Biotech Health Sci. 2015 August; 2(3): e28198. DOI: 10.17795/bhs-28198

Published online 2015 August 24. **Research Article**

Effect of Caffeic Acid and Low-Power Laser Light Co-Exposure on Viability of *Pseudomonas aeruginosa*

Nematolah Gheibi [1]; Nader Divan Khosroshahi [1,*]; Marzieh Habibi [2]

[1]Cellular and Molecular Research Center, Qazvin University of Medical Sciences, Qazvin, IR Iran
[2]Microbiology Department, Science and Research Branch, Islamic Azad University, Tehran, IR Iran

*Corresponding author: Nader Divan Khosroshahi, Cellular and Molecular Research Center, Qazvin University of Medical Sciences, Qazvin, IR Iran. Tel: +98-9125819521, Fax: +98-2833324971, E-mail: naderkhosroshahi@yahoo.com

Received: February 23, 2015; **Revised:** June 29, 2015; **Accepted:** July 6, 2015

Background: The resistance of *Pseudomonas aeruginosa* to antibiotics is a big problem, especially in burns and wound infections. Laser irradiation affects microorganisms by denaturing their proteins, which involves changes in the chemical or physical properties of the protein.
Objectives: The aim of this study was to investigate the effect of caffeic acid and low-power laser light co-exposure on *Pseudomonas aeruginosa* isolated from burn wounds.
Materials and Methods: Ten bacterial samples were collected from patients with burn wound infections at Shahid Motahhari medical center of Tehran. The He-Ne laser was used in this study with output power of 2 mW.
Results: The data significantly indicated that both the caffeic acid and laser treatment alone reduced the number of colony-forming units compared to control cultures. Co-exposure of bacterial suspensions to caffeic acid and laser at three time points showed the following number of colony-forming units 240.23 ± 60.15, 148.13 ± 52.66 and 84.57 ± 35, respectively. The best concentrations of caffeic acid to achieve countable colonies were 1.5 and 1.75 mM. At the concentration of 1.5 mM of caffeic acid, the number of colonies significantly reduced to 280.78 ± 59 (P = 0.008) while at 1.75 mM the number of colonies reduced to 234.07 ± 72.28 (P = 0.0001).
Conclusions: Caffeic acid treatment reduced bacterial growth and resulted in a decreased number of colony formation. The simultaneous effect of caffeic acid and laser at three time courses showed a synergic effect in reducing colony formation compared to the control and caffeic acid, and laser alone.

Keywords: Caffeic Acid; Laser Therapy; Low-Level; Minimum Inhibitor Concentration; *Pseudomonas aeruginosa*

1. Background

Pseudomonas aeruginosa is a non-fermentative, aerobic, gram-negative rod that normally lives in moist environments (1, 2). *Pseudomonas aeruginosa* is typically an opportunistic pathogen that seldom causes disease in healthy subjects. Normally, for an infection to occur, some disruption of physical barriers (skin or mucous membranes) or an underlying dysfunction of immune defense mechanisms, such as neutropenia, is necessary (3). The virulence mechanisms of *P. aeruginosa* are complex and only partially understood. Adherence mediated by pili and other adhesions appear to be important for the colonization of mucous membranes and other surfaces (4, 5). Furthermore, the production of a mucoid exopolysaccharide matrix that surrounds the cells and anchors them to each other and to the environment is important for growth as a biofilm, in which the bacterial cells are protected from the host innate and immune defenses and are overall less susceptible to antibiotics (6-9). A role for tissue damage and invasion has been recognized for a number of products secreted by *P. aeruginosa*, including elastase, alkaline protease, cytotoxin, phospholipase C and rhamnolipid (10, 11). Caffeic acid phenethyl esters (CAPE) (2-phenyl ethyl 3 (3, 4-dihydroxyphenyl) -2-propenoate), exhibits a broad spectrum of biological activities including antibacterial, anti-inflammatory, antiviral, antiatherosclerotic, antiproliferative, neuroprotective, and antitumoral actions (12, 13). Phenethyl caffeic acid esters, similar to frolic and chlorogenic acids, show antibacterial, anti-mutageneic, and antiviral activities (14). Several studies have shown the antibacterial effects of these components, e.g. diphenyl esters of caffeic acid act as highly effective antimicrobial agents against staphylococcal bacteria, *Bacillus subtilis*, and *Pseudomonas aeruginosa* (15). Low power laser irradiation (LPLI) has been used for a variety of clinical applications where it is thought to promote certain processes without inducing any thermal effects. Studies have demonstrated that laser-powered treatment will be useful for the treatment of many cases of infection caused by bacteria such as *P. aeruginosa* (16). Thus, we aimed to investigate the effect of low-power laser irradiation (LPLI)

on bacterial growth. Decrease in bacterial growth of *P. aeruginosa* after irradiation with 810 nm laser (0.03.W/cm^2) could potentially benefit wound infections (17).

2. Objectives

The aim of this study was to investigate the effect of low power laser irradiation (LPLI) at different time intervals and different doses of caffeic acid on resistance and viability parameters of *Pseudomonas aeruginosa*.

3. Materials and Methods

3.1. Isolation and Identification of Bacteria

Ten strains of *Pseudomonas aeruginosa* were collected from patients with burn wound infections using sterile swabs. The samples were inoculated on Mueller Hinton agar and incubated at 37°C for 24 hours for bacteria to multiply to form colonies. The strains were identified by routine laboratory tests (oxidase test, oF medium culture (oxidate fermenter test) and TSI reaction (biochemical reaction test for detection *Pseudomonas aeruginosa*)) and then tested with specific *Pseudomonas aeruginosa* polymerase chain reaction (PCR). In order to prepare the *Pseudomonas aeruginosa* DNA, specimens were first treated by proteinase K. Next, DNA was extracted using the phenol-chloroform method. The specific primers used in this study were gyr BR and gyr BF (Figure 1).

3.2. Bacterial Samples Preparation

For each strain suspensions were prepared using McFarland standards. Next, 1:10 dilutions were prepared from these standards. Furthermore, 100 µL of suspensions (1.5 × 10^2 CFU) were removed and cultured on Muller Hinton agar medium. Bacterial samples were incubated at 37°C for 24 hours and later the number of colonies were counted using a colony counter system. For caffeic acid treatment, the stock concentration of 10 mM caffeic acid (Merck) and filter-sterilized solution were prepared. The two concentrations below the minimum inhibitory concentration (MIC) of caffeic acid were prepared on Mueller Hinton agar in a way to obtain the most appropriate countable colonies comparable to the control group, for the preparation of bacterial suspensions. Furthermore, 100 µL of diluted (10^5 times) bacterial suspension was produced in Mueller Hinton (MH) agar medium and, by further addition of MH agar, the total mixture reached a final volume of 1 mL followed by homogenization using a magnetic stirrer. Later, a standard loop of 1.5 × 10^2 CFU of microbial suspension was transferred to MH agar medium containing two caffeic acid concentrations (1.5 and 1.75 mM); the loop was streaked throughout the culture surface and incubated for 24 hours at 37°C. Following the incubation period, colonies were counted using a colony counter and the results were further compared with those of the controls.

3.3. Irradiation Procedure

3.3.1. Laser Exposure

Primarily, 100 µL of bacterial suspension was mixed in a 96-well enzyme-linked immunosorbent assay (ELISA) plate and diluted with Muller Hinton broth by a magnetic stirrer and exposed to 630 nm of 2 mW He-Ne laser for one, two and three minutes. Furthermore, 100 µL of the laser-exposed bacterial suspension was cultured on Mueller Hinton agar medium and incubated at 37°C for 24 hours; the number of colonies that had grown, was then compared with the colony numbers counted on control plates.

3.3.2 Co-Exposure to Laser and Caffeic Acid

One hundred microliters of bacterial suspension was mixed with 1 mL of Muller Hinton broth and exposed to laser irradiation for one, two, and three minutes. Then, 100 µL (1.5 × 10^2 CFU/mL) of the microbial suspension was transferred to MH agar medium containing caffeic acid, and the culture was incubated for 24 hours at 37°C. A protocol analogous to that used above was used to compare the number of colonies with those of control plates. Minimum inhibitory concentration (MIC) for caffeic acid was determined by the broth macro dilution method for the *Pseudomonas aeruginosa* (ATCC 27853) standard strain and the ten strains isolated from patients. The inhibitory effect of caffeic acid and low power laser, individually and in combination was determined. Each sample was exposed to laser for one, two and three minutes and every experiment was carried out three times (Figure 2).

3.4. Statistical Analysis

One-way ANOVA, Turkey's post-hoc test, and Dunnett's test were performed using the SPSS software. In order to ensure the significance level, non-parametric kruskal-Wallis and Mann-Whitney tests were performed. P values of < 0.05 were considered statistically significant.

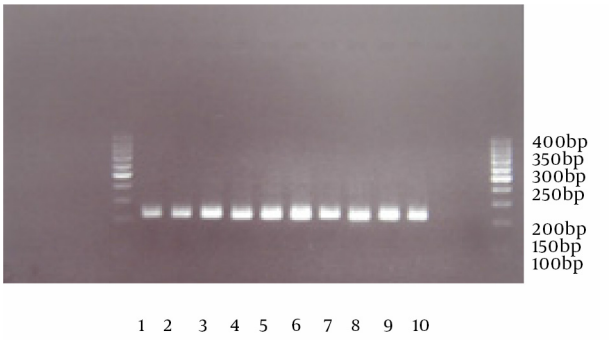

Figure 1. The 220 bp DNA bands of *gyrB* Gene Associated With Test and Standard Strains of *Pseudomonas aeruginosa* on 2% Agarose Gel

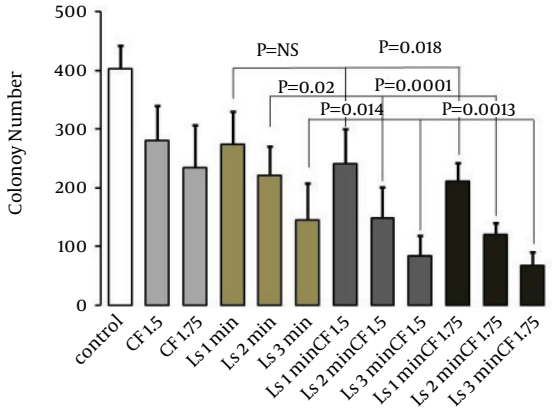

Figure 2. Colony Number of *Psudomonas aeroginosa* (Mean ± Standard Deviation) in the Control Group (n = 10; Without any Treatment), and Groups Under 1.5 and 1.75 mM Caffeic Acid (CF) Treatment, Under Laser (Ls) Exposure for One, Two, and Three Minutes, and Under Co-Exposure of Caffeic Acid and Laser; NS = Not Significant

4. Results

The minimum inhibitory concentration of caffeic acid through the macro dilution assay was obtained as 2 mM for the standard and sample strains of *P. aeruginosa*. Our results from caffeic acid and laser treatment on *P. aeruginosa* showed that the number of colonies in the presence of 1.5 and 1.75 mM of caffeic acid were 280.78 ± 59 (mean ± SD) and 234.07 ± 72.28, respectively. In bacterial suspensions exposed to low power (2 mW) laser for one, two, and three minutes the number of colonies were 273.12 ± 56.05, 220.57 ± 49.22 and 144.93 ± 63.42, respectively. Co-exposure to caffeic acid and laser of bacterial suspensions following three time points resulted in 240.23 ± 60.15, 148.13 ± 52.66 and 84.57 ± 35 colonies, respectively (Figure 1).

5. Discussion

The present results clearly showed a significant reduction in bacterial growth compared with the control group in presence of sub MIC (1.5 and 1.75 mM) concentrations of caffeic acid. The MIC for caffeic acid against *P. aeruginosa* at 2 mM was obtained by the micro dilution technique. After low-power He-Ne laser irradiation following three different exposure times (one, two, and three minutes), an elimination of colony formation compared with control colonies was observed. For laser-exposed bacterial suspensions the number of colonies showed significant reduction in a time-dependent manner (three minutes > two minutes > one minute)(Figure 2).

The resistance of *P. aeruginosa* to antibiotics is a big problem, especially in burn wounds infection. Laser irradiation affects microorganisms by denaturing their proteins, which involves changes in the chemical or physical properties of proteins. Denaturation includes structural alterations due to destruction of the chemical bonds holding proteins in a three-dimensional form. As proteins revert to a two-dimensional structure they coagulate (denature) and become nonfunctional; laser irradiation can enhance the release of growth factors from fibroblasts and stimulate the wounded cells to treat their functions and increase their in vitro proliferation (18).

Different molecular mechanisms may explain the effect of diode laser on the metabolism of *P. aeruginosa*; the first mechanism is the absorption of laser light wavelength by certain chromophore (CuA) that has a range of absorption in the infrared (IR) region. This CuA plays an important role in metabolism and production of ATP (19). The second possible mechanism is the conversion of a fraction of the excitation energy to heat, in which a local transient increase in the temperature of the absorbing chromophore occurs (20). Wilson and Pratten (21) applied low power gallium aluminum arsenide (GaAlAs) laser to meticilin resistance *staphylococcus aureus* (MRSA) in their study and reported a killing effect on bacteria through sensitization with aluminum disulfonated phthalocyanine. A study on microorganisms isolated from the mouth showed that the application of 905 nm diode laser for one minute induced a bactericidal effect, which was reinforced by the sensitizer combination (22). The same effect of low doses of He-Ne laser light was reported for various toluidine blue O (TBO) sensitized oral bacteria (23). Consistent with the studies mentioned above, in the present study the application of He-Ne low-power (2 mW) laser reduced the number of *P. aeruginosa* colonies and increasing the laser irradiation exposure time on bacteria induced even more reduction in colony formation. The effects of low-power laser therapy with 810, 660 and 630 nm wavelengths and energy level of between 1 and 50 J/cm^2 was also examined on *Staphylococcus aureus*, *P. aeruginosa* and *Escherichia coli* isolated from infected wounds and it was concluded that the highest antibacterial effect occurred at the wavelength of 630 nm and energy range of 1 - 50 J/cm^2 (24). The application of TBO as sensitizer with He-Ne laser against MRSA was reported to kill the bacteria in a dose-dependent manner (25). The results of the present study showed that using a suitable low-power (2 mW) He-Ne laser with no sensitizer had the highest killing effect on *P. aeruginosa* after three minutes of laser exposure time. Similar to the results of this study, using a 2 mW diode laser with 805 nm wavelength and power density of 7.07 W/cm^2 reduced the production of virulence factors and increased the sensitivity of *P. aeruginosa* to antibiotics, which may be an additional benefit of using light in the treatment of infectious disease (16). Furthermore a study on the effect of 35 mW He-Ne laser on the proteolytic activity of *P. aeruginosa* using TBO as a photosensitizer indicated that the reduction in proteolytic activity was 94%, at high doses of both light and TBO concentration (26). The application of a dye as a sensitizer has been the focus of some studies on photodynamic therapy (PDT), especially in mammalian cancer cells (27). The major objective of our study was to examine caffeic acid and its derivatives as an adjunct to other

compounds for the treatment of skin disorders, in particular melanoma (28, 29). Phenethyl caffeic acid esters, similar to frolic and chlorogenic acids show antibacterial, anti-mutageneic and antiviral activities and have no side effects (14). Thus, regarding caffeic acid, the effect of this chemical on cytoplasmic membrane or the possibility of damage to DNA in both prokaryotic and eukaryotic organisms needs to be evaluated. The results of this study showed that the antibacterial properties of He-Ne laser light depend on exposure time. Furthermore, reduction of bacterial growth, with the application of caffeic acid, was reflected by the number of colonies. The simultaneous effect of caffeic acid and laser at three time courses showed a synergic effect in reducing colony formation compared to the control, and groups that received either caffeic acid or laser alone.

Acknowledgements

This study was financially supported by the cellular and molecular research center, Qazvin University of Medical Sciences. The authors are thankful to the personnel working in the cellular and molecular research center and department of microbiology at the Qazvin University of Medical Sciences is appreciated.

Authors' Contributions

All authors helped with the design and analysis of the results and writing of the current manuscript.

Funding/Support

This work was supported by grants from the Qazvin University of Medical Sciences, Qazvin, Iran.

References

1. Goldberg JB. Pseudomonas: global bacteria. *Trends Microbiol.* 2000;**8**(2):55–7.
2. Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, et al. Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. *Nature.* 2000;**406**(6799):959–64.
3. NNIS system. National Nosocomial Infections Surveillance (NNIS) System Report, Data Summary from October 1986–April 1998, Issued June 1998. *American Journal of Infection Control.* 1998;**26**(5):522–33.
4. Ramphal R, Pier GB. Role of Pseudomonas aeruginosa mucoid exopolysaccharide in adherence to tracheal cells. *Infect Immun.* 1985;**47**(1):1–4.
5. Doig P, Todd T, Sastry PA, Lee KK, Hodges RS, Paranchych W, et al. Role of pili in adhesion of Pseudomonas aeruginosa to human respiratory epithelial cells. *Infect Immun.* 1988;**56**(6):1641–6.
6. Boyd A, Chakrabarty AM. Pseudomonas aeruginosa biofilms: role of the alginate exopolysaccharide. *J Ind Microbiol.* 1995;**15**(3):162–8.
7. Hoiby N, Krogh Johansen H, Moser C, Song Z, Ciofu O, Kharazmi A. Pseudomonas aeruginosa and the in vitro and in vivo biofilm mode of growth. *Microbes Infect.* 2001;**3**(1):23–35.
8. Drenkard E, Ausubel FM. Pseudomonas biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature.* 2002;**416**(6882):740–3.
9. Olson ME, Ceri H, Morck DW, Buret AG, Read RR. Biofilm bacteria: formation and comparative susceptibility to antibiotics. *Can J Vet Res.* 2002;**66**(2):86–92.
10. Berka RM, Vasil ML. Phospholipase C (heat-labile hemolysin) of Pseudomonas aeruginosa: purification and preliminary characterization. *J Bacteriol.* 1982;**152**(1):239–45.
11. Komori Y, Nonogaki T, Nikai T. Hemorrhagic activity and muscle damaging effect of Pseudomonas aeruginosa metalloproteinase (elastase). *Toxicon.* 2001;**39**(9):1327–32.
12. Ilhan A, Iraz M, Gurel A, Armutcu F, Akyol O. Caffeic acid phenethyl ester exerts a neuroprotective effect on CNS against pentylenetetrazol-induced seizures in mice. *Neurochem Res.* 2004;**29**(12):2287–92.
13. Hishikawa K, Nakaki T, Fujita T. Oral flavonoid supplementation attenuates atherosclerosis development in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol.* 2005;**25**(2):442–6.
14. Ida T, Okamoto R, Shimauchi C, Okubo T, Kuga A, Inoue M. Identification of aminoglycoside-modifying enzymes by susceptibility testing: epidemiology of methicillin-resistant Staphylococcus aureus in Japan. *J Clin Microbiol.* 2001;**39**(9):3115–21.
15. Nussbaum EL, Lilge L, Mazzulli T. Effects of 630-, 660-, 810-, and 905-nm laser irradiation delivering radiant exposure of 1-50 J/cm2 on three species of bacteria in vitro. *J Clin Laser Med Surg.* 2002;**20**(6):325–33.
16. Komerik N, Wilson M, Poole S. The effect of photodynamic action on two virulence factors of gram-negative bacteria. *Photochem Photobiol.* 2000;**72**(5):676–80.
17. Nussbaum EL, Lilge L, Mazzulli T. Effects of low-level laser therapy (LLLT) of 810 nm upon in vitro growth of bacteria: relevance of irradiance and radiant exposure. *J Clin Laser Med Surg.* 2003;**21**(5):283–90.
18. Anwer AG, Husien AS. Combination Effect of Laser, Antibiotics and Different Temperature on Locally Isolated Pseudomonas aeruginosa. 2007;**6**:21–30.
19. Hay M, Ang MC, Gamelin D, Solomon EI, Antholine WE, Ralle M, et al. Spectroscopic Characterization of an Engineered Purple CuA-Center in Azurin. *Inorganic Chemistry.* 1998;**37**(2):191–8.
20. Kujawa J, Zavodnik IB, Lapshina A, Labieniec M, Bryszewska M. Cell survival, DNA, and protein damage in B14 cells under low-intensity near-infrared (810 nm) laser irradiation. *Photomed Laser Surg.* 2004;**22**(6):504–8.
21. Wilson M, Pratten J. Sensitization of Staphylococcus aureus to killing by low-power laser light. *J Antimicrob Chemother.* 1994;**33**(3):619–24.
22. Haas R, Dortbudak O, Mensdorff-Pouilly N, Mailath G. Elimination of bacteria on different implant surfaces through photosensitization and soft laser. An in vitro study. *Clin Oral Implants Res.* 1997;**8**(4):249–54.
23. Wilson M, Dobson J, Sarkar S. Sensitization of periodontopathogenic bacteria to killing by light from a low-power laser. *Oral Microbiol Immunol.* 1993;**8**(3):182–7.
24. Nussbaum EL, Biemann I, Mustard B. Comparison of ultrasound/ultraviolet-C and laser for treatment of pressure ulcers in patients with spinal cord injury. *Phys Ther.* 1994;**74**(9):812–23.
25. Rassam YZ. The Effect of laser light on virulence factors and antibiotic susceptibility of locally isolated Pseudomonas aeruginosa. *Journal of Applied Sciences Research.* 2010;**6**(8):1298–302.
26. Wilson M, Yianni C. Killing of methicillin-resistant Staphylococcus aureus by low-power laser light. *J Med Microbiol.* 1995;**42**(1):62–6.
27. Karu T. Primary and Secondary Mechanisms of Action of Monochromatic Visible and IR Radiation on Cell. *J Photochem Photobiol B.* 1993;**49**(1):99–108.
28. Kudugunti SK, Vad NM, Whiteside AJ, Naik BU, Yusuf MA, Srivenugopal KS, et al. Biochemical mechanism of caffeic acid phenylethyl ester (CAPE) selective toxicity towards melanoma cell lines. *Chem Biol Interact.* 2010;**188**(1):1–14.
29. Kudugunti SK, Thorsheim H, Yousef MS, Guan L, Moridani MY. The metabolic bioactivation of caffeic acid phenethyl ester (CAPE) mediated by tyrosinase selectively inhibits glutathione S-transferase. *Chem Biol Interact.* 2011;**192**(3):243–56.