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RESEARCH AND EDUCATION

Biocompatibility of primers and an adhesive used for implant-retained maxillofacial prostheses: An in vitro analysis

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ABSTRACT

Statement of problem. Implant-retained maxillofacial prostheses should be biocompatible, regardless of the primers and adhesives used to bond the acrylic resin and facial silicone. The authors are unaware of any study evaluating the influence of these primers and adhesives on the biocompatibility of maxillofacial prostheses.

Purpose. The purpose of this in vitro study was to evaluate the cytotoxic effect of primers and an adhesive used to bond acrylic resin and facial silicone during the fabrication of implant-retained maxillofacial prostheses.

Material and methods. Twenty-eight circular specimens made of resin and silicone were fabricated, either bonded or nonbonded with primer and adhesive. The specimens were divided into 7 groups: resin; silicone; resin+silastic medical adhesive type A+silicone; resin+DC 1205 primer silicone; resin+Sofreliner primer+silicone; resin+DC 1205 primer+silastic medical adhesive type A+silicone; and resin+Sofreliner primer+silastic medical adhesive type A+silicone. Eluates of the materials tested were prepared by setting 4 specimens of each experimental group in Falcon tubes with medium and incubating at 37°C for 24 hours. The eluate cytotoxicity was evaluated by an assay of survival/proliferation ((3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide [MTT] test) in cultures of human keratinocytes. The levels of IL1, IL6, TNFα, and the chemokine MIP-1α were evaluated by enzyme-linked immunosorbent assay. The mRNA expressions for MMP-9, TGF-β, and collagen type IV were analyzed by the real time polymerase chain reaction. Data were submitted to analysis of variance with Bonferroni post hoc tests (α=.05).

Results. An increased cell proliferation was observed for the RAS group, with statistically significant differences ($P<.001$) compared with the unstimulated group. The RDCpS group showed the highest IL6 concentration values ($P<.001$). No significant statistical difference was found in the relative quantification of mRNA for collagen type IV, MMP9, or TGFβ between the groups ($P>.05$).

Conclusions. The RAS group showed the highest cell proliferation percentage, while the RDCpS group exhibited the highest IL6 concentration values. No detectable levels of IL1β, TNF α, or CCL3/MIP1α were observed. The tested materials showed no toxic effects on the HaCaT cell line. (J Prosthet Dent 2016;■:■-■)

Maxillofacial prostheses are devices used in the rehabilitation of patients with facial mutilations in an attempt to repair severe bone and tissue loss.[1,2] The purpose of these prostheses is to provide comfort and esthetics for patients to improve their self-esteem and quality of life.[1,3-5]

With the advance of implantology, maxillofacial prostheses may be stabilized and retained with osseointegrated implants.[4,6-9] However, the silicones commonly used in the fabrication of these prostheses show a reduced bond to the implant attachment system, allowing detachment from the silicone while still appearing connected to the implant.[7,10-12]

To improve the connection between prosthesis and implants, some authors have proposed using acrylic resin as

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Clinical Implications

Professionals should be aware of the available methods of fabricating biocompatible implant-retained maxillofacial prostheses. The materials tested showed no toxic effect on the HaCaT cell line. However, an inflammatory process can occur when DC 1205 primer is not used in conjunction with silastic medical adhesive type A.

an intermediary layer.[7,8,11] In this technique, the attachment system is embedded in acrylic resin, which is covered with silicone. However, the resin/silicone junction is a point of weakness because the materials have different compositions and there is no chemical bond between them. Therefore, the silicone may tear or separate from the resin during the removal of the prosthesis by the patient.[7] Some authors have proposed using primers and adhesive to improve the resin/silicone junction.[9,13]

However, byproducts of these materials, which contact the skin or mucous membrane, may act as irritants which trigger inflammatory processes and even be carcinogenic.[14,15] Biocompatible material with no undesirable effects for the user is a key factor in successful rehabilitation.[14,16] Different methods of evaluating the biocompatibility of dental materials have been reported.[14,15] The principle method is the in vitro cytotoxic analysis with cell cultures[15,17,18]; it is a relatively straightforward test to perform, has an adequate cost-benefit ratio, and can be controlled.[17,19] Primary culture or cell line can be used to perform in vitro tests, and the HaCaT keratinocyte line has been used extensively to analyze potential drug effects on the skin.[20]

Environmental risks such as carcinogens, chemical substances, and ultraviolet (UV) radiation can induce the release of inflammatory mediators by keratinocytes such as interleukin 6 (IL6), tumor necrosis factor α (TNFα), and IL1β. These mediators are the principle proinflammatory cytokines and can increase the local concentration of tissue repair cells.[21] Another important aspect related to the inflammatory process is tissue repair, a complex phenomenon that restores the morphological and functional integrity of injured tissues.[22] The repair occurs when the production of collagen, matrix metalloproteinase, and transforming growth factor β (TGFβ) is balanced.[23-25]

The use of a wide variety of materials for facial prostheses necessitates a biocompatibility test.[14,15] However, few studies have been published concerning biocompatibility tests for maxillofacial prosthesis materials.[14,15] The cytotoxicity of facial silicones,[14,15] acrylic resins,[16-18,26-28] and resilient materials,[18,29] have been assessed, but the authors know of no studies which evaluate the primers and adhesives used to promote adhesion between acrylic resin and silicone. Knowledge of the biocompatibility of these components is necessary to ensure the safe use of maxillofacial prostheses. Therefore, the purpose of this in vitro study was to evaluate the cytotoxic effect of primers and an adhesive used to bond acrylic resin and facial silicone during the fabrication of implant-retained maxillofacial prostheses by analyzing cell proliferation and the production of proinflammatory cytokines and extracellular matrix proteins by keratinocytes.

The null hypothesis was that the application of primers and an adhesive does not have cytotoxic effects on the cell line (HaCaT).

MATERIAL AND METHODS

Specimen preparation

Twenty-eight specimens of acrylic resin and/or facial silicone were fabricated and divided into the following groups (n=4): resin (R); silicone (S); resin+silastic medical adhesive type A+silicone (RAS); resin+DC 1205 primer+silicone (RDCpS); resin+Sofreliner primer+silicone (RSpS); resin+DC 1205 primer+silastic medical adhesive type A+silicone (RDCpAS); and resin+Sofreliner primer+silastic medical adhesive type A+silicone (RSpAS) (Table 1).

Resin disks were fabricated with autopolymerizing acrylic resin (Orto Clas; Artigos Odontológicos Clássico Ltda)[7,8] obtained from a metal matrix composed of an upper and lower portion. The upper portion was completely smooth, while the lower portion had circular compartments of 10-mm in diameter and 1-mm in thickness.[16]

The resin was proportioned and manipulated according to the manufacturer's instructions and was placed into the metal matrix. The upper portion was positioned over the lower portion, and the assembly was put in a hydraulic press (VH; Midas Dental Products Ltd), where a force of 12 MN was applied for 10 minutes. Subsequently, the matrix was placed under a hydrostatic pressure of 0.14 MPa in a resin polymerization device for 20 minutes (Metalvander). The matrix was opened, the disks were removed, and excess was removed with tungsten carbide burs (Maxi-Cut abrasive drill; Viking).[30]

The MDX4-4210 silicone disks were obtained from a metal matrix composed of circular compartments of 10 mm in diameter and 1 mm in thickness. For the other groups, the silicone disks were fabricated with a metal matrix over the acrylic resin disks. Thus, a metal matrix similar to the lower portion of the matrix for silicone disk preparation was used, but with a 2-mm-difference in thickness. The acrylic resin disks with 1 mm in thickness were cleaned with gauze and acetone and then placed into the matrix.

Table 1. Materials used for specimen preparation

Commercial Name	Manufacturer	Chemical Composition
Orto Clas	Artigos Odontológicos Clássico Ltda	Liquid: Methyl methacrylate monomer, acetone, hydrocyanic acid, and methyl alcohol Powder: Methyl-methacrylate polymers
Silastic MDX 4-4210	Dow Corning Corp	Dimethylsiloxane polymer, reinforcing silica, platinum catalyst, and siloxane crosslinking agent
Silastic Medical Adhesive Type A	Dow Corning Corp	Octamethylcyclotetrasiloxane, acetic anhydride
DC 1205 primer	Dow Corning Corp	Toluene, butanone, methoxymethylethoxy, propanol, and trimethoxysilyl propyl ethylenediamine
Sofreliner primer	Tokuyama Corp	Methylene chloride, PMMA with polyorganosiloxane

PMMA, poly(methyl methacrylate).

A thin layer of DC 1205 primer[30,31] or Sofreliner primer[13] was applied to the acrylic resin surfaces of the RDCpS, RSpS, RDCpAS, and RSpAS groups. The resin surface was exposed to the primer for 30 minutes to increase adhesive penetration.[30]

The MDX 4-4210 facial silicone was manipulated under a controlled temperature of 23°C ±2°C and humidity of 50% ±10% according to the manufacturer's instructions. Before placing the silicone mixture into the matrix, a thin layer of silastic medical adhesive type A[11,31] was applied directly on the prepared acrylic resin surface of the RAS, RDCpAS, and RSpAS groups. The silicone mixture was then inserted into the matrix compartments over resin, its surface was flattened with a steel spatula, and its thickness was standardized. The matrix was placed in a resin polymerization device with 0.14 MPa of pressure for 20 minutes. The silicone was polymerized for 72 hours at room temperature according to the manufacturer's instructions.[30]

Obtaining eluates

Eluates leached from specimens were used for the analysis of their cytotoxic effects.[18,19,32-34] Four specimens from each group were placed into a sterile vial with 9 mL of Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco)[27] and incubated at 37°C for 24 hours.

During this incubation period, substances were leached for the culture medium, forming the eluates used for cytotoxic analysis. Once solubilized, the eluates were filtered by 0.22 μm filters (Millex; Millipore) for sterilization, necessary, because of the ease of medium contamination due to the richness of nutrients.[14,16]

Cells, cultures, and cytology analysis

Any possible cytotoxic effects of the substances released from the tested materials were evaluated by using the cell culture method. Keratinocytes (HaCaT) were expanded in flasks with DMEM medium supplemented with 10% FBS, 10 μg/mL streptomycin (Gibco), 10 μg/mL penicillin (Gibco), 10 μg/mL gentamicin (Gibco), and 250 μg/mL amphotericin B (Fungizone; (Gibco)[17] and incubated (CO_2 incubator; Thermo Fisher Scientific Inc.) with 5% CO_2 and controlled humidity at 37°C.[35]

Cell suspensions of 1×10^5 cells/mL, predetermined by a pilot study, were prepared to perform the cytotoxic tests, and 1 mL of these suspensions was pipetted into each well of a 24- well plate. After 24 hours of incubation in 5% CO_2 and controlled humidity at 37°C, the medium was discarded, and 500 μL of eluates from different groups were added in each well. The unstimulated group (UG) remained in untreated wells and received DMEM with only 10% FBS. Tween 20 (Sigma-Aldrich) was added to the positive control wells (Tween). The same incubation and temperature conditions used to obtain the eluates were also used for this plate. This test quantified the activity of the mitochondrial succinate dehydrogenase enzyme by measuring the conversion of water-soluble tetrazolium salt in insoluble blue formazan.[16-18]

After 72 hours of eluate exposition to the cells, the culture medium was replaced with 500 μL of DMEM medium without FBS and with 0.5 mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich). It was then incubated with 5% CO_2 at 37°C for 4 hours.[14,16]

The culture medium was removed, and the intracellular formazan was released by solubilization with 1 mL of isopropanol (Synth) per well. The plates were shaken for 5 minutes before the absorbance was measured at 570 nm using a UV-visible spectrophotometer (SpectraMax 190; Molecular Devices), allowing the evaluation of cellular respiratory activity. The MTT assay was performed in triplicate.[14,17,18,26,27]

Enzyme-linked immunosorbent assay

The eluates obtained after 24 hours of specimen incubation were placed on the cell cultures, and the cell-free supernatants were collected after 72 hours. The collection aimed to dose the proinflammatory cytokines IL1β, IL6, and TNFα and the chemokine macrophage inflammatory protein 1α (CCL3/MIP1α), using enzyme-linked immunosorbent assay (ELISA; DuoSet ELISA Development Systems; R&D System).[35,36] A total volume of 100 μL of cell-free supernatant was used for the quantitative analysis of the specimen in triplicate, performed according to the recommendations of the manufacturer.[35,36]

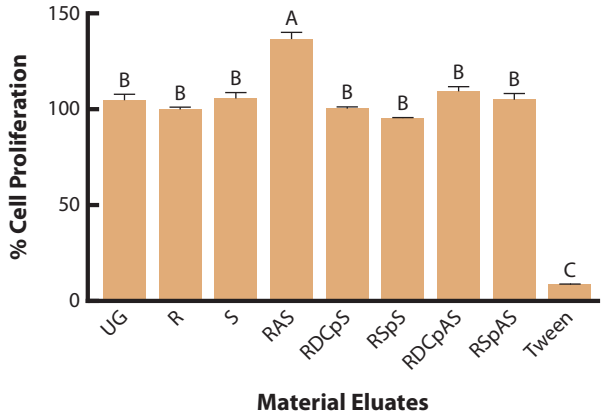

Figure 1. Percentage of cell proliferation for different eluates evaluated. Results show mean ±SE cell proliferation percentage. Different uppercase letters indicate statistical differences ($P<.05$) regarding the respective unstimulated group. R, resin; RAS, resin + silastic medical adhesive type a + silicone; RDCpAS, resin + DC 1205 primer + silastic medical adhesive type A + silicone; RDCpS, Resin + DC 1205 primer + silicone; RSpAS, resin + Sofreliner primer + silastic medical adhesive type A + silicone; RSpS, resin + Sofreliner primer + silicone; S, silicone; Tween, positive control; UG, unstimulated group.

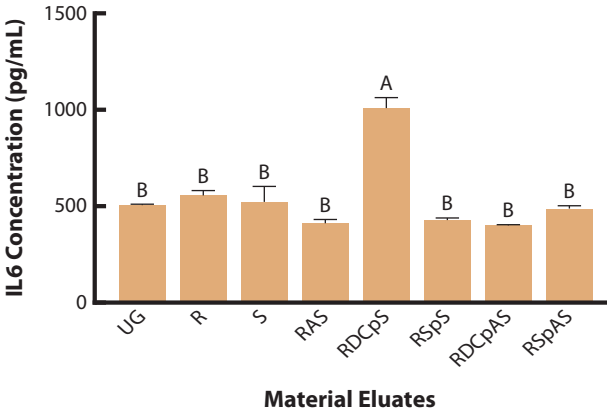

Figure 2. IL6 concentration for different eluates evaluated. Results show mean ±SE of IL6 concentration (pg/mL). Different uppercase letters indicate statistical differences ($P<.05$) between groups. IL6, interleukin 6; R, resin; RAS, resin + silastic medical adhesive type a + silicone; RDCpAS, resin + DC 1205 primer + silastic medical adhesive type A + silicone; RDCpS, Resin + DC 1205 primer + silicone; RSpAS, resin + Sofreliner primer + silastic medical adhesive type A + silicone; RSpS, resin + Sofreliner primer + silicone; S, silicone; Tween, positive control; UG, unstimulated group.

Real-time reverse transcription-polymerase chain reaction

The quantitative analysis of gene expression for collagen type IV (COL IV; COL4A3BP: Hs00178621_m1), matrix metalloproteinase 9 (MMP9; Hs00234579_m1) and TGFβ1 (Hs0099133_m1)[37] was performed by real-time reverse transcription-polymerase chain reaction (RT-PCR) assay.

For total RNA extraction after 72 hours of eluate exposition to the cells, TRIzol reagent (Invitrogen Life Technologies) was used according to the instructions of the manufacturer. The RNA concentration was measured by spectrophotometry. The first strands of cDNA were synthesized with 1 μg of total RNA and Superscript II RNase H^- reverse transcriptase (Invitrogen Life Technologies). Subsequently, the mRNA levels for COL IV, MMP9, and TGFβ and also their amplification obtained by using StepOnePlus real-time PCR system (Applied Biosystems; Invitrogen Life Technologies) were measured, The internal control used to detect mRNA was β-actin (ACTB: Hs03023880_g1). The reactions were performed using a volume of 20 μL, and each specimen was run in duplicate. The results were analyzed using the comparative threshold cycle (C_T) method.[35]

Statistical analysis

Data from MTT, ELISA, and RT-PCR assays were submitted to 1-way analysis of variance (ANOVA) followed by Bonferroni post hoc tests (α=.05).

RESULTS

Statistically significant differences were observed among the groups during the analysis of cell proliferation (df=8; F=281.905; $P<.001$ by ANOVA). The RAS group showed the highest cell proliferation percentage (137%), with a statistically significant difference ($P<.001$) compared with the unstimulated group (104%) (Fig. 1).

Detectable concentrations of IL1β, TNF α, and CCL3/MIP1α were not found. However, concentrations of IL6 were found in different groups. The RDCpS group showed the highest concentration of IL6 (1.005 pg/mL), statistically significant compared with other groups (df=7; F=37.130; $P<.001$ by ANOVA) (Fig. 2).

Concerning the relative quantification of mRNA for COL IV (df=7; F=.824; P=.577 by ANOVA) (Fig. 3), MMP9 (df=7; F=2.679; P=.340 by ANOVA) (Fig. 4), and TGFβ (df=7; F=1.940; P=.109 by ANOVA) (Fig. 5), no statistically significant difference was found among groups.

DISCUSSION

The null hypothesis that the application of primers and adhesives would not produce cytotoxic effects on the cell line (HaCaT) was accepted.

Before performing the present study, a pilot study was performed to determine the cell suspensions (1×10^5 cells/mL) for the cytotoxic test. The specimens were incubated for 24 hours to form the eluate: an increased release of subproducts present in the materials occurs in the first

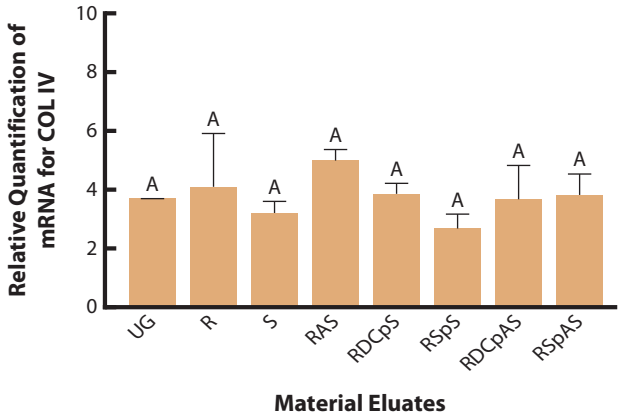

Figure 3. Relative quantification of mRNA for COL IV for different eluates evaluated. Results show mean ±SE of IL6 concentration (pg/mL). Different uppercase letters indicate statistical differences ($P<.05$) between groups. COL IV, collagen type IV; IL6, interleukin 6; R, resin; RAS, resin + silastic medical adhesive type a + silicone; RDCpAS, resin + DC 1205 primer + silastic medical adhesive type A + silicone; RDCpS, resin + DC 1205 primer + silicone; RSpAS, resin + Sofreliner primer + silastic medical adhesive type A + silicone; RSpS, resin + Sofreliner primer + silicone; S, silicone; Tween, positive control; UG, unstimulated group.

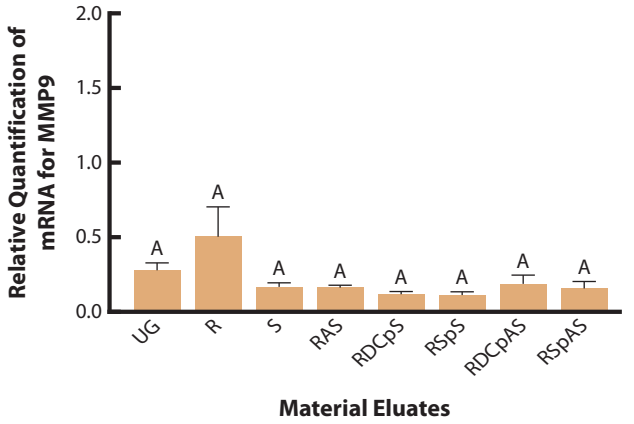

Figure 4. Relative quantification of mRNA for MMP9 for different eluates evaluated. Results show mean ±SE of IL6 concentration (pg/mL). Different uppercase letters indicate statistical differences ($P<.05$) between groups. IL6, interleukin 6; MMP9, matrix metalloproteinase 9; R, resin; RAS, resin + silastic medical adhesive type a + silicone; RDCpAS, resin + DC 1205 primer + silastic medical adhesive type A + silicone; RDCpS, resin + DC 1205 primer + silicone; RSpAS, resin + Sofreliner primer + silastic medical adhesive type A + silicone; RSpS, resin + Sofreliner primer + silicone; S, silicone; Tween, positive control; UG, unstimulated group.

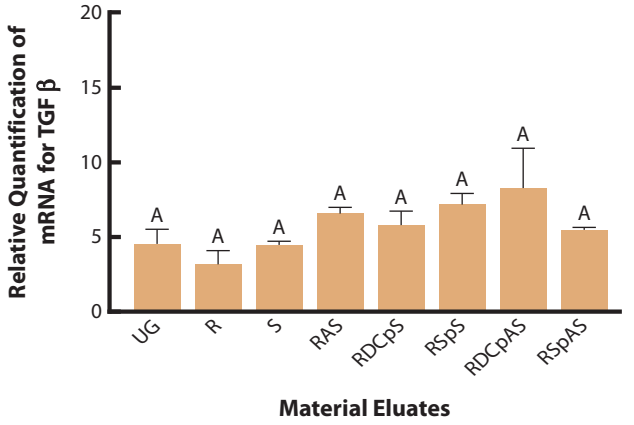

Figure 5. Relative quantification of mRNA for TGFβ for different eluates evaluated. Results show mean ±SE of IL6 concentration (pg/mL). Different uppercase letters indicate statistical differences ($P<.05$) between groups. IL6, interleukin 6; R, resin; RAS, resin + silastic medical adhesive type a + silicone; RDCpAS, resin + DC 1205 primer + silastic medical adhesive type A + silicone; RDCpS, resin + DC 1205 primer + silicone; RSpAS, resin + Sofreliner primer + silastic medical adhesive type A + silicone; RSpS, resin + Sofreliner primer + silicone; S, silicone; TGFβ, transforming growth factor-beta; Tween, positive control; UG, unstimulated group.

24 hours.[16,38] A period of 72 hours of eluate contact with cell cultures was selected for the cytotoxic assay to permit the formation of a monolayer of confluent cells.

According to the International Organization for Standardization 10993-5, in vitro methods for cytotoxic analysis can be classified as noncytotoxic (cell proliferation higher than 75%), slightly cytotoxic (proliferation between 50% and 75%), moderately cytotoxic (proliferation between 25% and 50%), and highly cytotoxic (lower than 25%).[33] All groups in this study showed cell proliferation higher than 75%, suggesting the tested materials were not cytotoxic to HaCaT cells (Fig. 1).

Even higher cell viability percentages than those with the RAS group (137%) were noticed, as statistically different from UG. The RAS group is believed to stimulate cell metabolism without changing the chemokine and cytokine levels, as can be seen in Figure 2. In vitro studies are necessary to analyze whether the RAS group stimulates this metabolism to the point of releasing inflammatory mediators responsible for keloid reactions, and if it also changes cytokine levels. The cytotoxicity of acrylic resin (R group) and silicone (S group) has been evaluated by different authors, many of whom have demonstrated the biocompatibility of these materials with a specific cell line.[14,15,27,39,40] However, the authors are unaware of studies evaluating the biocompatibility of primers and adhesives, hence the need for this investigation.

Although none of the tested materials were toxic, a greater statistically significant release of IL6 in the RDCpS group was observed. IL6 is an inflammatory mediator responsible for the increase of the local concentration of tissue repair cells,[37,41] suggesting that the DC 1205 primer used with resin and silicone may start the inflammatory process. However, when the primer was used with silastic medical adhesive type A in the RDCpAS group, the level of IL6 decreased, showing statistical similarity to the unstimulated group. This suggests always using both together for clinical use (Fig. 2). According to Gabay et al,[41] IL6 has a dual effect,

it is proinflammatory in chronic inflammation but also acts as a defense mechanism (anti-inflammatory), stimulating the production of IL1 (anti-inflammatory mediator). However, this did not happen in the present study because no detectable concentrations of IL-1β were found. There were no detectable concentrations of TNFα or CCL3/MIP1α, suggesting that the stimulus given by the eluates of the tested materials was not enough for HaCaT cells to secrete those inflammatory mediators.

No statistically significant differences were found in the relative quantification of mRNA for COL IV, MMP9, and TGFβ compared with the unstimulated group. This indicates that the line of HaCaT cells seems to produce these targets in physiological conditions. Similarity was seen in the increase of COL IV and TGFβ (protein responsible for stimulating the COL IV synthesis process) gene expressions.[25] The balance between synthesis and degradation of collagen is essential during the tissue repair process to avoid a fibrous reaction,[42] and the COL IV produced by epithelial cells is essential for the composition of its basal membrane.[43] It is critical for preservation of the cell structure that balance occurs in the mRNA expression between COL IV, MMP9, and TGFβ.

The choice of materials used in this study was based on an article that evaluated the bond strength of acrylic resin and silicone used with DC 1205 primer, Sofreliner primer, and/or silastic medical adhesive.[30] The authors observed that Sofreliner produced the best results and was not considered toxic. However, the silastic medical adhesive, which is the most suitable to promote the MDX Silicon bond, did not alter the bond strength[30] and was also nontoxic. As the bond of resin to silicone is a major problem for maxillofacial prostheses, we suggest applying the Sofreliner primer to bond these materials. However, this primer is expensive and is not sold separately from the soft lining material. An alternative could be the use of DC1205 primer with adhesive, which together showed good bond strength[30] and did not present a toxic effect.

In this study, cytotoxicity and cell activation were analyzed in vitro. Although the evaluation of material biocompatibility is a necessary step,[18] it presents some limitations since the results do not correspond completely with the cytotoxic properties of the materials in their clinical condition.[44,45] Future studies should be performed to assess different inflammatory mediators and to evaluate, separately, the primer and adhesive used in this study. In vivo studies, which have results closer to clinical practice should also be performed.

CONCLUSIONS

Based on the in vitro testing in this study, the following conclusions were drawn:

1. The tested materials showed no toxic effect on the HaCaT cell line.
2. An inflammatory process can occur when the DC 1205 primer is not used in conjunction with the silastic medical adhesive type A.
3. The RAS group was the most biocompatible.

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