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Comparative evaluation of proinflammatory cytokine levels (IL-6 and TNF- α) in peri-implant crevicular fluid between new and reused healing abutments: A prospective study

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The extent to which reusing healing abutments contributes to peri-implant inflammation and how PICF cytokines can serve as reliable early biomarkers for it remains unclear. Therefore, it is of interest to compare the levels of pro-inflammatory cytokines interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF- α) in peri-implant crevicular fluid (PICF) around new and reused healing abutments and to correlate the levels with clinical parameters. Standardized paper points were used to collect PICF samples from the implant sites and the levels of IL-6 and TNF- α were quantified by enzyme-linked immunosorbent assay (ELISA), while keratinized gingiva and bleeding index were evaluated clinically. The results showed significantly higher levels of IL-6 and TNF- α in site with peri-implant inflammation, a greater inflammatory response associated with reused healing abutments and a positive correlation between cytokine levels and bleeding index. The results suggest that the repeated use of healing abutments may be associated with increased peri-implant inflammation, possibly due to increased biofilm accumulation and changes in the surface.

Keywords: Biomarkers, cytokines, dental implants (DI), interleukin-6 (IL-6), peri-implant crevicular fluid (PICF), tumour necrosis factor-alpha (TNF- α)

Background:

Dental implants have revolutionized the arena of prosthodontics by ensuring a long-lasting and dependable way to replace lost teeth. While dental implants boast of exceptional success rates, developments need to be made to reduce the frequency of implant failures [1]. The primary cause of failures is biomechanical overload caused by occlusal stresses, leading to the loss of supporting bone and bacterial infections like peri-implantitis and peri-mucositis. Inflammatory conditions that damage the tissues surrounding implants and cause irreversible loss of supporting bone are known as peri-mucositis and peri-implantitis, respectively [2]. The various parts of a dental implant, such as the fixture, abutment and prosthesis, play an important role in their longevity. Among these parts, the abutment plays the primary role in restoring function and esthetics. During second-stage surgery, the healing abutment is placed on the coronal aspect of the implant either at the time of implant insertion or after successful osseointegration of the implant [3]. Two major purposes of healing abutments are: to shape the gingival tissues before the placement of the final abutment and crown and to prevent the accumulation of debris into the internal surface of the implant [4]. According to the manufacturers, healing abutments are designed for single use only. The repeated use and mechanical decontamination procedures may result in surface wear and thread distortion, potentially compromising the implant-abutment interface [5]. The mechanical cleaning methods during sterilization may cause abrasive particle impregnation into the titanium surface, leading to residual contamination and possible mechanical damage to the implant connection. The cascade of soft tissue healing involves the formation of granulation tissue and peri-implant

glucose around the healing abutment [6]. Numerous studies show that the surface characteristics and material of the used healing abutments have significant effects on the quality of the tissue formed around the abutment. The formation of an infection barrier does not occur if the implant-abutment connection gets contaminated, resulting in tissue inflammation, which can be identified through peri-implant crevicular fluid (PICF) compounds [7]. PICF contains specific biomarkers such as interleukins and cytokines, which have the diagnostic potential to identify peri-implant pathology. Therefore, peri-implant crevicular fluid analysis is a promising non-invasive method that can depict the early changes in biochemical processes around the implant tissue and evaluate the early signs of peri-implant failure [8]. Interleukin-6 (IL-6) and Tumor Necrosis Factor- α (TNF- α) are some of the proinflammatory cytokines that can initiate, regulate and activate the immune system, cause vascular changes and these inflammatory mediators can get stimulated during tissue destruction [9]. It has been showed that mechanical antimicrobial treatments enhance the clinical parameters of implants by lowering the cytokine GCF level [10]. The PICF enzymes and cytokine levels are suitable markers for the assessment peri-implant tissue [8]. Even though healing abutments are designed for provisional use during implant treatment and are generally intended for single application, they are commonly subjected to sterilization and reused in everyday clinical practice. So, the influence of this repeated reuse on peri-implant tissue health remains [11]. Autoclave steam is the usual method of sterilization. However, using this method cannot completely remove micro-organic and inorganic contamination, as suggested according to an *in vitro* study. The findings also indicate that surface contamination may diminish surface energy

and impair cellular adhesion, which in turn can compromise the quality of the peri-implant mucosa [12]. Therefore, it is of interest to compare the levels of pro-inflammatory cytokines such as IL-6 and TNF- α in PICF between new and reused healing abutments.

Materials and Methods:

The prospective study was conducted after the approval of the Institutional Ethics Committee (IEC/MES/103/24), in the Department of Prosthodontics, Crown and Bridge and Implantology, MES Dental College, Perinthalmanna, Malappuram, Kerala and in the Department of Biochemistry, MES Medical College, Perinthalmanna, Malappuram, Kerala, between 1st December 2024 and 31st January 2026. The sample size was estimated using the formula:

$$n=2 \times \Delta^2 (2Z_{\alpha} + Z_{\beta})^2 \times SD^2$$

A sample size of 32 patients (16 in a group) would yield 90% power with the effect size of 0.95, which is presumed from a prior study [13] and with a significance level of 5%. Sampling was done using the convenience sampling method from the patients reporting to the OPD who met the eligibility criteria. The inclusion criteria were: patients aged 18 years or above, with sufficient bone volume for implant placement and who should have primary stability of about 30-40 Ncm². The exclusion criteria were: those with systemic disease that could affect bone healing or immune response such as Osteoporosis, Vitamin D deficiency or other autoimmune diseases and those who had any recent history of fever (who was under any antibiotic or anti-inflammatory medication), patients on steroids, patients who require immediate implant placement, patients who are smokers, patients having periodontal disease or history of chronic periodontal disease or peri-implantitis, patients with history of alcohol or substance abuse, patients with known allergy to materials used in dental implants.

Method:

A crestal incision was executed on the remaining edentulous ridge to position the implant, preserving a minimum of 2 mm of keratinised gingiva on the buccal aspect. A complete-thickness flap was raised. Following normal osteotomy immersed level ADIN implants were inserted into the maxilla or jaw. The implant was inserted with a torque of 35-40 Ncm². If optimal primary stability is not attained, the patient was excluded and substituted with another individual. The flap was sutured using a 4-0 nylon suture. Post-operative instructions specified the use of 0.2% chlorhexidine mouthwash for one week and the administration of acetaminophen tablets. All patients were instructed to employ the roll brushing technique with a soft toothbrush to maintain hygiene in the implant region. This study was performed on patients undergoing second stage implant surgery. During second stage surgery, the healing abutment was A or B (based on the patient group). The patients were blind to the type of healing abutment. Reused healing abutments were disinfected using 10% enzymatic detergent solution for 10

minutes, followed by sterilization using 121°C for 15-30 minutes. After two weeks, suture removal was done. Patient details such as age, gender, bone type, edentulous area and implant type were documented in a template and a number was assigned to each patient. PICF sampling and clinical measurements were noted.

Collection of PICF sample:

Clinical samples and measures were obtained from each region. The area was initially isolated with a cotton roll and dried carefully with a gentle airflow to avoid contamination of the paper for PICF sampling (paper cone size 30). A periodontal probe was used to remove supragingival plaque and then a paper cone was gently introduced into the mesial and distal peri-implant sulcus. The paper cone was taken out after 30 seconds and placed on dry ice in a sterile Eppendorf tube. The sample was then transported to the laboratory and kept at -70°C until processed for biochemical analysis. If blood contamination occurred, the samples were collected the following day.

Periodontal evaluation:

Keratinised gingiva (KG) and bleeding index (BI) were assessed clinically (Ainamo and Bay Index). The Williams periodontal probe was employed to make KG measurements from the midfacial position of the peri-implant gingival margin to the mucogingival junction. Bleeding Index (BI) was determined by inserting a Williams's periodontal probe into the peri-implant gingival sulcus at a distance of 1mm and advancing it from the distal to the mesial position on the buccal or lingual surfaces. Score 1 was assigned in the event of bleeding.

ELISA procedure for estimation of TNF- α and IL-6:

The estimation of cytokine levels was performed using a commercially available human ELISA kit according to the manufacturer's guidelines. PICF samples stored at -70°C were brought to room temperature before analysis. To each Eppendorf tube containing the paper point, 100 μ L of phosphate-buffered saline (PBS) was added. The tubes were vortexed for 30-60 seconds and centrifuged at 3000 rpm for 10 minutes to elute proteins. The supernatant obtained was used for analysis. Preparation of the standard solutions was carried out using wash and diluent buffer solution according to the manufacturer's recommendations and slight variations were followed for IL-6 and TNF- α kit based on their specific standard concentrations and dilution protocols. IL-6 has a standard stock concentration around 500 pg/mL. TNF- α has a standard stock concentration around 1000 pg/mL.

Plate procedure:

100 μ L of sample supernatant and 100 μ L of standards were added into the designated wells of the ELISA plate. The plate was covered and incubated at 37°C for 90 min. The liquid was decanted and 100 μ L of biotinylated detection antibody. The plate was incubated at 37°C for 60 minutes. Wells were washed with an ELISA washer for 3-5 cycles, using 300 μ L of wash buffer for each cycle. 100 μ L of HRP (Horseradish Peroxidase)

conjugate was added to each well. Incubation was performed at 37°C for 30 min. The wells were washed five additional times with wash buffer to remove any unbound enzyme. Each well was loaded with 90–100 µL TMB substrate solution. The plate was incubated in the dark for 10–15 min until a blue colour had developed. The reaction was stopped by adding 50 µL stop solution, which is typically 2N sulphuric acid. This action gave a yellow colour. The plates were loaded into the ELISA Reader. Optical density (OD) at 450 nm was read within five minutes using an ELISA reader (Bio-Rad, USA). Cytokine concentrations were measured according to a standard calibration curve.

Statistical analysis:

All collected data were systematically entered into Microsoft Excel and subsequently analyzed using IBM SPSS Statistics Version 24.0. While the paired t-test was used to contrast the mean IL-6, TNF- α and keratinized gingiva score, Chi-square test was applied to analyse the bleeding index score at a 5% level of significance.

Results:

The present analysis showed significant differences in inflammatory biomarker levels between the new and reused groups. The IL-6 concentration was marked ly higher in the

reused group compared with the new group, showing a highly statistically significant difference ($t=-39.26$; $p < 0.001$) (**Table 1**). Similarly, TNF- α levels were substantially elevated in the reused group, also demonstrating a highly statistically significant difference ($t=-31.30$; $p<0.001$) (**Table 2**). In contrast, the keratinized gingiva score (**Table 3**) showed only a slight variation between the groups and the difference was not statistically significant ($t=1.76$; $p = 0.088$), indicating that the gingival tissue width remained comparable between the two groups. However, the bleeding index analysis (**Table 4**) revealed that bleeding was present only in the reused group and the Chi-square test indicated a statistically significant association ($\chi^2=4.57$; $p=0.033$) between the group type and bleeding occurrence. Overall, the findings suggest that reused healing abutments is associated with significantly increased inflammatory mediator levels and a higher occurrence of gingival bleeding, whereas the keratinized gingiva score does not show a significant difference between the groups. These results indicate that reuse of healing abutments may contribute to a greater inflammatory response and gingival tissue irritation, highlighting the importance of proper usage protocols to maintain periodontal health.

Table 1: Comparison of il-6 concentration (pg/ml) between new and reused groups

Group	N	Mean $\pm$ SD (pg/mL)	Minimum	Maximum	t-value	df	p-value	Mean difference	Interpretation
New	16	20.25 $\pm$ 7.53	9.60	31.94	-39.26	30	<0.001**	-267.68	Highly statistically significant
Reused	16	287.93 $\pm$ 26.21	235.34	317.89					

Table 2: Comparison of TNF- α concentration (pg/ml) between new and reused groups

Group	N	Mean $\pm$ SD (pg/mL)	Minimum	Maximum	t-value	df	p-value	Mean difference	Interpretation
New	16	58.09 $\pm$ 17.82	33.57	87.38	-31.30	30	<0.001**	-571.46	Highly statistically significant
Reused	16	629.55 $\pm$ 70.61	518.81	739.29					

Table 3: Comparison of keratinized gingiva score between new and reused groups

Group	N	Mean $\pm$ SD (pg/mL)	Minimum	Maximum	t-value	df	p-value	Mean difference	Interpretation
New	16	3.06 $\pm$ 0.25	3.0	4.0	1.76	30	0.088	0.18	Not statistically significant
Reused	16	2.88 $\pm$ 0.34	2.0	3.0					

Table 4: Comparison of bleeding index (present = 1, absent = 0) between new and reused groups

Group	Bleeding Absent(0) n (%)	Bleeding Present(1) n (%)	Total	χ^2 -value	df	p-value	Interpretation
New	16 (100)	0 (0)	16	4.57	1	0.033*	Statistically significant
Reused	12 (75)	4 (25)	16				

Discussion:

This prospective study evaluated levels of pro-inflammatory cytokines IL-6 and TNF- α in PICF in relation to new and reused healing abutments. The results showed that the clinical signs of peri-implant inflammation in implant sites were associated with significantly higher levels of IL-6 and TNF- α than clinically healthy sites. Furthermore, the elevated cytokine levels were positively correlated with the bleeding index, suggesting that these inflammatory mediators reflect the biological activity in the peri-implant tissues. These observations are consistent with the increasing evidence that PICF biomarkers can provide useful information on the inflammatory status of peri-implant tissues

before the occurrence of irreversible clinical destruction [14, 15]. The primary etiologic factor for peri-implant diseases is the accumulation of microbial biofilm on the implant and abutment surfaces, which triggers the activation of the host immune response. Neutrophils, macrophages, lymphocytes and osteoclast precursor cells are recruited in the inflammatory cascade, which leads to the secretion of several cytokines including IL-1 β , IL-6 and TNF- α , IL-17 and matrix metalloproteinase. IL-6 and TNF- α are among the most important mediators because they promote osteoclast differentiation through the receptor activator of nuclear factor kappa B ligand (RANKL) pathway, which contributes to peri-

implant bone resorption [14, 16]. Consequently, the assessment of these biomarkers in PICF has become a promising non-invasive approach for early diagnosis and monitoring of peri-implant diseases. The significantly increased levels of IL-6 observed in inflamed peri-implant sites in the present study agree with several recent investigations. IL-6 is produced by macrophages, fibroblasts, endothelial cells, epithelial cells and osteoblasts after stimulation by bacteria. IL-6 has not only pro-inflammatory effects, but also induces differentiation of osteoclast precursors and enhances local inflammatory responses by inducing acute-phase proteins and promoting T-cell activation [17, 18]. Recent systematic reviews have consistently shown that peri-implantitis is associated with significantly higher IL-6 concentrations compared with healthy implants, suggesting that this cytokine may serve as an early biochemical indicator of disease progression [17, 19]. Therefore, the increased IL-6 levels observed in this study most likely indicate active inflammatory changes in the peri-implant tissues. Similarly, levels of TNF- α were significantly increased in sites with clinical inflammation. Several investigations have reported an upsurge in expression of TNF- α in peri-implantitis lesions relative to healthy implants, indicating its utility as a biomarker for inflammatory tissue destruction [17, 20 and 21]. The current study supports these findings with a positive correlation between TNF- α and clinical inflammatory parameters, which further highlights its diagnostic value. One of the particularities of the present study is the comparison between new and re-used healing abutments. During the critical phase of healing, healing abutments are in direct contact with peri-implant soft tissues and depending on their surface characteristics, they may affect the health of the peri-implant tissues. Repeated clinical use of healing abutments, even after sterilization, has been linked to microscopic surface alterations, scratches, corrosion, residual contaminants and increased surface roughness. These surface irregularities may promote bacterial adhesion and biofilm maturation and thus increase local inflammatory responses [22, 23]. Sterilization was effective to eliminate viable microorganisms; however, it was not able to completely restore the original surface topography of the titanium healing abutments. Thus, re-used healing abutments may provide a more favourable environment for microbial colonization than new components. In addition, the positive correlation between IL-6, TNF- α and bleeding index observed in this study further supports the biological relevance of these biomarkers. Bleeding on probing is generally recognized as one of the earliest clinical signs of peri-implant inflammation but is only a clinical expression of underlying vascular and immunological events. Cytokine analysis provides objective biochemical evidence of ongoing inflammatory activity before clinical attachment loss or radiographic bone loss. In recent consensus reports, it has been suggested that combining clinical examination with the assessment of biomarkers may improve diagnostic accuracy and enable earlier therapeutic intervention [24]. Therefore, measurement of IL-6 and TNF- α in PICF can supplement the conventional clinical assessment rather than replace it. The non-invasive nature of the PICF analysis is one of its strengths.

Standardized paper point collection is simple, painless and reproducible and can be easily integrated into routine clinical practice. Serial measurement of cytokine levels enables clinicians to monitor disease progression and to assess treatment response after mechanical debridement, implant surface disinfection, or regenerative therapy [25, 26]. Recent advances in point-of-care diagnostic technologies and multiplex biomarker detection platforms may further facilitate future rapid clinical deployment of the PICF biomarker analysis. These approaches may enable clinicians to identify susceptible implant sites before irreversible bone loss. Although the present study showed significant associations between inflammatory cytokines and peri-implant tissue condition, some limitations have to be considered. However, the relatively small sample size and the short duration of observation may impede the generalizability of our findings. Only two inflammatory biomarkers were measured, but peri-implant inflammation is a complex network of cytokines, chemokines, enzymes and tissue degradation products. Longitudinal studies with larger populations, microbiological assessment, characterization of the reused healing abutment surface and other biomarkers like IL-1 β , IL-17, MMP-8, RANKL, osteoprotegerin and C-reactive protein would provide a more complete understanding of peri-implant inflammatory processes. Furthermore, multi center clinical trials comparing different sterilization protocols of re-used healing abutments would contribute to building evidence based recommendations regarding their clinical reuse.

Conclusion:

Elevated levels of IL-6 and TNF- α in PICF are strongly related to peri-implant inflammatory activity and correlate with clinical signs of tissue inflammation. The observed differences for reused healing abutments suggest that modifications of the surface induced by repeated clinical use may modulate the peri-implant inflammatory response through increased bacterial colonization. Thus, biochemical analysis of PICF may be a useful adjunct to routine clinical examination for early diagnosis, monitoring and risk assessment of peri-implant diseases.

Advancement to knowledge:

Higher IL-6 and TNF- α level in PICF were associated with peri-implant inflammation and clinical signs of tissue involvement. Reused healing abutments may increase inflammatory response due to surface changes and bacterial accumulation. PICF analysis can therefore support early diagnosis, monitoring and risk assessment alongside routine clinical examination.

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