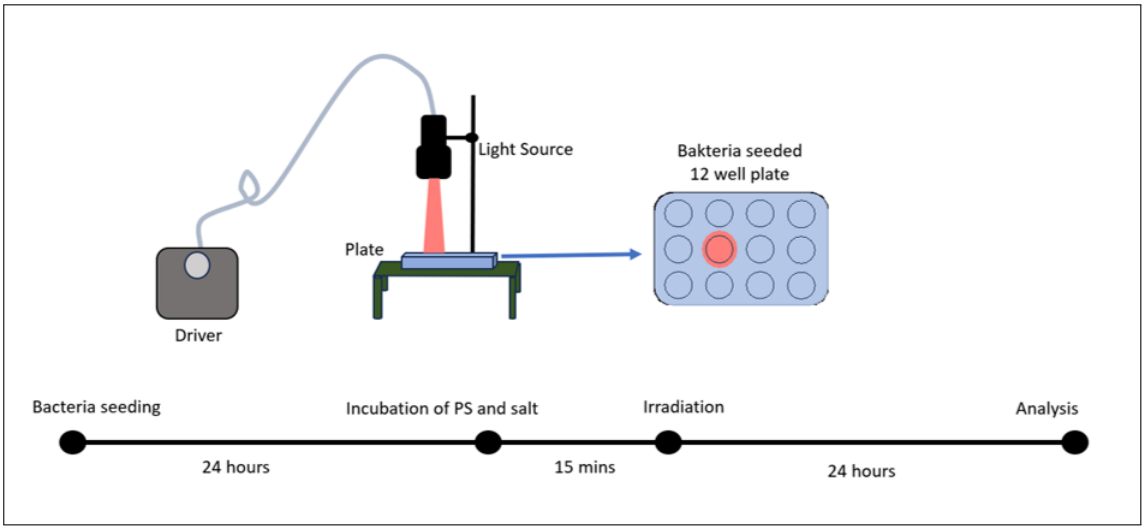


Figure 3.5 PDT experimental setup and experimental process.

3.5 Statistical Analysis

All experiments were repeated at least twice (two biological replicates). During each repetition, more than one technical repetition was taken, and when making statistical calculations, the average of the technical repetitions was taken and included in the statistical calculation. While making group comparisons, if more than two groups were compared, Dunnett's test was applied as multiple comparison after the One-way ANOVA method. In cases where two groups were compared, t-test was used. Statistically significant differences ($p<0.05$) are indicated with a (*) sign in the graphs.

4. RESULTS

S. aureus bacteria and different concentrations of KI and KCl inorganic substances were incubated with ALA and Ce6 photosensitizers and irradiated with 633 nm diode laser and 660 nm LED light sources and the results were evaluated.

4.1 Cytotoxicity of KI on Fibroblast Cells

In the literature, KI concentrations of 200-400 mM have been used for similar purposes, but no study has been found in the literature examining the toxicity of these concentrations on fibroblast cells. Therefore, first, the toxicity of KI concentrations between 200-800 mM on L929 cells was investigated on previous search. As shown in Figure 4.1,

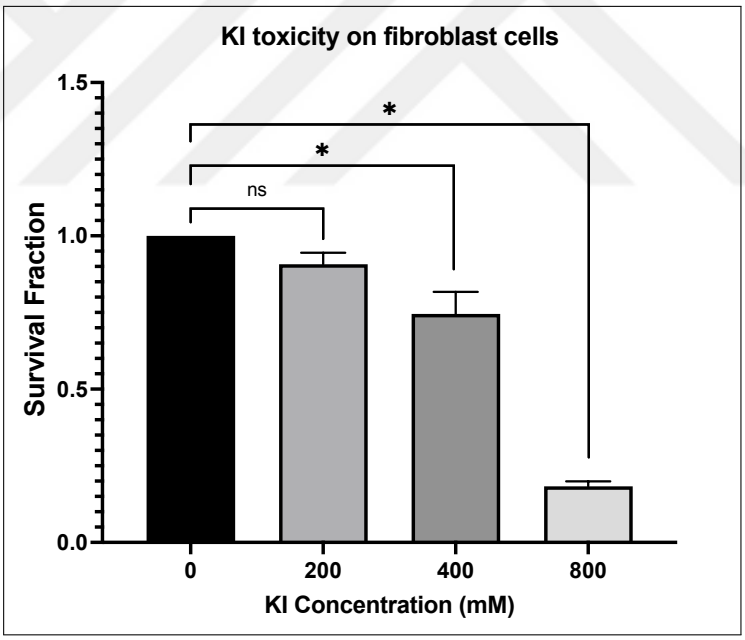


Figure 4.1 KI toxicity on fibroblast cells (ns: not significant, *: $p<0.05$).

400 and 800 mM KI concentrations caused statistically significant toxicity compared to no treatment control. However, in order to continue in line with the literature, it was decided to test 400 mM KI concentration in the next experiment in addition to 200 mM KI, which does not cause toxicity.

4.2 Effect of KI Addition on ALA-PDT Activity

As seen in Figure 4.2, KI addition did not increase the effectiveness of ALA-mediated PDT application on bacteria. The reason for this may be that the iodide atom in the inorganic salt used did not undergo the reactions stated in the project text and/or that the PpIX produced by the bacteria after ALA application produced toxicity largely as a result of Type II reactions instead of Type I, that is, single oxygen-based reactions. In the next stage, our research group continued as stated in the project text as plan B, and tested whether potassium chloride (KCl), a different inorganic salt, increases the effect of PDT.

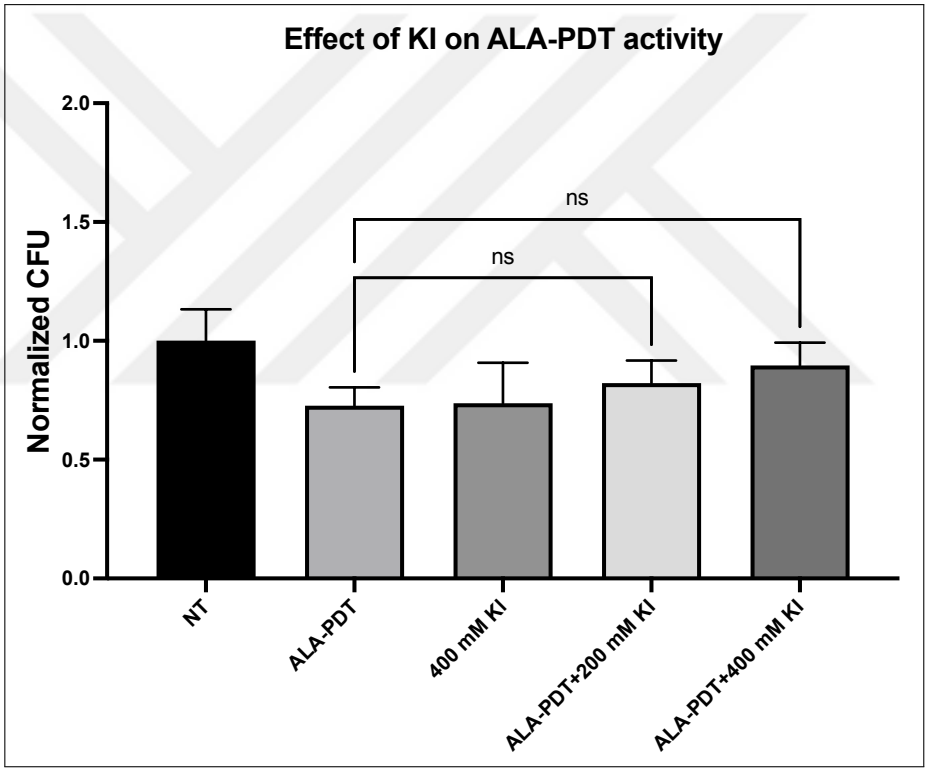


Figure 4.2 Effect of KI addition on ALA-PDT activity (ns:not significant, NT: NonTreated group).

4.3 Cytotoxicity of KCl on Fibroblast Cells

Since there is no similar use of KCl in the literature, the toxicity of 0-1600 mM KCl concentrations on L929 cells was investigated (Figure 4.3). As seen in Figure 4.3, 800 and 1600 mM KCl concentrations caused statistically significant toxicity compared to no treatment control. In the next experiment, it was decided to try 400 mM KCl, which does not cause toxicity.

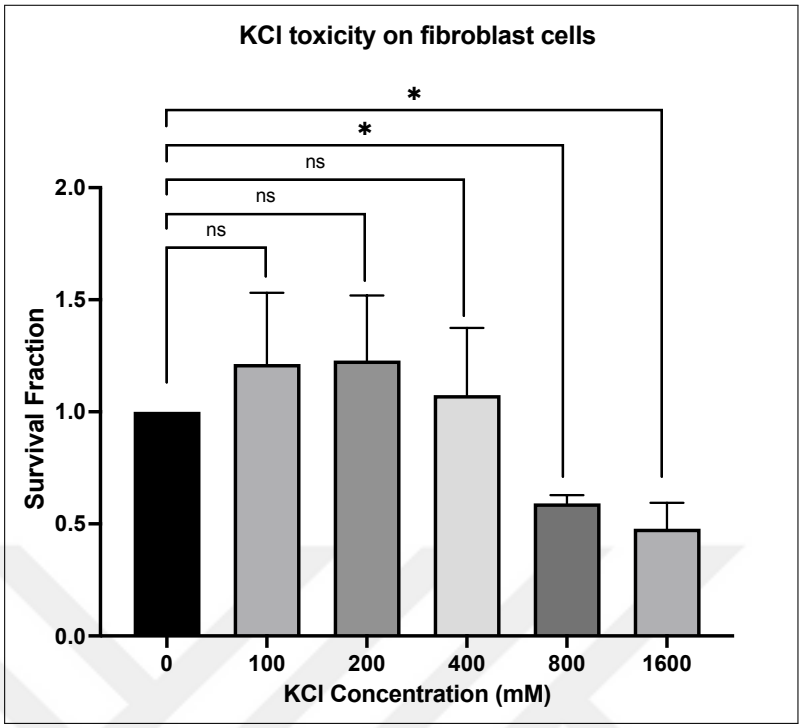


Figure 4.3 KCl toxicity on fibroblast cells (ns: Not Significant, *: $p<0.05$).

4.4 Effect of KCl Addition on ALA-PDT Activity

As seen in Figure 4.4, the addition of KCl did not increase the effectiveness of ALA-mediated PDT application on bacteria. In the next stage, our research group decided to try different photosensitizers for PDT instead of endogenous PpIX, and included Chlorine e6 (Ce6) photosensitizer, which is known to be used for PDT in the literature, in the study.

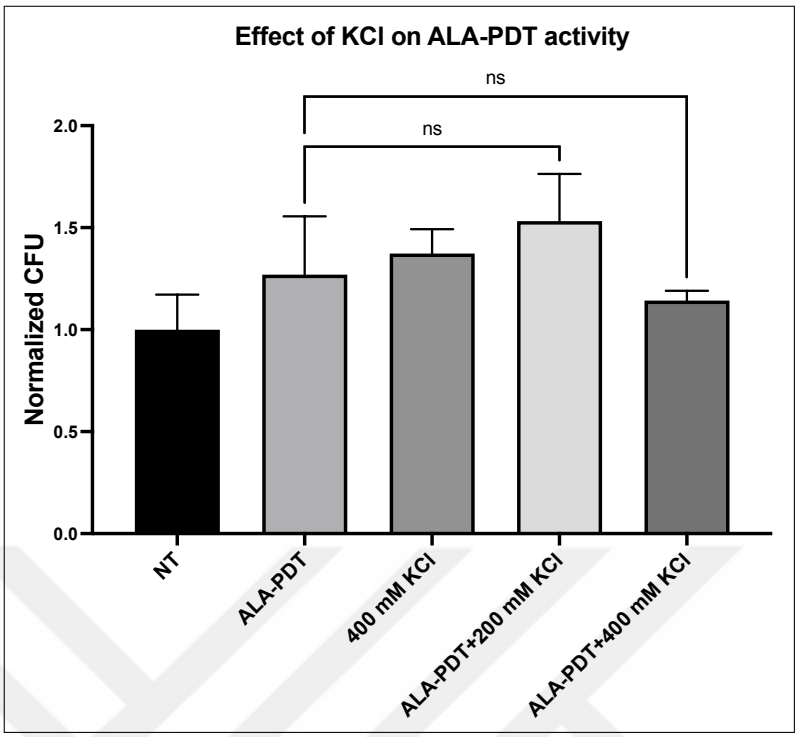


Figure 4.4 Effect of KCl addition on ALA-PDT activity (NT: NonTreated, *: $p<0.05$).

4.5 Ce6-mediated PDT Results and the Effect of KCl Addition on Ce6-Mediated PDT Activity

The Ce6 and light energy densities that were selected for aPDT application was determined based on the literature, as well as the studies previously conducted by our research group. Figure 4.5 reveals that Ce6-mediated PDT killed statistically significantly more bacteria when used with KCl than without KCl, meaning that the KCl concentration, which is non-toxic to bacteria and L929 cells, enhances the effect of Ce6-mediated aPDT application.

4.6 Absorption Spectra of Ce6-KCl

The absorption spectra of Ce6 and KCl were presented in Figure 4.6 The results show that, unlike Ce6, KCl does not have any absorption at 660 nm, which indicates that the potentiation effect of KCl was not based on photochemical interactions.

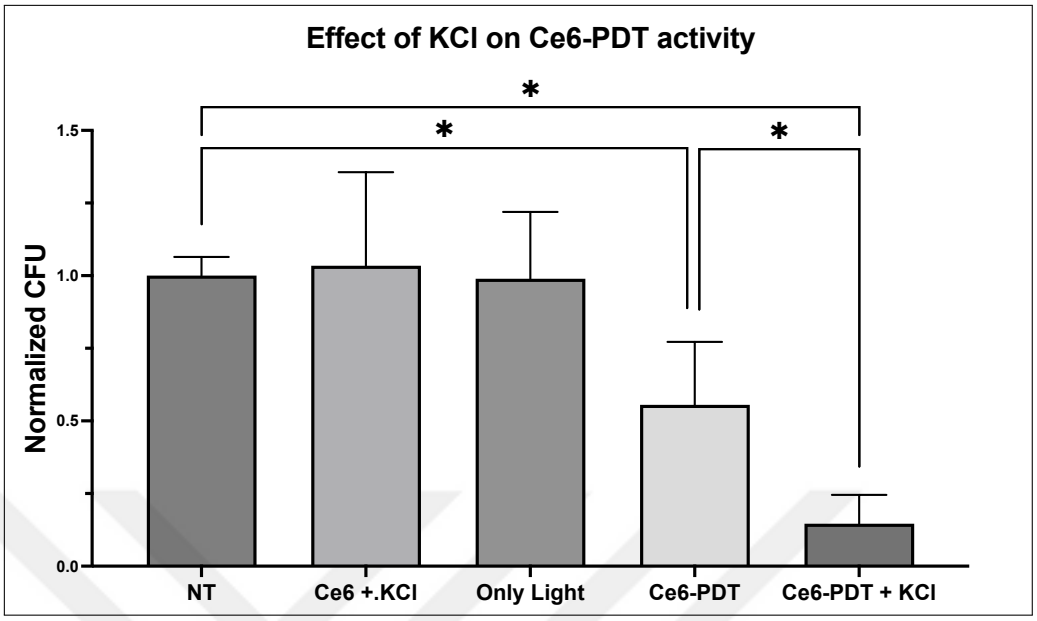


Figure 4.5 Efficacy of Ce6-mediated PDT (25 μM Ce6 and 30 J/cm^2 energy density) on *S. aureus* and the effect of KCl addition on Ce6-mediated PDT activity (NT: NonTreated group, *: p<0.05).

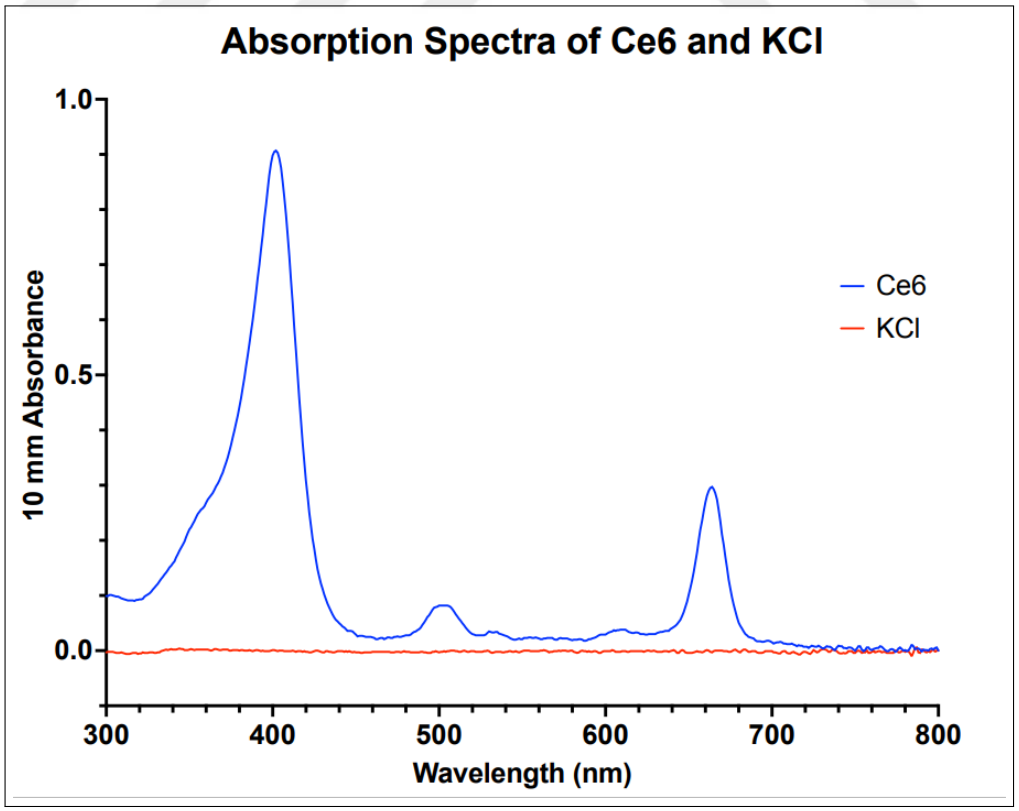


Figure 4.6 Absorption spectra of KCl-Ce6.

5. DISCUSSION

Antibiotics are still the main therapeutic method to treat infections. However, drug resistance of bacteria is a well-known problem preventing their effectiveness. PDT leads to cell death when photosensitizers group are excited by light. Although the mechanism is still being studied, it is proposed that exceeding a certain amount of ROS, which are formed during the irradiation of the photosensitizer molecules accumulated on the target tissue. Although PDT has been shown to be efficient in the inactivation of antibiotic-resistant bacteria, active research is going on to increase its efficiency in order to accelerate the translation of this method into clinics. Potentiation of aPDT using inorganic salts, including KI and sodium azide has been reported. This study aimed to investigate both KI and KCl as potentiation agents for aPDT. *S. aureus* bacterial suspension was used as the *in vitro* model.

This project was designed as a study to increase the effectiveness of a previously untested PDT protocol, ALA-mediated PDT, with the use of inorganic salt. ALA is a precursor to an endogenous photosensitizer, PpIX, and when bacteria are incubated with ALA, they produce this endogenous photosensitizer [24], [43]. While ALA-mediated aPDT has shown potential for bacterial inactivation, the studies indicate that this method often requires long irradiation times and varying concentrations of ALA, which limits its clinical application. For instance, *E. coli* did not respond to the treatment in some studies, and other bacteria required 2-3 hours of irradiation for inactivation [12–14]. These findings suggest that enhancing the process with inorganic salts could lead to more efficient bacterial inactivation with shorter irradiation times. For this purpose, first experiments were conducted to investigate the dark toxicity of KI and KCl on both *S. aureus* and mouse fibroblast cells. Subsequently, the endogenous protoporphyrin IX (PpIX) produced by *S. aureus* bacteria due to the incubation with ALA was used for aPDT and inorganic salts were used to increase the effectiveness of this method. Results showed that neither the use of KI nor KCl statistically significantly increased the efficacy of PpIX-mediated PDT application. Since KI was able to increase the efficacy of exogenous PS-mediated aPDT in the previous studies,

the results we obtained were unexpected and not in accordance with our hypothesis.

In the following part of the study, we decided to seek the potential of using 400 mM KCl as an aPDT-enhancing agent using another photosensitizer, Ce6, for the first time. Ce6, a second-generation PS, has an absorption peak in the near-infrared range, has high singlet oxygen quantum yield, and low toxicity in the absence of light, making it popular in cancer research [39]. Antibacterial potential of Ce6 has been also researched. For example, in a study comparing methylene blue (MB) and Ce6 on Streptococcus mutans biofilms, showed that both PSs were effective, however, while MB was more effective than Ce6 at lower concentrations, Ce6-based PDT showed higher efficacy at higher concentrations of the PS, achieving a 5-log bacterial reduction [41]. Another study on methicillin-resistant Staphylococcus aureus (MRSA) showed that Ce6-PDT with added ethanol provided better antibacterial outcomes, especially at lower Ce6 concentrations and energy densities [48]. In our study, in order to observe the strengthening effect of KCl more clearly, a low-dose PDT protocol that would not inhibit bacteria completely was chosen (25 μM Ce6 and 30 J/cm^2 energy density). According to the results of the study, it was shown for the first time that using a non-toxic concentration of KCl with Ce6-mediated aPDT significantly enhances the inactivation of *S. aureus*.

Based on the literature, the suggested mechanism for KI-mediated potentiation is associated with ROS production through the following reactions [17].

$$^1O_2 + 3I^- + 2H_2O \rightarrow I_3^- + 2H_2O_2 \tag{5.1}$$

$$^1O_2 + 2I^- + H_2O \rightarrow HOOI_2^- + HO^- \tag{5.2}$$

$$HOO_2^- \rightarrow I_2.^- + HOO^{\cdot} \tag{5.3}$$

Therefore, reactive species including I_3^-, $2H_2O_2$, I_2^- and $HOO^{\cdot}$ which are toxic for

bacteria accumulate in the environment. We speculate that adding KCl increases ROS by leading to similar chemical reactions as KI.

To sum, this study showed that KCl could be a promising potentiator for aPDT, however, further research is needed to investigate the mechanism of action of KCl-mediated aPDT potentiation, as well as the efficacy of this method on other experimental models [49].

6. CONCLUSION

This study hypothesized that the administration of a non-toxic concentration of KI would potentiate the efficacy of ALA-mediated aPDT. Unexpectedly, the results did not show any significant difference between the experimental groups that included KI or not. In a follow-up study, we showed for the first time that KCl could be a promising potentiator for aPDT because the addition of 400 mM KCl significantly inhibited bacteria in Ce6-mediated aPDT. Further research is needed to investigate the mechanism of action of KCl-mediated aPDT potentiation, as well as the efficacy of this method on other experimental models, including *in vitro* cultures of gram-negative bacteria and animal models.

6.1 APPENDIX A. Publication Produced From the Thesis

Potassium Chloride Potentiates Chlorin e6-Mediated Antimicrobial Photodynamic Inactivation (2023).

Potassium Chloride Potentiates Chlorin e6-Mediated Antimicrobial Photodynamic Inactivation

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***Abstract*— Photodynamic therapy (PDT) is being researched as an alternative for the inactivation of antibiotic-resistant bacteria. Despite promising results, only a few clinical applications involve antimicrobial PDT (aPDT), particularly for localized infections. One of the main reasons for this slow transition into clinics is that the effectiveness of PDT may vary depending on the type of the bacteria, their strains, and the photosensitizing agent (PS) used. Therefore, methods to increase the efficacy of antimicrobial photodynamic inactivation protocols, including combining PSs with natural compounds and inorganic salts, is an active area of research. Previous research has shown that inorganic salts, such as sodium azide and potassium iodide, can potentiate the efficacy of aPDT. This is the first study that uses another inorganic salt, potassium chloride (KCl), to potentiate aPDT, and the results of this in vitro study revealed that chlorin e6 (Ce6)-mediated PDT can kill significantly more bacteria when combined with a non-toxic concentration of KCl.**

Keywords—antimicrobial photodynamic therapy; chlorin e6; potassium chloride.

I. INTRODUCTION

Antibiotics are used in the prevention and treatment of bacterial infections. However, unnecessary, mis/overuse of antibiotics may cause drug-resistant bacteria, which pose a significant threat to human health [1]. According to the World Health Organization (WHO), diseases caused by antibiotic-resistant bacteria causes around 700,000 deaths per year in the world, and this number is expected to reach 10 million by 2050 [2]. PDT is researched as an alternative bacterial infection treatment method. In PDT, photosensitive molecules (photosensitizer, PS) are excited by light with an appropriate wavelength to create reactive oxygen species (hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) through Type I and singlet oxygen ($^1O_2$) through Type II reactions) that are toxic for bacteria [3, 4]. PDT has advantages over antibiotics, including selectivity and low risk of resistance. PDT can also be used to treat a wide range of infections, including localized bacterial infections, viral infections, and fungal infections [5, 6].

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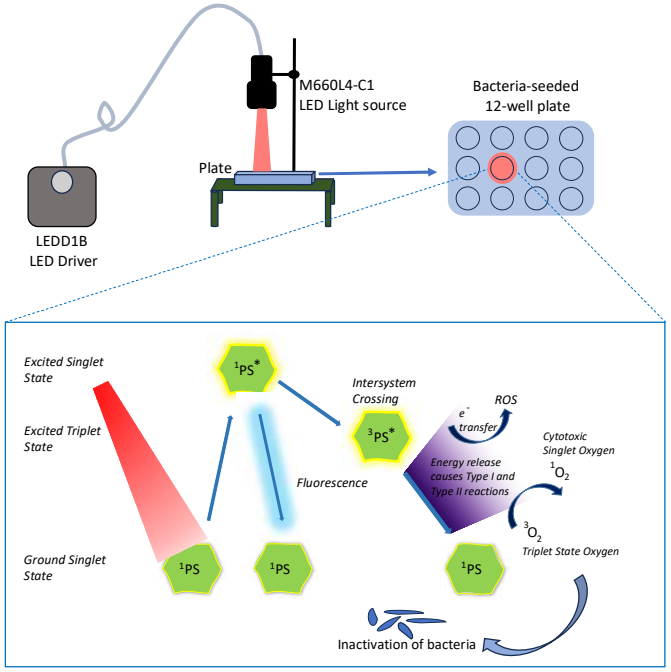


Fig. 1: Mechanism of aPDT. Irradiation of PS molecules by appropriate light leads to the excitation of PS molecules to the excited singlet state. Excited molecules may relax to the ground singlet state by emitting fluorescence. Another possibility is to make a transition to the excited triplet state by intersystem crossing. From excited triplet state, PS molecules can return to the ground singlet state by interacting with other molecules via Type I and Type II reactions. This interaction leads to the formation of $^1O_2$ or other reactive oxygen species (ROS) that are toxic for bacteria [6].

Besides the advantages, there are also situations that limit the effectiveness of PDT. For example, the wavelength of the applied light that is used to excite the PS molecules may not penetrate deep tissues effectively, which makes it hard to treat deeper or internal infections. Another limitation of aPDT is that the effectiveness of PDT may vary depending on the specific bacterium and the photosensitizing agent used [3-6]. Therefore, strategies to increase the efficacy of antimicrobial photodynamic inactivation protocols is an active area of research and necessary for the increased application of this

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method in clinics. Potentiation of antimicrobial photodynamic inactivation by inorganic salts has been researched by multiple studies including our research group. For example, Wen *et al.* showed that addition of 100 mM potassium iodide potentiates rose bengal-mediated PDT on Gram-negative and Gram-positive bacteria by initiating iodide-mediated chemical reactions that leads to formation of ROS [7]. In another aPDT study on Gram-positive and Gram-negative bacteria, Kasimova *et al.* determined that the efficacy of methylene blue (MB)-mediated aPDT can be increased by addition of sodium azide due to electron transfer to the MB triplet state from azide anion, which leads to production of azidyl radical [8]. One of the studies from our group on *E. faecalis* suspensions and biofilms also showed that toluidine blue-mediated aPDT can be potentiated using potassium iodide [9]. Another recent study by Topaloğlu *et al.* showed that photoinactivation using Ce6, which was also preferred in this current study, did not provide high mortality rate on *P. aeruginosa*, yet, combining this protocol with potassium iodide could significantly increase its efficacy [10]. Building on the literature and considering that chlorine (Cl) is a commonly used disinfectant for bacteria, in this study, potassium chloride (KCl) has been used as a potentiator for aPDT, for the first time, to potentiate the efficacy of Ce6-mediated PDT on *S. aureus*.

II. Materials and Methods

A. Bacteria and Culture Conditions

A single colony was taken from S. aureus (ATCC 43300) stock and incubated in 10 ml Tryptic Soy Broth overnight at 37°C to allow them to grow. The following day, the tubes were centrifuged, the supernatant was discarded, and the pellets were dissolved in phosphate buffered saline (PBS) to obtain a bacterial concentration of 10^7-10^8 CFU/ml. The optical density of the suspensions was measured at 600 nm before experiments to make sure that the number of bacteria is optimal.

B. Absorption Spectra of Ce6 and KCl

The absorption spectra of Ce6 and KCl between 300-800 nm were recorded using a Nanodrop 2000c UV–vis spectrophotometer.

C. Determination of Non-toxic Ce6 and KCl Concentrations

One of the concentrations of Ce6 that is considered as low in the literature, 25 µM, was preferred in this study to be able to show the potentiation by KCl [10-12]. To decide on the highest non-toxic KCl concentration, a cytotoxicity study was conducted on L-929 mouse fibroblast cells, which are considered as a model for dermal cells in similar studies [13, 14]. Briefly, cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C with 5% CO_2. On the first day of experiment, cells were seeded on each well of a 96-well plate at 10^4 cell/well density and incubated overnight. The next day, cells were incubated with 0, 100, 200, 400, 800 and 1600 mM KCl in PBS for 30 minutes. At the end of the experiments, the medium of the cells were refreshed and plates were placed into the incubator for another overnight incubation. The cytotoxicity of KCL concentrations on L-929 cells were determined by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay.

D. In Vitro Studies

A Thorlabs M660L4-C1 660nm LED light source was used to irradiate the samples at 100 mW/cm^2 power density. Bacteria that were seeded on the wells of a 12-well plate were treated as follows:

No treatment (negative control)
Only light (300 s irradiation to achieve 30 J/cm^2 energy density)
25 µM Ce6 + 400 mM KCl (No light)
25 µM Ce6 + light (PDT)
25 µM Ce6 + 400 mM KCl + light (Potentiated PDT)

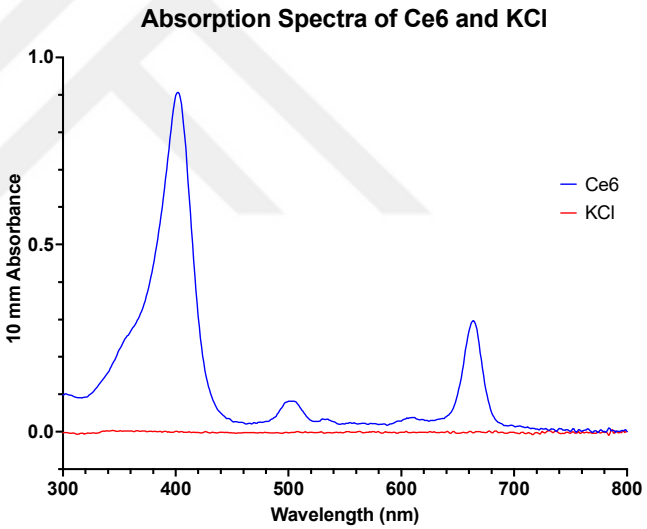
At the end of the treatments, bacterial suspensions were serially diluted, spread on agar plates and incubated overnight. Bacterial colonies were counted the following day to determine the number of colony forming units (CFU).

III. Results

A. Absorption Spectra

The absorption spectra of Ce6 and KCl were presented in Fig. 2. The results show that, unlike Ce6, KCl does not have any absorption at 660 nm, which indicates that the potentiation effect of KCl was not based on photochemical interactions.

Fig. 2: Absorption spectra of Ce6 and KCl



B. Determination of Experimental Parameters

According to the results of a preliminary experiment, 300 seconds of irradiation using 660 nm LED light source at 100 mW/cm^2 power density (i.e. 30 J/cm^2 light energy density) do not cause a detrimental effect on *S. aureus* bacteria (data not shared). The results of the cytotoxicity assay on L-929 cells showed that 400 mM KCl was not toxic for cells when incubated for 30 minutes (Fig. 3). Therefore, 30 J/cm^2 light energy density, 25 µM Ce6 concentration and 400 mM KCl concentration were selected for this study.

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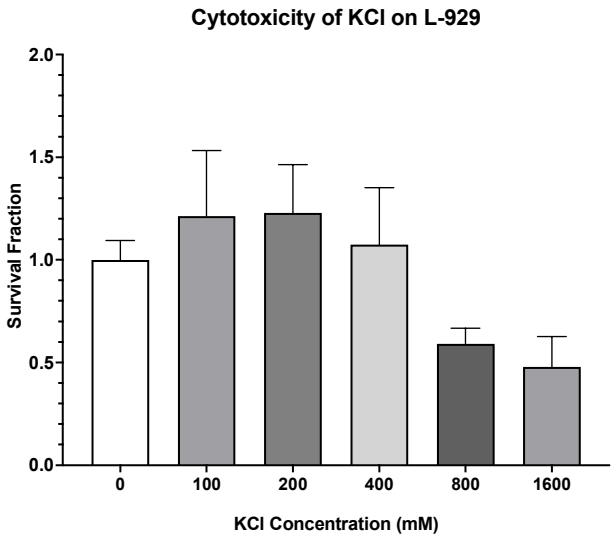


Fig. 3: Cytotoxicity of different concentrations of KCl on L-929 cells. 400 mM KCl is determined as the highest non-toxic concentration.

C. KCl Potentiates Ce6-Mediated Antimicrobial Photodynamic Inactivation

Fig. 4 shows that Ce6-mediated aPDT combined with KCl (Ce6+KCl+light) kills significantly more bacteria than Ce6-mediated aPDT without KCl (Ce6+light). This reveals that a concentration of KCl that is non-toxic for L-929 cells can potentiate Ce6-mediated aPDT application.

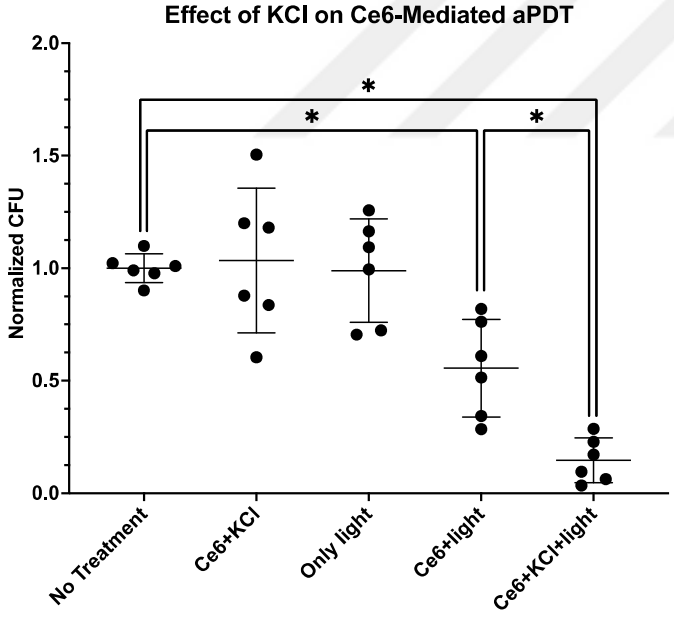


Fig. 4: Results of the aPDT study. Comparison of the means of Ce6+Laser and Ce6+KCl+Laser groups reveals that addition of KCl significantly potentiates Ce6-mediated aPDT (*: $p<0.05$).

IV. DISCUSSION

Antibiotic resistant bacteria are considered as one of the biggest concerns for human health. Although photodynamic therapy has been shown to be efficient in the inactivation of antibiotic-resistant bacteria, active research is going on to increase its efficiency in order to accelerate the translation of this method into the clinics. Potentiation of aPDT using inorganic salts, including potassium iodide (KI) and sodium azide has been reported. In this study, we aimed to investigate KCl as a potentiation agent for aPDT, for the first time. *S. aureus* bacterial suspension was used as the in vitro model. To be able to observe the potentiation effect of KCl more clearly, a relatively low dose aPDT protocol has been selected based on the literature. Each dot in Fig. 4 represents technical replicates from three independent biological replicates. Other experiments were repeated at least two times. The results of the study show that inactivation of *S. aureus* by Ce6-mediated aPDT was potentiated using 400 mM KCl. Based on the literature, the suggested mechanism for KI-mediated potentiation is associated to ROS production through following reactions [15].

$$^1O_2 + 3I^- + 2H_2O \rightarrow I_3^- + 2H_2O_2 \quad (1)$$

$$^1O_2 + 2I^- + H_2O \rightarrow HOOI^-_2 + HO^- \quad (2)$$

$$HOOI^-_2 \rightarrow I_2^{\bullet -} + HOO^{\bullet} \quad (3)$$

We speculate that the addition of KCl increases ROS by leading to similar chemical reactions as KI. To sum, this study showed that KCl could be a promising potentiator for aPDT, however, further research is needed to investigate the mechanism of action of KCl-mediated aPDT potentiation, as well as the efficacy of this method on other experimental models.

ACKNOWLEDGMENT

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