PHOTOTOXIC EFFECT OF VISIBLE BLUE LIGHT ON AGGREGATIBACTER ACTINOMYCETEMCOMITANS AND PORPHYROMONAS GINGIVALIS ISOLATED FROM PATIENTS WITH CHRONIC PERIODONTITIS: AN IN-VITRO EXPERIMENTAL STUDY

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ABSTRACT

Chronic periodontitis is a quite common disease in adult patients characterized by pocket formation and/or recession while progressive loss of periodontal attachment occurs slowly to moderately local risk factors, e.g. bacterial plaque. Wide array of microorganisms have been associated with periodontal disease, out of which Aggregatibacter actinomycetemcomitans (Aa) and Porphyromonas gingivalis (Pg) have been predominantly associated with periodontal diseases. The objectives of this study were to determine phototoxic effect of visible blue light on Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis clinical isolates from chronic periodontitis patients, and to study their antibiotic sensitivity against selected antibiotics. The test was carried out on 15 strains of Aggregatibacter actinomycetemcomitans and 15 strains of Porphyromonas gingivalis isolated from pockets of chronic periodontitis patients aged between 30-50 years old with pocket depths of 5-6 mm. The bacteria cultured, isolated, and identified by standard bacteriological methods, then subjected to visible blue light at different periods of time exposures. After light exposure, the bacterial killing rates were calculated from colony forming unit (CFU) counts after 48 hours of anaerobic incubation. There was a decrease in CFU for both microorganisms as we proceeded from zero, 20, 40 and 60 seconds of blue light exposure. In conclusions, there was a phototoxic effect for the visible blue light emitted from the light curing device against the anaerobic periodontal pathogens and blue light exposure is effective in reducing periodontal pathogens. It is recommended that an adjunctive exogenous photosensitizer be used and that pathogens be exposed to visible light for clinical antimicrobial periodontal therapy.

Keywords: Anaerobic periodontal pathogen, blue light, CFU.
instrumentation is still considered the gold standard and allows the sufficient cleaning of the periodontal pockets. Anatomical peculiarities like root curvatures or invaginations can make it difficult to remove bacterial deposits and biofilms completely from root surfaces by means of mechanical methods. Several treatment options are available to support the efficacy of instrumentation, for example the usage of local antibiotics or antimicrobials or photodynamic therapy (PDT). Different types of antibiotics have been used to avoid this obstacle. But another problem was noted as biofilm showed antibiotic resistance mechanisms. One of the problems that tackle the use of chemical agents is the failure in maintaining therapeutic concentrations in the targeted site and disruption of the oral microflora. Photodynamic Therapy (PTD) thus was introduced to open a new path in treating periodontal diseases without being hindered by the obstacles and problems mentioned above. As an innovative non-antibiotic approach, antimicrobial blue light (aBL) in the spectrum of 400–470 nm has demonstrated its intrinsic antimicrobial properties resulting from the presence of endogenous photosensitizing chromophores in pathogenic microbes. It is envisioned that microbes are less able to develop resistance to aBL than to traditional antibiotics, because of the multi-target characteristics of aBL. In addition, it is well accepted that aBL is much less detrimental to host cells than UVC irradiation. The objectives of this study were to determine phototoxic effect of visible blue light on Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis clinical isolates from chronic periodontitis patients, and to study their antibiotic sensitivity against selected antibiotics.

MATERIALS AND METHODS
Patient selection and sampling
Fifteen systemically healthy patients of age range between 30-50 years old participated in this study. They had chronic periodontitis with at least one pocket of 5-6mm depth. A piece of plaque from periodontal pocket was excavated by gracey curette without touching adjacent tissue. Plaque sample was spread on blood agar solid media supplied with selective antibiotics (Clindamycin and Metronidazole). Colonies were subcultured again on the same media anaerobically for 72 hours under the same condition, using the same method, to obtain pure cultures of both Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis.

The application of light exposure using a serial dilution technique on microtiter plates
After incubation period, a serial dilution procedure was performed for standardization of the amount of bacteria using 10⁶ as bacterial initial concentration, and to decrease the numbers of colonies into a countable one. A standard volume of Thioglycollate broth which is liquid media used to culture bacteria anaerobically containing special reducing agents to be dispersed in each well of micro-titer 96 well, (150 μl), then a single colony of each micro-organism was carefully chosen and mixed into the well of broth, from that well. We proceeded in dilution on 1:10 rate until we reached the 5th dilution. Four plates of enriched solid blood agar media were prepared for each bacteria; spreading broth taken from 5th dilution well on each plates then exposed to different periods of light exposure, a light beam of blue light was directed on the plate, starting from zero/seconds (no light exposure) for the first plate, then 20, 40, 60 for the 2nd, 3rd and 4th plate respectively; tip of the light cure devise is standardized with the center of light beam was directed towards the center of plate for all experiments. The visible blue light emitted from commercially available light cure devise (LED curing light); that emits blue light (400-500nm) of 1000mw energy. After light exposure, the bacterial killing rates were calculated from colony forming unit (CFU) counts after 48hours of anaerobic incubation. The total colonial count was achieved on day 13; the CFU’s was counted by direct vision. The plate that has no light exposure (zero second groups) for each micro-organism considered the control plate which with we compared the results of the remaining 3 plates. The whole procedure was repeated for each one of the 15 samples of patients who participated in the study.

RESULTS
It is very obvious that the values of CFU decreased significantly (p=0.001) as the time of exposure difference increased between groups until reaching the highest value when the difference was 60 seconds. There was significance effect of visible blue light on the CFU of anaerobic periodontal pathogens Aggregatibacter actinomycetemcomitans in-vitro at different light exposure time. There was increase in the inhibition of growth in which the inhibition rates for 20 sec exposure, 40 sec exposure and 60 sec exposure were 41.2%, 54.2%, and 64.5% respectively. Also, there was decrease of Mean±SD of CFU as we proceed from A: zero seconds of light exposure, B: 20S, C: 40S and D: 60S [305.6±36.9 to 179.7±18.6 (20S), 140±15.9 (40S) and 108.6±13.3 (60S)]. In intergroup comparison CFU of the bacteria at each period of light exposure time was compared to the CFU at all the periods of
light exposure. There was a high significant statistical difference between the control group (had no light exposure) and the 60 second group (p=0.001) (Table 1, 2). Table 3 and Table 4 shows the phototoxic effect of visible blue light on the CFU of anaerobic periodontal pathogens Porphyromonas gingivalis at different light exposure time. It is very obvious that the values of CFU decreased significantly (p<0.001) by the increase of exposure time compared to the control group. By comparing with zero exposure, the inhibition rates for 20 sec exposure, 40 sec exposure and 60 sec exposure were 22.04%, 35.4%, and 49.7% respectively. Also, there was decrease of Mean±SD of CFU as we proceed from A: zero seconds of light exposure, B: 20S, C: 40S and D: 60S (236.8±28.8 at zero time to 184.6±14.7, 153.1±15.4, and 119±9 respectively).

DISCUSSION
In the current study there was significance effect of visible blue light on the CFU of anaerobic periodontal pathogens Aggregatibacter actinomycetemcomitans in-vitro at different light exposure time in culture media in which the inhibition rates for 20 sec exposure, 40 sec exposure and 60 sec exposure were 41.2%, 54.2%, and 64.5% respectively in comparing with zero exposure time (Table1). Also the Mean±SD of CFU was significantly (p<0.05), decreased from 305.6±36.4 at zero time to 179.7±18.6 (20 sec), 140.1±15.9 (40 sec), and 108.±13.8 (60 sec) respectively (Table 2). These results confirmed the toxic effect of visible blue light on Aggregatibacter actinomycetemcomitans. This effect can be explained by the fact that the function of the exogenous photosensitizers is to absorb the visible light that matches the wavelength of their peak absorption, then causing a photochemical mechanism that kills bacterial pathogens. The current study results are similar to that reported by Henry et al. in which they showed that visible blue light was proven to have phototoxic effects on Porphyromonas; Prevotella species. Also similar effects were observed when utilizing visible light against Porphyromonas gingivalis and Fusobacterium nucleatum without an exogenous photosensitizer.

In addition, results regarding Porphyromonas gingivalis obtained from this research is in agreement with a study done by Feuerstein et al. who suggested a phototoxic effect of visible blue light on Gram negative anaerobic periodontal pathogens without use of exogenous photosensitizer. Results regarding Porphyromonas gingivalis came in agreement also with a study done by Hyun-Hwa Song et al. but in disagreement with the same study as much as it’s concerned with Aggregatibacter actinomycetemcomitans results where they found no significant phototoxic effect of visible blue light against Aggregatibacter actinomycetemcomitans. Also, they found that there was a phototoxic effect of visible blue light emitted from a halogen light curing device source on planktonic anaerobic periodontal pathogens.

A high significant statistical difference (p<0.05) was observed in comparing the CFU of Aggregatibacter actinomycetemcomitans at different periods of time of blue light exposure, and there was a significant statistical difference was observed in comparing the CFU of Porphyromonas gingivalis at different periods of time of blue light exposure. That’s mean more exposure time leads to more bacterial death. This can be explained by the decrease of bacterial CFU of both Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis directly with the period of exposure to the curing blue light. Also the decrease of bacterial CFU is explained by the killing ability of light and temperature against these bacteria. Visible light (408-750 nm) has been found to be mutagenic and to cause genotoxic effects on relevant host cells such as superoxide anion, hydrogen peroxide, and hydroxyl radicals that damage proteins, DNA, lipid, and cell membrane. As well light sources have considerably stronger effects with reactive oxygen radicals which occurs in combined form with natural photosensitizers, such as humic acid or protoporphyrin. It was also found that Enzyme synthesis of bacteria such as Super Oxide Dismutase and catalyse of bacteria have been shown to decrease with the effects of light independently.

The current study results suggest clearly that the effect of blue light exposure increases as the time of exposure increases, whenever the difference of blue light exposure time between groups increases, the difference between CFU’s was more significant, and the best results were obtained when there was a (60 seconds) difference, and the results of comparison was high significant in both organisms. As conclusion, there was a phototoxic effect for the visible blue light emitted from the light curing device against the anaerobic periodontal pathogens. Furthermore, one of the advantage of photodynamic therapy (PDT) as visible blue light is its safety, as it be confirmed by Dai et al. a, b, Ramakrishnan et al. and Zhang et al. in which they showed that under certain exposures, no cytotoxic or genotoxic effects on relevant host cells. In addition, no evidence of genotoxicity of visible blue light (aBL) was observed in mouse skin in vivo when exposed to the therapeutic aBL exposure for inactivating mature biofilms.

CONCLUSION
In conclusions, there was a phototoxic effect for the visible blue light emitted from the light curing device against the anaerobic periodontal pathogens and blue light exposure is effective in reducing periodontal pathogens. It is recommended that an adjunctive exogenous photo-sensitizer be used and that pathogens be exposed to visible light for clinical antimicrobial periodontal therapy.

ACKNOWLEDGMENTS
The authors would like to acknowledge Faculty of Dentistry, Sana’a University and the Microbiology Department of the National Center of Public Health...
Laboratories (NCPHL) Sana’a, Yemen for support and provided working space and materials.

CONFLICT OF INTEREST

"No conflict of interest associated with this work”.

AUTHOR'S CONTRIBUTION

This research work is part of MSc thesis. The candidate is the second author (AAA) who conducted the works and the experiments and wrote up the thesis. The corresponding author (HAA) supervised the experimental work, revised and edited the thesis draft and the manuscript. (MAA) was co-advisor of the work, and (AA) helped in dental works and laboratory works.

REFERENCES

1. Petersen PE, Ogawa H. The global burden of periodontal disease: towards chronic integration with clinical disease prevention and control. Periodontology 2012; 2000 15-39.
2. Schmidt J, Jentsch H, Stingu CS, Sack U. General immune status and oral microbiology in patients with different forms of periodontitis and healthy control subjects. PLoS ONE 2014; 9(10): e109187.
3. Kah Yun How KY, Song KP, Chan KG. Porphyromonas gingivalis: An Overview of Periodontopathic Pathogen below the Gum Line. Front Microbiol 2016; 7:53 62.
4. Praveen NC, Rajesh A, Madan M, Chaurasia VR, Hiremath NV, Sharma AM. In vitro evaluation of antibacterial efficacy of pineapple extract (Bromelain) on Periodontal Pathogens. J Int Oral Health 2014; 6(5): 96–98.
5. Berakdar M, Callaway A, Fakhr Eddin M, Roß A, Willershansen B. Comparison between scaling-root planing (SRP) and SRP/photodynamic therapy: six month study. Head Face Med 2012; 8: 12.
6. del Pozo JL, Patel R. The challenge of treating biofilm-associated bacterial infections. Clin Pharmacol Ther 2007; 82: 204–9.
7. Anderson GG, O'Toole GA. Inmate and induced resistance mechanisms of bacterial biofilms. Curr Top Microbiol Immunol 2008; 322: 85–105.
8. Wilson M. Lethal photo-sensitisation of oral bacteria and its potential application in the photodynamic therapy of oral infections. Photochem Photobiol Sci 2004; 3: 412–8.
9. Takasaki AA, Aoki A, Mizutani K, Schwarz F, Sculean A, Wang CY, et al. Application of antimicrobial photodynamic therapy in periodontal and peri-implant diseases. Periodontol 2009; 51:109–140.
10. Dai T, Gupta A, Murray CK, Vrahos MS, Tegos GP, Hamblin MR. Blue light for infectious diseases: Propionibacterium acnes, Helicobacter pylori, and beyond? Drug Resist Updat 2012; 15:223–236.
11. Kleinpenning MM, Smits T, Frunt MH, Van Erp PE, Van de Kerkhof PC, Gerritsen RM. Clinical and histological effects of blue light on normal skin. Photo-dermatology, photo-immunology and photo-medicine 2010; 26:16–21.
12. Liebmann J, Born M, Kolb-Bachofen V. Blue-light irradiation regulates proliferation and differentiation in human skin cells. J Invest Dermatol 2010; 130:259–269.
13. Sharman WM, Allen CM, van Lier JE. Photodynamic therapeutics: basic principles and clinical applications. Drug Discov Today 1999; 4: 507–517.
14. Maisch T, Szeimies RM, Jori G, Abelis C. Antibacterial photodynamic therapy in dermatology. Photochem Photobiol Sci 2004; 3: 907–17.
15. Maisch T. Anti-microbial photodynamic therapy: useful in the future? Lasers Med Sci 2007; 22: 83–91.
16. Henry CA, Judy M, Dyer B, Wagner M, Matthews JL. Sensitivity of Porphyromonas and Prevotella species in liquid media to argon laser. Photochem Photobiol 1995; 61:410–13.
17. Henry CA, Dyer B, Wagner M, Judy M, Matthews J. Photo-toxicity of argon laser irradiation on biofilms of Porphyromonas and Prevotella species. J Photo chem Photobiol B 1996; 34: 123–8.
18. Feuerstein O, Persman N, Weiss EI. Phototoxic effect of visible light on Porphyromonas gingivalis and Fusobacterium nucleatum in vitro study. Photo-chem Photobiol 2004; 80: 412–15.
19. Feuerstein O, Ginsburg I, Dayan E, Veler D, Weiss EI. Mechanism of visible light photo-toxicity on Porphyromonas gingivalis and Fusobacterium nucleatum. Photochem Photobiol 2005; 81: 1186–9.
20. Hyun-Hwa Song, Jae-Kwan Lee, Heung-Sik Um, Beom-Soon Chang, Si-Young Lee, Min-Ku Lee. Phototoxic effect of blue light on the planktonic and biofilm state of anaerobic periodontal pathogens. J Periodontal Implant Sci 2013; 43(2): 72–8.
21. Karim E, René S. A comparative study of the photo-inactivation of bacteria by meso-substituted cationic porphyrin, rose Bengal and methylene blue. Desalination 2009; 246(1–3):353-362.
22. Dai T, Gupta A, Huang YY, Sherwood ME, Murray CK, Vrahos MS, Kielian T, Hamblin MR. Blue light eliminates community-acquired methicillin-resistant Staphylococcus aureus in infected mouse skin abrasions. Photomed Laser Surg. 2013a; 31:531–538.
23. Dai T, Gupta A, Huang YY, Yin R, Murray CK, Vrahos MS, Sherwood ME, Tegos GP, Hamblin MR. Blue light rescues mice from potentially fatal Pseudomonas aeruginosa burn infection: efficacy, safety, and mechanism of action. Antimicrob Agents Chemother 2013b; 57:1238–1245.
24. Ramakrishnan P, Maclean M, MacGregor SJ, Anderson JG, Grant MH. Cytotoxic responses to 405nm light exposure in mammalian and bacterial cells: Involvement of reactive oxygen species. Toxicology in vitro: an international journal published in association with BBIRA. 2016;33:54–62.
25. Zhang Y, Zhu Y, Gupta A, Huang Y, Murray CK, Vrahos MS, Sherwood ME, Baer DG, Hamblin MR, Dai T. Antimicrobial blue light therapy for multidrug-resistant Acinetobacter baumannii infection in a mouse burn model: implications for prophylaxis and treatment of combat-related wound infections. J Infect Dis 2014;209:1963–1971.
26. Wang Y, Wu X, Chen J, Amin R, Lu M, Bhayana B, Zhao J, Murray CK, Hamblin MR, Hooper DC, Dai T. Antimicrobial blue light inactivation of gram-negative pathogens in biofilms: in vitro and in vivo studies. J Infect Dis, 2016; 213:1380-1387.
### Table 1: The phototoxic effect of visible blue light on the CFU of anaerobic periodontal pathogens

*Aggregatibacter actinomycetemcomitans* at different light exposure time

| CFU Values | Time of exposure (sec) | Zero | 20 sec | 40 sec | 60 sec |
|------------|------------------------|------|--------|--------|--------|
| Mean       | 305.6                  | 179.7| 140.1  | 108.6  |
| Variance   | 1366.6                 | 346  | 253.6  | 190.6  |
| Standard division | 36.9           | 18.6 | 15.9   | 13.8   |
| Standard error | 9.5              | 4.8  | 4.11   | 3.5    |
| Minimum    | 208                    | 140  | 102    | 80     |
| Maximum    | 370                    | 210  | 165    | 125    |
| Median     | 305                    | 180  | 143    | 110    |
| Mode       | 300                    | 190  | 130    | 100    |
| Sum        | 4584                   | 2696 | 2101   | 1629   |
| student test | 32                | 37.4 | 30.4   | 30.4   |
| P value    | <0.001                 | <0.001| <0.001| <0.001 |
| Inhibition growth rate | Ref | 41.2%| 54.2% | 64.5%  |

### Table 2: The significance of the phototoxic effect of visible blue light on the mean ±SD CFU of anaerobic periodontal pathogens *Aggregatibacter actinomycetemcomitans* at different light exposure time

| Time of exposure (sec) | CFU of tested bacteria, Mean ±SD | $\chi^2$ | P value |
|------------------------|----------------------------------|--------|---------|
| Control (Zero)         | 305.6±36.9                       | Reference |
| 20 sec                 | 179.7±18.6                       | 12.02  | 0.002   |
| 40 sec                 | 140±15.9                         | 16.2   | 0.0007  |
| 60 sec                 | 108.6±13.8                       | 19.79  | 0.0003  |

### Table 3: The phototoxic effect of visible blue light on the CFU of anaerobic periodontal pathogens *Porphyromonas gingivalis* at different light exposure time

| CFU Values | Time of exposure (sec) | Zero | 20 sec | 40 sec | 60 sec |
|------------|------------------------|------|--------|--------|--------|
| Mean       | 236.8                  | 184.6| 153    | 119    |
| Variance   | 832.1                  | 218  | 237.2  | 81     |
| Standard division | 28.8           | 14.7 | 15.4   | 9      |
| Standard error | 7.4              | 3.8  | 3.9    | 2.3    |
| Minimum    | 200                    | 168  | 130    | 100    |
| Maximum    | 317                    | 215  | 180    | 135    |
| Median     | 230                    | 185  | 153    | 120    |
| Mode       | 210                    | 170  | 130    | 120    |
| Sum        | 3552                   | 2769 | 2295   | 1786   |
| student test | 31.8             | 48.3 | 38.4   | 51.2   |
| P value    | <0.001                 | <0.001| <0.001| <0.001 |
| Inhibition growth rate | Ref | 22.04%| 35.4% | 49.7%  |

### Table 4: The significance of the phototoxic effect of visible blue light on the mean ±SD CFU of anaerobic periodontal pathogens *Porphyromonas gingivalis* at different light exposure time

| Time of exposure (sec) | CFU of tested bacteria, Mean±SD | $\chi^2$ | P value |
|------------------------|----------------------------------|--------|---------|
| Control (Zero)         | 236.8±28.8                       | Reference |
| 20 sec                 | 184.6±14.7                       | 6.3    | 0.01    |
| 40 sec                 | 153±15.4                         | 10.16  | 0.004   |
| 60 sec                 | 119±9                            | 15.5   | 0.001   |