RSBO

ISSN:
Electronic version: 1984-5685
RSBO. 2025 Jul-Dec;22(2):169-78

Original Research Article

Inflamatory response of screw joint sealing agents used at the implant-abutment interface

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Received for publication: December 7, 2023. Accepted for publication: February 6, 2025.

Keywords:
biocompatible materials; methacrylates; dental prosthesis, implant-supported.

Abstract

Introduction: Adequate contact and passive fit between the components that form the implant-abutment interface promote greater screw joint stability in implant-supported prostheses. The maintenance of the preload is an indispensable mechanical factor for the stability of the complex. **Objective:** This study evaluated the rat subcutaneous tissue response to a commercial methacrylate-based sealing agent to be employed in the sealing of the implant-abutment interface of screw joints in implant-supported prostheses. **Material and methods:** Twenty-four male Wistar rats were submitted to the surgical procedure for insertion of polyethylene tubes into the subcutaneous tissue of the anterior region of the dorsum. On the right side the tube was implanted filled with the sealer and on the left side the tube was empty. After the experimental periods of 7, 15 and 30 days postoperatively, the animals were euthanized and the samples were dissected. The specimens were post-fixed, processed in a conventional manner and the histological sections were submitted to hematoxylin-eosin staining (HE) for histopathological analysis or to immunohistochemical processing to detect the main cytokines with proinflammatory activity: tumor necrosis factor-alpha (TNFα) and interleukin-1 beta (IL-1β) via immunolabeling density. **Results:** The

magnitude of the local inflammatory response was similar between the control and the test site and underwent a gradual reduction over the experimental periods. There was no statistically significant difference in TNFα and IL-1β immunolabeling density between the control and the test site, except at 7 days postoperatively where the TNFα immunolabeling was greater at the test site when compared to the control site. There was a gradual reduction in the immunolabeling for both TNFα and IL-1β over the experimental periods. **Conclusion:** The m ethacrylate-based sealing agent showed a reduction in the inflammatory response throughout the experimental periods, which indicates that it may be employed as a sealing agent at the implant-abutment interface without resulting in undesirable tissue response in the adjacent tissues.

Introduction

Adequate contact and passive fit between the components that form the implant-abutment interface promote greater screw joint stability in implant-supported prostheses by resisting vibrations and the action of repeated external cyclical loads generated mainly by masticatory forces [12, 20]. This process of bonding the screw joint is generated after a torque is applied to the retaining screw, creating an internal tension (the preload) that keeps this system stable [25]. The constant maintenance of the preload is an indispensable mechanical factor for the stability of the complex [7]. When external loads exceed the value of the preload, the stability of the fitting between the prosthetic components reaches a critical level, leading to screw loosening, which leads to instability of the prosthetic component and, in more extreme cases, to the fracture of the retaining screw [3, 37].

Furthermore, the loss of the sealing and interlocking between the parts enhances micromovements and microleakage, promoting the flow of oral fluids at this implant-abutment interface, thus creating a microbial reservoir that generates biological damage to tissues (e.g., mucositis and/or periimplantitis), and in severe cases, may result in fracture or even implant loss [4, 22, 23, 31].

To test the effectiveness of materials that can seal this interface against microorganisms, the efficacy of a commercial sealing agent at the abutment/implant interface against microleakage of single and dual-species biofilms of *Candida albicans* and *Enterococcus faecalis* into external hexagon and Morse taper prosthetic connections was evaluated. Their favorable results show that the presence of the sealing agent reduces or eliminates the infiltration of biofilms into the implants [2].

It is interesting to use a material that contributes to the stabilization of the screwed joint in implant-supported prostheses, thus avoiding the consequences of misfitting in this system. It was showed that a sealing agent is highly effective in maintaining the preload on the interface of platform matching systems and also in platform-switched interfaces even at low torque conditions. It is important to emphasize that the material evaluated retained its reversibility, which is a fundamental mechanical property of screw-retained implant-supported prostheses [12, 33].

In general, studies on the biological behavior of sealing agents are scarce, the biocompatibility of these materials is not widely known and whether these materials will elicit toxic reactions, such as irritation, inflammatory or allergic responses, or whether they are mutagenic or carcinogenic [9]. The animal model is well accepted in the scientific community for the analysis of the inflammatory response of tissues to biomaterials [6].

Considering the need to better understand the biological responses of this methacrylate-based sealing agent intended for usage in the implant-abutment interface of screw joints of implant-supported prostheses, the aim of this study was to evaluate the rat subcutaneous tissue response after the insertion of a sealing agent specimen.

Material and methods

Animals

In the present study twenty-four (2-month-old) male rats (Wistar – *Rattus norvegicus),* weighing between 200-250 g were used. The animals were obtained and maintained throughout the experimental period in the dental school under the following conditions: 12h of light and 12h of dark at 22 ± 2ºC, ventilation/exhaustion of 20 cycles per hour and relative humidity of 55 ± 5%, maintained in plastic cages (41 × 34 × 18 cm) with four animals per cage, where they had free access to water and food. All appropriate measures to minimize the number of animals used and to avoid their suffering were taken. The experimental protocol followed the guidelines established by "Guide for the Care and Use of Laboratory Animals" and was approved by the Ethics Committee on Animal Use (Protocol n. 00173-2018).

Anesthesia

Surgical procedures (polyethylene tubes insertion and euthanasia) were performed under general anesthesia through intramuscular injection with ketamine hydrochloride (80 mg/kg, Francotar®, Virbac, SP, Brazil) and xylazine hydrochloride (10 mg/kg, Rompum®, Bayer, RS, Brazil).

Control and test sample preparation

Polyethylene tubes (Mac Med, São Paulo, Brazil) with a 1.5-mm external diameter, and 10.0-mm length were used as specimens [8, 17]. The tubes were previously sterilized in ethylene oxide. The control group consisted of empty polyethylene tubes and the test group consisted of polyethylene tubes filled with the sealing agent (HS Bond, Araçatuba, Brazil). No other material was placed at the ends of the tubes, thus there was direct contact between the material and the subcutaneous tissue.

Surgical procedure for samples implantation into the subcutaneous tissue

Tricotomy was performed on the back of each rat, after antisepsis with 1% polyvinylpyrrolidone (Riodente, Rioquímica, São José do Rio Preto, Brazil) a 2,0 cm incision was made in a head–tail orientation and tissue divulsion with blunt tip scissors. The samples were introduced into the subcutaneous tissue with a college tweezer. Then, the tissues were repositioned, sutured with interrupted stitches using 5-0 nylon thread (Mononylon®, Etchicon®, Johnson & Johnson®, São José dos Campos – SP, Brazil) and a new antisepsis of the region. Each animal received two specimens: on the test site (right side) the polyethylene tube was filled with the sealing agent and on the control site (left side) the polyethylene tube was empty; the tubes were positioned at an adequate distance so that there was no interference between the groups. Euthanasia was carried out after 7, 15 or 30 days postoperatively.

Euthanasia and tissue sample collection

The animals were deeply anesthetized and submitted to transcardiac perfusion with with 0.9% sodium chloride added with 0.1% heparin (100 ml), followed by fixative solution (800 ml) of 4% formaldehyde (Sigma, Saint Louis, MO, USA) in phosphate buffered saline (PBS – Sigma, St Louis, MO, USA), 0.1 M, 4ºC, pH 7.4. The samples containing the tubes and adjacent tissues were carefully dissected and submitted to post-fixation in the same fixative solution for 24 hours.

Histological processing of tissue samples

The samples were embedded in paraffin using conventional histological processing methods. Histological sections (4 μm thick) following the long axis of the polyethylene tube were collected. The histological sections of the central portion of the sample were then mounted on silanized glass coverslips.

For histopathological analysis the histological sections were submitted to hematoxylin-eosin (HE) staining.

For immunohistochemical analysis, the histological sections were divided into two batches and submitted to indirect immunoperoxidase technique for detection of tumor necrosis factor (TNF)α and interleukin (IL)- 1β. The histological sections were cleared in xylene and hydrated in a graded ethanol series (100º–100º–100º–95º–70º GL). Antigenic recovery was performed by immersing the histological sections in citrate buffer, pH 6 (Spring Bioscience®, Pleasanton, CA, EUA), and pressure chamber (Decloaking Chamber®, Biocare Medical, Concord, CA, EUA) at 95ºC, for 20 min. At the end of each step of the immunohistochemical reaction, the histological sections were washed with phosphate buffered saline (PBS) 0,1 M, pH 7,4 and blocking of endogenous peroxidase and non-specific binding sites were done using 3% hydrogen

peroxide for 1 hour and 1% bovine serum albumin for 12 hours, respectively. Each batch was incubated with one of the following primary antibodies: goat anti-TNFα (SC-1350, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit anti-IL-1β (SC-7884, Santa Cruz Biotechnology, Santa Cruz, CA, USA). For signal amplification, the histological sections were incubated with universal biotinylated secondary antibodies for 2 hours and streptavidin conjugated with horseradish peroxidase (HRP) for 1 hour (Universal Dako Labeled HRP Streptavidin-Biotin Kit®, Dako Laboratories, CA, USA). The reaction was developed using the chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB chromogen Kit®, Dako Laboratories, CA, USA). Then, the histological sections were dehydrated in ethanol, cleared in xylene and covered in mounting medium (Permount, Fisher Scientific, San Diego, CA, USA) and glass coverslips. As negative control, the specimens were submitted to the same procedures described above, eliminating the use of the primary antibody.

Region of interest (ROI)

For both histopathological and immunohistochemical analyses, the region of interest (ROI) consisted of the connective tissue at the opening of the polypropylene tube, which was tissue that was in direct contact with the sealing agent. All analyzes were performed by a certified histologist, previously calibrated and blinded to treatments.

Histopathological analysis

A semi-quantitative analysis on the ROI was performed in light microscopy evaluating the following parameters: intensity of local inflammatory response; extent of inflammatory infiltrate and; cellularity pattern and extracellular matrix structure pattern of connective tissue. These parameters and their respective scores were based on and adapted from the criteria established in ISO/TR 7405-1997(E) [19] and are shown in table I.

Immunohistochemical analysis of TNFα and IL-1β

For the immunohistochemical analysis of TNF-α and IL-1β, images of the region of interest were captured using a digital camera (AxioCam® Carl Zeiss, Gottingen, Germany) coupled to an optical microscope (AxioLab® Carl Zeiss, Gottingen, Germany) and connected to a microcomputer. With the aid of the Image J program the area corresponding to immunolabeling was delineated using the color threshold tool to obtain the immunolabeling optical density, expressed as random unit percentage of optical density (mean ± standard deviation).

Statistical analysis

For the statistical analysis the GraphPad Prism 6 program was used. The sample size was calculated to ensure 95% statistical test power ($p < 0.05$). For histopathological analyses, nonparametric Kruskal-Wallis analysis of variance test followed by Student-Newnan-Keuls post-test were used. Shapiro-Wilk test was used for analysis of the normal distribution of data. For immunohistochemistry, analysis of variance (Anova) and Tukey's post-test were used. The significance level was set at 5% ($p<0.05$).

Results

The magnitude of the local inflammatory response was similar between the control and the test site and underwent a gradual reduction of the inflammatory infiltrate at both sites over the experimental periods. The cellular pattern and the extracellular matrix structure pattern did not differ between the test site and the control site (figure 1 and table I).

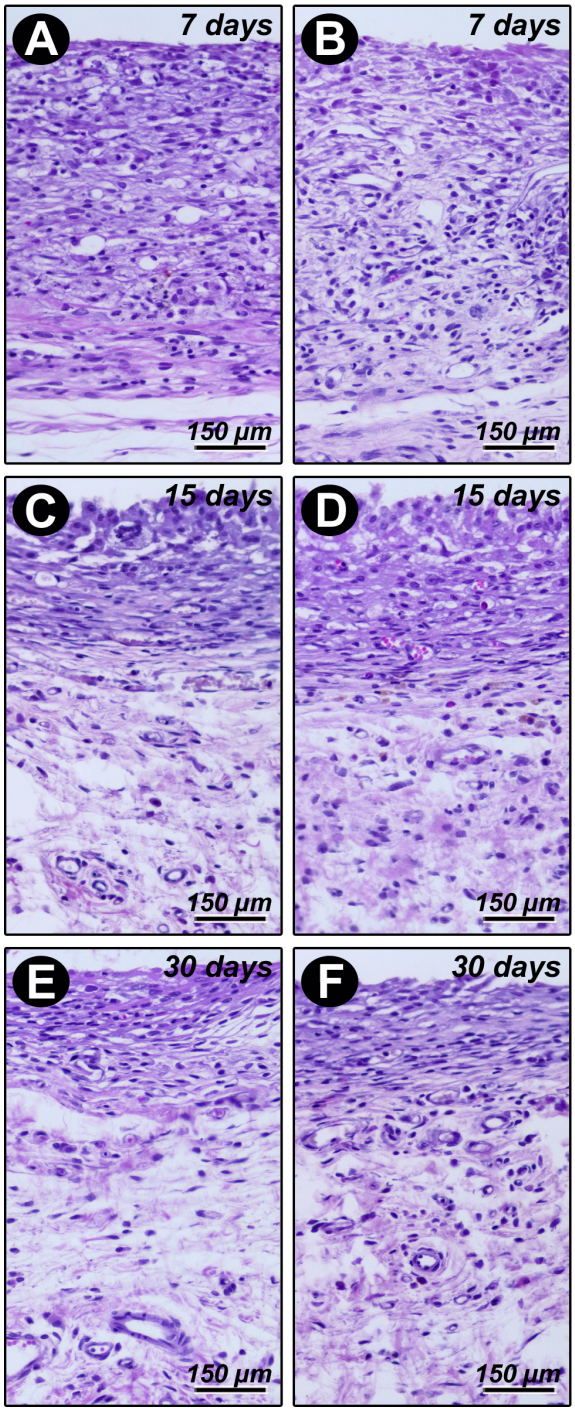

Figure 1 – Histological aspect of the connective tissue at the control site (A, C, E) and test site (B, D, F) at 7 (A-B), 15 (C-D), and 30 (E-F) days pospoperatively

Staining: HE. Scale bars: 150 µm

Table I – Parameters, scores and distribution of specimens according to histopathological analysis in control site and test site at 7, 15 and 30 postoperative days

Histological analysis						
Parameters and respective scores	**Number of specimens**					
	Experimental groups					
	Control site			**Test site**		
	7d	**15d**	**30d**	**7d**	**15d**	**30d**
Intensity of local inflammatory response						
(1) absence of inflammation	-	3	8	-	3	8
(2) mild quantity of inflammatory cells	3	5	-	5	5	-
(3) moderate quantity of inflammatory cells	5	-	-	3	-	-
(4) severe quantity of inflammatory cells	-	-	-	-	-	-
median	**3**	**2†**	**1†**	**2¶**	**2†**	**1†§**
Extent of inflammatory infiltrate						
(1) extending for up to 1/4 of ROI	3	8	8	3	8	8
(2) extending for up to 1/2 of ROI	5	-	-	5	-	-
(3) extending for up to 3/4 of ROI	-	-	-	-	-	-
(4) extending the entire ROI	-	-	-	-	-	-
median	**2**	**1†**	**1†**	**2‡¶**	**1†§**	**1†§**
Cellularity pattern and extracellular matrix structure pattern of connective tissue						
(1) moderate quantity of fibroblasts and large quantity of collagen fibers	-	-	3	-	3	3
(2) moderate quantity of both fibroblasts and collagen fibers	3	8	5	5	5	5
(3) small quantity of both fibroblasts and collagen fibers	5	-	-	3	-	-
(4) severe tissue disorganization with necrosis areas	-	-	-	-	-	-
median	**3**	**2**	**2†**	**2¶**	**2†§**	**2†§**

†: statistically significant difference in relation to the control site at 7 days postoperatively; ‡: statistically significant difference in relation to the control site at 15 days postoperatively; ¶: statistically significant difference in relation to the control site at 30 days postoperatively; §: statistically significant difference in relation to the test site at 7 days postoperatively

There was no statistically significant difference in TNFα immunolabeling optical density between the control and the test site, except at 7 days postoperatively where the TNFα immunolabeling was greater at the test site. There was a gradual reduction in the immunolabeling density for both control and test site over the experimental periods, except between the 15 and 30 days postoperatively in the test group, where there was no statistically significant difference (figure 2).

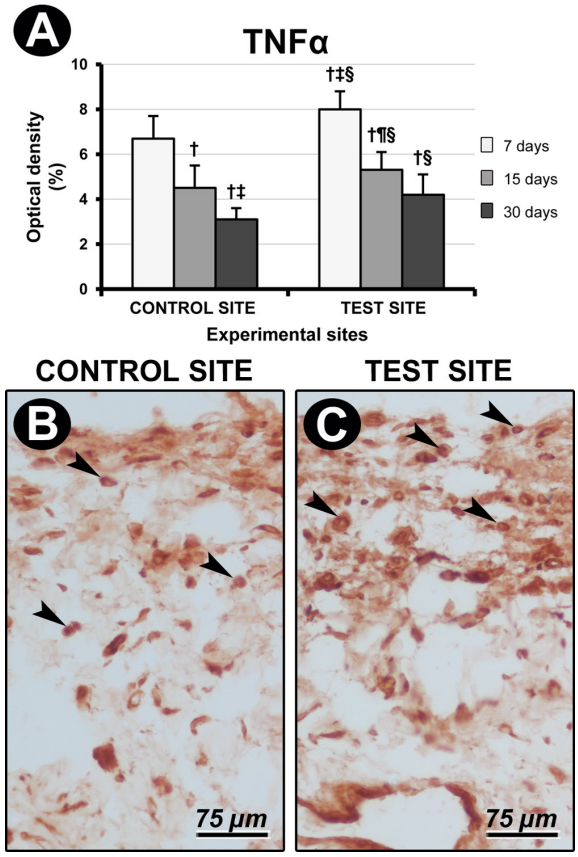

Figure 2 - Immunolabeling pattern for TNFα in the connective tissue in the control site and in the test site at 15 days postoperatively. A: Graphic presenting immunolabeling data for TNFα in the different experimental groups and periods. B-C: Photomicrographs of the connective tissue showing immunolabeling pattern for TNFα in the control site (B) and test site (C).

Arrows: immunolabeling cells; †: statistically significant difference in relation to the control site at 7 days postoperatively; ‡: statistically significant difference in relation to the control site at 15 days postoperatively; ¶: statistically significant difference in relation to the control site at 30 days postoperatively; §: statistically significant difference in relation to the test site at 7 days postoperatively; |: statistically significant difference in relation to the test site at 15 days postoperatively. Scale bars: 75 µm

There was no statistically significant difference in IL-1β immunolabeling optical density between the control and the test site in all experimental periods. In the control site there was a gradual and statistically significant reduction in the immunolabeling density along the experimental periods, which was significantly lower at 15 and 30 days postoperatively when compared to the 7 postoperative days (figure 3).

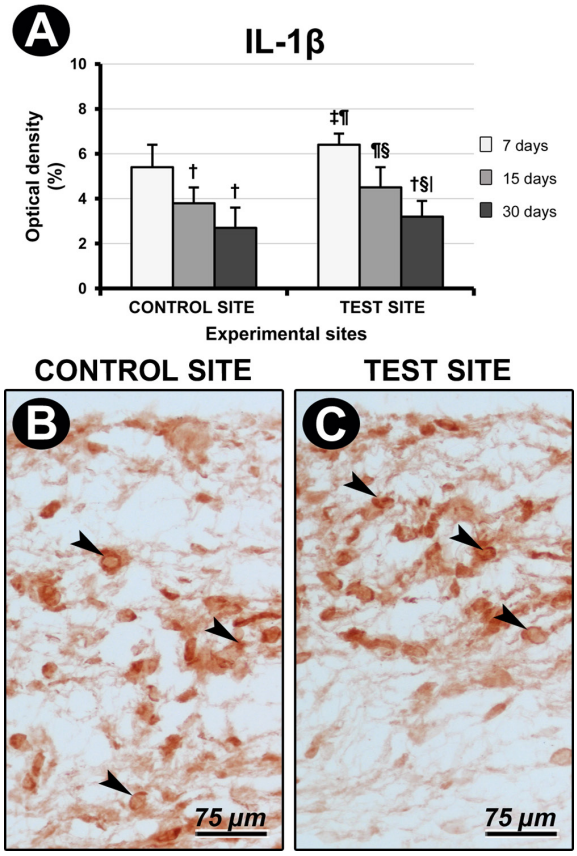

Figure 3 - Immunolabeling pattern for IL-1β in the connective tissue in the control site and in the test site at 15 days postoperatively. A: Graphic presenting immunolabeling data for IL-1β in the different experimental groups and periods. B-C: Photomicrographs of the connective tissue showing immunolabeling pattern for IL-1β in the control site (B) and test site (C)

Arrows: immunolabeling cells; †: statistically significant difference in relation to the control site at 7 days postoperatively; ‡: statistically significant difference in relation to the control site at 15 days postoperatively; ¶: statistically significant difference in relation to the control site at 30 days postoperatively; §: statistically significant difference in relation to the test site at 7 days postoperatively; |: statistically significant difference in relation to the test site at 15 days postoperatively. Scale bars: 75 µm

Discussion

Inflammation is a process regulated by specialized signaling molecules called cytokines. When materials such as methacrylate come into contact with tissues, they interact with different cells of the immune system, and those cells produce various cytokines that elicit different effects on adjacent tissues. Some of these cytokines promote inflammation while others limit it. Among the proinflammatory cytokines, the main ones are TNFα and IL-1β [1].

Our methodology aimed to evaluate the inflammatory response and its changes through different time periods (7, 15 and 30 days). We have demonstrated that the sealing agent may be used safely for the adjacent tissues of the implant in both the short and long term, because the material showed good compatibility throughout its application.

We evaluated the inflammatory response of a mono component resin, thixotropic, that set at room temperature in the absence of oxygen intended for use inside dental implants, specifically around the retaining screw of the prosthetic abutment. The material is commonly being used industrially to adjust screws and with low resistance to disassembly. This methacrylate-based product (2-hydroxyethyl methacrylate – HEMA) is suitable for locking and sealing threaded surfaces. During curing, when the product is confined between metal surfaces in the absence of air, it prevents loosening and leakage caused by impacts and vibration. It is especially suitable for applications on less active surfaces, such as stainless steel and treated surfaces.

Investigating the biocompatibility of dental materials is essential to avoid possible irritation or degeneration of tissues that will come into contact with the material [24]. The methacrylate present in the sealing agent evaluated in this study is a synthetic polymer 2-hydroxyethyl methacrylate (HEMA) that has been widely applied in the medical-dental and tissue engineering fields [14, 21, 26]. In dentistry, methacrylate-based polymers are used in dental prostheses, maxillofacial prostheses, restorations, and adhesive systems; these polymers are in the form of polymethyl methacrylate (PMMA) and HEMA [27]. Numerous studies have shown adverse reactions to methacrylate-based composites and their effects in oral cavity [29, 30, 32]. Due to the natural wear and the release of unpolymerized monomers, these composites may enter the oral cavity and diffuse through oral tissues [15, 16, 28]. However, some studies have showed non-significant DNA damage and nuclear changes with the use of more dilute concentrations of these methacrylates, which would resemble clinical conditions [5, 36].

Considering the small diameter of retaining screw, it should be emphasized that the amount of material used inside the implant is very low, thus the amount of material that may spill out of the implant-abutment interface region is almost insignificant. Therefore, even if the material does cause any kind of irritation (which has been proven not to happen), it can be easily eliminated. It is also important to note that one of the main advantages of the sealing agent used in this study is its reversibility with the screwed prostheses, which makes it possible to loosen the prosthetic part for modifications, repairs, adjustments, and maintenance [11, 33].

The biocompatibility of HEMA-based materials implanted into the subcutaneous tissue of rats was evaluated. The materials were considered biocompatible when the intensity of the inflammatory response of the connective tissue adjacent to the implanted tube decreased over the experimental periods. At 7 days, the inflammatory response ranged from mild to moderate; but at 90 days, the connective tissue had been repaired, thus showing the biocompatibility of all tested materials [34]. These results corroborate with the findings in this study. The initial inflammatory response is likely due to surgical trauma caused during the implantation of the experimental materials into the subcutaneous tissue [10].

Tests exposing subcutaneous tissue to different methacrylate/acrylate monomers have found that different monomers affect the production, increase, and decrease of different cytokines [1]. While, poly 2-hydroxyethyl methacrylate (pHEMA) has desirable characteristics that allow its use in the field of skin regeneration [35]. Other authors have investigated a new biodegradable bioactive bone cement (comparable to PMMA cement) composed of ternary glass particles and a photocurable hydroxyethyl methacrylate resin; this material elicits minimal immunological reactions when it is used to treat bone defects [18].

The commercial sealing agent tested appear to be a biocompatible material. However, important studies still need to be carried out to test microbial infiltration through connection implants, to evaluate the properties of this material in relation to its cellular biocompatibility, and to perform human studies to confirm its safety and effectiveness, before recommending it for clinical use.

Conclusion

The methacrylate-based sealing agent showed a reduction in the inflammatory response throughout the experimental periods. This indicates that it may be employed as a sealing agent at the implant-abutment interface without causing undesirable responses in the adjacent tissues.

Acknowledgements

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (Capes) – Finance Code 001.

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