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Modulation of inflammatory pathway in human gingival fibroblasts exposed to resinous materials

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Abstract. The goal of this work was to evaluate the anti-inflammatory effects of two resins, Bis-GMA-based resin (ProTemp 4™) and PMMA-based resin (Coldpac), used in dentistry for temporary prosthetics treatments, in the modulation of the inflammatory pathway NFkBp65/NLRP3/IL-1β. The protein expression of inflammatory markers was evaluated in an *in vitro* model of primary human gingival fibroblasts (hGFs) by immunofluorescence analysis while the study of the ultra-morphological analysis was performed through scanning electron microscopy. Taken together these results may suggest that ProTemp 4™ resin exerts a better performance in terms of inflammatory modulation.

Keywords: hGFs, ProTemp 4™, Coldpac, inflammation, biocompatibility.

INTRODUCTION

The advent of new technologies in the dental field has allowed the development of more resistant and easy-to-use resinous materials with innovative features.

One of the most important uses of composite resins is the development of temporary restorations, which is a fundamental step in the prosthetic treatment plan. Each type of resinous material has specific properties depending on the structure and molecular composition [1].

The main problem using these resinous materials is the incomplete polymerization process that leads to leakage of monomers [2] the substances released by these resinous materials may have important adverse reactions such as irritation or allergy to the oral mucosa [3-5]Previous studies reported that the toxicity of dental resin composite can be due to the release of monomers during the auto-polymerization process [6]. For this reason, there is an increased interest in the development of novel biocompatible resinous mate-

rials in the dental field [7-9] the present work aimed to evaluate the biological effects of the ProTemp 4™ resin, containing bisphenol A glycidyl methacrylate (Bis-GMA), and of the Coldpac resin, containing polymethyl methacrylate (PMMA), in an *in vitro* model of hGFs.

Protein expression and scanning electron microscopy were performed to understand the biological effects of provisional resins in contact with the oral fibroblasts. The expression of the inflammatory pathway nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), the inflammasome protein NOD-, LRR- and pyrin domain-containing 3 (NLRP3), and the pro-inflammatory cytokine interleukin-1 beta (IL-1β) [10,11] were evaluated using the immunofluorescence analysis.

MATERIALS AND METHODS

Preparation of resin disc samples to evaluate cell adhesion

Two materials used for temporary restorations were compared: ProTemp 4™ (ProtempTM 4 Temporization Material, 3M ESPE, St. Paul, USA) and Coldpac self-curing PMMA (Coldpac tooth acrylic, Yates Motloid, Chicago, USA).

After mixing and polymerization according to the manufacturer's instructions, round section bars of 10 mm in diameter were produced.

Discs with a thickness of 0.3mm were obtained using two glass plates, while using a core drill bit with an internal diameter of 0.5mm, approximately 50 disks were obtained for each resin under examination. Each disc was then finished and polished according to the procedures described by the manufacturer. To eliminate any finishing residues, the disks were placed in distilled water and sonicated for 30 minutes. The samples were dried and placed in an autoclave at 134 °C for 50 minutes to obtain complete disinfection.

Cell culture of hGFs

Human gingival fibroblasts (hGFs PCS-201-018 ATCC, Manassas, Virginia, US) were cultured in basal medium (Fibroblast Growth Kit-Low Serum, (PCS-201-041, ATCC), containing 5 ng/mL rh FGF-β (fibroblast growth factor beta), 7.5 mM L-glutamine, 50 μg/mL ascorbic acid, 1 μg/mL hydrocortisone hemisuccinate, 5 μg/mL rh insulin and 2% fetal bovine serum [12,13]. The culture was maintained in an incubator at 37°C in a humidified atmosphere of 5% CO2 and 95% air [13,14]

Once the cells reached 75-80% confluency, subcultures were made.

Confocal Microscopy Analysis

The hGFs cells were seeded at 8500/well on 8-well culture glass slides (Corning, Glendale, Arizona, USA) and treated with ProTemp 4™ and Coldpac resins, for 24 hours and 1 week, replacing the medium every 2 days. The cells were then fixed for 10 min at room temperature (RT) with 4% paraformaldehyde in 0.1 M PBS (pH 7.4); after washing, samples were processed for immunofluorescence staining.

The Confocal Microscopy analysis was performed using NFkB (1:500, sc-8008, Santa Cruz Biotechnology, CA), NLRP3 (1:500, NBP1 77080, Novus, Milan, Italy), and Il-1b (1:500, sc-32294, Santa Cruz) as primary mouses monoclonal antibodies [16] and Alexa Fluor 568 red fluorescence conjugated goat anti-mouse antibody (A11031, Invitrogen, Eugene, OR, USA) as a secondary antibody. The microscope used is Zeiss LSM800 confocal system (Zeiss, Jena, Germany) [17].

SEM

SEM analyses were then performed to evaluate the relationship between hGFs and the resin disks.

After 24 hours and 1 week of culture, the samples were fixed for 1 hour 4 °C in 2.5% glutaraldehyde (Electron Microscopy Sciences, EMS, Hatfield, PA, USA), in 0.1 M sodium phosphate buffer (PB), pH 7.3, rinsed three times with PB, and post-fixed for 1 h in 1% aqueous osmium tetroxide (EMS) at 4 °C. The cells were dehydrated through an ethanol series (30%, 50%, 70%, 90%, 95%, and two times 100%) followed by drying in air and carbon. Specimens were mounted on aluminum stubs and gold-coated in an Emitech K550 sputter-coater (Emitech Ltd., Ashford, UK). SEM EVO 50 (Zeiss, Jena, Germany) was used for analysis [20].

Design of the experimental study

The experimental steps featured in this study were performed in triplicate with hGFs (Figure 1):

- hGFs cultured alone as negative control for 24 hours and 7 days.
- hGFs cultured with ProTemp 4™ resin disk for 24 hours and 7 days
- hGFs cultured with Coldpac resin disk for 24 hours and 7 days

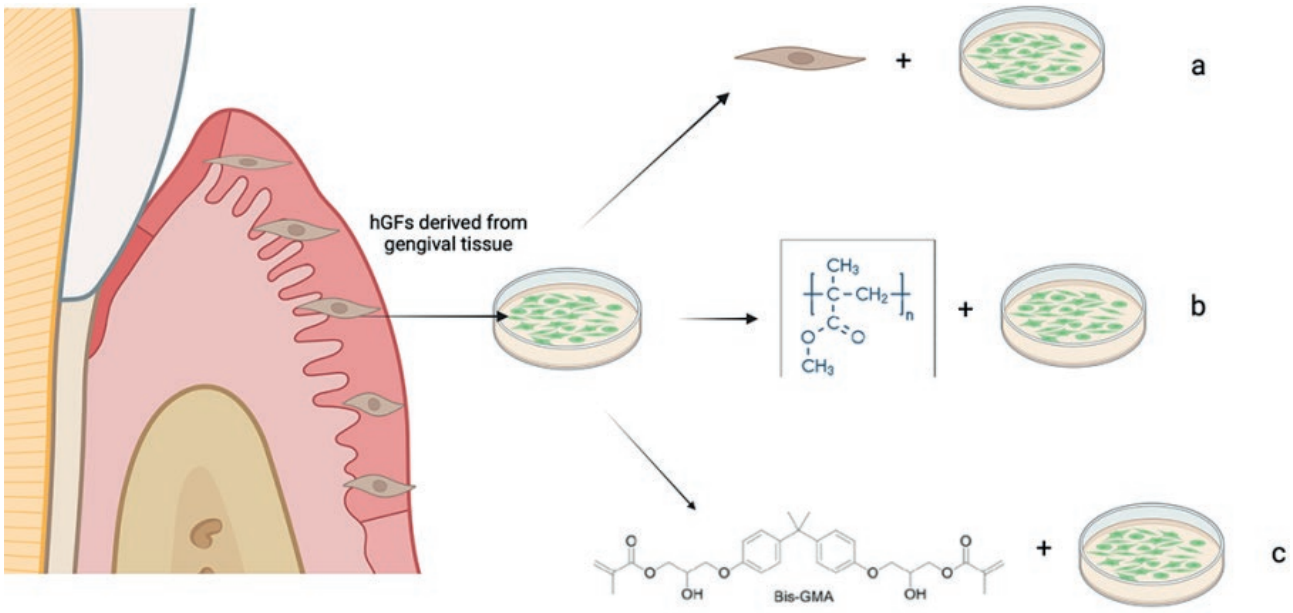

Figure 1. Design of the experimental study. 1a) hGFs cultured alone; 1b) hGFs cultured with Coldpac; 1c) hGFs cultured with ProTemp 4™.

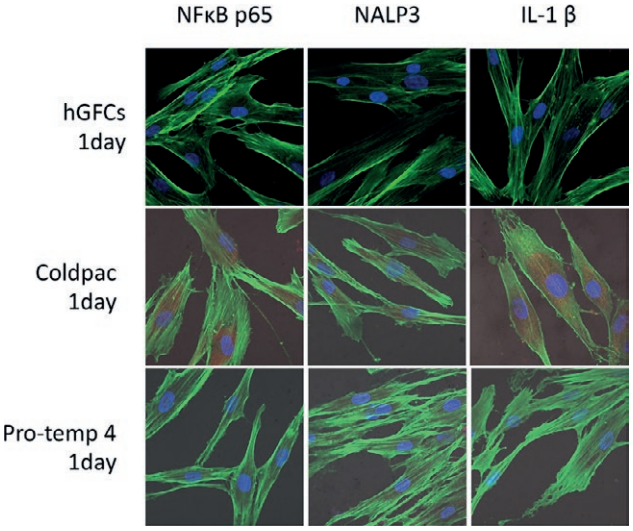

Figure 2. Protein Expression evidenced by CLSM in hGFs, in hGFs cultured on Coldpac and in hGFs cultured on ProTemp 4™ after 24 h.

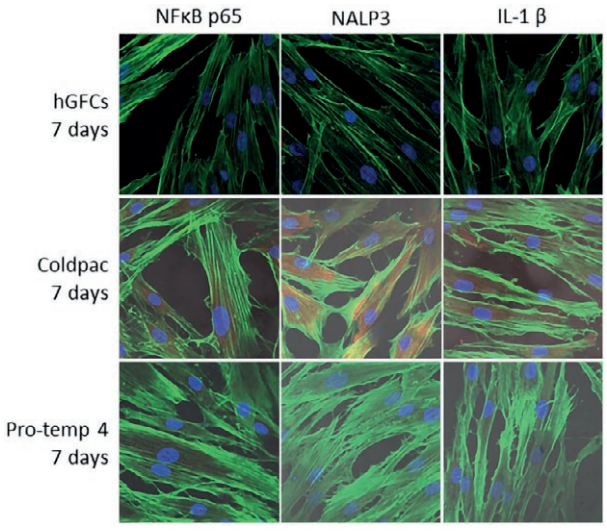

Figure 3. Protein Expression evidenced by CLSM in hGFs, in hGFs cultured on Coldpac, and in hGFs cultured on ProTemp 4™ after 1 week.

RESULTS

The immunofluorescence analysis showed that the pathway NFkBp65/NLRP3/IL-1β was significantly downregulated in hGFs alone and in hGFs cultured with ProTemp 4™ disks compared to hGFs cultured with Coldpac disks, after 24 h (Figure 2) and 1 week of treatment (Figure 3).

The immunofluorescence figures show the NFκB p65/NLRP3/IL-1β expression in hGFs alone, cultured with Coldpac and with ProTemp 4™ for 24 hours. The results show that the NFκBp65/NLRP3/IL-1β pathway was significantly upregulated in hGFs cultured with Coldpac after 24h compared to hGFs cultured with Pro-Temp 4™ and hGFs alone.

The immunofluorescence figures show the NFκB p65/NLRP3/IL-1β expression in hGFs alone, cultured with Coldpac and with ProTemp 4™ after 1 week. The results

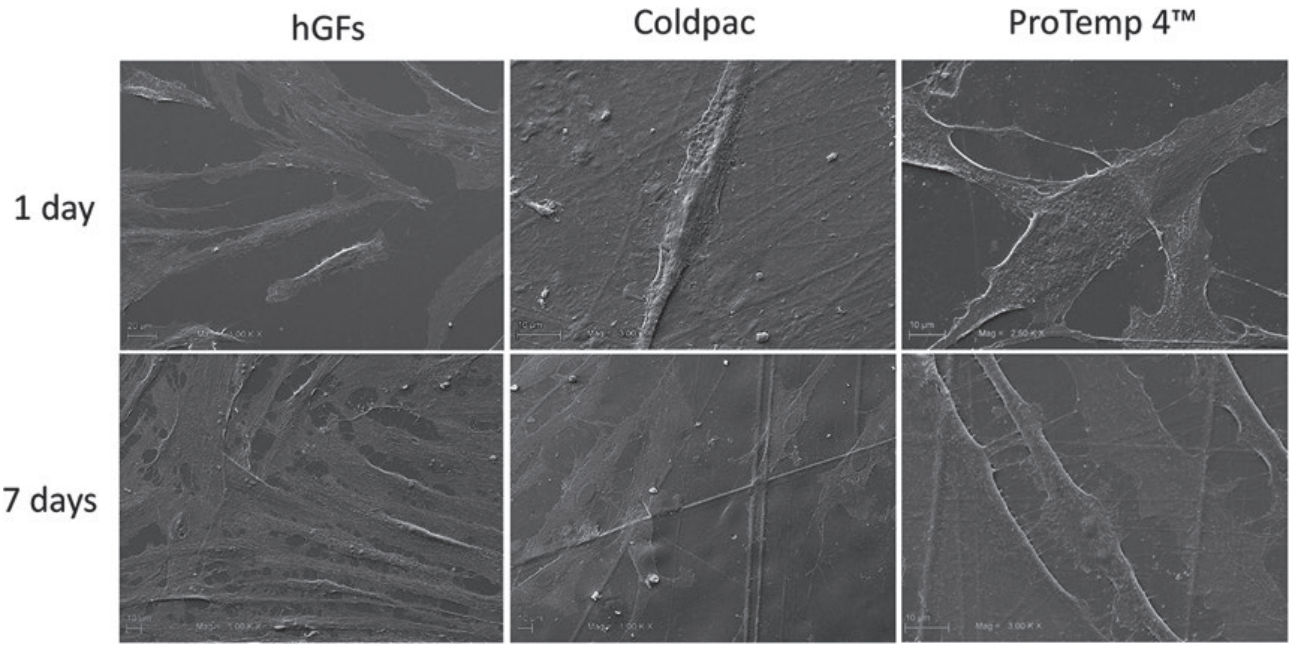

Figure 4. Representative SEM images of hGFs cultured alone, with Coldpac, and with ProTemp 4™ after 24 hours and 7 days.

show that the NFκBp65/NLRP3/IL-1β pathway was significantly upregulated in hGFs cultured with Coldpac after 1 week compared to hGFs cultured with ProTemp 4™ and hGFs alone.

Regarding the morphological analysis, hGFs cultured on ProTemp 4™ showed a similar morphology as hGFs evidencing nucleo and nucleoli, after 24h and 1 week; on the contrary, cells cultured on Coldpac showed a different morphological feature (Figure 4).

DISCUSSION

Over the last few years, new technologies have developed biologically compatible resins with optimum safety profiles and physical properties.

In our study, we focused on the ProTemp 4™ and Coldpac resins that are particularly used for the development of prosthetic implants [21]. The incomplete polymerization process of these materials may induce toxic effects on the oral cavity cells [22].

The *in vitro* model hGFs was used to understand which provisional resin can lead to better biocompatibility, after 24 hours and 1 week of culture.

Scanning electron microscopy was performed to understand the cell adhesion capacity on the resin disks, on the other hand, confocal laser scanning microscopy was performed to analyze inflammatory modulation.

The NFkB p65/NLRP3/ IL-1β inflammatory pathway was found to be downregulated in hGFs cultured with ProTemp 4™ resin when compared with cells cultured with Coldpac resin, after 24h and 7 days of cultured. In parallel, SEM analysis showed that the fibroblastic morphology was preserved in hGFs treated with Protemp 4™ while compared with hFGs treated with Coldpac.

According to our results, ProTemp 4™ resin could be less inflammatory when compared to Coldpac resin; this could mean that the ProTemp 4™ resin could lead to better biocompatibility and better performance in terms of cell/material interaction.

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