Dental Materials xxx (xxxx) xxx

Contents lists available at ScienceDirect

Dental Materials

journal homepage: www.elsevier.com/locate/dental

Guidance on the assessment of biocompatibility of biomaterials: Fundamentals and testing considerations

Vinicius Rosa [a,b,*], Nikolaos Silikas [c], Baiqing Yu [a], Nileshkumar Dubey [b,d], Sriram Gopu [a,b], Spiros Zinelis [e], Adriano F. Lima [f], Marco C. Bottino [g], Joao N. Ferreira [h], Gottfried Schmalz [i,j], David C. Watts [k,**]

[a] Faculty of Dentistry, National University of Singapore, Singapore
[b] ORCHIDS: Oral Care Health Innovations and Designs Singapore, National University of Singapore, Singapore
[c] Dental Biomaterials, Dentistry, The University of Manchester, Manchester, United Kingdom
[d] Division of Cariology and Operative Dentistry, Department of Comprehensive Dentistry, University of Maryland School of Dentistry, Baltimore, United States
[e] School of Dentistry National and Kapodistrian University of Athens (NKUA), Greece
[f] Dental Research Division, Paulista University, Sao Paulo, Brazil
[g] School of Dentistry, University of Michigan, Ann Arbor, USA
[h] Center of Excellence for Innovation for Oral Health and Healthy Longevity, Faculty of Dentistry, Chulalongkorn University, Thailand
[i] Department of Conservative Dentistry and Periodontology, University Hospital Regensburg, Regensburg, Germany
[j] Department of Periodontology, School of Dental Medicine, University of Bern, Bern, Switzerland
[k] School of Medical Sciences and Photon Science Institute, University of Manchester, United Kingdom

ARTICLE INFO

Keywords:
Tissue engineering and regeneration
Dental pulp and periodontium
Bioactive materials
Biocompatibility and proliferation
Cell membrane damage, death and apoptosis
Restorative and regenerative materials
Nanoparticles

ABSTRACT

Background: Assessing the biocompatibility of materials is crucial for ensuring the safety and well-being of patients by preventing undesirable, toxic, immune, or allergic reactions, and ensuring that materials remain functional over time without triggering adverse reactions. To ensure a comprehensive assessment, planning tests that carefully consider the intended application and potential exposure scenarios for selecting relevant assays, cell types, and testing parameters is essential. Moreover, characterizing the composition and properties of biomaterials allows for a more accurate understanding of test outcomes and the identification of factors contributing to cytotoxicity. Precise reporting of methodology and results facilitates research reproducibility and understanding of the findings by the scientific community, regulatory agencies, healthcare providers, and the general public.

Aims: This article aims to provide an overview of the key concepts associated with evaluating the biocompatibility of biomaterials while also offering practical guidance on cellular principles, testing methodologies, and biological assays that can support in the planning, execution, and reporting of biocompatibility testing.

Abbreviations: **AgNPs**, Silver Nanoparticles; **Akt**, Protein Kinase B; **aMw**, Apparent Molecular Weight; **BCA**, Bicinchoninic Acid; **BisGMA**, Bisphenol A-glycidyl methacrylate; **BMI-1**, B lymphoma Mo-MLV insertion region 1; **CCK-8**, Cell Counting Kit-8; **ClO$^-$**, Hypochlorite Ion; **CQ**, Camphorquinone; **DMSO**, Dimethyl Sulfoxide; **DPSC**, Dental Pulp Stem Cells; **DSB**, Double-Strand Break; **ECM**, Extracellular Matrix; **ERK1/2**, Extracellular Signal-Regulated Kinases 1 and 2; **EthD-1**, Ethidium Homodimer-1; **GSH**, Glutathione; **GSK3α/β**, Glycogen Synthase Kinase 3 alpha/beta; **H_2O_2**, Hydrogen Peroxide; **HEMA**, 2-Hydroxyethyl Methacrylate; **IkB-α**, NFKB Inhibitor α; **IL**, Interleukin; **iPSCs**, Induced Pluripotent Stem Cells; **LDH**, Lactate Dehydrogenase; **MAPKs**, Mitogen-Activated Protein Kinases; **MG-63**, Human Osteosarcoma Cell Line; **MTS**, 3-(4,5-Dimethylthiazol-2-yl)−5-(3-carboxymethoxyphenyl)−2-(4-sulfophenyl)−2 H-tetrazolium; **NRU**, Neutral Red Uptake; **O2$^-$**, Superoxide Ion; **OH**, Hydroxyl Radical; **p38-MAPK**, p38 Mitogen-Activated Protein Kinase; **PDL**, Periodontal Ligament; **PDLSC**, Periodontal Ligament Stem Cells; **PI**, Propidium Iodide; **ROS**, Reactive Oxygen Species; **SHED**, Stem Cells from Human Exfoliated Deciduous Teeth; **SSB**, Single-Strand Break; **TEGDMA**, Triethylene Glycol Dimethacrylate; **TNF-α**, Tumor Necrosis Factor Alpha; **TPO**, Diphenyl-(2,4,6-trimethylbenzoyl)phosphine Oxide; **WST-1**, Water-Soluble Tetrazolium Salt 1.

* Correspondence to: Faculty of Dentistry, National University of Singapore, 9 Lower Kent Ridge Road, 119085, Singapore.
** Correspondence to: School of Medical Sciences, Division of Dentistry, University of Manchester, Coupland 3 Building, Oxford Road, Manchester M13 9PL, United Kingdom.
E-mail addresses: vini@nus.edu.sg (V. Rosa), nikolaos.silikas@manchester.ac.uk (N. Silikas), baiqing_yu@u.nus.edu (B. Yu), nileshd@nus.edu.sg (N. Dubey), sriram@nus.edu.sg (S. Gopu), szinelis@dent.uoa.gr (S. Zinelis), lima.adf@gmail.com (A.F. Lima), mbottino@umich.edu (M.C. Bottino), joao.f@chula.ac.th (J.N. Ferreira), gottfried.schmalz@klinik.uni-regensburg.de (G. Schmalz), david.watts@manchester.ac.uk (D.C. Watts).

https://doi.org/10.1016/j.dental.2024.07.020
Received 9 June 2024; Received in revised form 22 July 2024; Accepted 24 July 2024

1. Toxicity of biomaterials: considerations

Assessing material's biocompatibility is essential to ensure the safety and well-being of patients without triggering sensitivities, immune, or allergic reactions, and that the materials can remain functional without triggering adverse reactions over time. In addition, regulatory bodies require biocompatibility testing of biomaterials as a part of the approval process for market access.

Researchers have often adopted testing standards as guidelines for testing the biocompatibility of biomaterials. The standards offer structured testing methodologies that allow worldwide data comparison and also suggest safety thresholds often recognized by industry and regulatory bodies [1]. For instance, ISO 7405 outlines procedures and assessments for evaluating the biocompatibility of dental medical devices, including classification, specimen preparation techniques, testing methods, and recommendations for reporting results [2]. Likewise, it provides recommendations for testing the biocompatibility of light-curing and chemically setting materials and outlines the experimental setup for the dentin barrier cytotoxicity test, Minucells device, and ADA-perfusion chamber [2]. Nonetheless, the standards are not exhaustive in covering many potential scenarios related to the clinical application of new biomaterials [3–5]. For instance, ISO 10993 suggests that cell lines established from recognized repositories are preferred for testing in vitro cytotoxicity during the biological evaluation of medical devices [6]. Although the use of these cell lines has supported many material developments, they also have limitations. For instance, the ISO-endorsed cell lines that are derived from the subcutaneous connective tissue of mice (L929) or lung tissue of hamsters (V-79 379 A), are arguably relevant cell models for characterizing materials that will be used in the mouth [6].

Therefore, testing protocol customization - through critical consideration of the intended application, exposure scenarios (such as direct contact with the tooth or surrounding tissues or through diffusion), composition, type, frequency, and duration of the contact - are essential for the selection of relevant cell types, testing parameters, and interpretation of tests that will yield a fair assessment of the biocompatibility of the materials relevant to their potential clinical application.

Given the growing interest in the materials science community for developing biomaterials and evaluating their biological properties, it has become increasingly important to discuss the principles and parameters that guide a comprehensive understanding of the characteristics of materials and biological properties that underpin the definition of biocompatibility. The aim of this article is to provide an overview of the key concepts associated with evaluating the cytocompatible properties of biomaterials while also offering practical guidance on cellular principles, testing methodologies, and biological assays that can aid in the planning, execution, and reporting of biocompatibility testing in biomaterial research.

2. Relevant cytotoxicity mechanisms activated by biomaterials

Understanding the cellular responses within each tissue is critical for the design and application of biomaterials [6–8]. When biomaterials are used inside the oral cavity, they are exposed to bacteria, saliva, food elements, enzymes etc., which can influence their biompatibility by changing temperature, pH, chemical composition, and promoting degradation through wear and tear.

The biocompatibility of dental biomaterials can change as they remain in contact with the dental pulp or oral epithelial tissues for an extended time via direct or indirect contact [9–14]. Biomaterials can also exhibit chemical leakage, which may induce DNA strand breaks, cell cycle arrest, and reactive oxygen species (ROS) production that can lead to innate immune responses, partly driven by macrophages, triggering a cascade of cytokine production [15–18]. The interactions between biomaterials and oral tissues or fluids can trigger biological reactions that may elicit both immune response and toxic effects, typically locally and rarely systemically [6–8,17].

2.1. Dental pulp reactions

In dentistry, one of the major concerns of biomaterials is their cytotoxic effects on pulp tissues induced by the initial release of free monomers or degradation of polymers (e.g., resin-based composites, polymer-modified ceramics, , adhesives, and epoxy components), releasing leachable components over time [8,11,15,17]. The free monomers can negatively impact cellular metabolism (Fig. 1) by reducing the level of GSH, which maintains cell redox homeostasis and triggers an innate immune response [15,17]. In addition, the generation of ROS may increase, leading to oxidative stress, DNA strand breaks, and DNA damage responses, which can trigger autophagy or a caspase-driven apoptosis cascade, ultimately leading to cell death. For example, bisphenol A-glycidyl methacrylate (BisGMA) can stimulate ROS production and inhibit gene expression, subsequently leading to cell cycle arrest and apoptosis in dental pulp cells [19,20]. Likewise, the toxicity of dental adhesives is influenced by the presence of monomers such as triethylene glycol dimethacrylate (TEGDMA) and 2-hydroxyethyl methacrylate (HEMA) as well as the solvent content [21,22]. Solvated adhesives may significantly affect dental pulp cells more than their nonsolvated counterparts, while including ethanol as a solvent can influence the release of cytokines such as interleukin (IL) −6 and TNF-α from dental pulp cells [21]. Finally, low TEGDMA concentrations can potentially hinder the mineralization process initiated by dental pulp cells, thereby impeding the formation of reparative dentin [22].

Oxidative stress conditions regulate kinases and transcription factors and can lead to the inhibition of specific odontoblast functions, including differentiation pathways and mineralization patterns. Furthermore, other variables, such as concentration and aging, may influence the toxicity of leached monomer components[9,11].

Photoinitiators can alter cell metabolism and the structure of lipids, thereby affecting membrane integrity and permeability. An evaluation of the biocompatibility of resins containing different initiators revealed a higher toxicity level for diphenyl-(2,4,6-trimethylbenzoyl)phosphine oxide (TPO) compared to camphorquinone (CQ) towards dental pulp cells [23].

Pulp sealants have the potential to be toxic in a dose- and time-dependent manner [24,25]. Zinc-oxide eugenol can activate complement-mediated immune responses, inhibit macrophage adhesion, and trigger cytotoxicity [26]. Generally, resin-based sealers possess limited biocompatibility when they are not adequately polymerized and can exhibit genotoxic properties. This can be attributed to the presence of residual monomers and formaldehyde [26–28]. In contrast, glass ionomer- and tricalcium silicate-based sealers that lack resins in their composition exhibit significantly lower cytotoxicity and possess acceptable biocompatibility [27,29].

Bioactive materials are often used in pulpal and other endodontic procedures to enhance soft and hard tissue healing outcomes [27,30,31]. These materials are typically formulated by combining various compounds (e.g., tricalcium silicate, aluminates, lithium carbonate, silicon oxide, zirconium dioxide and others), which influence the properties of the final product [27,30,32,33]. Cements are often used to promote the healing of periapical and pulp tissues by modulating genetic expression, alkaline phosphate activity, and mineralization potential by releasing ions and compounds and changing the pH of the microenvironment. The effects of compound dosage and stimulus duration can result in different cellular responses ranging from tissue necrosis to regeneration [31,34]. Hence, assessing the biocompatibility of cement is crucial for ensuring its safety in clinical applications. The studies on the biocompatibility of cements often present varying outcomes, yet the trend suggests that experimental and commercially available cements are generally biocompatible [30,32,33,35,36]. Nonetheless, some cements can induce lower biocompatibility in vitro because they are more prone to release calcium ions and increase the alkalinity of the

microenvironment [33]. In addition, changes in cement composition, such as changing the liquid or adding medicines and compounds to improve biological outcomes, can interfere with setting reactions and ion release, and pottentially change the biocompatibility of the cement [37,38]. It must be noted that the biocompatibility outcomes can be related to the test setups, which can differ in terms of specimen dimensions, duration of time cells are exposed to treatment, and other variations in the experimental design [1,39], which will be discussed in detail in subsequent sections of this article. Although various studies have pointed out the potential damage that biomaterials can cause to the pulp, it is important to emphasize that the presence of a dentin barrier is a critical factor in protecting the pulp tissue. In fact, Studies have suggested a negative correlation between dentin layer thickness and toxicity experienced by cells exposed to potentially harmful biomaterials [40,41].

2.2. Oral mucosa cytotoxicity

Persistent exposure to substances leached from biomaterials may render certain individuals more susceptible to oral lesions and delayed-type hypersensitivity reactions mediated by immune cells. In particular, this may affect the oral epithelial tissues [10,17,42–47].

The application of dental biomaterials in the oral cavity for a prolonged time can cause toxic or allergic reactions in the oral mucosa [10,43,46]. The presence of free monomers can cause allergic reactions triggered by the recruitment of T lymphocytes, which are activated by antigen-presenting cells to produce cytokines that mediate local inflammation [15,17,46]. Cytotoxic metallic ions released from dental materials and formaldehyde formed as byproducts of unreacted monomers of dental resins can cause adverse allergic reactions [46]. Despite the risk of mercury release from amalgam restorations, clinical trials have not shown renal toxicity or neurotoxicity, and studies have confirmed an acceptable safety profile for these restorations [42,43,48]. Allergic reactions include clinical manifestations, such as red rash, swelling, urticaria, and rhinorrhea. Life-threatening conditions such as anaphylaxis, laryngeal edema, and cardiac arrhythmias are possible, but rarely occur [49].

2.3. Novel nanoparticles for dental applications and their cytotoxicity potential

Nanoparticles, specifically silver nanoparticles (AgNPs), have raised interest due to their potential applications in restorative and prosthetic dentistry, endodontics, implantology, and periodontology[50,51]. AgNPs have in vitro antimicrobial, antiviral, and antifungal properties and can enhance the mechanical properties of dental materials. Despite these advantages, the interactions of nanoparticles with living cells are complex and not well understood. AgNPs can exert cytotoxic effects and induce a DNA damage response, leading to apoptosis and necrosis, and may also damage neighboring normal cells. [52,53]. Nanoparticles can enter cells at the extracellular sites of accumulation and reach distant organs through multiple mechanisms.

Moreover, nanoparticle-induced cytotoxic effects are correlated with nanoparticle composition, size, concentration, surface area and charge, presence of functional groups, and others. Based on their concentrations, chemical and physical properties, nanoparticles may trigger ROS production, regulate autophagy via oxidative stress, and ultimately modulate different cell fates, including apoptosis, necrosis, necroptosis, and mitotic catastrophe. However, the modulation of these mechanisms is not fully understood and long-term in vitro and in vivo studies are scarce. In addition, the poor translation of AgNPs from bench-top to chair-side clinical studies requires more in vivo findings with long-term outcomes to unveil the potential interactions between AgNPs and different body tissues and organs and to inform the design of in vitro studies using appropriate cell types or lines [50,51].

3. Standards for testing the biocompatibility of materials

Standards, specifications, and guidelines developed by international organizations aim to provide guidelines for methods, criteria, and thresholds for evaluating the biocompatibility of materials [1,2,6]. Standardized testing methods enable the comparison of data from various laboratories in a nearly timeless manner, because the standards do not frequently undergo significant and critical modifications that could hinder future comparisons. In addition, international standards can also be part of the compliance documentation required for market access by regulatory agencies (see [1] for a detailed discussion of standards in biomaterials research). There are various standards for testing the safety, and effectiveness of materials for oral care and medicine, both in the preclinical and clinical phases. Research questions may not always be addressed using only the experimental setups, designs, and reporting tools described in conventional standards, particularly when they are aligned with clinical scenarios not covered by the guidance proposed in the standard [2,3,6,39,54]. Nonetheless, the standards offer an advantage for the initial screening of the biological properties of materials and allow comparisons of biocompatibility with results available in the literature obtained using the same methodology. Another important consideration is that many of the cells recommended in the standard are immortalized cell lines that can be acquired from repositories and commercial suppliers [6,55]. Thus, the standards provide a competitive advantage in toxicity assessments, as the recommended cell lines are accessible worldwide, enabling comprehensive analyses and comparisons of the biocompatibility of biomaterials.

Studies have shown that immortalized cell lines display similar trends as primary cells in side-by-side analyses. However, immortalized cells may exhibit greater resistance to toxicity than their primary counterparts [56,57]. Despite the apparent paradox that immortalized cells are typically more resistant to "toxic materials" than other cell

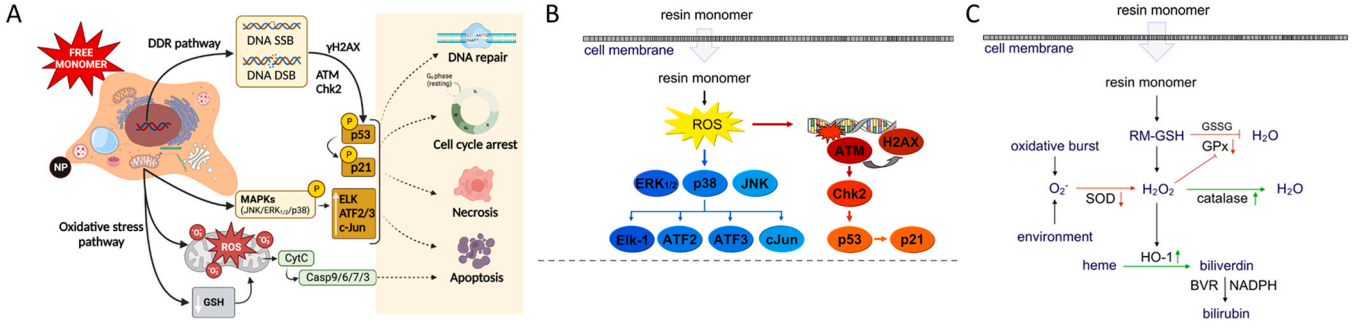

Fig. 1. (A) Overview of the biological pathways involved in the cytotoxicity of free resin monomers and nanoparticles (NP) leaked from dental biomaterials (DDR, DNA damage response; SSB, single-strand breaks; DSB, double-strand breaks; created with BioRender.com).
(B) Dental resin monomers elicit various cellular adaptive responses in relation to oxidative stress (adapted with permission [17]).

types, researchers can still benefit from their use, as indicated by various standards. This is because substances that pose a threat to immortalized cells are likely to have a negative impact on primary cells. Therefore, materials that fail to meet the standards during the initial screening process may not require further testing with primary cells to improve their biocompatibility.

Hence, the standards provide important guidance for initial biocompatibility screening that can support subsequent experimental efforts better suited to capturing the potential clinical applicability of the tested materials.

4. Considerations for specimen preparation and experimental testing design

The experimental design for assessing a material's biocompatibility must consider the elements related to how humans will be exposed to materials, such as the nature, type, frequency, and duration of contact or exposure. Although previous publications and standards can serve as guidelines, it is crucial to emphasize that they should not be viewed as strict prescriptions for determining experimental design parameters that can be adapted to align with the research question's objectives [3].

1. The exposure route encompasses the pathway or means through which biomaterials interact with living cells, such as direct contact with teeth or surrounding tissues, through salivary exposure, or even potential inhalation and ingestion during dental procedures. Understanding the primary exposure route is essential for designing experiments that simulate real-world conditions. For instance, one can opt for either a direct contact assay or use eluents to test the biocompatibility of calcium silicate cement, as these materials interact directly and indirectly with pulp cells while treating vital dental pulp [6,30]. However, a permeation assay assisted by a dentin barrier is recommended to assess the biocompatibility of unbound monomers or sodium diamine fluoride, as these materials are not applied directly to the dental pulp and cytotoxic effects may only be observed when the molecules can diffuse through dentin [41,58]. The route of exposure to biomaterials can significantly influence cytotoxicity. For instance, the bicinchoninic acid (BCA) assay showed no decrease in the viability of periodontal ligament cells (PDL) upon indirect stimulation with RelyX Ultimate, but significantly decreased after direct stimulation (eluent obtained from 24 h specimen incubation) [59]. In addition, materials deemed to be biocompatible as substrates can provoke cytotoxic reactions when tested in suspension [60,61]. Hence, the testing route of exposure must be selected based on intended use.
2. Duration of contact (exposure) refers to the time the biomaterial is in contact with living cells or tissues during the testing process. Evaluating the effects of short- and long-term exposure is crucial to ensure that the materials do not exhibit cytotoxicity over time. Nonetheless, determining the duration of contact in an experimental design is more likely to reveal potential short-term cytotoxicity and effects than the adverse reactions arising from real-life long-term exposure. Typically, researchers evaluate biocompatibility within a time frame of one to a few days. Nonetheless, short-term exposure is often insufficient to evaluate the potential of a material to compromise cellular differentiation and functional outcomes (such as mineralization) or chronic exposure (which is often characterized in vivo) [30,62] because mammalian cells can adapt to exposure to subtoxic concentrations of elements [63] owing to the activation of cytoprotective mechanisms, such as modulation of ROS production [61,64]. In scenarios where long-term exposure is required, pilot tests to determine the critical concentrations at which materials and compounds inhibit biological functions without promoting extensive cell death (e.g., IC50 followed by cell counting and characterization of gene expression at different time points) offer important insights for the final experimental design. In addition, these findings should be interpreted in light of the potential adaptive mechanisms exhibited by cells in response to subtoxic concentrations of substances administered over an extended period [65].
3. Specimen dimensions and leachate concentration can indicate the amount of substances released from biomaterials that affect biocompatibility. Ideally, the leachable concentration should reflect clinical scenarios. However, the reference values are difficult (or even impossible) to determine clinically. Hence, a range of relevant testing concentrations that yield a dose-response relationship is preferred to ensure that cytotoxic effects are accurately assessed. In addition, experimental designs with broad testing ranges are likely to include "non-cytotoxic" to "very cytotoxic" concentrations that support the definition of safe exposure thresholds, as some substances elicit cytotoxic effects or interfere in cell cycles only at higher concentrations [61,66,67]. Herein, the inclusion of concentrations or compounds that do and do not induce cell death (e.g., basal growth media or varying concentrations of dimethyl sulfoxide (DMSO)) is essential to ensure the functionality of the reagents, accuracy of testing procedures, and correctness of calculations [68]. Researchers often refer to ISO standards that outline the temperature, thickness, and extraction ratio used to prepare the extract [6,69]. Nonetheless, these standards may not apply to all testing materials owing to the geometric constraints or costs involved in specimen fabrication and analyses. Indeed, the test specimen geometry influences the elution rate and biocompatibility, and lower toxicity was observed with smaller-volume cement discs (0.09 cm^3) than with larger ones (1.96 cm^3) [39]. Thus, it is interesting to characterize the testing solution obtained via the eluent technique using liquid chromatography, inductively coupled plasma mass spectrometry, and other techniques before adding them to cell cultures, because the amount of leached compounds varies according to the extraction method, specimen dimensions, and elution time [37,54,70,71]. Furthermore, quantification enables concentration standardization and offers insightful information on the origins of cytotoxic effects [72].

In addition to the experimental materials being tested and the relevant controls, it is beneficial to include reference materials within the set of test materials. Reference materials should have similar chemistry, the same indication for use, and a documented clinical history of acceptable safety and performance (e.g., commercially available resin composites, cements, etc.). Although not always required, adding a reference material to the test is particularly beneficial when evaluating novel materials, unconventional compounds, or using non-standardized cells, as this practice provides an important context for the measured data, especially regarding the comparative toxicity of established parameters and materials.

Finally, one aspect that requires attention is the decontamination of the specimens used to prepare extracts or that will be in contact with the cells. It can be assumed that several steps involved in preparing the materials needed are not carried out in environments such as biosafety cabinets (e.g., cutting teeth for dentinal barrier assays, or performing chemical reactions for material synthesis). Therefore, different strategies, such as autoclaving specimens, filtering solutions, and using antibiotics and antifungals, can be used to decontaminate materials and prevent cell contamination. Despite the acceptable logic behind such strategies, they have shortcomings. For instance, sterilizarion with autoclave, ethylene oxide, and gamma radiation can affect the chemical composition and mechanical properties of biomaterials and promote protein denaturation [73–75]. An alternative method that can be employed to prevent contamination is filtration of the culture media after removal of the soaked specimen. This method does not alter the chemical composition of the extracts. However, if this step is performed, it must be justified, and the procedures and materials used must be reported in the articles, as specified by ISO 10993–5 [6]. Finally, it must be noted that antibiotics and their combinations can alter cell proliferation [76,77]. Thus, it is advisable to include additional groups (e.g., cells

grown in basal growth media without antibiotics) to control the potential impact of antibiotics on biocompatibility. Moreover, reporting the use of antibiotics to produce the extracts and in culture conditions is essential to prevent misinterpretation and provide the necessary context for comparing results between studies.

5. Cellular models and their relevance to the testing of biomaterials

In vitro studies with diverse cell types serve as the initial phase for assessing the biocompatibility of biomaterials, facilitating the examination of their effects on cellular functions, and aiding in the selection of materials that require further development and evaluation, including in vivo and clinical studies. This strategy can ultimately reduce the overall cost and time required for biomaterial development. Cellular functions vary across tissues. Therefore, the selection of cells must be aligned with the intended applications of the biomaterial. For instance, if the intent is to assess the biocompatibility of an endodontic material, pulp-derived or immortalized cells are often used [21,78–80]. However, if the intent is to check whether the toxicity of a biomaterial negatively impacts the potential for pulp mineralization, tests could be performed with cells that undergo mineralization in response to appropriate stimuli, such as dental pulp stem cells (DPSC) [22,30,33,35]. In addition, some materials can be tested with different types of cells, as tissues can be exposed to materials in various ways. For example, resin-based materials can affect both periodontal tissues (e.g., class II restorations or implant abutments) and dental pulp (e.g., bonding procedures); therefore, cells from these tissues are relevant for testing their biocompatibility. Similarly, bone cells can be used to evaluate the toxicity of resin cement, considering cases of cemented implant-supported prostheses with a potential overflow of the material reaching the periodontal tissue.

Consequently, selecting relevant cells that align with the intended material application site is critical for robust study design in biocompatibility assessments. In vitro toxicity studies can be performed using immortalized or primary cells [81].

Immortalized cell lines are modified cells with the advantage of continuous proliferation, making them readily available and cost effective for use in biocompatibility studies. Nonetheless, they can exhibit genetic and phenotypic variances compared to their tissue of origin, display modified cytomorphology, or lose critical markers that impact their reactivity to external stimuli [81–83]. Furthermore, immortalized cells may allow for more cost-effective testing, not only because of their ability to keep expanding, but also because they are likely to be preserved in the terminal stage of differentiation compared with the differentiation of specialized cells, such as odontoblasts from human induced pluripotent stem cells (iPSCs) [84]. Nonetheless, several immortalized cell lines have been derived from dental/odontogenic tissues of animal origin, which differ physiologically and genetically from those of humans [85]. This is not a deterrent to the use of animal-derived cell lines to test the biocompatibility of dental materials. However, assumptions from the results must be made considering the non-human cell origin, and further testing with human cells is advisable.

Primary cell lines can be obtained from tissues and tested after isolation and purification [86]. Although primary cells can retain tissue-specific functions and responses, thus enhancing the physiological relevance of biocompatibility assessments, they also present restricted proliferation potential, finite lifespan, and variable characteristics depending on the donor type, which can influence cell responses [81,87,88]. Moreover, primary cells may exhibit a reduced capacity to respond to biomaterial stimuli as they undergo multiple passages in culture [84]. Notably, primary cells of similar types from different sources may share nearly identical fundamental characteristics. For instance, human gingival and dermal fibroblasts differ by 5 % in their gene profiles, with 164 and 114 genes uniquely expressed in gingival and dermal fibroblasts, respectively [89]. Nonethelsss, they can be prone to significant inter-batch and site-origin-to-origin variability.

Finally, it is important to note that the conclusions drawn from the cell culture studies cannot be directly extrapolated to clinical scenarios. Furthermore, studies based on cell cultures test cells under conditions different from those found in the target tissue, lacking connections between different cell types and signaling mediators, even when tested in co-culture studies. These limitations must be considered when interpreting the results.

5.1. Selection of cell types for the evaluation of dental materials in the light of targeted tissue and intended application

5.1.1. Cells to evaluate the effect of materials on the dental pulp

Dental pulp can be exposed to the components of various dental materials (e.g., endodontic cements, and components released from resins). One of the most commonly used cell types is DPSC, which can be obtained non-invasively from extracted human teeth, typically wisdom teeth or premolars, otherwise discarded. These cells offer several advantages over other cell types in the body as they are easily accessible, abundant, and exhibit a remarkable capacity for self-renewal and differentiation into various cell types, including neurons, odontoblasts, and fibroblasts, which are relevant for dental pulp regeneration [90,91]. These cells are versatile models for assessing biocompatibility of dental materials. Nonetheless, they experience disruption of the biological activity and differentiation potential of DPSC after long-term in vitro expansion (14 passages) [84,92]. DPSC undergo senescence and lose their proliferative potential after being cultured for 64 population doubling periods, accompanied by progressive shortening of telomere DNA and a decrease in *BMI-1* expression, which is essential for maintaining the differentiation and regenerative capacities of stem cells during replication [92]. These aspects of cell passage and culture time must be considered when using DPSC to evaluate biocompatibility of materials.

Another cell type is stem cells from human exfoliated deciduous teeth (SHED), which can differentiate into odontoblasts and generate dental pulp-like tissues in vivo [70–72]. These cells also present several of the advantages listed for DPSC, representing a more immature population than DPSC demonstrated by higher self-renewing capacity, proliferation rate, and increased cell-population doublings [93,94].

Notably, SHED exhibits rapid growth kinetics compared with DPSC [95,96], exhibiting a higher proliferation rate at early passage (P4), eventually stabilizing over long-term growth (P20) [95]. Therefore, the experimental design should consider the differences in proliferation potential when assessing the biological properties of the materials used in primary and permanent teeth.

In addition, immortalized dental pulp cells (e.g., MDPC-23 cells and odontoblast-lineage cells) can undergo mineralization when exposed to an osteogenic differentiation medium and present oxidative stress responses when exposed to unpolymerized fractions of resin cements [97,98]. Also, dental pulp fibroblasts play an important role in regulating immunity and inflammation within the pulp. Some specialized contractile fibroblasts can be a source of newly differentiated odontoblast-like cells that can synthesize reparative dentin [99]. Similar to DPSC, dental pulp fibroblasts experience variable cell death when cultured in the presence of glass ionomer cements and $CaOH_2$ specimens on permeable membranes (non-contact) [100]. Dental pulp cells can also be used to evaluate the biocompatibility of resin components. For instance, diphenyl-(2,4,6-trimethylbenzoyl)phosphine oxide (TPO) and camphorquinone (CQ) exhibit varying degrees of toxicity towards dental pulp cells, with TPO presenting greater toxicity than CQ [23]. Odontoblast-like cells and dental pulp cells have been used to show that an antioxidant agent (sodium ascorbate) can counteract the adverse effects of bleaching agents [79,101], and that low-level laser therapy can reduce or reverse the damage caused by bleaching procedures through increased cell metabolism and release of alkaline phosphatase from odontoblast-like cells after treatment [102].

5.1.2. Cells to evaluate the effect of materials on the periodontium

The periodontium, comprising both soft tissues (gingiva and periodontal ligament) and hard tissues (cementum and alveolar bone), provides vital support and maintains the teeth within the oral cavity [103]. The periodontium is composed of various cell types, including gingival fibroblasts, periodontal ligament cells, cementoblasts, and osteoblasts, which can come into contact with different dental materials and substances, such as bone grafts, membranes, and disinfectants, whichmay cause cellular toxicity.

Gingival fibroblasts, the predominant cells in gingival connective tissue, are vital for periodontal health and exhibit heterogeneity in their spindle-shaped morphology and phenotypic subpopulations [104]. These cells are sensitive to dental materials, which often come into direct contact with gingival tissue, for example, in the case of class V filling, bone grafting materials, and implants. Their response to these materials can influence periodontal health, with some materials eliciting inflammatory or cytotoxic effects, whereas others promote cell proliferation and extracellular matrix (ECM) synthesis, highlighting the need to assess gingival fibroblast responses to evaluate the biocompatibility of dental materials [105]. Gingival fibroblasts are responsible for synthesizing and maintaining ECM constituents, contributing to the structural integrity, repair, regeneration, and wound healing of periodontal and oral mucosal tissues [106,107]. Moreover, gingival fibroblasts possess cellular reprogramming, self-renewal, and multipotency capabilities, making them valuable for experimental in vitro studies [108]. Understanding their reactions to dental materials is essential to ensure the safety and effectiveness of dental treatments in clinical practice, particularly concerning substances released from restorations due to corrosion or solubility, which may cause gingival fibroblast damage. A recent study showed that components released from dental composites can affect the oxidant status of gingival fibroblasts more than those released from dental amalgam, compomers, and glass ionomer cement. This difference could be due to the release of monomers from the composites during the first 24 h [109].

PDL is a specialized connective tissue that anchors teeth to the surrounding alveolar bone and plays a crucial role in maintaining periodontal health and integrity. Cells derived from the PDL can have either an osteoblastic or fibroblastic function, which plays an important role in the formation of various periodontal structures [110]. Periodontal ligament stem cells (PDLSC) are a unique population of stem cells residing within PDL tissue. These cells possess self-renewal capacity and multilineage differentiation potential, making them valuable for periodontal tissue regeneration and repair. Together, these cells play a crucial role in maintaining periodontal homeostasis and are involved in processes such as collagen synthesis, mineralization, and tissue remodelling. When evaluating the biocompatibility of dental materials, it is essential to consider the impact of mechanical loading on the proliferative and regenerative capacities of PDLSC [110]. Studies have shown that PDLSC subjected to mechanical loading exhibit enhanced proliferation, emphasizing the need to assess how dental materials affect this crucial aspect of periodontal health [111]. These assessments provide valuable insights into the biocompatibility and safety of dental materials for clinical use. Periodontal ligament fibroblasts are the primary cell type found in the periodontal ligament, comprising approximately 60–65 % of its cellular content, and are responsible for synthesizing and maintaining the ECM within the PDL. Similar to PDLSC, these cells have been shown to possess osteoblast-like properties in vitro, with the capacity to form mineralized nodules when treated with osteogenic media [112].

Cementoblasts play a crucial role in periodontal health by contributing to the formation and maintenance of cementum, a mineralized tissue that anchors teeth to the alveolar bone. Although cementum tissue exhibits limited or no regeneration potential compared to bone tissue under pathological conditions, cementoblasts are pivotal in periodontal healing [113]. Studies have demonstrated that the compositions of different dental materials can influence the biocompatibility of cementoblasts. For example, research has shown that cementoblasts may proliferate more effectively on the surface of white mineral trioxide aggregate (MTA) than on gray MTA [114].

5.1.3. Cells to evaluate the effect of materials on the bone

Numerous biomaterials have been developed specifically for bone regeneration, including grafts, scaffolds, implants, and delivery systems for drugs and growth factors [115]. Different types of cells can be used to test the biocompatibility of materials that interact with bony tissue. For example, the MG-63 human osteosarcoma cell line is perhaps the most widely used cell line for studies evaluating the properties of biomaterials for bone applications. They are derived from human osteosarcoma cell lines and have a short doubling time (38 h), high proliferation capacity, and are easy to culture [116,117]. They present functional characteristics of the pre-osteoblastic stage and similarities to primary osteoblasts, including the expression of several integrin subunits, comparable organization of internal cellular structures, and ability to adhere to biomaterial surfaces [117,118]. A remarkable attribute of this cell type lies in its stability upon repeated culturing, exhibiting consistent expression of signaling proteins, such as Akt, p38-MAPK, GSK3α/β, IkB-α, ERK1/2, and intracellular calcium ion mobilization for up to 30 passages [119]. This stability enables researchers to establish a continuous cell stock and sustain uninterrupted work by using cells from a single source for several years.

Alternatively, primary osteoblasts isolated directly from bone tissue offer a more physiologically relevant model than immortalized cell lines do. Primary osteoblasts, derived directly from bone tissue, maintain many characteristics of native bone cells and offer valuable insights into how biomaterials interact with bone tissue in vitro. However, utilizing primary cell cultures can be technically demanding and cultures may exhibit donor-to-donor variability [120].

6. Planning and reporting biocompatibility assays

Clear and direct planning and reporting of the methodology and results are essential to enable an understanding of the testing conditions that lead to the observed results and guide the discussion of the findings. An effective report should comprise the following elements: i) a description of the cell types and conditions used for culturing and maintaining the cells before testing; ii) a detailed description of the conditions employed for preparing the specimens and culture media used to assess cytotoxic effects, including the controls used, reference materials, and treatment conditions applied to the cells; and iii) the assays used to evaluate the toxicity of the biomaterial, along with a concise summary of the experiment, any modifications made to established protocols, and a detailed account of the statistical analyses carried out (Fig. 2). A checklist to facilitate the reporting of the methodology and results is presented in Supplementary Information.

During the planning phase, it is essential to establish a well-defined objective (e.g., to evaluate specific cell responses to materials, such as a decrease in cell proliferation, or to assess fundamental biological processes triggered by materials, such as genotoxicity). The rationale for choosing a specific cell type must be consistent with the evaluation stage, whether initial screening, application-specific, or focusing on functional outcomes (e.g., effects of material toxicity on mineralization). The intended use of the material and potential mechanisms by which it interacts with cells, should also be identified (Fig. 3 A). For example, the toxicity of materials that are indirectly in contact with the pulp tissue (such as adhesives and resins due to the presence of a dentin barrier) can be tested using the indirect method, where cells are treated with culture medium containing leachable products and tested with the cell counting kit-8 (CCK-8) because unreacted monomers are expected to interfere with cell metabolism and not disrupt the cell membrane, which is typically characterized using the lactate dehydrogenase (LDH) assay [11,61]. It is critical to consider the potential interactions between materials and reagents during this phase. This is particularly important in colorimetric and fluorescence assays, as various materials can cause

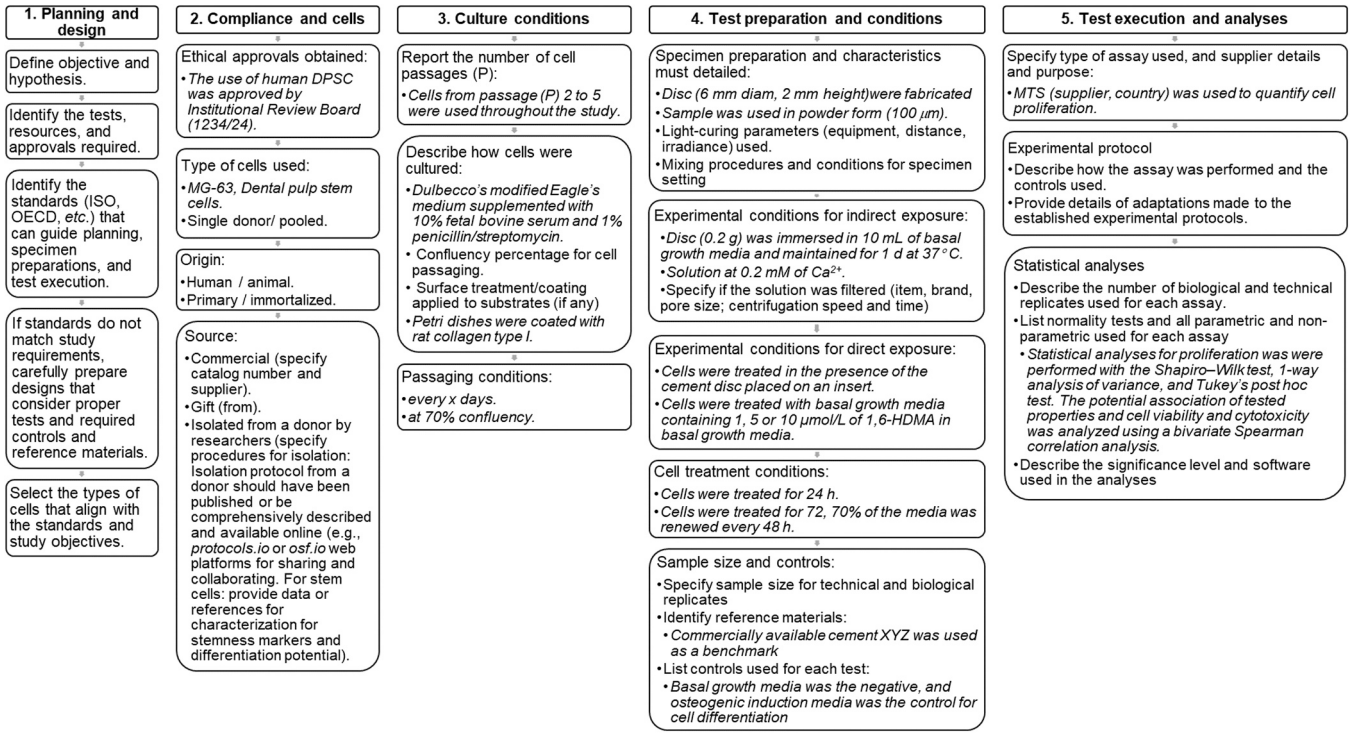

Fig. 2. Overview of the technical and biological factors that must be considered when planning, conducting, and reporting the biocompatibility of dental biomaterials. Examples of such factors (text in *italics*) can be added to the Methods and Results.

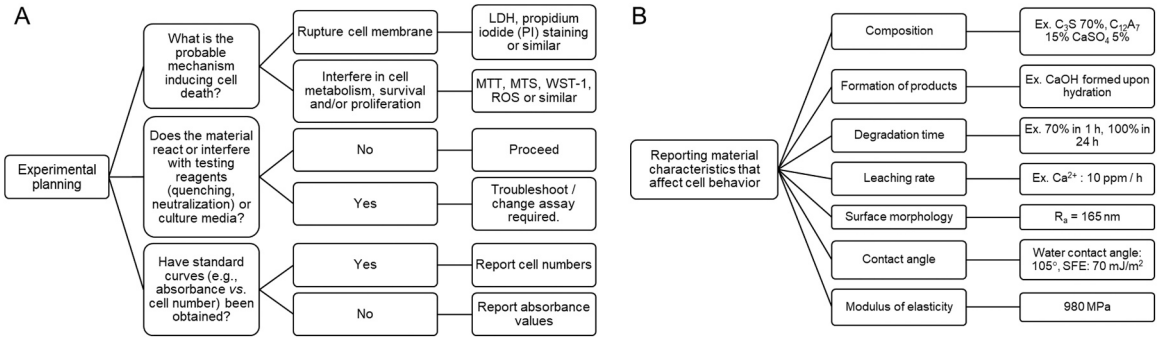

Fig. 3. (A) Technical aspects that must be considered when selecting appropriate assays and conducting tests. (B) The characterization of chemical and mechanical properties can support the interpretation of factors contributing to potential cytotoxicity and facilitate the discussion of the results (left). Examples of how to report such information in the methods and results sections are also provided (right).

pH changes, exhibit autofluorescence, or even undergo chemical reactions with reagents, thereby inhibiting the ability to emit quantifiable signals [121].

The selection of biocompatibility tests must consider the mode of exposure and potential routes of damage that may arise from interactions between materials, cells, and tissues. For instance, materials with a higher chemical reactivity can increase ROS production. In contrast, nanoparticles can promote membrane damage, cell lysis, and LDH release. Therefore, appropriate test selection can also support elucidation of the mechanisms involved in material-induced cell death. The next sections present a non-exhaustive list of the common types of cytotoxic effects that can be induced by biomaterials, along with potential tests that can be used to assess them:

1. Membrane rupture can be induced by biomaterials that contain sharp edges or irregular surfaces that can physically puncture the cell membrane, biomechanical stress, differences in osmotic pressure, triggers that lead to the release of inflammatory mediators, or the presence of toxic chemicals that can disrupt cell membranes [61, 122–124]. The ability of a material to promote membrane rupture can be characterized by quantifying LDH released from damaged or lysed cells. Alternatively, ethidium homodimer-1 (EthD-1) and propidium iodide (PI) are impermeable to intact cell membranes, but can penetrate compromised cell membranes to produce fluorescent signals. Finally, fluorescently labeled Annexin V can bind to exposed phosphatidylserine during apoptosis as lipid asymmetry is lost and can stain necrotic cells with ruptured membranes by staining the inner leaflet of the plasma membrane [124]. It must be emphasized that the detection of LDH is a less technologically demanding technique as it requires a spectrophotometer. In contrast, fluorescence-based techniques require flow cytometry, fluorescence microscopes, or fluorometry to locate and/or quantify the signals. Notably, cell death and lysis can occur independently during programmed cell death triggered by proinflammatory signals and are associated with inflammation [122]. Hence, Annexin V must be combined with a cell-impermeant stain during the differentiation of

apoptotic and dead cells to mitigate the occurrence of false positives. Likewise, one should avoid making strong assumptions about the biocompatibility of biomaterials based solely on the absence of LDH in the culture media [61,124]. The neutral red uptake (NRU) assay is valuable for evaluating cell viability and cytotoxicity due to its practicality, cost-effectiveness, and sensitivity [2,6]. This assay is contingent upon the functionality of lysosomes, as the cationic dye penetrates the cell membranes and accumulates in these acidic organelles. Cells with healthy lysosomal function retain the dye within their lysosomes, whereas compromised lysosomal function reduces dye uptake. Nonetheless, certain compounds can interfere with the dye uptake, leading to potential discrepancies. Therefore, including all the controls (e.g., test compound plus neutral red without cells) is indispensable for the tests.

2. Intracellular toxicity caused by biomaterials: the use of biomaterials for a short or long time can promote foreign body responses and induce the production of highly reactive ions or free radicals [125], such as hydrogen peroxide (H_2O_2), hydroxyl radicals (·OH), and superoxide (O_2^-)hypochlorite ions (ClO^-). Therefore, it is essential to characterize whether a biomaterial can provoke undesirable intracellular responses, such as ROS production, which could aggravate oxidative stress and lead to DNA/RNA damage, protein dysfunction, unbalanced immune system cell responses, apoptosis, inflammation, and impaired tissue regeneration. Intracellular mechanisms can be characterized using a myriad of commercially available chemiluminescent, fluorescent, reduction, and absorbance-based assays and probes, depending on the mechanism likely to be activated by the material. Despite the simplicity and convenience of such tests, caution should be exercised as the intrinsic properties of dental materials or experimental setups can lead to erroneous quantification of ROS and false-positive/negative outcomes. Quantifying ROS induced by complex materials, such as polymers, antibiotics, anti-inflammatories, or materials without a defined molecular weight and chemical structure, is challenging and may require protocol optimization. For instance, no false-negative predictions (or 100 % sensitivity) were observed for compounds with apparent molecular weight (aMw) of 250–350. Nonetheless, false positive predictions can occur for compounds with an increased aMw of 350 aMw. Hence, the determination of aMw (or equivalent in mass/mL) is recommended for quantifying ROS in complex materials to prevent the occurrence of false negatives or positives [126].

Another aspect that must be considered is that materials can exhibit intrinsic fluorescence or interact with fluorescent probes (leading to the quenching of the emitted signal) which can interfere with the results [121]. Hence, it is prudent to characterize the autofluorescence properties of materials under different wavelengths and include controls (e.g., materials and probe, no cells) in assessments using fluorescence-based assays (Fig. 4 A and B). Finally, combining fluorescence labeling with a cell quantification assay is recommended to confirm whether a potentially low fluorescence value is related to material/reagent compatibility rather than being an artifact caused by a reduced cell count due to material toxicity, which leads to cell detachment. This can result in an incorrect interpretation that adherent cells produce less fluorescence (suggesting higher biocompatibility, as in the ROS assay), whereas the decrease in fluorescence may be attributable to a reduction in the number of live cells, as detached cells do not emit fluorescence and are not included in the measurement [127]. Similarly, experiments that assess cell metabolism by altering the color of the culture medium promoted by byproducts of cell metabolism (e.g., WST-1 and MTS) can provide inaccurate outcomes if biomaterials present in the medium cause color changes due to changes in pH. Therefore, it is advisable to test the stability of the reagents and culture medium in contact with the biomaterials and to include the necessary controls to ensure that color changes are promoted only by cellular metabolism (Fig. 4 C and D).

Another factor that must be considered when reporting results is the characterization of the physical and chemical properties of the materials, as these can cause alterations in the cell behavior. For example, many endodontic cements based on calcium silicate are known to increase the alkalinity of the microenvironment, which can lead to both cell differentiation and death depending on the intensity and kinetics of ion release [39]. Similarly, the surface roughness and elastic modulus of the substrates influence the duration of cell surface anchoring and proliferation [128]. Therefore, reporting the basic characteristics of the materials is important for supporting the interpretation and discussion of the observed results.

7. Emerging testing methodologies for assessing biomaterials cytotoxicity

Traditional cytotoxicity testing is primarily based on in vitro monolayer cell cultures such as fibroblasts, dental pulp, and epithelial cells. This method involves exposing cells to biomolecules or biomaterials or to a culture medium conditioned by the tested biomaterial [39,129]. This approach is known for its practicality and cost-effectiveness, and provides insights into cell biocompatibility. Although these cell-based assays remain fundamental, recent

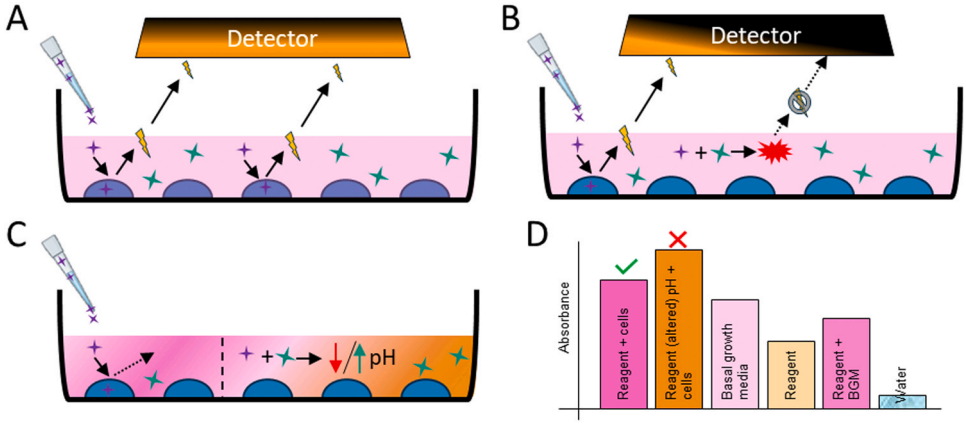

Fig. 4. (A) Various reagents (purple stars) are used to assess the cytotoxicity of biomaterials (green stars) by measuring and quantifying metabolic byproducts or fluorescent emissions. (B) The reaction between reagents and biomaterials can decrease the emitted signal, resulting in false-negative toxicity results. (C) Colorimetric assays change the color of the medium through the metabolism of the reagent by the cells (left). In cases where the reaction of the reagent with biomaterials leads to a pH alteration (right), it can result in a significant change in the color of the culture medium and a false positive result in cytotoxicity assays (false negative is marked as × in D).

developments such as 3D organotypic cultures, in vitro pulp chambers, ex vivo tooth slice cultures, and microfluidic or microphysiological systems have enhanced the precision and physiological relevance of biocompatibility assessments [41,130].

Three-dimensional organotypic cultures of the oral mucosa, gingiva, and dentin-pulp complex mimic intricate structures and microphysiological interactions within the tissues. These models involve the culture of cells in a 3D matrix, allowing physiological cell-cell and cell-extracellular matrix interactions. They provide valuable insights into how dental biomaterials and oral care products influence tissue responses and help predict biocompatibility in vivo [131,132].

Three-dimensional organotypic gingival and oral mucosa equivalents display native tissue-like, multi-layered epithelia, which have been employed to assess the mucosal irritation and corrosion potential of mouthwash, toothpaste, dental restoratives, and other oral/dental care formulations [41,129,133,134]. Histological examination of tissue substitutes offers valuable insights into cellular and morphological alterations, as demonstrated by the visualization and quantification of epithelial disruption following exposure to silver diamine fluoride, phosphoric acid, and eugenol [41,134]. Similarly, cytokine analysis of the culture media derived from tissue equivalents provided cues for surrogate markers of tissue viability and cytotoxic and immune responses. Cytokines such as IL-1α and IL-1β are commonly used as surrogate biomarkers for mucosal irritation potential [129,133,134]. Also, commercially available organotypic analogues of the human gingival and oral mucosal tissues (EpiGingival™, EpiOral™, SkinEthic™ HOE, SkinEthic™ HGE), are commonly utilized for the biocompatibility studies, screening and early safety assessment of actives and excipients [131,134,135]. Similarly, organotypic cultures of dentin disks stacked over hydrogels incorporated with pulpal and endothelial cells within a hanging insert have also been used to reconstruct 3D vascularized dentin-pulp complex analogs [132]. These analogs of 3D dentin/pulp tissue with vasculature provide a sophisticated 3D in vitro tool for understanding the cytotoxicity of resin monomers (HEMA and TEGDMA) [136] and commercially available HEMA/TEGDMA-based adhesive resin cements [132]. Despite the presence of vasculature, the absence of active perfusion (reminiscent of blood circulation) does not facilitate clearance of noxious stimuli and immune responses [137,138].

Cell perfusion chambers play a pivotal role in replicating the dentin-pulp interface and evaluating the biocompatibility of dental materials. These chambers consist of dentin discs serving as dentin analogs and 3D scaffolds infused with dental pulp cells as pulp analogs sandwiched between two chambers [75,80,130,139]. Active perfusion of culture media on the pulpal side is an effective method for simulating pulpal blood flow, which is useful for evaluating the response of the pulp tissue to dental materials applied to the dentin surface. This approach is advantageous due to its cost-effectiveness and accessibility. However, there are limitations associated with this method. The thickness and morphology of dentin discs can vary, leading to differences in the distribution and density of dentinal tubules [75,80]. This variability may affect the permeation of the test substances and ultimately influence the results of the experiment. Furthermore, sterilization, typically involving autoclaving, can cause denaturation of proteins, particularly collagen, which may affect the diffusion of the test substances [75].

The dentin-pulp tooth slice model is based on the use of thin dentin slices (usually 1 or 2 mm) containing native pulp tissue to recapitulate the physiology of the dentin-pulp complex and investigate the responses of pulp tissues to resin adhesive systems, calcium hydroxide, resin composites, and bioceramic cements [84,140–142]. Despite its ease of fabrication and cost-effectiveness, the tooth slice model is susceptible to contamination and limitations in simulating the nutritional constraints of human dental pulp. An alternative method of ex vivo culture provides an opportunity to recapitulate the native pulp microenvironment. Notably, this approach has been utilized for biocompatibility assessment and evaluation of dental pulp response to bioceramic cements following pulp-capping procedures [138,143–145].

The convergence of microfluidic technology and 3D culture models has led to the recent emergence of microphysiological systems, which are referred to as microfluidic organ-on-chip devices. These devices offer new possibilities for emulating complex features of the host tissue microenvironment within confined microchambers and microchannels. Furthermore, they enable precise control over fluid flow, shear stress, and chemical and physical gradients, thereby fostering the potential to emulate the 3D microenvironment of native tissues. These advances are gaining significant traction in the dental, oral, and craniofacial fields [132,146–148]. This includes replication of the intricate features of the host tissue microenvironment and gaining insights into the impact of mechanical forces [134,149], interstitial fluid flow [150,151], gingival crevicular fluid flow [150], dentin barrier [41,152] and saliva flow on the host material [41,134,152–155], and host-microbe interactions [150,156,157]. In vivo, odontoblasts responsible for transmitting external stimuli exhibit unique morphological characteristics that are typically lost in conventional cell culture. To address this issue, the technology of “dentin-on-a-chip” has the potential to recreate the dentinal architecture, which includes narrow microchannels that restrict the movement of odontoblasts, ultimately resulting in the formation of projections that resemble those found in natural odontoblasts [158]. This approach offers promising insights, although the use of silicon-based microchannels instead of real dentin limits the representation of the dentin-pulp environment in the system. The “tooth--on-a-chip” mimics the pulp-dentin complex, with a native dentin disc sandwiched between two channels: one simulating pulp cells and another allowing the introduction of dental biomaterials [41,152]. The native dentin disc serves as a semipermeable barrier, and this model facilitates step-by-step testing of restorative materials. When applied through dentin, the materials demonstrated a reduced potential to compromise cell viability in contrast to direct exposure [41,152]. Furthermore, this tooth-on-a-chip can be used to explore the interactions between calcium silicate cement and DPSC, revealing correlations between pH changes and growth factor release. Vertical bilayer tooth-on-chip is a valuable tool for examining the influence of dentin thickness on the permeation of silver diamine fluoride and its effects on the viability of DPSCs under physiological flow conditions. In this design, the dentin disc is clamped over a rhomboid-shaped microchannel to culture dental pulp cells. This design allows simultaneous analysis of cellular viability on the unexposed (internal control) and exposed (experimental) sides of the same microchannel [41]. Similarly, “gingiva-on-a-chip” may be utilized to evaluate the biocompatibility of mouthwash using controlled bidirectional flow of the solution over reconstructed gingival tissues. By employing this approach, it was found that the observed increase in tissue disruption and cytotoxic effects surpassed those observed under static conditions, thereby providing a more accurate representation of mouth rinsing actions and allowing for the physiological assessment of mucosal irritation potential [134]. Lastly, with the potential to fabricate vascularized tissue analogs in vitro, such as vascularized dentin-pulp and gingiva tissues, incorporation of immune cells and their potential integration with microfluidic platforms opens opportunities to study systemic dissemination of biomaterials and microbes and associated oral-systemic effects [159,160].

8. Conclusion

Assessing the biocompatibility of materials is a critical step in the development of biomaterials, as only those deemed safe can perform their intended functions in clinical settings. Evaluating the biocompatibility of a material involves numerous aspects, including the selection of cell type and origin, specimen preparation, choice of tests, and performance of assays, all of which must be aligned with the objectives of the study and the intended future application of the biomaterial. Despite the seemingly trivial nature of many tests, each stage of preparation, execution, and reporting contributes to the biocompatibility "character" of a biomaterial. Therefore, the information provided in this paper is

non-exhaustive, and researchers must further optimize the testing parameters and troubleshoot potential interferences to prevent false positives and negatives, and characterize material properties that could significantly impact cell viability.

Future developments in testing methodologies, models, and strategies will enhance the precision and applicability of testing, and provide more streamlined methods for screening potential materials. The integration of conventional cell-based assays, high-throughput screening, organ-on-a-chip models, noninvasive imaging, computational modeling, and multi-omics analysis holds great promise for achieving a more complete understanding of cytotoxicity, ultimately leading to the development of safer and more biocompatible dental materials.

Disclosure statement

Given his role as Editor in Chief, Dr David Watts had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to Dr George Eliades, Editor.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.dental.2024.07.020.

References

[1] Schmalz G, Jakubovics N, Schwendicke F. Normative approaches for oral health: Standards, specifications, and guidelines. J Dent Res 2021;101:489–94. https://doi.org/10.1177/00220345211049695.
[2] International Standard Organization. ISO 7405:2018: Dentistry. Evaluation of biocompatibility of medical devices used in dentistry 2018.
[3] Darvell BW. Misuse of ISO standards in dental materials research. Dent Mater 2020;36:1493–4. https://doi.org/10.1016/j.dental.2020.10.014.
[4] Gruber S, Nickel A. Toxic or not toxic? The specifications of the standard ISO 10993-5 are not explicit enough to yield comparable results in the cytotoxicity assessment of an identical medical device. Front Med Technol 2023;5. https://doi.org/10.3389/fmedt.2023.1195529.
[5] Schmalz G, Watts DC, Darvell BW. Dental materials science: research, testing and standards. Dent Mater 2021;37:379–81. https://doi.org/10.1016/j.dental.2021.01.027.
[6] International Standard Organization. ISO 10993–5:2009 Biological evaluation of medical devices — Part 5: Tests for in vitro cytotoxicity. 2022.
[7] Paula AB, Laranjo M, Coelho AS, Abrantes AM, Goncalves AC, Sarmento-Ribeiro AB, Ferreira MM, Botelho MF, Marto CM, Carrilho E. Accessing the cytotoxicity and cell response to biomaterials. J Vis Exp 2021. https://doi.org/10.3791/61512.
[8] Bapat RA, Parolia A, Chaubal T, Dharamadhikari S, Abdulla AM, Sakkir N, Arora S, Bapat P, Sindi AM, Kesharwani P. Recent update on potential cytotoxicity, biocompatibility and preventive measures of biomaterials used in dentistry. Biomater Sci 2021;9:3244–83. https://doi.org/10.1039/d1bm00233c.
[9] Bouillaguet S, Shaw L, Gonzalez L, Wataha JC, Krejci I. Long-term cytotoxicity of resin-based dental restorative materials. J Oral Rehabil 2002;29:7–13. https://doi.org/10.1046/j.1365-2842.2002.00804.x.
[10] Ditrichova D, Kapralova S, Tichy M, Ticha V, Dobesova J, Justova E, Eber M, Pirek P. Oral lichenoid lesions and allergy to dental materials. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 2007;151:333–9. https://doi.org/10.5507/bp.2007.057.
[11] Ferracane JL. Elution of leachable components from composites. J Oral Rehabil 1994;21:441–52. https://doi.org/10.1111/j.1365-2842.1994.tb01158.x.
[12] Karabucak B, Stoopler ET. Root canal treatment on a patient with zinc oxide allergy: a case report. Int Endod J 2007;40:800–7. https://doi.org/10.1111/j.1365-2591.2007.01275.x.
[13] Porto IC, Oliveira DC, Raele RA, Ribas KH, Montes MA, De Castro CM. Cytotoxicity of current adhesive systems: in vitro testing on cell cultures of primary murine macrophages. 22128 Dent Mater 2011;27. https://doi.org/10.1016/j.dental.2010.10.006.
[14] Kloukos D, Pandis N, Eliades T. Bisphenol-A and residual monomer leaching from orthodontic adhesive resins and polycarbonate brackets: a systematic review. e1-2 Am J Orthod Dentofac Orthop 2013;143:S104–12. https://doi.org/10.1016/j.ajodo.2012.11.015.
[15] Chang HH, Guo MK, Kasten FH, Chang MC, Huang GF, Wang YL, Wang RS, Jeng JH. Stimulation of glutathione depletion, ROS production and cell cycle arrest of dental pulp cells and gingival epithelial cells by HEMA. Biomaterials 2005;26:745–53. https://doi.org/10.1016/j.biomaterials.2004.03.021.
[16] Eckhardt A, Harorli T, Limtanyakul J, Hiller KA, Bosl C, Bolay C, Reichl FX, Schmalz G, Schweikl H. Inhibition of cytokine and surface antigen expression in LPS-stimulated murine macrophages by triethylene glycol dimethacrylate. Biomaterials 2009;30:1665–74. https://doi.org/10.1016/j.biomaterials.2008.09.024.
[17] Krifka S, Spagnuolo G, Schmalz G, Schweikl H. A review of adaptive mechanisms in cell responses towards oxidative stress caused by dental resin monomers. Biomaterials 2013;34:4555–63. https://doi.org/10.1016/j.biomaterials.2013.03.019.
[18] Schweikl H, Hartmann A, Hiller KA, Spagnuolo G, Bolay C, Brockhoff G, Schmalz G. Inhibition of TEGDMA and HEMA-induced genotoxicity and cell cycle arrest by N-acetylcysteine. Dent Mater 2007;23:688–95. https://doi.org/10.1016/j.dental.2006.06.021.
[19] Chang MC, Chen LI, Chan CP, Lee JJ, Wang TM, Yang TT, Lin PS, Lin HJ, Chang HH, Jeng JH. The role of reactive oxygen species and hemeoxygenase-1 expression in the cytotoxicity, cell cycle alteration and apoptosis of dental pulp cells induced by BisGMA. Biomaterials 2010;31:8164–71. https://doi.org/10.1016/j.biomaterials.2010.07.049.
[20] Krifka S, Hiller KA, Bolay C, Petzel C, Spagnuolo G, Reichl FX, Schmalz G, Schweikl H. Function of MAPK and downstream transcription factors in monomer-induced apoptosis. Biomaterials 2012;33:740–50. https://doi.org/10.1016/j.biomaterials.2011.10.026.
[21] Massaro H, Zambelli L, Britto A, Vieira R, Ligeiro-de-Oliveira A, Andia D, Oliveira M, Lima A. Solvent and HEMA increase adhesive toxicity and cytokine release from dental pulp cells. Materials 2019;12. https://doi.org/10.3390/ma12172750.
[22] Galler KM, Schweikl H, Hiller KA, Cavender AC, Bolay C, D'Souza RN, Schmalz G. TEGDMA reduces mineralization in dental pulp cells. J Dent Res 2010;90:257–62. https://doi.org/10.1177/0022034510384618.
[23] Van Landuyt KL, Krifka S, Hiller K-A, Bolay C, Waha C, Van Meerbeek B, Schmalz G, Schweikl H. Evaluation of cell responses toward adhesives with different photoinitiating systems. Dent Mater 2015;31:916–27. https://doi.org/10.1016/j.dental.2015.04.016.
[24] Vouzara T, Dimosiari G, Koulaouzidou EA, Economides N. Cytotoxicity of a new calcium silicate endodontic sealer. J Endod 2018;44:849–52. https://doi.org/10.1016/j.joen.2018.01.015.
[25] Zhou HM, Du TF, Shen Y, Wang ZJ, Zheng YF, Haapasalo M. In vitro cytotoxicity of calcium silicate-containing endodontic sealers. J Endod 2015;41:56–61. https://doi.org/10.1016/j.joen.2014.09.012.
[26] Huang FM, Tai KW, Chou MY, Chang YC. Cytotoxicity of resin-, zinc oxide-eugenol-, and calcium hydroxide-based root canal sealers on human periodontal ligament cells and permanent V79 cells. Int Endod J 2002;35:153–8. https://doi.org/10.1046/j.1365-2591.2002.00459.x.
[27] Primus CM, Tay FR, Niu LN. Bioactive tri/dicalcium silicate cements for treatment of pulpal and periapical tissues. Acta Biomater 2019;96:35–54. https://doi.org/10.1016/j.actbio.2019.05.050.
[28] Ersev H, Schmalz G, Bayirli G, Schweikl H. Cytotoxic and mutagenic potencies of various root canal filling materials in eukaryotic and prokaryotic cells in vitro. J Endod 1999;25:359–-63. https://doi.org/10.1016/s0099-2399(06)81172-6.
[29] Benetti F, Queiroz IOA, Cosme-Silva L, Conti LC, Oliveira SHP, Cintra LTA. Cytotoxicity, biocompatibility and biomineralization of a new ready-for-use bioceramic repair material. Braz Dent J 2019;30:325–32. https://doi.org/10.1590/0103-6440201902457.
[30] Cahyanto A, Rath P, Teo TX, Tong SS, Malhotra R, Cavalcanti BN, Lim LZ, Min KS, Ho D, Lu WF, Rosa V. Designing calcium silicate cements with on-demand properties for precision endodontics. J Dent Res 2023;102:1425–33. https://doi.org/10.1177/00220345231198185.
[31] Giraud T, Jeanneau C, Rombouts C, Bakhtiar H, Laurent P, About I. Pulp capping materials modulate the balance between inflammation and regeneration. Dent Mater 2019;35:24–35. https://doi.org/10.1016/j.dental.2018.09.008.
[32] Ali M, Okamoto M, Watanabe M, Huang H, Matsumoto S, Komichi S, Takahashi Y, Hayashi M. Biological properties of lithium-containing surface pre-reacted glass fillers as direct pulp-capping cements. Dent Mater 2022;38:294–308. https://doi.org/10.1016/j.dental.2021.12.011.
[33] Koutroulis A, Kuehne SA, Cooper PR, Camilleri J. The role of calcium ion release on biocompatibility and antimicrobial properties of hydraulic cements. Sci Rep 2019;9. https://doi.org/10.1038/s41598-019-55288-3.
[34] Gluskin AH, Lai G, Peters CI, Peters OA. The double-edged sword of calcium hydroxide in endodontics. J Am Dent Assoc 2020;151:317–26. https://doi.org/10.1016/j.adaj.2020.01.026.
[35] Queiroz MB, Torres FFE, Rodrigues EM, Viola KS, Bosso-Martelo R, Chavez-Andrade GM, Guerreiro-Tanomaru JM, Tanomaru-Filho M. Physicochemical, biological, and antibacterial evaluation of tricalcium silicate-based reparative cements with different radiopacifiers. Dent Mater 2021;37:311–20. https://doi.org/10.1016/j.dental.2020.11.014.
[36] Mocquot C, Colon P, Fernando D, Jackson P, Pradelle-Plasse N, Grosgogeat B, Attik N. The influence of experimental bioactive glasses on pulp cells behavior in vitro. Dent Mater 2020;36:1322–31. https://doi.org/10.1016/j.dental.2020.07.006.
[37] Natu VP, Dubey N, Loke GCL, Tan TS, Ng WH, Yong CW, Cao T, Rosa V. Bioactivity, physical and chemical properties of MTA mixed with propylene glycol. J Appl Oral Sci 2015;23:405–11. https://doi.org/10.1590/1678-775720150084.
[38] Lee S-Y, Min K-S, Choi G-W, Park J-H, Park S-H, Lee S-I, Kim E-C. Effects of simvastain and enamel matrix derivative on Portland cement with bismuth oxide–induced growth and odontoblastic differentiation in human dental pulp cells. J Endod 2012;38:405–10. https://doi.org/10.1016/j.joen.2011.12.025.
[39] Phang V, Malhotra R, Chen NN, Min K-S, Yu VSH, Rosa V, Dubey N. Specimen shape and elution time affect the mineralization and differentiation potential of

dental pulp stem cells to biodentine. J Funct Biomater 2023;15. https://doi.org/10.3390/jfb15010001.
[40] Schmalz G, Hiller K, Nunez L, Stoll J, Weis K. Permeability characteristics of bovine and human dentin under different pretreatment conditions. J Endod 2001; 27:23–30. https://doi.org/10.1097/00004770-200101000-00007.
[41] Hu S, Muniraj G, Mishra A, Hong K, Lum JL, Hong CHL, Rosa V, Sriram G. Characterization of silver diamine fluoride cytotoxicity using microfluidic tooth-on-a-chip and gingival equivalents. Dent Mater 2022;38:1385–94. https://doi.org/10.1016/j.dental.2022.06.025.
[42] Bellinger DC, Daniel D, Trachtenberg F, Tavares M, McKinlay S. Dental amalgam restorations and children's neuropsychological function: the New England Children's Amalgam Trial. Environ Health Perspect 2007;115:440–6. https://doi.org/10.1289/ehp.9497.
[43] Bellinger DC, Trachtenberg F, Barregard L, Tavares M, Cernichiari E, Daniel D, McKinlay S. Neuropsychological and renal effects of dental amalgam in children: a randomized clinical trial. JAMA 2006;295:1775–83. https://doi.org/10.1001/jama.295.15.1775.
[44] Bruzell Roll EM, Jacobsen N, Hensten-Pettersen A. Health hazards associated with curing light in the dental clinic. Clin Oral Invest 2004;8:113–7. https://doi.org/10.1007/s00784-003-0248-x.
[45] Ferreira JN, Ko CC, Myers S, Swift J, Fricton JR. Evaluation of surgically retrieved temporomandibular joint alloplastic implants: pilot study. J Oral Maxillofac Surg 2008;66:1112–24. https://doi.org/10.1016/j.joms.2007.09.013.
[46] Hensten-Pettersen A. Skin and mucosal reactions associated with dental materials. Eur J Oral Sci 1998;106:707–12.
[47] Worthington HV, Khangura S, Seal K, Mierzwinski-Urban M, Veitz-Keenan A, Sahrmann P, Schmidlin PR, Davis D, Iheozor-Ejiofor Z, Rasines Alcaraz MG. Direct composite resin fillings versus amalgam fillings for permanent posterior teeth. Cochrane Database Syst Rev 2021;(8):CD005620. https://doi.org/10.1002/14651858.CD005620.pub3.
[48] Rodríguez-Farre E, Testai E, Bruzell E, De Jong W, Schmalz G, Thomsen M, Hensten A. The safety of dental amalgam and alternative dental restoration materials for patients and users. Regul Toxicol Pharmacol 2016;79:108–9. https://doi.org/10.1016/j.yrtph.2015.12.015.
[49] Schmalz G, Arenholt-Dindslev D. Biocompatibility of Dental Materials. Springer,; 2009. https://doi.org/10.1007/978-3-540-77782-3.
[50] Foldbjerg R, Olesen P, Hougaard M, Dang DA, Hoffmann HJ, Autrup H. PVP-coated silver nanoparticles and silver ions induce reactive oxygen species, apoptosis and necrosis in THP-1 monocytes. Toxicol Lett 2009;190:156–62. https://doi.org/10.1016/j.toxlet.2009.07.009.
[51] Schmalz G, Hickel R, van Landuyt KL, Reichl F-X. Nanoparticles in dentistry. Dent Mater 2017;33:1298–314. https://doi.org/10.1016/j.dental.2017.08.193.
[52] Chairuangkitti P, Lawanprasert S, Roytrakul S, Aueviriyavit S, Phummiratch D, Kulthong K, Chanvorachote P, Maniratanachote R. Silver nanoparticles induce toxicity in A549 cells via ROS-dependent and ROS-independent pathways. Toxicol Vitr 2013;27:330–8. https://doi.org/10.1016/j.tiv.2012.08.021.
[53] Mohammadinejad R, Moosavi MA, Tavakol S, Vardar DO, Hosseini A, Rahmati M, Dini L, Hussain S, Mandegary A, Klionsky DJ. Necrotic, apoptotic and autophagic cell fates triggered by nanoparticles. Autophagy 2019;15:4–33. https://doi.org/10.1080/15548627.2018.1509171.
[54] Behnke R, Stahl F, Duske K, Warkentin M, Schwartz M, Hinz B, Walther U. Influence of test specimen geometry and water soaking on the in vitro cytotoxicity of Orthocryl®, Orthocryl® LC, Loctite® EA 9483 and polypropylene. Molecules 2022;27. https://doi.org/10.3390/molecules27123949.
[55] Rosa V, Toh WS, Cao T, Shim W. Inducing pluripotency for disease modeling, drug development and craniofacial applications. Expert Opin Biol Ther 2014;14: 1233–40. https://doi.org/10.1517/14712598.2014.915306.
[56] Macnair R, Rodgers EH, Macdonald C, Wykman A, Goldie I, Grant MH. The response of primary rat and human osteoblasts and an immortalized rat osteoblast cell line to orthopaedic materials: comparative sensitivity of several toxicity indices. J Mater Sci - Mater Med 1997;8:105–11. https://doi.org/10.1023/a:1018510900941.
[57] Ferreira Lopes S, Vacher G, Ciarlo E, Savova-Bianchi D, Roger T, Niculita-Hirzel H. Primary and immortalized human respiratory cells display different patterns of cytotoxicity and cytokine release upon exposure to Deoxynivalenol, Nivalenol and Fusarenon-x. Toxins 2017;9. https://doi.org/10.3390/toxins9110337.
[58] Mahdhaoui K, Fournier B, Derbanne MA. Unbound monomers do diffuse through the dentin barrier. Dent Mater 2017;33:743–51. https://doi.org/10.1016/j.dental.2017.04.007.
[59] Diemer F, Stark H, Helfgen E-H, Enkling N, Probstmeier R, Winter J, Kraus D. In vitro cytotoxicity of different dental resin-cements on human cell lines. J Mater Sci Mater Med 2021;32. https://doi.org/10.1007/s10856-020-06471-w.
[60] Chang Y, Yang S-T, Liu J-H, Dong E, Wang Y, Cao A, Liu Y, Wang H. In vitro toxicity evaluation of graphene oxide on A549 cells. Toxicol Lett 2011;200: 201–10. https://doi.org/10.1016/j.toxlet.2010.11.016.
[61] Malhotra R, Halbig CE, Sim YF, Lim CT, Leong DT, Neto AHC, Garaj S, Rosa V. Cytotoxicity survey of commercial graphene materials from worldwide. npj 2D Mater Appl 2022;6. https://doi.org/10.1038/s41699-022-00330-8.
[62] Radunovic M, Pavic A, Ivanovic V, Milivojevic M, Radovic I, Di Carlo R, Pilato S, Fontana A, Piattelli A, Petrovic S. Biocompatibility and antibiofilm activity of graphene-oxide functionalized titanium discs and collagen membranes. Dent Mater 2022;38:1117–27. https://doi.org/10.1016/j.dental.2022.04.024.
[63] Santa-Gonzalez GA, Gomez-Molina A, Arcos-Burgos M, Meyer JN, Camargo M. Distinctive adaptive response to repeated exposure to hydrogen peroxide associated with upregulation of DNA repair genes and cell cycle arrest. Redox Biol 2016;9:124–33. https://doi.org/10.1016/j.redox.2016.07.004.
[64] Wuersching SN, Hickel R, Edelhoff D, Kollmuss M. Initial biocompatibility of novel resins for 3D printed fixed dental prostheses. Dent Mater 2022;38:1587–97. https://doi.org/10.1016/j.dental.2022.08.001.
[65] Miller MA, Zachary JF. Mechanisms and morphology of cellular injury, adaptation, and death. e19 Pathol Basis Vet Dis 2017:2–43. https://doi.org/10.1016/b978-0-323-35775-3.00001-1.
[66] Frasheri I, Grimm A, Ern C, Hickel R, Folwaczny M. In-vitro cytocompatibility of self-adhesive dual-curing resin cements on human mesenchymal stem cells (hMSC) and periodontal ligament cells (PDL-hTERT). Dent Mater 2022;38: 376–83. https://doi.org/10.1016/j.dental.2021.12.020.
[67] Moghaddamejafari S, Mantellini M, Botero T, McDonald N, Nor J. Effect of ProRoot MTA on pulp cell apoptosis and proliferation in vitro. J Endod 2005;31: 387–91. https://doi.org/10.1097/01.don.0000145423.89539.d7.
[68] Mantellini MG, Botero T, Yaman P, Dennison JB, Hanks CT, Nör JE. Adhesive resin and the hydrophilic monomer HEMA induce VEGF expression on dental pulp cells and macrophages. Dent Mater 2006;22:434–40. https://doi.org/10.1016/j.dental.2005.04.039.
[69] International Standard Organization. ISO 10993–12:2021 Biological evaluation of medical devices — Part 12: Sample preparation and reference materials. 2021.
[70] Bural C, AktaŞ E, Deniz G, Ünlüçerçi Y, Bayraktar G. Effect of leaching residual methyl methacrylate concentrations on in vitro cytotoxicity of heat polymerized denture base acrylic resin processed with different polymerization cycles. J Appl Oral Sci 2011;19:306–12. https://doi.org/10.1590/s1678-77572011005000002.
[71] Moharamzadeh K, Van Noort R, Brook IM, Scutt AM. HPLC analysis of components released from dental composites with different resin compositions using different extraction media. J Mater Sci Mater Med 2007;18:133–7. https://doi.org/10.1007/s10856-006-0671-z.
[72] International Standard Organization. ISO 10993–17:2023 Biological evaluation of medical devices — Part 17: Toxicological risk assessment of medical device constituents. 2023.
[73] de Sousa Iwamoto LA, Duailibi MT, Iwamoto GY, de Oliveira DC, Duailibi SE. Evaluation of ethylene oxide, gamma radiation, dry heat and autoclave sterilization processes on extracellular matrix of biomaterial dental scaffolds. Sci Rep 2022;12. https://doi.org/10.1038/s41598-022-08258-1.
[74] White JM, Goodis HE, Marshall SJ, Marshall GW. Sterilization of teeth by gamma radiation. J Dent Res 2016;73:1560–7. https://doi.org/10.1177/00220345940730091201.
[75] Jiang RD, Lin H, Zheng G, Zhang XM, Du Q, Yang M. In vitro dentin barrier cytotoxicity testing of some dental restorative materials. J Dent 2017;58:28–33. https://doi.org/10.1016/j.jdent.2017.01.003.
[76] Skubis A, Gola J, Sikora B, Hybiak J, Paul-Samojedny M, Mazurek U, Łos MJ. Impact of antibiotics on the proliferation and differentiation of human adipose-derived mesenchymal stem cells. Int J Mol Sci 2017;18. https://doi.org/10.3390/ijms18122522.
[77] Nygaard UH, Niehues H, Rikken G, Rodijk-Olthuis D, Schalkwijk J, van den Bogaard EH. Antibiotics in cell culture: friend or foe? Suppression of keratinocyte growth and differentiation in monolayer cultures and 3D skin models. Exp Dermatol 2015;24:964–5. https://doi.org/10.1111/exd.12834.
[78] Rosa V, Xie H, Dubey N, Madanagopal TT, Rajan SS, Morin JLP, Islam I, Castro Neto AH. Graphene oxide-based substrate: physical and surface characterization, cytocompatibility and differentiation potential of dental pulp stem cells. Dent Mater 2016;32:1019–25. https://doi.org/10.1016/j.dental.2016.05.008.
[79] Lima AF, Lessa FC, Hebling J, de Souza Costa CA, Marchi GM. Protective effect of sodium ascorbate on mdpc-23 odontoblast-like cells exposed to a bleaching agent. Eur J Dent 2010;4:238–44.
[80] Galler K, Hiller KA, Ettl T, Schmalz G. Selective influence of dentin thickness upon cytotoxicity of dentin contacting materials. J Endod 2005;31:396–9. https://doi.org/10.1097/01.don.0000145428.26880.e5.
[81] Czekanska EM, Stoddart MJ, Ralphs JR, Richards RG, Hayes JS. A phenotypic comparison of osteoblast cell lines versus human primary osteoblasts for biomaterials testing. J Biomed Mater Res A 2014;102:2636–43. https://doi.org/10.1002/jbm.a.34937.
[82] Kaur G, Dufour JM. Cell lines. Spermatogenesis 2014;2:1–5. https://doi.org/10.4161/spmg.19885.
[83] Hynds RE, Giangreco A. Concise review: The relevance of human stem cell-derived organoid models for epithelial translational medicine. Stem Cells 2013; 31:417–22. https://doi.org/10.1002/stem.1290.
[84] Xie H, Dubey N, Shim W, Ramachandra CJA, Min KS, Cao T, Rosa V. Functional odontoblastic-like cells derived from human iPSCs. J Dent Res 2017;97:77–83. https://doi.org/10.1177/0022034517730026.
[85] Zeng Y, Liu L, Huang D, Song D. Immortalized cell lines derived from dental/odontogenic tissue. Cell Tissue Res 2023;393:1–15. https://doi.org/10.1007/s00441-023-03767-5.
[86] Yildirim S. Isolation methods of dental pulp stem cells. Dent Pulp Stem Cells 2013:41–51. https://doi.org/10.1007/978-1-4614-5687-2_5.
[87] Phinney DG, Kopen G, Righter W, Webster S, Tremain N, Prockop DJ. Donor variation in the growth properties and osteogenic potential of human marrow stromal cells. J Cell Biochem 1999;75:424–36.
[88] Siegel G, Kluba T, Hermanutz-Klein U, Bieback K, Northoff H, Schafer R. Phenotype, donor age and gender affect function of human bone marrow-derived mesenchymal stromal cells. BMC Med 2013;11:146. https://doi.org/10.1186/1741-7015-11-146.
[89] Ebisawa K, Kato R, Okada M, Sugimura T, Latif MA, Hori Y, Narita Y, Ueda M, Honda H, Kagami H. Gingival and dermal fibroblasts: their similarities and

differences revealed from gene expression. J Biosci Bioeng 2011;111:255–8. https://doi.org/10.1016/j.jbiosc.2010.11.014.
[90] Rosa V, Zhang Z, Grande RHM, Nör JE. Dental pulp tissue engineering in full-length human root canals. J Dent Res 2013;92:970–5. https://doi.org/10.1177/0022034513505772.
[91] Huang GT, Sonoyama W, Chen J, Park SH. In vitro characterization of human dental pulp cells: various isolation methods and culturing environments. Cell Tissue Res 2006;324:225–36. https://doi.org/10.1007/s00441-005-0117-9.
[92] Mehrazarin S, Oh JE, Chung CL, Chen W, Kim RH, Shi S, Park NH, Kang MK. Impaired odontogenic differentiation of senescent dental mesenchymal stem cells is associated with loss of Bmi-1 expression. J Endod 2011;37:662–6. https://doi.org/10.1016/j.joen.2011.02.009.
[93] Sakai VT, Zhang Z, Dong Z, Neiva KG, Machado MA, Shi S, Santos CF, Nor JE. SHED differentiate into functional odontoblasts and endothelium. J Dent Res 2010;89:791–6. https://doi.org/10.1177/0022034510368647.
[94] Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, Shi S. SHED: stem cells from human exfoliated deciduous teeth. Proc Natl Acad Sci USA 2003;100:5807–12. https://doi.org/10.1073/pnas.0937635100.
[95] Wang H, Zhong Q, Yang T, Qi Y, Fu M, Yang X, Qiao L, Ling Q, Liu S, Zhao Y. Comparative characterization of SHED and DPSCs during extended cultivation inï ¿½vitro. Mol Med Rep 2018. https://doi.org/10.3892/mmr.2018.8725.
[96] Wang X, Sha X-J, Li G-H, Yang F-S, Ji K, Wen L-Y, Liu S-Y, Chen L, Ding Y, Xuan K. Comparative characterization of stem cells from human exfoliated deciduous teeth and dental pulp stem cells. Arch Oral Biol 2012;57:1231–40. https://doi.org/10.1016/j.archoralbio.2012.02.014.
[97] Arany S, Kawagoe M, Sugiyama T. Application of spontaneously immortalized odontoblast cells in tooth regeneration. Biochem Biophys Res Commun 2009;381:84–9. https://doi.org/10.1016/j.bbrc.2009.02.025.
[98] Alvarez MMP, Carvalho RGd, Barbosa SCdA, Polassi MR, Nascimento FD, D'Alpino PHP, Tersariol ILdS. Oxidative stress induced by self-adhesive resin cements affects gene expression, cellular proliferation and mineralization potential of the MDPC-23 odontoblast-like cells. Dent Mater 2019;35:606–16. https://doi.org/10.1016/j.dental.2019.02.008.
[99] Edanami N, Yoshiba N, Ohkura N, Takeuchi R, Tohma A, Noiri Y, Yoshiba K. Characterization of dental pulp myofibroblasts in rat molars after pulpotomy. J Endod 2017;43:1116–21. https://doi.org/10.1016/j.joen.2017.02.018.
[100] Modena KCdS, Calvo AM, Sipert CR, Colombini-Ishikiriama BL, Dionísio TJ, Navarro MFdL, Atta MT, Santos CF. Molecular response of pulp fibroblasts after stimulation with pulp capping materials. Braz Dent J 2020;31:244–51. https://doi.org/10.1590/0103-6440202003523.
[101] Lima AF, Lessa FC, Mancini MN, Hebling J, Costa CA, Marchi GM. Transdentinal protective role of sodium ascorbate against the cytopathic effects of H2O2 released from bleaching agents. Oral Surg Oral Med Oral Pathol Oral Radio Endod 2010;109. e70-6. S1079-2104(09)00937-8 [pii] 10.1016/j.tripleo.2009.12.020.
[102] Lima AF, Ribeiro AP, Basso FG, Bagnato VS, Hebling J, Marchi GM, de Souza Costa CA. Effect of low-level laser therapy on odontoblast-like cells exposed to bleaching agent. 1533-8 Lasers Med Sci 2014;29. https://doi.org/10.1007/s10103-013-1309-2.
[103] Aytac Z, Dubey N, Daghrery A, Ferreira JA, de Souza Araújo IJ, Castilho M, Malda J, Bottino MC. Innovations in craniofacial bone and periodontal tissue engineering - from electrospinning to converged biofabrication. Int Mater Rev 2022;67:347–84. https://doi.org/10.1080/09506608.2021.1946236.
[104] Häkkinen L, Larjava H, Fournier BPJ. Distinct phenotype and therapeutic potential of gingival fibroblasts. Cytotherapy 2014;16:1171–86. https://doi.org/10.1016/j.jcyt.2014.04.004.
[105] Fujioka-Kobayashi M, Miron RJ, Lussi A, Gruber R, Ilie N, Price RB, Schmalz G. Effect of the degree of conversion of resin-based composites on cytotoxicity, cell attachment, and gene expression. Dent Mater 2019;35:1173–93. https://doi.org/10.1016/j.dental.2019.05.015.
[106] Páez J, Hernández R, Espinoza J, Rojas L, Martínez CE, Tobar N, Martínez J, Smith PC. Uncoupled inflammatory, proliferative, and cytoskeletal responses in senescent human gingival fibroblasts. J Periodontal Res 2020;55:432–40. https://doi.org/10.1111/jre.12727.
[107] Smith PC, Cáceres M, Martínez C, Oyarzún A, Martínez J. Gingival wound healing: an essential response disturbed by aging? J Dent Res 2015;94:395–402. https://doi.org/10.1177/0022034514563750.
[108] Ali HRW, Suliman S, Osman TA, Carrasco M, Bruland O, Costea DE, Ræder H, Mustafa K. Xeno-free generation of human induced pluripotent stem cells from donor-matched fibroblasts isolated from dermal and oral tissues. Stem Cell Res Ther 2023;14:199. https://doi.org/10.1186/s13287-023-03403-7.
[109] Celik N, Binnetoglu D, Ozakar Ilday N, Hacimuftuoglu A, Seven N. The cytotoxic and oxidative effects of restorative materials in cultured human gingival fibroblasts. Drug Chem Toxicol 2021;44:502–7. https://doi.org/10.1080/01480545.2019.1620265.
[110] Tomokiyo A, Wada N, Maeda H. Periodontal ligament stem cells: regenerative potency in periodontium. Stem Cells Dev 2019;28:974–85. https://doi.org/10.1089/scd.2019.0031.
[111] Jin SS, He DQ, Wang Y, Zhang T, Yu HJ, Li ZX, Zhu LS, Zhou YH, Liu Y. Mechanical force modulates periodontal ligament stem cell characteristics during bone remodelling via TRPV4. Cell Prolif 2020;53:e12912. https://doi.org/10.1111/cpr.12912.
[112] Liu Z, He Y, Xu C, Li J, Zeng S, Yang X, Han Q. The role of PHF8 and TLR4 in osteogenic differentiation of periodontal ligament cells in inflammatory environment. J Periodo 2021;92:1049–59. https://doi.org/10.1002/JPER.20-0285.
[113] Grzesik WJ, Narayanan AS. Cementum and periodontal wound healing and regeneration. Crit Rev Oral Biol Med 2002;13:474–84. https://doi.org/10.1177/154411130201300605.
[114] Oviir T, Pagoria D, Ibarra G, Geurtsen W. Effects of gray and white mineral trioxide aggregate on the proliferation of oral keratinocytes and cementoblasts. J Endod 2006;32:210–3. https://doi.org/10.1016/j.joen.2005.10.036.
[115] Rosa V, Della Bona A, Cavalcanti BN, Nör JE. Tissue engineering: From research to dental clinics. Dent Mater 2012;28:341–8. https://doi.org/10.1016/j.dental.2011.11.025.
[116] Heremans H, Billiau A, Cassiman JJ, Mulier JC, de Somer P. In vitro cultivation of human tumor tissues II. Morphological and virological characterization of three cell lines. Oncology 1978;35:246–52. https://doi.org/10.1159/000225298.
[117] Dubey N, Ellepola K, Decroix FED, Morin JLP, Castro Neto AH, Seneviratne CJ, Rosa V. Graphene onto medical grade titanium: an atom-thick multimodal coating that promotes osteoblast maturation and inhibits biofilm formation from distinct species. Nanotoxicology 2018;12:274–89. https://doi.org/10.1080/17435390.2018.1434911.
[118] Saldaña L, Bensiamar F, Boré A, Vilaboa N. In search of representative models of human bone-forming cells for cytocompatibility studies. Acta Biomater 2011;7:4210–21. https://doi.org/10.1016/j.actbio.2011.07.019.
[119] Staehlke S, Rebl H, Nebe B. Phenotypic stability of the human MG-63 osteoblastic cell line at different passages. Cell Biol Int 2018;43:22–32. https://doi.org/10.1002/cbin.11073.
[120] Chen F, Zhang Y, Peng P, Huang X-T, Qiu Z-H, Liu B-C, Yang T-L, Yang B, Guo Y. Isolation and culture of human primary osteoblasts: comparing the effects of differences in method details on osteoblast characteristics. Genes Dis 2024;11:546–9. https://doi.org/10.1016/j.gendis.2023.03.024.
[121] Zou L, Harkey MR, Henderson GL. Effects of intrinsic fluorescence and quenching on fluorescence-based screening of natural products. Phytomedicine 2002;9:263–7. https://doi.org/10.1078/0944-7113-00121.
[122] DiPeso L, Ji DX, Vance RE, Price JV. Cell death and cell lysis are separable events during pyroptosis. Cell Death Discov 2017;3. https://doi.org/10.1038/cddiscovery.2017.70.
[123] Laplaca MC, Lee VMY, Thibault LE. An in vitro model of traumatic neuronal injury: Loading rate-dependent changes in acute cytosolic calcium and lactate dehydrogenase release. J Neurotrauma 1997;14:355–68. https://doi.org/10.1089/neu.1997.14.355.
[124] Crowley LC, Marfell BJ, Scott AP, Waterhouse NJ. Quantitation of apoptosis and necrosis by Annexin V binding, propidium iodide uptake, and flow cytometry. Cold Spring Harb Protoc 2016;2016. https://doi.org/10.1101/pdb.prot087288.
[125] Liu WF, Ma M, Bratlie KM, Dang TT, Langer R, Anderson DG. Real-time in vivo detection of biomaterial-induced reactive oxygen species. Biomaterials 2011;32:1796–801. https://doi.org/10.1016/j.biomaterials.2010.11.029.
[126] Ohtake H, Tokuyoshi Y, Iyama Y, Nukaga T, Nishida H, Ohtake T, Hirota M, Yamada K, Seto Y, Sato H, Kouzuki H, Onoue S. Reactive oxygen species (ROS) assay-based photosafety screening for complex ingredients: modification of the ROS assay protocol. J Toxicol Sci 2022;47:483–92. https://doi.org/10.2131/jts.47.483.
[127] Azqueta A, Stopper H, Zegura B, Dusinska M, Møller P. Do cytotoxicity and cell death cause false positive results in the in vitro comet assay? Mutat Res/Genet Toxicol Environ Mutagen 2022;881. https://doi.org/10.1016/j.mrgentox.2022.503520.
[128] Kim MH, Kino-oka M, Taya M. Designing culture surfaces based on cell anchoring mechanisms to regulate cell morphologies and functions. Biotechnol Adv 2010;28:7–16. https://doi.org/10.1016/j.biotechadv.2009.08.002.
[129] Klausner M, Ayehunie S, Breyfogle BA, Wertz PW, Bacca L, Kubilus J. Organotypic human oral tissue models for toxicological studies. Toxicol Vitr 2007;21:938–49. https://doi.org/10.1016/j.tiv.2007.01.024.
[130] Schmalz G, Schuster U, Nuetzel K, Schweikl H. An in vitro pulp chamber with three-dimensional cell cultures. J Endod 1999;25:24–9. https://doi.org/10.1016/S0099-2399(99)80394-X.
[131] Klausner M, Handa Y, Aizawa S. In vitro three-dimensional organotypic culture models of the oral mucosa. Vitr Cell Dev Biol Anim 2021;57:148–59. https://doi.org/10.1007/s11626-020-00539-1.
[132] Hadjichristou CAI, Koidis P, Bakopoulou A. Advanced in vitro experimental models for tissue engineering-based reconstruction of a 3D dentin:Pulp complex a literature review. Stem Cell Rev Rep 2021;13:785–802. https://doi.org/10.1007/s12015-020-10069-8.
[133] Moharamzadeh K, Franklin KL, Brook IM, van Noort R. Biologic assessment of antiseptic mouthwashes using a three-dimensional human oral mucosal model. J Periodo 2009;80:769–75. https://doi.org/10.1902/jop.2009.080610.
[134] Muniraj G, Tan RHS, Dai Y, Wu R, Alberti M, Sriram G. Microphysiological modeling of gingival tissues and host-material interactions using gingiva-on-chip. Adv Health Mater 2023;12:e2301472. https://doi.org/10.1002/adhm.202301472.
[135] Schmalz G, Arenholt-Bindslev D, Hiller KA, Schweikl H. Epithelium-fibroblast co-culture for assessing mucosal irritancy of metals used in dentistry. Eur J Oral Sci 2007;105:86–91. https://doi.org/10.1111/j.1600-0722.1997.tb00185.x.
[136] Hadjichristou C, Papachristou E, Bonovolias I, Bakopoulou A. Three-dimensional tissue engineering-based Dentin/Pulp tissue analogue as advanced biocompatibility evaluation tool of dental restorative materials. Dent Mater 2020;36:229–48. https://doi.org/10.1016/j.dental.2019.11.013.
[137] Pedano MS, Li X, Jeanneau C, Ghosh M, Yoshihara K, Van Landuyt K, About I, Van Meerbeek B. Survival of human dental pulp cells after 4-week culture in human tooth model. J Dent 2019;86:33–40. https://doi.org/10.1016/j.jdent.2019.05.023.

[138] Tecles O, Laurent P, Aubut V, About I. Human tooth culture: a study model for reparative dentinogenesis and direct pulp capping materials biocompatibility. J Biomed Mater Res B Appl Biomater 2008;85:180–7. https://doi.org/10.1002/jbm.b.30933.

[139] Sengun A, Yalcin M, Ulker HE, Ozturk B, Hakki SS. Cytotoxicity evaluation of dentin bonding agents by dentin barrier test on 3-dimensional pulp cells. e83-8 Oral Surg Oral Med Oral Pathol Oral Radio Endod 2011;112. https://doi.org/10.1016/j.tripleo.2011.02.023.

[140] Goncalves SB, Dong Z, Bramante CM, Holland GR, Smith AJ, Nor JE. Tooth slice-based models for the study of human dental pulp angiogenesis. J Endod 2007;33:811–4. https://doi.org/10.1016/j.joen.2007.03.012.

[141] Poimenova A, Kitraki E, Kakaboura A, Rahiotis C. Early responses of human pulp to direct capping with resin adhesive systems and calcium hydroxide. Dent Mater 2018;34:e73–82. https://doi.org/10.1016/j.dental.2018.01.018.

[142] Saw TY, Cao T, Yap AU, Lee Ng MM. Tooth slice organ culture and established cell line culture models for cytotoxicity assessment of dental materials. Toxicol Vitr 2005;19:145–54. https://doi.org/10.1016/j.tiv.2004.08.006.

[143] Laurent P, Camps J, About I. Biodentine(TM) induces TGF-beta1 release from human pulp cells and early dental pulp mineralization. Int Endod J 2012;45:439–48. https://doi.org/10.1111/j.1365-2591.2011.01995.x.

[144] Pedano MS, Li X, Camargo B, Hauben E, De Vleeschauwer S, Yoshihara K, Van Landuyt K, Yoshida Y, Van Meerbeek B. Injectable phosphopullulan-functionalized calcium-silicate cement for pulp-tissue engineering: An in-vivo and ex-vivo study. Dent Mater 2020;36:512–26. https://doi.org/10.1016/j.dental.2020.01.011.

[145] Chakka LRJ, Vislisel J, Vidal CMP, Biz MT, Cavalcanti AKS. BN. Application of BMP-2/FGF-2 gene-activated scaffolds for dental pulp capping. Clin Oral Invest 2020;24:4427–37. https://doi.org/10.1007/s00784-020-03308-2.

[146] Franca CM, Balbinot GS, Cunha D, Saboia VPA, Ferracane J, Bertassoni LE. In-vitro models of biocompatibility testing for restorative dental materials: From 2D cultures to organs on-a-chip. Acta Biomater 2022;150:58–66. https://doi.org/10.1016/j.actbio.2022.07.060.

[147] Huang C, Sanaei F, Verdurmen WPR, Yang F, Ji W, Walboomers XF. The application of organs-on-a-chip in dental, oral, and craniofacial research. J Dent Res 2023;220345221145555. https://doi.org/10.1177/00220345221145555.

[148] Rosa V, Sriram G, McDonald N, Cavalcanti BN. A critical analysis of research methods and biological experimental models to study pulp regeneration. Int Endod J 2022;55(Suppl 2):446–55. https://doi.org/10.1111/iej.13712.

[149] Lee E-J, Kim Y, Salipante P, Kotula AP, Lipshutz S, Graves DT, Alimperti S. Mechanical regulation of oral epithelial barrier function. Bioengineering 2023;10:517.

[150] Makkar H, Zhou Y, Tan KS, Lim CT, Sriram G. Modeling crevicular fluid flow and host-oral microbiome interactions in a gingival crevice-on-chip. Adv Health Mater 2023;12:e2202376. https://doi.org/10.1002/adhm.202202376.

[151] Mishra A, Kai R, Atkuru S, Dai Y, Piccinini F, Preshaw PM, Sriram G. Fluid flow-induced modulation of viability and osteodifferentiation of periodontal ligament stem cell spheroids-on-chip. Biomater Sci 2023. https://doi.org/10.1039/d3bm01011b.

[152] Franca CM, Tahayeri A, Rodrigues NS, Ferdosian S, Puppin Rontani RM, Sereda G, Ferracane JL, Bertassoni LE. The tooth on-a-chip: a microphysiologic model system mimicking the biologic interface of the tooth with biomaterials. Lab Chip 2020;20:405–13. https://doi.org/10.1039/c9lc00915a.

[153] Ly KL, Rooholghodos SA, Rahimi C, Rahimi B, Bienek DR, Kaufman G, Raub CB, Luo X. An oral-mucosa-on-a-chip sensitively evaluates cell responses to dental monomers. Biomed Micro 2021;23:7. https://doi.org/10.1007/s10544-021-00543-6.

[154] Rahimi C, Rahimi B, Padova D, Rooholghodos SA, Bienek DR, Luo X, Kaufman G, Raub CB. Oral mucosa-on-a-chip to assess layer-specific responses to bacteria and dental materials. Biomicrofluidics 2018;12:054106. https://doi.org/10.1063/1.5048938.

[155] Adelfio M, Bonzanni M, Callen GE, Paster BJ, Hasturk H, Ghezzi CE. A physiologically relevant culture platform for long-term studies of in vitro gingival tissue. Acta Biomater 2023;167:321–34. https://doi.org/10.1016/j.actbio.2023.06.008.

[156] Rodrigues NS, Franca CM, Tahayeri A, Ren Z, Saboia VPA, Smith AJ, Ferracane JL, Koo H, Bertassoni LE. Biomaterial and biofilm interactions with the pulp-dentin complex-on-a-chip. J Dent Res 2021;100:1136–43. https://doi.org/10.1177/00220345211016429.

[157] Jin L, Kou N, An F, Gao Z, Tian T, Hui J, Chen C, Ma G, Mao H, Liu H. Analyzing human periodontal soft tissue inflammation and drug responses in vitro using epithelium-capillary interface on-a-chip. Biosens (Basel) 2022;12. https://doi.org/10.3390/bios12050345.

[158] Niu L, Zhang H, Liu Y, Wang Y, Li A, Liu R, Zou R, Yang Q. Microfluidic chip for odontoblasts in vitro. ACS Biomater Sci Eng 2019;5:4844–51. https://doi.org/10.1021/acsbiomaterials.9b00743.

[159] Hadjichristou C, Papachristou E, Vereroudakis E, Chatzinikolaidou M, About I, Koidis P, Bakopoulou A. Biocompatibility assessment of resin-based cements on vascularized dentin/pulp tissue-engineered analogues. Dent Mater 2021;37:914–27. https://doi.org/10.1016/j.dental.2021.02.019.

[160] Zhang L, Han Y, Chen Q, Dissanayaka WL. Sema4D–plexin-B1 signaling in recruiting dental stem cells for vascular stabilization on a microfluidic platform. Lab a Chip 2022;22:4632–44. https://doi.org/10.1039/d2lc00632d.