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MODULATION OF THE ELECTROMAGNETIC PULSE ON THE ORAL BIOFILM OF INDUCED GINGIVITIS AND MUCOSITIS: PROTEOMIC AND MICROBIAL EFFECTS

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MODULATION OF THE ELECTROMAGNETIC PULSE ON THE ORAL BIOFILM OF INDUCED GINGIVITIS AND MUCOSITIS: PROTEOMIC AND MICROBIAL EFFECTS

Research project to the Research Ethics Committee of Univeritas-UNG University

Area of concentration: Implantology

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ABSTRACT

As in natural teeth, bacterial colonization of the implant surface can trigger a reversible inflammatory process in the peri-implant soft tissue-defined mucositis. However, differences in the organization of peri-implant tissues may combat the progression of lesions associated with bacterial biofilm, resulting in a broader inflammatory infiltrate compared to periodontal tissues. Some previous studies have used electromagnetic pulses (PEMF-pulse electromagnetic fields) to modulate bacterial biofilm. Thus, the objective of this study will be to evaluate the host response pattern (proteomics, microbiome, and clinical) during the induction and progression of experimental gingivitis and mucositis in humans with the action of the electromagnetic pulse. Forty systemically healthy individuals will be included, who must present the need for two implant-supported restorations adjacent to the teeth. Individuals will receive oral hygiene instruction and professional prophylaxis fortnightly, 30 days before implant installation. The implants will be installed in time -60 days. After 60 days, at baseline, the healing abutment will be installed on the two implants, one conventional and the other with an electromagnetic pulse. At the same time, everyone will receive acetate plates to protect the selected areas (tooth and implants) during brushing, to be used for 21 days. Clinical periodontal parameters, microbiological (supragingival and subgingival biofilm), and immunological (crevicular fluid) collections will be obtained at 0, 3, 7, 14, 21, 42, and 60 days. The results will be evaluated for normality using the D'Agostino test. Then, parametric or non-parametric tests will be applied to compare the means/medians obtained between the gingivitis and mucositis groups in the different periods of the study.

Keywords: Oral Surgery, Dental Implant, Microbiome, Proteome, Gingivitis, Mucositis.

SUMMARY

1. INTRODUCTION

Prosthetic rehabilitation by means of osseointegrated implants is currently a well-established method in dentistry, offering excellent aesthetic and functional results with high predictability. However, it is known that numerous implants are lost early or late (Zitzmann; Berglundh, 2008; Shibli et al. 2015). Among the main causes of these losses are: the patient's systemic health condition, smoking, bone quality, the experience of the dental surgeon, inadequate prosthetic planning, occlusal overload, parafunctional activity, and bacterial infection at the time of or after surgery (Zitzmann; Berglundh, 2008).

As with natural teeth, bacterial colonization of the implant surface can trigger a reversible inflammatory process in the peri-implant soft tissue (Salvi et al., 2012), called mucositis. When not identified and treated, this inflammatory process can intensify and progress apically, leading to clinical signs very similar to those of periodontitis. Peri-implantitis leads to loss of bone support followed by remodeling of the supporting periodontium during the healing process (American Academy of Periodontology, 2013).

The main risk indicators for mucositis involvement, according to Lindhe and Meyle (2008), are: poor oral hygiene, history of periodontitis, smoking, diabetes, alcohol consumption and genetic factors, with the first three mentioned having a higher degree of evidence.

It is known that the interface between soft tissue and osseointegrated implant has several similarities with the tissue around natural teeth, but some differences should be considered. The absence of the periodontal ligament, the main one, can limit the peri-implant blood supply, especially on the surface immediately adjacent to the implant surface (Carranza et al., 2012) Histologically, the collagen fibers around the implants are oriented in parallel without insertion on their surface, while in the teeth the collagen fibers are perpendicular and insert into the cementum (Hanisch et al., 1997).

The evaluation of the formation of dental bacterial biofilm on the surface of dental implants by scanning electron microscopy indicates that the formation patterns identified in implants are very similar to those observed in teeth (Lang et al., 2000).

In a clinical study, Zitzmann et al. (2001) demonstrated that the changes that occurred in the gingiva and peri-implant mucosa during the experimental period of biofilm accumulation induction did not present statistically significant differences,

which, for the authors, indicates that the host response to dental plaque or peri-implant is, in many aspects, comparable.

Although few, clinical studies that have compared the response of gingival and peri-implant tissues to biofilm formation through the analysis of clinical signs (plaque index, gingival bleeding index, and depth of probing and bleeding on probing) of the host response have reached very similar results (McKenna; Borzabadi-Farahani; Lynch, 2013; Tawse-Smith et al., 2012; Berglundh et al. 1992; Ericsson et al., 1992; Pontoriero et al., 1994; Zitzmann et al., 2001). However, differences in the organization of peri-implant tissue mean that combating the progression of lesions associated with bacterial plaque results in a broader inflammatory infiltrate, when compared to gingival tissue (Melo, 20019). As can be seen in the study by Salvi et al. (2012) who, in addition to the clinical signs, evaluated the crevicular gingival fluid of their sample and observed that the soft tissues around the implants developed a more intense inflammatory response during the experimental period of plaque accumulation, when compared to that of their gingival counterparts. Zitzmann et al. (2001) and Ziztimann et al. (2002) found similar results in their studies.

Recently, however, Albrektsson et al. (2014), suggested that the basic mechanism behind marginal bone loss around implants cannot be compared to that described for periodontal diseases, but rather, with a foreign body reaction. This assumption, based only on a few explanations from isolated cases, has been widely disseminated without being questioned.

Few studies evaluating the effects of biofilm formation around implants (McKenna, Borzabadi-Farahani, Lynch, 2013; Salvi et al., 2011; Zitzmann et al., 2001, 2000; Pontorieiro et al., 1994) compared the peri-implant response to periodontal disease with bacterial biofilm accumulation (McKenna, et al., 2013; Salvi et al., 2011; Zitzmann et al., 2001; 2000; Tawse-Smith et al., 2001), but were inconclusive or did not analyze the host's immune response to this experimental model. However, studies evaluating the host's immune response to this colonization are still scarce and often inconclusive. Regardless of gingival health and subgingival microbiota, inflammatory cytokines produced within peri-implant tissues may be different from those present in periodontal tissues (Nowzari et al., 2012). Therefore, more specific studies correlating the microbiological findings to the analysis of cytokines and proteins around teeth and implants using the experimental gingivitis model should be performed.

In addition, previous studies using *pulse electromagnetic fields (PEMF*) in the modulation of bacterial biofilm have drawn attention (ZHOU et al. 2017). Although **PEMF** has been used more frequently to increase bone density and osseointegration (BUZZA et al. 2003; BARAK et al. 2016), recent advances in the miniaturization of the equipment have allowed the entire apparatus to be inserted into an implant healer (BARAK et al. 2016; BARAK et al. 2019).

Using this PEMF-emitting healer, our group evaluated the effect of the activated healer (PEMF+) on periodontal bacterial biofilm with 31 species in *an in vitro model* (Faveri et al. 2020). In this study, PEMF healers modulated the bcaterian biofilm, reducing some bacterial species of the orange complex and increased one species of the blue complex (beneficial species). In the meantime, it would be very interesting to evaluate the effect of this technology during the induction of experimental mucositis, on the modulation of the biofilm and consequently the host reaction.

Therefore, there is a need to evaluate a greater number of markers around the periodontal and peri-implant fluids, as well as their correlation with the microorganisms present during biofilm maturation through the induction of experimental gingivitis/mucositis.

2. OBJECTIVE

To evaluate the host response pattern (proteomics, microbiome, and clinical) during induction and progression of gingivitis and experimental mucositis in humans with electromagnetic pulse action.

3. MATERIAL AND METHODS

3.1. Experimental design

This prospective, controlled study will induce experimental gingivitis and mucositis in humans using the suspension of hygiene of the elements involved in the research: one tooth and 2 implants. The model used will be one in which only the teeth and implants involved in the research will not be cleaned while the entire oral cavity of

the study participant will be cleaned. To this end, the participants will be molded and will receive an acetate plate that will cover the teeth involved in the research, protecting them from the toothbrush. Participants selected for the study must sign the Informed Consent Form (ICF) after verbal and written explanation of the project. Subjects will receive oral hygiene instruction and biweekly professional prophylaxis within 30 days prior to implant placement. The implants will be installed in time -60 days. After 60 days, that is, at time 0, the healers will be installed in the two implants, one being a conventional healer and the other with an electromagnetic pulse. At the same time, everyone will receive acetate plates to protect the selected areas (tooth and implant) during brushing, to be used for 21 days. Periodontal clinical examination, microbiological (supragingival and subgingival biofilm) and immunological (crevicular fluid) collections will be obtained at times 0, 3, 7, 14, 21, 42 and 60 days. The choice between the implant that will receive the conventional healer or the one with electromagnetic pulse will be made randomly, by means of a randomization table. At the end of the study, they will again receive professional prophylaxis and control of oral biofilm after the experiment. Figure 1 shows the study design.

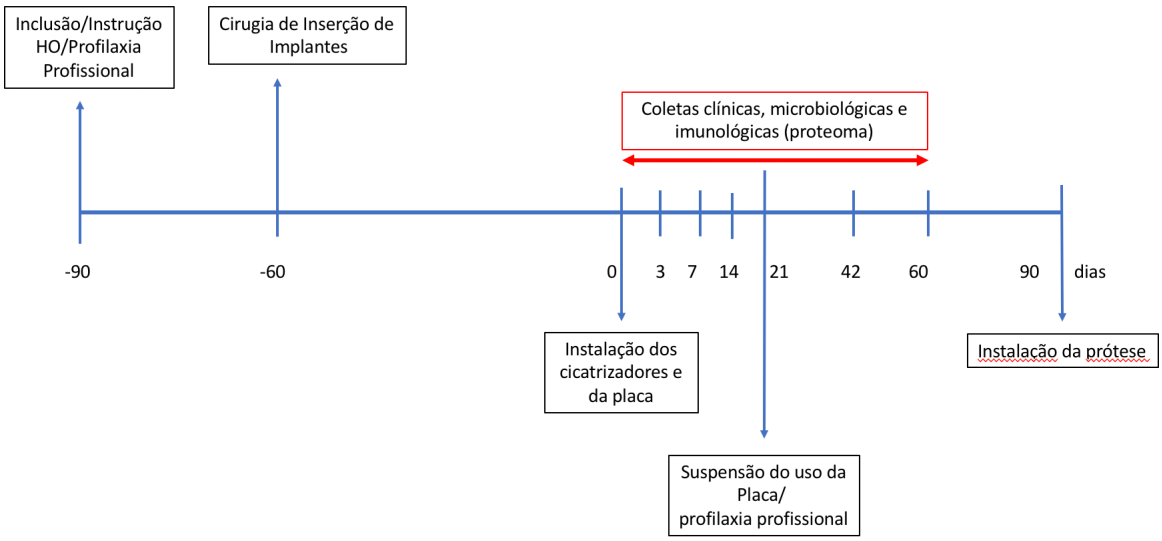

Figure 1. Study design.

3.2. Sample selection

This clinical study will be submitted for approval by the Ethics

Committee of the University of Guarulhos (CEP-UnG). The 40 individuals will be recruited and evaluated at the Implantology clinic of the University of Guarulhos and must need 2 implants adjacent to teeth, in the same hemiarch. This sample was obtained according to the power test so that the study had a sampling power of 85%, and was therefore subject to subsequent publication in an impactful journal.

3.3. Sample inclusion criteria

The sample will include 40 systemically healthy individuals, over 18 years of age, who have at least 2 contiguous edentulous spaces; more than 15 dental elements in the oral cavity; have not undergone periodontal treatment for at least 6 months prior to the beginning of the study; agree to participate in the study and agree to sign the ICF.

3.4. Sample exclusion criteria

Individuals with extensive dental restorations; with periodontal disease stages II to IV; with peri-implantitis and/or mucositis (Shibli et al. 2015); with clinical aspects of occlusal trauma on implant-supported restorations; individuals requiring previous grafting for implant insertion; diabetics; smokers or former smokers; pregnant women; breastfeeding women; use of antibiotics or oral antiseptics in the last 6 months and those who do not agree to sign the ICF.

3.5. Preparation of participants

3.5.1. – Insertion of osseointegrated implants

The selected subjects, after hygiene instruction, will be submitted to surgery to insert the osseointegrated implants according to surgical-prosthetic planning. The implants will be inserted (Time -60 days) according to the surgical technique recommended by the manufacturer, respecting anatomy and three-dimensional positioning. After insertion of the implants, participants will receive analgesics and postoperative care during the 90 days of healing. At the end of the preparation period, individuals must present clinical parameters compatible with those established for the health condition. In the preparation stage, they will also receive instructions on how to clean the oral cavity during the biofilm formation

period.

After 60 days (Time 0), the implants will receive conventional or electromagnetic pulse healers. The choice between the implant that will receive the conventional healer or the one with electromagnetic pulse will be made randomly, by means of a randomization table. They will also be molded so that individualized acetate plates, which will be used during the period of bacterial biofilm formation (21 days), can be made. Everyone will receive reinforcement instructions on how they should clean the oral cavity during the period of biofilm formation.

3.5.2. Induction of gingivitis and experimental mucositis

Subjects will be instructed to use an individualized acetate plate, for a period of 21 days. The acetate plate will cover the selected sites (both the tooth and the selected implants), preventing the accumulated biofilm from being accidentally disorganized during the cleaning of the oral cavity. This plate will not hinder or prevent the other teeth and implants in the oral cavity from being cleaned.

During the period of induction of bacterial biofilm accumulation, individuals (Hallström et al., 2012; Arweiler et al., 2010 Pereira et al., 2010) will be evaluated and monitored weekly, in order to ensure the necessary conditions for the feasibility of the research and its integrity.

After the final collection of samples, individuals will undergo periodontal and peri-implant therapies necessary to reestablish the health conditions of the oral cavity. Any complications caused during the induction period will be remedied at this stage.

3.5.3. Clinical analysis

Initial and final periapical radiographs will be performed to analyze bone remodeling around the teeth and implants.

The selected sites will also have the visible plaque index (presence or absence), the gingival bleeding index (presence or absence of gingival bleeding after removal of visible plaque), the probing depth (distance between the gingival margin and the bottom of the groove/pocket in millimeters) and the presence of bleeding on probing (presence or absence of bleeding after probing the groove/pocket), and evaluated at the beginning and end of the induction period.

These data will be obtained by an examiner trained and calibrated to perform the clinical examinations (Shibli et al. 2008).

3.6. Obtaining samples

Samples of bacterial biofilm and crevicular gingival fluid (CGF) will be collected at baseline (day 0), and at 3, 7, 14, 21, 42 and 60 days on the mesial surface of the selected tooth and implant sites.

3.6.1. Collections of bacterial biofilm samples

Supra- and subgingival biofilm samples will be taken from the mesial sulci/pouches with Gracey Curettes, minifive type 11-12 sterile. They will be positioned in the most apical portion of the sites and in a single scraping movement in the apic-coronal direction they will be collected. Samples will be deposited in 1.5 ml polypropylene tubes containing 100µl of TE RNAse-free solution (10mM Tris-HCl, 0.1mM EDTA, pH 7.6). The collected material will be stored at a temperature of -80^{o}C, for a maximum of 48 hours, when the total nucleic acids (ANT) will be extracted.

3.6.2. *Microbiological monitoring*

3.6.2.1. Extraction of Total Nucleic Acids

The ANT of the samples will be extracted using the Epicentre Masterpure DNA & RNA Purification kit (Epicentre, Madison, WI, USA) according to the manufacturer's instructions. Initially, the surfaces of the benches and apparatus will be treated with ribonuclease inhibitors (RNaseAWAY® – Invitrogen, San Diego, CA, USA) to minimize the risk of nucleic acid degradation. The tube containing the sample will be taken out of the freezer at -80^{o}C and kept on ice for 10 minutes for thawing. The collection tube will be shaken in the vortex for 1 minute and the solution will be transferred to a new tube. For the lysis of the collected material, 1.0µL of proteinase K solution (50µg/µL) will be added to the 100µL of the sample's TE solution, and then it will be incubated at 65^{o}C for 15 minutes. The samples will be kept on ice for 5 minutes, 175µL MCP reagent will be added for protein precipitation and centrifugation for 10 minutes at 12°C. The supernatant will be transferred to a new tube where 500 µL of isopropanol will be added, the tubes will be stirred for 2 minutes and will be subjected to new centrifugation for 10 minutes at 4°C. The isopropanol will be discarded and the pellets will be washed twice with 70% ethanol. The pellets will be dried for 10 minutes and then resuspended in 25 µL TE and stored at -80^{o}C.

3.6.2.2 Preparation of the probes *16S rRNA*

Oligonucleotide probes for species not yet cultured should have about 18-22 nucleotides and minimal secondary structures in the conserved region of the *16S* rRNA gene for prokaryotic organisms. The sequence of the probes was kindly provided by Prof. Bruce Paster and has recently been published (PREZA et al., 2009). All probes will be checked in the *Ribosomal Database Project* (http://rdp.cme.msu.edu/probematch/search.jsp) system and subsequently synthesized (Invitrogen, São Paulo, SP, Brazil). The controls will consist of sequences complementary to the probes. The probes will be labeled with digoxigenin using a specific oligonucleotide kit, according to the manufacturer's specifications at the final concentration of 4.5ρM/µL (DIG Oligonucleotide 3´- End labeling kit, 2nd Generation – Roche, Indianapolis, IN, USA). In general, 100 M of the probe will be added to a sterile 200µL tube ρfor a final volume of 10µL. Subsequently, 4µL of a buffer solution, 4µL of a 5mM solution of $CoCL_2$, 1µL of a 0.05mM solution of DIG-ddUTP and 1µL of the transferase enzyme (20U/µL) will be added. This solution will be incubated at 37oC for 30 minutes. Subsequently, the solution will be withdrawn and placed on ice for 5 minutes and 2µL of a 0.2mM EDTA solution (pH 8.0) will be added to stop the reaction.

3.6.2.3. Collection of crevicular fluid samples ***Liquid chromatography coupled to Tandem mass spectrometry LC-MS/MS (Proteome)***

Gingival and peri-implant fluid samples will be collected at the same sites and at the same time as the sites selected for the bacterial sample (see item 3.8.1 ***Collection of bacterial biofilm samples).*** The selected periodontal and peri-implant sites will be isolated with cotton rollers and dried with an air jet so that there is an influx of gingival fluid. The samples will be collected using standardized sterile strips of paper (PerioPaper, Oraflow, Smithtown, NY), gently introduced into the gingival and peri-implant sulcus, until they meet resistance, and held in position for 30 seconds, and then transferred to a 1.5mL dry plastic tube. The samples will then be immediately frozen at -80ºC until they are processed.

Liquid chromatography coupled to tandem mass spectrometry will be performed in periods o, 3, 7, 14, 21, 42 and 60 days. The elution of the adsorbed protein will be performed according to a previous protocol (Siqueira and Oppenheim 2009). The sample set will be vortex-stirred for 1 min, sonicated at 7W (4$^{oC)}$ for 5 min, vortex-stirred for 1 min, and stored at -80$^{oC.}$ Then, the samples will be lyophilized and resuspended in 50 ml of urea (8 M). The Bradford method will use samples for total protein quantification, using bovine serum albumin (BSA) as standard. The proteins will be reduced, alkylated and digested with trypsin (1:50 w w-1), and the extracted proteins will be subjected to LC-MS/MS. The samples will be dried in a vacuum concentrator and reconstituted in 22.5ul of 0.1% formic acid. A 4.5ul aliquot containing 2 mg peptides will be analyzed in an LTQ Orbitrap Velos mass spectrometer connected to the EASY-nLC system through a Proxeon ion nanoelectrospray source. The peptides will be separated by a gradient of 2-90% acetonitrile in 0.1% formic acid in an analytical PicoFrit column (20 cm ID75 lm, particle size 5 lm) at a flow rate of 300 ml min-1 over 27 min. The voltage of the nanoelectrospray will be adjusted to 2.2 kV, and the temperature of the source will be 275$^{oC.}$ All instrument methods will be configured in data-dependent acquisition mode. After accumulation, full-scan MS spectra (m/z 300-1,600) will be acquired on the Orbitrap analyzer for a target value of 1 106. The resolution in Orbitrap will be set to r.60,000 and the 20 most intense peptide ions with charge states will be isolated sequentially to a target value of 5,000 and fragmented in the linear ion trap by low energy CID (normalized collision energy of 35%). The signal threshold to trigger an MS/MS event will be set to 1,000 counts. A dynamic delete will be enabled with a deletion size list of 500, a deletion duration of 60 s, and a retry count of 1. A q.0.25 activation and a 10ms activation time will be used. Each sample will be subjected to three readings. Acquired peptide sequences will be identified using MaxQuant software (v.1.3.0.3) and the Uniprot Human Protein Database. With the Perseus v.1.5 application software, the list of identified peptides will be filtered by a minimum localization probability of 0.75, and reverse entries and contaminants will be excluded from further analysis.

The influence of the gingival tissue of the implants and the comparison of the periodontal and peri-implant tissues will be analyzed and compared with the proteomic profile to identify the unique/exclusive proteins adsorbed in each group. A Venn diagram will be constructed to show the differences between the groups. The

Uniprot database will also verify the molecular function and biological process in which the identified proteins are involved. Each group will be characterized according to the ten molecular procedures and biological processes with the most proteins. Heatmaps will be used to show the intensity of LFQ (protein intensity/expression) for each protein identified according to the groups.

4. ETHICAL CONSIDERATIONS

4.1. Description of methods that affect the research subjects

In this study, the impact of electromagnetic pulse and the difference between teeth and implants for oral bacterial biofilm accumulation will be evaluated. Clinical examinations will be performed on all implants and teeth evaluated, as well as collection of submucosal biofilm from the tooth and implant test sites.

4.1.1 – **Gengivite e mucosite experimental**

Experimental gingivitis has been an efficient, well-documented, and accepted tool in clinical research with different objectives in the last 20 years, as shown in Table 1. There are no reports of any local and/or systemic problems associated with its realization. It is known, however, that a small local and reversible inflammation, compatible with gingivitis (periodontal pathology found in a large part of the world population), can be observed at the end of the biofilm formation period, without permanent damage to the periodontal and peri-implant health of the volunteers and reversed immediately after the resumption of oral hygiene procedures.

Table 1 - Research that has used experimental gingivitis as an analysis methodology in the last 20 years.

Authors	**Location, year.**	**Objective of the Study**
McKenna et al.	United Kingdom, 2013	To evaluate the effect of subgingival ozone and/or hydrogen peroxide on the development of periimplant mucositis.
Salvi et al.	Switzerland, 2011	To evaluate the clinical, microbiological, and immunological factors involved in the pathogenesis of gingival inflammation and experimental perrimplantation and to compare the sequence of resolution of inflammation around the tooth and implants after restitution of mechanical biofilm removal.

Zitzmann et al.	Switzerland, 2001	To analyze the expression of endothelial cell adhesion in the alveolar mucosa, gingiva, and periimplant mucosa in humans.
Tawse-Smith et al.	Nova Zealand, 2001	To compare the clinical efficacy of manual toothbrush (Oral-B Squish-grip) and electric toothbrush (Braun Oral-B Plaque Remover 3-D) in a group of elderly patients with implant-supported mandibular overdentures.
Zitzmann et al.	Switzerland, 2000	To evaluate soft tissue reactions to biofilm accumulation in teeth and implants.
Pontoriero et al.	Switzerland, 1994.	To compare clinical and microbiological parameters during the development of experimental gingivitis and mucositis, 6 months after implant installation in humans.
Branco et al.	Brazil, 2012.	To assess supra and subgingival plaque formation in the dentogingival area using the experimental model of gingivitis and a scoring plate system that considers the presence of a biofilm-free zone in smokers and non-smokers.
Keukenmeester et al.	Netherlands, 2011	To test the effect of chewing gum with xylitol or maltitol compared to using chewing gum alone as a negative control, on plaque development and gingivitis.
Hallström et al.	Sweden, 2012.	To evaluate whether daily oral administration of probiotic bacteria can influence the inflammatory response and supragingival plaque composition, in an experimental model of gingivitis.
Slawik et al.	Germany, 2011.	To determine the effects of a probiotic consumed for 28 days, with regard to the expression of inflammatory clinical parameters in the gingival tissue during the various stages of biofilm-induced gingivitis.
Zanatta et al.	Brazil, 2011.	To compare the effects of 0.12% chlorhexidine gluconate on staining and stone formation on surfaces with and without biofilm.
Arweiler et al.	Germany, 2010.	To evaluate the clinical effect of a new formulation of toothpaste with S. *baicalensis extract* (0.5%).
Pereira et al.	Brazil, 2010.	To evaluate the antiplaque and antigingivitis effect of Copaifera spp (Cp).
Rodrigues et al.	Brazil, 2008.	To evaluate *in vivo* the anti-plaque and anti-gingivitis effect of a gel containing *Lippia sidoides*.
Baumgartner et al.	Switzerland, 2009.	To evaluate oral microbiota and clinical data in individuals without access to traditional oral hygiene methods and who ate a diet compatible with that available in the Stone Age.
Versteeg et al.	Netherlands, 2007.	To test the potential of a tapered filament toothbrush (TFTB), compared to a control toothbrush, with regard to gum abrasion and to evaluate plaque removal and gum condition improvement before treatment begins.
Konradsson e van Dijken.	Sweden, 2005.	To evaluate the hypothesis that there are higher levels of IL-1 adjacent to the composite resin, compared to calcium aluminate cement (CAC) and enamel.

Sekino et al.	Sweden, 2005.	To assess the effect of systemic administration of ibuprofen on gingivitis and plaque formation.
Eberhard et al.	Germany, 2004.	To evaluate the antimicrobial and anti-inflammatory capabilities of 45S5 bioactive glass, using the human model of experimental gingivitis.
Sekino et al.	Sweden, 2004.	To evaluate the effect of a therapeutic regimen combining the administration of a chlorhexidine gel and mouthwash on the recolonization of various microbiological species in biofilm and saliva over a 4-day period of induced plaque formation.
Trombelli et al.	Italy, 2004.	Characterize the clinical behavior of gingivitis in response to a regimen of cleaning the teeth with amine and stannous fluoride.
Putt et al.	United States, 2001.	To compare the clinical effectiveness of three electric toothbrushes in reducing plaque and improving gum condition.
Wright et al.	England, 2003.	To investigate changes in TGF-p1 levels in gingival fluid, serum, and plasma during a 21-day period of experimental gingivitis.
Claydon et al.	England, 2001.	Determine if PVP can be added to chlorhexidine-based mouthwashes while maintaining their effectiveness and reducing their coloration.
Weidlich et al.	Brazil; 2001.	To analyze the pattern of supragingival plaque formation in the dentogingival region over a period of 4 days and to clinically evaluate the gingival inflammatory response in this period.
Brägger et al.	Switzerland, 1998.	To evaluate *in vivo* the method errors when using digital subtraction CADIA images in periodntally healthy patients, and to determine a threshold that can be used to exclude false positives of changes in density.

These studies are divided as to the methodology used to induce gingivitis/mucositis. Some authors have completely interrupted ***the mechanical means of oral hygiene*** (Branco et al., 2012; Keukenmeester et al., 2011; Li et al., 2012; Kumar et al., 2012; Slawik et al., 2011; Zanatta et al., Baumgartner et al., 2009; Versteeg et al., 2007; Lorenz et al., 2006; Versteeg et al., 2005; Rosema et al., 2005; Sekino, Ramberg; 2005; Konradsson, van Dijken, 2005; Eberhard et al., 2004; Sekino et al., 2004; Tsalikis et al., 2002; Putt et al., 2001; Nogueira-Filho et al., 2002; Rüdiger et al., 2002; Zitzmann et al., 2001; Lang et al., 2002; Quirynen et al., 2001; Claydon et al., 2001; Weidlich, Lopes de Souza, Oppermann, 2001; Weidlich, Lopes de Souza, Oppermann, 2001; Gonzáles et al., 2011; Zitzmann et al., 2000; Nogueira-Filho, Toledo, Cury, 2000; Gründemann et al., 2000; Fransson et al., 1999; van Dijken, Sjöström, 1998; Deinzer et al., 1999; Brägger et al., 1998; Campan, Planchand, Duran, 1997; Johnson et al., 1997; Fransson, Berglund, Lindhe, 1996; Ramberg et al., 1996)

while others ***used acrylic plates that prevented the accidental disorganization of the biofilm on the teeth analyzed during the cleaning of the elements not included in the research and thus allowed the individual not to remain without performing oral hygiene*** (Hallström et al., 2012; Arweiler et al., 2010 Pereira et al., 2010; Rodrigues et al., 2008; Gleissner et al., 2006; Salgado et al., 2006; Witt et al., 2005; Shearer et al., 2004; Rosin et al., 2004; Trombelli et al., 2004; Wright et al., 2003; Yates et al., 2003; Eberhard et al., 2002; Kobayashi et al., 1998; Daly, Highfield, 1996).

The present research will use acetate plates made individually for each study participant and for each element (tooth and implant) included in the study, thus allowing the hygiene of the oral cavity to be maintained.

4.1.2. Risk description with severity assessment

Study participants will not have systemic diseases that interfere with the performance of routine clinical procedures as well as the surgical procedure of implant insertion.

Risks with the surgical procedure: All procedures, in addition to using sterile and disposable instruments to prevent contamination, will be performed by specialists trained for such a procedure. Possible risks are those inherent to common dental care during the use of local anesthesia. These are rare adverse events, such as hypersensitivity reaction and paresthesia. However, dentists and dental clinics are prepared to provide the necessary care. In the case of hypersensitivity, administration of immediate medication (antihistamine) and in the case of paresthesia, administration of medication (vitamin B) or application of laser, when necessary. It is important to note that dental clinics linked to educational institutions are frequently inspected by the Sanitary Surveillance of the municipalities to receive permission to operate and one of the requirements is the demonstration of capacity to respond to emergency situations. Radiographic shots will be performed by properly calibrated devices and with a lead apron for patient protection. Finally, the main direct benefit that the study participant will receive will be implant treatment and implant-supported restoration, as well as biofilm control procedures and oral hygiene instructions.

Risks with data collection and absence of oral hygiene: Participation in this study involves minimal risk. Non-invasive, painless and quick techniques will be used, both for the clinical examination and for the collection of the materials to be evaluated. The

risk and discomfort related to periodontal examination is minimal. The only way to control such discomfort, in individuals with lower tolerance, is to perform the exam more slowly. In the last case, the individual may decide not to participate in the study. The researchers involved in this study will contact the participant to monitor periodontal indices once a week throughout the study.

In addition, the study participant will receive verbally and also in writing the Informed Consent Form (ICF; Appendix I: Informed Consent Form) the recommendation to contact the principal investigator in case of the occurrence of any undesirable effect. If necessary, the researcher will make the decision to resume oral brushing in the 3 sites evaluated (2 implants and 1 tooth) and in this case, the participant will be immediately removed from the study, without prejudice to the basic treatment of hygiene or prosthetic restorations of the implants (receiving the crowns/teeth), that is, they will continue to receive this treatment free of charge. Participants will be followed up until the end stipulated by the research. After this period, participants will be instructed to look for the Clinical Studies Center of the Dentistry course at Univeritas-UNG University or specialized centers in Implantology, if they need any assistance. This return protocol will be the same used for other patients in specialization courses.

4.2. Risk protection and confidentiality measures

Clinical investigators will use the biosafety precautions inherent in common clinical approaches in dentistry to avoid cross-infection, using physical barriers (masks, caps, gloves, goggles, *face-shield*) and sterilization of material in an autoclave.

The surgical procedure involves risks related to local anesthesia that will be avoided through the selection of the anesthetic base and the most appropriate constrictor vessel for the participant. This choice is based on the information he gives us in the anamnesis and in his preoperative exams. Another risk, uncommon, but associated with this type of surgery is the occurrence of hemorrhages. If it cannot be avoided, during the procedure, surgical techniques (local compression and clamping and/or suturing of the bleeding site) and specific medications (local hemostatic) will be used to contain it. If it happens in a postoperative moment, a rare situation, the team of researchers will give all the necessary support to the participant, this support includes clinical procedures to control bleeding, prescription of medications or even referral for medical/hospital care, if necessary.

Postoperative infections may also occur. In this case, the region will be properly sanitized and systemic antibiotics and individualized sites appropriate to each case will be prescribed. If these antibiotics, or even the anti-inflammatory drugs and analgesics prescribed soon after surgery cause nausea, headache, gastrointestinal upset or any other type of discomfort for the participant, the medication can be replaced immediately after the complaint and the prescription of gastric protectors (Omeprazole) will be considered along with diet counseling, after careful evaluation by the researchers, without prejudice to the treatment. Edema (swelling) and bruising (purple spots) at the surgical site are common and expected. These clinical signs are controlled with the medication that will be prescribed on the day of surgery and tend to disappear between the 7th and 10th postoperative day, a period that coincides with one of the returns to which the participant must attend to evaluate the postoperative conditions.

The privacy of those who participate in the survey is very important. All information collected in this study will be confidential. If the results of this study are published, there will be no information or data that can identify the participants. Clinical records that may identify participants will be kept confidential, as required by law. The participant will not be identified by RG or CPF number, address, telephone number or any other data that directly identifies him/her in the study records that are revealed outside the Faculty of Dentistry of Univeritas-UNG University. Data that is recorded and revealed outside the study will be assigned a unique numerical code that will not identify the participant. The identification of the code will be kept at the Faculty of Dentistry of Univeritas-UNG University.

4.3. Forecast of reimbursement of expenses

All expenses related to the project will be borne by the person responsible for the research. The costs concern: the study drugs, including anti-inflammatories, antihistamines, antibiotics and analgesics; oral hygiene products; costs related to the insertion of implants and prostheses, and transportation and food for the visits provided for in the project. Participants will not receive any payment for participating in this study.

4.4. Critical risk and benefit analysis

As previously described, the surgical procedure for implant insertion as well as the clinical evaluation procedures that participants will undergo may generate temporary discomfort, which will be avoided through the use of anesthetics and analgesics. The main benefit to the research participants will be the instruction in oral hygiene and the restoration of edentulous spaces through implants. All volunteers will also benefit from periodontal condition maintenance therapy that will be performed monthly until the end of the study. Finally, volunteers who need it will receive referral for treatment in other dental specialties at the UNG undergraduate clinic.

4.5. Criteria for suspending or terminating the survey

The survey will be suspended for participants who are unable to attend appointments or who no longer wish to participate in the study. Other eventualities that may interfere with the inclusion criteria and the integrity and well-being of the participants may be considered grounds for suspending the research (e.g., medical impediment to undergo the research procedures). Whatever the reason that suspends or terminates the research for a given participant, this will not be detrimental to his or her rehabilitative treatment. In addition, the volunteer will not have any loss in referrals for treatment in other dental specialties at Univeritas-UNG University.

4.6. Place of realization of the various stages and infrastructure

The research will be carried out entirely at Univeritas-UNG University. The stages of care for the participants of the research will be carried out at the Center for Clinical Studies in Dentistry, which has all the necessary infrastructure (dental equipment and instruments). The laboratory stages will be carried out in the Dental Research Laboratory II, which already has the equipment and methodologies that will be carried out in this research.

5. BUDGET

Budget proposal: This study is sponsored by the partner companies *Plenum (Brazil)* and MagDent (Israel) in addition to the CNPq grant that will allow the acquisition of inputs after approval by the Research Ethics Committee of Univeritas-UNG University.

COST TABLE

Area of analysis	VALUE (reais)
Clinical, Surgical (implants and healers) and prostheses (funded by Plenum Bioengenharia, Jundiaí, SP, Brazil)	R$ 90,000.00
Microbiological (funded by Magdent, Israel)	R$ 250,000.00
Proteome (funded by MagDent, Israel)	R$ 250,000.00
Medicines (including anti-inflammatory and antihistamine drugs, analgesics) and acetate plates (funded by CNPq Project 311368/2019-0)	R$ 2.0000,00
Transportation (10 visits x 40 patients x R$ 10.00 + 15% of the amount allocated to possible complications + Light food/snack R$ 10.00) (funded by the CNPq Project 311368/2019-0)	R$ 8.600,00
Total Value	**R$ 600,600.00**

6. SCHEDULE

STEP IDENTIFICATION	START (dd/mm/yyyy)	TERM (dd/mm/yyyy)
Patient selection	01/03/2025	01/05/2025
Clinical evaluation/implants	01/03/2025	01/06/2025
Treatment	30/05/2025	20/08/2025
Maintenance	05/08/2025	05/10/2025
Microbiome/Proteome	05/08/2025	05/02/2026
Analysis of the results	10/10/2025	05/04/2026
Preparation of publications	10/10/2025	05/05/2026
Final report	10/10/2025	10/05/2026

7. FORM OF ANALYSIS OF THE RESULTS

The results will be evaluated as to their normality using the D'Agostino test and, then, parametric or non-parametric tests will be applied to compare the

means/medians obtained between the gingivitis and mucositis groups in the different periods of the study. If the data do not fit the normality curve, the data will be evaluated using the Mann-Whitney test for independent variables and the Wilcoxon test, in the case of dependent variables. If the data follow normality, the unpaired t-test (non-dependent samples) and paired t-test (dependent samples) will be used to test the interaction between the study groups. Linear regressions and Pearson's correlation test will also be performed to verify the association between the study variables. The significance level will be 95% (α=0.05) for all tests used.

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