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Investigation of the Effect of Electrode Material on Antibacterial Activity in Plasma Treatment with Dielectric Barrier Discharge

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by

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This is to certify that we have read the thesis **Investigation of the Effect of Electrode Material on Antibacterial Activity in Plasma Treatment with Dielectric Barrier Discharge** submitted by **Merve Gül**, and it has been judged to be successful, in scope and in quality, at the defense exam and accepted by our jury as a MASTER'S THESIS.

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Investigation of the Effect of Electrode Material on Antibacterial Activity in Plasma Treatment with Dielectric Barrier Discharge

Abstract

Plasma is an ionized gas generated under an electric field that contains photons, free electrons, ions, free radicals, and reactive oxygen/nitrogen species (RONS). Cold atmospheric plasma (CAP) is generated in the cold form under a high electric field at atmospheric pressure. CAP is generated using two methods, Dielectric Barrier Discharge (DBD) and plasma jet. In the DBD method, the electrode configuration, shape, material, and substance from which the dielectric barrier is made are important. Beyond the electrode design and geometry, the barrier and electrode materials can also impact the reactivity of the discharge by modifying the discharge electrical power. Plasma discharge at various durations in CAP applications is hypothesized to alter because of a difference in conductivity of the conductive material utilized depending on capacitive resistance and permittivity. Therefore, in this study, plasma activated waters (PAWs) are evaluated using three alternative electrode materials (copper, stainless steel, and aluminum) to quantify physical quantities that can differ significantly such as RONS levels, pH, and RONS types. The study aims to investigate the effect of the antibacterial activity of CAP treatment using different electrode materials on microbial inactivation of *E.coli* and the biological outcome of CAP treatment on skin cells. The results revealed that the difference in electrode materials affects the amount of, RONS, pH, and antibacterial activity in CAP treatments, the electrode material with the highest antibacterial activity was observed to be stainless steel according to ZOI results. The results of the antibacterial activity of CAP treatment were divided into groups; direct treatment and by PAWs. According to the colony counting results, it can be said that the aluminum electrode is the most effective group for antibacterial efficacy on *E.coli*. Therefore, the aluminum electrode has the highest cell proliferation effect compared to others with respect to cell viability assay results. By adding one more parameter to which factors are important in antimicrobial experimentations in the field of plasma medicine, this research will carry light on future studies and which RONS types are more effective for required microbial inactivation.

Keywords: Cold Atmospheric Plasma, Plasma Medicine, Electrode Material, Antibacterial Activity

Dielektrik Bariyer Deşarjı ile Plazma Arıtmasında Elektrot Malzemesinin Antibakteriyel Aktiviteye Etkisinin Araştırılması

Öz

Plazma, fotonlar, serbest elektronlar, iyonlar, serbest radikaller ve reaktif oksijen/azot türleri (RONS) içeren bir elektrik alanı altında üretilen iyonize olmuş gazdan oluşur. Soğuk atmosferik plazma (CAP), atmosferik basınçta yüksek bir elektrik alanı altında soğuk formda üretilir. CAP, Dielektrik Bariyer Deşarjı (DBD) ve plazma jeti olmak üzere iki yöntem kullanılarak üretilir. DBD yönteminde dielektrik bariyerin yapıldığı elektrot konfigürasyonu, şekli, malzemesi ve maddesi önemlidir. Elektrot tasarımı ve geometrisinin ötesinde, bariyer ve elektrot malzemeleri de deşarjın elektrik gücünü değiştirerek deşarjın reaktivitesini etkileyebilir. CAP uygulamalarında çeşitli sürelerdeki plazma deşarjının, kapasitif direnç ve geçirgenliğe bağlı olarak kullanılan iletken malzemenin iletkenliğindeki bir farklılıktan dolayı değiştiği varsayılmaktadır. Bu nedenle, bu çalışmada, RONS seviyeleri, pH ve RONS türleri gibi önemli ölçüde farklılık gösterebilen fiziksel miktarları ölçmek için, bu çalışmada plazma ile aktifleştirilmiş sular (PAW'ler) üç alternatif elektrot malzemesi (bakır, paslanmaz çelik ve alüminyum) kullanılarak değerlendirilmiştir. Çalışmanın amacı, farklı elektrot malzemeleri kullanılarak yapılan CAP tedavisinin antibakteriyel aktivitesinin, E.coli bakterisinin mikrobiyal inaktivasyon üzerindeki etkisini ve CAP tedavisinin cilt hücreleri üzerindeki biyolojik sonucunu araştırmaktır. Sonuçlar, elektrot malzemelerinin farklılığının CAP tedavisinde RONS miktarı, pH ve antibakteriyel aktivite üzerinde etkili olduğunu ve ZOI sonuçlarına göre çoğu grupta antibakteriyel aktivitenin en yüksek olduğu elektrot malzemesinin paslanmaz çelik olduğu gözlemlendi. CAP tedavisinin antibakteriyel etkinliğinin sonuçları gruplara ayrıldı; doğrudan tedavi ve PAW'ler tarafından. Koloni sayımı sonuçlarına göre *E.coli* üzerinde antibakteriyel etkinlik açısından en etkili grubun alüminyum elektrot olduğu söylenebilir. Bu nedenle alüminyum elektrot, hücre canlılığı tahlil sonuçları açısından diğerlerine göre en yüksek hücre çoğalma etkisine sahiptir. Bu çalışma, plazma tıbbı alanında yapılan antimikrobiyal çalışmalarda hangi parametrelerin etkili olduğu ve gerekli mikrobiyal inaktivasyon için hangi RONS tiplerinin daha etkili olduğuna bir parametre daha ekleyerek bundan sonraki çalışmalara ışık tutacaktır.

Anahtar Kelimeler: Soğuk Atmosferik Plazma, Plazma Tıbbı, Elektrot Malzemesi, Antibakteriyel Aktivite

To my lovely family...

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İçindekiler

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List of Abbreviations

KI	Potassium Iodide
CAP	Cold Atmospheric Plasma
İKÇÜ	İzmir Kâtip Çelebi University
DIW	Deionized Water
ORCID	Open Researcher and Contributor ID
DBD	Dielectric Barrier Discharge
OES	Optical Emission Spectroscopy
PAW	Plasma Activated Water
UV	Ultraviolet
OES	Optical Emmission Spectroscopy
EMF	Electromagnetic Field
RONS	Reactive Oxygen/Nitrogen Species
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
COVID-19	Coronavirus
DNA	Deoxyribonucleic Acid
NO_2	Nitrogen Oxide
ETO	Ethylene Oxide
PALs	Plasma-activated Liquids
TNF	Tumor Necrosis Factor
VEGF	Vascular Endothelial Growth Factor
KI	Potassium Iodide
TSB	Tryptic Soy Broth
TSA	Tryptic Soy Agar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

FBS	Fetal Bovine Serum
ZOI	Zone of Inhibition

List of Symbols

g	Grams
mL	Milliliter
s	Seconds
μL	Microliter
F	Force [N]
m	Mass [kg]
N	Newtons
A	Ampere
μL	Microliter
Ar	Argon
He	Helium
O_2	Oxygen
mm	Millimeter
s	Second
CFU/mL	Colony Forming Units/Milliliter

Chapter 1

1 Introduction

1.1 Cold Atmospheric Plasma

Plasma is the fourth state of matter and a partially ionized gas containing ions, free electrons, reactive oxygen and nitrogen species, ultraviolet (UV) radiation, and particles such as radicals [1]. The term of plasma was first defined by Langmuir in 1928. Plasma has had applications in many fields since then, especially in medicine [2]. Plasma can be classified as natural and artificial plasmas. Aurora lights, thunder, sun, and star systems are examples of natural plasmas. In addition, plasma can be produced at atmospheric pressure or under a vacuum [3]. The examples of plasma light and CAP applications are shown in : Plasma light, and CAP application in medicine [4].

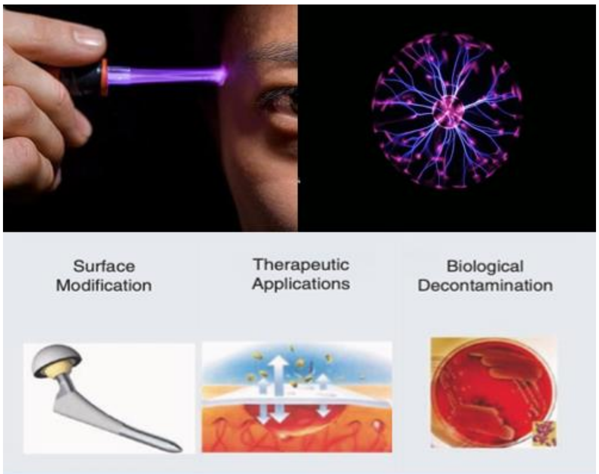

Figure 1.1: Plasma light, and CAP application in medicine [4].

Plasma is a quasi-neutral ionized gas and without any external forces, the net electrical charge of plasma is zero. It contains the same amount of positively and negatively charged particles [5]. Plasma can be created in two different forms; thermal or hot and non-thermal or cold plasma. There are quite distinctive physical differences between these two forms of plasma. In thermal plasma, the electrons and heavy gas molecules are at the same temperature. Since the electrons receive the external energy much faster than heavy gas molecules in cold plasma and their temperature can be reached thousands of degrees before the external environment heats up. Overall, in cold plasma, the ambient temperature can be stabilized at room temperature [6], [7]. Cold atmospheric plasma (CAP) can be created at atmospheric pressure and room temperature. CAP is an emerging technology in medical and biomedical applications nowadays. CAP applications in medicine can be divided into three main classes; surface modifications, therapeutic applications, and biological decontamination [4].

1.1.1 Generation Types of Plasma

There are two types of generation of plasma methods; direct and indirect plasma treatment. In direct plasma treatment, the target material serves as one of the electrodes and it becomes in the active part of plasma discharge. In indirect plasma treatment, the active plasma content is transferred into a fluid with gas flow, and after that, this fluid can be applied to a specified target [8], [9]. Plasma devices used in indirect plasma therapy include plasma jets, needles, and torches. Fluid-mediated plasma treatments can get involved in indirect plasma treatment methods [10]. Figure 1.1.1 shows the generation types of plasma as direct and indirect plasma treatment.

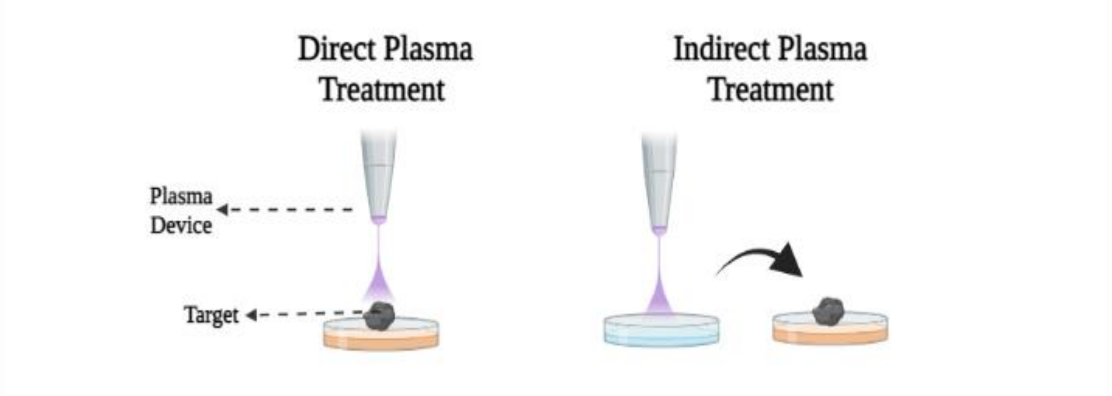

Figure 1.1.1: Different types of plasma generation – direct and indirect treatment (Drawn by BioRender)

There are a lot of generation methods of CAP, and even generation types of plasma can be redesigned for specific targets. In clinical treatments, CAP can be used for skin diseases, and a specific CAP treatment application can be made by specifying the generation type of plasma and calculating surface area according to the design of the plasma device, electrode design, healing efficiency according to the places that cannot be easily reached in the body [11]. The images of generation types of plasma are demonstrated in Figure 1.1.2.

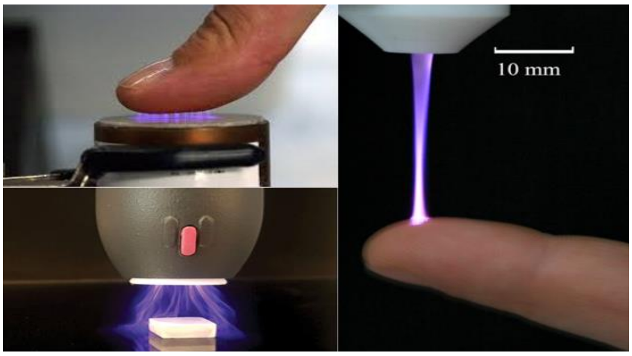

Figure 1.1.2: The images of generation types of plasma [12].

In fluid-mediated plasma treatment, a liquid is treated with plasma and the active content of the plasma is transferred to this liquid. Thanks to this transferred active content, chemical modifications occur in the liquid and then the treated liquid is transferred to the target material. These applications include both direct and indirect treatment and are considered a different method in experimental research studies [10], [13].

1.1.2 Dielectric Barrier Discharge

Dielectric barrier discharge (DBD), commonly known as quiet discharge or ozone discharge, is a method of generating plasma that involves an electrical discharge between two electrodes separated by an electrically insulating dielectric barrier material such as glass, ceramic, or quartz [14]. It consists of a high voltage electrode and ground electrode in the electrical configuration of the DBD method. DBD mostly is used in the medical and biomedical applications of CAP. The schematic of the electrical configuration of DBD method and direct plasma treatment is shown in Figure 1.1.2.1.

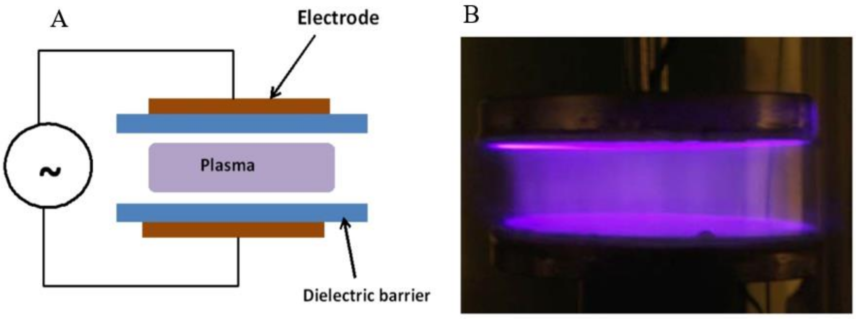

Figure 1.1.2.1: A) Schematic of electrical configuration of DBD method, B) direct plasma treatment [15].

1.1.3 Electrode Materials and Their Effect on Plasma Discharge

Electrode materials used for CAP treatment must be conductive. The electrical power passing through the conductive material provides the energy required for the ionization of the gas. Changing the electrode material may also cause the discharge characteristics to change in general. Changing discharge characteristics play an important role in the antimicrobial and biological activity of plasma. Conductive materials have electrical behavior properties such as conductivity, resistivity, permittivity, permeability, capacitive reactance, and capacitive resistance. Conductivity is the ability of a substance to conduct electric current, while resistivity is the ability to resist the electric current. Electrical resistivity and conductivity can be expressed by the following formulas;

$$\rho = R\,\frac{A}{l} \quad \text{and} \quad \sigma = \frac{1}{\rho}$$

where; ρ = electrical resistivity; R = electrical resistance of material; A = cross-sectional area of material; l = length of material.

Permeability is a measurement of magnetization obtained as a material's response to an applied magnetic field, and its symbol is newtons per ampere squared (N/A^2). The permeability (μ) of the material is the ratio of the movement of the charges in the magnetic flux density medium, the amount of bending (B) to the magnetic field measurement (H) in the medium. In plasma, it is physically produced under an electric field and is a matter of magnetization. The behavior of charged particles is very important for determining the permeability of a material. The permeability of a material is expressed by the following formula;

$$B = \mu H$$

where; B = magnetic flux density; H = magnetic field.

Permittivity- dielectric constant is a coefficient that measures the ability of a material to store charge on it. It is a metric that indicates how easily a substance can be polarized by passing it through an electric field. The following formula expresses a material's permittivity;

$$\varepsilon r = \varepsilon / \varepsilon 0$$

where εr = relative permittivity, ε = permittivity of substance ($C^2/(N\ m^2)$), ε_0 = permittivity of vacuum or free space. The material's permittivity is a purely static property, while its conductivity involves its dynamics. There is a relationship between a material's permittivity and electrical conductivity and this relationship can be expressed by this formula below;

$$\varepsilon = \varepsilon r \varepsilon o + j * \sigma/\omega$$

where; εr = relative permittivity; εo = permittivity of free space; σ = electrical conductivity; ω = angular frequency = 2πf

Also, j * σ/ω is the dielectric loss factor, ie complex permittivity in linear media and how it correlates to the dielectric constant and conductivity. In the limited case of linear media, the Kramers-Kronig relations express conductivity as an integral of the dielectric constant across all frequencies, and dielectric constant as an integral of conductivity over all frequencies [16].

The electrode design, geometry, material, and composition from which the dielectric barrier is created are all essential in the DBD process [17]. Many research with on various electrode configurations has been published in the literature. Aside from electrode arrangement and form, the barrier and electrode materials can also influence discharge reactivity via varying discharge electrical power. Plasma discharge at different times in CAP applications is hypothesized to fluctuate because to a variation in the conductivity of the conductive material utilized depending on capacitive resistance. As a result of these changes, the electrode deposit of each electrode will be different, and the reactive species and radicals formed may be different or cause different chemical processes [18]. *Hepburn et al*. reported that the chemical reactions occurring on the surface of the electrode material can vary according to the material from which the electrode material is made, and that electrical discharge can create chemical stress on the discharge insulation by affecting the gas environment [19]. *Watson et al.*, investigated the dielectric materials on DBD method and the electrical characterizations of these materials, dielectric barrier material also can affect the electrical characteristics of the discharge in plasma treatment. As a result of these changes, it is concluded that the characteristic properties of the electrode material used, such as CAP treatment (OES, EMF, etc.), as well as the characteristic properties of the target applied (pH, contact angle, surface structure, etc.) can be changed [18]. Table 1.1 shows the electric conductivities of conductive materials which are used as electrode materials.

Table 1.1: Electric conductivity values of electrode materials [20].

Electrode Material	**Electric Conductivity (10E6 Siemens/m)**
Copper	58.7
Aluminum	36.9
Stainless Steel	1.32

1.1.4 Plasma Chemistry

Plasma consists of ionized gas and contains almost equal numbers of positively and negatively charged particles. While producing the plasma, an electric field is applied to the ambient gas so that these charged particles collide. Elastic and inelastic collisions occur due to the effect of electronegative and electropositive forces. These colliding charged particles cause several chemical reactions in the treated target. In addition, these charged particles gain energy at the atomic level and they can gain kinetic energy as well as they already have static energy. Thanks to the electrons in the plasma, ionization becomes easier, and plasma chemistry is activated when ions suppress the chemical reaction activation barrier [2]. Ionization is the essential component of these plasma-chemical processes. Dissociation is the other main reaction type of plasma–chemical processes. Reactive oxygen/nitrogen species (RONS) in the active content of the plasma interact with the molecules in the target material and lead to biological activity [11]. As a result of the interactions of these reactive oxygen species (ROS) and the molecules in the treated target, it can be formed such as hydrogen peroxide (H_2O_2), ozone (O_3), hydroxyl radical (•OH), singlet oxygen ($^1O_2$), superoxide (O_2^-), hydroperoxyl radical (HO_2•) and as a result of the interaction of reactive nitrogen species (RNS) and other molecules, it can be formed nitric oxide (NO), nitrite (NO_2), nitrate (NO_3), peroxynitrous acid (HNO_3), peroxynitrite ($ONOO^-$) [21]. With the production of many hydroxyls (OH-) and reactive oxidants in the environment, the biocompatibility of materials can increase and reduce the pH of the target material. In short, plasma performs the generation of different reactive gas species like ions (H_3O^+, O^+, O^-, OH^-, N_2^+), molecular species (O_3, H_2O_2), and reactive radicals (O •, OH •, NO •) on the applied surface [22]. Plasma–chemical processes with treatment of air gas can be examined under two categories, ROS and RNS, and the chemical reactions that occur are shown in Table 1.2.

Table 1.2: Chemical reactions and its equations in plasma treatment, according to ROS or RNS model [23].

ROS model	**RNS model**
Ionization: $e + O_2 \rightarrow O_2^+ + 2e$	**Ionization:** $e + N_2 \rightarrow N_2^+ + 2e$
Dissociation: $e + O_2 \rightarrow 2O + e$	
Dissociative attachment: $e + O_2 \rightarrow O^- + O$	**Dissociative recombination:** $e + N_2^+ \rightarrow N_2$
Recombination: $O^- + O_2^+ \rightarrow O + O_2$	
Dissociative recombination: $O + O_2^+ \rightarrow O_2 + e$	**Dissociation:** $e + N_2 \rightarrow 2N + e$

1.1.5 Plasma Medicine

Plasma is an emerging technology in medicine and biomedical applications nowadays. Plasma has many advantages due to it has no proven side effects on the body up-to-date. CAP is used in medicine and biomedical applications because it does not harm the tissues in general and is produced at ambient temperature [7]. CAP is generally used in the sterilization, wound healing, cancer therapy, and orthopedic implants in medicine and biomedical fields and it is promising for many patients [24]. In addition, CAP has quite important applications in dentistry such as tooth whitening, removal of biofilms, adhesive restorations, and disinfection of dental implants [25].

CAP, which is widely used in sterilization, is a cost-effective and basic method that can be used to eliminate hospital infections and intensive care infections [26]. CAP can inactivate many microorganisms, mainly bacteria, fungi and viruses, thanks to its active content. Microorganisms can easily attach to hydrophobic surfaces, a material can be made hydrophilic by CAP treatment; so that microorganisms cannot adhere to the material surface and cannot proliferate themselves on this material anymore [27]. Based on this important and basic method, sterilization of materials used in many catheters, implants and surgical instruments with CAP treatment has become widespread in clinical practice [28]. In addition, CAP can also selectively kill cancerous cells by RONS [9]. Thanks to its active content, which can also be used in diabetic foot wounds, pressure sores and severe burns, it provides effective and complete wound closure by triggering the wound healing pathways of skin cells [29].

1.1.6 Plasma in Disinfection and Sterilization

The sterilization of pathogen-contaminated medical instruments is critical in preventing serious infections. There are some chemical and physical sterilization methods of medical instruments to avoid hospital infections. CAP is the alternative promising method used for disinfection and sterilization compared to traditional methods [30]. The sterilization of pathogen-contaminated medical instruments is critical in preventing serious infections. CAP can reduce the microorganism load on contaminated surfaces, thus reducing the risk of contamination and infection. CAP is used for COVID-19 virus inactivation, today, there is a lot of study on this topic [31]. The chemically active content of plasma triggers various physical and chemical processes. The degradation of organic molecules and inactivation of living microorganisms is achieved by the oxidizing effect of reactive oxygen/nitrogen species on the one hand, by bombarding them with electrons, ions and short-lived neutral species. Ions and electrons have high energies and they can break hydrogen bonds of organic molecules which form the cell membrane. CAP treatment methods may vary due to the different types, strains, and metabolisms of microorganisms [32]. Types of plasma treatment to microorganism inactivation can be classified as follows; direct treatment which discharges contact with treated material like DBD, indirect treatment which discharging is not in contact with treated material like plasma jet and fluid-mediated treatment which transfers the plasma active content to liquid [33].

1.2 Microbial Inactivation

Microorganism is an organism that which has a microscopic size, and may exist in its single cell form or as a colony of cells. There are two types of organisms; unicellular and multicellular organisms.

1.2.2 Advantages & Disadvantages of Traditional Microbial Inactivation Methods

These disinfection and sterilization methods, which have been used for years, have advantages and disadvantages. However, with a general approach, it can be said that the disadvantages of traditional methods are more because they damage the sterilized material. These advantages and disadvantages may vary according to the sterilization method and purpose. Moreover, the type and metabolic behavior of the microorganism to be inactivated have a very important place in the discussion of advantages and disadvantages, since the inactivation of endospores and protozoans and resistant microorganisms is inconvenient. In physical methods, they are harmless to anybody and the environment, easy to control and monitor and they have a fast microbicidal effect and high penetration capability. They have several disadvantages of physical sterilization methods such as they are not suitable for heat sensitive materials for heat sterilization and filtration, it is not suitable for some solid materials, and the radiation is harmful to the human body [30], [38].

In chemical sterilization methods, they have a good penetration capability to material and are compatible with many medical instruments. Also, they are easy to use and suitable for heat sensitive materials. They have various disadvantages some of them carcinogenic and toxic and it is required ventilation after the sterilization process. In general, traditional methods are costly, not environmentally friendly, and harmful to the health of people [39].

1.2.3 Microbial Inactivation by CAP Treatment

CAP treatment can provide microbial inactivation thanks to its active content, which includes ions, electrons, radicals, and reactive species [40]. There are many studies examining the antimicrobial effect of CAP treatment. The antimicrobial effect of CAP treatment can be achieved by two types of plasma treatment, direct and plasma-activated liquids (PALs) [13]. In the PALs method, the active plasma content is transferred to the liquid which is used and added to the microorganism suspension, while in direct treatment, microorganisms are treated by direct contact. There are a lot of studies, including both of them or only one of them for achieving microbial inactivation by CAP treatment. *Laroussi et al*., reported the effect of air plasmas content such as UV radiation, reactive species, and heat for bacterial inactivation at atmospheric pressure [41]. *Guo et al*, described the effect of experimental conditions on microbial inactivation, the relationship between plasma types and plasma agents, and the interactions of microorganisms with charged particles [42]. *Hahn et al*. investigated the antimicrobial effect of direct and indirect high voltage CAP for the inactivation of very common microorganisms that cause diseases such as *E.coli* and *S. aureus*, and as a result, DNA damage and cell membrane integrity were compromised after 1 minute of CAP treatment [43]. CAP treatment, PALs especially plasma-activated water (PAWs) have applications in many areas such as food hygiene, hospital sterilization, surface decontamination, sterilization in the packaging of medical instruments, and decontamination of contaminated surfaces or objects [44]. The formation of an antimicrobial agent rich in reactive species by transferring the active content of the plasma of a liquid can provide a strong microbial inactivation. *Boxhammer et al.*, reported a result of up to a 5-log bacterial reduction in liquid mediated CAP treatment on microbial inactivation *E.coli* in less than 3 minutes [45].

1.2.1 Microbial Inactivation Methods

Microbial inactivation is a method to destroy the microorganisms that have been infecting the human body for years and the pathogens adhering to the surface, is called decontamination and there are many methods in the literature. Sterilization and disinfection are the most important of these methods. Disinfection is the elimination or reduction of hazardous bacteria from inanimate items and surfaces, whereas sterilization is the complete removal of all microorganisms [30]. There are several conventional sterilization methods and mainly divided into two classes which are physical and chemical sterilization methods. Examples of physical sterilization methods are sunlight, dry heat, moist heat like autoclave, filtration, and radiation. Heat is mostly used for sterilization and microbial inactivation and its mode of action is denaturation of protein, oxidative damage, and toxic effect due to the high amount of electrolytes. In addition, the dry heat can damage the DNA of the microorganism. Several factors affect the sterilization process such as temperature, time, characteristics of microorganisms and type of sterilized material [34], [35]. Figure 1.2.1.3 shows the physical sterilization methods illustration.

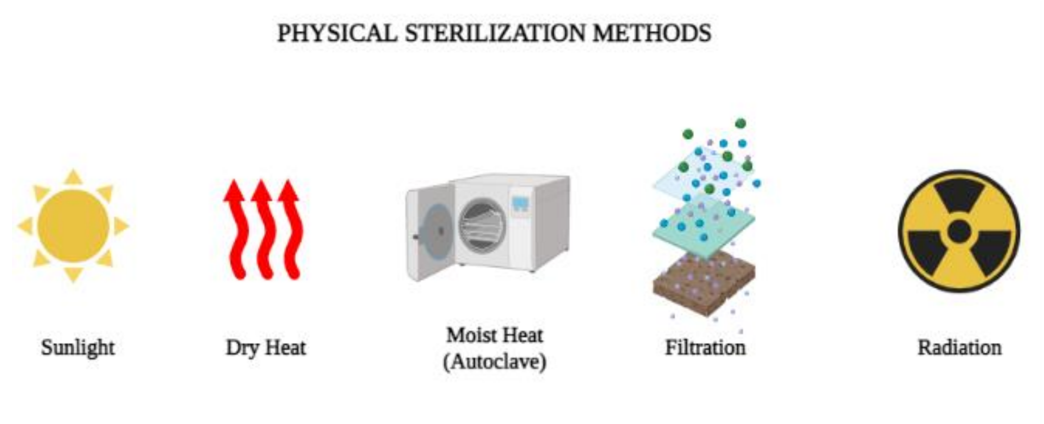

Figure 1.2.1.3: Physical sterilization methods (Drawn by BioRender).

The most common examples of chemical sterilization methods are alcohols, aldehydes, phenols, chlorhexidine, halogens, oxidizing agents like hydrogen peroxide, peracetic acid, salts, etc. and vapor phase disinfectants like ethylene oxide (ETO), formaldehyde, nitrogen dioxide (NO_2) and ozone [30]. Antiseptics and disinfectants can be used as various chemical agents, and their mode of action is similar to physical methods. Their common features are; they must behave like the presence of organic matter, have strong penetration power, be chemically stable in acidic environments, non-toxic and non-corrosive. ETO is the most widely used method among chemical methods and it interrupts DNA and RNA by interacting with radicals of protein molecules [36].

Figure 1.2.1.4: Images of conventional methods of sterilization – formaldeyhde and ETO [37].

1.2.4 CAP Treatment Parameters for Microbial Inactivation

There are a lot of parameters that affect the required microbial inactivation by CAP treatment related to plasma treatment parameters such as frequency, power, duty cycle, discharge gap, gas type, plasma generation type, exposure time, and electrode characteristics [46]. Also, the conditions of the environment such as temperature, humidity, and atmospheric pressure are quite significant for CAP discharge. *Das et al.* described the role of CAP in microbial inactivation and the factor affecting its efficacy. This paper emphasizes that gas composition and flow rate, variation of source frequency, voltage and power, distance gap, treatment times, initial concentration of microorganism suspension, growth media, and the nature of microorganisms can affect the antimicrobial efficacy of CAP [47]. *Fallon et al.* reported the operating parameters on the production of RONS for antibiofilm and the antibacterial activity of CAP. This study demonstrates that the set-up of plasma systems and CAP operating parameters how relate to the antibacterial and antibiofilm activity of CAP [48]. According to the generation type of plasma, it can add new parameters to these parameters. For instance, fluid-mediated plasma treatment, requires the treatment of a liquid, and this liquid is transferred into the microorganism suspension. The microbial strain, PALs/microorganism ratio, contact time, and liquid type are quite important parameters for microbial inactivation by PALs [49]. Especially, the antibacterial activity of PAWs is reported in a lot of studies in this field for required microbial inactivation. *Ozdemir et al*. reported a study that predicting microbial inactivation by CAP treatment with machine learning and parameters which affects the antibacterial activity of PALs [46]. *Lou et al.* investigated the parameters affecting the antimicrobial properties of CAP as plasma jet application [50]. *Shen et al.* investigated the bactericidal affects of CAP on *S.Aureus* and the physicochemical properties of PAWs at several temperatures of the environment [51]. *Ma et al.* observed the antibacterial effect of PAW on *E.coli* and *S. Aureus* by CAP at different times and reactive species which are most effective for microbial inactivation [52].

1.2.5 Effect of Electrode Materials on Antimicrobial Activity of CAP

The electrode arrangement, shape, material, and properties of material play an important role in the discharge, microbial inactivation, and biological outcome of CAP [53]. In the DBD method, which is the most used example of the direct treatment methods, the electrode configuration, shape, electrode material, and the material from which the dielectric barrier is made. Several studies have been published that use various electrode setups. Aside from the electrode design and structure, the barrier and electrode materials can influence the reactivity of the discharge by varying the discharge's electrical power. According to the principle of DBD plasma generation, the discharge electric power is a direct function of the input voltage and the electric current material on the efficiency and reactivity of the plasma accordingly. According to the Maxwell equations, it can be said that there is a relationship between material permittivity and conductivity [54], [55]. This may affect the types of generated RONS (long-lived and short-lived) during CAP treatment. Previous studies in the literature also determined the effect of electrode materials on DBD plasma generation and plasma chemistry. The capacitive resistance of conductive electrode materials changes, resulting in varying conductivity. As a result, the conductivity of electrodes constructed of different materials varies, as does the current permeability [56], [57]. The study of *Ragni et al.* examined the decontamination efficiency of electrode materials consisting of four distinct materials that can be altered in bacterial models such as *L. Monocytes* and *E. Coli* by the gas plasma reactor produced by the dielectric barrier discharge (DBD) method. As a result of this study, the highest decrease was observed in plasma treatment with electrodes consisting of silver and brass alloy [17]. In addition, in a study by *Pontiga et al*., in the DBD plasma method, the effect of several electrode materials such as stainless steel, steel, and aluminum on ozone formation was compared. While the creation of ozone was largest in the steel with the highest conductivity, it was weakest in the aluminum with the minimum conductivity [58].

1.3 Biological Activity of CAP treatment

1.3.1 Cell Biology

Cell is the smallest structure of a living thing that can display functional characteristics. Cells are the building blocks of tissues and they consist of cytoplasm and organelles in the membrane surrounding the nucleus. In the nucleus, there is some genetic material for necessary vital functions such as cell division, proliferation, and migration [59]. Cells are divided into 4 types of categories based on their morphology; i) epithelial, ii) endothelial, iii) neuronal, and iv) fibroblast [60]. The morphology of the cells may vary depending on their location and functions. Fibroblasts are the cells that building blocks of connective tissue. They are generally found in the skin of the human body [61].

1.3.2 Effect of CAP treatment on skin cells

CAP has biological effects on biological surfaces by inducing physical and chemical changes. In recent years, various studies have reported the biological outcome of CAP. CAP has a selective effect on cancerous cells [62]. Cancer cells show lower antioxidant mechanisms when compared to normal healthy cells. This property can facilitate selective attack of cancer cells by CAP mediated by the extracellular RONS, resulting in severe oxidative damage, apoptosis pathways, and cell death [63]. CAP causes oxidative stress on cells and also CAP is effective in wound healing, it can cause faster pathways of wound healing. It has a proliferative effect on skin cells and in this way, the healthy cells can proliferate and the immune cells can migrate the wound area. CAP treatment cause microholes in the cellular membrane of cells and cells try to heal these microholes there are some effects of CAP on gene expression [29], [64], [65]. *Zhong et al.* assessed a study about CAP-induced cell death and gene expression in human keratinocytes. As a result of this study, the release of several interleukin proteins (IL-6 and IL-8), tumor necrosis factor (TNF)-α, and vascular endothelial growth factor (VEGF) induced after 2 min CAP treatment [66].

1.3.3 Parameters Affecting the Biological Activity of CAP Treatment

Many studies have been conducted to measure the biological activity of CAP on epidermal (melanomas) and dermal (keratinocytes) skin cells *in vitro*. In these studies, it can be said that CAP treatment parameters have an effect on the biological activity of CAP and even an overdose has a cytotoxic effect on cells [40]. They have shown that 2 minutes or less of CAP treatment is not associated with cytotoxicity and apoptosis of cells, but an increase or decrease in plasma treatment time can stimulate or induce the proliferation and migration of melanomas or keratinocytes. Also, the type of plasma source (direct or indirect treatment), plasma source (DBD, jets, torches), gas type (Ar, air, He or O_2), frequency, gas flow rate, power voltage, and target are quite important parameters for CAP treatment on skin cells. These parameters may affect the biological activity of CAP treatment on skin cells, and the target skin disease is significant for these types of treatments [67].

In recent studies, it is reported that PAWs can promote wound healing by regulating inflammatory responses and can inactivate a variety of common wound infection bacteria. It can undergo the expression levels of the proinflammatory factors interleukin (IL)-1β and IL-6, the anti-inflammatory factor IL-10, and VEGF [68]. In addition, the parameters which affect the biological activity of CAP are plasma treatment type, gas type, treatment time, liquid type, the volume of treated liquid, contact ratio, and contact time between cells and PAWs [69].

There are a lot of parameters that affect the discharge of CAP. By keeping any of these parameters constant, the effect of changing one of them on the antibacterial and biological effectiveness of CAP treatment can be investigated [70]. In particular, the effect of dielectric barrier and electrode material changes on the discharge has been investigated in the DBD method in recent years [71]. Changing the electrical properties of materials changes plasma chemistry and leads to the differentiation of the chemical processes it causes [72]. As a result of these changes, the antibacterial and biological activity of CAP treatment on microorganisms and cells change.

1.4 Aim of the Study

This study aims to characterize the physical quantities such as RONS amount, pH, conductivity, magnetic field, and wettability of different electrode materials (copper, stainless steel, aluminum) used in dielectric barrier discharge (DBD) method in CAP applications used in biomedicine and medicine, and its effect on antimicrobial inactivation and biological activity in vitro. Thanks to this study, the characterization of the mentioned properties of PAW treated with different electrode materials will be realized. In this way, the effectiveness of different materials on many outputs will be determined, apart from the electrode material copper, which is generally used in biomedical and medical applications of CAP.

For this purpose, it is thought that this thesis will provide a foresight to the studies to be done in this context, thanks to the discharge with different electrode materials for plasma medicine applications, which are rarely seen in the literature, and have the potential to pave the way for other studies that can be done in this field. It is anticipated that the results of this thesis will contribute to the literature by comparing different electrode materials and examining them for different outputs.

The objectives of this thesis can be listed as follows;

(i) Determining the amount of reactive oxygen/nitrogen of PAWs according to the different plasma treatment times with different (copper, stainless steel, and aluminum) electrodes;

(ii) Determination of the pH values of PAWs treated with different plasma treatment times with different electrodes;

(iii) Measurements of electric fields generated by plasma treatments with different electrodes;

(iv) Wettability measurements of plasma treatments on titanium implants according to the plasma treatment times with different electrodes;

(v) OES measurements to determine the types of RONS formed during plasma treatment with different electrodes;

(vi) Performing antibacterial experiments to determine the antimicrobial effect of plasma treatments as direct and indirect treatment (with PAWs) with different electrodes;

(vii) Treatment with PAWs on skin cells and viability tests in skin cells to determine the biological activity of CAP with different electrodes;

(viii) Statistical analysis of all outputs obtained.

Chapter 2

2 Material and Methods

In this chapter, all the materials and procedures required to carry out the experimental research are mentioned.

2.1 Production of DBD electrodes

The special hand-made manufactured electrodes were used to generate a discharge by CAP and observe these discharges. The electrodes contain three main parts;

- **Insulating Cover:** The cylindrical form 40mm length and 35mm diameter cover material produced with polyethylene was used. Polyethylene was used to insulate the discharge and was designed to fit the conductive metal inside of electrodes. The insulating cover was formed on three different stages of electrodes; the upper side, middle side, and end side of the electrode. At the upper side, a cylindrical hollow 10 mm in length and diameter was done for wire connection. On the middle side, another cylindrical hallow was fitted at the end of the prior one with 29 mm length and 25 mm diameter where the conductive metal would fit. At the end of the electrode, the third cylindrical hallow was made of 1 mm length and 31 mm diameter where the barrier would fit.
- **Conductive Metal:** Different metals such as copper, stainless steel, and aluminum were used as conductive materials in the electrodes. All metals were designed with identical physical properties which have a length of 28mm and a diameter of 25 mm. Each metal was fitted in the middle hallow and the stabilization of two materials is provided by silicone.

- **Dielectric Barrier:** It is an insulating material that is used for the generation of the discharge between two electrodes (high voltage and ground electrodes). Materials such as glass, ceramic, and quartz can be used as a dielectric barrier. In this study, glass was used as the barrier material and the glass was adhered to the polyethylene material on the end stage using epoxy glue.

2.2 Generation of PAW by CAP treatment

The DBD method was used for CAP treatment, and PAW was activated using PVM500 DBD air plasma. In the CAP treatment setup, adjustable optical holders were used to locate the position of the electrode along the X, Y, and Z axes. 500 µL of DIW was treated using the DBD method with CAP treatment for 0, 5, 15, 30, 45, 60, 90, 120, 150, and 180 s, using specially produced electrodes made of copper, aluminum, and stainless steel. The discharge gap is adjusted to 2mm for all the experiments with treatment by CAP. The generation method of CAP treatment and special setup is shown in Figure 2.2.1.

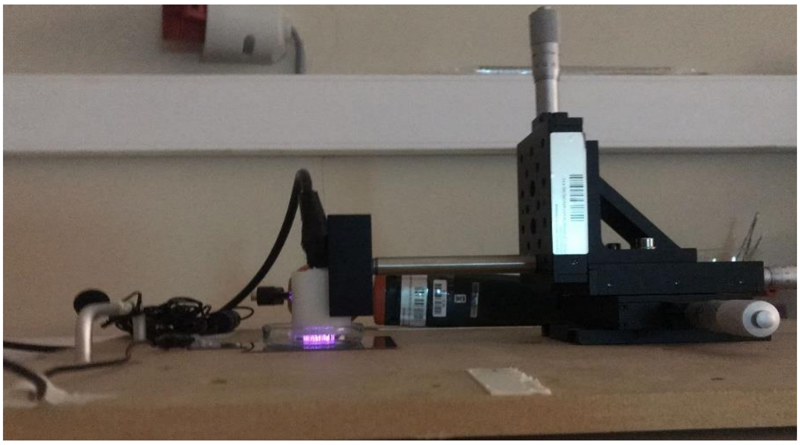

Figure 2.2.1: Special hand-made setup for CAP treatment.

2.3 Determination of RONS by KI-Starch test

To prepare the solution, KI (TEKKIM Chemical Industry Trade Limited Company) soluble potato starch (Sigma Aldrich) was used. 240 g of KI and 120 g of starch were weighed with a precision scale. The starch was dissolved in 20 mL of warmed deionized water (DIW) and mixed in the magnetic stirrer until homogenous.

After heating the prepared solution to room temperature, KI was added and stirred until it was completely homogeneous again. 100 μL of prepared KI-Starch solution was transferred onto the 96-well plate by plasma treatment time groups. After plasma treatment, 100 μL of PAW was added to each well. Due to the interaction of RONS and KI, a color change close to blue-violet was observed with respect to the plasma treatment time points as shown in Figure 2.3.1 for the aluminum electrode.

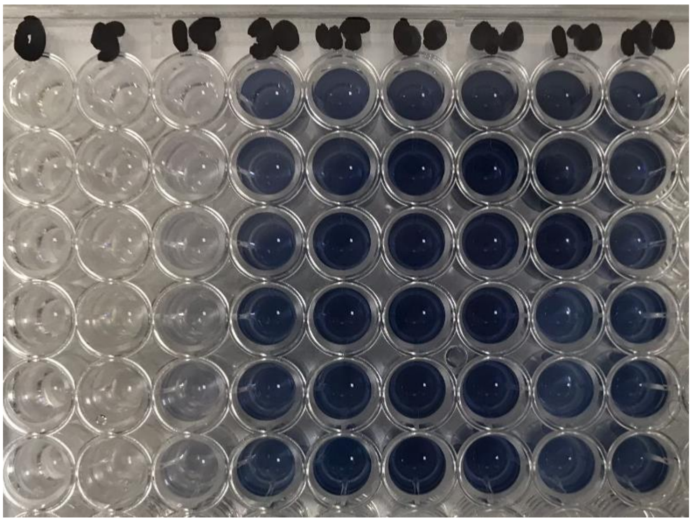

Figure 2.3.1: Determination the amounts of RONS in PAWs by KI-Starch test.

The samples prepared to determine the amount of RONS generated in CAP-treated groups were examined at a wavelength of 680 nm in a microplate reader (Biotek, Synergy HTX, USA). Only DIW was added to the KI-Starch solution for the control groups, and measurements were performed.

2.4 pH Measurements of PAW Groups

A bench-top Conductivity/TDS/Salinity/pH Meter (SperScientific, USA) was used to determine the acidity/basicity degree of PAW groups according to the plasma treatment times. After the CAP treatment of 500 μL of DIW at 0, 5, 15, 30, 45, 60, 90, 120, 150, and 180 s, the pH values were measured immediately. This process was repeated separately for copper, aluminum, and stainless steel electrodes.

2.5 Wettability Measurements of PAW Groups

Titanium discs used in the wettability test were supplied by Metrosan End. and Elk. Med Devices and Medical Malz San Tic Ltd Sti, consisting of a medical grade-5 Ti6Al4V alloy with a diameter of 10 mm and a length of 3 mm. In the wettability test, the surface contact angle of each group will be photographed by dropping 4 μL of water and measured with the help of the Image J program. Measuring the contact angle (θ), is aimed to determine the hydrophilic or hydrophobic properties of the surfaces. These measurements were repeated three times for all plasma treatment time groups and three electrodes.

2.6 Optical emission spectroscopy (OES) Measurements of Electrodes

OES measurements are performed for all electrodes and OES is the method that shows which RONS types are formed when discharge is formed by CAP. These measurements were done with fiber optic cable and a special optic spectroscopy device by special software. These measurements were repeated three times for all plasma treatment time groups.

2.7 Electric Field Measurements of Electrodes

The electric field of electrodes was measured by using a Radio-Frequency EMF strength meter (Extech Company; 480836). Each electrode has placed a position according to the marked orientations. The distance between the electrode which is measured and the device was kept constant. The electric fields of three electrodes were measured at three different times. First, as a control no plasma discharge generation was found, second was the initial point of discharge generation, and third was the end point of discharge. The operating parameters of CAP are 20 kV voltage and 1.67 kHz frequency.

2.8 Investigation of Antibacterial Activity of CAP Treatment

In this section, the effect of CAP treatment of different electrode materials on the antibacterial activity was investigated by two different methods, measurement of the zone of inhibition (ZOI) and colony counting. While microbial inactivation of plasma was observed in a certain area with ZOI measurements, colony counting was done in two separate procedures, direct and with PAWs.

2.8.1 ZOI Measurements

The ZOI was used to investigate the antibacterial activity of the CAP treatment utilizing different electrode materials. The model bacterium *E.coli ATCC 25922* was cultured overnight in tryptic soy broth (TSB, Sigma Aldrich). Bacterial suspensions of all groups were diluted to 10^7 CFU/mL in PBS using the serial dilution method. 100 µL of diluted bacterial samples were spread on tryptic soy agar (TSA, Sigma Aldrich) and agar plates treated with CAP by DBD method using three different electrodes at 0, 5, 15, 30, 45, 60, 90, 120, 150 and 180s.

2.8.2 Colony Counting Method

2.8.2.1 Direct Treatment

E.coli (ATCC 25922) which is the model bacteria was used for the colony counting method. The overnight bacterial suspension was diluted at a dilution ratio of 1:10 to a concentration of $2x10^5$ CFU/mL. 500 µL of bacterial suspension was treated with CAP treatment for three electrodes separately and for different specified times. Then, the CAP-treated bacterial suspensions were re-diluted 1:10 ratio and spread on 100 µL plates at a concentration of 10^3 CFU/mL. Each experiment was repeated three times for each electrode and plasma treatment times.

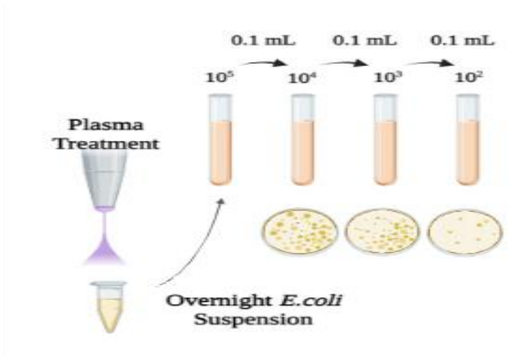

Figure 2.8.2.1: The illustration of the colony counting method by direct CAP treatment (Drawn by BioRender).

2.8.2.2 CAP Treatment by PAWs

E.coli (ATCC 25922) which is the model bacteria was used for the microbial inactivation method using liquid treated with CAP. 500 µL of DIW CAP was treated with different electrodes at the specified plasma treatment times. The PAWs were then mixed with 10^5 CFU/mL bacterial suspension at a ratio of 1:5 and contacted for 30 min. This incubated solution was then further diluted 1:10 and spread on 100 µL plates at a concentration of 10^3 CFU/mL. Each experiment was repeated 3 times for each electrode and plasma treatment times.

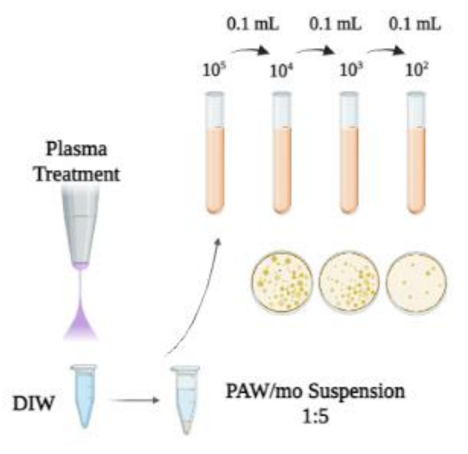

Figure 2.8.2.2: The illustration of colony counting method CAP treatment by PAWs (Drawn by BioRender).

2.9 Investigation of Biological Activity of CAP Treatment

2.9.1 Cell Culture

Mouse fibroblast cell line (L929 Sigma Aldrich) was used to determine the biological activity of CAP treatments with different electrodes. Cells were taken from -80 degrees and cultured in T75 flasks for one week in a medium prepared with RPMI 1640 Medium with Stable Glutamine + Fetal Bovine Serum (FBS) to ensure proliferation. The medium of the cell suspensions was changed every two days.

2.9.2 Indirect CAP treatment with PAWS on cells

Mouse fibroblast cells (L929) were seeded at 10^4 cells/well in 96-well plates. The seeded cells were mixed at a 1:1 concentration ratio with groups of PAWs after one day of incubation. Then, the mixed suspensions of cells and PAWs were incubated for 30 min. The incubated suspension was removed from the wells and renewed with the growth medium.

2.9.3 MTT Assay

MTT Assay was used to measure cell viability. The MTT assay was a 96-well plate fast colorimetric assay created for cell viability high screening. The conversion of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to an insoluble blue formazan product by mitochondrial succinate dehydrogenase is measured in this test [73]. The solutions prepared at the content of 90% MTT solution and 10% growth medium were incubated for 2 hours after adding to each group. Then, the MTT solutions were eliminated and DMSO was added to each well and the absorbance values were measured in a multimode reader at 570 nm. MTT assay was repeated until 1,3,5 and 7 days. Figure 11 shows the illustration of the cell viability experiment for each electrode.

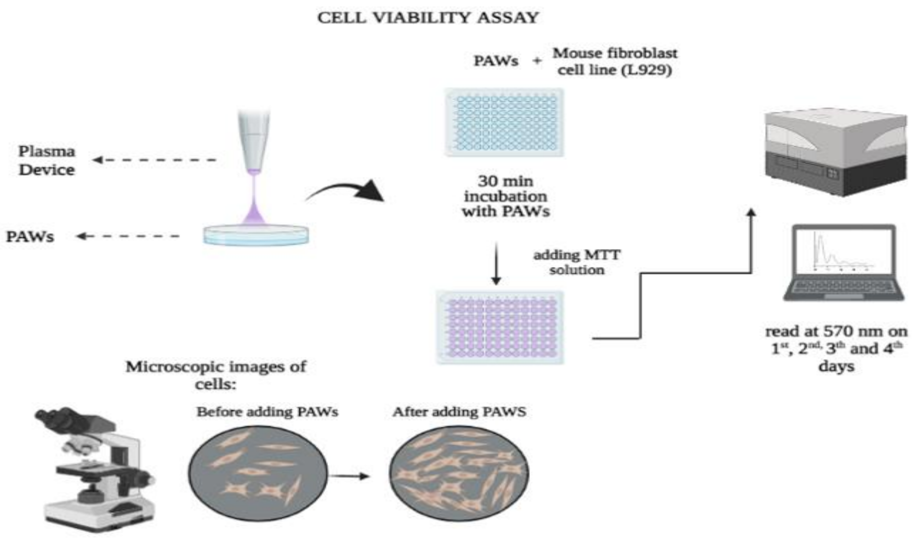

Figure 2.9.3.1: The illustration of cell viability assays for investigating the biological activity of indirect CAP treatment (Drawn by BioRender).

Chapter 3

3 Results

3.1 Determination of RONS by KI-Starch Test

The amount of RONS in PAW groups is determined by KI-Starch test and Figure 3.1.1 shows the absorbance values according to the plasma treatment times for each electrode.

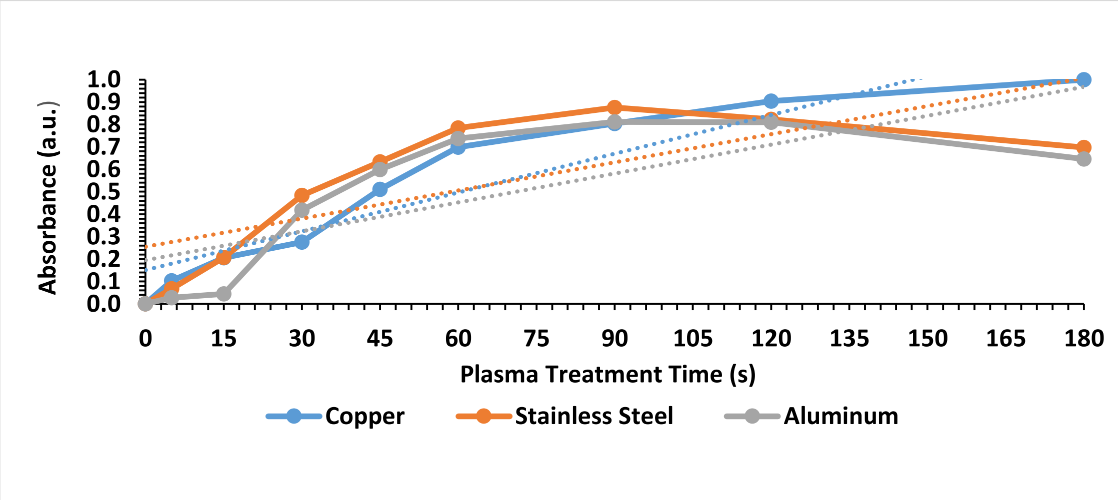

Figure 3.1.1: Absorbance values of RONS measurement via KI-starch test.

The darkest color shows that this group has the highest absorbance value and amount of RONS. When the color is seen among the groups, the 180 s CAP treatment group has the darkest color, and the 0 s CAP treatment group has the lightest color. For three distinct electrode materials, the absorbance values increased as the plasma treatment period increased. This rise in copper continues until the final plasma treatment time point. The absorbance value of the remaining two electrodes (stainless steel and aluminum) rose to 90 s after CAP treatment. The copper electrode has the highest absorbance value, whereas the aluminum electrode has the lowest absorbance value. The absorbance plots, on the other hand, exhibited an increasing slope about the conductivities of the electrode material, resulting in a significant difference observed.

3.2 pH Measurements of PAW Groups

The pH measurement results of PAW groups are demonstrated in Figure 3.2.1. Acidification is an important parameter for antibacterial activity. Although no significant acidification was noticed following the shortest CAP treatment, the pH value dropped to 3.00. The copper group had the greatest pH, while the aluminum group had the lowest pH. It was discovered that changing the electrode material affected the pH measurements. The findings demonstrated that the electric conductivity of the electrode materials has a significant relationship with pH change. The "decreasing trend" was observed in pH values as plasma treatment time increased for all groups.

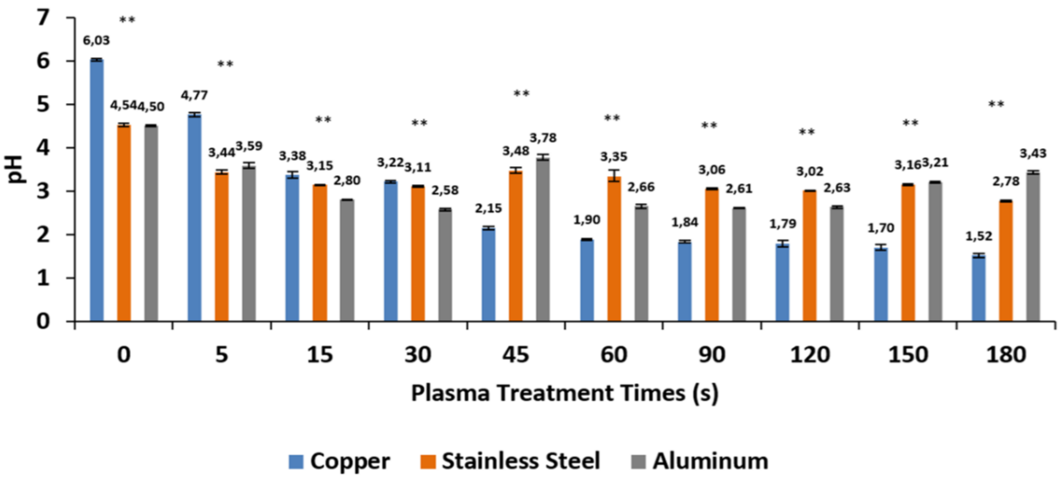

Figure 3.2.1: pH measurement results of groups ($p < 0.05$ and $p < 0.01$ is statistically significant and show the * and **).

3.3 Wettability Measurements of PAW Groups

The wettability measurements results are shown in Figure 3.3.1 and the contact angle values on titanium disks were measured by Image J software. Wettability is quite important for bacterial adhesion, bacteria adhere to hydrophobic surfaces more easily, and the hydrophilic nature of the surface causes the bacteria to be unable to adhere to the surface and growth.

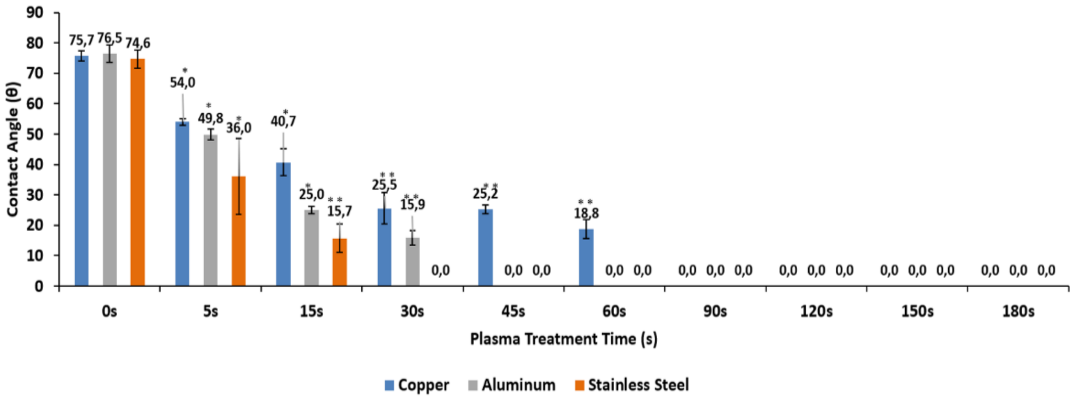

Figure 3.3.1: The contact angle values of groups on titanium implants according to the plasma treatment times ($p < 0.05$ and $p < 0.01$ is statistically significant and showing the * and **).

After 15s, the contact angle value of the titanium disc which is treated by stainless steel electrode is zero. This means that the stainless steel electrode is the most effective electrode on the wettability of titanium implant. The copper electrode is the least effective electrode on the wettability of titanium implant due to the contact angle value of this electrode dropping to zero after 60s CAP treatment. For all electrodes, it can be said that the plasma can make a super hydrophilic surface on titanium implants after the 60s.

3.4 OES Measurements of Electrodes

OES measurements of electrodes are shown in Figure 3.4.1. OES results indicate that types of RONS when a discharge formed and intensity of peaks at specific wavelengths.

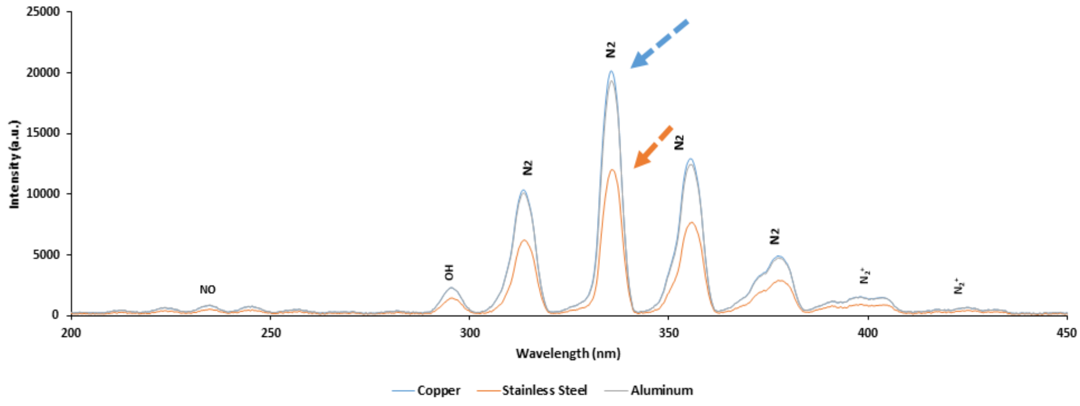

Figure 3.4.1: OES results of different electrode materials which show which types of reactive species formed.

According to the OES results, NO, OH, N_2, and N_2^+ species were formed during the discharge of all electrodes. The species formed are similar in all electrodes. When the intensity values at specific wavelengths are compared, they are generally copper electrode, aluminum electrode, and stainless steel electrode, respectively, from highest to lowest. Here, although no change was observed in terms of species difference, a significant change was observed in signal magnitude.

3.5 EMF Measurements of Electrodes

The electric field measurement results of each electrode are shown in Figure 3.5.1. As can be seen from Figure 3.5.1, the highest electric field occurred during the discharge of the copper electrode. The lowest electric field was formed during the discharge of the aluminum electrode. There is a direct relationship between the amounts of the electric field when they discharged the electrodes and the conductivities of these electrodes. It can be said that plasma can easily be generated by the copper electrode.

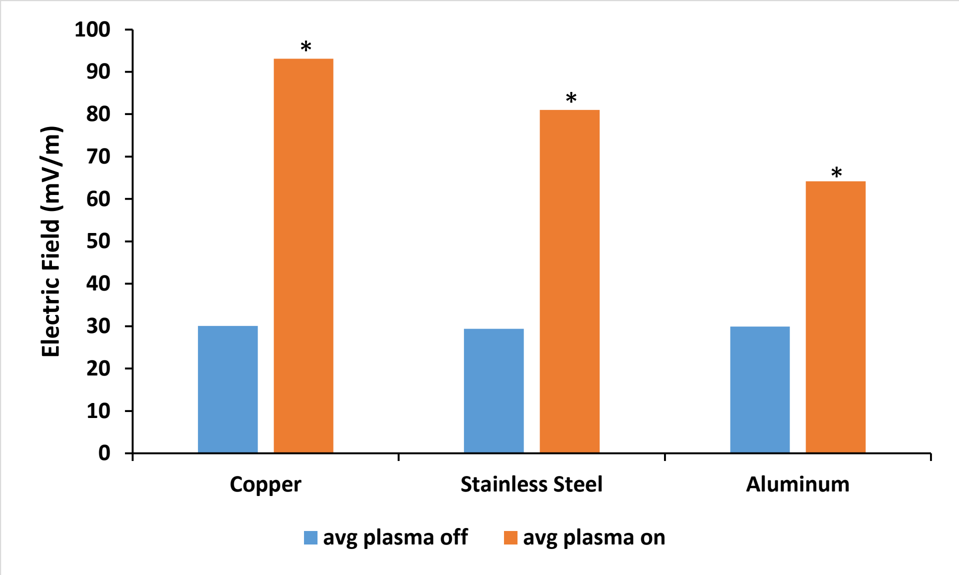

Figure 3.5.1: EMF measurement results of different electrodes when plasma discharge is off and on.

Also, “avg plasma off” and “avg plasma on” means the average amount of electric field when plasma is discharged or not discharged. Plasma off value can be accepted as a control value. According to the average plasma off values, the amount of electric field measured in the discharge of all electrodes is quite high compared to the control.

3.6 Antibacterial Activity of CAP Treatment

After characterization of all electrodes during discharge or on PAW, ZOI measurements and colony counting were made for antibacterial activity. ZOI measurements were analyzed using the Image J program. Colony counting was analyzed manually.

3.6.1 ZOI Measurements

The images of agar plates that are used for ZOI measurements of electrodes according to the plasma treatment times are illustrated in Figure 3.6.1.1.

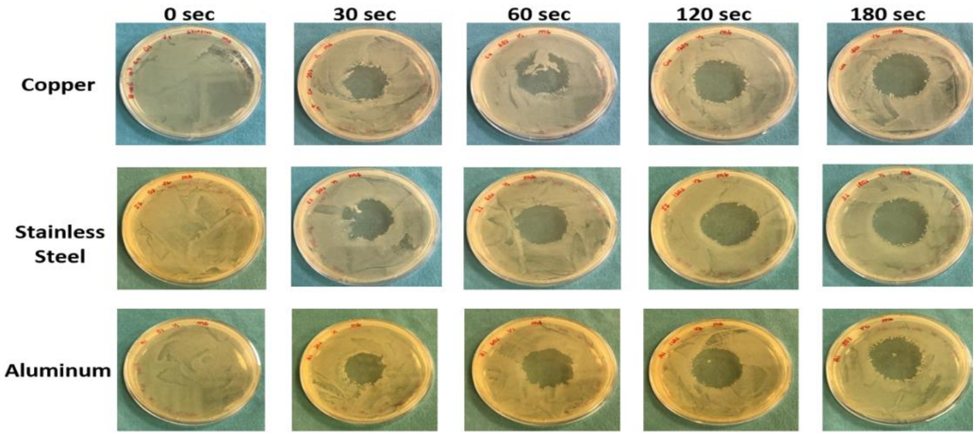

Figure 3.6.1.1: Images of agar plates indicating the ZOI of groups.

The groups 0, 30, 60, 120, and 180 s were investigated for optimization between plasma treatment periods. Bacteria could not grow in the CAP-treated areas, and ZOI on agar plates formed as a result. The groups with the highest ZOI for all three electrodes were observed at the increased treatment time points, as expected. Because the antibacterial activity is proportional to plasma treatment time, the groups' ZOI increased as the plasma treatment times increased. When the agar plate photographs were examined as a qualitative method, the electrode with the highest ZOI was observed as stainless steel for all plasma treatment times, and the electrode with the lowest ZOI was observed as aluminum.

The results of ZOI measurement for optimization with ImageJ software are shown in Figure 3.6.1.2 and this optimization process is required for specifying the optimum duration of plasma treatment for effective microbial inactivation by all electrodes. There are significant results for 0, 30, 60, 120, and 180s. The mean of ZOI measurements for all electrodes, stainless steel electrode has highest ZOI result and aluminum electrode has lowest ZOI result. In the literature, the optimum plasma treatment time for microbial inactivation generally is 60 s CAP treatment. However, the optimum plasma treatment time depends on electrode material, so it is quite necessary for determining the antibacterial activity of CAP treatment by different electrode materials.

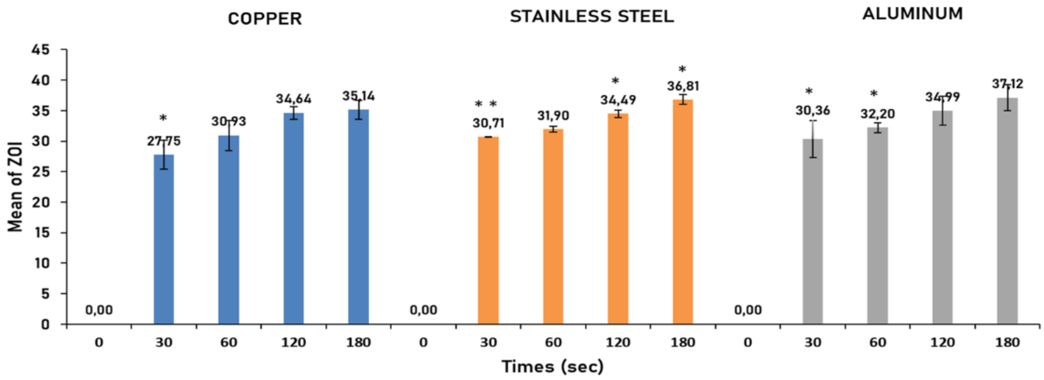

Figure 3.6.1.2: Graph of ZOI according to the optimization of plasma treatment times ($p < 0.05$ and $p < 0.01$ is statistically significant and showing the * and **).

The results of ZOI measurement with ImageJ software are shown in Figure 3.6.1.3 and this method is a quantitative method which showing which electrode is more effective As a consequence, as expected, increasing the duration of treatment enhanced the efficacy of the CAP treatment. When the groups were compared, stainless steel had the greatest mean ZOI value and aluminum had the lowest. According to ZOI data, the electrode materials with the highest to lowest antibacterial activity were stainless steel, aluminum, and copper. After 180 seconds of CAP treatment, copper was found to be less efficient than the other materials in determining a ZOI of groups. The ZOI results demonstrate that the electrode material difference affects its antibacterial activity and may be an essential parameter for future research. After the 150s, there is a reducement in stainless steel and aluminum electrodes slightly.

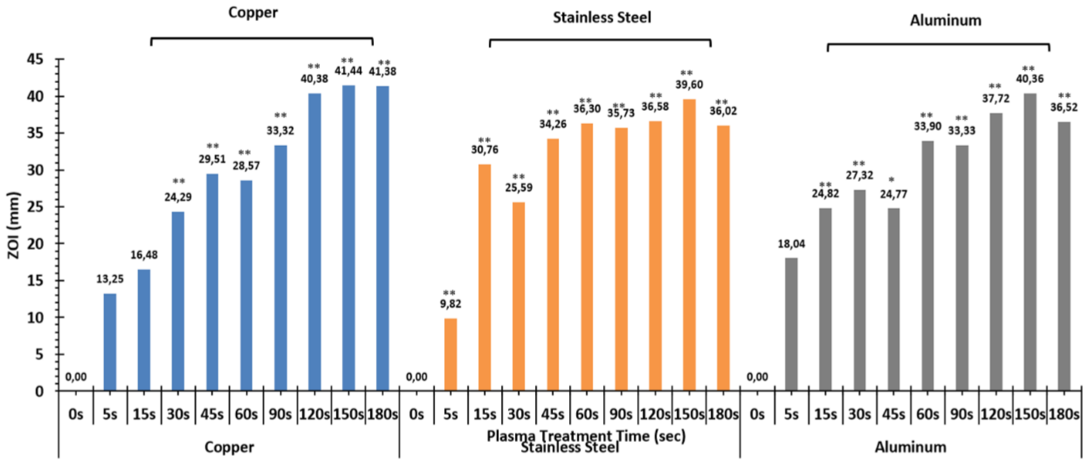

Figure 3.6.1.3: Graph of ZOIs according to all plasma treatment times ($p < 0.05$ and $p < 0.01$ is statistically significant and showing the * and **).

3.6.2 Colony Counting Method Results

Colony counting results were analyzed by using two different methods as direct and CAP treatment by PAWs. For direct treatment, CAP treatment was applied to bacteria suspended with all electrodes. In the CAP treatment by PAWs method, CAP treatment was first applied to the DIW, and then the colonies were counted by mixing with the bacterial suspension at certain ratio. Since the mode of action of these methods is different, different results are expected from these experiments.

3.6.2.1 Direct Treatment

Figure 3.6.2.1 shows the graph of bacterial reduction by direct CAP treatment by all electrodes with initial and final count log10 mean. For stainless steel electrode, initial concentration of bacteria is 4.54 log CFU/mL. After 30s CAP treatment by stainless steel electrode, the log reduction is around 5-log which is most effective antibacterial activity on *E.coli*. The log reduction with aluminum electrode is around after 30s CAP treatment. However, the copper electrode killed all the bacteria after 60s CAP treatment which has 4log reduction. It can be said that while the copper electrode killed the bacteria in 60 s, the aluminum electrode killed the bacteria in 30 s.

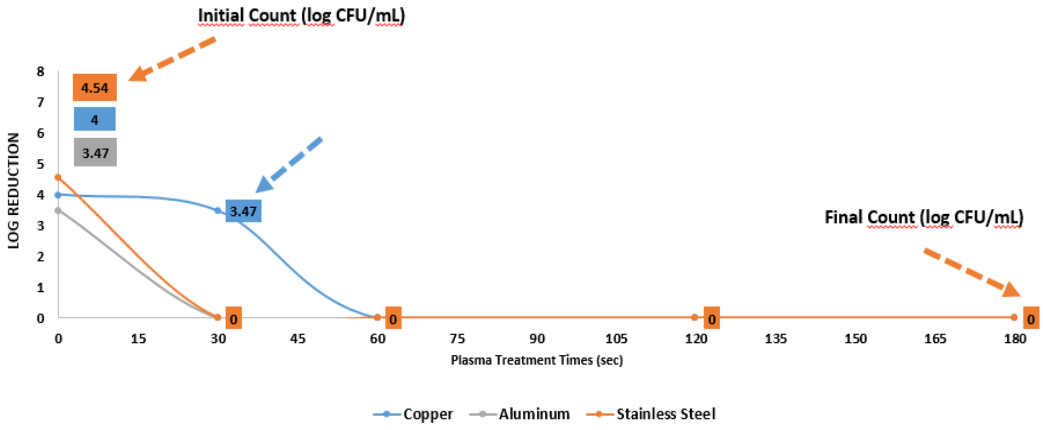

Figure 3.6.2.1: Graph of bacterial reduction by direct treatment with initial and final count log10 mean ($p < 0.05$ for all groups ; Dilution Factor: 1/1000th or 10^3).

All initial concentrations are very close values for all electrodes and all experiments repeated three times for all electrodes and plasma treatment times.

3.6.2.2 CAP Treatment by PAWs

Figure 3.6.2.2 shows the graph of bacterial reduction by CAP treatment by PAWs all electrodes with initial and final count log10 mean. For PAWs treated by aluminum electrode, the initial concentration of the bacteria is 4.32 log CFU/mL. After 180s CAP treatment by aluminum electrode, the log reduction is approximately 4log and it could kill all the bacteria. It is the most effective group for antibacterial efficacy on *E.coli* with indirect treatment. For PAWs treated by copper electrode, there is a 0.7log reduction and for PAWs treated by stainless steel electrode, there is a 2log reduction after 180s CAP treatment. It is the least effective group for antibacterial efficacy on *E.coli* with indirect treatment. All initial concentrations are close to each other for electrodes and comparing with direct treatment, it was observed that for PAWs have less antibacterial activity than direct treatment. All experiments are repeated three times for all electrodes and plasma treatment times. There are significant results for direct and indirect treatment by different electrodes. For direct and indirect CAP treatment, plasma has an antibacterial effect on *E.coli* and this activity increases with increasing plasma treatment times.

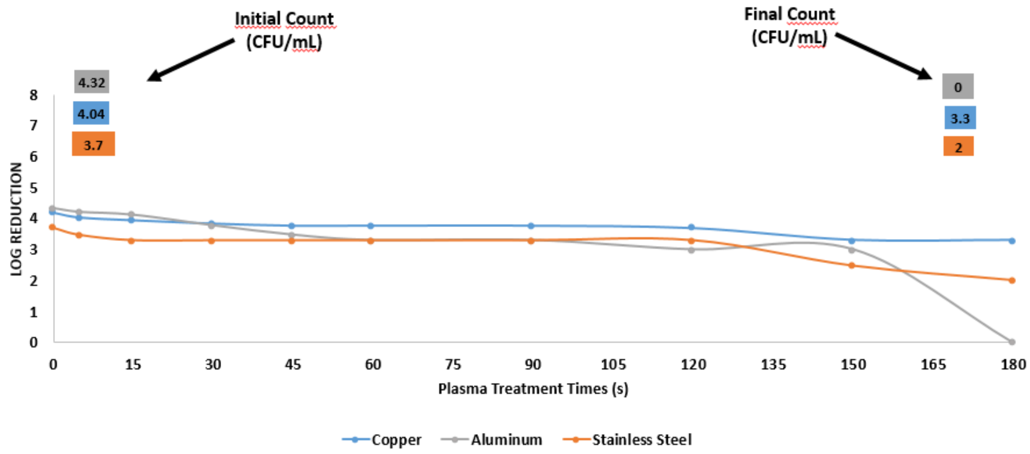

Figure 3.6.2.2: Graph of bacterial reduction by indirect treatment- PAWs with initial and final count log10 mean ($p < 0.05$ for all groups ; |Dilution Factor: 1/1000th or 10^3).

3.7 Biological Activity of CAP Treatment

The effect of different electrodes on biological activity with CAP treatment was tested with cell viability assays. Cell viability analyzes were performed on days of 1, 3, and 5 after CAP treatment.

3.7.1 Cell Viability

Figure 3.7.1, Figure 3.7.2, and Figure 3.7.3 demonstrate that cell viability assay results of PAWs treated with copper, aluminum and stainless steel electrodes until day of 5. As a result, 150s and 180s show that cytotoxic results for skin cells and cell number of wells reduced and cells apoptated for all days.

The highest amount of viable cells are 100, 87 and 123 percent in the 120s, 60s and 120s groups at the first day, third day and fifth day, respectively. After 60s and 120s CAP treatment by copper electrode on cells, biological activity of CAP treatment increased at day of 1 and 5. However, after 180s, there is a significant reduction on amount of viable cells. This means that as the optimum plasma treatment time increases, it can stimulate the apoptotic pathways of cells and induce apoptosis.

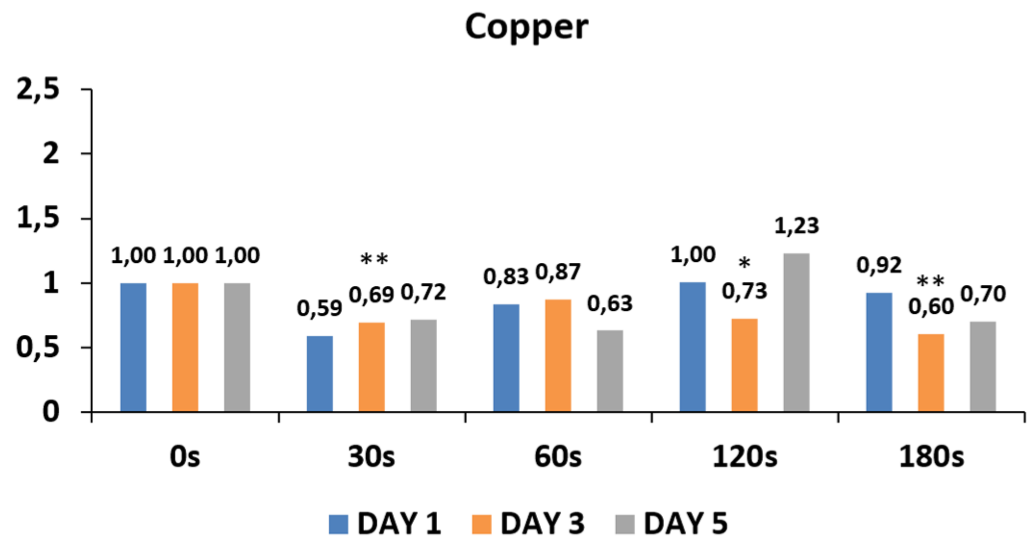

Figure 3.7.1: Cell viability results according to the plasma treatment times for copper electrode at day of 1, 3, and 5 (Initial Cell Count: 10^4 cell/ cm^2 ; $p < 0.05$ and $p < 0.01$ is statistically significant and showing the * and **).

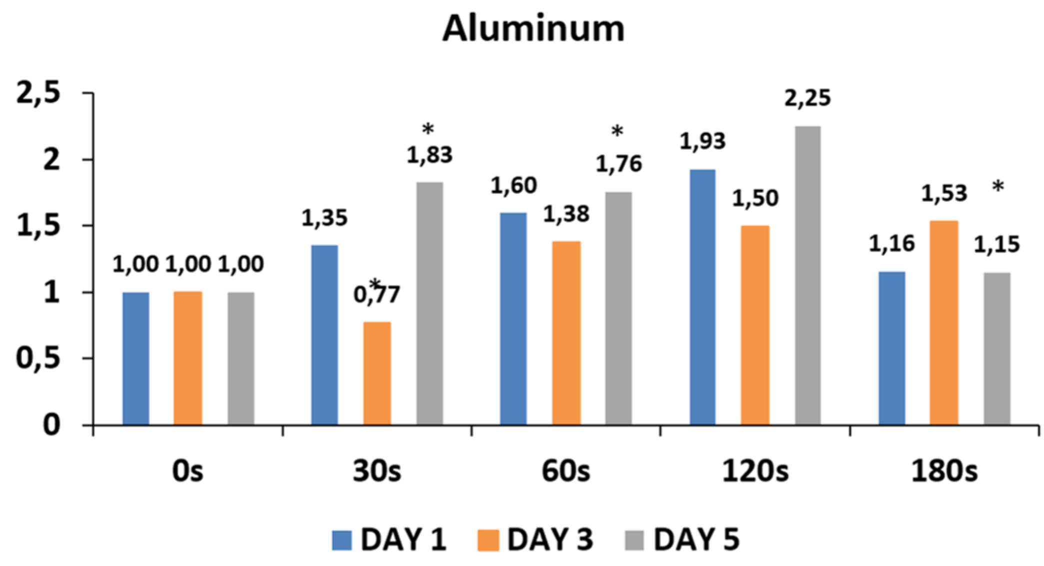

Figure 3.7.2: Cell viability results according to the plasma treatment times for aluminum electrode at day of 1, 3, and 5 (Initial Cell Count: 10^4 cell/ cm^2 ; $p < 0.05$ and $p < 0.01$ is statistically significant and showing the * and **).

For aluminum electrode, the highest amount of viable cells are 193, 153 and 225 percent in the 120s, 180s and 30s groups at the first day, third day and fifth day, respectively.

After 180s CAP treatment shows that cytotoxic results for skin cells and cell number of wells reduced and cells apoptated according to after 120s CAP treatment.

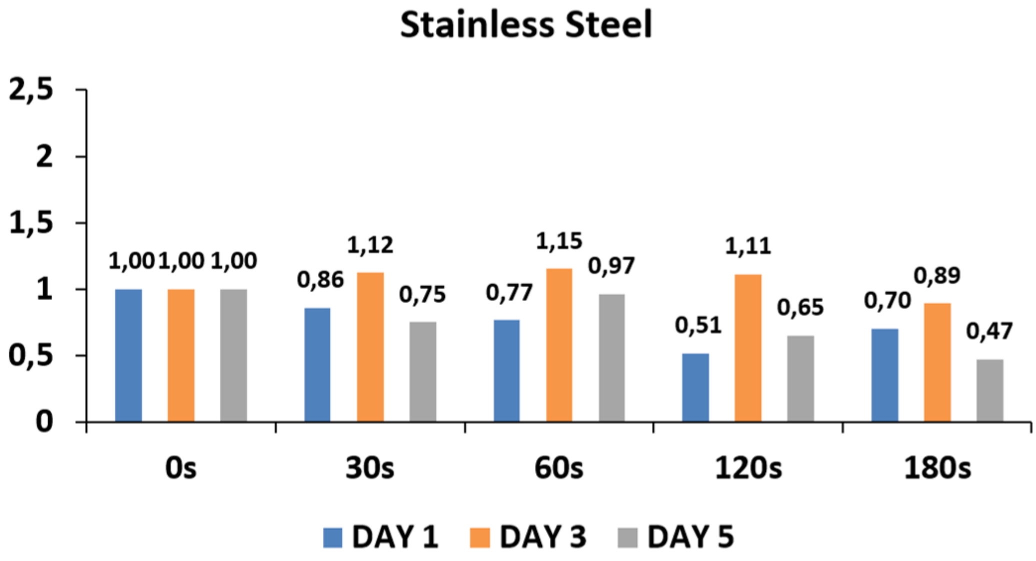

Figure 3.7.3: Cell viability results according to the plasma treatment times for stainless steel electrode at day of 1, 3, and 5 (Initial Cell Count: 10^4 cell/ cm^2 ; $p < 0.05$ and $p < 0.01$ is statistically significant and showing the * and **)

For stainless steel electrode, the highest amount of viable cells are 86, 115 and 97 percent in the 30s, 60s and 60s groups at the first day, third day and fifth day, respectively. After 180s CAP treatment shows that cytotoxic results for skin cells and cell number of wells reduced and cells apoptated according to after 120s CAP treatment.

Chapter 4

4 Discussion

To specify the parameters which affecting the CAP treatment and its applications, this study presents a new parameter which related to electrical characteristics of electrode material. According to the literature, there are a lot of factors that affects the microbial inactivation by CAP treatment and this factors can be related with CAP devices parameters (electrical power, frequency, discharge gap, duration of treatment etc), used gas, concentrations, properties of microbial strain and environmental conditions [74]. In addition, the generation type of plasma and the microbial strain used are very important factors in the biological and antibacterial activity of CAP [8], [75]. On the other hand, the electrode configuration, shape, material, and substance from which the dielectric barrier is made are important. Beyond from electrode arrangement and design, the barrier and electrode materials can also influence discharge reactivity via varying discharge electrical power. Some research in the literature investige that electrode properties and its effect on discharge of plasma. *Laroussi et al*. reported the resistive barrier discharge which the resistivity of the electrode material can change the electrical signals of discharge of CAP treatment and this leads to expansion of applications of CAP treatment [76]. *Govaert et al.* demonstrated a study which related to comparing the influence of electrode configuration and gas composition for microbial inactivation by CAP treatment and as a result, the electrode configuration of CAP treatment is the more important parameter than gas composition [77]. In addition the these studies, some studies investigating the effect of electrode material on microbial inactivation by CAP treatment. In addition, the generation types of CAP treatment is quite important to achieve the intended results for microbial inactivation. There are a lot of studies in the literature which investigated the antibacterial activity of PALs. Liquid type, PAL/microorganism ratio, and contact time are considerable parameters for required microbial inactivation. There are a few studies about investigating the electrode material on microbial inactivation by CAP treatment. For instance, *Pontiga et. al* measured the amount of ozone formed as a result of CAP treatments with different electrode materials, but its effect on biological output was not discussed [58]. *Ragni et. al.* investigated the effect of different electrode materials and CAP treatments on antibacterial activity, but not all of the electrode materials used are conductive materials [17]. These studies in the literature show that the electrode material is a significant effect on CAP treatments. To the best of our knowledge, no studies have previously relating the electrical characteristics of electrode materials on antibacterial and biological activity of CAP treatment. This study compares and investigates the effect of conductive electrode materials on plasma discharge with DBD method and associate the electrical properties of electrode materials and outcomes of plasma discharge on biological and antibacterial experiments.

In this study, the effect of three different electrode materials (copper, aluminum, stainless steel) of CAP treatment with DBD method on antibacterial activity was demonstrated by preliminary experimental studies. According to the determination of amounts of RONS, copper electrode has highest absorbance value and aluminum has least absorbance value in the Figure 7. It can be said that the amount of RONS is directly proportional to the electrical conductivity of the electrode material. However, the types of RONS that occur are not known to be long-lived or short-lived. OES results shows that which types of RONS are generated by CAP treatment during discharge. According to the OES results, NO, OH, N_2 and N_2^+ types were formed during the discharge of all electrodes. Some of these species are short-lived species and some of them these species are long-lived species. For instance, OH and NO are the examples of short-lived species and they can leads to inactivate the microorganisms [78]. N_2 and N_2^+ are the long-lived species and they can lead to selective inhibition of cancer cells or microbial inactivation [79]. As expected, in the wettability measurement results, the contact angles of all groups on titanium implants dropped with increasing plasma treatment times as in the literature [80]–[82]. For all electrodes, it can be said that the plasma can make a superhydrophilic surface on titanium implants after 60s.

In the literature, there are some studies about EMF and DBD plasma discharge. *Cretu et al.* investigated the effects of parameters such as discharge gap and frequency on the electromagnetic field created by the plasma. This study shows that as the values of parameters such as discharge gap and frequency increase, the size of the magnetic field decreases [83]. Beniugă et al. demonstrated a study which measuring the magnetic flux density of DBD plasma treatment on air or air-water mix at 20 cm discharge and in 60 kHz. As a result, the difference of target can affect he magnitude of magnetix flux density when plasma discharged [84]. All parameters reported in the literature show that any change in the plasma device or parameters may affect the magnitude of electromagnetic field generated by plasma. In this study, the effect of different electrode material investigated when plasma discharge is on or off in average. According to the EMF results, there is a directly relationship with conductivities of electrodes which related with permittivity and permeability of materials as mentioned previously. Also, EMF is related to antibacterial effects of plasma activated water in the literature [85]. When the plasma discharge is off, average values of EMF are same for three electrodes.

For interpretation of investigation the antibacterial activity of CAP treatments, it can divided into two groups as direct and PAWs groups. All ZOI measurements are conducted by direct treatment. The most effective electrode material is stainless steel respect to the ZOI measurement results. It is predicted that this result may be directly due to the difference of chemical reactions caused by RONS for microbial inactivation of CAP treatment on bacteria on agar plates. However, the antibacterial activity results of PAWs were measured by the colony counting method. According to the colony counting results, the group with antibacterial activity from the highest to the lowest was determined as aluminum, stainless steel, copper. This result shows that it has an inverse relationship with the electrical conductivity values of the electrode materials.

Finally, according to the biological outcome of this study, the MTT results show that aluminum, stainless steel, copper have the highest to lowest cell proliferation effect on skin cells. Also it can be said that after 120s and 180s generally observed the cytotoxic effects on cells and this issue may cause apoptosis of cells. Furthermore, this study can be developed with further experiments like cytotoxicity assays, determination of antibacterial activity on biofilm, and using different microbial strains.

Chapter 5

5 Conclusion

The effect of three alternative electrode materials of CAP treatment with DBD technique on antibacterial activity was demonstrated in this study. According to ZOI findings, the difference in electrode materials affects the amount of RONS, pH, and antibacterial activity in CAP treatment, and the electrode material with the highest antibacterial activity was found to be stainless steel in most groups. The most unexpected results in terms of electrode material on antibacterial activity were reported after 180s of treatment, with the stainless steel electrode displaying a higher reduction than the copper and aluminum electrodes. According to the colony counting results, it can be said that aluminum electrode is the most effective group for antibacterial efficacy on *E.coli* with indirect treatment after 180s CAP treatment, the log reduction is approximately 4log and it could kill all the bacteria. Moreover, the results showed that this behavior was not related to pH. Also the antibacterial activity of CAP treatment depends on generation types of plasma, this leads to difference in the results of antibacterial experiments. The inversely proportional relationship between antibacterial experiment results and RONS determination shows that each electrode material may form different types (long-lived and short-lived) and amounts of reactive species. The cell viability results show that aluminum, stainless steel, copper have the highest to lowest cell proliferation effect on skin cells. Along with the further experiments in the future, this study will be developed and will fill the gap in the literature and can be used in many areas, especially in the field of plasma physics and plasma medicine and which RONS types are more effective for required microbial inactivation.

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Curriculum Vitae

MERVE GUL

WORK EXPERIENCE

Intern (Clinical Engineering)

Manisa Celal Bayar University Hafsa Sultan Hospital [01/07/2017-01/08/2017]

- Sales, maintenance-repair and quality processes of medical devices in clinical engineering

Intern (Biomechanical Engineering)

Dokuz Eylul University Hospital [01/07/2018 – 01/08/2018]

- Biomechanical analysis of implants
- Production and use of 3D printers
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EDUCATION AND TRAINING

Bachelor Degree in Biomedical Engineering

Izmir Katip Celebi University [15/09/2015 – 16/07/2020]

Field(s) of study: Engineering, manufacturing and construction

Final grade : 3.36/4.0

Thesis: The Effect of Atmospheric Cold Plasma on Corrosion Resistance in Titanium Dental Implants

Master Degree in Biomedical Engineering

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Field(s) of study: Natural sciences, mathematics and statistics : Biological and related sciences not further defined Inter-disciplinary programmes and qualifications involving natural sciences, mathematics and statistics

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PUBLICATIONS

1. Gul M, Ozdemir GD, Ercan UK. Investigation of the Effect of Electrode Material on Antibacterial Activity in Plasma Treatment with Dielectric Barrier Discharge. 2022 Med Technol Congr [Internet]. 2022 Oct 31 [cited 2022 Dec 18];1–4.
2. Ozdemir, M. A., Ozdemir, G. D., Gul, M., Guren, O., & Ercan, U. K. (2022). Machine Learning to Predict the Antimicrobial Activity of Cold Atmospheric Plasma-Activated Liquids. http://arxiv.org/abs/2207.12478