UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

LARISSA ÁLAMO

Biological assessment of 3D printed resins for interim restorations using an organotypic model of oral mucosa cells

Avaliação biológica de resinas impressas em 3D para restaurações provisórias usando um modelo organotípico de células da mucosa oral

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"Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota"

Madre Teresa de Calcutá

ABSTRACT

Biological assessment of 3D printed resins for interim restorations using an organotypic model of oral mucosa cells

Statement of the problem: 3D printed resins are a new class of materials for making temporary restorations with great commercial appeal; however, little is known about the parameters of the confection technique that can influence the biological compatibility with oral tissues. **Purpose:** The objective of the present study was to evaluate the effect of the post-cure time on the cytotoxicity of two resins for printing interim restorations in a 3D organotypic model of the oral mucosa. **Material and methods:** Cylindrical samples were prepared with conventional acrylic resin (AR) and CAD-CAM resin (CC), composite resin (CR), and two biocompatible resins for 3D printing (3DP), submitted to post-curing in a UV light chamber for 1, 10 or 20 min (90 W, 405 nm). Standardized samples of the materials were incubated for 1, 3 and 7 days in close contact with an organotypic model of keratinocytes (NOK-Si) in co-culture with gingival fibroblasts (HGF-1) in a 3D collagen matrix, or directly with 3D HGF cultures with viability (Live / Dead n = 2) and cell metabolism (Alamar blue n = 4) being evaluated. Spectral scanning of the culture medium was performed to detect the release of resin components (n = 6) (ANOVA/Tukey; α = 5%). **Results:** Severe reduction in metabolism (> 70%) and viability of keratinocytes was observed for 3DP resin post-cured for 1 min in all periods of analysis in a time-dependent manner. This cytotoxic effect was moderate for the 3D culture of HGFs in both experimental models, being correlated to the intense leaching of components in the culture medium. The post-cured resins for 10 and 20 min promoted a mild-moderate cytotoxic effect in the period of 1 day, similar to AR; however, recovery of viability was observed at 7 days. The 3DP resins submitted to post-cure for 20 min showed a pattern similar to CR and CC at the end of the experiment. **Conclusions:** It was concluded that the cytotoxic potential on oral mucosa cells of the tested 3DP resins is influenced by post-printing processing, which may be related to the leaching of residual components.

Key words: cytotoxicity, 3D printing, oral mucosa cells

RESUMO

Avaliação biológica de resinas impressas em 3D para restaurações provisórias usando um modelo organotípico de células da mucosa oral

Declaração do problema: As resinas de impressão 3D são uma nova classe de materiais para confecção de restaurações provisórias com grande apelo comercial; no entanto, pouco se sabe sobre os parâmetros da técnica de confecção que podem influenciar com a compatibilidade biológica com os tecidos orais. **Objetivo:** Objetivou-se avaliar o efeito do tempo de pós-cura sobre a citotoxicidade de duas resinas para impressão de restaurações provisórias em modelo organotípico 3D de mucosa oral. **Materiais e métodos:** Amostras cilíndricas foram preparadas com resina acrílica (RA) convencional e CAD-CAM (CC), resina composta (CR), e duas resinas biocompatíveis para impressão 3D (3DP), submetidas à pós-cura em câmara de luz UV durante 1, 10 ou 20 min (90 W, 405 nm). Amostras padronizadas dos materiais foram incubados por 1, 3 e 7 dias em íntimo contato com um modelo organotípico de queratinócitos (NOK-Si) em co-cultura com fibroblastos gengivais (HGF-1) em matriz de colágeno 3D, ou diretamente com cultura 3D de HGFs, sendo a viabilidade (Live/Dead n = 2) e metabolismo celular (Alamar blue n = 4) avaliados. Varredura espectral do meio de cultura foi realizada para detectar a liberação de componentes das resinas (n = 6) (ANOVA/Tukey; α = 5%). **Resultados:** Redução severa no metabolismo (>70%) e viabilidade dos queratinócitos foi observada para as resinas 3DP pós-curadas por um 1 min em todos os períodos de análise, de forma tempo-dependente. Este efeito citotóxico foi moderado para a cultura 3D das HGFs em ambos os modelos experimentais, sendo correlacionado à intensa lixiviação de componentes no meio de cultura. As resinas pós-curadas por 10 e 20 min promoveram efeito citotóxico leve-moderado no período de 1 dia, similar à RA; no entanto, recuperação da viabilidade foi observada aos 7 dias. As resinas 3DP submetidas à pós-cura por 20 min apresentaram padrão similar à RC e CC ao término do experimento. **Conclusões:** Concluiu-se que o potencial citotóxico sobre células da mucosa oral das resinas para 3DP testadas é influenciado pelo processamento pós-impressão, podendo estar relacionado com a lixiviação de componentes residuais.

Palavras-chave: citotoxicidade, impressão 3D, células da mucosa oral

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LIST DE ABBREVIATIONS AND ACRONYMS

GS	Glass Slide
AR	Acrylic Resin
CR	Composite Resin
CC	Prensed Acrylic Resin
PZ-3D	Three-Dimensional Printing Resin (Prizma 3D – MakertechLabs)
CS-3D	Three-Dimensional Printing Resin (Cosmos DLP – Yller)
3DP	3D Printing
UV	Ultraviolet
NOK-Si	Keratinocytes
HGF-1	Fibroblasts
DLP	Digital Light Processing
SLA	Stereolithography
UDMA	Urethane Dimethacrylate
Bis-GMA	Bisphenol-A Glycidyl Methacrylate
Bis-EMA	Ethoxylated Bisphenol-A-dimethacrylate
TEGDMA	Triethylene Glycol Dimethacrylate
DMEM	Dulbecco´s Modified Eagle´s Medium
FBS	Fetal Bovine Serum
%CMr	Percentage Of Cell Metabolism Reduction
%CMi	Percentage Of Individual Cell Metabolism
PBS	Phosphate Buffered Saline
PMMA	Polymethylmethacrylate
PEMA	Polyethylmethacrylate

Bis – Acryl	Bisphenol-A-Glycidyl Dimethacrylate
CAD/CAM	Computer-Aided Design/Computer-Aided Manufacturing
STL	Standard Tessellation Language
G-Code	Geometric Code
FDA	Food and Drug administration
EU	European Union

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1 INTRODUCTION

1 INTRODUCTION

Temporary restorations are a fundamental component of indirect restorations treatments. Therefore, the quality of materials selected for interim restorations fabrication can be considered a key factor for the success of rehabilitation treatment.[28] The provisional phase allows aesthetics re-establishment, maintenance of occlusal relations, protection of teeth mechanical stability, phonetics restoration, and support against masticatory forces [20], in addition to serving as a reference for future permanent restorations. [62] Temporary restorations also play a relevant biological role, as they must act in the protection of dentin-pulp complex and maintenance of periodontal health.[64]

The establishment of a marginal seal around indirect restorations on teeth or implants is essential to obtain an adequate emergency profile, as well as to prevent gingival recession and food impaction. [51, 61] In many clinical situations, this process is established at the interim restorations stage. Thus, to obtain an aesthetic gingival profile after permanent restoration placement, an adequate response of gingival tissues during the interim phase is necessary. This interaction can be considered as a critical factor for the success of rehabilitation treatment, especially in aesthetic areas. [17, 66] Besides, this process becomes even more relevant when there is a need of positioning the finish line of the preparation apically to the gingival margin, as well as when gingival conditioning is necessary. [60, 15, 77, 12] Thus, the biocompatibility of interim restoration materials is crucial, allowing the establishment and maintenance of healthy periodontal and peri-implant tissues around the restoration. [66]

Three main parameters must be observed for interim restoration in relation to gingival tissue: (1) good adaptation and marginal integrity; (2) satisfactory surface roughness; and (3) biocompatibility with the surrounding gingival tissues.[5] When these requirements are not met, a clinical condition of gingival irritation and biofilm accumulation can be observed, resulting in a local inflammatory reaction. [73, 44, 15, 28, 48, 68, 13] According to the literature, the release of unreacted residual toxic monomers directly affects the biocompatibility of the resins used for temporary restorations. [22, 72, 25, 41] Thus, diverse cytotoxic levels can be observed at different types of commercially

available materials, since the release of residual monomers is related to the chemical composition of the resin, degree of conversion, surface energy, and surface topography, and these parameters can be influenced by manufacture method. [25] The most widely used temporary restoration materials are acrylic resins, such as polymethylmethacrylate (PMMA), polyethylmethacrylate (PEMA), and bisphenol-A-glycidyl dimethacrylate, also known as bis-acrylic resin (Bis-Acryl). [47] It is possible to classify these resins based on their polymerization process into chemically activated, by heat, by light, or by dual reaction (by light and chemically). Acrylic resins are traditionally used in the direct preparation of interim restorations in a clinical scenario; however, they are also used in the laboratory for manufacturing indirect restorations by analogic or CAD/CAM (computer-aided design/computer-aided manufacturing) approaches.[47] In general, the literature indicates that indirect methods have better biological outcomes, since the polymerization is more effective, resulting in less release of toxic residual monomers.

Despite the widespread use of PMMA and PEMA for manufacturing temporary restorations by a direct method, there are many disadvantages, such as poor marginal adaptation, the high release of cytotoxic residual monomers, and high surface roughness, which benefits microorganisms adhesion and facilitates biofilm establishment.[32, 26, 33, 82, 14, 48, 4, 34] Bis-acrylic resin-based materials have been considered as an excellent alternative for the production of temporary restorations, with the advantage of easy handling, low polymerization contraction, and limited exothermic reaction, which can cause pulp and periodontal biological damage. [81, 65, 31, 37] Laboratory studies demonstrate that bis-acrylic resin is biocompatible to human gingival cells seeded in aqueous media with products released by it. [25, 66]

Another alternative for temporary restorations are resins manufactured using the CAD/CAM method. This technique uses prefabricated blocks that are machined with drills in a specific equipment until obtaining the prosthetic component. [47] The literature indicates that gingival cells can adhere more intensely on the surface of interim restorations obtained from prefabricated CAD/CAM blocks than on conventional PEMA and PMMA acrylic resins, or Bis-Acryl resin. [66] Shim et al. (2019) attributed this result to the highest surface energy and the highest degree of conversion observed on the CAD/CAM resins. [66]

With the emergence of 3D printing (3DP), a new category of materials for interim restorations has been introduced in Restorative Dentistry in recent years, creating an innovative treatment modality in the restorative clinic. [70] 3D printing is a terminology used to describe an additive manufacturing method [49], where a given object is constructed by sequential addition of layers. Because of the great expansion of 3DP applications in Dentistry, there is a wide variety of 3DP methods and materials in use, resulting in classification difficulty. [58] For Restorative Dentistry applications, high resolution, precision, and repeatability are required. These characteristics can be achieved using stereolithographic printers (stereolithography - SLA), where a liquid resin contained in a tank (VAT) is photo-polymerized layer by layer by a UV (ultraviolet) laser. The layers adhere to a platform that moves up or down, determining their thickness based on the range of the movement, which can be between 12.5 to 100 µm. At the end of the process, the object must receive an isopropyl bath for uncured resin removal, and a post-curing process in a UV light chamber and/or thermal chamber. The use of 3DP allows the creation of complex geometries, however, it requires structural supports for its manufacture, which consumes material and increases the time of production and post-processing. [56, 58]

SLA printers use a UV laser that moves from point to point tracing the geometry of each layer of the object to polymerize the printing material. The laser is focused using a set of lenses that are reflected on motorized mirrors. The depth of polymerization is determined by the photoinitiator and the conditions of exposure to irradiation, as well as by the presence of dyes, pigments, or other UV absorbers. On the other hand, in the DLP (digital light processing) method, a stationary light source projects the full image of one layer of the 3D model onto the surface of the liquid photopolymer, decreasing the printing time. Besides, in the DLP method, the projector is closer to the VAT, reducing the size of the projected pixels, and increasing the resolution. Thus, DLP printers have obtained prominence within Restorative Dentistry due to their high precision, reproducibility, and speed. [19]

The process of manufacturing temporary restorations using 3DP involves the acquisition of data through intraoral scanning devices, followed by image processing using specific CAD software to obtain the object's design in STL (Standard Tessellation Language) file. The object is imported in a 3D slicer software and the printing parameters are set, creating a G-code (Geometric Code) file that is responsible for

coordinating the 3D printer functions. After, the object needs to be cleaned and post-cured to complete the 3D printing process. [19] The parameters that define the efficiency of 3DP vary according to the printer, the additive manufacture method, and the printing material. An important characteristic that must be taken into consideration is the equipment resolution, which determines the printer's ability to reproduce fine details defined by the x-y and z axes, representing horizontal and vertical dimensions, respectively. The printer precision refers to the ability to manufacture objects with the same dimensions over and over again. Besides, accuracy refers to the discrepancy between the printed object and the actual dimensions. [56, 58]

Alharbi, et al. (2018), evaluated the marginal and internal adjustment of provisional full coverage restorations, noticing that the restorations obtained by the 3DP showed lower estimated marginal and internal gap values than restorations milled by CAD/CAM. Park et al. (2016) also found better results from marginal, intermarginal, axiogingival, axioocclusal, and occlusal discrepancy for temporary restorations on implant abutments made using the DLP printing method compared to thermoplastic resin and CAD-CAM system. As for mechanical strength, studies have shown that printed composites have less resistance to bending, however, greater microhardness when compared to current interim dental materials. It is also known that the print orientation interferes with the mechanical strength of the composites. Vertically printed composites with layers oriented perpendicular to the load direction showed greater resistance to compression than horizontally printed composites with layers parallel to the load direction. [56]

Despite the great current emphasis on the use of 3DP with resins called "biocompatible", there is a limited number of polymers available and approved for intraoral use, as well as little information on all chemical compounds present in these materials. Among some components already reported in the literature, we can mention the multifunctional acrylic monomers, acrylic acid esters, tetrahydrofurfuryl methacrylate, urethane dimethacrylate,2-hydroxy-3-phenoxypropyl acrylate, tricyclodecane dimethanol diacrylate, bisphenol-A epoxy acrylate, among others. According to a recent literature review [54, 56], the materials available on the market are approved by the food and drug administration (FDA) and/or European Union (EU). However, the authors point out that the EU certification establishes these materials within class IIa, which considers materials for medical use with medium risk, which are

certified to be installed in the body between 60 minutes to 30 days. [56] However, the literature is scarce regarding the biological evaluation of resins for 3DP [56, 58]. Thus, it is evident the need, at this moment, to carry out studies that demonstrate biological compatibility with oral tissues in the category of resins called “biocompatible”.

2 ARTICLE

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The article presented in this Dissertation was written according to the Journal of Prosthetic Dentistry instructions and guidelines for article submission.

Biological assessment of 3D printed resins for interim restorations using an organotypic model of oral mucosa cells

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ABSTRACT

Biological assessment of 3D printed resins for interim restorations using an organotypic model of oral mucosa cells

Statement of the problem: 3D printed resins are a new class of materials for making temporary restorations with great commercial appeal; however, little is known about the parameters of the confection technique that can influence the biological compatibility with oral tissues. **Purpose:** The objective of the present study was to evaluate the effect of the post-cure time on the cytotoxicity of two resins for printing interim restorations in a 3D organotypic model of the oral mucosa. **Material and methods:** Cylindrical samples were prepared with conventional acrylic resin (AR) and CAD-CAM resin (CC), composite resin (CR), and two biocompatible resins for 3D printing (3DP), submitted to post-curing in a UV light chamber for 1, 10 or 20 min (90 W, 405 nm). Standardized samples of the materials were incubated for 1, 3 and 7 days in close contact with an organotypic model of keratinocytes (NOK-Si) in co-culture with gingival fibroblasts (HGF-1) in a 3D collagen matrix, or directly with 3D HGF cultures with viability (Live / Dead n = 2) and cell metabolism (Alamar blue n = 4) being evaluated. Spectral scanning of the culture medium was performed to detect the release of resin components (n = 6) (ANOVA/Tukey; α = 5%). **Results:** Severe reduction in metabolism (> 70%) and viability of keratinocytes was observed for 3DP resin post-cured for 1 min in all periods of analysis in a time-dependent manner. This cytotoxic effect was moderate for the 3D culture of HGFs in both experimental models, being correlated to the intense leaching of components in the culture medium. The post-cured resins for 10 and 20 min promoted a mild-moderate cytotoxic effect in the period of 1 day, similar to AR; however, recovery of viability was observed at 7 days. The 3DP resins submitted to post-cure for 20 min showed a pattern similar to CR and CC at the end of the experiment. **Conclusions:** It was concluded that the cytotoxic potential on oral mucosa cells of the tested 3DP resins is influenced by post-printing processing, which may be related to the leaching of residual components.

Key words: cytotoxicity, 3D printing, oral mucosa cells

INTRODUCTION

The establishment of adequate marginal sealing around indirect restorations on teeth or implants is essential to obtain an adequate emergency profile, as well as to prevent gingival recession and food impaction [38, 46]. In many clinical situations, this process is established under interim restorations phase. Thus, to achieve ideal gingival profile, an adequate response of gingival tissues to interim restoration is necessary, which has been considered as a critical factor for the success of prosthetic reahbilitation, especially on aesthetic areas. [14,48] These characteristics become even more relevant when preparation margin is placed on gingival sulcus[58, 11]. Thus, the biocompatibility of the interim restorations materials with the gingival and periodontal tissues becomes essential, as it allows the establishment and maintenance of a healthy tissue around definitive restoration [48].

Three main parameters are essential for interim restorations: (1) good adaptation and marginal integrity; (2) satisfactory surface roughness; and (3) biocompatibility with surrounding gingival tissues. [4] When these requirements are not met, a clinical condition of gingival irritation and biofilm accumulation can be established, resulting in a local inflammatory reaction. [35, 50, 12] According to literature, the biocompatibility of resins used for temporary restorations with periodontal tissues cells is directly related to the release of unreacted residual toxic monomers. [17, 54, 20, 29] Thus, the cytotoxic potential can vary according to chemical composition and degree of conversion, and these parameters are directly influenced by preparation method. [20] In general, the literature demonstrates that indirect methods have better biological results since the degree of polymerization is more effective, resulting in release of a lower amount of toxic residual monomers, in addition to providing a surface with better finish and less roughness. [17, 20, 34, 51, 48]

With the emergence of 3D printing (3DP), a new category of materials for interim restorations has been introduced in restorative dentistry in recent years, creating an innovative treatment modality. [52] 3DP is a terminology used to describe an additive manufacturing method, where a given object is built by the sequential addition of layers. [36] Despite the great emphasis on the use of 3DP with resins called “biocompatible”, the literature is scarce with regard to biological evaluation, as well as there is little information on all chemical compounds present in these materials and the ideal manufacturing protocol that minimize the possibility of adverse effects. [41, 43] Thus,

it is evident the need, at this moment, to carry out studies that demonstrate the biological compatibility with oral tissues of this category of temporary resins in order to understand the interaction of gingival cells on the surface of these new materials.
In the present study, the biological compatibility of two 3DP resins submitted to different post-curing protocols was evaluated using an organotype cell culture model of oral mucosa cells. Our hypothesis is that the incorrect post-printing processing can influence the cytotoxic potential of these materials.

MATERIALS AND METHODS

Sample preparation: For the present study, three resinous materials used in the clinic (acrylic resin, composite resin and pressed acrylic resin for CAD / CAM) and two 3D printing resins were selected. As a negative control group, a glass coverslip was used. The description of the experimental groups together with their composition is described in Table 1. All resinous materials were initially prepared with 14 mm in diameter and 1 mm in thickness, following the recommendations of each manufacturer. After completing sample preparation, they were dry stored and protected from contact with light for a period of 24 h.

Acrylic resin (AR) and composite resin (CR):): For making the samples, hollow metal matrices were used, which were previously isolated with a thin layer of lubricating gel (K-Med; Cimed, Pouso Alegre, MG, Brazil) and interposed between two glass slides (Exacta, São Paulo , SP, Brazil). The acrylic resin (Dencôr cor 66; Artigos Odontologicos Classico Ltda, Sao Paulo, SP, Brazil) was manipulated (15 seconds) and inserted inside the matrices with digital pressure until the polymerization was completed (4 minutes). To obtain composite resin samples (Filtek™ Z350XT; 3M, Saint Paul, MN, USA), the resin was inserted in a single increment, subjected to manual pressure between the glass slides, and light cured for 40 s on each side with continuous pulse (wavelength 440 - 480nm and light intensity 1,200mW / cm²; DB 685, Dabi Atlante, Ribeirão Preto, SP, Brazil).

CAD/CAM Resin (CC): Initially, the pressed acrylic resin CAD / CAM block (Vipiblock; TRILUX GmbH & Co., Arnsberg, Germany) was transformed into a cylinder after being

cut using a cup saw bit. Next, the 1 mm samples were obtained by sectioning the cylinder on a diamond disk coupled to micro-grinding equipment (Dremel, Racine, WI, USA).

3D Printed Resins (CS-3D e PZ-3D): To print the samples, a CAD project was carried out using the Autodesk MeshMixer software (Autodesk Inc., Mill Valley, CA, USA), the stl file was sliced using the FlashDLPrint software (FlashForge Corporation, Zhejiang Province, China) and the printing code was imported into the DLP FlashForge Hunter printer (FlashForge Corporation) to obtain cylindrical specimens. The samples were printed with layer thickness set at 50 µm and exposition time standardized at 5 seconds with 100% light intensity following the manufacturer recommendations. The printed samples were immersed in isopropyl alcohol for 10 minutes under agitation, and then subjected to post-curing for periods of 1, 10 or 20 min in a UV LED light chamber (Done 3D®, Ribeirão Preto, SP, Brazil) with a wavelength of 405 nm and 90 W of power, under rotation of 15 turns/ min, obtaining the following groups: PZ-3D-1' , PZ-3D-10 'and PZ-3D-20': Prizma 3D Smart Print Bio A1 resin (MakertechLabs, Tatuí, SP, Brazil) submitted to post-curing for 1, 10 or 20 min, respectively; and CS-3D-1', CS-3D-10' and CS-3D-20': Resin Cosmos DLP Temp A1 (Yller, Pelotas, RS, Brazil), submitted to post curing for 1, 10 or 20 min, respectively.

Standardization of samples: Before carrying out the biological experiments, standardization of the surface of the resins was performed. For this, 24 hours after preparation, the samples had their surface polished using 600 and 1200 grit sandpaper (T469-SF-Norton, Saint-Gobam Abrasivos Ltda., Jundiaí, SP, Brazil), respectively. Each sample was sanded 20 times on each side in a pre-determined circuit, with this process being repeated for each sandpaper. The final average thickness of the samples was 0.34 mm, which was determined with the aid of a digital caliper (Model HDCD01150, INGCO, Lençóis Paulista, SP, Brazil), providing a surface area of 3.2 cm^2. The samples were washed separately with deionized water in an ultrasonic vat at 40 kHz and 155 W (Unique, USC - 1800, Indaiatuba, SP, Brazil) for 10 minutes and then sterilized by exposure to ultraviolet (UV) light for 30 minutes on each side. For the glass coverslips used as a control, no treatment was performed, being only sterilized prior to the experiments for 30 min in 70% alcohol, followed by 3 washes in Phosphate

Buffered Saline solution (PBS; ThermoFisher®, Waltham, MA, USA), for 10 minutes each.

Evaluation of surface roughness: The surface roughness was evaluated immediately after obtaining the samples, after surface polishing procedure and after 7 days of immersion in the culture medium (transwell model) (n = 4). The discs (14 mm diameter x 0.34 mm thickness) were stabilized with dense silicone and evaluated using a portable rugosimeter (Hommel Tester T1000; Jenoptik AG, Jena, TH, Germany). Five readings were taken in each period, randomly over the entire surface of the samples, with the values of surface roughness obtained through the arithmetic mean (Ra). The Ra parameter translates the value of the arithmetic mean of all the absolute distances of the roughness profile (R), from the central line within the Lm measurement extension. The parameters employed were: Lc = 0.25 mm, Lt = 4.8 mm, Lm = 1.25 mm, being: Lc = cutt-off (filtering, minimizing the surface ripple interference); Lt = tracing limit (actual extent traveled by the probe tip); Lm = measurement limit (considered extent of reading).

***In vitro* biological evaluation:** Two experimental models were performed using immortalized normal oral keratinocytes spontaneously immortalized (NOK-Si; RRID:CVCL_BW57) and a 3D culture of immortalized normal human gingival fibroblasts (HGF-1; ATCC CRL-2014). The cells were cultured in 100 cm^2 ventilated petri dishes (CELLSTAR®; Greiner Bio-One, Americana, SP, Brazil), containing Dulbecco´s Modified Eagle´s Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO®, Invitrogen, Carlsbad, CA, USA), 100 IU/mL L-glutamine (GIBCO®), 100mg/mL penicillin-streptomycin (GIBCO®) and 2 mmol/L of glutamine (GIBCO®), being maintained at 37°C and 5% CO_2 until reaching 80% confluence.

Organotypic model of oral mucosa cells: In order to simulate the tissue organization of oral mucosa and to create an *in vitro* organotypic model to evaluate the cytotoxicity of resinous materials, an experimental model of co-culture was performed using transwell inserts (0.4 µm pore polystirene membrane; Corning®; New York, NY, USA). Prior to the experiment, the transwells were distributed inside 24-well plates (Greiner Bio-One) and incubated in complete DMEM culture medium at 37°C and 5% CO_2 for 60 min.

After this period, the medium was aspirated and the transwells were placed inverted in sterile petri dishes (Greiner Bio-One), in order to keep the lower membrane facing upwards. NOK-Si cells were seeded (5 x 10^4 cells/membrane) in a single drop of 30 μl on the lower membrane surface, and the cell/transwell constructs were incubated at 37°C and 5% CO_2 for 30 min to allow initial cell adhesion. Next, the transwells were placed on 24-well plates and 1 ml of complete DMEM culture medium was added, followed by incubation for 24 hours to allow cell growth.

After this period, 3D cultures of HGFs were obtained in an environment at 4°C, from the dissolution of type 1 collagen (3.7 mg/mL; Corning®) in 10x concentrated culture medium, in a 4: 1 ratio, followed by neutralizing the pH in 7.2 with 5 M sodium hydroxide. A total of 1 x 10^5 HGFs in 10 µL were incorporated into the final volume of 200 µL of the 3D collagen culture. Then, 100 µL of the resulting solution was applied in the upper compartment of the transwells, previously seeded with NOK-Si cells, followed by incubation at 37°C and 5% CO_2 for 30 min to allow the gelation of collagen matrix. At the end, a total of 450 μL of DMEM culture medium was added to the upper compartment of the transwells, and the co-culture was incubated for 48 h. After this period, the transwells containing the co-culture were transferred to 24-well plates containing the resin samples (14 mm in diameter and 0.34 mm in thickness) so that they remained in close contact with the lower membrane of the transwells where NOK-Si were previously seeded. Glass slides were placed at the bottom of the compartments in the negative control group (GS group). The set was incubated in 1 mL of culture medium in order to standardize an extraction ratio at 3 cm^2/mL following the recommendations of ISO 10993-12: 2012. Figure 1A illustrates this experimental model.

Direct contact model of gingival fibroblasts in 3D culture: This experimental model was carried out in order to simulate a condition of exposure of gingival connective tissue in direct contact with resins. The 3D culture was formulated as previously described. The samples were placed on the bottom of 24-well plates (Greiner Bio-One) and stabilized with a silicone matrix. The 3D culture of the HGF-1 was positioned on the surface of the materials, with a volume of 1 mL of culture medium added for cell culture (extraction ratio at 3 cm^2 /mL; ISO10993-12: 2012) (Figure 1B).

Cell metabolism and proliferation: To evaluate cell growth and quantify metabolically viable cells, the Alamar blue assay (Life Technologies, Carlsbad, CA, USA) was performed in periods of 1, 3 and 7 days of culture (n = 4). Therefore, the 3D culture and the transwells containing NOK-Si on the lower membrane were separately incubated for 4 hours at 37°C and 5% CO_2 in DMEM solution without FBS containing the Alamar Blue reagent, in a 10:1 ratio. After this period, the supernatant was transferred to 96-well plates and the fluorescence measured at 540 nm excitation and 590 nm emission (Synergy H1, Biotek, Winooski, USA). The mean fluorescence value obtained in the control group (GS) in the period of 1 day was used as a parameter of 100% of cellular metabolism to indirectly calculate cell proliferation. The percentage of cell metabolism reduction was calculated to graduate the cytotoxicity based on ISO 10993-5:2009 recommendations into: **Non-cytotoxic**: < 20% of cell metabolism reduction; **Slight**: 21-30% of cell metabolism reduction; **Mild:** 31-50% of cell metabolism reduction; **Moderate:** 51-70% of cell metabolism reduction; **Severe:** > 70% of cell metabolism reduction. For that, the mean absorbance value of the GS group was considered as 100% of cell metabolism at each time-point; then, % of cell metabolism reduction was calculated by using the following equation: %CMr = 100 – %CMi, where %CMr means percentage of cell metabolism reduction and %CMi is percentage of individual cell metabolism value for each sample.

Cell viability: To assess the presence of viable cells at 1, 3 and 7 days of culture, the cells under the lower membrane of transwells and in the 3D cultures were washed in PBS and incubated separately for 45 min at room temperature in culture medium supplemented with 4 mM Calcein AM (green fluorescence = viable cells) and 2 mM Ethyl Homodimer-1 (red fluorescence = dead cells) (Live / Dead cell viability / cytotoxicity kit; Invitrogen, San Francisco, CA, USA). Next, the transwells and 3D cultures were washed with PBS, and placed on glass slides for analysis of viable and dead cells, using a fluorescence microscope (FLoid Cell Imaging Station, Applied Biosystems, Frederick, MD, USA).

Spectral evaluation of the culture medium: The culture medium in which the resins were immersed to perform the co-culture model in transwells was evaluated using a UV-Vis spectrophotometer (Synergy H1, Biotek) to detect the presence of components released by the resins. This test was carried out in order to complement the obtained

cytotoxicity data, since most components of 3D printing resins have patent confidentiality, and it is not possible to carry out an accurate identification of the components released in the culture medium. For this purpose, the culture medium of the upper and lower compartments of the transwell were collected separately for each medium change performed during the Alamar Blue test, being frozen at -20ºC until analysis. A volume of 200 µL was transferred to 96 wells ultra-clear reading plates (Corning® UV-Transparent Microplates) with a spectral scan from 240 to 450 nm, with 10 nm intervals in order to identify the absorbance peaks. Next, a comparative analysis at 270 nmm was performed to compare experimental groups. In all the analyses carried out, the culture medium of the GS group was used as a blank. To validate the analysis of the culture medium, a pilot study was initially performed in ultra-pure water (ThermoFisher Scientific), with the release of components in the same range being observed as those detected on culture medium, indicating that the absorbance detected came from components of the resins, without interference from the cells or the culture medium.

Statistical Analysis

Three independent experiments were performed. Data were compiled and analyzed by two-way ANOVA and Tukey's test for observation of the significant differences between the study groups ($p < 0.05$ = statistically significant).

RESULTS

Roughness analysis: Figure 2 shows the results of the surface roughness. It is observed that immediately after the preparation of the samples, there is a great variability between the different resinous materials. After the polishing procedure, there was an effective standardization of the surface roughness, which remained unchanged for all groups after 7 days of immersion in culture medium, with no significant differences between the experimental groups.

3D co-culture model in transwells - NOK-Si cells: The Alamar Blue assay for NOK-Si cells (Figure 3) demonstrated no significant difference between the CR and CC groups with the negative control (GS) in all periods of analysis, being categorized as non-cytotoxic (Table 2). Among the resins used as a control, only the AR group showed

a significant reduction compared to the GS in the periods of 1 and 3 days, presenting mild and slight cytotoxicity, respectively. The GS, AR, CR, CC groups showed a significant increase in cell metabolism between the periods of 1 and 3 days, demonstrating the proliferative capacity of the cells in contact with the evaluated materials. As for 3D printing resins, only the PZ-3D-20 'group did not show a significant difference with the GS in the periods of 1 and 3 days, also showing cellular metabolism values significantly higher than the other 3D printing resin groups, with the exception of the PZ-3D-10 ' in 1 day. The PZ-3D-1' and CS-3D-1' groups showed the lowest values of cell viability in all periods of analysis, with a significant difference with all other experimental groups. According to the gradation of cytotoxicity, 3D printing resins showed severe, slight/ moderate, and non-cytotoxic/slight cytotoxicity for the 1, 10 and 20 min post-cure protocols, respectively (Table 2). In the evaluation over time, a significant reduction in cell metabolism was observed at 7 days for post-cured resins for 1 min, while the others showed an increase in metabolism in this period of analysis. The images obtained by the Live / Dead test corroborate the data from the Alamar Blue, showing that the reduction in cellular metabolism was, at least in part, a result of the reduction in the viability of cells in contact with the resinous materials. The presence of viable NOK-Si cells covering the transwell surface was observed in all periods of analysis for the GS, CR and CC groups. A slight reduction in the number of adhered cells can be seen in the AR compared to these groups (Figure 4). Regarding 3D printing resins, there is a noticeable reduction in viable NOK-Si cells that remained adhered to transwell membrane after 1 day of incubation with the resins subjected to post-cure for 1 min, with a gradual reduction over time and the presence of cells positively labeled for Ethyl Homodimer-1. For the 10 min post-cure period, a similar pattern can be observed between the two resins tested, with a reduction in the number of viable cells that remained adhered to the transwell in the period of 1 day, and a gradual increase over time. A pattern similar to AR can be observed for the PZ-3D-20' group, while the CS-3D-20' was similar to the GS, CR and CC groups (Figure 5).

Significant reduction in cellular metabolism (Figure 6) relative to GS was observed only for the PZ-3D-1' and CS-3D-1' groups, in all periods of analysis, and were categorized as slight/mild. The PZ-3D-10' group showed a slight reduction in cellular metabolism at 3 and 7 days compared to GS. The resins post-cured for 20 min were non-cytotoxic in all analysis periods (Table 2).

3D co-culture model in transwells - 3D culture HGFs: Significant reduction in cellular metabolism (Figure 6) in relation to the GS was observed only for the PZ-3D-1 'and CS-3D-1' groups in all periods of analysis, being categorized as slight /mild. The PZ-3D-10 ’group showed a reduction in cell metabolism in the periods of 3 and 7 days compared to the GS. In the analysis over time, a significant reduction in cell metabolism at 7 days was also detected only for the 3D printing resin groups with 1 min post-cure, while the other groups did not show significant differences in cell metabolism values between the analysis periods. The post-cured resins for 20 min were non-cytotoxic in all periods of analysis (Table 2). The 3D culture images of the HGFs shown in Figures 7 and 8 demonstrated a similar pattern of viability for the GS, CR, AR, CC, PZ-3D-10', PZ-3D-20', CS-3D-10 'and CS-3D-20' groups in all periods of analysis. Presence of less viable cells and positive Ethyl Homodimer-1 labeling can be observed for PZ-3D-1 'and CS-3D-1', especially in the periods of 3 and 7 days.

3D culture model in direct contact: In the direct contact assay, the Alamar Blue results demonstrated that only the PZ-3D-1 'and CS-3D-1' groups promoted a significant reduction in the metabolism of HGF cells in 3D culture compared to the GS and all others experimental groups (Figure 9). The resins in these groups were categorized as moderate cytotoxicity, while all other resinous materials tested were non-cytotoxic (Table 2). The Live / Dead assay (Figures 10 and 11) demonstrates the presence of viable cells in the 3D culture in all periods of analysis and in a similar way for the GS, CR, AR, CC, PZ-3D-10 ', PZ -3D-20 ', CS-3D-10' and CS-3D-20' groups. The PZ-3D-1' and CS-3D-1' groups showed a notable reduction in the amount of viable cells present in 3D culture in all the periods and in comparison with the other experimental groups.

Culture medium analysis: The spectral evaluation demonstrated the presence of absorbance area in the range of 240-280 nm for the PZ-3D-1 'and CS-3D-1' groups in all periods of analysis and in both extracts evaluated (upper and lower transwell compartments) (Figure 12A). Figure 12B shows the results for the absorbance at 270 nm. There was a significant difference in the release of components from 3D printing resins when they were post-cured for 1 and 10 min, this release being proportional to the post-curing time and more intense for the PZ-3D group. It is also possible to verify

that there was a reduction in the release of resin components over time, with the release being more intense at 1 and 3 days.

DISCUSSION

The incorporation of 3D printing (3DP) in dentistry is already a reality, and the technique adds to the rehabilitation treatment greater autonomy, replicability, lower cost, practicality and less work time, while allowing the manufacture of prosthetic components with mechanical properties and adequate adaptation. [27, 1, 40, 39] However, information about the chemical composition and biological parameters of these materials is still scarce. [52, 41, 25] In addition, the variety of techniques and resins for 3DP, as well as the different printing protocols used, make it difficult to classify and compare the properties of these materials. [52] This is particularly critical when considering applications where the printed devices will remain in contact with the patient for long periods, as in the case of temporary prostheses, as the behavior of these materials in the oral environment over time is not well described. According to a literature review carried out by Revilla-León et al. (2019), 3DP dental resins for clinical use are classified as medium risk by regulatory agencies (Class II), showing an indication of continuous use over a maximum period of 30 days. Thus, the biological characteristics of 3DP resins need to be better understood to enable their use in long-term treatments. [15, 55, 60]

In the present study, the cytotoxicity of 3DP resins submitted to different post-printing treatments was tested, along with resinous materials widely used in clinical practice (acrylic resin, composite resin and temporary resin pressed for CAD / CAM). The methods traditionally used to evaluate the cytotoxicity of dental materials are based on the ISO10993-5 standard, highlighting the indirect contact model, where extracts collected for a certain period of time from standardized specimens are applied for short periods in cells seeded in monolayers. [19] Another recommended method is the direct contact test, where monolayer cells are seeded directly onto materials surface, which approximates, at least in part, the laboratory experimentation with the clinical application. [51] However, it has been shown that the behavior of cells maintained in monolayer differs from that observed *in vivo.* [33, 8, 6] The use of 3D cultures has been considered as a suitable model to mimic the clinical situation as it allows 3D interaction

with neighboring cells and the extracellular matrix. Direct contact tests of dental materials with 3D cultures have been used to evaluate the biological effects of dental materials with oral and dental tissues. According to them, the interface material/3D culture allows direct cell interaction with material surface morphology, and the establishment of a kinetic release pattern of soluble components at this interface as it is expected that release of soluble material compounds will be initially high and decrease over time, thus creating a realistic microenvironment. [47, 56] Collagen type I has been considered the ideal scaffolding material for 3D culture formulation as it simulates composition connective tissues, providing a stable matrix that allows cell adhesion and migration within its structure, and cell growth in a 3D network. [47, 56, 8, 13] Organotypic models [24, 5] of epithelium and skin based on acellular dermal matrix have been widely accepted in the literature as a substitutive model to animals in testing dermatological products [26] Basso et al., (2018a) translated this technology to create a 3D full oral epithelium cell culture model, in which gingival keratynocites were seeded onto acellular dermal matrices. In a sequential study, they were able to propose different organotypic full-thickness oral mucosa strategies in which gingival keratynocites and fibroblasts were co-cultured. [6] The authors found out that a collagen 3D matrix allowed for better gingival fibroblasts infiltration and distribution than dermal matrices, exhibiting similar cytoplasmatic projections as normal gingival tissue. This collagen 3D model also allowed adhesion and establishment of a keratinocytes layer. In the present study, an *in vitro* organotypic model was proposed, where a monolayer of human keratinocytes (NOK-Si cells) was established in the lower membrane of the transwell device, and a 3D culture of human gingival fibroblasts in a type 1 collagen matrix was placed in intimate contact on the opposite side of this membrane. This structure allowed the easy handling of the 3D co-culture, as well as evaluating cells separately. In addition, we performed a direct contact assessment of the 3D culture of the HGFs on the samples in order to mimic the condition of exposed connective tissue. In order to standardize the surface roughness and thickness of the samples, they were subjected to a standard polishing protocol [51], and this surface remained stable after the entire cell culture period in our experimental model. It is recommended that the materials used for making prostheses have the most polished surface possible to favor both aesthetics and periodontal health, since rough surfaces favor the retention of biofilm. [31] In this respect, 3DP resins are capable of presenting values similar to conventional materials for temporary prostheses, as observed in the present study,

without showing differences in biofilm formation. [62, 3, 49] However, according to Revilla-León et al. (2020), this parameter is highly dependent on the additive manufacturing technique, the material and the printing and post-printing protocol used.

According to our results, the composite resin and acrylic resin pressed for CAD / CAM did not have a cytotoxic effect in both study models. The acrylic resin significantly reduced cellular metabolism in relation to the control group by 37.8% and 30.5% at 1 and 3 days, respectively, only for the NOK-Si cells, which were in close contact with the sample surface. These results corroborate the findings by Souza et al. (2020), where NOK-Si cells seeded directly on samples of conventional acrylic resin and bisacrylic resin showed reduced metabolism and cell viability, while cells seeded onto samples of pressed acrylic resin CAD / CAM showed no changes in these parameters. According to the authors, this positive effect may be related to lower release of residual monomers for CAD / CAM resins in comparison to chemically cured acrylic resins. [32, 16] With regard to composite resins, the literature demonstrates that cytotoxicity is directly related to resin composition and degree of polymerization. Nanoparticulate and nanohybrid resins constantly show low cytotoxicity with gingival cells due to the limited release of residual monomers [30, 61, 10,] as observed in our study.

With regard to 3DP resins, it is possible to observe an intense reduction in the metabolism and viability of NOK-Si cells on PZ-3D 1 'and CS-3D 1' groups at all times of analysis. This impact was even more striking at 7 days, suggesting cumulative damage over time. Taking into account the proposed cytotoxicity gradation, we can consider that both resins submitted to post-curing for 1 min were severely cytotoxic throughout our experimental period. These groups also showed the most intense cytotoxic effects in HGF 3D culture, leading to a significant reduction in cell metabolism and cell viability, which has intensified over time. However, this effect was milder than that observed for the NOK-Si, varying between slight and mild. Resins post-cured for 10 min showed similar biological behavior for the NOK-Si cells, leading to a significant reduction in cell metabolism compared to the control, varying from slight to moderate, associated with a reduction in the amount of viable cells present in the transwell membrane. However, the PZ-3D-10 'group was slightly more cytotoxic to HGFs, while the CS-3D-10' was non-cytotoxic. In general, both resins post-cured for 20 min showed better biological results in both cells of the co-culture, with the CS-3D-20' being the one that presented parameters similar to the negative control, composite resin and pressed acrylic resin for the CAD-CAM. In the experimental 3D culture direct contact

model, we could observe that only the resins subjected to post-curing for 1 min promoted a significant reduction in cell metabolism and viability, categorized as moderate to severe.

Chen et al. (2020) recently performed an evaluation of the cytotoxicity of two resins for interim restorations (Enlighten AA temp and NextDent C&B), printed by the DLP system and subjected to different post-curing processes. The authors incubated mouse fibroblasts (L929) for 24 hours with the extracts collected from the samples (3 cm^2 / mL), according to the recommendation of the ISO10993-5 standard (24 hours of incubation). Using this experimental model, the authors observed that only resins not submitted to the post-cure procedure showed a reduction in cell metabolism above 70%, while the other processes, including times of 1, 5, 10, 15 and 30 minutes in a post-curing chamber with parameters similar to that used in our study, did not show a significant reduction in cellular metabolism (> 20%). The authors suggest that in the absence of post-curing, a surface seal of the samples was not obtained to prevent the diffusion of resin from the interior of the pieces to the culture medium. Additionally, the authors tested a post-curing chamber with heating (60ºC) and did not observe significant differences in the cytotoxicity of the resins compared to the conventional chamber.

Other studies carried out biological evaluations of 3D printing resins used in dentures. Tzeng et al. (2021) evaluated five formulations of composite resins for 3D printers (DLP) based on urethane acrylate and compared their results with a commercially available 3D printing acrylic resin for dental prosthesis. The tested materials and the control group received different post-curing times (0, 15 and 30 min) and the authors were able to see improvements in the mechanical properties with 15 min post-cure; however, no statistically significant differences were found between the 15 min and 30 min post-cure. The authors also evaluated cytotoxicity for L929 cells cultured in printed resin extracts showing relative cell viability above 70% for all groups tested. Bayarsaikhan et al. (2021) evaluated different post-cure times and temperatures of printed teeth on the viability of human fibroblasts and were able to find that the increase in post-cure time and temperature reduced the cytotoxic effects in all evaluated materials. This result is compatible with our findings, since cell viability increased with the time of post-curing.

In the present study, the additive manufacturing technique used was Digital Light Processing (DLP), which emits a cross-sectional image of the object through a

projector, polymerizing an entire layer instantly. [22] With this, DLP printers are shown to be faster, while providing high quality prints, being widely recommended for printing in the dental area. [15] The curing of 3DP resins occurs through the polymerization of methacrylate through free radicals generated by the activation of the photoinitiator from the printer's energy source. [2] However, not all the resin of the object will have gone through this process at the end of printing, being of paramount importance the removal of residual monomers, since they can be released in the oral environment due to mechanical degradation and exposure to saliva, bacterial enzymes and the hydrolysis process. [21, 57, 63, 18] For this reason, post-printing treatment is an important phase of the additive manufacturing process, being responsible for detoxifying and strengthening the printed part. Thus, immediately after printing, the piece is washed with alcohol to remove all unpolymerized material from the object's surface, followed by post-curing to enhance their mechanical and biological properties such as UV-light and thermal polymerization. [37] As a result, post-curing can be considered as an essential step to promote complementary polymerization of the printed material through the conversion of residual monomers into polymers in the outermost layers. [37]

Cell viability and proliferation depend not only on post-print processing, but also on the components of the resins. The depth of cure is directly linked to these components, the intensity of the light and the time of exposure of the object to the light source. [23] In the label of 3DP resins tested in this experiment, oligomers, monomers, photoinitiators, stabilizer and pigment are listed, however, they are not specified. Thus, in the present study, a spectral evaluation of the culture medium in contact with the resins was carried out in order to try to detect the presence of components leached by the resinous materials. It was possible to observe an absorbance pattern between 240-280 nm for the post-cured resins for 1 and 10 min, being more intense for the PZ-3D-1 'group. In addition, we found that the release of these components was time-dependent, with a gradual reduction over time; however, it is important to note that there was a continuous release for the post-cured resins for 1 minute, which can be related to the greater cytotoxicity observed for these experimental groups in all evaluated models. The other resins that showed component leaching showed an intense drop in release after 24 hours, which may be related to better metabolism recovery capacity and cell viability in the periods of 3 and 7 days. Rogers et al. (2021) found severe reproductive cytotoxicity in oocytes of female mice exposed directly and indirectly to two oral surgical guides 3DP resin. The authors performed detected by mass spectroscopy

substances considered dangerous. They are: methacrylic oligomers, phosphine oxides, glycol methacrylate and pentamethyl-piperidyl sebacate. This study shows the importance of clarifying the chemical components present in resins for 3DP.

It is important to note that despite the improvement of the biological aspects found in our results, excessive post-curing time in a UV light chamber can lead to distortions in the piece printed by the DLP method, making it necessary to make adjustments to the printing parameters: print intensity UV, printing time of a single layer, height of a single layer, intensity of UV post-curing and thickness of the piece to be printed. [59] Chen et al. (2020) also detected a tendency to increase the cytotoxicity of printed resins with excessive post-cure times, such as 30 minutes which was related to sample distortion. The post-cure times of 10 and 20 minutes selected in the present study were based on the manufacturers' recommendations for the evaluated resins, with the time of 1 minute being included to assess a critical post-processing situation of the resins in a non-recommended manner. According to our results, we can see that an inadequate post-cure time can negatively influence the biological compatibility of resins, serving as a warning for professionals who intend to use this category of material in the restorative clinic. In this way, provisional printed restorations have biological behavior *in vitro* similar to nanoparticulate composite resins and restorations milled in CAD / CAM, when the post-processing is carried out properly. However, further studies are still needed to ensure its routine use in clinical practice.

CONCLUSION

The biological compatibility of 3D printing resins for interim restorations can be negatively influenced by inadequate post-cure processing, which may be related to intense leaching of unreacted components when these materials are immersed in a humid environment. Thus, the post-processing of this category of resins must strictly follow the manufacturer's recommendations to ensure biocompatibility with the oral tissues that will remain in close contact with these materials.

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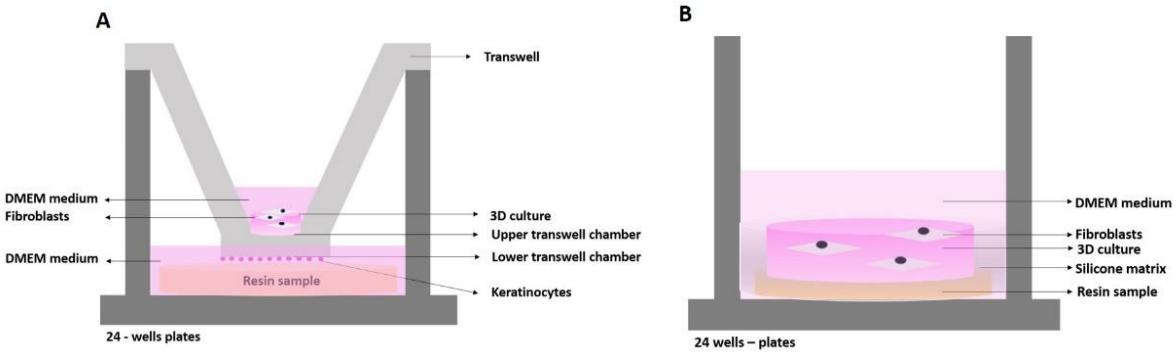

Figure 1. Schematic representations of 3D co-culture model in transwell (A) and 3D culture model in direct contact with samples (B).

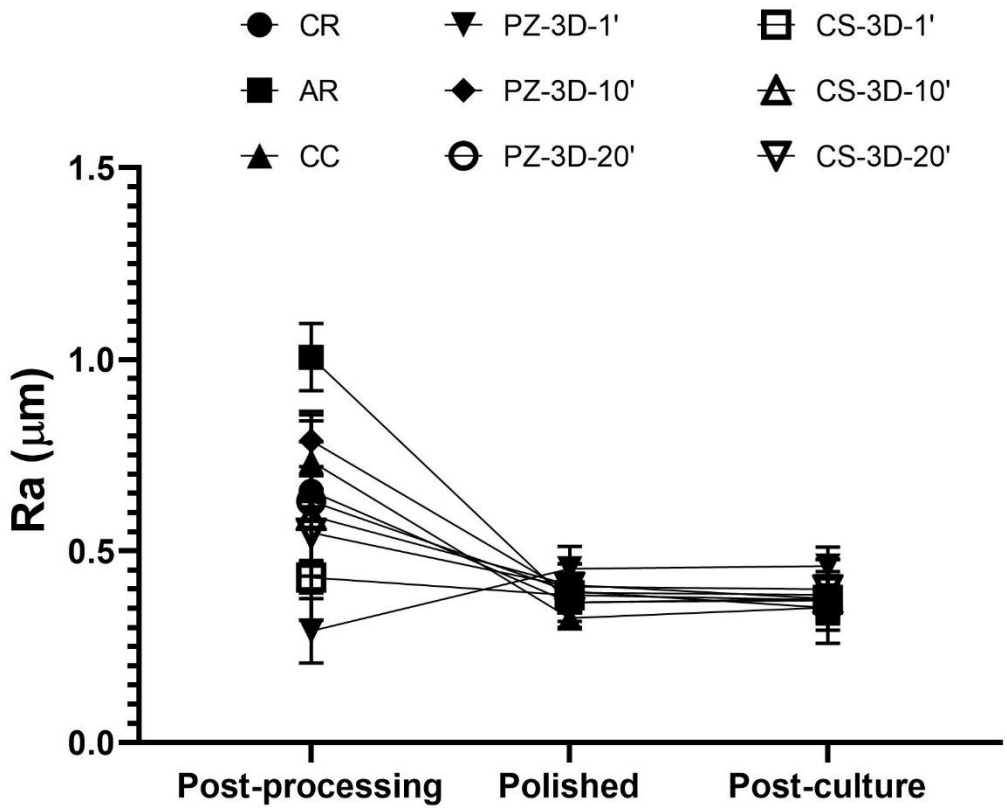

Figure 2. Graph of mean and standard deviation Ra values of resinous samples throughout periods of analysis

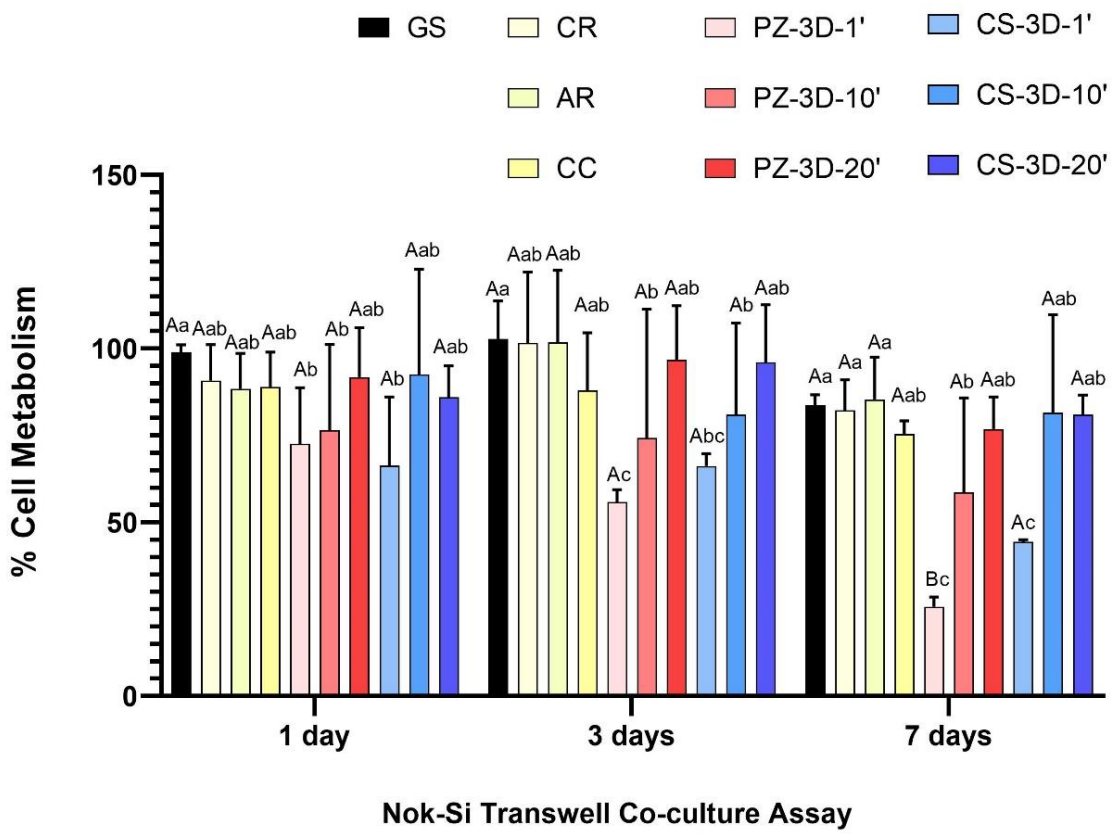

Figure 3. Cell metabolism analysis (% GS) of Nok- Si cells (n=6). Data are mean values and standard deviation. Upper-case letters allows comparison among time-points for each group; lower-case letters allows comparison among groups, at each time-point (Two-way ANOVA; Tukey´s test. $P < 0.05$).

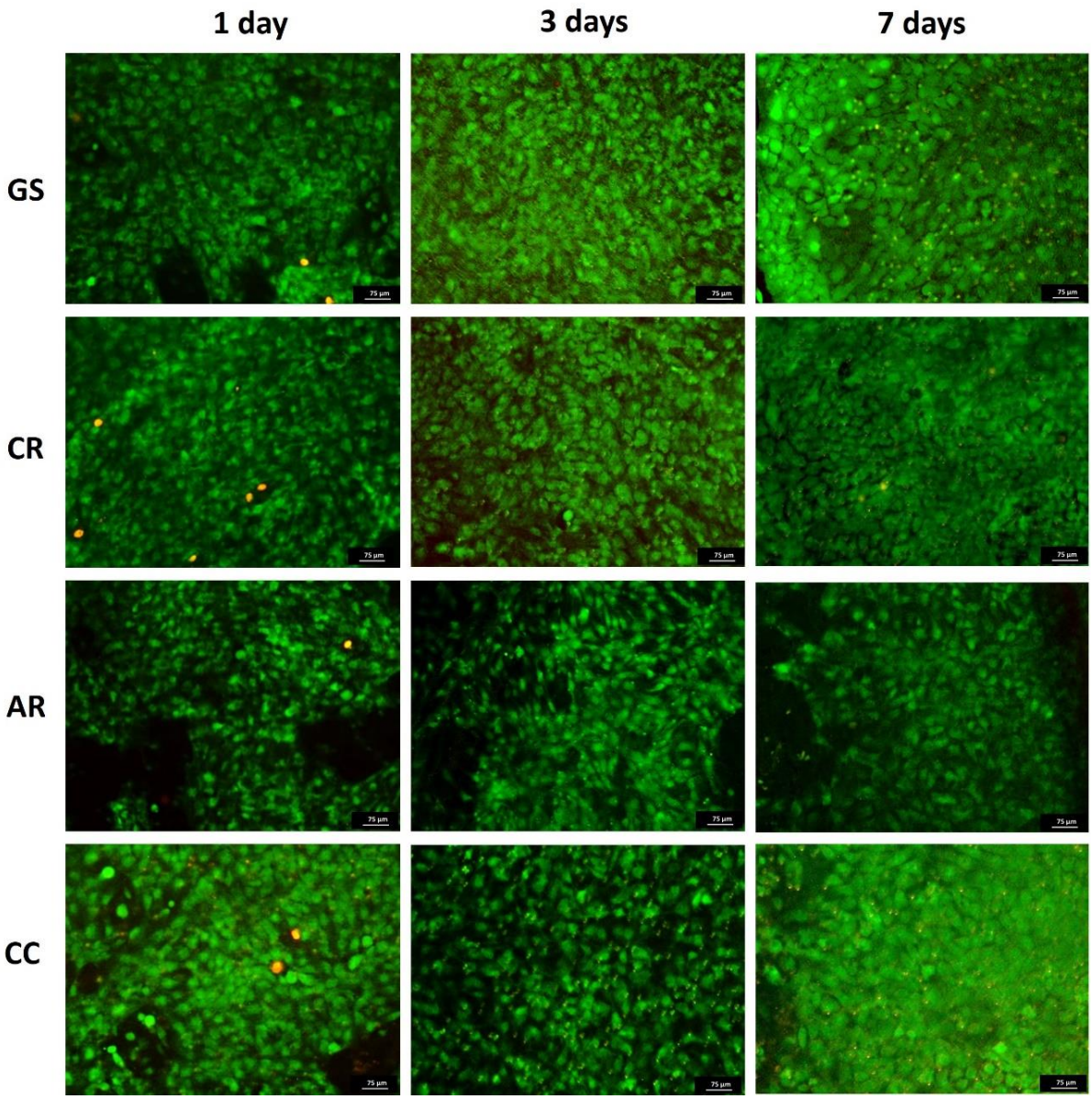

Figure 4. Panel of Live/Dead assay for Nok-Si cells – control samples. Live cells = green; dead cells = red. Original magnification ×20.

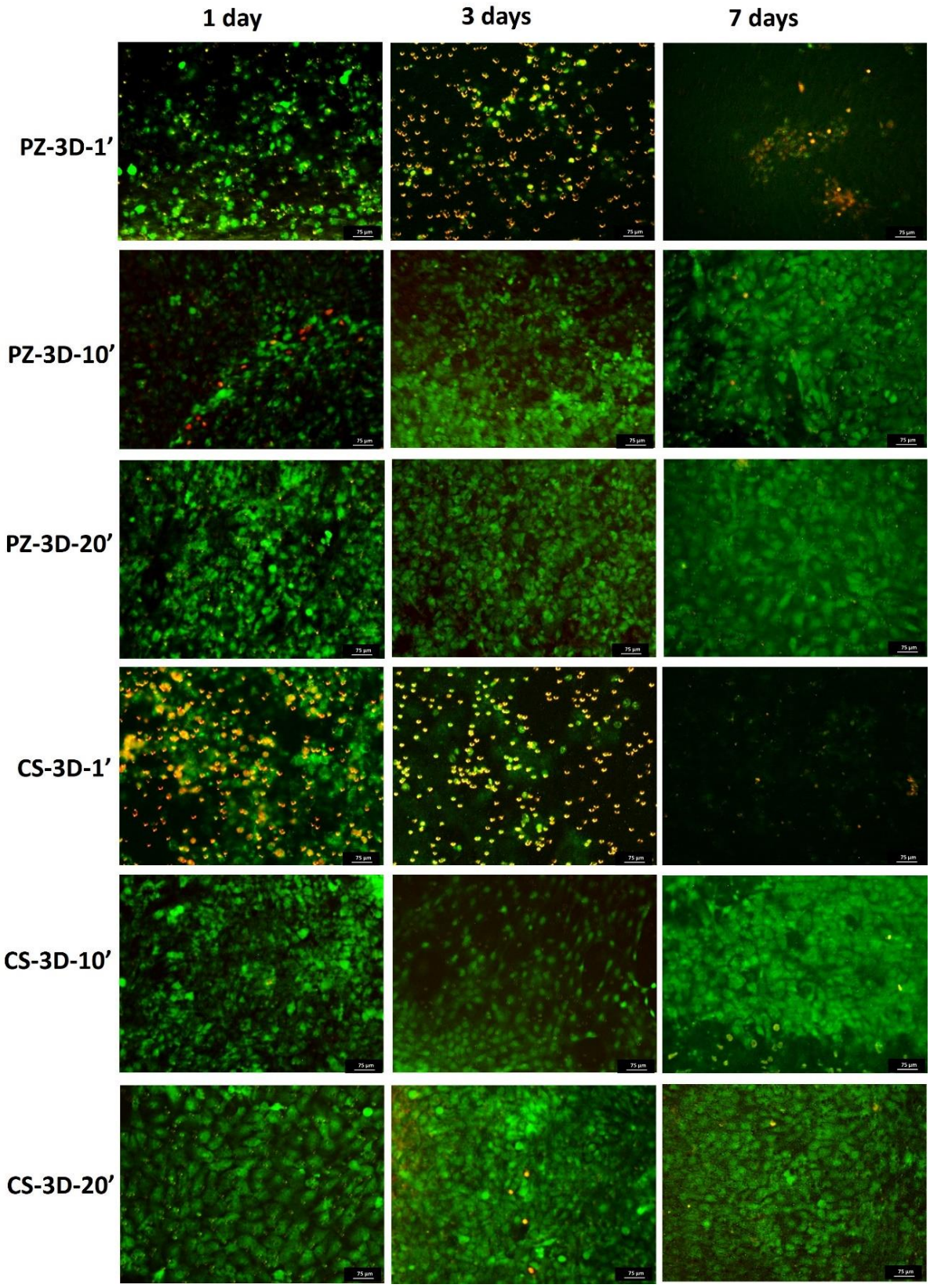

Figure 5. Panel of Live/Dead assay for Nok-Si cells – 3DP samples. Live cells = green; dead cells = red. Original magnification ×20.

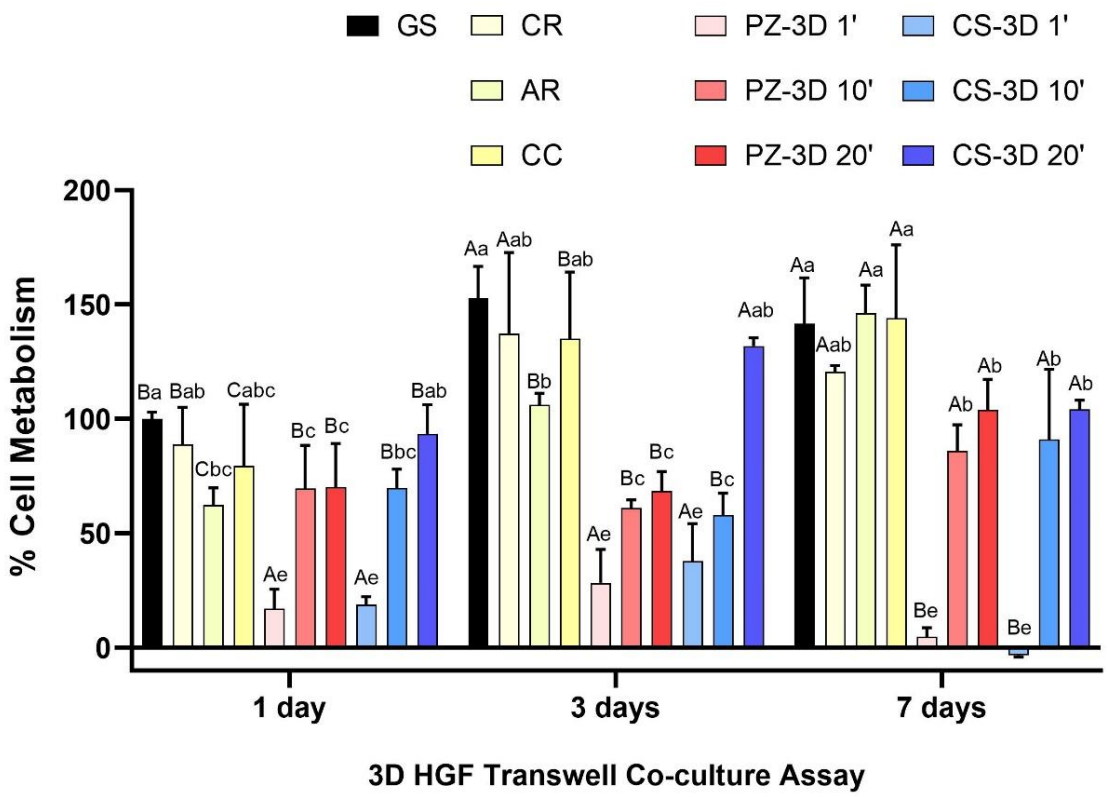

Figure 6. Cell metabolism analysis (% GS) of HGF 3D culture on transwell assay (n=6). Data are mean values and standard deviation. Upper-case letters allow comparison among time-points for each group; lower-case letters allow comparison among groups, at each time-point (Two-way ANOVA; Tukey´s test. $P < 0.05$).

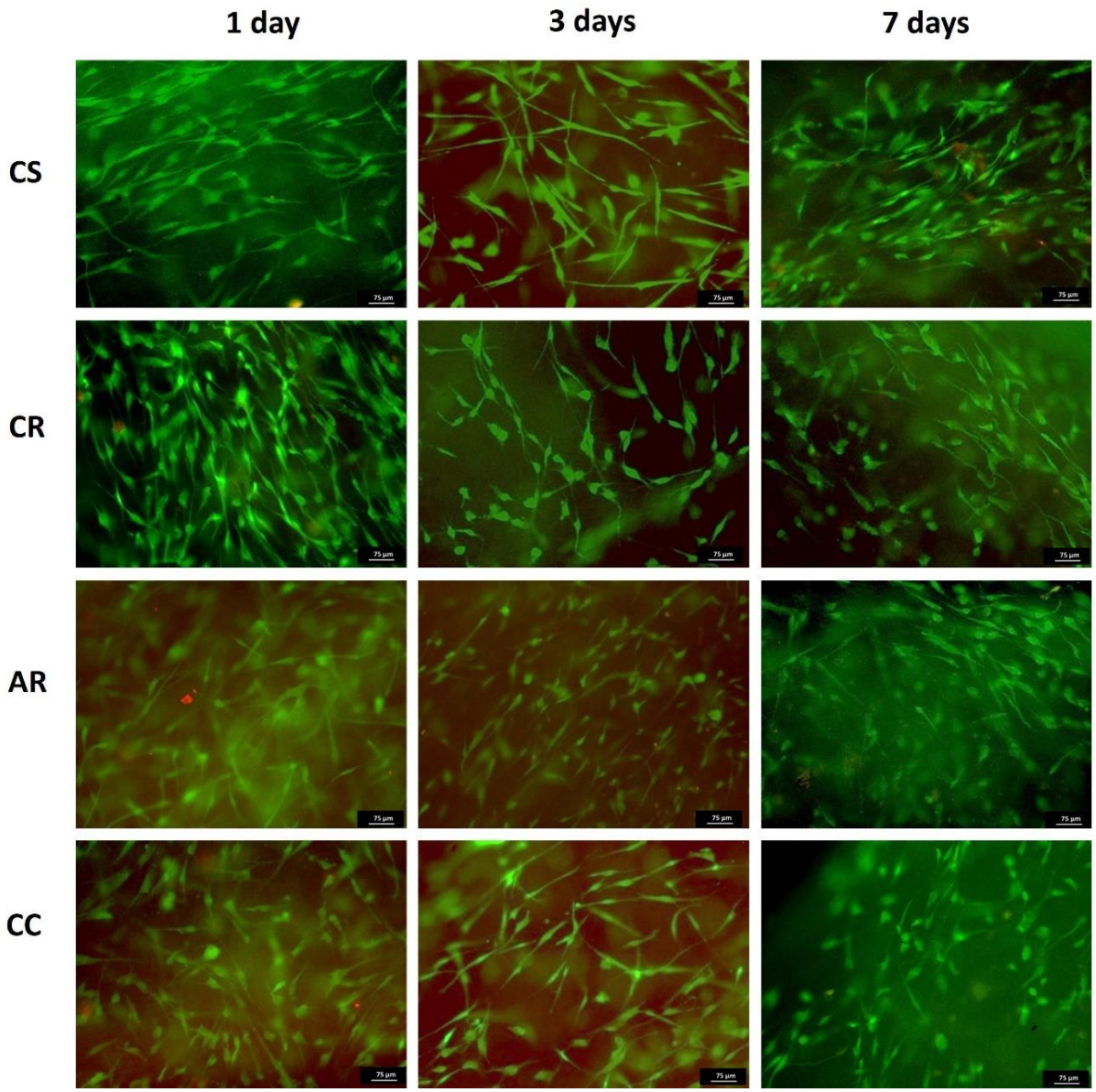

Figure 7. Panel of Live/Dead assay for HGF 3D culture on transwell assay – control samples. Live cells = green; dead cells = red. Original magnification ×20.

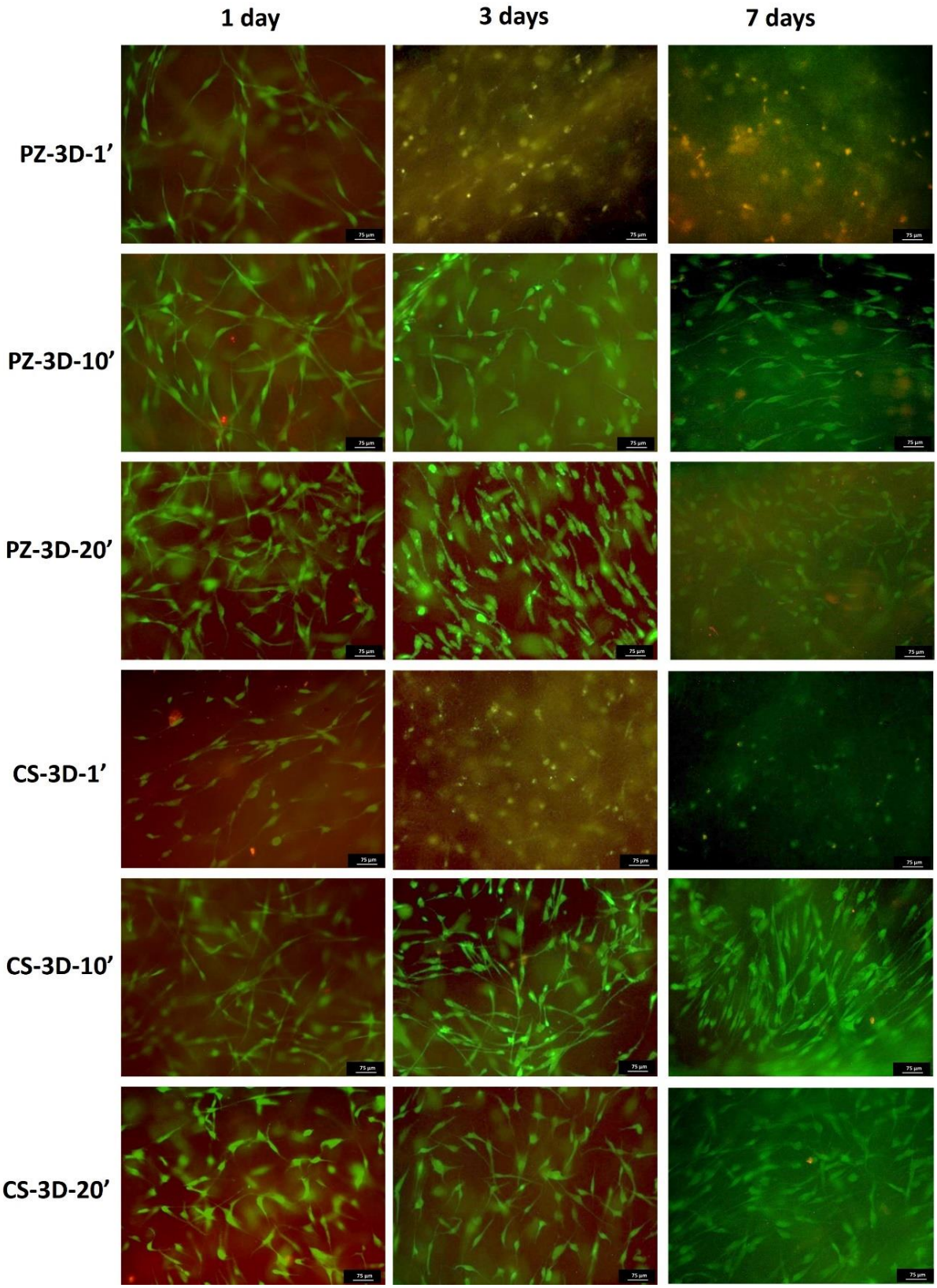

Figure 8. Panel of Live/Dead assay for HGF 3D culture on transwell assay – 3DP samples. Live cells = green; dead cells = red. Original magnification ×20.

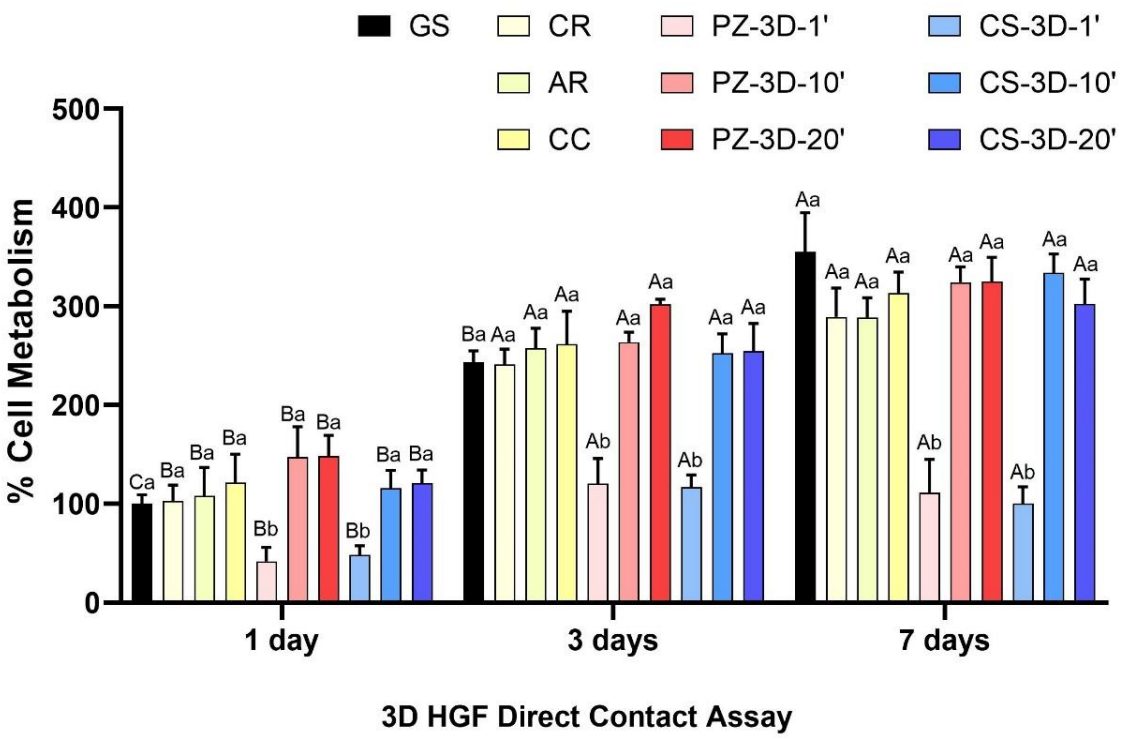

Figure 9. Cell metabolism analysis (% GS) of HGF 3D culture on direct contact assay (n=6). Data are mean values and standard deviation. Upper-case letters allow comparison among time-points for each group; lower-case letters allow comparison among groups, at each time-point (Two-way ANOVA; Tukey´s test. $P < 0.05$).

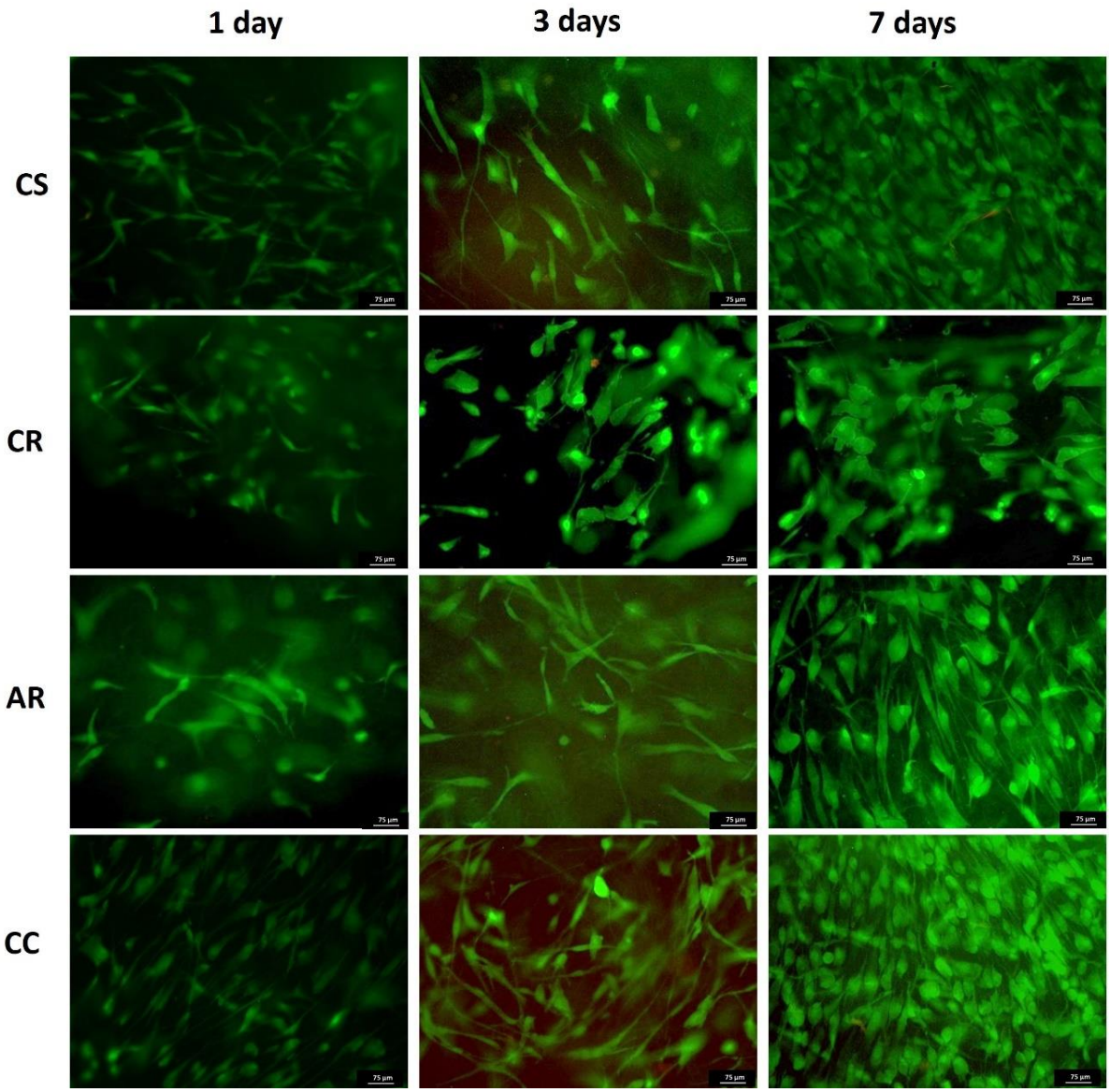

Figure 10. Panel of Live/Dead assay for HGF 3D culture on direct contact assay – control samples. Live cells = green; dead cells = red. Original magnification ×20.

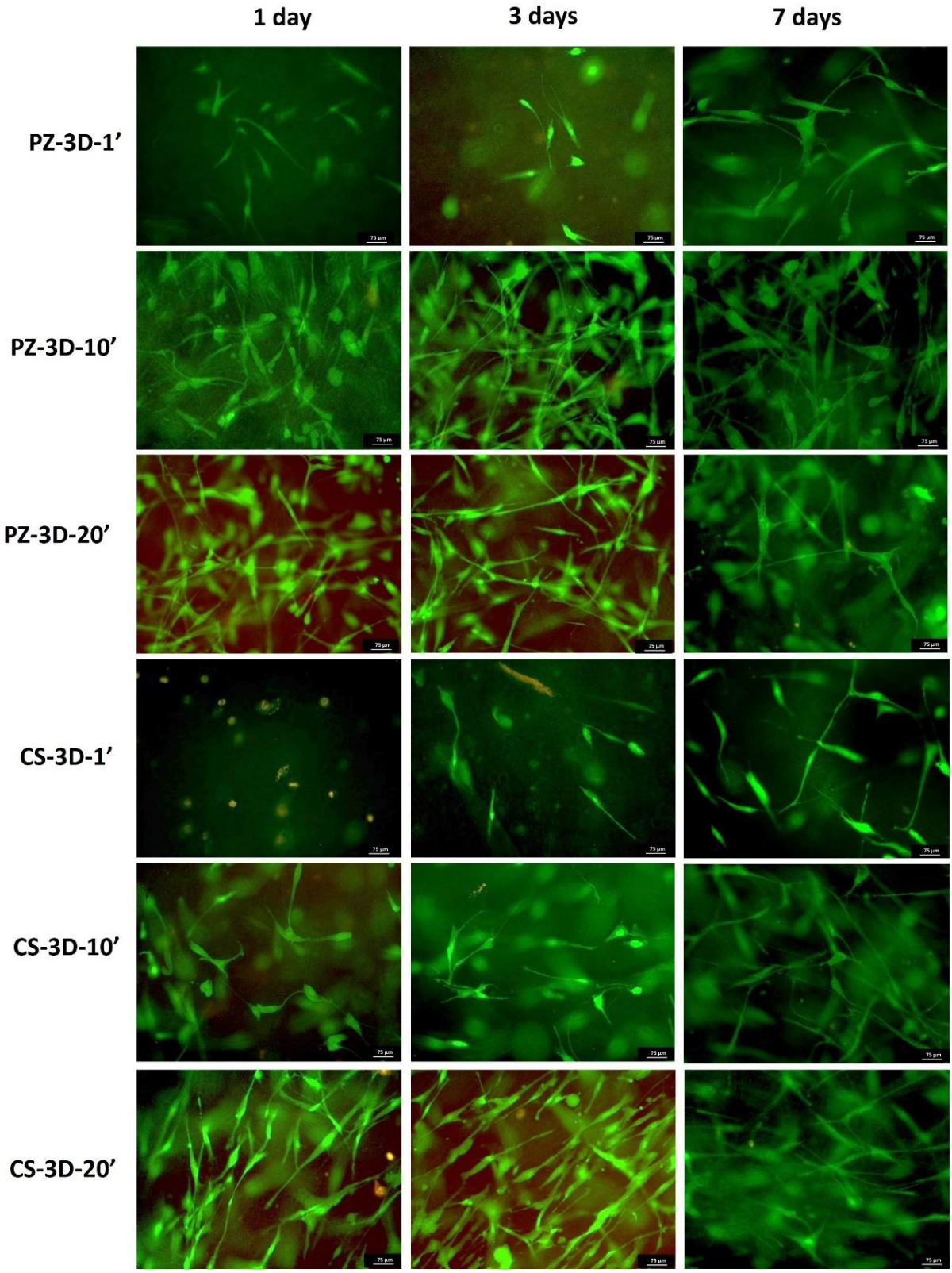

Figure 11. Panel of Live/Dead assay for HGF 3D culture on direct contact assay – 3DP samples. Live cells = green; dead cells = red. Original magnification ×20.

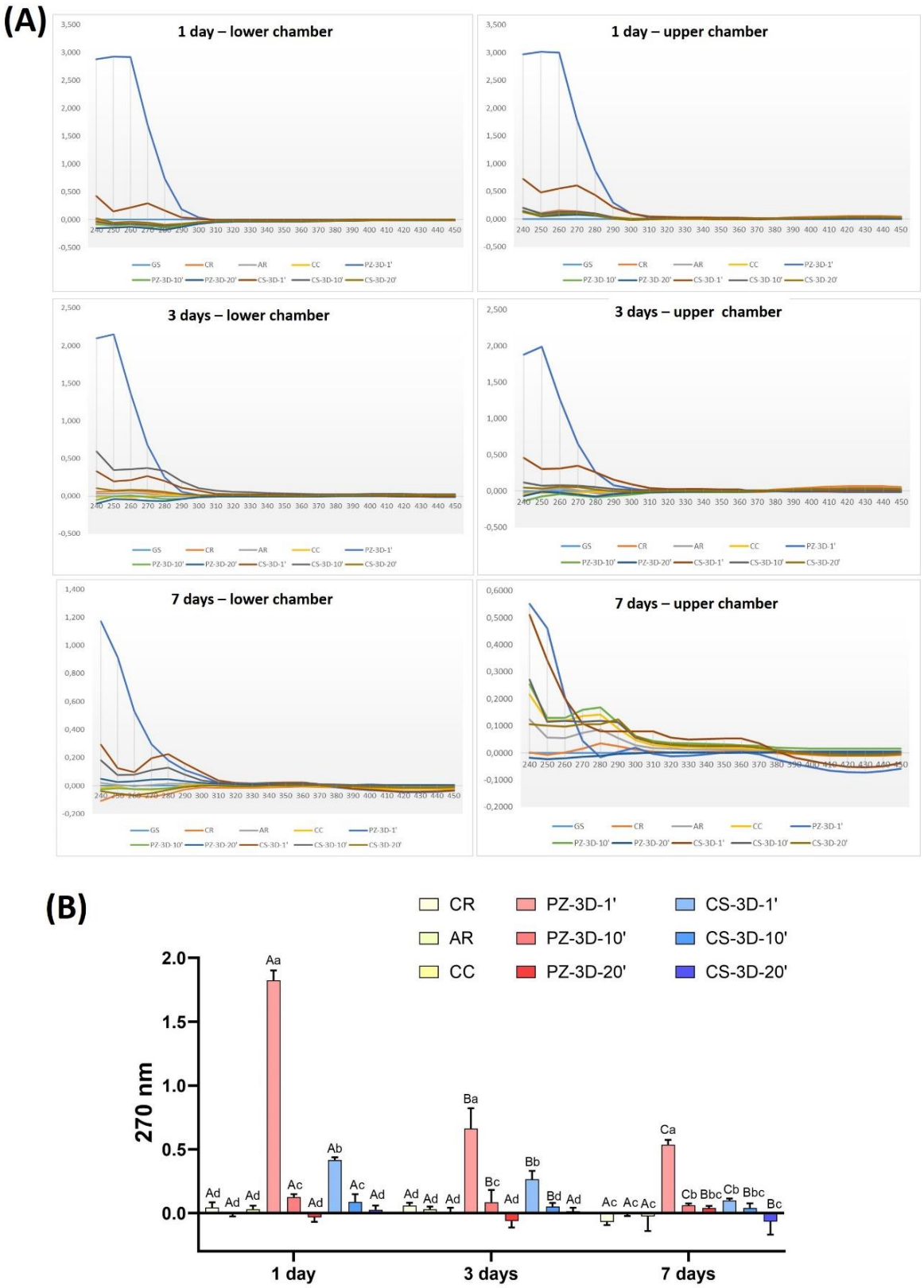

Figure 12. A – Graph of spectral evaluation of the culture medium at 1, 3 and 7 days, on both upper and lower transwell compartments. B – Absorbance values at 270 nm (n=6). Data are mean values and standard deviation. Upper-case letters allow comparison among time-points for each group; lower-case letters allow comparison among groups, at each time-point (Two-way ANOVA; Tukey´s test. P < 0.05).

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Table 1. Relationship between experimental groups and control according to their respective features.

GROUPS	MATERIALS	COMPOSITION
GS (negative control)	Glass Slide Perfecta, São Paulo, SP, Brazil	Polished alkaline glass
AR	Acrylic Resin Dencôr; Artigos Odontológicos Clássico Ltda, São Paulo, SP, Brazil	Powder: methyl methacrylate copolymer. Liquid: methyl methacrylate monomer
CR	Composite Resin Filtek™ Z350XT 3M, Saint Paul, MN, EUA	bis- GMA, UDMA, TEGDMA e bis – EMA
CC	Prensed acrylic resin CAD/CAM; VIPI BLOCK TRILUX Pirassununga, SP, Brazil	Polymethylmethacrylate) (PMMA), biocompatible pigments, EDMA and fluorescente
PZ-3D	Three-dimensional printing resin Prizma 3D Smart Print Bio A1, MakertechLabs, Tatuí, SP, Brazil	Oligomers, Monomers, Photoinitiators, Stabilizer, Pigment
CS-3D	Three-dimensional printing resin Cosmos DLP Temp A1, Yller, Pelotas, RS, Brazil	Oligomers, Monomers, Photoinitiators, Stabilizer, Pigment

Table 2. Percentage of cell metabolism reduction and graduation of cytotoxicity based on ISO 10993-5:2009(E)

Group		Nok-Si co-culture			3D HGF co-culture			3D HGF direct contact		
		1 day	**3 days**	**7 days**	**1 day**	**3 days**	**7 days**	**1 day**	**3 days**	**7 days**
CR	***% reduction***	11.1±10.2*	10.1±20.0	14.9±1.4	9.2±8.0	1.0±16.6	1.9±7.5	-2.7±12.9	0.9±5.2	18.5±6.5
	Grade	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic
AR	***% reduction***	37.8±4.4	30.5±2.9	-3.3±6.9	11.6±7.6	0.9±16.4	-1.8±11.6	-8.0±23.5	-5.9±6.1	18.7±3.8
	Grade	Mild	Slight	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic
CC	***% reduction***	20.5±18.6	11.7±15.9	-1.7±18.6	11.0±7.6	14.4±12.1	10.0±3.9	-21.5±22.2	-7.6±11.8	11.6±4.8
	Grade	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic
PZ-3D-1'	***% reduction***	83.0±5.8	81.5±8.2	96.7±2.3	27.4±13.9	45.7±2.8	42.4±26.3	58.3±11.7	50.5±8.8	68.6±7.7
	Grade	Severe	Severe	Severe	Slight	Mild	Mild	Moderate	Moderate	Moderate
PZ-3D-10'	***% reduction***	30.4±10.5	60.1±2.0	39.3±7.0	8.5±13.2	27.7±31.2	30.1±28.1	-47.3±26.4	-8.4±3.5	8.6±3.5
	Grade	Slight	Moderate	Mild	Non-cytotoxic	Slight	Slight	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic
PZ-3D-20'	***% reduction***	29.7±10.9	55.2±4.4	26.6±6.7	8.2±9.9	5.7±11.3	8.3±8.8	-48.0±17.9	-24.4±1.6	8.4±5.3
	Grade	Slight	Moderate	Slight	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic
CS-3D-1'	***% reduction***	81.3±2.1	75.3±8.7	100.0±0.4	33.8±17.1	35.5±2.5	29.1±17.3	52.0±7.5	52.0±4.4	71.8±3.8
	Grade	Severe	Severe	Severe	Mild	Mild	Slight	Moderate	Moderate	Severe
CS-3D-10'	***% reduction***	30.1±5.5	62.1±5.4	35.8±18.6	7.4±24.1	21.1±22.0	2.8±28.9	-15.5±15.6	-3.9±6.7	5.9±4.2
	Grade	Slight	Moderate	Mild	Non-cytotoxic	Slight	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic
CS-3D-20'	***% reduction***	6.6±8.7	13.7±2.0	26.5±2.0	14.0±6.0	6.5±12.3	3.3±5.7	-21.0±10.3	-4.7±10.0	14.7±5.4
	Grade	Non-cytotoxic	Non-cytotoxic	Slight	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic

* mean values for % of cell metabolism reduction ± mean standard deviation. Negative values denotes increase on cell metabolism.

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APPENDIX

APÊNCIDE A - DECLARAÇÃO DE USO EXCLUSIVO DE ARTIGO EM DISSERTAÇÃO/TESE

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN DISSERTATION/THESIS

We hereby declare that we are aware of the article (Biological assessment of 3D printed resins for interim restorations using an organotypic model of oral mucosa cells) will be included in (Dissertation/Thesis) of the student (Larissa Álamo) and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, may 28th, 2021.

Larissa Álamo	_____
Author	Signature
Diana Gabriela Soares dos Passos	_____
Author	Signature