"COMPARATIVE EVALUATION OF EFFICACY OF DIFFERENT DECONTAMINATION METHODS ON REUSE OF IMPLANT HEALING ABUTMENTS - AN IN VITRO STUDY"

BY

REG. NO- IM0218005

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Head of Department

Dr. ANANDKUMAR G. PATIL M.D.S.
Professor & Head,
Department of Prosthodontics
and Crown & Bridge,
KAHER Vishwanath Katti Institute
of Dental Sciences, Belagavi.

Date:
Place: Belagavi

Principal

Dr. ALKA D. KALE M.D.S.
Principal,
KAHER Vishwanath Katti Institute of
Dental Sciences,
Belagavi.

Date:
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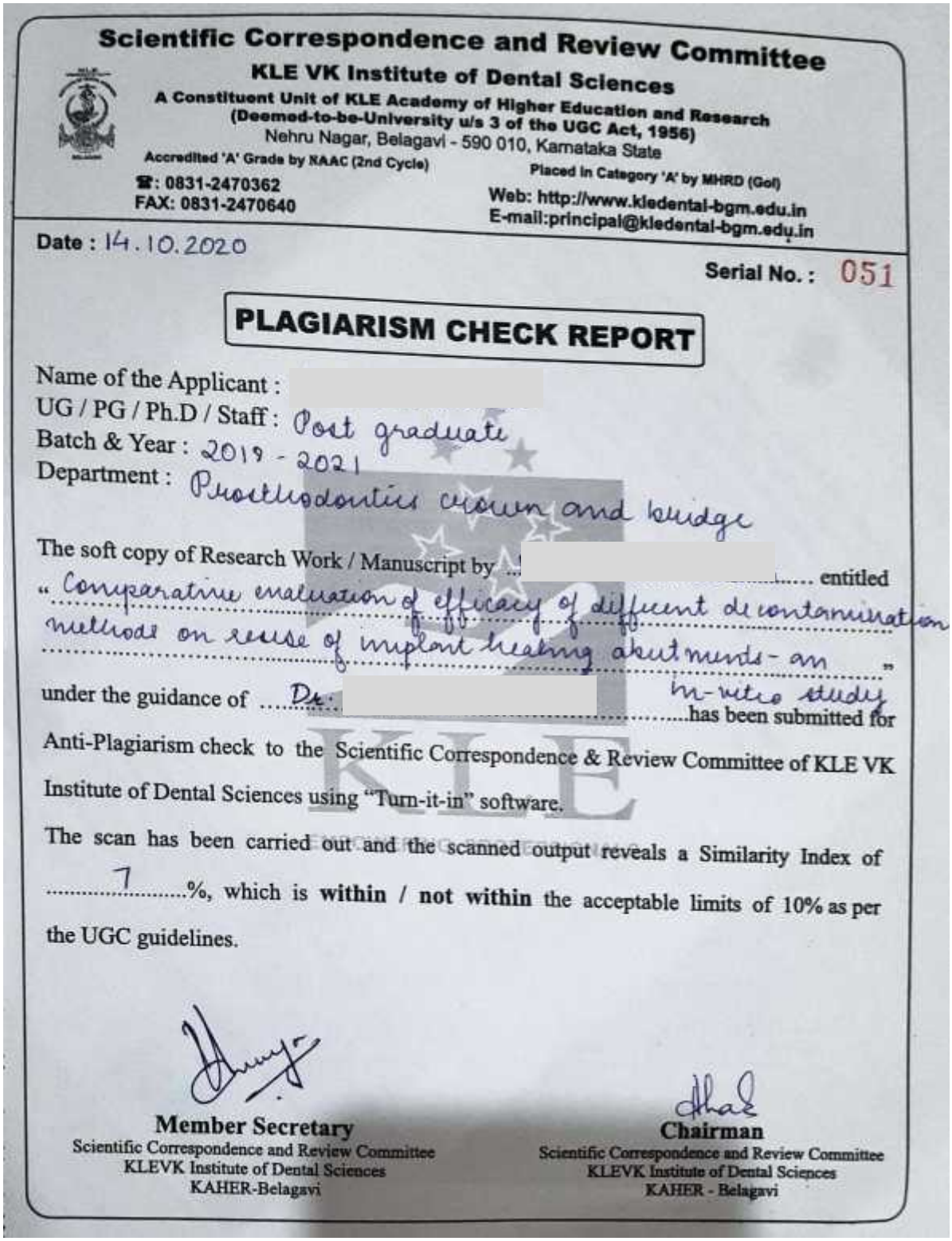

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KLE VK Institute of Dental Sciences

A Constituent Unit of KLE Academy of Higher Education and Research
(Deemed-to-be-University u/s 3 of the UGC Act, 1956)
Nehru Nagar, Belagavi - 590 010, Karnataka State

Accredited 'A' Grade by NAAC (2nd Cycle) — Placed in Category 'A' by MHRD (GoI)

☎: 0831-2470362
FAX: 0831-2470640

Web: http://www.kledental-bgm.edu.in
E-mail:principal@kledental-bgm.edu.in

Date : 14.10.2020

Serial No. : 051

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UG / PG / Ph.D / Staff : Post graduate
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KLE V.K. Institute of Dental Sciences
(A Constituent unit of KLE Academy of Higher Education & Research
Deemed-to-be-University u/s 3 of the UGC Act, 1956)
Nehru Nagar, Belagavi-590 010 INDIA
Re-Accredited 'A' grade by NAAC (2nd Cycle) & Placed in Category 'A' by MHRD (GoI)
☎ 0831-2470362
FAX: 0831-2470640
Web: http://www.kledental-bgm.edu.in
E-mail: principal@kledental-bgm.edu.in

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ABSTRACT

The success of the dental implants mainly depends upon the osseointegration process which increases bone to implant contact. They are widely used for the treatment of partially and fully edentulous patients. After implant placement, there is loss of marginal bone due to surgical trauma. To reduce or to prevent further bone loss, special importance must be given to healing abutments. Contour and action of peri-implant tissue and its acknowledgement to enhance esthetics have been given importance since years. Healing abutments allows growth of gingival tissue and controls tissue shrinkage before final restoration of an implant. When it is made up of biocompatible material such as titanium or titanium alloys, it allows spread of soft tissue and its maturation during healing. It also protects internal part of the implant body from accumulation of debris during healing phase. So, a sterile environment is essential for dental implant placement. Scientific knowledge on the fundamental process of reuse of implant healing abutments is poor and therapy recommendations on peri-implantitis are mainly based on empirical values rather than on well-founded research results.

PURPOSE

The aim of the present study was to evaluate the efficacy of different decontamination methods on the reuse of titanium implant healing abutments.

MATERIALS AND METHODS

Forty-eight titanium implant healing abutments were procured and contaminated by three different microorganisms namely *Porphyromonas gingivalis* ATTC322, *Aggregatibacter actinomycetemcomitans* ATTC33384, *Prevotella intermedia ATTC25611* and were divided into 3 sub-groups based on 3 different

decontamination methods used. Surface profile of all the healing abutments were analysed to evaluate any surface flaw present on their surfaces. If any healing abutments with surface pitting, scratches, worn out threads were excluded from the study. The stereomicroscope with 10X magnification was used to evaluate the surface profile of all the specimens. For adhesion experiment, the healing abutments were immersed with a suspension of the bacterial cultures in 2ml test tubes with 1.5ml of BHI broth. The contamination of the healing abutments was confirmed with staining of 0.5ml of phloxine B staining solution. The decontamination for the specimens in group A (n=16) were done with steam autoclave sterilization at 121^0C for 15-30 mins, in group B (n=16) by ethylene oxide sterilization and in group C (n=16) by low temperature gas plasma sterilization. The samples were vortexed in PBS solution for 1 min and colonies were grown on agar plates. Resultant colony forming units (CFU) were counted by using semiquantitative methods and were tabulated, which formed the basic data for the study.

RESULTS

The results of the study indicated that Low Temperature Gas Plasma decontamination (Group C) showed the least CFU count as compared to other decontamination procedures. Hence, it can be regarded as a better decontamination procedure than Ethylene Oxide and Steam Autoclave for decontamination of titanium implant healing abutments.

CONCLUSION

Within the limitations of this in-vitro study, it was concluded that out of the three decontamination methods, Low temperature gas plasma sterilization showed lowest total CFU counts as compared to other methods and Steam autoclave

sterilization showed highest total CFU counts. Low temperature gas plasma sterilization is better as compared to Ethylene oxide and Steam autoclave sterilization as decontamination method for the reuse of titanium implant healing abutments.

LIST OF ABBREVIATIONS USED IN THE STUDY

Group A	Steam Autoclave sterilization
Group B	Ethylene Oxide sterilization
Group C	Low Temperature Gas Plasma sterilization
CFU	Colony Forming Unit
Pg	Porphyromonas gingivalis
Aac	Aggregatibacter actinomycetemcomitans
Pi	Prevotella intermedia
Mins	minutes
sec	seconds
ml	millilitres
hrs	hours
S.D	Standard Deviation
ANOVA	Analysis of variance
mg	milligrams

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INTRODUCTION

The main objective of modern dentistry is to restore the health, function and aesthetics of patient regardless of a disease or any injury to the system. Now adays for a treatment of any missing tooth, dental implants can be surgically placed into the jaw for fixed replacement. They are left undisturbed for 3 to 6 months where bone healing takes place in which bone grows in and around the implant which is termed as osseointegration. The success of the dental implants mainly depends upon the osseointegration process which increases bone to implant contact. They are widely used for the treatment of partially and fully edentulous patients. After implant placement, there is loss of marginal bone due to surgical trauma. To reduce or to prevent further bone loss, special importance must be given to abutments. [1]

Contour and action of peri-implant tissue and its acknowledgement to enhance esthetics have been given importance since many years. Healing abutments allows growth of gingival tissue and controls tissue shrinkage before final restoration of an implant. These are made up of biocompatible material such as titanium or titanium alloys, which allows spread of soft tissue and its maturation during healing. They also protects internal part of the implant body from accumulation of debris during healing phase. So, a sterile environment is essential for dental implant.[2]

Manufacturers generally recomend single use of these components and few encouraged their reuse for cost efficiency.[3] Many manufacturers recommend reuse of impression copings, implant analogs for the same but only if proper sterilization protocols for these components are followed. Lack of proper sterilization of the healing abutments may result in transmission of pathogens and debris among patients leading to inflammation of the peri-implant tissues and may compromise healing.[2]

The contamination of healing abutments comes from different sources like saliva, epithelial cells, food debris, blood, etc. Properties like surface contamination and wettability have shown to affect the behavior of soft tissue cells leading to reduction of fibroblast cell attachment and spreading.[4]

Contaminants and debris were seen on the surface and at the implant-abutment connection of customized titanium abutments. Presence of such contaminants at the platform-abutment level were suggested to cause tissue inflammation. Titanium microparticles confirmed activation of osteoclast activity showed detrimental effect on the implant-abutment fit. This fit was also suggested to achieve tight soft tissue seal around the implant to prevent soft tissue downgrowth and infection.[4] The early formation of this seal is very important. This seal is divided into two zones:- a marginal zone that consists of junctional epithelium and, apical zone made of connective tissue.[5]

Complications such as peri-implant infections always gave serious challenges to the practitioners. Microbial adhesion leads to biofilm formation which is one of the major reason for failing implant. Biofilms have been associated with majority of infectious diseases like peri-implantitis and periodontitis. It is generally formed around implants in similar way as on teeth. It first forms pellicle, then bacterial contamination with primary and then with secondary colonization. Gram positive cocci and rods and gram-negative bacteria are associated with healthy implants which may also be found in smaller proportions.[6]

As the peri-implant tissues undergo changes from healthy state to diseased one, there is a shift from gram positive to gram negative anaerobic microflora. Most periodontal microorganisms were identified just after 6 months of loading and in

particular, Porphyromonas gingivalis was significantly associated with the implant loss. A greater proportion of red (Treponema denticola, Porphyromonas gingivalis) and orange (Prevotella Intermedia) complexes and Aggregatibacter Actinomy-cetemcomitans were found in failing implant.[6]

Steam autoclave sterilization was shown to be effective in removal of contaminated residue on the abutment surfaces as a conventional sterilization method but also alters the surface topography. Therefore, it has been suggested to use other sterilization methods for their reuse such as Ethylene oxide which is a chemiclave sterilization.[7] It is considered as an effective sterilization method due to its bactericidal activity and its sensitivity for moisture and heat sensitive materials[8]. Studies have shown that Low temperature gas plasma sterilization has been successful to decontaminate the surfaces more effectively as well as modifying the hydrophilicity and wettability of the surfaces.[9] Thus considering the significance of different sterilization methods, this study was taken up to investigate the effect of different decontamination methods for the reuse of implant healing abutments.

NEED FOR THE STUDY

Replacing missing teeth by titanium dental implants is a very promising method.[10] Dentistry needs use of some components that can be reused and decontaminated frequently. These components may cause risk of cross-infection among patients if proper decontamination protocol is not carried out. This occurs due to their direct contact with human tissues. During healing period, healing abutments which are composed of titanium or titanium alloys act as intermediate components between dental implant and oral cavity. This component protects internal aspect of implant screw threads and develops a seal to prevent microbial contamination. It also permits spreading of soft tissue along with their maturation for forming a gingival collar. Once osseointegration and soft tissue maturation is achieved, the healing abutment is replaced with a definitive abutment and prosthesis. Major problem arising on the reuse of healing abutments is efficacy of their decontamination procedures. Any remaining microbial contamination or debris on the surface of the healing abutment can cause deleterious effect on healing of soft tissue and may lead to inflammatory reaction.[11]

Healing abutments are generally established by manufacturers for single use but in general clinical practice many clinicians sterilize them and reuse due to economical constraints.[11] Recent studies showed the that contamination may comes from several sources including epithelial cells, saliva and food debris and without proper disinfection protocol becomes challenging to clean the contaminated surfaces of healing abutment for their reuse. The contamination site can alter mechanical properties of implant-abutment connection. It can cause friction in contaminated screw thread when tightened. It reduces preload level within the screw threads which

decreases clamping forces in implant abutment joint. Contamination also results in violation of hermatic seal which prevents microbial contamination developed by direct contact of the abutment to implant.[11]

According to Mombelli et al[12], Salcetti et al[13] and Persson et al[14], peri-implant pocket nurtures microflora similar to ones found in periodontal diseases like porphyromonas gingivalis, prevotella intermedia, aggregatibacter actinomycetemcomitans, prevotella nigrescens, tannerella forsythia and campylobacter rectus.[9] Porphyromonas Gingivalis, Aggregatibacter Actinomycetemcomitans and Prevotella Intermedia are majority of species found in periodontitis.[15] Due to dental plaque accumulation, development of biofilm occurs consisting of anaerobic bacteria of red complex. This complex contains group of bacteria that are grouped together based on their association with extreme forms of periodontal diseases.[15]

Decontaminating these components to remove the microflora can also modify the surface chemical composition. Studies showed that steam autoclaving and gamma irradiation reduces the surface energy of the components as hydrocarbons react with the surface chemicals. On metals, adhesion increases with increase in hydrophilicity of the surface. Increase in surface hydrophilicity promotes mineralization of bone leading to rapid osteointegration in early bone loading. Higher surface energy is also suggested to produce local factors that promote osteogenic microenvironment.[16]

Some manufacturers of dental implant supports conventional sterilization methods and reusing components of implant but other studies showed that conventional decontamination methods have detrimental effects on the titanium implant surfaces. This may impart major effect on long term success of implants.[17]

Therefore, this study intends to evaluate the efficacy of different decontamination methods on reuse of titanium implant healing abutments.

HYPOTHESIS

NULL HYPOTHESIS: There is no difference in efficacy of different decontamination methods on reuse implant healing abutments.

ALTERNATIVE HYPOTHESIS: There is difference in the efficacy of different decontamination methods on reuse implant healing abutments.

AIM AND OBJECTIVES

AIM OF THE STUDY:

- To evaluate the efficacy of different decontamination methods on the reuse implant healing abutments.

OBJECTIVES:

- To evaluate the efficacy of steam autoclave sterilization, Ethylene oxide sterilization and Low temperature gas plasma sterilization on decontamination of reuse implant healing abutments.
- To compare the efficacy of Steam Autoclave, Ethylene Oxide and Low Temperature Gas plasma sterilization methods on the reuse implant healing abutments.

REVIEW OF LITERATURE

1. **James H.Doundoulakis (1987)** measured and studied comparison of five different sterilization methods on titanium surfaces of implants. Titanium metal samples were manufactured from commercially pure titanium. Each sample (n=50) was ultrasonically cleaned for 10 minutes with a detergent and dried in a clean air room. The samples were processed through a series of analytical technique including "contact angle measurements" and "scanning electron microscopy" with a resolution to $60A^0$. The five sterilization methods evaluated were 1) Endodontic glass bead sterilizer at 425^0F, submerged for 20 seconds in extra fine commercially packaged glass beads. 2) Conventional steam autoclave at 250^0F under 15 psi gauge pressure for 20 minutes. 3) Dry heat at 320^0f for 60 minutes. 4) Ultraviolet radiation 150 watt/second;360 to 450 nm;8 second each side and 5) Radiofrequency glow discharge treatment (Plasma cleaner) with frequency of 13.18 MHz. According to the results of this study ,Dry heat sterilization did not provide a higher surface energy to the specimen. Decontamination of low surface energy materials by radiofrequency glow discharge treatment and Ultraviolet treatment may be suggested for providing a clean and high surface energy that corresponds with induced cell adhesion and implant fixation.[18]

2. **Ong et al (1992)** selected 19 patients for evaluation of periimplant space with age ranged between 22 to 77 years with a minimum requirement of two osseointegrated branemark titanium implants. All clinical variables were assessed for Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis and Prevotella intermedia and total of 37 sites were examined. Patients were

prescribed 0.2% chlorhexidine gluconate mouthwash daily as oral home care hygiene regimen. The results showed 22 of 37 sites having more proportion of anaerobes than aerobes.[19]

3. **Emel Okte et al (1999)** evaluated the adherence of Aggregatibacter actinomycetemcomitans serotypes on the titanium implants. Serotypes a, b and c and two clinical variants d and e strains were taken along with commercially available titanium blade implants. Identical concentration of bacterial suspension of each strain was prepared and 0.5ml of each was added on the implant surfaces coated with glycine-bovine serum albumin(BSA) and incubated at 37^0C for 60mins in 5%CO_2. Implants with adhered microorganisms were evaluated under scanning electron microscope(SEM). The SEM results showed serotype A having highest adherence affinity whereas serotype e showed lowest among all. Serotypes b, c and d showed equal affinity for adhesion to the titanium surfaces.[20]

4. **Olsen et al (1999)** described in detail "taxonomy and biochemical" characteristics of A. actinomycetemcomitans, P. gingivalis and P. intermedia. According to the author, A. actinomycetemcomitans is a member of *Pasteurellaceae* family. It comes under gram -ve, facultative and fermentive anaerobic group of bacteria. Its morphological characteristics were first explained by Klinger. It showed star shaped inner structure in colonies grown under selective medium and cells showed bacillary or short rod shaped cells. It can cause systematic diseases by spreading through oral cavity. One study showed hemolytic colonies grown on blood agar plates under anaerobic environment. According to Takazoe et al, P. gingivalis showed inhibition of growth of other

species such as Prevotella melaninogenica and prevotella intermedia. Okuda et al also showed inhibitory effect on Prevotella loescheii and Prevotella intermedia.[21]

5. **Laurie Ann Ximenez Fyvie et al (2000)** compared supra and subgingival plaque microbial contamination in 22 healthy and 23 adult periodontitis subjects.2358 samples of supra and separately subgingival plaque were collected from the mesial aspect of all teeth excluding third molars in each subject. Results showed porphyromonas gingivalis, bacteriodes forsythus and treponema denticoli could be detected in supragingival plaque samples of both healthy and periodontitis subjects. Actinomyces species were found to be the dominant taxa in both supra and subgingival plaque from healthy and periodontitis subjects.[22]

6. **Van winkelhoff AJ et al (2000)** studied putative periodontal pathogens early colonization in peri-implant pockets in twenty partially edentulous patients. Firstly, levels of the putative microflora were established in periodontal pockets and saliva. Then after immediate loading of an implant and after 6 and 12 months of implant osseointegration, putative periodontal pathogen levels were detected. Authors also detected bacterial contamination on the implant sites and inside of it. 20 patients were included in this study consisting of 13 males and 7 females. Results of this study showed presence of early colonizing microflora like P.intermedia, P.micros and F.nucleatum just after one month of implant placement. They also concluded that P.intermedia was found in high frequency 1 month after implant placement. Leonaedt et al detected presence of P.gingivalis and A. actinomycetemcomitans 6 months after abutment connection in 19 partially edentulous patients.[23]

7. **M.Laroussi et.al (2004)** evaluated heat and ultraviolet radiation in the passivation of bacterial cells by air plasmas at atmospheric pressure. It concluded that in the case of low temperature air plasmas, the reactive species such as O, OH and NO2 that play the most crucial role in the destruction of the microorganisms. Heat and ultraviolet radiation may play secondary role, but expected their effects to be either minimal or indirect.[24]

8. **Gisela C.C. Mendes et.al (2007)** reviewed use of ethylene oxide sterilization for medical equipments. It is an effective bactericidal and sporicidal agent which makes it an exceptional sterilizing agent. Ethylene Oxide is strong alkylating agent which kills micro-organisms by reacting with their cellular constituents. There are three microbiological approaches in decreasing order of utilization namely (i)Overkill method (ii)Combined biologic indicator and (iii) Bioburden method.[8]

9. **Gregory Fridman et al (2007)** suggested two different approaches for atmospheric pressure non-thermal plasma-based sterilization of living tissues. These two approaches were categorized as Indirect treatment, which uses a jet of products(plasma after glow) generated in remotely located plasma discharge and direct treatment which uses the tissue as an electrode that takes part in creating the plasma discharge. The study evaluated the effectiveness of direct and indirect sterilization treatment by “non-thermal atmospheric pressure plasma” generated using the discharge setup, and demonstrated that the direct treatment can provide sterilization much faster without any thermal effects. The comparing results showed that plasma which comes in direct exposure with bacteria is able to sterilize significantly faster than afterglow or jet. Complete sterilization occurs

within 15 seconds when the direct treatment is employed and only partial disinfection can be achieved within the same time frame with the indirect treatment.[25]

10. **Jamil.A.Shibli et al (2008)** compared the supra and subgingival biofilm microbial composition in subjects with and without peri-implantitis. 44 subjects with minimum one implant restored and functional for at least two years were assigned to two groups- a peri-implantitis group(n=22) with subjects presenting peri-implant sites with radiographic defects>3mm, and/or suppuration, and a control group(n=22) of subjects with healthy implants. Supra and subgingival biofilm samples were collected from deepest sites of each implant and examined for microorganisms. Results showed increased counts of porphyromonas gingivalis, treponema denticola and tannerella forsythia observed in the peri-implantitis group for both supra and subgingivally.[15]

11. **Angie Lee et al and Hom-Lay Wang et al(2010)** evaluated biofilm related to implants. It concluded that biofilm formed around implant occurs as similar as of natural teeth. The micro-organisms associated with healthy implants are predominantly gram-positive rods and cocci. Gram negative bacteria can be found in little proportions like Prevotella intermedia, Tannerella forsythia and Porphyromonas gingivalis. As there is a shift from the gram-positive dominated flora to the gram negative one, the peri-implantitis is characterized by greater proportions of red(porphyromonas gingivalis,etc) and orange(Prevotella intermedia) complexes, and as well as Aggregatibacter actinomycetemcomitans. Van Winkelhoff et al analyzed the early colonization of the peri-implant pockets by putative periodontal pathogens in partially edentulous patients and found that

most periodontal pathogens were already identified 6 months early after loading. In particular, P.gingivalis was found to be associated with the presence of fistulas and implant loss.[6]

12. **Yoshiki Oshida et al (2010)** reviewed 500 published articles to throw some light on complicated issues for placing dental implants in patients. According to his study, implants are known as artificial tooth root which are made up of biocompatible materials like titanium and are surgically inserted in the patients jaws to restore missing teeth. Author concluded that the dental implant system should include surface physics, modification and surface chemistry. It should include other important factors such as:- (i) Material biocompatibility to surrounding tissues, (ii) It should transfer stresses smoothly between bone and implant [mechanical compatibility], (iii) Accommodate surface roughness and promote osseointegration [morphological compatibility]. [1]

13. **S.Rupf et al (2012)** investigated destructive potential of "cold atmospheric plasma" on oral biofilms formed in-situ on micro-structured titanium (mTi) surfaces. 120 mTi discs (sand blasted and etched) were exposed to oral environment by two healthy male volunteers for 24 hours. These Ti discs were secured in their oral cavity in maxillary premolar and molar areas with silicone impression material on custom fabricated plastic splint. 72 discs covered with biofilm and 24 discs without the biofilm were treated with plasma. Decontamination of the biofilms were evaluated by contact agar samples and by fluorescence microscopy. The biofilm morphology was assessed by scanning electron microscope and biofilm coverage was measured by fluorescence microscopy. Results showed reduction in biofilm viability and total protein and

mTi surface of the samples were not affected by plasma treatment. Authors also concluded that "cold atmospheric plasma technology"combined with air or water spray allowed complete elimination of oral biofilm from mTi.[26]

14. **Duske K et al (2012)** studied effect of atmospheric plasma on surface wettability and spreading of cell on dental implant metals. Different topological titanium discs i.e. machined, SLA, SLActive, diamond bur-treated discs were used to treat them with "argon plasma jet" with different oxygen admixtures. Before and after treating the specimen with plasma, water contact angles were measured and the spreading of human osteoblastic cells were investigated. Results of this study showed reduced contact angle of titanium discs after argon plasma application with 1.0% oxygen admixture for 60s or 120s. The size of osteoblastic cells was larger on plasma treated specimen than non-treated surfaces.[27]

15. **Maria Eugenia Portillo et al (2013)** studied improved ability of vortexing-sonication procedure in diagnosis of implant associated infections. Inclusion criteria of this study were patients from whom joint prosthesis or a part of it was excised and other criterias like acute inflammation of peri-prosthetic tissue, pus accumulation around prosthesis and growth in the prosthetic tissue. Blood agar plates were inoculated by periprosthetic and synovial cultures and then incubated at 37^0C for 7 days with 5% CO_2 aerobically and 14 days anaerobically. Thioglycolate broth was added and vortexed for 1 min. Sonication of container with prosthesis was done for 5 mins and then subjected to vortexing for 1 min. Results of this study showed that vortexing-sonication procedure had higher efficiency in removing biofilm than vortexing alone.[28]

16. **Simonetta D'Ercole et al (2013)** examined biofilm formation on healing screws and the presence of periodontal pathogens in peri-implant crevicular fluid (PCF) quantitative and qualitative. Thirty patients were selected for this study. Healing abutments were left in-situ for 20, 30 and 90 days. At regular times, the bleeding on probing (BOP) was checked. After 20, 30 and 90 days, the healing screws were removed and evaluated for the total bacterial count by a culture method. Results showed that after 20 days, the counts of anaerobic flora were more than aerobic oral flora and at 30 days, the mean counts of anaerobic flora underwent a significant decrease. After 90 days, the anaerobic counts were significantly greater than 20 and 30 days. The analyzed data revealed that the periodontal pathogens were present in every sample of PCF. The sites with a positive presence of A.actiomycetemcomitans, F.nucleatum , P.gingivalis, P.intermedia and T.forsythia were increased.[29]

17. **May Korachi et al(2013)** studied low temperature atmospheric gas plasma for microbial decontamination. Plasma is a ionized gas which contains balanced ions and electrons. Its use is mostly in textiles surface modifications or biomedical devices or for water sterilization and for wound healing. It is classified into "thermal plasma" (TP) and "non-thermal plasma" (NTP). NTP is having ability to be effective without affecting the physical properties of the materials. Atmospheric pressure NTP is observed to have a lethal effect on many different kinds of microflora and is nowadays becoming the technology of choice for decontamination processes. Studies showed the mechanism of cell death by plasma as:- (1) Etching of cell surface which is induced by reactive species formed during plasma generation. (2) Volatilization of compounds and, (3) Destruction of genetic material. These reactive species which are formed have

higher decontamination potential than conventional chemical agents giving plasma its potential anti-microbial effect. Laroussi (1996) first observed NTP to be capable of destroying bacteria.[30]

18. **Mcdonnell et al (2013)** evaluated different decontamination methods against prions which are proteins and are inactive and difficult to remove. They have silent and long incubation periods. It can spread to humans via cross contamination of medical equipments. 316 stainless steel wire segments were contaminated in this study with infected brain homogenate which was obtained from hamsters infected with scrapie strain 263K and kept to dry. All wires were implanted into prefrontal subcortical region of hamsters and monitored over designated incubation time period of at least an year. All animals were euthanized at the end. All samples were screened for PrP, a specific marker of the disease. Various decontamination methods were conducted following immersion in 1N NaOH for 1 hour at 25^0C, 2.5% NaOH for 1 hour at 25^0C, and porous load steam sterilization at 134^0C for 18 mins. Partial responses were also investigated that includes 0.15N NaOH and steam sterilization at 134^0C for 4 mins. The results showed higher concentrations of NaOH were effective against prions. Lower concentrations of NaOH(0.15N) was also partially effective. Steam sterilization showed least effectiveness.[31]

19. **Luigi canullo et al (2013)** conducted a study on how plasma of argon could affect fibroblast growth on titanium discs at different time periods to enhance soft tissue healing around titanium dental implant abutments. 60 sterile machined titanium discs were divided into control group (n=30) and test group sterilized using plasma of argon. Both groups were immersed in culture of murine

fibroblasts(L292) for 2,8 and 48 hours to simulate clinical conditions during soft tissue healing around titanium abutments,. This study concluded that removal of organic and inorganic contaminants from the surfaces of titanium discs using argon plasma accelerated fibroblast adhesion in the early stages of colonization at 2 to 8 hours.[32]

20. **Luigi Canullo et al (2014)** conducted a study on steaming, ultrasonic and plasma cleaning treatments on customized titanium abutments to characterize pollution micro-particles and bacterial growth. Thirty implant abutments after customization were divided into 3 groups of 10 each and were sterilized by steam, ultrasonic cleaning and plasma of argon. The study confirmed plasma and ultrasonic treatments can be successfully used for abutment cleaning process to improve soft tissue healing and implant-prosthetic connection stability. It also concluded that cleaning with argon plasma is also supposed to have effect on the state of titanium. It activates the surfaces at molecular level , producing hydrophilic surfaces and enhancing wettability.[33]

21. **Luigi Canullo et al (2014)** evaluated soft tissue adhesion on titanium healing abutments which were subjected to different decontamination procedures and effect of plasma cleaning on cell adhesion at early healing time. Eighteen patients with osseointegrated implants were included and divided into 3 groups with different clinical conditions and decontamination processes:(1)no treatment,(2) steam sterilization and (3)cleaning by plasma of argon. Scanning electron microscope was used to analyze cell adhesion to the abutment surface. The results demonstrated plasma of argon may enhance cell adhesion to titanium abutments even at early stage of soft tissue healing. [5]

22. **Arora.V et al (2014)** showed as matter mostly includes solid, liquid and gases. The fourth matter discovered named "Plasma" by Irving Langmuir in 1929. The plasma can get changed into gaseous state if the energy gets dissipated and electron gets reattached. The plasma exists at different ranges of temperatures for example ice cool, lightning, neon, fluorescent lights. As it is controlled by magnetic and electric fields, it can be shaped into any useful structures. It is generally classified into thermal and non-thermal. Nowadays, cold atmospheric plasma(CAP) at below 40^0C are introduced that provides us to extend plasma treatment to living tissues. Due to its high bactericidal effect and easy access to narrow and confined spaces, it can be used for sterilization of medical equipments, implants, wound healing, blood coagulation, etc. It is also useful in dentistry like sterilization, removal of biofilms and bleaching. Whittaker et al showed that plasma cleaning is very beneficial to reduce amount of protein debris that may cause cross contamination between patients when endodontic files are used.[34]

23. **Umut Cakan, Cagri Delilbasi, Sevda ER and Merih Kivanc (2015)** conducted a study to evaluate the sterile nature of used implant healing abutments which were decontaminated by dealers of dental implant manufacturers. In this study, 60 healing abutments previously used were obtained from dealers of 6 implant manufacturers. They were grouped from A to F according to manufacturer (10 in each group). All were examined for presence of perforation or any surface flaw . Each abutment was removed and placed 10 ml brain heart infusion (BHI) broth and microbial colonies were grown on them. This study concluded reuse of healing abutments can be economical in dental practice but might become a source of cross infection. Enterococcus faecalis and Enterococcus faecium were

identified from the test samples. Enterococci are important human pathogens causing life threatening infections in humans especially in nosocomial environment.[2]

24. **Jung-hwan Lee et al (2015)** applied air atmospheric pressure plasma jet (AAPPJ) treatment to titanium discs to evaluate the efficacy for early peri-implant soft tissue seals on titanium implant abutment. After titanium discs were treated with AAPPJ for 10 seconds, surface analysis was performed. The control group includes air only. Human gingival fibroblasts were cultured on the specimens for assessing cell attachment and proliferation. Results showed in AAPPJ treated specimens, there is decrease in water contact angle according to increased flow rate. Oxygen composition increased but no topographical changes were observed. The AAPPJ treatment at 1000sccm was apparent 2mm from the treated area, with increase in early cell attachment and proliferation. Human gingival fibroblasts cultured on AAPPJ treated specimens displayed more vinculin formation than the control group.[10]

25. **C.Wadhwani et al (2016)** conducted a study to evaluate the contamination remaining on used dental implant healing abutments even after sterilizing in dental practices. This study evaluated used and sterilized healing abutments with respect to residual contamination and identified any particular type of healing abutment or site which was more susceptible to contamination remnants. Hundred healing abutments which were used were collected from 8 dental offices. They were cleaned by mechanical wiping using disinfection sponges. These abutments were stained by phloxine B and each surface was photographed to check the contamination. The results showed a reddish-orange color in areas

where residual protein contamination was present. Contamination of healing abutments is mostly by bacterial plaque/epithelial attachment on titanium that tears during abutment removal/blood/food debris and saliva. This study gives us highlights about following proper sterilization protocol if one wants to reuse the healing abutments[4]

26. **Marco Annuziata et al (2016)** assessed the argon plasma treatment effect on titanium discs with turned, sandblasted/acid-etched and titanium plasma sprayed surfaces. Aggregatibacter actinomycetemcomitans (Aac) strain was grown at 37^0C under anaerobic conditions and were transferred on 6 discs. After 24 hours, the control group was evaluated for colony forming unit and the test group were treated in argon plasma chamber for 12 mins at room temperature before analyzed for colony forming unit counting. The results advocated that argon plasma technology could be effectively used to decontaminate previously infected titanium disc surfaces.[35]

27. **Berta Garcia et al (2017)** evaluated cleaning efficiency of the titanium abutment surfaces with plasma of argon on cell adhesion at an early healing time. Patients with submerged implants were divided into control group and test group (cleaned with plasma of argon). After 2 weeks, a small biopsy with the abutment and soft tissues around it were performed and analyzed using SEM to access cell adhesion to the abutment surface. The results concluded that plasma of argon induces cell adhesion and influences collagen fiber orientation.[9]

28. **Luigi Canullo et al (2017)** performed randomized controlled trial to evaluate radiographic changes at 6 years time period around customized abutments which were treated with or without plasma of argon treatment. 20 patients were selected

and each one received one implant in between maxillary premolars. Before commencing 2nd stage surgery, patients were randomly divided into control group(steam sterilization) and test group(plasma of argon treatment). Peri-apical radiographs were taken at crown connection(T_0) and at 12(T_1), 24(T_2), 48(T_4), 60(T_5) and 72(T_6) after final restoration. Primarily, success rate of the implant and prosthesis and occurrence of any biological complications during follow up period were analyzed. Later, secondary outcomes such as peri-implant marginal bone loss and plaque scores were analyzed. The results showed that the radiographic analysis revealed increased mean bone loss in control group than the test group which concluded that using plasma of argon could minimize the peri-implant marginal bone resorption and stabilize esthetics outcome.[36]

29. **Christian Mehl et al (2017)** evaluated adhesion properties of gingival fibroblasts on three different abutment materials after five different decontamination procedures. Lithium disilicate, Zirconium dioxide and Titanium alloy discs were fabricated. These specimens were cleaned by one of five different methods:- argon plasma, steam, ultrasound and disinfection, ultrasound and sterilization in an autoclave and photofunctionalization with ultraviolet light. Adhesion of gingival fibroblasts was measured by single cell force spectroscopy, which quantify cell adhesion at the single cell level. Results concluded that all decontamination methods provide comparable cell detachment forces for Lithium disilicate abutments. Argon plasma or ultrasonic cleaning were the most suitable methods for strong cell adhesion on Zrirconium dioxide and Ultrasound and disinfection provided the best cell adhesion for Titanium.[37]

30. **Claudio Stacchi et al (2018)** evaluated the efficacy of cleaning between novel device and conventional cleaning procedures. Two healing abutments with same dimensions from each patient were taken of at least 1 month of clinical use. They are divided into test and control groups. Control group was cleaned by mechanical wiping with disinfected sponges and 30 min ultrasonic bath. Test group was sterilized by automatic cleaning system. The results concluded that contaminated areas were observed with different frequencies in both two groups. It showed uniform distribution of cells in test group whereas areas without adhering cells as a common finding in control group.[3]

31. **Michelle chew et al (2018)** conducted a study to assess contamination remaining on used implant healing abutments after autoclaving them and compared the effectiveness of two additional decontamination methods. 120 used and autoclaved healing abutments were divided into 3 groups (n==40). First group includes used healing abutments after autoclaving only, second group includes used implant healing abutments after autoclaving and air flow polishing and third group includes used healing abutments after autoclaving and sodium hypochlorite treatment. The results concluded that the autoclaving as the only procedure was insufficient for successful decontamination while additional decontamination procedures significantly helped in reducing remaining contaminants.[11]

32. **Angeles Sanchez-Garces et al (2019)** evaluated the survival of microorganisms on the sterilized abutments. 53 healing abutments used previously in patients were collected from 8 different dental clinics after completion of their osseointegration which was around 3 to 6 months. Once retrieved, they were cleaned and disinfected with detergent for 2 to 5 mins followed by ultrasonic bath for 10-15 mins and dried. The abutments were sealed in sterilization bags and

sterilization process is carried out in an autoclave at 134^0C. Then, each specimen was removed and placed in BHI (Brain Heart Infusion) broth containing test tubes. For control group, one consist of 3 new unused healing abutments and other one containing just bacteriological medium. Incubation of these test tubes was done for 10 days under 5%CO_2 at 37^0C and examined everyday for turbidity. Petri dishes containing BHI and blood agar were inoculated with 100ul of the bacteriological medium from each test tube to test for its sterile nature and determination of total organic carbon(TOC) was done later. The results concluded that sterilization was satisfactory in removing live bacteria and spores as no bacterial growth was detected. Notable amounts of organic carbon may still be recovered after sterilization.[7]

33. **Myung-Jin Lee et.al (2019)** evaluated the effect of Nonthermal atmospheric pressure plasma jet(NTAPPJ) treatment and its effect on adhesion of gram positive and gram negative bacteria. Commercially pure titanium discs were used in this study. NTAPPJ device was used and test samples were treated for 2 to 10 mins. Control samples were not exposed to plasma. The test and control groups were evaluated by optical surface profilometry for surface roughness. The change in their surface energy and hydrophilicity were measured by using video contact angle goniometer. 4 bacterial strains were used – Gram +ve bacterias as S.mutans and S.aureus and gram -ve bacterias as klebsiella oxytoca and klebsiella pneumoniae. For adhesion, bacterial culture suspension of each strain was placed on treated and non-treated Ti specimens and incubated at 37^0C for 24 hours. The results concluded increase in hydrophilicity and surface energy of titanium surfaces after NTAPPJ treatment without altering their topographies. chemical composition change was observed by fluorescent imaging on the NTAPPJ treated

titanium surfaces compared with the untreated samples. In between gram positive and gram negative bacteria, both adhesion and the biofilm formation rate were significantly lower for gram negative bacteria than gram positive on samples treated with the NTAPPJ.[38]

34. **Luigi canullo et al (2020)** performed in-vitro study to verify the ability of plasma treatment to change the implant surface characteristics. Implants from 9 different companies were procured in this study. SEM surface analysis of each implant was done. For microbiological analysis, 10 implants from each company were used and randomly divided into test or control group. To replicate the surgical workflow, the test and the control samples were left for 60 seconds in clinical environment and bacterial growth analysis was performed. Colony forming units (CFU) after 24 hours was evaluated. Results of this study concluded that plasma of argon maintains a substantial sterility of the implant independently of its morphology.[39]

35. **B.Eggers et al (2020)** showed in this study the effects of "cold atmospheric plasma (CAP)" on hard tissues as human osteoblast like cells MG-63 . As CAP is the ionized gas and has many applications in the field of dentistry. Many authors described that CAP is useful in wound healing, killing microorganisms, etc. In this study, MG-63 cells were cultured and exposed to CAP for 60 seconds. The effects of CAP were studied for the molecules responsible for wound healing using polymerase chain reaction (PCR), ELISA and immunocytochemistry. Cell migration was also examined using in vitro wound healing assay. The results from this study concluded that CAP exposure effects genes and protein regulation in osteoblast like cells and might have a positive effect on hard tissue regeneration.[40]

MATERIALS AND METHODOLOGY

SOURCE OF DATA:

This in vitro study was conducted in KAHER Dr.Prabhakar Kore Basic Science Research Centre, KAHER V.K. Institute of Dental Sciences, KLE Dr.Prabhakar Kore Hospital, Belagavi and Ruby Hospital, Pune. This study was taken up to comparatively evaluate the efficacy of different decontamination methods on reuse of implant healing abutments.

METHOD OF COLLECTION OF DATA.

SAMPLE SIZE:

Forty-eight commercially available titanium implant healing abutments were procured and contaminated by three different microorganisms namely Porphyromonas gingivalis ATTC322, Aggregatibacter actinomycetemcomitans ATTC33384, Prevotella intermedia ATTC25611 and were divided into 3 sub-groups for 3 different decontamination methods used.

- Group A consist of (n=16) healing abutments decontaminated with Steam Autoclave Sterilization.
- Group B consist of (n=16) healing abutments decontaminated with Ethylene Oxide (ETO_2) Sterilization.
- Group C consists of (n=16) healing abutments decontaminated with Low Temperature Gas Plasma Sterilization.

INCLUSION CRITERIA:

1. Standard healing abutments supplied by single manufacturer.
2. All healing abutments made up of titanium.
3. All healing abutments are uniform in dimension.

EXCLUSION CRITERIA:

1. Healing abutments with any surface flaw.

Table 1:- Armamentarium used in the study:- (Fig 1)

Armamentarium	Description	Manufacturer
Titanium implant healing abutment	LOT No.:- 0018816	NORIS Medical Engineered FOR HEALTH
Sonicating Unit	Model No.:- CPX1800H-E	Branson Ultrasonic Corporation, USA
Vortexing Unit	CAT No.50141002	Riviera Glass Pvt Ltd, Mumbai
Bacteriological Incubator	Model No.:-BDI-54	B.D.Instrumentation
Laminar Air Flow	Model.:- Vertical	Quesst International, Bangalore
Electric Loop Sterilization	Model.:-i-therm A1-401	Hi-Media, Mumbai
Steam Autoclave Unit	GENTINE Model No. VS1-1,5	GENTINE Groups , Mumbai
Ethylene Oxide Unit	3M Steri-Vac (GS8-1D)	Mumbai
Low Temperature Gas Plasma Unit	STERRAD #160402	STERRAD^{M} 100S STERILIZER, PUNE
Anaerobic Jar	LOT No.:- 14-1016	Hi-Media, Mumbai
Micropipette	Model No.:-299932	Riviera Glass Pvt. Ltd., Mumbai
Centrifuge Tube	CAT No.:-546021 10 ml	Tarsons Products Pvt. Ltd., Kolkata
Micro-centrifuge Tube	Batch No.:- JW-16200413 2ml	Tarsons Products Pvt. Ltd., Kolkata
Stereomicroscope	Model. No.:- 97036	LABOMED, Mumbai
Weighing Machine	UW6200H	Uni-Bloc, Mumbai

Table 2: Materials used in the study

Materials	Description	Manufacturer
Blood Agar Media	LOT No.:-0000155548	Hi-Media, Mumbai
Brain Heart Infusion Broth	LOT No.:-0000149333	Hi-Media, Mumbai
Distilled Water	Batch No.:-007M15	Rankem Chemicals, Avantor, India
70% Ethanol	LOT No.:-20151011	Changshu Hongsheng Fine Chemicals Co., LTD
Phosphate Buffer Saline (PBS)	LOT No.:-0000237353	Hi-Media, Mumbai
Phloxine B stain	RM835-25G	Hi-Media, Mumbai

METHODOLOGY:-

A) **Preparation of experimental specimens**:-

Forty-eight healing abutments made of titanium of diameter 4.6mm and height 4.0mm were procured from NORIS Medical Engineered FOR HEALTH. (Fig.2) All the specimens were mechanically cleaned with sponges followed by 30 min ultrasonic bath and dried according to specifications.(Fig.3)

B) **Surface profile analysis:-**

Surface profile of all the healing abutments was analyzed to evaluate any surface flaw present on their surfaces. If any healing abutments with surface pitting, scratches, worn out threads were excluded from the study. The stereomicroscope with 10X magnification was used to evaluate the surface profile of all the specimens at the following sites:- (a) main body, (b) connection to implant fixture and (c) threaded portion.(Fig.4) After surface profile evaluation, all the healing abutments were subjected to microbiological contamination.

C) Microbial adhesion and evaluation:-

The bacterial strains used for adhesion were anaerobic gram negative bacteria on the test specimens were namely:- Porphyromonas gingivalis(Pg), Prevotella intermedia(Pi) and Aggregatibacter actinomycetemcomitans(Aac). The strains were subcultured in 60ml of BHI broth of 2 ml of each strain . These diluted sub-cultures were then incubated at 37^0C in anaerobic jar for *Porphyromonas gingivalis(Pg), Prevotella intermedia(Pi)* and *Aggregatibacter actinomycetemcomitans(Aac)* for 48 hours.(Fig.5) For adhesion experiment, the healing abutments were immersed with a suspension

of the bacterial cultures in 2ml test tubes with 1.5ml of BHI broth and 20 ul of each bacterial strain and were incubated at 37^0C in anaerobic jar for 48 hours.(Fig.6) The contamination of the healing abutments was confirmed with staining with 0.5ml of phloxine B staining solution.(Fig.7) It is the fluoresin derivative stain used as a protein and peptide highlighting stain. The microbial contamination was observed with stereomicroscope with 10X magnification.(Fig.8) The contaminated areas showed reddish-orange color due to residual protein and amino acid contamination for the above mentioned microflora.

D) <u>Procedure for decontamination</u>:- (Fig.9)

All the contaminated healing abutments were divided into 3 groups (n=16) depending on the decontamination methods used. The decontamination for the specimens in group A (n=16) were done with steam autoclave sterilization at 121^0C for 15-30 mins. It is sealed device that kills microorganisms using saturated steam under pressure. The use of moist heat facilitates the killing of microorganisms, including heat-resistant endospores which is achieved by heating the material inside the device at temperatures above the boiling point. The specimens in group B (n=16) were decontaminated by ethylene oxide sterilization. In this method, the healing abutments were packed into gas permeable packaging. All the air present in stainless steel chamber was emptied. All the packed healing abutments were placed inside the chamber. Then, the steam was inoculated into the chamber as it helps sterilant (i.e ETO gas) to penetrate the packaging easily and also easy adsorption on the healing abutments. Finally, the sterilant was injected to a final gas concentration of approximately 600-800 mg/l typically at 40^0C-50^0C.

For the specimens in group C (n=16), the decontamination was done by low temperature gas plasma sterilization. The healing abutments were packed into gas permeable packing and kept into sterilization vessel. Gas molecules were excited by radiofrequency under deep vacuum in an enclosed vessel so that gas plasma was formed. Then, it was ionized when subjected to electric field where decontamination took place. Its cycle was of 55 mins 29 seconds which includes: Vacuum stage (23min 7sec), Injection stage (6mins 2sec), Diffusion stage (2mins), Plasma stage (7mins 57sec), Injection stage (6mins 1sec), Diffusion stage (2mins 1sec), Plasma stage (7mins 57sec) and Vent stage. Packaging available for the instruments to be sterilized in gas plasma sterilization has a biological indicator which turns from white to yellow after sterilization procedure indicating successful decontamination of the specimens.

E) **Microbial analysis:-**

The decontaminated specimens were collected after 48 hours and then they were processed by washing 4 times in phosphate buffer saline (PBS) solution to remove unattached bacteria and then placed in test tubes containing 1ml of PBS solution.(Fig.10) The tubes were submerged in an ultrasonic bath for 3 mins, vigorously vortexed for 1 min and then were treated ultrasonically again for 3 mins to release the surface attached bacteria.(Fig.11) Serial dilutions of these suspensions were used to inoculate on agar plates,(Fig.12) which were incubated at 37^0C in anaerobic jar for *Porphyromonas gingivalis and Prevotella intermedia* and in CO_2 jar for *Aggregatibacter Actinomycetemcomitans* for 48 hours. The PBS solution was then streaked on the agar medium containing nutrient agar and blood with a swab. The colonies

grown on agar plates were counted and results were expressed as colony forming units (CFU)/ml/implant healing abutment.(Fig.13,14) It was calculated by using following formula:-

CFU = number of colonies X 2^n (where n is the dilution factor)

The micro-organisms were differentiated by difference in morphology of Porphyromonas gingivalis, Prevotella intermedia and Aggregatibacter actinomycetemcomitans.(Fig.15,16,17)

Aggregaibacter Actinomycetemcomitans refers to star-shaped inner structure seen in the colonies on selective medium. Porphyromonas gingivalis are rod shaped, non-motile, anaerobic bacteria and can be easily differentiated on culture media. It dominates the growth on Prevotella intermedia when grown in the same culture. Prevotella intermedia show small, rod shaped, smooth colonies which sometimes look black, light grey or brown color in the selective medium.[21]

The resultant colony forming units for different organisms in different subgroups were tabulated. The resultant data for all the specimen in each group was subjected to statistical analysis to draw the conclusion from the resultant data.

FLOWCHART:-

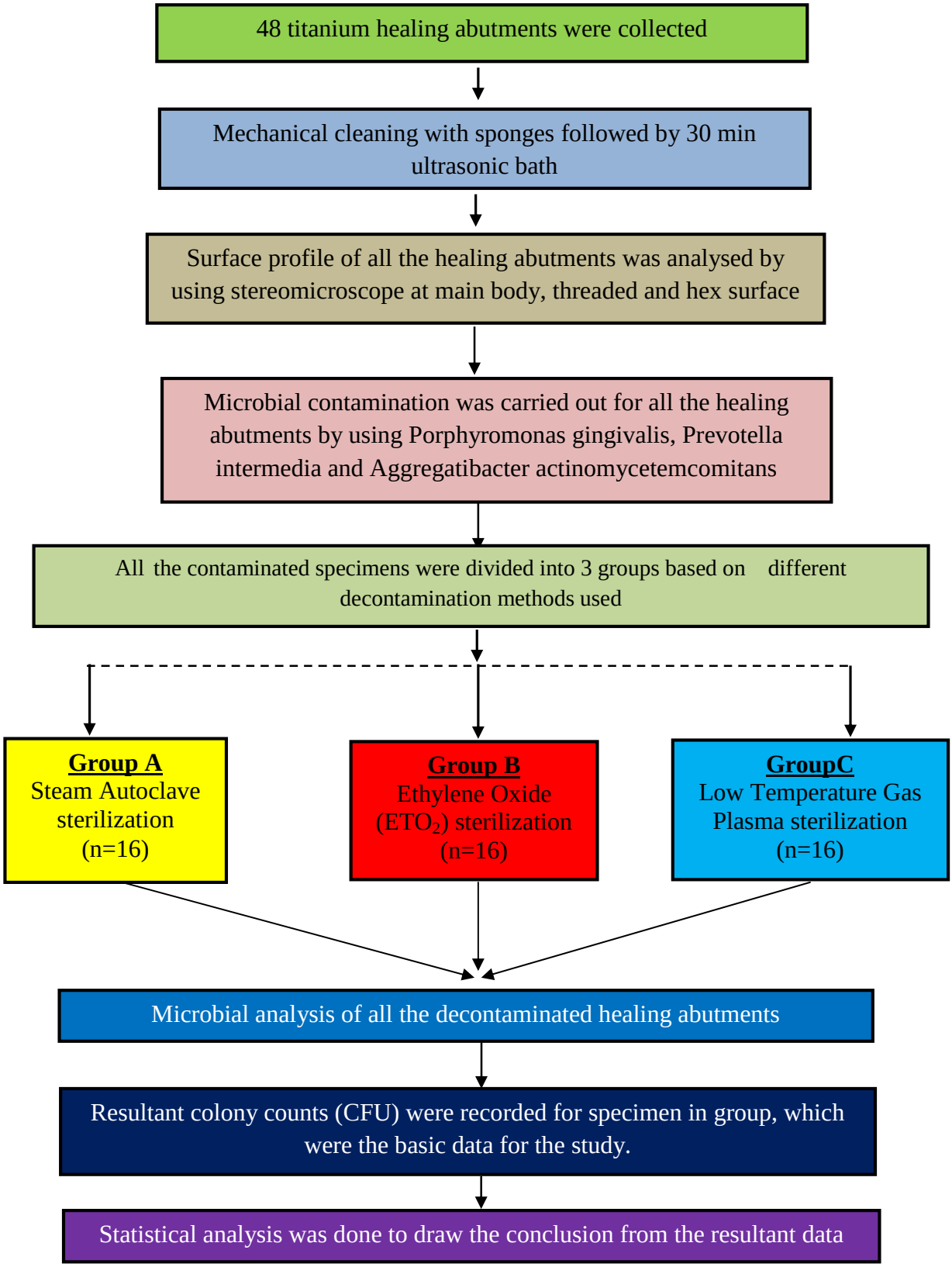

48 titanium healing abutments were collected
Mechanical cleaning with sponges followed by 30 min ultrasonic bath
Surface profile of all the healing abutments was analysed by using stereomicroscope at main body, threaded and hex surface
Microbial contamination was carried out for all the healing abutments by using Porphyromonas gingivalis, Prevotella intermedia and Aggregatibacter actinomycetemcomitans
All the contaminated specimens were divided into 3 groups based on different decontamination methods used
Group A
Steam Autoclave sterilization
(n=16)
Group B
Ethylene Oxide (ETO_2) sterilization
(n=16)
GroupC
Low Temperature Gas Plasma sterilization
(n=16)
Microbial analysis of all the decontaminated healing abutments
Resultant colony counts (CFU) were recorded for specimen in group, which were the basic data for the study.
Statistical analysis was done to draw the conclusion from the resultant data

FIGURE1:- ARMAMENTARIUM USED IN THIS STUDY

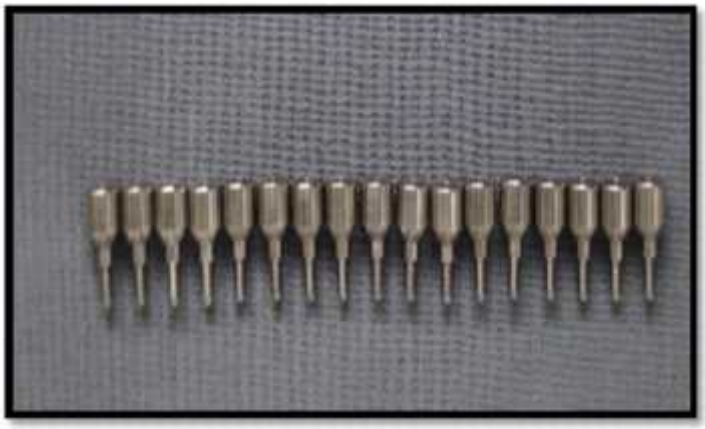


FIGURE2:- TITANIUM IMPLANT HEALING ABUTMENTS USED IN THIS STUDY

FIGURE3:- ULTRASONIC BATH USED FOR CLEANING HEALING ABUTMENTS

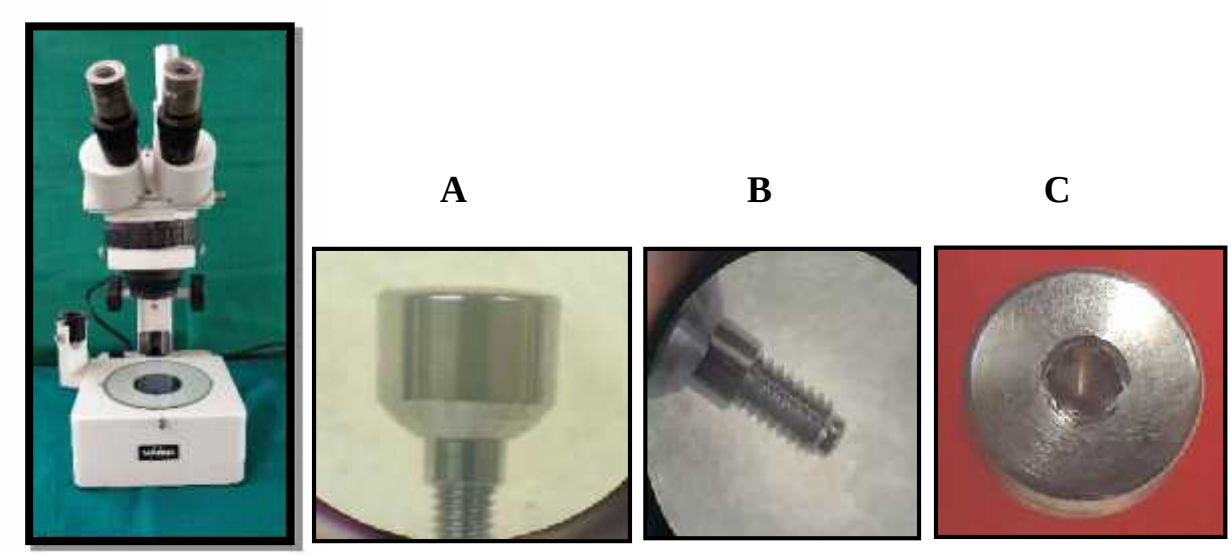


FIGURE4:- SURFACE PROFILE EVALUATION OF HEALING ABUTMENTS DONE BY USING STEREOMICROSCOPE (10X MAGNIFICATION) AT (A) BODY, (B) THREADED AND (C) HEX SURFACES

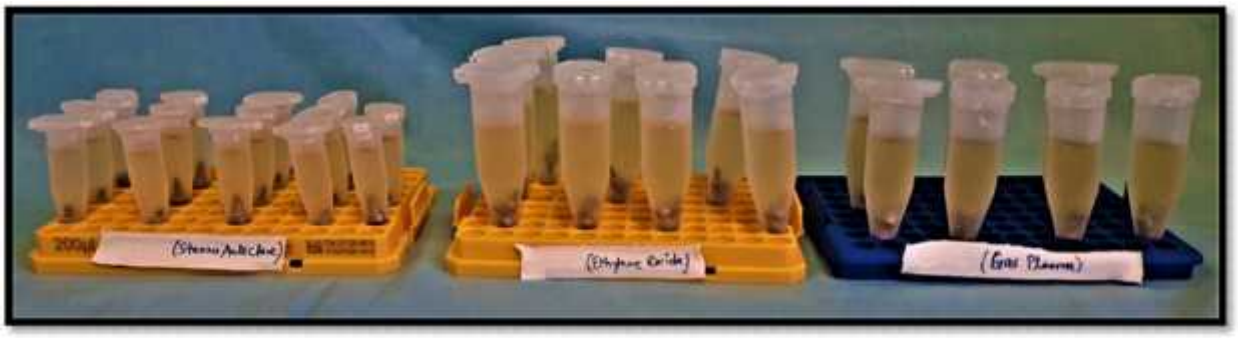


FIGURE5:- SPECIMENS IN BHI BROTH FOR GROUP A, B AND C WITH PORPHYROMONAS GINGIVALIS, AGGREGATIBACTER ACTINOMYCETEMCOMITANS AND PREVOTELLA INTERMEDIA

FIGURE6:-INCUBATION OF CONTAMINATED SPECIMENS FOR 48 HOURS

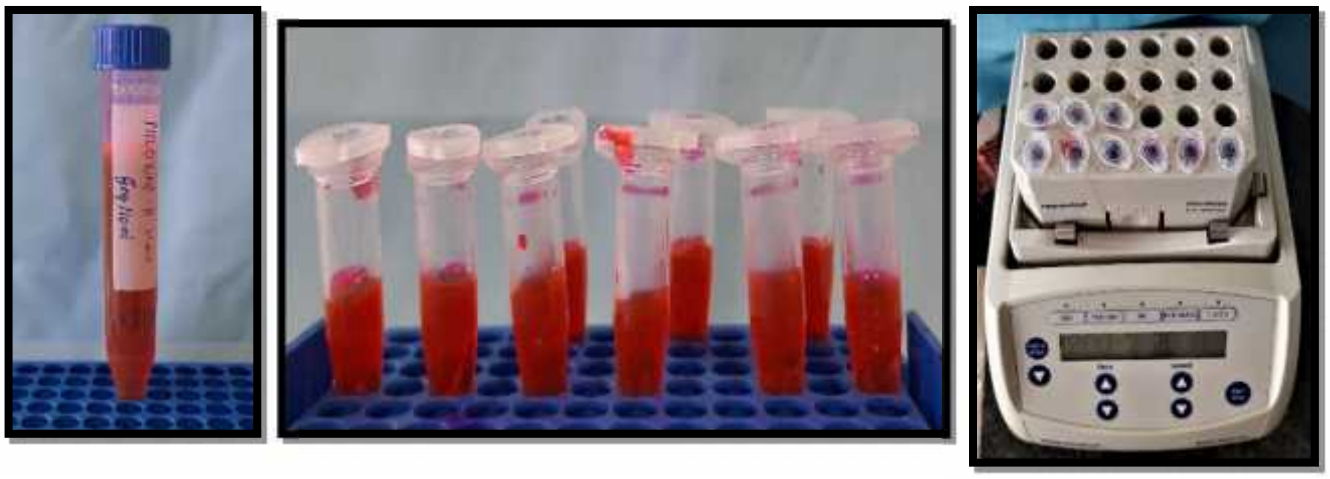


FIGURE7:- ARMAMENTARIUM USED FOR PHLOXINE B STAINING

A **B** **C**

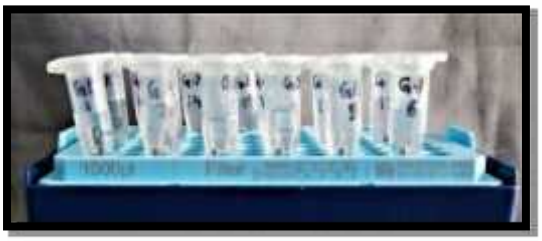
FIGURE8:- EVALUATION OF CONTAMINATION ON ABUTMENT SURFACES USING STEREOMICROSCOPE (10X) (A) BODY, (B) THREAD AND (C) HEX SURFACE

GROUP A **GROUP B** **GROUP C**

FIGURE9:- DECONTAMINATION PROCEDURES FOR SPECIMEN IN GROUP A, B AND C

GROUP A

GROUP B



GROUP C

FIGURE10:- DECONTAMINATED SPECIMENS IN PBS SOLUTION

FIGURE11:-VORTEXING OF DECONTAMINATED SPECIMENS INTO PBS SOLUTION

FIGURE12:- PREPARATION OF BLOOD AGAR PLATES FOR GROUP A, B AND C

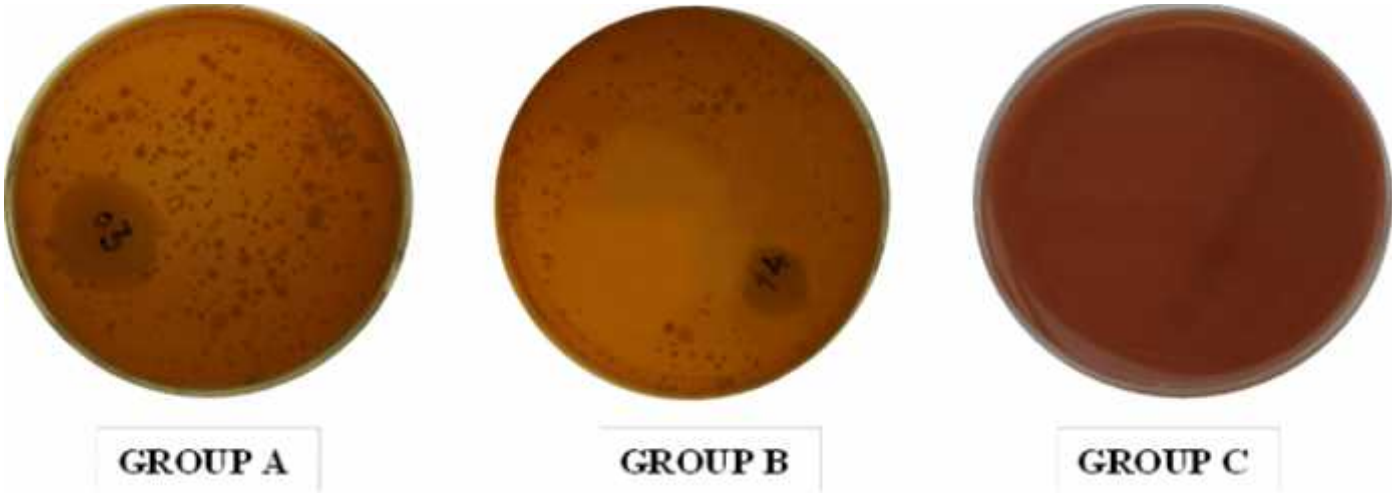


FIGURE13:- COLONY FORMING UNITS WITH RESPECT TO GROUP A, B AND C

FIGURE14:-SUBCULTURING OF BLOOD AGAR PLATES FOR CFU IN GROUP A, B AND C

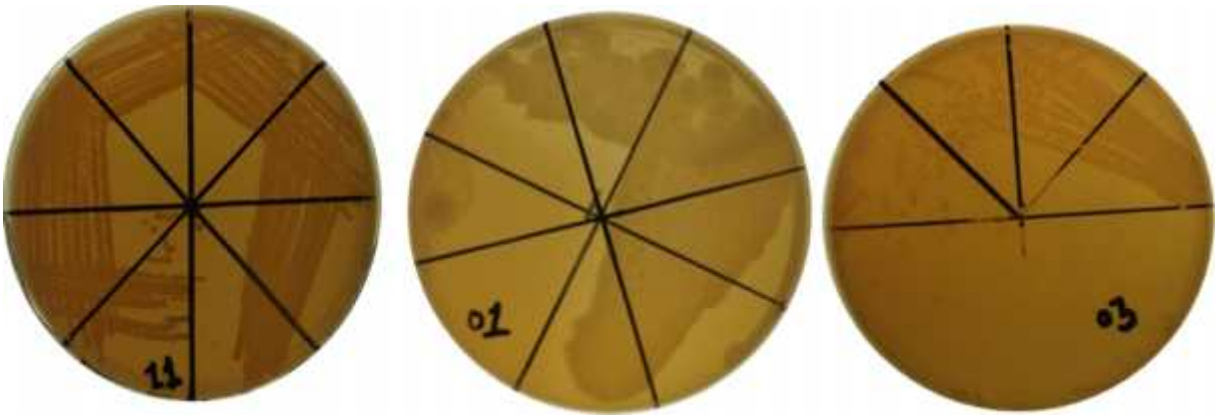


FIGURE15:- SUBCULTURE SHOWING CFU FOR *PORPHYROMONAS GINGIVALIS*, *A. ACTINOMYCETEMCOMITANS* AND *PREVOTELLA INTERMEDIA* IN STEAM AUTOCLAVED SPECIMENS (GROUP A)

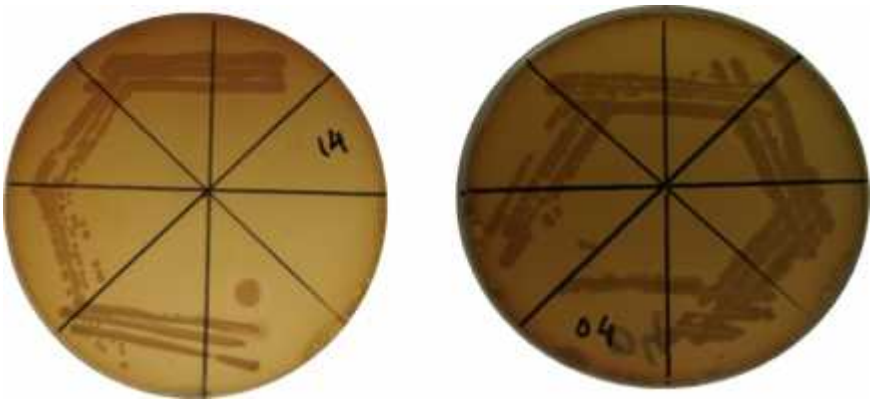


FIGURE16:- SUBCULTURE SHOWING CFU FOR *PORPHYROMONAS GINGIVALIS, A. ACTINOMYCETEMCOMITANS* AND *PREVOTELLA INTERMEDIA* IN ETHYLENE OXIDE STERILIZED SPECIMENS (GROUP B)

FIGURE17:- SUBCULTURE SHOWING NO CFU FOR *PORPHYROMONAS GINGIVALIS, A. ACTINOMYCETEMCOMITANS* AND *PREVOTELLA INTERMEDIA* IN LOW TEMPERATURE GAS PLASMA STERILIZED SPECIMENS (GROUP C)

RESULTS

This present study evaluated and compared the efficacy of three different decontamination methods, namely Steam Autoclave sterilization, Ethylene Oxide sterilization and Low Temperature Gas Plasma sterilization on titanium implant healing abutments. Microbial adhesion was performed using sonicating and vortexing method for all the group specimens. Post-decontamination microbial assessment was done qualitatively for all the specimens in the three test groups.

Table No.3 and Graph No. 1: Comparison of three groups (A, B, C) with respect to total CFU counts by Kruskal Wallis ANOVA

Table No.4 : Pair wise comparisons of three groups (A, B, C) with respect to total CFU counts by Mann-Whitney U test

Table No.5 and Graph No.2 : Comparison of three groups (A, B, C) with respect to CFU counts in Porphyromonas gingivalis by Kruskal Wallis ANOVA

Table No.6: Pair wise comparisons of three groups (A, B, C) with respect to CFU counts in Porphyromonas gingivalis by Mann-Whitney U test

Table No.7 and Graph No.3 : Comparison of three groups (A, B, C) with respect to CFU counts in Aggregatibacter actinomycetemcomitans by Kruskal Wallis ANOVA

Table No.8: Pair wise comparisons of three groups (A, B, C) with respect to CFU counts in Aggregatibacter actinomycetemcomitans by Mann-Whitney U test

Table No.9 and Graph No.4 : Comparison of three groups (A, B, C) with respect to CFU counts in Prevotella intermedia by Kruskal Wallis ANOVA

Table No.10 : Pair wise comparisons of three groups (A, B, C) with respect to CFU counts in Prevotella intermedia by Mann-Whitney U test

The resultant values of Colony Forming Unit (CFU) of specimens in group A, Group B and Group C were subjected to statistical analysis to draw conclusion from the experimental data. Descriptive statistical measures such as Mean, Standard Deviation and Mean Rank were computed for all the study groups. Comparison of the test groups with respect to total colony counts was done with Kruskal Wallis ANOVA ($p<0.05$) and pairwise comparison of the test groups with respect to total CFU counts was done using Mann-Whitney U test ($p<0.05$).

The Mean, Standard Deviation and Mean Rank were calculated for the CFU counts of *Porphyromonas gingivalis, Prevotella intermedia* and *Aggregatibacter actinomycetemcomitans* organisms in each of the three groups. The mean value for Group A was 454.94(±692.89), which was significantly higher than Group B-30.06(±66.58) and Group C-0.00 with respect to total CFU count with p value of 0.0070* showing statistically significant difference among them (Table No.3 and Graph No.1) ; In pair-wise comparison between Groups A, B and C with respect to total CFU count, there was statistically significant difference between Group A and Group C with p value of 0.0348 (Table No.4).

The mean value of CFU count for Group A was 388.38 (±573.57), which was significantly higher than Group B – 13.88 (±33.64) and Group C – 0.00 with respect to *P. gingivalis* with p value of 0.0170* showing statistically significant difference among them (Table No.5 and Graph No.2) ; In pair-wise comparison between Groups A, B and C with respect to *p.*gingivalis, there was no statistically significant

difference in CFU count with p value of 0.2136 (Group A and B), 0.0704 (Group A and C) and 0.3657 (Group B and C) (Table No.6).

The mean value of CFU count for Group A was 81.00 (±300.45), which was significantly higher than Group B – 12.94 (±40.28), which in turn was significantly higher than Group C 0.00 with respect to *Aggregatibacter actinomycetemcomitans* with p value of 0.0640 showing no statistically significant difference among the groups (Table No.7 and Graph No.3) ; In pair-wise comparison between Groups A, B and C with respect to *Aggregatibacter actinomycetemcomitans*, there was no statistically significant difference in CFU count with p values of 0.5591 (Group A and B), 0.1317 (Group A and C) and 0.3657 (Group B and C) (Table No.8).

The mean value for Group A was 53.06 (±151.93), which was significantly higher than Group B – 3.25 (±7.33) and Group C 0.00 with respect to *Prevotella intermedia* with p value of 0.0180* showing statistically significant difference among them (Table No.9 and Graph No.4); In pair-wise comparison between Groups A, B and C with respect to *Prevotella intermedia*, there was no statistically significant difference in CFU count with p values of 0.2278 (Group A and B), 0.0704 (Group A and C) and 0.3657 (Group B and C) (Table No.10).

The results of the study indicated that Low Temperature Gas Plasma decontamination (Group C) showed the least CFU count as compared to other decontamination procedures. Hence, it can be regarded as a better decontamination procedure than Ethylene Oxide and Steam Autoclave for decontamination of reuse titanium implant healing abutments.

Table No. 3: Comparison of three groups (A, B, C) with respect to total CFU counts by Kruskal Wallis ANOVA

Groups	Mean	SD	Mean rank
Group A	454.94	692.89	30.47
Group B	30.06	66.58	23.53
Group C	0.00	0.00	19.50
H-value	9.9720		
p-value	0.0070*		

*p<0.05

It means that there was significant difference between total CFU counts in between the Groups A, B and C (p<0.05).

Table No. 4: Pair wise comparisons of three groups (A, B, C) with respect to total CFU counts by Mann-Whitney U test

Groups	Mean	SD	Mean rank	U-value	Z-value	p-value
Group A	454.94	692.89	18.97			
Group B	30.06	66.58	14.03	88.50	-1.4887	0.1366
Group A	454.94	692.89	20.00			
Group C	0.00	0.00	13.00	72.00	-2.1106	0.0348*
Group B	30.06	66.58	18.00			
Group C	0.00	0.00	15.00	104.00	-0.9045	0.3657

*p<0.05

This table shows that there was statistically significant difference between Group A and Group C (p<0.05).

Table No. 5: Comparison of three groups (A, B, C) with respect to CFU counts in Porphyromonas gingivalis by Kruskal Wallis ANOVA

Groups	Mean	SD	Mean rank
Group A	388.38	573.57	29.56
Group B	13.88	33.64	23.94
Group C	0.00	0.00	20.00
H-value	8.1330		
p-value	0.0170*		

*p<0.05

This table demonstrates statistically significant correlation between the CFU counts in all the Groups (A, B and C) with respect to Porphyromonas gingivalis (p<0.05).

Table No. 6: Pair wise comparisons of three groups (A, B, C) with respect to CFU counts in Porphyromonas gingivalis by Mann-Whitney U test

Groups	Mean	SD	Mean rank	U-value	Z-value	p-value
Group A	388.38	573.57	18.56			
Group B	13.88	33.64	14.44	95.00	-1.2437	0.2136
Group A	388.38	573.57	19.50			
Group C	0.00	0.00	13.50	80.00	-1.8091	0.0704
Group B	13.88	33.64	18.00			
Group C	0.00	0.00	15.00	104.00	-0.9045	0.3657

There was no statistically significant difference in CFU counts in between the Groups (A, B and C) with respect to Porphyromonas gingivalis.

Table No. 7: Comparison of three groups (A, B, C) with respect to CFU counts in Aggregatibacter actinomycetemcomitans by Kruskal Wallis ANOVA

Groups	Mean	SD	Mean rank
Group A	81.00	300.45	27.97
Group B	12.94	40.28	25.03
Group C	0.00	0.00	20.50
H-value	5.4850		
p-value	0.0640		

There was no statistically significant difference in CFU counts in between the Groups (A, B and C) with respect to Aggregatibacter actinomycetemcomitans.

Table No. 8: Pair wise comparisons of three groups (A, B, C) with respect to CFU counts in Aggregatibacter actinomycetemcomitans by Mann-Whitney U test

Groups	Mean	SD	Mean rank	U-value	Z-value	p-value
Group A	81.00	300.45	17.47			
Group B	12.94	40.28	15.53	112.50	-0.5842	0.5591
Group A	81.00	300.45	19.00			
Group C	0.00	0.00	14.00	88.00	-1.5076	0.1317
Group B	12.94	40.28	18.00			
Group C	0.00	0.00	15.00	104.00	-0.9045	0.3657

This table did not show any statistically significant difference in CFU counts in between the Groups (A, B and C) with respect to Aggregatibacter actinomycetemcomitans.

Table No. 9: Comparison of three groups (A, B, C) with respect to CFU counts in Prevotella intermedia by Kruskal Wallis ANOVA

Groups	Mean	SD	Mean rank
Group A	53.06	151.93	29.50
Group B	3.25	7.33	24.00
Group C	0.00	0.00	20.00
H-value	8.0100		
p-value	0.0180*		

*p<0.05

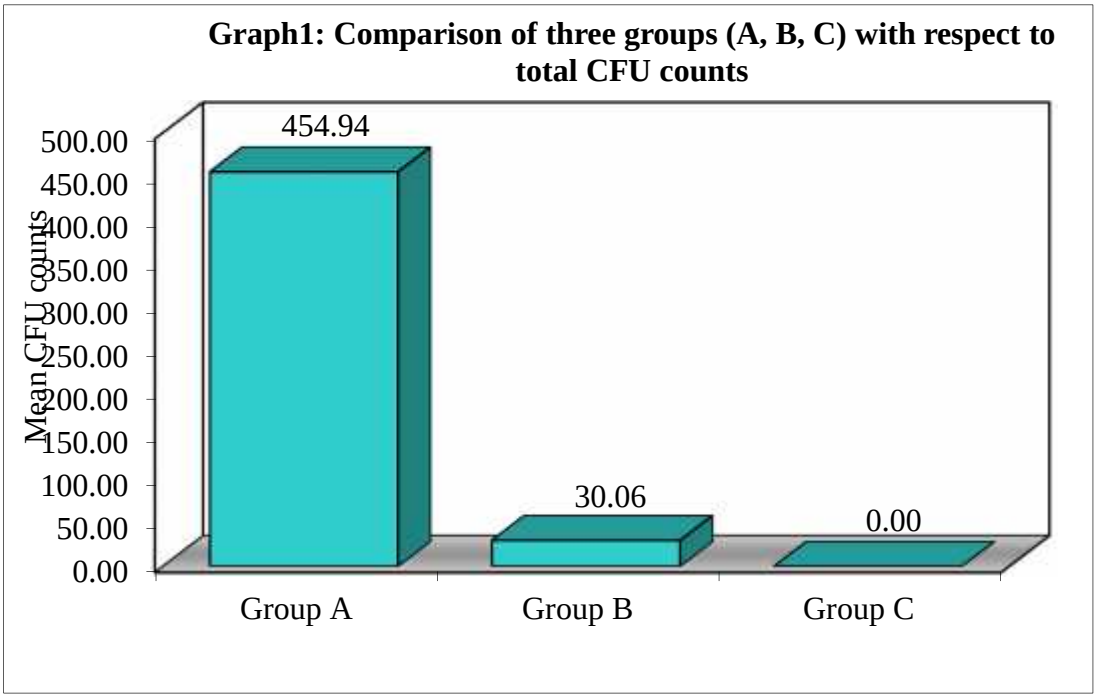
This table demonstrates statistically significant correlation between CFU counts in all the Groups (A, B and C) with respect to Prevotella intermedia (p<0.05).

Table No. 10: Pair wise comparisons of three groups (A, B, C) with respect to CFU counts in Prevotella intermedia by Mann-Whitney U test

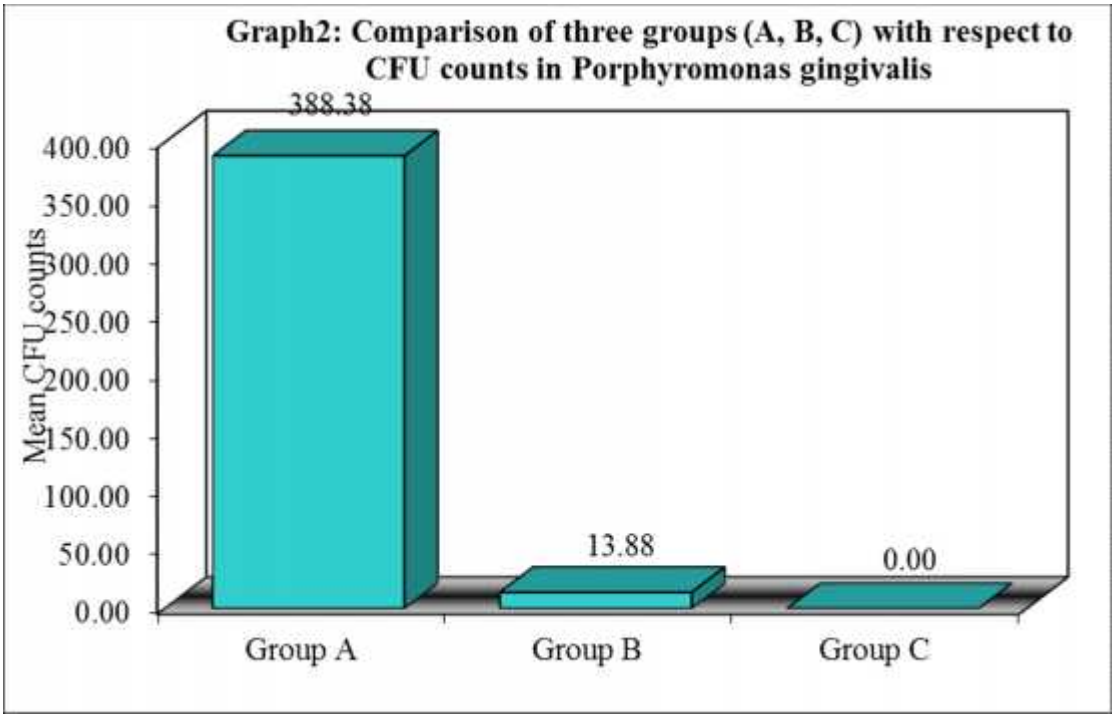
Groups	Mean	SD	Mean rank	U-value	Z-value	p-value
Group A	53.06	151.93	18.50			
Group B	3.25	7.33	14.50	96.00	-1.2060	0.2278
Group A	53.06	151.93	19.50			
Group C	0.00	0.00	13.50	80.00	-1.8091	0.0704
Group B	3.25	7.33	18.00			
Group C	0.00	0.00	15.00	104.00	-0.9045	0.3657

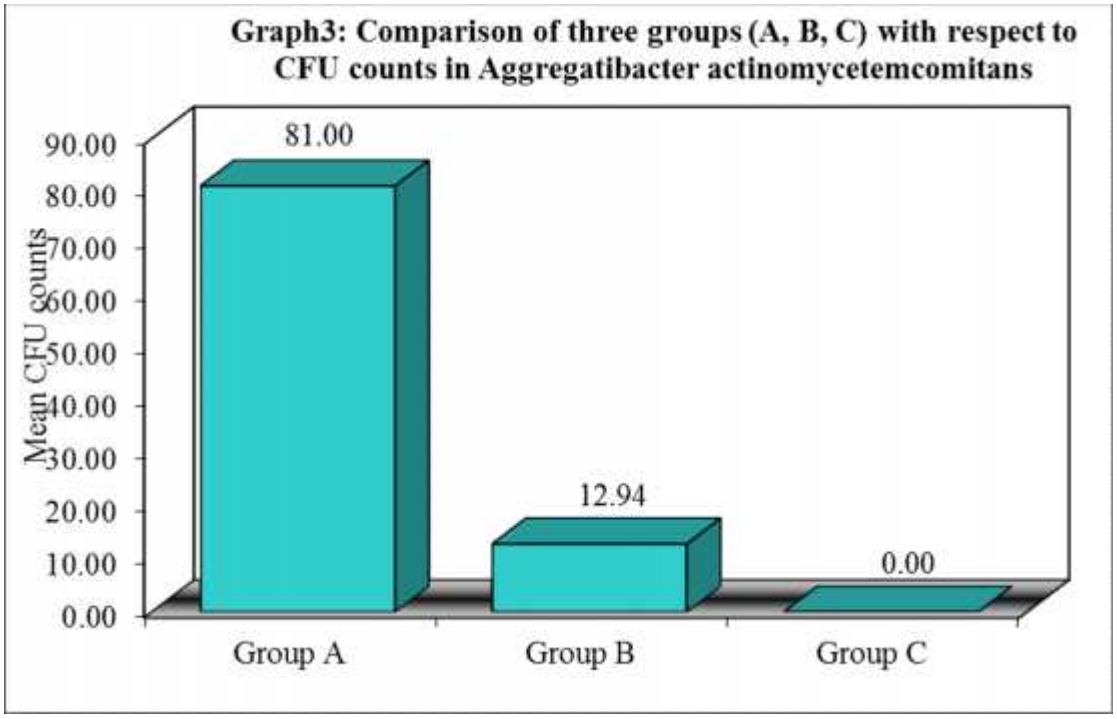
This table demonstrates no statistically significant difference in CFU counts in between Group A and Group C with respect to Prevotella intermedia.

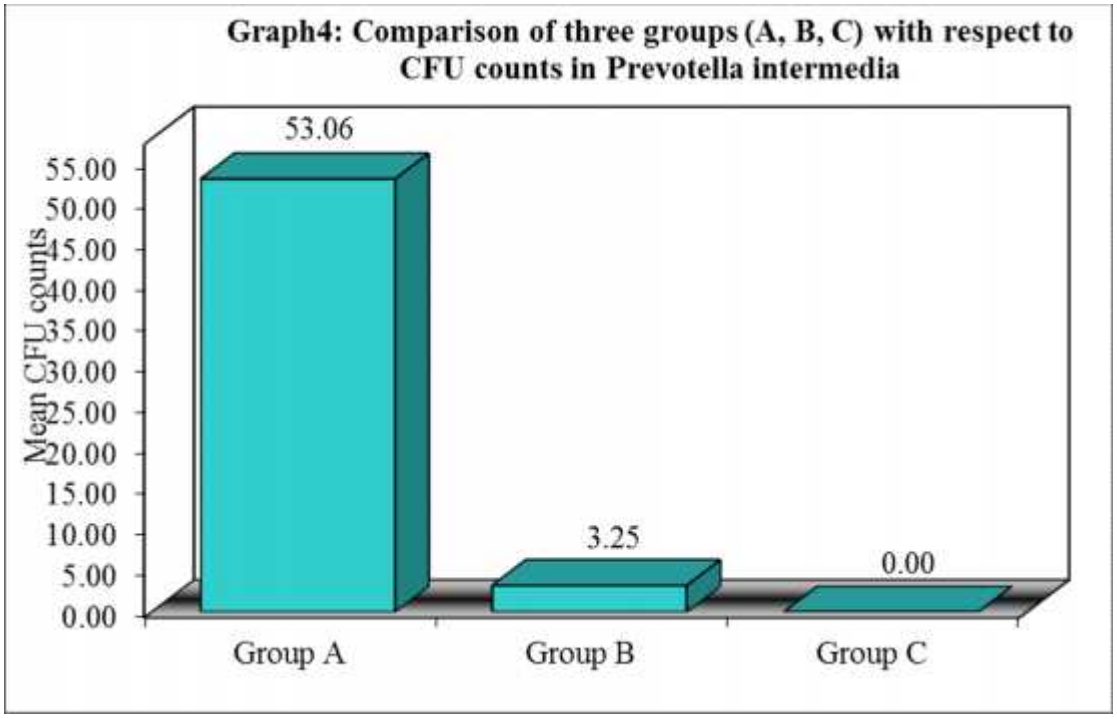
Graph1: Comparison of three groups (A, B, C) with respect to total CFU counts



Graph2: Comparison of three groups (A, B, C) with respect to CFU counts in Porphyromonas gingivalis




Graph3: Comparison of three groups (A, B, C) with respect to CFU counts in Aggregatibacter actinomycetemcomitans
Mean CFU counts
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
81.00
12.94
0.00
Group A
Group B
Group C


Graph4: Comparison of three groups (A, B, C) with respect to CFU counts in Prevotella intermedia
Mean CFU counts
55.00
50.00
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
53.06
3.25
0.00
Group A
Group B
Group C

DISCUSSION

In modern dentistry, dental implants are classic option for replacing teeth which are lost due to injury or because of any other reasons. They are made up of biocompatible material which are surgically inserted in jaw bone that supports artificial crown. It is then left undisturbed for 3-6 months or more for osseointegration. It is direct bone-to-implant contact without interruption from other tissues. This healing process must not be disturbed or else might lead to implant failure. Failure can happen due to microbial infection or overloading. Peri-implantitis caused by microbial biofilm on abutment sub-gingivally is considered to be a major supporter to loss of dental implant.[1] Failures of implant can be categorized as early or late failure. Early failures are due to intra-osseous infection, bacterial contamination and surgical trauma whereas late failures are due to peri-implantitis.[15]

Healing abutments are placed on implants during osseointegration and are allowed to keep there for adequate gingival cuff formation after second stage surgery. It allows epithelial maturation and prevents tissue shrinkage before placement of definitive prosthesis. Sterile environment is very crucial for implant placement.[2] Healing abutments are also designed to protect internal parts within dental implant body from debris accumulation during osseointegration. Once bone-to-implant contact and soft tissue maturation are achieved, healing abutment is removed and definitive abutment and prosthesis are placed. Studies showed that titanium healing abutments can be sterilized and these decontamination processes increases soft tissue cell adhesion. Any remaining surface contamination may lead to biological and mechanical consequences.[4]

Implant and its components are recommended for single use by manufacturers for each patient to prevent cross-infection among them. For cost management, reuse of healing abutments and impression copings are common in dental clinical practice but improper management of sterilization and cleaning of these components results in cross-infection and can cause peri-implantitis.[2]

When the healing abutment is exposed to oral cavity, there is development of pellicle on implant surface. This pellicle leads to lower plaque formation around implants due to its low concentration of albumin. The preferred method of growth for most bacteria are biofilms as they ease interchange of nutrients and guard bacterial community from microorganisms that compete.[6] Abrahamsson et al[41] stated abutment material has valuable role to halt soft tissue and crestal bone recession. Abrahamsson et al[41] and Glauser et al[42] found that composition of the soft tissue seal formed in contact with abutments was not influenced by surface roughness but may affect because of contamination and wettability. In-vitro studies demonstrated that contamination of titanium surfaces decreases fibroblast cell attachment and spreading.[5]

The most common microflora, a greater proportion of red complex (P.gingivalis,T.denticola and T.forsythia) and orange complex (P.intermedia, Fusobacterium nucleatum) and Aggregatibacter actinomycetemcomitans in lower proportions were found in failing implants.[6] Van winkelloff et al[23] found most of the periodontal pathogens(P.gingivalis, F.nucleatum, P.intermedia and T.forsythia) were recognized 6 months just after loading.[6]Mombelli et al[12], Salcetti et al[13], Persson et al[14] and Renvert et al[43] stated in their microbiological studies of implants showing Porphyromonas gingivalis, Prevotella intermedia and Aggregatibacter

actinomycetemcomitans present in clinically healthy peri-implant soft tissues in humans.[15] Generally healthy gingival sulcus consists of gram positive rods and cocci but after periodontitis showed higher amount of gram negative rods and increased levels of Prevotella intermedia, Porphyromonas gingivalis and Treponema species.[22,44] Even after cleaning these components, contaminants like carbon and aluminium traces can be found on abutment surfaces. Such debris can minimize soft tissue adhesion and could initiate inflammatory response.[45]

Therefore, the main objective of the present study was to investigate the efficacy of three different decontamination methods namely Steam Autoclave, Ethylene Oxide and Low Temperature Gas Plasma Sterilization on reuse of titanium implant healing abutments.

The contamination of specimens was done by three different gram-negative microorganisms namely *Porphyromonas Gingivalis ATCC 322 , Aggrigatibacter Actinomycetemcomitans ATCC 33384* and *Prevotella Intermedia ATCC 25611.* The contamination was confirmed by Phloxine B staining and the areas which got contaminated were observed under stereomicroscope in 10X magnification. It shows areas having reddish-orange staining which confirmed the contamination of the specimens.[3, 4]

While considering different decontaminating procedures to be used on the healing abutments, their probable effects on titanium surface must be considered.[11] As Conventional decontamination methods such as steam autoclave can cause damage to the material but do not cause problem in cases where preservation of the material is not an issue. In situations where it is necessary not to cause damage to the materials to be decontaminated, conventional methods can be either not suitable or might offer

tedious solutions.[24] As abutments are mostly made of titanium, it was assumed that steam autoclave sterilization gave assurance of safety of its reuse but it can change the composition of its surface when the autoclave procedure is repeated many times. [16] In a study by Chew et al[11], they found that more than half of surface area can stay contaminated as different parts of healing abutments like top, body and bottom responds differently to the decontamination procedures.

Body surface of abutment was decontaminated more readily than by bottom and top of their surfaces. This is due to difference in approachability of specific areas of the healing abutments.[9] As primary goal of implantology is to avoid cross-infection among patients, several authors concluded that new procedures should be adopted to decontaminate them for reuse.[7]

Stacchi et al[3] and Chew et al[11] suggested that steam autoclaving procedure was ineffective in decontaminating used healing abutments. Contaminants were most commonly found on the body and screw engagement site of the healing abutments.[2,9]

Ethylene Oxide has an effective virucidal, bactericidal and sporicidal activity which makes it exceptional sterilizing agent but may cause potential hazards of ethylene oxide to patients, staff and environment must be handled carefully as it is a flammable gas. According to Gisela et al, Ethylene oxide gas may be having a carcinogenic potential.[8]

Plasma is a collection of electrons removed from atoms and also known as the fourth state of matter. Based on its existence in wide range of temperatures, it is classified as thermal and non-thermal plasma. Its application in dentistry includes elimination of biofilms, removal of dental caries and root canal disinfection.[34]Non-thermal gas plasma do not affect mechanical properties of the material and is

considered to be more effective than thermal gas plasma.[34]According to Vezeau et al[17], Autoclaving reduces cell attachment and in recent in-vitro studies plasma cleaning showed dual effect on titanium implant abutments as disinfecting them and increasing the cell attachment simultaneously.[5] Coelho et al[46] performed study on dogs with primary objective of studying effect of plasma on osseointegration and found out improved interaction between connective tissue and plasma treated titanium implants after one week.[5] According to Duske et al[27], treatment done by plasma increases surface energy and promotes cell spreading. Swart et al[47] stated that enhancing hydrophilicity and wettability are due to activation of the surfaces by plasma treatment at atomic and molecular level.[9] Plasma treatment activates surface energy resulting in increased adsorption of osteoblastic cells on titanium surface has been confirmed by Hauser et al,[48] Junker et al[49] and Tavares et al[50]. It interacts with intrinsic components of the materials to eliminate the contamination effectively.[33] It can be used in biomedical applications to decrease degradation of titanium surfaces and to improve its protein adhesion.[39]It also had an great influence in soft and hard tissue wound healing by increasing the cell viability and wound closure rate.[40]

After decontamination of all the specimen, microbial adhesion was assessed. Sonication and vortexing method, is the standard method to access the microbiological adhesion on different prosthetic materials. It has got improved ability to diagnose implant-related infections. Therefore, in this present study, Sonication and vortexing method was used to check microbial adhesion. Its main advantage is simplicity and high accuracy of the procedure and the results obtained from this technique are accurate, reliable and reproducible.[28]

The results of CFU counts of the present study demonstrated that Group C (Low Temperature Gas Plasma Sterilization) showed least mean total CFU counts amongst all the groups. While Group A showed highest mean total CFU counts amongst all the groups.

In comparison between all the three groups, *Prevotella intermedia* showed statistically significant difference between Group A and Group C. There was no difference between all the groups with respect to *Porphyromonas gingivalis and Aggregatibacter Actinomycetemcomitans.*

The results are in accordance with the similar study conducted by Drake et al[51], which showed contaminated discs sterilized by steam autoclave had shown notably greater CFU count than Ethylene Oxide and Low Temperature Gas Plasma sterilization methods. This significant increase in CFU count was due to surface discoloration because of addition of oxide thickness on the surface and deposition of elements such as carbon and iron. This leads to increased hydrophobic nature of the material.[51] Surfaces decontaminated by Ethylene Oxide sterilization showed low CFU count as compared to Steam Autoclave with minimum change in surface characteristics. This was due to unknown after-effects of elements of ethylene oxide gas which was used in decontamination process. Low Temperature Gas Plasma cleaned surfaces showed low CFU count in this study compared to the other decontamination procedures as it eliminates the surface contaminants and makes the surface hydrophilic which explained the low colonization of the micro-organisms.[51]

The present study showed that Low Temperature Gas Plasma Sterilization is a better decontamination process than Ethylene Oxide and Steam Autoclave sterilization. There is reduction in bacterial counts for decontamination with Low

Temperature Gas Plasma indicating positive correlation between decontamination methods and their removal of microbial contamination from the titanium surfaces.

SCOPE OF THE STUDY

The present study evaluated the efficacy of steam autoclave, ethylene oxide and low temperature gas plasma decontamination procedures on contaminated titanium implant healing abutments. Microbial adhesion and evaluation were done using sonicating and vortexing method for all the specimens. Qualitative assessment of remaining microbial analysis was done of the specimens after decontaminating them with the above procedures.

This study can be further expanded by doing in-vivo studies in patients. Healing abutments of different materials such as gold-platinum alloy, commercially-pure titanium and polyetheretherketone (PEEK) can be used which will enhance the results of this present study.

Newer sterilization methods such as ozone (O_3), chlorine dioxide, universal homogenous ultraviolet (UHUV) rays can be used for the decontamination of the healing abutments which will add more value to this study.

Further investigations assessing these features against different types of abutment materials by fluorescence microscopy, scanning electron microscopy and molecular methods analysis may add new information.

LIMITATIONS OF THE STUDY

- This is an in-vitro study; all clinical parameters have not been studied.
- Only titanium implant healing abutments were studied, inclusion of various other implant abutment materials might differ the results.
- Three different anaerobic microbial species were studied, including other aerobic and anaerobic microbial species in the study might give different results.
- Newer decontamination methods such as Ozone(O_3), universal homogenous ultraviolet(UHUV) rays and gamma irradiation should be tested which are gaining popularity in the market to add new information in this study.
- In the present study, Sonicating and vortexing method was performed for culture media method. Other methods like fluorescence microscopy, scanning electron microscopy and molecular methods were not studied which might have added new insights to this topic.

CLINICAL IMPLICATIONS

As dental implant manufacturers provide healing abutments sterilized by conventional decontamination methods, it could not remove all the contaminants and reduces the hydrophilic nature of the titanium healing abutment surfaces leading to poor epithelial attachment on their surfaces. This gave us an opportunity to focus on other decontamination methods which will provide more efficacy on the reuse of these components. In this study, the results showed efficacy of different decontamination methods on contaminated titanium implant healing abutments. Out of all the decontamination methods used, Low temperature gas plasma sterilization method showed to be more effective method than ethylene oxide and steam autoclave sterilization in removing microbes that are commonly associated with peri-implantitis. Therefore, Low temperature gas plasma sterilization can be considered as an effective method for decontamination of titanium implant healing abutments. This study will help clinicians to provide better esthetic results in prosthetic rehabilitation of implants and to minimize the risk of peri-implantitis .

CONCLUSION

Within the limitations of this in-vitro study the following conclusions were drawn.

1) Out of the three decontamination methods compared, Low temperature gas plasma sterilization showed lowest total CFU counts as compared to other methods and Steam autoclave sterilization showed highest total CFU counts.
2) Low temperature gas plasma sterilization is better as compared to Ethylene oxide and Steam autoclave sterilization as decontamination method for the reuse of titanium implant healing abutments.

SUMMARY

The present in-vitro study was carried out to evaluate the efficacy of decontamination methods namely Steam Autoclave (Group A), Ethylene Oxide (Group B) and Low Temperature Gas Plasma Sterilization (Group C) on reuse of titanium implant healing abutments.

A total of 48 titanium implant healing abutments (n=16) were procured from Norris company manufacturer of diameter 4.6mm and height 4.0mm and were divided into 3 subgroups (n=16) based on 3 different decontamination methods . The stereomicroscope with 10X magnification was used to evaluate the surface profile of all the specimens at the following sites:- (a) main body, (b) threaded portion and (c)hex. After surface profile evaluation, all the healing abutments were subjected to microbiological contamination.

The bacterial strains used for adhesion were anaerobic gram negative bacteria namely:- *Porphyromonas gingivalis* strain ATCC 322, *Prevotella intermedia* strain ATCC 25611 and *Aggregatibacter actinomycetemcomitans* strain ATCC 33384. The contamination of the healing abutments was confirmed with phloxine B staining solution. The microbial contamination was observed in stereomicroscope with 10X magnification.

The decontamination for the specimens in group A were done with Steam Autoclave sterilization at 121^0C for 15-30 mins. Specimens in group B were decontaminated by Ethylene Oxide sterilization and in Group C by Low Temperature Gas Plasma sterilization. Highly reliable and accurate Sonicating and Vortexing technique was

used for this purpose. CFU count was calculated using formula: CFU = No. of colonies X 2^n (Dilution factor).

The resultant data was tabulated and then subjected to statistical analysis to draw conclusion from experimental data. Kruskal-Wallis test was performed for comparison of three groups, followed by Mann-Whitney test for pair-wise comparison between groups. ($p<0.05$)

The results showed that Low Temperature Gas Plasma sterilization showed lowest CFU count and Steam Autoclave sterilization showed highest CFU count in comparison between all three groups. The efficiency of decontamination methods was inversely proportional to CFU counts. Hence, Low Temperature Gas Plasma sterilization may be a suitable method for decontaminating titanium implant healing abutments.

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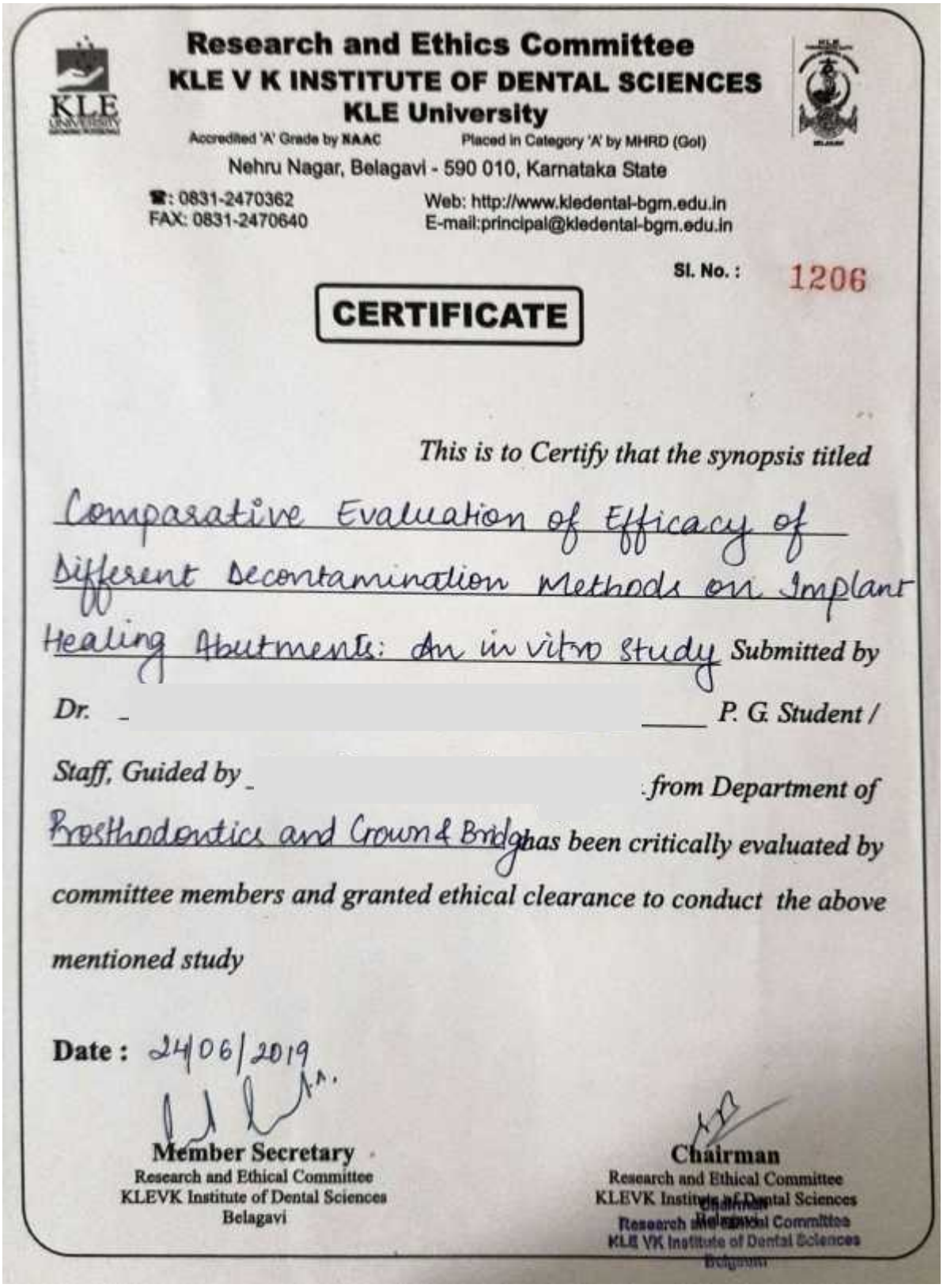
ANNEXURE - I - ETHICAL CLEARANCE



Research and Ethics Committee
KLE V K INSTITUTE OF DENTAL SCIENCES
KLE University
Accredited 'A' Grade by NAAC Placed in Category 'A' by MHRD (GoI)
Nehru Nagar, Belagavi - 590 010, Karnataka State
☎: 0831-2470362
FAX: 0831-2470640
Web: http://www.kledental-bgm.edu.in
E-mail:principal@kledental-bgm.edu.in

Sl. No. : 1206

CERTIFICATE

This is to Certify that the synopsis titled

Comparative Evaluation of Efficacy of Different Decontamination Methods on Implant Healing Abutments: An in vitro Study *Submitted by*

Dr. _____ *P. G. Student /*

Staff, Guided by _____ *from Department of*

Prosthodontics and Crown & Bridge *has been critically evaluated by committee members and granted ethical clearance to conduct the above mentioned study*

Date : 24/06/2019

Member Secretary
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

Chairman
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

ANNEXURE - II

Table 1:- Total Colony Forming Unit (CFU) count in Group A

GROUP A	TOTAL CFU
A1	1291
A2	-
A3	1034
A4	-
A5	-
A6	-
A7	1050
A8	-
A9	1187
A10	-
A11	2267
A12	-
A13	113
A14	337
A15	-
A16	-

Table 2:- Total Colony Forming Unit (CFU) count in Group B

GROUP B	TOTAL CFU
B1	-
B2	-
B3	-
B4	208
B5	-
B6	-
B7	-
B8	-
B9	122
B10	-
B11	-
B12	-
B13	-
B14	151
B15	-
B16	-

Table 3:- Total Colony Forming Unit (CFU) count in Group C

GROUP C	TOTAL CFU
C1	-
C2	-
C3	-
C4	-
C5	-
C6	-
C7	-
C8	-
C9	-
C10	-
C11	-
C12	-
C13	-
C14	-
C15	-
C16	-

GROUP A:- Steam Autoclave sterilization
GROUP B:- Ethylene Oxide sterilization
GROUP C:- Low Temperature Gas Plasma sterilization

Table4:-COLONY FORMING UNIT (CFU) of three Groups A, B, and C with respect to Porphyromonas gingivalis CFU count (CFU/ml) = Number of colonies X 2 ^{n} (n = dilution factor)

GROUP A	CFU/ml	GROUP B	CFU/ml2	GROUP C	CFU/ml3
A1	1143	B1	-	C1	-
A2	-	B2	-	C2	-
A3	976	B3	-	C3	-
A4	-	B4	38	C4	-
A5	-	B5	-	C5	-
A6	-	B6	-	C6	-
A7	1027	B7	-	C7	-
A8	-	B8	-	C8	-
A9	1143	B9	63	C9	-
A10	-	B10	-	C10	-
A11	1624	B11	-	C11	-
A12	-	B12	-	C12	-
A13	-	B13	-	C13	-
A14	301	B14	121	C14	-
A15	-	B15	-	C15	-
A16	-	B16	-	C16	-

Table 5:-COLONY FORMING UNIT (CFU) of three Groups A, B, and C with respect to Aggregatibacter actinomycetemcomitans CFU count (CFU/ml) = Number of colonies X 2 ^{n} (n = dilution factor)

GROUP A	CFU/ml	GROUP B	CFU/ml	GROUP C	CFU/ml
A1	1207	B1	-	C1	-
A2	-	B2	-	C2	-
A3	22	B3	-	C3	-
A4	-	B4	159	C4	-
A5	-	B5	-	C5	-
A6	-	B6	-	C6	-
A7	23	B7	-	C7	-
A8	-	B8	-	C8	-
A9	13	B9	41	C9	-
A10	-	B10	-	C10	-
A11	31	B11	-	C11	-
A12	-	B12	-	C12	-
A13	-	B13	-	C13	-
A14	-	B14	7	C14	-
A15	-	B15	-	C15	-
A16	-	B16	-	C16	-

Table 6:-COLONY FORMING UNIT (CFU) of three Groups A, B, and C with respect to Prevotella intermedia CFU count (CFU/ml) = Number of colonies X 2 ^{n} (n = dilution factor)

GROUP A	CFU/ml	GROUP B	CFU/ml	GROUP C	CFU/ml
A1	21	B1	-	C1	-
A2	-	B2	-	C2	-
A3	36	B3	-	C3	-
A4	-	B4	11	C4	-
A5	-	B5	-	C5	-
A6	-	B6	-	C6	-
A7	-	B7	-	C7	-
A8	-	B8	-	C8	-
A9	31	B9	18	C9	-
A10	-	B10	-	C10	-
A11	612	B11	-	C11	-
A12	-	B12	-	C12	-
A13	113	B13	-	C13	-
A14	36	B14	23	C14	-
A15	-	B15	-	C15	-
A16	-	B16	-	C16	-