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EVALUATION AND COMPARISON OF THE EFFECT OF INDOCYANINE GREEN, PHOTOTHERMAL THERAPY AND PHOTODYNAMIC THERAPY ON TITANIUM ADHERENTBIOFILM OF PORPHYROMONAS GINGIVALIS - AN IN VITRO STUDY

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ABSTRACT

Introduction: Peri-implantitis is an oral disease that is known to cause inflammation around osseointegrated implants and its supporting structures due to the presence of plaque (dental biofilm). It is caused by the accumulation of dental plaque which is a structurally and functionally well-organized biofilm that normally maintains a homeostatic relationship with the human host. A disturbance in this balance causes a microbial shift from commensal to pathogenic periodontal pathogens that mark the beginning of the peri-implant disease. The frontline treatment for peri-implant diseases includes scaling and root planning (SRP) that effectively removes the plaque and restores it to a healthy state and the use of antimicrobial agents such as Chlorhexidine that are often used as an adjunct to SRP to aid and maintain the healthy state of tissues. However, these antimicrobial agents have side effects such as alteration of taste, discoloration of teeth and development of antimicrobial resistance. The conventional treatment doesn't eliminate the biofilm completely due to the complex structure of the implant. Hence, there has been a shift in research towards new non- invasive technique as LASER. However, the laser may cause some detrimental thermal effects on the surrounding periodontal tissues that lead to potential and unexpected side effects. Recently, an alternative approach named antimicrobial photodynamic therapy (aPDT) has been developed for the decontamination of implant surfaces. In dentistry, the application of PDT is growing rapidly. The purpose of the current study is to assess and compare the antimicrobial activity of Indocyanine green, Photothermal therapy and Photodynamic therapy on the titanium adherent biofilm of Porphyromonas gingivalis. **Aim:** To assess and compare the antimicrobial activity of Indocyanine green, Photothermal therapy and Photodynamic therapy on titanium adherent biofilm of Porphyromonas gingivalis. **Materials and Methods:** This is an experimental in-vitro microbial study. 120 pre-sterilized titanium of 8mm and thickness of 2mm was obtained and the disc were inoculated with strain of porphyromonas gingivalis and kept in anaerobic chamber for 48 hours. The inoculated disc was randomly allocated into four groups. Group 1: control group; group 2: photosensitizer (Test group 1); group 3: Photothermal therapy (Test group 2); group 4: Photodynamic therapy (Test group 3). The dye used was Indocyanine green dye. Diode laser was used with 940 nm at 0.1 power watt at 5J/cm2 for 30-40 sec. The data were entered in Excel and analyzed statistically using the SPSS software version. Inter group comparisons was done by One way ANOVA. Pairwise Comparison of four groups was also carried out using LSD post hoc test. All statistical tests were performed at a significance level of 5% ($p<0.05$). **Results and Conclusion:** In test groups there was a significant reduction in P. gingivalis colony count in ICG, PTT and PDT group. The maximum reduction in P.gingivalis bacterial colony count was noted with PDT (Mean ±SD is $20.7\pm2.7 \times 10^4$) while minimum reduction was noted in ICG group (Mean±SD is $51 \pm 2.6 \times 10^4$). Photothermal therapy also showed reduction in P.gingivalis bacterial colony count with $34.3 \pm 4.2 \times 10^4$) Therefore, it is described that Photodynamic therapy (PDT) showed maximum reduction in P. gingivalis bacterial colony count when compared to other treatment protocol and shows there is statistically significant result of P value <0.001

KEYWORDS: Implant, Indocyanine green, Laser, Peri-implantitis, Photodynamic therapy.

INTRODUCTION

Peri-implantitis is an inflammatory process that affects the tissue around an osseointegrated implant and results in the loss of supporting bone. Mouhyi et al (2012) proposed that predisposing factor of peri-implantitis includes the presence of aggressive bacteria, excessive mechanical stress, and corrosion. Each was documented as a factor that could act synergistically with biofilm to worsen the condition.[1]

Numerous surgical and non-surgical approaches have been proposed to treat peri-implantitis with the primary intention of eliminating bacterial contamination and impeding bone resorption. However, current roughened implant body surfaces have made the removal of the biofilm from the surface extremely challenging via mechanical decontamination alone.[2] Thus, antibiotics, antiseptics, and laser treatments have been advocated as therapeutic supplement alternatives in the non-surgical and surgical treatment of peri-implantitis.[3]

Laser is an acronym for Light amplification by stimulated emission of radiation. A laser has a direct effect on gram- negative anaerobic bacteria Hence using of diode laser (940 nm) can be clinically valuable for the treatment of peri- implantitis.[4]

Photosensitizer molecules can play a significant role in killing both Gram-positive bacteria and Gram-negative bacteria. Indocyanine green (ICG) is an anionic photosensitizer, which is water soluble and relatively non-toxic. It has both a photothermal effect and a photochemical effect. It can efficiently eliminate bacteria from deep periodontal pockets.[5]

Photodynamic therapy (PDT) has emerged in recent years as a non–invasive therapeutic modality for the treatment of various infections by bacteria, fungi, and viruses.[6] Photodynamic antimicrobial chemotherapy represents an alternate antibacterial, antifungal, and antiviral treatment against drug-resistant organisms.[7] Applications of PDT in dentistryare growing rapidly. They are also used in the treatment of oral cancer, bacterial and fungal infections, and in the photodynamic diagnosis of the malignant transformation of oral lesions.[8]

Thus, the aim of the present study is to evaluate and compare the effect of Indocyanine green, photothermal therapy, and photodynamic therapy on the titanium adherent biofilm of *Porphyromonas gingivalis.*

MATERIALS AND METHODS

120 Pre-sterilized titanium discs of diameter 8 mm and thickness of 2 mm was obtained from Indident™ (Indident
Medical Devices) and randomly allocated to four different group. Each group contains 30 discs.
Group 1 - Control group; Group 2 – Photosensitizer (Test group 1);
Group 3 – Photothermal therapy (Test group 2);
Group 4 – Photodynamic therapy (Test group 3)

Blood agar was prepared in Petri-plates and the titanium discs are placed in the prepared petri -plates and the strains *Porphyromonas gingivalis* (PG) – ATCC 33277 which was obtained from depository BSRC was inoculated on thirty titanium discs. The discs are kept in an anerobic chamber for 48 hours and 72 hours.Bacterial colonization or biofilm formation was taken places in all discs. These biofilm formation on titanium discs was assessed by Phloxin – B stain.

All these inoculated discs in Petri plates were randomly allocated to four different groups that includes
Group 1: Control group (no treatment);
Group 2: Photosensitizer (Indocyanine green dye ICG);
Group 3: Photothermal Therapy (Diode Laser);
Group 4: Photodynamic Therapy (ICG + Diode Laser)

Laser protocol

Titanium discs were irradiated with GaAlAs Diode Laser (BIOLASE, the Diode Laser Therapy System) with 400µm fiber optic handpiece at a wavelength of 940 nm operated at power – 0.1 W, with a pulse length of 200µm and pulse interval of 200µm in the noncontact mode for 30-60s.

Indocyanine green

A solution of Indocyanine green was prepared by dissolving it in 5 mL of sterile water to prepare an initial 5 mg/ mL ICG stock solution. This stock solution was further diluted in saline solution at ratio of 1:5 to achieve a final ICG concentration of 5mg/mL before implementation.

Photodynamic therapy procedure

Application - Titanium discs which are randomly allocated with a solution for 2 minutes.
Soaking phase – The solution with active ingredient attaches the bacterial cell membrane and dyes them; sensitizesthe bacteria.
Rinsing phase – Rinsing off excessive active ingredients. Green dye bacteria remain on titanium discs.
Activation - It is activated by laser light energy for 30-60 s with 5J/$cm^{2.}$

Statistical analysis

The data were entered in Microsoft Excel and analyzed statistically using the SPSS software, version 21; SPSS Inc., (Chicago, IL, USA). The normality of the data was assessed prior to analysis using the Shapiro-Wilk's test/Kolmogorov-Smirnov test. Data were found to be normally distributed. Thus, the parametric test was chosen. Descriptive analysis was calculated.

Intergroup comparisons were done by One way ANOVA. Pairwise Comparison of four groups was also carried out using LSD post hoc test. All statistical tests were performed at a significance level of 5% ($p<0.05$).

RESULT AND OBSERVATIONS

This in-vitro study was conducted on 120 sterile titanium discs contaminated with *P. gingivalis.* The antimicrobial effect of different treatment protocols on the biofilm formation of *P. gingivalis* is presented in Table 1. It shows the count of colony forming unit/ml in all the groups (i.e., Control group, Indocyanine Group (ICG), Photothermal therapy (PTT) and Photodynamic therapy (PDT). It was observed that the most contaminated groups was control group (ster-C) in which no treatment

protocol was applied. In the present study it was observed that there was a significant reduction in P. gingivalis colony count in ICG, PTT, and PDT groups when compared with the control group (Figure 1). The maximum reduction in bacterial colony count was noted with PDT while the minimum reduction was noted in ICG group. Therefore, it is described that Photodynamic therapy (PDT) showed a maximum reduction in *P. gingivalis* bacterial colony count when compared to other treatment protocol.

Table 1: Descriptives Analysis of CFU/ml of all groups. DYE: Indocyanine green dye; PTT: Photothermal therapy; PDT: Photodynamic therapy; Ster-C: Sterile control group.

Groups	n	Mean ± SD	95% Confidence Interval	
			Lower Bound	Upper Bound
STER -C	30	$62.3 \pm 3.2\times10^4$	60.3×10^4	63.4×10^4
DYE	30	$51 \pm 2.6 \times10^4$	50.9×10^4	52.1×10^4
PTT	30	$34.3 \pm 4.2\times10^4$	32×10^4	35.7×10^4
PDT	30	$20.7 \pm 2.7 \times10^4$	19.4×10^4	21×10^4

* Shows a statistically significant result.

The descriptive analysis of all groups was done to estimate the mean and standard deviation. (Table 2). All test groups show significant differences compared to control group. Among test groups; Photodynamic therapy (PDT) protocol showed statistically significant reduction in microbial load compared to PTT and Dye groups, demonstrating that PDT was superior in reducing microbial load compared to other test groups. In comparison with mean and standard deviation of all test groups; the mean and standard deviation in PDT group is $20.7 \pm 2.7 \times10^4$; stating that PDT is superior in reducing microbial load. With PTT and ICG (PS) the mean and standard deviation is $34.3 \pm 4.2\times10^4$ and $51 \pm 2.6 \times10^4$ respectively; stating that PTT protocol is better in reducing the microbial load compared to ICG(PS); (Table 2).

Table 2: ANOVA.

CFU	Sum of Squares	df	Mean Square	F	p-value
Between Groups	3.07E+12	3	1.02E+12	847.65	<0.001
Within Groups	1.4E+11	116	1.21E+09		

Analysis was done to see the difference between inter and intra group comparison. ANOVA was carried out to determine the difference between and within groups (Table 3). Between the groups the mean difference was 3.07+12 and within groups the mean difference was 1.4+11. For individual result, inter group comparisons (i.e., multiple comparison) were carried out to determine the mean differences of control and test groups. It shows that there is a statistically significant *P value* of <0.001 between groups. (Table 2).

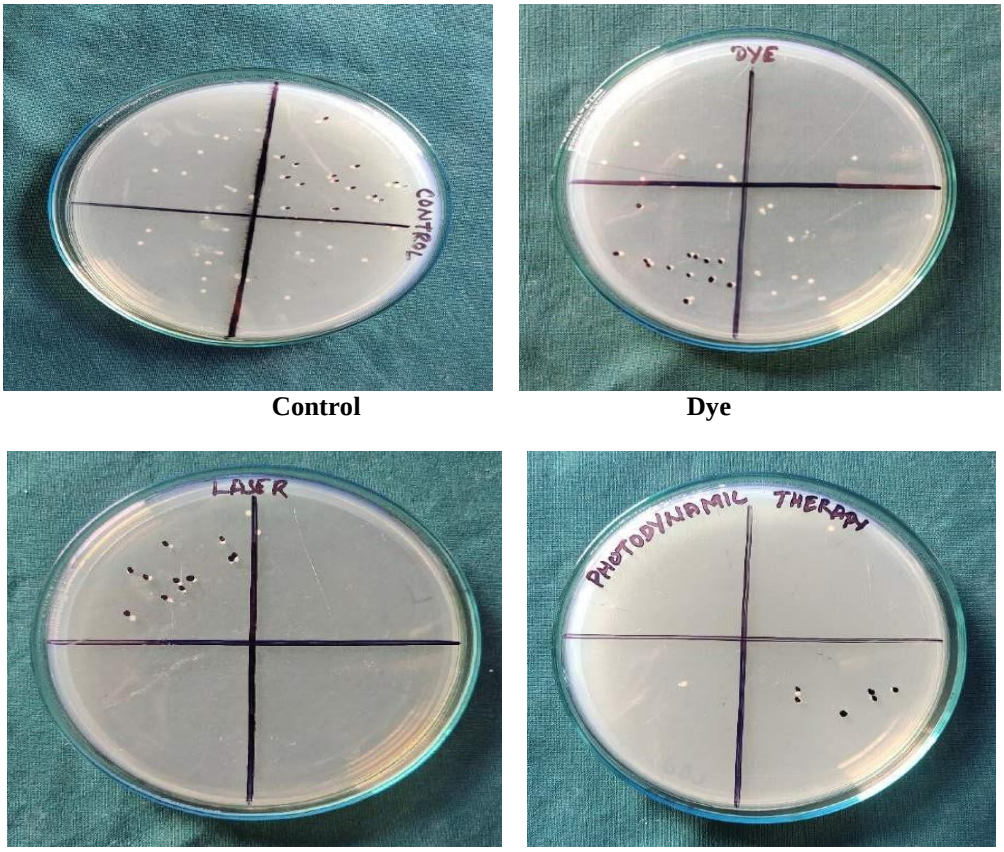
Table 3: Inter group comparisons of Control and Test groups.
Multiple Comparisons Dependent Variable: CFULSD

Status	Status	Mean difference	Std. Error	Sig.	95% Confidence interval	
					Lower Bound	Upper Bound
STER-C	DYE	10.3	8975.06	<0.001	91557.1	127109.6
STER-C	PTT	28	8975.06	<0.001	262223.8	297776.2
STER-C	PDT	41.6	8975.06	<0.001	400890.4	436442.9
DYE	PTT	17.6	8975.06	<0.001	152890.4	188442.9
DYE	PDT	30.3	8975.06	<0.001	291557.1	327109.6
PTT	PDT	13.6	8975.06	<0.001	120890.4	156442.9

Result of the Post hoc test between experimental groups according to *P-value*. The level of significance was set to 0.5. DYE: Indocyanine green dye; PTT: Photothermal therapy;
PDT: Photodynamic therapy; Ster-C: Sterile control group
*The mean difference is significant at the 0.05 level.

The inter group comparisons between Control and other test groups (Dye, PDT, PTT) showed mean difference of Dye – 10.3; PDT - 41.6; PTT – 28 compared to control groups. In comparison with Photosensitizer (ICG) and Photothermal therapy (PTT), PTT showed significant mean difference of 17.6; stating that PTT is better in reducing microbial load compared to ICG. Then comparing photosensitizer dye and photodynamic therapy, PDT showed significant mean difference of 30.3; stating that PDT is superior in reducing the microbial load compared to Photosensitizer dye (ICG).

In comparison with Photodynamic therapy and photothermal therapy; PDT showed significant mean differenceof 13.6 stating that PDT is superior in reducing the microbial load compared to PTT. Therefore, the intergroup comparison shows that photodynamic therapy is superior in reducing the bacterial load when compared to other treatment groups and has statistically significant *P value* (<0.001) (Table 3)



Control **Dye**

Laser(PTT) **PDT**

Figure 1: Efficacy of different treatment modalities in the reduction of colony counts (CFU/ml) of *P.gingivalis.* (PTT- Photothermal therapy; PDT- Photodynamic therapy).

DISCUSSION

Several studies and investigations have been carried out to find an alternative method for conventional treatment for peri-implantitis, which consists of mechanical debridement of the involved implant surfaces. In periodontal and peri-implant diseases, of all the bacteria engaged in peri-implant diseases, mainly *P. gingivalis* is a major periodontal pathogen for developing periodontitis and peri-implant disease.

The present study was conducted to compare and investigate the effect of Indocyanine green (ICG), Photothermal therapy (PTT) and Photodynamic therapy (PDT) against *P.gingivalis*. The results showed significant reductions in colony counts of *P.gingivalis* on the titanium surfaces in all studied groups. The highest reduction was observed with Photodynamic therapy and the lowest rate was seen in ICG group. It was also found that the PTT also showed significant reduction in *P.gingivalis* colony count compared to ICG group.(Table2).

It was observed that there was a statistically significant reduction in *P.gingivalis* colony count in ICG, PTT,PDT group when compared with the control group (Table 2). The greatest reduction in colony count was noted with PDT while minimum reduction was noted in ICG group. These findings were in accordance with the study done by Alagl et al[9] states that PDT was superior in reducing the microbial load compared to laser therapy.

Evidence shows that aPDT decreases the level of tumor necrosis factor alpha and interleukin-1B.[10] This is in accordance to the present study stating that the efficacy of aPDT with ICG and diode laser showed a statistically significant reduction of *P.gingivalis* bacterial colony count. Clinical studies have shown that in application of PDTthere is a reduction in level of cytokines in gingival crevicular fluid.[11] It also resolves inflammation and enhances peri-implant soft and hard tissue healing.[12] Evidence shows that cationic, anionic and neutral photosensitizer molecules play a significant role in killing of Gram-positive bacteria and Gram-negative bacteria.[13] Gram-negative bacteria have an internal cytoplasmic membrane along with an external membrane, which decrease the penetration of photosensitizer. ICG is an anionic photosensitizer, which is water soluble and relatively non-toxic.[14] Its

photothermal effect is greater than its photochemical effect.[15] It can efficiently reduce the bacteria from deep periodontal pockets due to its photothermal effect. Its absorbance peak should be at 800 -940 nm.[16] Thus, Diode laser used in this current study is suitable for ICG i.e., PDT[17] (Table 2).

Bohem et al.[18] demonstrated that *A. actinomycetemcomitans* had the highest absorbance peak in presence of 10 µm concentration of ICG for 5 min. In his study; diode laser used with 810 nm with 0.1W and 0.5 W power at 80 and400 W/cm2 energy density for time period of 5s caused a significant reduction in *A. actinomycetemcomitans* in culture medium. Only laser therapy did not show reduction in the bacterial count. Because power of laser (0.1W) used was of lower Watt and time period of application was of shorter duration(5s).

In our present study, we used diode laser with 940nm at power (150 mW) of time period of 30s. Our study showed a significant reduction of *P. gingivalis* colony count on titanium disc (Table 2); stating that use of higher power watt and longer duration showed statistically significant reduction in bacterial count with both Photothermal therapy and Photodynamic therapy. (Table 2)

Pourhajibagher et al.[19] demonstrated that ICG at 62.5 µg/mL concentrations for 5 min caused 23.2% reduction in *P. gingivalis* count. Diode laser irradiation with 810 nm wavelength and 62.5 J/cm2 energy density for 2 min causeda reduction in *P. gingivalis* count by 37%.

In our present study, we used ICG at 1000 µg/mL concentration for 5 mins caused significant reduction in *P. gingivalis* count and its Mean ± SD value is 20.7 ± 19.4. Diode laser irradiation with 940nm wavelength and 6 J/cm^2 energy density for 60 sec caused reduction in *P. gingivalis* count with Mean±SD value of 34.3 ± 4.3 and shows statistically significant result in both Laser and PDT group *(P value* < 0.001). (Table 2).

Saffarpour et al.[20] Moslemi et al[21] and Mattiello et al[22] have reported that aPDT with 810 nm diode laser with 300mW power and 2.38 W/cm2 power density caused a significant reduction in *A.actinomycetemcomitans* count on implant surfaces. These findings were in accordance with the present study stating that aPDT caused a statistically significant reduction in *P.gingivalis* colony count on implant surfaces (P<0.001) in within group and between the groups.(Table 3)

Birang et al[23] in the year 2017 conducted a randomized clinical trial on peri-implantitis patients, both groups in his study i.e., Control (LT) and Test group (PDT) had showed statistically significant improvements in clinical parameters (i.e., bleeding on probing, probing pocket depth (PPD) and modified plaque index). The number of *Aggregatibacter actinomycetemcomitans* (*P* = 0.022), *Tannerella forsythia* (*P* = 0.038) and *Porphyromonas gingivalis* (P = 0.05) in the test group and *Porphyromonas gingivalis* (*P* = 0.015) in the control group had significantly decreased the bacterialcount.

In our study the photosensitizer used was ICG with diode laser and their findings were generally in accordance to the present study and got a statistically significant reduction of *P.gingivalis* colony count in Photodynamic therapy (P<0.001). (Table 4)

Giannelli et al[24] done a study on various laser to evaluate the efficacy of its anti-microbial property of photodynamic therapy on titanium coated biofilm of *P.gingivalis.* The laser includes in the study are Er:YAG (with 2,940-nm wavelength , power density of 75.4 w/cm^2 and pulse energy of 100 mJ for 1 minutes), Nd:YAG (with 1064 nm wavelength and maximum power density of 75.4 W/cm^2 for 1 minute), and 810 nm diode laser (in continuous mode with 1W power and power density of 175.4 W/cm^2 for 1 minutes in photoablation mode.

It had showed that Diode laser in conjunction with Photosensitizer (methylene blue) showed high efficacyin reducing *P.gingivalis* on surface of titanium discs compared with other lasers and confirmed the superiority of a PDT. These findings were in accordance to the present study shows that PDT has significant results (P<0.001) over lasers. (Table 4). The advantages of using PS for treatment of periodontitis or peri-implantitis is these materials can penetrate the implant and root surface porosities that are not accessible by mechanical debridement's.

Thus, the bactericidal effect of Photosensitizer (ICG) is mainly due to its photothermal effect. However, the photodynamic therapy of this material gained a less attention since the oxygen pressure is low. The photothermal mechanism is effective in conjunction with PS(ICG) for reducing the microbial load from implant surfaces. As per the knowledge at present no studies has been done in evaluating the ICG – PDT on peri implantitis.

In the present in-vitro study, it states that there was a statistically significant reduction in *P.gingivalis* colony count on titanium disc PDT states that ICG mediated photodynamic therapy (ICG-PDT) is effective in reducing antimicrobial-resistant strains causing periodontitis and peri-implantitis. Collectively, all this evidence indicates that ICG-PDT provides a clinical benefit than the conventional treatment. Main advantages of using ICG-PDT are a non-invasive surgical procedure which can penetrates into a deep tissue and can reduce the microbial load in the affected sites and has no side effects and it is suitable for medically compromised patients who are not indicated for surgical procedures. It can be act as an alternative approach for decontamination of implant surface and could be used in association with

conventional treatment.

The present study mainly focused on the bactericidal efficacy of laser and PDT on *P.gingivalis* biofilm coated on titanium discs. Thus, to generalize the result of this study, more studies should be conducted on the effect of treatment modalities on the biofilm of other periodontal pathogens causing periodontitis and peri-implantitis. Further studies with different photosensitizers and different laser parameters are required to determine the most efficient combination for aPDT.

None of the techniques in the present study was capable of the complete elimination of *P.gingivalis* bacteria on the implant surface. There was a failure in the complete elimination of *P. gingivalis* on implant surfaces, stating that these methods are inadequate for decontamination of the implant surface, so it is still used as an adjunct to conventional treatment to eliminate the *P. gingivalis* biofilm. Finally, the result of in vitro studies may not be generalized to in vivo conditions. Environmental factors such as variable plaque accumulation, salivation, immune system, limited accessibility, etc., cannot be established in in-vitro studies, so the result of this study could be established by in-vivostudies with large sample size.

CONCLUSION

Within the limitation of this present study, Photodynamic therapy had superior efficacy in reducing the*P.gingivalis* biofilm than the other methods investigated in the study. Simultaneously, photothermal therapy was also found to be a suitable method for disinfecting and reducing the colony count of *P.gingivalis* biofilm on titanium discsin comparison with photosensitizer alone. Therefore, PDT is an effective alternative treatment method for the decontamination of dental implant surfaces without damaging the surface topography and also could be an appropriate adjunct to conventional treatment. The result of this research requires to be further corroboration with long-term prospective in-vivo clinical trials.

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