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Effect of Plasma-Activated Water and Direct Plasma on *Enterococcus faecalis* Bacteria for Disinfection of Tooth Root Canal

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Abstract

This research studies the effect regarding two plasma types, plasma jet and plasma-activated water (PAW), on tooth root canal bacteria Enterococcus faecalis. The plasma jet works with argon gas, and it is generated by a power supply that operates at alternating high voltages in the form of a sinusoidal wave with peak-to-peak value of about 12 kV at a frequency of 30 KHz and its power is about 200 watts. This plasma was utilized directly to treat the tooth canal and indirectly by activating the water that was used later for treating the *Enterococcus faecalis* bacteria that are present in the tooth root. Pure distilled water was treated by plasma jet for one hour at flow rate 1 L/min. Plasma water activated by plasma contains NO_2, NO_3, and H_2O_2 with a concentration (10,100,200) ppm, respectively. It was noted, as a result of direct exposure with the two values of gas flow rate and for different exposure times, an ideal killing rate (from 298 x 10^8 to zero)CFU/ml was obtained when the exposure time changed from (5-30) minutes with a constant gas flow rate of 2.5 L/min; while in the case of changing the gas flow rate from (0.5-2.5) L/min and fixing time to 5 min, the killing rate decreased from (298 $\times 10^8$ to 30×10^8) CFU/ml. In the case of (PAW) when the time changed (5-25) min, an ideal killing rate was obtained (298 x 10^8 to zero) CFU/ml; adopted one factor, where the results indicated that plasma-activated water is good in disinfecting the root canal of the tooth and inactivating bacteria.

Keywords: plasma jet, plasma activated water, Enterococcus faecalis, Reactive species, PH concentration

تأثير الماء المنشط بالبلازما والبلازما المباشرة في البكتيريا المعوية البرازية لتطهير قناة جذر السن

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الخلاصة:

يدرس هذا البحث تأثير نوعين من البلازما ، البلازما النفث والماء المنشط بالبلازما (PAW) ، على بكتيريا قناة جذر الأسنان *Enterococcus faecalis*. تعمل البلازما نفث التي تعمل بغاز الأرجون ، وتتولد

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البلازما عن طريق مصدر تيار متناوب. الفولتية العالية على شكل موجة جيبية تبلغ قيمتها من الأعلى إلى الأعلى حوالي 12 كيلو فولت بتردد 30 كيلو هرتز وقوتها حوالي 200 واط. استُخدمت هذه البلازما لمعالجة قناة السن بشكل مباشر ، كما تم استخدامها في عملية تنشيط الماء ، والتي ستستخدم لاحقًا في علاج قناة جذر السن بكتريا *Enterococcus faecalis*. تمت معالجة الماء المقطر النقي بواسطة البلازما نفث لمدة ساعة بمعدل تدفق 1 لتر / دقيقة. تحتوي مياه البلازما التي يتم تنشيطها بواسطة البلازما على NO_2 و NO_3 و H_2O_2 بتركيز (200،100،10) جزء في المليون على التوالي. ونلاحظ نتيجة التعرض المباشر للمتغيرين تدفق الغاز والوقت ، تم الحصول على معدل قتل مثالي عند تغير زمن التعريض من (5−30) دقيقة (298×10^8 إلى صفر) مستعمرة/مل بمعدل تدفق غاز ثابت 2.5 لتر / دقيقة ، ولكن في حالة تغير معدل تدفق الغاز من (0.5 − 2.5) لتر / دقيقة وثبت الوقت على 5 دقائق ، وانخفض معدل القتل من (298×10^8 إلى 30×10^8)مستعمرة/مل. وفي حالة (PAW) عند تغير الزمن من (5−25) دقيقة، تم الحصول على معدل قتل مثالي من (298×10^8 إلى صفر) مستعمرة/مل ، حيث اشارت النتائج إلى أن الماء المنشط بالبلازما فعال في تطهير قناة جذر السن وفي تعطيل البكتيريا.

1. Introduction

Plasma is a partially ionized gas with ions, electrons, and uncharged particles such as atoms, molecules, and radicals. There are two types of plasma: thermal and non-thermal or cold atmospheric plasma[1]. Cold Atmospheric Plasma (CAP) is said to be non-thermal because it has electrons at a hotter temperature than the heavy particles that are at room temperature[2]. There are several methods to produce (CAP) such as Dielectric Barrier Discharge (DBD), Atmospheric Pressure Plasma Jet (APPJ), plasma needle, and plasma pencil. Several different gases can be used to produce (CAP) such as helium, argon, nitrogen, heliox (a mix of helium and oxygen), and air. Due to the ability of CAP to deactivate microorganisms, causing cell detachment, and causing death in cancer cells, it has been of interest in many applications in dentistry and oncology[3]. Plasma elements like single oxygen and free radicals have anti-bacterial characteristics [4]. Those species are capable of inactivating cells and causing cell lysis through oxidation that leads to sterilization and decontamination [5-7]. Plasma sterilization is frequently used in dentistry for plaque removal [8], teeth whitening [9], sterilization of dental equipment, implants [10], also root canal disinfection [11]. It can, for example, be utilized for root-canal disinfection, which is impossible with a typical plasma device. This is one of the reasons why atmospheric-pressure plasma-jet systems have recently attracted a lot of interest [12]. The main procedures for root-canal disinfection are mechanical cleaning, irrigation, laser irradiation, ultrasound, hypochlorite, and other antibacterial chemicals [13]. According to clinical studies, traditional disinfection treatments result in around 10% of treatment failures. The failures are primarily caused by germs, which cannot be thoroughly sterilized using the procedures previously mentioned [4]. *Enterococcus faecalis* is a regularly isolated pathogen linked to persistent periapical lesions, according to multiple investigations. In the case when nutrients are low, the facultative gram-positive bacteria *E. faecalis* could persist for extended periods of time in dentinal tubules. Those bacteria are more resistant to antibiotics and antibodies compared to planktonic bacteria. *E. faecalis* is simply eradicated in an open environment, but when it develops in the system of the root canal, it becomes more resilient. *E. faecalis* from persistent infections of the root canal must be removed using more powerful disinfection techniques [4].

As a new and adaptable antimicrobial, plasma-activated water (PAW) was the subject of substantial investigation. Effectiveness of PAW against a range of bacteria was demonstrated [14]. At the gas-liquid interface, energetic particles from plasma phase are trapped in the solution throughout PAW formation, starting a series of processes that result in the dissolution of numerous reactive secondary and primary species dissolved in water. Reactive oxygen

species (ROS) are produced primarily as a result of the presence of oxygen in the environment [15]. A breeze primary ROS interacts with one another producing hydroxyl radicals which when combined with water produce singlet oxygen (H_2O_2), and hydrogen peroxide (OH). (O_2), superoxide, and ozone are three gases that can be found in the atmosphere. Due to the nitrogen in the air and gaseous/aqueous reactions, reactive nitrogen species (RNS) are generated in the forms of nitrates (NO_3), nitrites (NO_2) and peroxynitrites ($ONOO^-$) [16]. Peroxynitrites are a type of peroxynitrite that occurs naturally in the environment [17]. The bactericidal property of PAW is attributed to the synergetic effects of all of the reactive species and the acidic environment [17, 18].

Most of the researchers conducted their research on bacteria by direct exposure to plasma in the tooth's root canal [5]. Our current study aims to kill bacteria in two ways: direct exposure and activated water with plasma. The comparison between these two ways is presented, in addition to, the effect of time and gas flow rate.

2. Experimental Setup

2.1. Plasma Jet System Setup

A schematic diagram of the setup of non-thermal atmospheric pressure plasma jet is shown in Figure 1. It includes Pyrex glass tube wrapped-up with 10 mm wide aluminum foil 20mm away from its end. A high voltage power supply was connected to the aluminum foil. The high voltage power supply produces high voltage with a sinusoidal shape of 30 kV peak-to-peak and frequency of 20 kHz. Compressed argon gas at a flow rate of roughly 2.5 L /min was used in this system. A plasma jet with a visible length of around ≤1.5 cm was produced under such operating circumstances. Utilizing a thermometer, the gas temperature, 1 cm away from the nozzle, was measured to be approximately 34°C. As the distance between the sample and the plasma torch increases, the measured temperature of the gas gradually decreases.

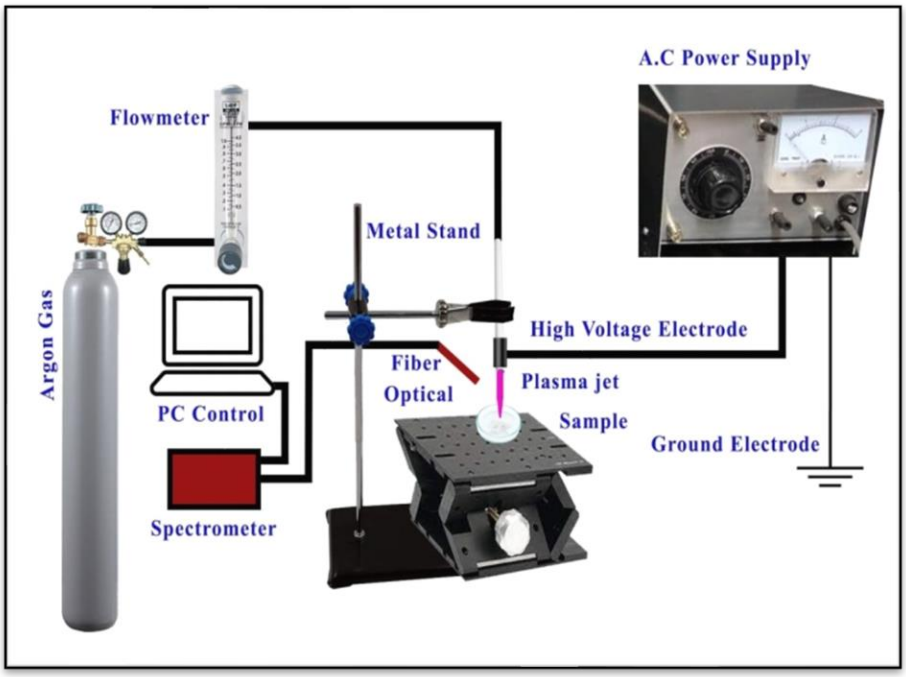

Figure 1: The device of plasma jet sustained in argon gas

2.2 Root canal samples and Bacterial Growth

Before the experiment, single-rooted extracted, intact permanent teeth were chosen and preserved in a 0.1% thymol solution at a temperature of 4 Celsius. Using the step-back

approach, root canals were prepared using Ni-Ti hand files (Mani Inc., Japan) up to #40 size. Debris was removed by irrigating each time the file size was changed. For injecting bacteria inside the root canal, every one of the apical foramens was sealed with composite resin (Clearfill AP-X, Kuraray Dental, Japan). Following this process, the root canals create a cone-shaped cavity (volume of about 10µL) with a narrow bottom that is sealed. All the samples were sterilized in an autoclave before any additional treatments. *Enterococcus faecalis* was cultured in Luria-Bertani (LB) medium for 18 hours to get them into the growth phase. Root canals received 10µL of a fresh, diluted suspension of *Enterococcus faecalis* that contained 10^7 CFU per ml. Such concentration of bacteria was selected because it is comparable to the actual situation in clinics.

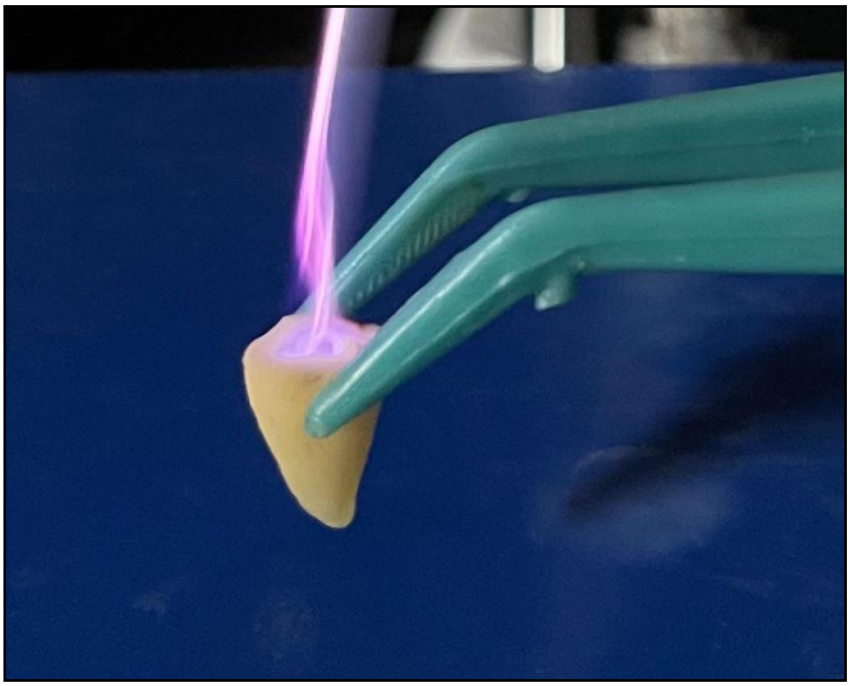

Figure 2: Picture of plasma jet treatment on an extracted single-root human tooth

2.3Plasma activated water

10 Milliliters of pure distilled water, of measured temperature and acidity, in a Petri dish was exposed to the jet plasma for one hour. After completing the water treatment, the concentrations of H_2O_2, NO_3, NO_2 were measured using test strips USA (Bartovation), as shown in Table 1.

Table 1: The concentrations of H_2O_2,NO_3,NO_2

PH	H_2O_2	NO_2	NO_3	T(^{0}C)before	T(^{0}C)after
3	200	10	100	28	34

2.4. Treatment method

There are two approaches to treat bacteria in root canal teeth: direct plasma and indirect plasma (plasma-activated water). In the first technique, the bacteria-infected teeth are exposed to a plasma jet that uses Ar gas. Time and gas flow rate factors are used to guide the treatment. The gas flow was fixed at 2.5 L/min) and the teeth were exposed at various intervals of time (5,10,15,20,25) min. The treatment was also carried out by varying the gas flow rate (0.5, 1, 1.5, 2, 2.5 L/min) and fixing time to 5 min. Additionally, test strips were used to measure the

amounts of NO_2, H_2O_2, and NO_3. In the second approach, water was exposed to the plasma jet for an hour at a stable gas flow rate of L/min. The contaminated teeth were placed in the activated water for different time intervals of (5, 10, 15, 20, and 25 minutes. After the teeth have been exposed, 100 microliters of Luria-Bertani broth was added and allowed to sit for 20 minutes. Then, 100 microliters of bacteria was added to a petri dish and the bacteria were distributed with the use of a swap, and the dish is then implanted with Luria-Bertani agar for 18 hours, or until the bacteria have reached the growth phase and bacterial colonies were counted.

2.5 Statistical Analysis:

To determine how various factors affected the study parameters, Statistical Analysis System-SAS (2012) application was employed. The means in the present work were compared significantly using the LSD test (ANOVA).

4. Results and Discussion

Figures (3-a and 3-b) shows the relationship between the rate of killing of bacteria in the tooth root canal and exposure time to plasma where the gas flow rate was 2.5 l/min, and the number of colonies was 298×10^8 before exposure. The percentage of killing bacteria after (5, 10, 15, 20, 25, 30) minutes of exposure was (30×10^8, 27×10^8, 25×10^8, 20×10^8, 0, 0), respectively. The results showed that the time factor has an important effect on the inhibition of bacteria and this is clear when the teeth were exposed to plasma at different times. There was an apparent decrease after the treatment for 5 minutes. The disinfection rate of the tooth root canal increases gradually with the increase of the exposure time, reaching 98% at 20 min; the results for a 30 min period led to a complete killing rate. The results showed a complete elimination of bacteria present in the tooth's root canal, which is difficult to reach through medicine. The plasma and reactive species' atoms and ions can interact directly with the bacteria. The accumulating charges on the cell membrane cause the membrane to rupture due to Coulomb force. Some reactive species live for a long time, and decomposition occurs in water on the tooth's surface. Those reactive species interact with the bacteria. Reactive species, when interacting with water, produce effective oxygen compounds. OH, reactive species can be transported into cytoplasm through a series of mechanisms. This result is consistent with that of Wang et al. [19]. Bacterial inactivation in plasma jets can occur through two different mechanisms: physical and chemical. Heat, ultraviolet radiation, and charged particles are examples of physical factors, while active species are examples of chemical agents. The gas temperature reached 34°C, at this temperature heat has no effect on inactivating microorganisms. On the outside of the cell membrane, charged particles might accumulate due to plasma jet [20]. These charges combine to create an electrical force that can break the tensile force of the cell membrane and cause it to burst. Because of the rapid recombination of electrons and ions, the concentration of charged particles decreases when the plasma displays bacteria indirectly [21]. Increasing the time of exposing bacteria to the plasma leads to an increase in the accumulation of charges, which results in an increase in the rate of killing of bacteria, and from this it becomes clear that the exposure time has a very important effect on the increase in the rate of killing (298×10^8 to zero). Since all bacteria samples in this investigation were indirectly exposed to the plasma column. As a result, the inhibition effect of ultraviolet radiation on microorganisms in this range is largely related to DNA damage [22].

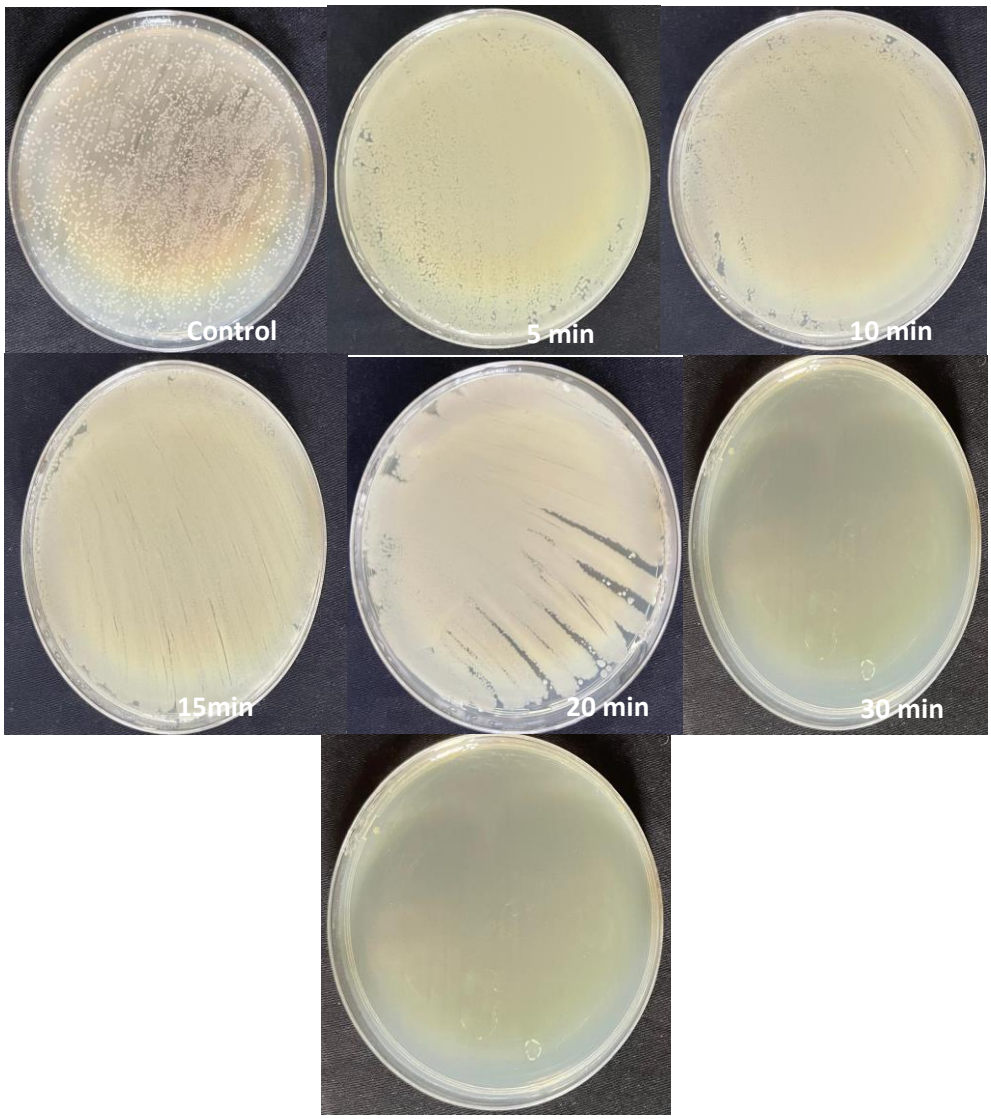

Figure 3: (a) *Enterococcus faecalis* bacteria inactivation at different exposure times.

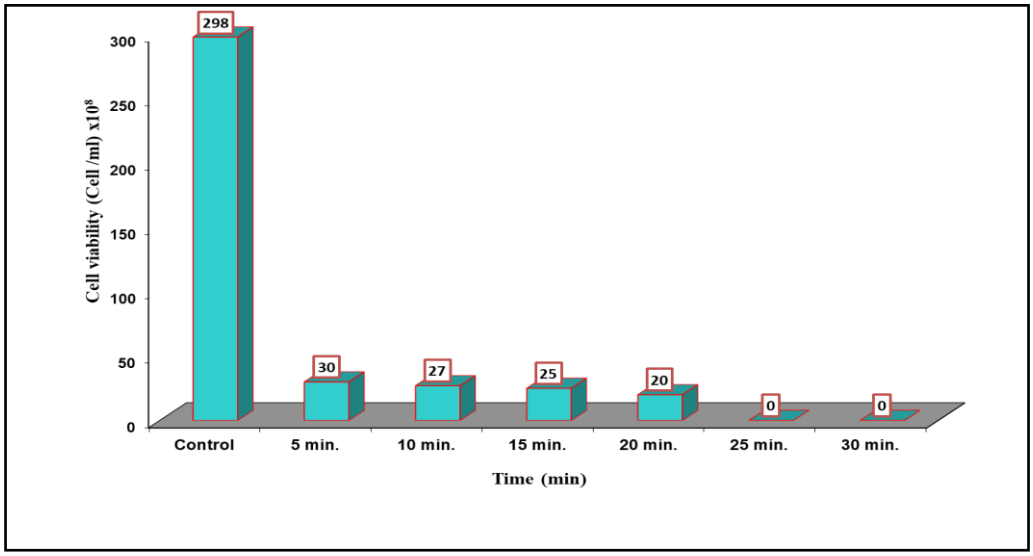

Figure 3: (b) Enterococcus faecalis cell viability affected by plasma jet of various time, as shown in the following histogram. All data have been represented as mean ± standard deviation LSD test (ANOVA). Statistical significance has been considered as P<0.010.

In the other case, the teeth were exposed to direct plasma with an increase in the gas flow rate in the range (0.5-2.5) L/min for a time of 5 minutes. Figure (4-a and 4-b) shows the relationship between the argon gas flow rate and bacteria inhibition rate. It shows the number of bacteria that were killed by. (37×10^8, 36×10^8, 35×10^8, 32×10^8, 30×10^8) for different gas flow rate of (0.5, 1, 1.5, 2, 2.5) L/min, respectively with a constant flow of 2.5 L/min of argon. It can be noted that as the gas flow rate increases, the rate of killing increases, which indicates an increase in the generation of effective nitrogen and oxygen compounds (RONS)which contribute to cell death through DNA damage. This indicates that a high gas flow rate is essential in killing bacteria that contribute to disinfecting the tooth root canal.

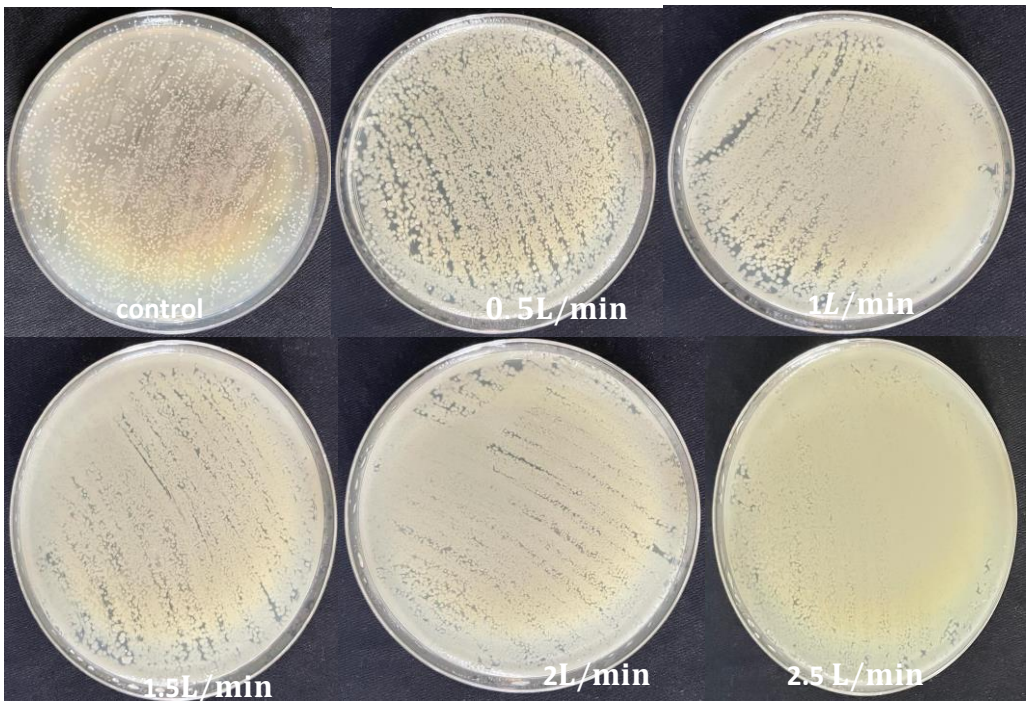

Figure 4: (a) Enterococcus faecalis bacteria inactivation at different gas flow rates.

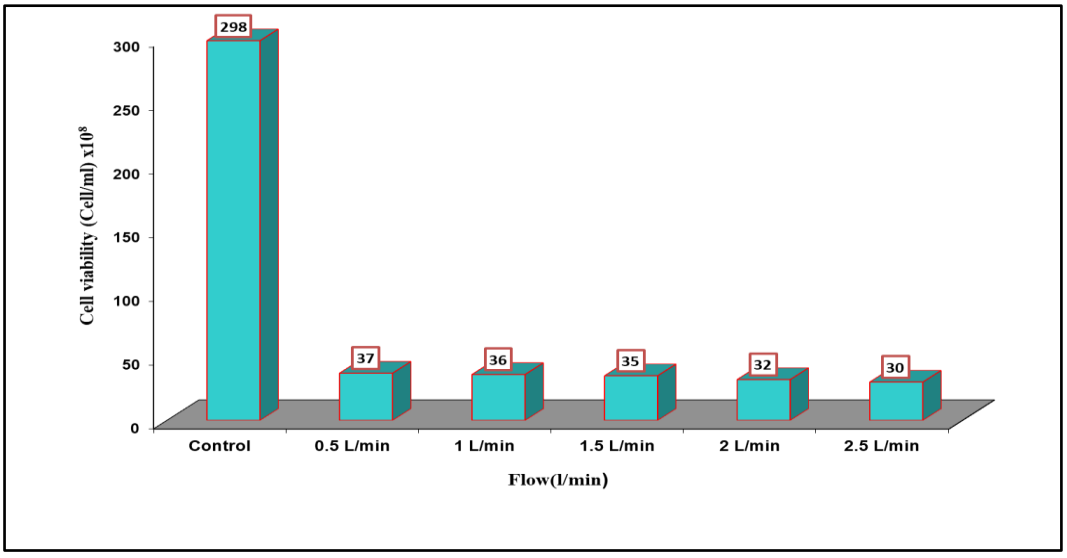

Figure 4: (b) Enterococcus faecalis cell viability affected by plasma jet of various gas flow rates. All data have been represented by mean ± standard deviation LSD test (ANOVA) .Statistical significance has been considered as P<0.010.

The second approach for disinfecting the tooth root canal was using plasma activated water with a time change from (5-25) min. Figure (5-a and 5-b) shows the relationship between the percentage of bacteria killing in the tooth root canal and the change in time with a constant gas flow rate of 2.5 L/min using plasma-activated water. The teeth were treated with plasma activated water at different times (5,10,20,25) min. Table (1) shows the concentrations of the activated water. The longer the duration of the therapy for bacteria-infected teeth and the longer the exposure, the more probable it is that reactive species will interact with Enterococcus faecalis bacteria cells. Moreover, the bacteria showed greater sensitivity to PAW than the direct exposure to the teeth. During the generation of PAW, charged particles are absorbed in the gas phase and interact with water molecules, resulting in long-lived and short-lived reactive species. It was also observed that the pH of the activated water in the plasma decreased from 7 to 3, as shown in Table (1), which is consistent with the results [23]. As the acidic pH plays an indispensable role in inhibiting the growth of bacteria.

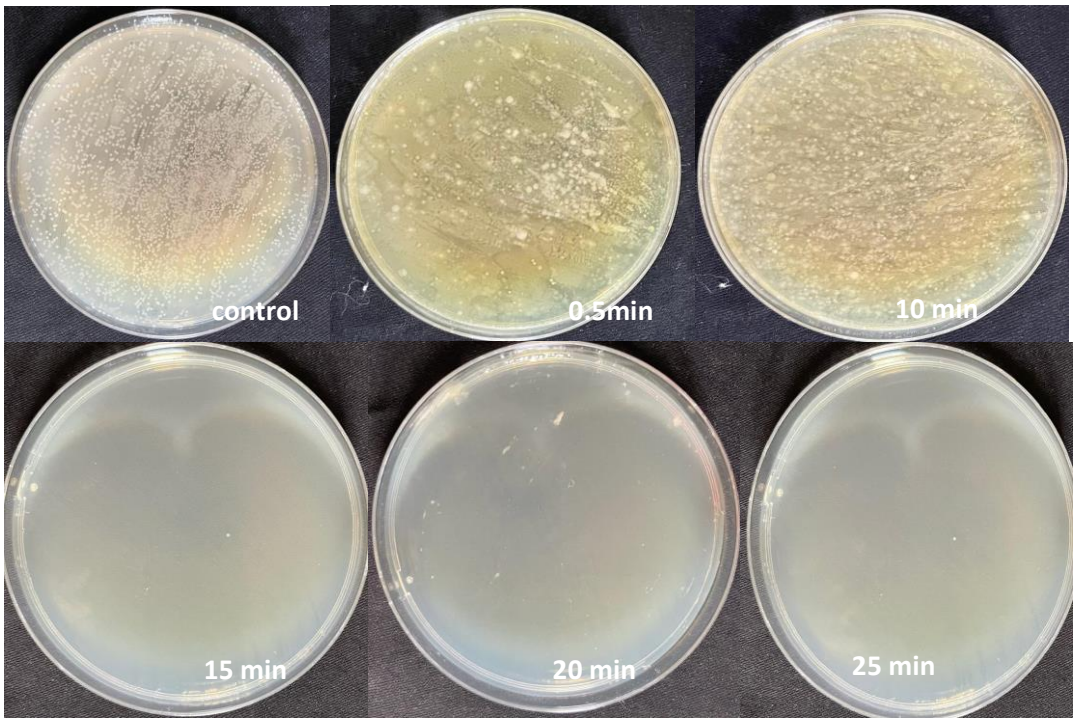

Figure 5: (a) *Enterococcus faecalis* bacteria inactivation at different time.

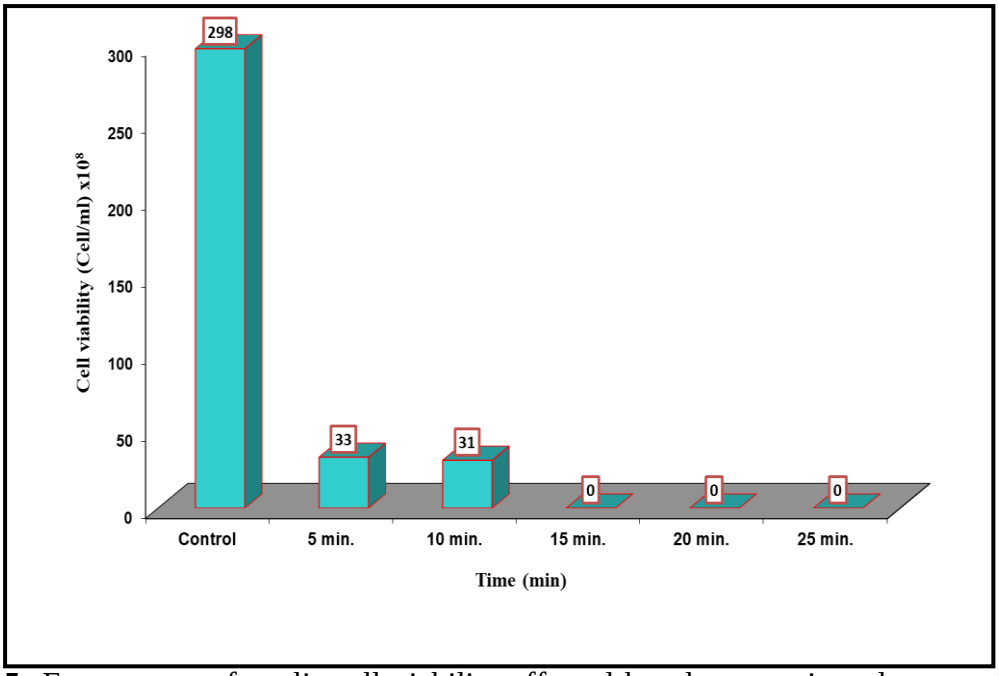

Figure 5: Enterococcus faecalis cell viability affected by plasma-activated water of various time, All data have been represented by a mean ± standard deviation LSD test (ANOVA). Statistical significance has been considered as P<0.010.

5. Conclusion

In this research, non-thermal plasma jet system was built and employed in disinfecting tooth root canal *Enterococcus faecalis* bacteria. The results showed the effectiveness of using plasma by direct and indirect method (PAW). The time factor was significant in killing bacteria, as the killing rate increased with the gas flow rate increase in the range (0.5-2.5)L/min. It increased the percentage of free radicals and RONS, which had a significant effect in inhibiting bacteria. The results showed that increasing the acidity of distilled water contributes to the process of disinfecting bacteria. Moreover, the bacteria showed greater sensitivity to PAW exposure to the teeth .The results of the two methods in the case of direct and indirect were similar in terms of rates of killing colonies. From these results, it is concluded that the activated water method is a method that can be adapted to disinfect the root canal of the tooth, and it needs more research.

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