ASSESSMENT OF CYTOTOXICITY CAUSED BY DENTURES ON HaCaT CELL LINE

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ABSTRACT

Aim of the study: This study aimed to evaluate the in vitro cytotoxicity of four types of complete denture materials—milled, injected, classical (heat-cured), and 3D-printed—on HaCaT human keratinocyte cells after exposure to artificial saliva with varying pH levels, simulating oral environmental conditions. **Materials and methods:** Artificial saliva solutions with acidic (pH 3), neutral (pH 7.05), and basic (pH 9.12) values were prepared and used to incubate each denture type for 7 days. The resulting extracts were applied to HaCaT keratinocyte cultures. Cell viability was assessed using the MTT assay, a standard colorimetric test for cytotoxicity, with absorbance measured at 570 nm. **Results:** None of the four denture types showed significant cytotoxicity in any pH condition. Minor variations in cell viability were observed, with classical and printed prostheses showing slightly reduced viability under acidic conditions, while milled prostheses exhibited stable or improved viability. All samples maintained cell viability within acceptable biological limits. **Conclusions:** All tested denture materials demonstrated good biocompatibility with HaCaT keratinocytes in vitro, even under varying pH conditions mimicking the oral environment. These findings support the safe use of modern and conventional denture fabrication methods and highlight the relevance of HaCaT cells as a predictive model for mucosal compatibility. Further long-term and in vivo studies are recommended.

Key words: Removable dentures; Biocompatibility; HaCaT keratinocytes; Cytotoxicity; Artificial saliva; pH variation; MTT assay; Denture materials; 3D-printed prosthesis; In vitro study.

INTRODUCTION

Removable dentures are widely used in dental practice, particularly among the elderly, due to their effectiveness in restoring oral function, aesthetics, and quality of life after tooth loss. Tooth loss is a common issue in aging populations, primarily resulting from periodontal disease, dental caries, trauma, or systemic health conditions. As the global population ages, the demand for prosthetic solutions like removable dentures continues to grow [1].

Edentulism (complete tooth loss) is significantly more prevalent in individuals over the age of 65. In many countries, a substantial percentage of older adults are either partially or completely edentulous. Removable dentures provide an accessible and functional

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solution for these patients [2].

Compared to implant-supported prostheses or fixed bridges, removable dentures are more cost-effective, making them an attractive option for elderly patients, many of whom may be on fixed incomes or lack dental insurance coverage [3].

Removable dentures do not require surgical procedures, which is particularly beneficial for older patients who may have contraindications to surgery due to systemic conditions such as diabetes, cardiovascular disease, or compromised immune systems [4].

Dentures can be tailored to individual anatomical and functional needs. They are also relatively easy to modify or reline as oral tissues change over time, which is common among the elderly due to ongoing alveolar bone resorption.

Removable dentures help restore essential functions such as mastication, speech, and facial aesthetics. These improvements contribute significantly to psychosocial well-being and nutritional health in older adults.

For patients undergoing phased treatment or awaiting implant placement, removable partial or complete dentures can serve as temporary prosthetic devices.

Despite their advantages, removable dentures present several clinical challenges, especially in elderly patients. These include: progressive bone resorption affecting fit and retention, compromised oral mucosa that may be more susceptible to pressure and friction, potential for decreased dexterity or cognitive function, which can impair the ability to maintain hygiene or manage removable prosthetics. Ongoing dental monitoring and maintenance are crucial to ensuring long-term success with removable dentures [5].

Material biocompatibility is a critical consideration in prosthetic dentistry, particularly in the fabrication and use of removable dentures, because these devices remain in prolonged and often continuous contact with the oral mucosa. Ensuring that the materials used in dentures are biocompatible is essential for maintaining oral and systemic health, especially in elderly patients, who often have compromised immune responses and mucosal integrity [6, 7].

The oral mucosa is highly vascular and sensitive, making it vulnerable to irritation, inflammation, or allergic reactions when in contact with incompatible materials. Poorly biocompatible denture base resins, such as polymethyl methacrylate (PMMA) with residual monomers, can lead to conditions like denture stomatitis, mucosal erythema, and ulcers.

Dentures are typically worn daily for many hours, and sometimes continuously. This extended exposure increases the likelihood of chronic reactions if the materials are not adequately biocompatible. Leaching of substances, such as plasticizers, unpolymerized monomers, or metal ions, can have local and systemic effects over time.

Biocompatible materials help maintain a healthy balance of the oral microbiota. Certain materials can promote the adherence and proliferation of pathogenic microorganisms such as Candida albicans, increasing the risk of fungal infections, particularly under the denture base [8-11].

As patients age, oral tissues often become thinner, less resilient, and slower to heal. The use of biocompatible materials minimizes the risk of trauma and supports tissue health, which is especially important in elderly denture wearers with systemic comorbidities like diabetes or xerostomia.

Hypersensitivity reactions to denture materials, although relatively rare, can have significant consequences. Nickel and other metals used in some partial denture frameworks may elicit allergic responses. Therefore, selecting hypoallergenic and inert materials is essential for long-term patient safety [12].

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Recent advancements in dental material science have focused on enhancing biocompatibility through: high-degree polymerization PMMA to reduce residual monomer content, thermoplastic resins (e.g., polyamides and PEEK) that offer flexibility and lower allergenic potential, surface treatments and coatings that resist microbial adhesion, or 3D-printable biocompatible resins tailored for digital dentistry applications [13].

Material biocompatibility is foundational to the success of removable dentures in prosthetic dentistry. It influences not only the comfort and health of the oral tissues but also the longevity and acceptability of the prosthesis. Selecting and using biocompatible materials is thus a central responsibility of the dental professional, ensuring safety, function, and quality of life for patients—especially within the aging population.

While numerous studies have extensively evaluated the cytotoxicity of dental adhesives, liners, and restorative materials, direct evidence regarding the cytotoxic effects of complete denture materials—particularly those produced using different fabrication techniques such as milling, injection molding, and 3D printing—remains relatively limited. This gap in the literature is significant, considering the increasing adoption of digital and alternative fabrication methods in prosthetic dentistry.

Each fabrication technique introduces different chemical and physical characteristics to the final denture base material, such as varying degrees of polymerization, surface roughness, and residual monomer content—all of which can influence the biological response of the surrounding oral tissues. For instance, 3D-printed resins may have different elution profiles compared to conventionally polymerized or milled PMMA, yet comprehensive cytotoxicity data comparing these methods under clinically relevant conditions is still lacking [14-16].

Moreover, while many dental materials meet established biocompatibility standards set by regulatory bodies, these standards are often based on controlled laboratory conditions that do not fully replicate the dynamic and variable oral environment. Intraoral factors such as fluctuating pH levels in saliva, enzymatic activity, temperature changes, and mechanical wear can significantly alter material behavior, potentially leading to degradation, leaching of residual monomers, or changes in surface properties that promote microbial colonization.

Therefore, there is a growing need for in vitro and in vivo studies that assess the long-term biological effects of complete denture materials in conditions that closely simulate the oral cavity, particularly in vulnerable populations such as the elderly. Such research is essential to better understand the real-world biocompatibility and to inform material selection and clinical protocols in prosthetic dentistry.

The HaCaT human keratinocyte cell line is widely recognized as a relevant and reliable in vitro model for assessing oral mucosal toxicity, including the evaluation of denture base materials. Its use is well-justified based on both biological relevance and practical advantages in toxicological research [17-19].

Keratinocytes are the predominant cell type in the oral epithelium, forming the first line of defense against mechanical, chemical, and microbial insults. They are critically involved in maintaining mucosal barrier integrity, wound healing, and immune responses. Therefore, studying the effects of denture materials on keratinocytes directly reflects potential impacts on the oral mucosa.

HaCaT cells are an immortalized human keratinocyte line derived from adult skin but exhibit many morphological and functional characteristics of normal human oral

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keratinocytes, including their capacity for differentiation and expression of keratin proteins similar to those found in oral tissues. Their ease of culture, reproducibility, and availability make them particularly useful for standardized cytotoxicity assays such as MTT, LDH release, and reactive oxygen species (ROS) generation.

Numerous previous studies have employed HaCaT cells to evaluate the cytotoxicity of dental materials, including resin-based composites, bonding agents, and denture base resins. These studies support the suitability of HaCaT cells as a surrogate for primary oral keratinocytes due to their consistent responses to known toxicants.

The aim of this study is to evaluate the in vitro cytotoxicity of four types of complete denture materials—milled, injected, classical (heat-cured), and 3D-printed—on HaCaT human keratinocyte cells following incubation in artificial saliva with varying pH levels.

This investigation seeks to simulate intraoral conditions more accurately by considering the influence of salivary pH fluctuations on material behavior and potential cytotoxic effects, thereby contributing to a more comprehensive understanding of the biocompatibility of modern and conventional denture fabrication techniques.

The working hypothesis of this study is that the tested denture materials—milled, injected, classical (heat-cured), and 3D-printed—will not exert significant cytotoxic effects on HaCaT human keratinocyte cells following immersion in artificial saliva across a range of pH conditions.

It is anticipated that, despite differences in fabrication methods and potential material composition, all denture types will demonstrate acceptable biocompatibility and maintain cell viability under simulated oral environmental conditions.

MATHERIALS AND METHODS

Reagents and instruments

In the current study, the chemicals utilized for the preparation of artificial saliva were: hydrochloric acid (HCl) 37% and calcium chloride dihydrate (CaCl2 ·2H2O) from Honeywell Fluka™ (Charlotte, NC, U.S.A.); urea from Sigma-Aldrich (St. Louis, MO, USA); KCl and NaOH pellets procured from Chimreactiv (Bucharest, Romania) and NaCl acquired from Chimopar S.A (Bucharest, Romania).

For the in vitro experiment Dulbecco's Modified Eagle's medium (DMEM), penicillin-streptomycin solution, and fetal bovine serum (FBS) were procured from The American Type Culture Collection (ATCC) (Manassas, VA, USA) and MTT kit, trypsin-EDTA solution, phosphate buffer saline (PBS) were acquired from Sigma-Aldrich, Merck KGaA (Darmstadt, Germany). The Cytation 5 device and Gen5™ Microplate Data Collection and Analysis Software (Version 3.14) from BioTek Instruments Inc. (Winooski, VT, USA) were used to quantify the results and read the absorbances.

Cell culture

The line chosen for cytotoxicity evaluation of the samples was HaCaT cell line-immortalized human keratinocytes (CVCL_0038), healthy cells, procured from, CLS Cell Lines Service (Eppelheim, Germany), as frozen vial. Cells were grown in a specific culture medium DMEM supplemented with 10% FBS and 1% antibiotic mix (penicillin-streptomycin) to avoid contamination.

Throughout the experiments, the cells were kept under standard temperature and pressure conditions in the incubator at 5% CO2 and 37°C.

Artificial saliva and samples preparation

Artificial saliva solutions with different pH were obtained, the initial solution with neutral

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pH (7.05) and solutions with acid pH (3) and basic pH (9.12), according to the technique outlined by Damian et al. [20]. In the first stage, the saliva solution with a neutral pH was prepared by dissolving in a liter of distilled water 0.80 mg CaCl2 × 2H2O, 1 mg CO(NH2)2 and 0.40 mg KCl and respectively NaCl; after which were obtained two other types of saliva. Saliva with a basic pH was prepared by treating neutral saliva with NaOH 10 N until the desired pH (pH 9.12). In the same way, saliva with acidic pH was obtained by adding HCl 37% in drops to neutral saliva up to a pH of 3. The pH of the artificial saliva solutions was recorded utilizing a Thermo Scientific Eutech pH 150 electrode pH meter (Thermo Scientific, Waltham, MA, USA).

Four different types of dentures were individually placed in sterile falcon tubes in 15 ml of artificial saliva (acidic, neutral, and basic pH) and were kept in an incubator with an orbital shaker at standard conditions at 37°C for 7 days. After 7 days, the dentures were extracted and each sample (artificial saliva in which the prostheses were immersed) was diluted 1:1 with the specific cell culture medium for testing. The samples were named as follows: P1 (milled prosthesis), P2 (injected prosthesis), P3 (classical prosthesis), and P3 (printed prosthesis).

Cell viability evaluation

To assess the impact on the cell viability of the four samples, the MTT test was performed. Cells were cultured in 96-well plates (1x104 cells/well) and treated with the 4 dilutions when the desired confluence was reached. After 24 hours with the samples of interest, the culture medium was replaced with 100 µL fresh medium, and 10 µL of MTT kit-1 was added to each well. The plate was incubated for 3 hours, and then the MTT kit-2 was added in a volume of 100 µL/well. The absorbance was read at 570 nm using a Cytation 5 device.

RESULTS

Cell viability

To detect the cellular impact of the samples in vitro, the MTT assay was performed 24 hours after stimulation with the four solutions on HaCaT cells. The results of the samples shown in Figure 1 were reported to the control representing HaCaT cells treated with the vechicle used for the samples, namely artificial saliva at the 3 pH values – acidic, neutral, and basic

According to Figure 1A) in acidic pH, slight changes brought P1 and P3 samples which decreased cell viability to about 75%. At neutral pH, as can be seen in Figure 1B), all samples gave similar results with a cell viability of about 80% after 24 hours of treatment. The results obtained from samples placed in basic pH (Figure 1C) showed the least changes, the lowest viability being reported by P3 with a percentage of 86%, while sample P1 at the same pH seems to increase cell viability. None of the 4 samples significantly affected cell viability.

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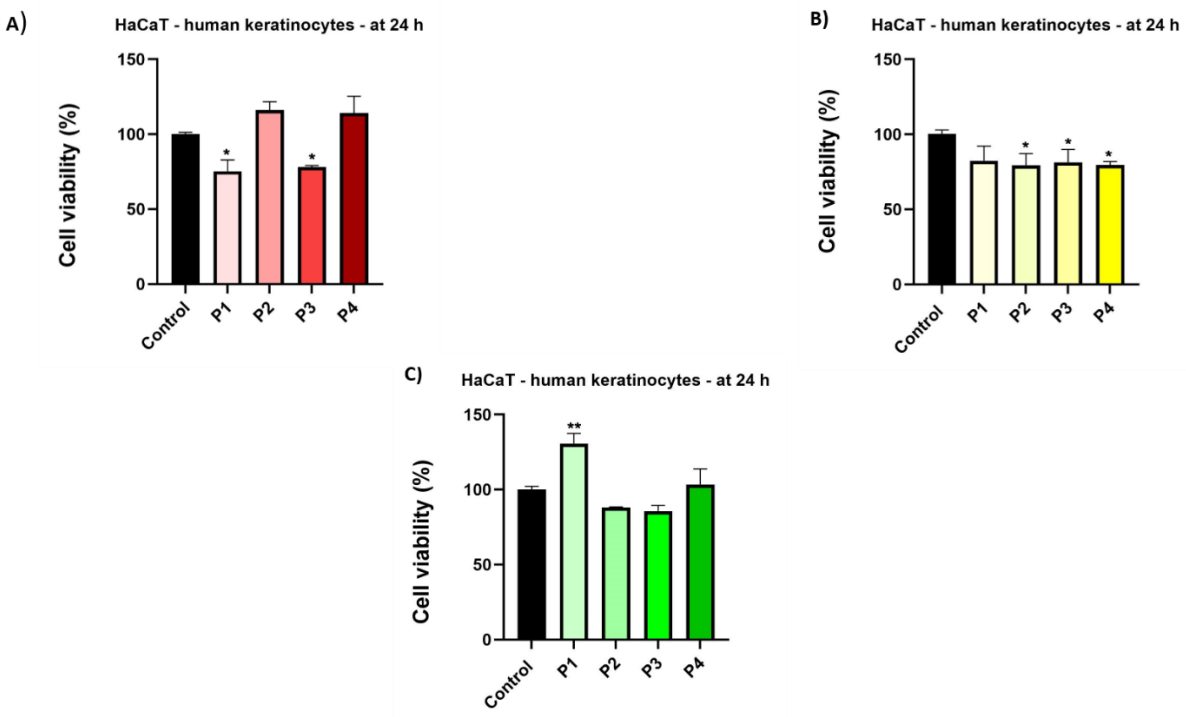

Figure 1. Graphical representation of cell viability percentages 24 hours after stimulation with the samples (P1-milled prosthesis, P2-injected prosthesis, P3-classical prosthesis, and P4-printed prosthesis) in 1:1 dilution. The current results are presented as percentages (%) normalized to the control, representing the cells treated only with artificial saliva (artificial acidic saliva-Figure 1A, neutral-Figure 1B, and basic-Figure 1C pH). All data are expressed as mean values ± SD of three independent experiments performed in triplicate. For statistical analyses, the One-way ANOVA test was performed, followed by Dunnet's multiple comparison post hoc test. "*" marks statistical significance (* $p < 0.05$; ** $p < 0.01$).

DISCUSSIONS

Keratinocytes are found on both the surface of the skin and the oral mucosa, their function being of interest and essential for wound re-epithelialization [21]. They are also a major component in the structure of the epidermis [22] and for this reason, HaCaT cells have also been used to evaluate the impact of certain dental materials in vitro [23]. The safety profile at the cellular level is also important for dental materials, as many of them come into direct contact with the skin or mucosa. To assess the impact of dentures at the cellular level, we performed the MTT assay. The MTT method is ubiquitous in toxicity studies. The reagent can pass through the cell membrane and the mitochondrial inner membrane of living cells due to its lipophilic structure, and for this reason, it has a broad utility and applicability [24].

To analyze the in vitro influence with the greatest accuracy, the cell viability of the 4 dentures immersed in artificial saliva was determined on HaCaT cells (artificial saliva at acidic-Figure 1A, neutral-Figure 1B, and basic-Figure 1C pH). Thus, the artificial saliva was initially tested and the results were perceived as a control for reporting the dentures. According to Figure 1, none of the four samples to be analyzed caused considerable decreases in cell viability 24 hours after stimulation. The P3 sample was the one that maintained approximately the same results under all 3 pH conditions on HaCaT cells. For the P4 sample, however, stimulation of the cells subjected to acidic (Figure 1A)) and basic (Figure 1C)) pH conditions was observed. The results of the in vitro

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experiment show that the four types of dentures do not seem to have a cytotoxic effect on healthy cells. Similar information on the direct cytotoxicity of dentures is today quite limited, but other assessments have included different dental materials to observe their effect on cells. Sobolewska et. al have evaluated cytotoxicity including for denture adhesives, some of them showing moderate to significant toxic effects on gingival fibroblasts according to MTT test results [25,26].

This study aimed to assess the in vitro cytotoxicity of four types of denture materials—milled, injected, classical (heat-cured), and 3D-printed—on HaCaT human keratinocyte cells following immersion in artificial saliva with varying pH values, simulating conditions of the oral environment. The experimental approach utilized the MTT assay to evaluate cell viability and thus infer the biocompatibility of each denture type.

The findings indicate that none of the tested denture materials exerted significant cytotoxic effects on HaCaT cells, regardless of the pH condition (acidic, neutral, or basic) in which they were incubated. While minor variations in cell viability were observed—particularly with the classical (P3) and printed (P4) dentures in acidic and basic conditions—these changes were not statistically or biologically significant in the context of acute cytotoxicity. Notably, milled dentures (P1) showed stable or even slightly enhanced cell viability across conditions, suggesting a favorable biological profile.

These results support the working hypothesis and provide preliminary evidence of acceptable biocompatibility for all four denture fabrication methods, under simulated salivary conditions. Furthermore, the use of HaCaT keratinocytes—a well-established in vitro model for oral epithelial tissue—proved effective in evaluating potential mucosal responses to denture materials.

Given the limited cytotoxicity data available for complete dentures fabricated using modern technologies such as CAD/CAM milling and 3D printing, this study contributes valuable insight into their biological safety. However, further research is needed to assess long-term effects, including chronic exposure, degradation byproducts, and interactions with oral microbiota.

CONCLUSIONS

This study evaluated the in vitro cytotoxicity of four denture materials—milled, injected, classical (heat-cured), and 3D-printed—on HaCaT keratinocytes after immersion in artificial saliva at different pH levels. The results showed no significant cytotoxic effects from any material, with cell viability remaining within acceptable limits across all conditions.

These findings suggest that all tested denture types are biocompatible with oral keratinocytes, even under pH variations that mimic the oral environment. The use of HaCaT cells provided a reliable model for assessing mucosal safety. Further long-term studies are recommended to confirm these results in more complex conditions.

In conclusion, all four tested denture types demonstrated non-cytotoxic behavior toward human keratinocytes in vitro, under a range of pH conditions representative of the oral environment. These findings reinforce the importance of continued evaluation of material biocompatibility as dental technologies evolve, with an emphasis on maintaining patient safety, especially in vulnerable populations such as the elderly.

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