A critical analysis of research methods and experimental models to study biocompatibility of endodontic materials

Sepanta Hosseinpour, Alexis Gaudin, Ove A Peters

Received: 6 October 2021 | Accepted: 4 February 2022

DOI: 10.1111/iej.13701

INTERNATIONAL ENDODONTIC JOURNAL | WILEY

REVIEW

A critical analysis of research methods and experimental models to study biocompatibility of endodontic materials

Sepanta Hosseinpour[1] | **Alexis Gaudin**[2,3] | **Ove A. Peters**[1]

[1]School of Dentistry, The University of Queensland, Herston, Queensland, Australia

[2]Inserm, UMR 1229, RMeS, Regenerative Medicine and Skeleton, Université de Nantes, ONIRIS, Nantes, France

[3]Université de Nantes, UFR Odontologie, Nantes, France

Correspondence
Ove A. Peters, Oral Health Centre, University of Queensland, 288 Herston Rd, Herston, QLD 4006, Australia.
Email: o.peters@uq.edu.au

Abstract

Materials used for endodontics and with direct contact to tissues have a wide range of indications, from vital pulpal treatments to root filling materials and those used in endodontic surgery. In principle, interaction with dental materials may result in damage to tissues locally or systemically. Thus, a great variety of test methods are applied to evaluate a materials' potential risk of adverse biological effects to ensure their biocompatibility before commercialization. However, the results of biocompatibility evaluations are dependent on not only the tested materials but also the test methods due to the diversity of these effects and numerous variables involved. In addition, diverse biological effects require equally diverse assessments on a structured and planned approach. Such a structured assessment of the materials consists of four phases: general toxicity, local tissue irritation, pre-clinical tests and clinical evaluations. Various types of screening assays are available; it is imperative to understand their advantages and limitations to recognize their appropriateness and for an accurate interpretation of their results. Recent scientific advances are rapidly introducing new materials to endodontics including nanomaterials, gene therapy and tissue engineering biomaterials. These new modalities open a new era to restore and regenerate dental tissues; however, all these new technologies can also present new hazards to patients. Before any clinical usage, new materials must be proven to be safe and not hazardous to health. Certain international standards exist for safety evaluation of dental materials (ISO 10993 series, ISO 7405 and ISO 14155-1), but researchers often fail to follow these standards due to lack of access to standards, limitation of the guidelines and complexity of new experimental methods, which may cause technical errors. Moreover, many laboratories have developed their testing strategy for biocompatibility, which makes any comparison between findings more difficult. The purpose of this review was to discuss the concept of biocompatibility, structured test programmes and international standards for testing the biocompatibility of endodontic material biocompatibility. The text will further detail current test methods for evaluating the biocompatibility of endodontic materials, and their advantages and limitations.

KEYWORDS

animal testing, biocompatibility, cytotoxicity, endodontic materials, genotoxicity, mutagenicity

INTRODUCTION

The need for safe materials in Endodontics has generated a demand for experimental assays to screen compositions and characterize the potentially adverse effects of these materials on oral tissues before clinical application, in other words, to assess their biocompatibility. Although adverse reactions are rare, considering the number of endodontic treatments provided, many patients may potentially be affected (Mjör, 1992). Moreover, in some cases, the dental team is at higher risk of adverse reactions to the dental materials (Sadoh et al., 1999; Scott et al., 2004). Consequently, biocompatibility is by law an aspect that must be tested before a dental material is permitted to be released to the market. However, the legal regulations do not release dentists from their responsibility to inform patients independent of the manufacturers' interest and choosing materials with adequate biocompatibility. Traditionally, the biocompatibility concept is regarded as a lack of substantial harmful reaction between materials and host (Browne, 1988). An updated definition of biocompatibility describes 'the ability of a material to function in a specific application in the presence of an appropriate host response' (Donaruma, 1988), which means a biocompatible material may not be entirely inert and the appropriateness of the host reaction is decisive. The term 'host response' encompasses a wide range of various biological responses from cytotoxicity and allergic reaction to biostimulatory effects on tissues.

For the biomedical application of materials, they must undergo rigorous testing to specify their biocompatibility and safety when they have contact with the human body, regardless of their physical and chemical properties based on International Organisation for Standardization protocols (Black, 2005).

Safety concerning the assessment of endodontic materials means freedom from unacceptable risk. Therefore, it does not stand for a complete absence of risks. Evaluating the biocompatibility of endodontic materials may be seen as a risk assessment exercise, where the aim is to minimize risk whilst maximizing benefit to patients. As with the definition of biocompatibility, appropriateness has an imperative function with respect to safety.

For each of the biological reaction areas, numerous *in vitro* and *in vivo* tests have been used in the literature. It is imperative to note that certain international standards exist for biocompatibility tests including ISO 10993 series (10993-5, 10993-12) and ISO 7405, which include well-known and commonly used tests. However, researchers also frequently fail to precisely follow these protocols, possibly due to lack of access to these standards and their inherent limitations. Many laboratories utilize their own experimental methods for biocompatibility testing, rendering the comparison of findings challenging. In addition, the presence of numerous and diverse methods to explain biological mechanisms behind clinical responses accentuates the importance of a systematic approach in the selection of the test methods and considering the rationality of tests based on specific scientific objectives.

Clinical relevance of the data and interpretation of the findings of biocompatibility tests is the other important point that should be considered. The clinical relevance of a test depends on its specific degree of simulation. For instance, the clinical relevance of data from a simple screening test such as an *in vitro* cytotoxicity test is not entirely achieved. On the contrary, when the test is applied to unravel the biological mechanism of a particular reaction, clinical relevance is of reduced interest and the method must be selected and designed based on the specific scientific enquiry.

Finally, the physical and chemical properties of a material and its contact with the oral tissues should be considered for designing biocompatibility testing. Endodontic materials can be generally classified as those applied for vital pulp therapy, root canal filling, root canal disinfection, regenerative endodontics, management of endodontic complications and aesthetic and functional restoration of teeth. Each of these endodontic materials is in contact with host tissues, which is worth acknowledging during the investigations (Figure 1). The biocompatibility of these materials is characterized by various aspects including cytotoxicity, genotoxicity and mutagenicity, histocompatibility, general toxicity, allergic reactions, systemic effects and microbial effects. In the following, a comprehensive review of the methodology and experimental models involved in biocompatibility testing of endodontic materials is provided.

LITERATURE SEARCH

For this review, a comprehensive search of the MEDLINE, Scopus, Embase, Web of Science and Google Scholar online information sources was undertaken, using biocompatibility tests on dental materials, particularly endodontic materials. All original research papers on biocompatibility testing for endodontic materials that were located using the following keywords ("Biocompatibility" OR "cell viability" OR "cytotoxicity" OR "toxicity" OR "proliferation" OR "differentiation") AND ("endodontic material" OR "root canal filling" OR "sealer" OR "Endodontic")) in the title or abstract were retrieved. The majority of the articles only assess *in vitro* cytotoxicity using cell lines or primary cells. After omitting duplicates, titles and abstracts were screened to include related articles, and in the next step, full texts of the papers were analysed according to

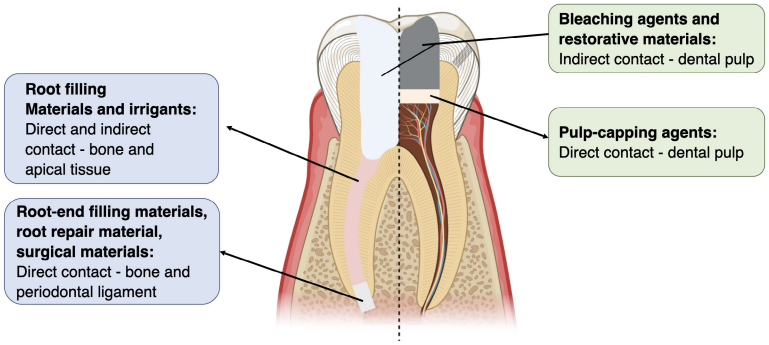

FIGURE 1 Endodontic materials can be broadly categorized as those used to maintain pulp vitality and those used in root canal treatment for disinfection of the pulp space (irrigants and intracanal medicaments) and root canal filling (solid materials and sealers). Biocompatibility of these endodontic materials is characterized by many parameters. Information regarding the clinical applications of test materials including location of the treatment and type of contact is key factors for selecting appropriate testing methods

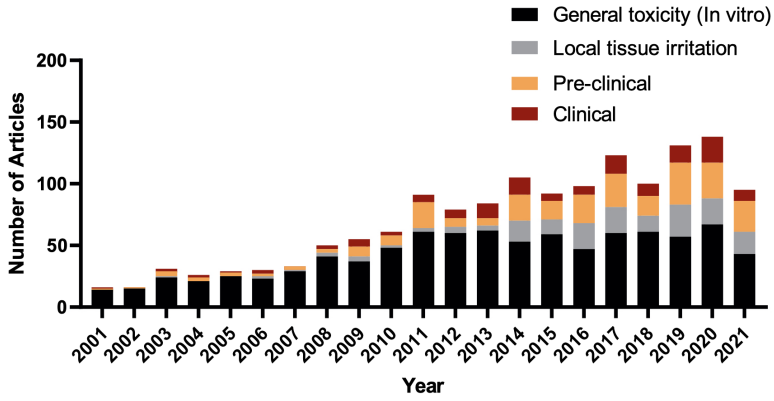

FIGURE 2 Distribution of the articles during past 20 years in Scopus. A total of 1377 original articles and 145 review articles with searching these keywords ("Biocompatibility" OR "cell viability" OR "cytotoxicity" OR "toxicity" OR "proliferation" OR "differentiation") AND ("endodontic material" OR "root canal filling" OR "sealer" OR "Endodontic")) in the title or abstract, have been retrieved. Majority of the articles only assess in vitro cytotoxicity using cell lines or primary cells.

TABLE 1 ISO testing protocols for biocompatibility of dental biomaterials including endodontic materials

Test evaluation	***In vitro***				
Assay type	**Agar diffusion test**	**Filter diffusion test**	**Direct contact or extract tests**	**Dentine barrier cytotoxicity test**	**Antioxidant responsive element (ARE) reporter assay**
Test element	Established fibroblast or epithelial cell line	Established fibroblast or epithelial cell line	An established cell line	An established cell line Dentine slice	HepG2-AD13 cell
Suggested follow-up (days)	≥1 day	≥1 day	≥1 day	14 ± 2	≥1 day
Test suitability for:					
Cytotoxicity and dentinal injury	Yes	Yes	Yes	Yes	Yes
Hypersensitivity	No	No	No	No	No
Carcinogenic or mutagenic	No	No	No	No	No
Tissue irritation and inflammation	No	No	No	No	No

Note: Data extracted from 'ISO 7405. Dentistry - Preclinical evaluation of biocompatibility of medical devices used in dentistry - Test methods for dental materials. International Standards Organization; 1996' and 'ISO 10993. Biological evaluation of dental devices. International Standards Organization. 1992'.

the inclusion criteria. All *in vitro*, *in vivo* and clinical studies were included. Conference papers, systematic reviews, meta-analyses, narrative reviews, letters to the editor, book chapters, technical notes and theses were excluded. At the time of searching, all records had to be in the final or 'in press' stage to be included. Figure 2 shows the distribution of the articles during the past 20 years in one of the databases. In Scopus alone, there were 1377 original articles plus 145 reviews. Key articles based on the subheadings of the research were selected and analysed for this review.

BIOCOMPATIBILITY TESTING: CONSIDERATIONS AND ASPECTS

Assessing the biocompatibility of endodontic materials is a complex and comprehensive area since a vast range of undesirable tissue reactions may occur. Any single test method is applicable just for assessing one specific type of unwanted reaction out of many possible reactions. In addition, single test methods are commonly adequate only to document or demonstrate a single aspect of unwanted reactions, which might not be transferred to clinical conditions without limitation. The other important consideration for biocompatibility testing is determining the most adequate exposure duration for assessment. Tissue can be directly/indirectly exposed to the material or only an extract that has been released in a liquid under specific conditions for a certain time at a specific temperature. These types of exposures will be discussed in detail for *in vitro* methods.

One of the major aspects of biocompatibility testing is toxicity assessment. The toxicity of material reveals its ability to damage a biological system. It starts at the cell level and local tissue toxicity and in higher organisms also includes systemic effects. In endodontics, local reactions primarily occur in the pulp, periradicular tissues and occasionally gingivae. The most common toxicity assessment is the cytotoxicity test that defines the effect of material on cellular viability. Cytotoxicity tests are primary biocompatibility tests that specify the lysis of cells and the inhibition of cell proliferation. Numerous cytotoxicity screening methods are available for studying endodontic materials. The administration of various methods of cytotoxicity screening has been shown to display a spectrum of findings for the same material (Henstenpettersen, A. & Helgeland, K., 1977; Mittal et al., 1995; Wennberg et al., 1983; Witte et al., 1996). Therefore, determining the biocompatibility of material only by using an *in vitro* cell culture assay and, from this, attempting to anticipate *in vivo* pulpal and periradicular responses are controversial (Mjör, 1980). It has been shown that the cytotoxicity findings provided by cell culture assays have not necessarily agreed with the results of *in vivo* animal usage (Hanks et al., 1981; Mjör et al., 1977; Schmalz et al., 1996), which should be considered for the interpretations of cytotoxicity assays.

Endodontic materials may release substances into the local tissues (local toxicity), the oral cavity, blood via

In vivo			
Pulp and dentine usage test	**Pulp capping test**	**Endodontic usage test**	**Endosseous dental implant usage test**
Extracted human tooth/Animal in situ tooth nonrodent mammals	Extracted human tooth/ Animal in situ tooth Nonrodent mammals	Animal in situ tooth a minimum of four nonrodent mammals	Intraosseous implant No particular animal model has yet been validated.
5 ± 2 25 ± 5 70 ± 5	25 ± 5 70 ± 5	28 ± 3 90 ± 5	-
Yes	Yes	Yes	Yes
Yes	Yes	Yes	Yes
No	No	No	No
Yes	Yes	Yes	Yes

circulation or even the respiratory system via inhalation. Therefore, the application site may be different from the location of the effect and these materials may cause systemic toxicity. Based on the time frame, system toxicities are categorized into acute (up to 24 h post-exposure), subacute (up to 3 months) and chronic (longer than 3 months). Although the literature has clearly identified only rare chronic general health complaints after exposure to dental materials (Schmalz, 2009), there is still a potential risk that needs to be taken into account during testing.

Genotoxicity or mutagenicity is another aspect of biocompatibility testing. Genotoxicity is defined as the presence of a DNA-reactive component that can result in carcinogenicity and mutagenicity (Leyhausen, 1995). The ISO 10993-3: 2003 (tests for genotoxicity, carcinogenicity and reproductive toxicity) recommends several methods for evaluation of genotoxicity (Schmalz, 2009). *In vitro* tests for genotoxicity are categorized into prokaryotic (e.g. *umu* test, which is a common test of DNA-damaging chemicals in environmental genotoxicity field) and eukaryotic (e.g. DNA synthesis inhibition test) tests (Heil et al., 1996; Leyhausen, 1995; Oda et al., 1985; Stea et al., 1994). Since some endodontic materials have strong antibacterial activity (Cheng et al., 2020), prokaryotic tests cannot be the only basis for the assessment of their DNA-damaging activity.

Due to the diversity and complexity of toxicity assessments, a structured approach of test methods is necessary (Schmalz, 1998). Traditionally, the structured approach has three levels (Autian, 1970; Autian & Dillingham, 1975): 1. unspecific toxicity tests including cell cultures and small animals; 2. specific toxicity by using biological models (e.g. usage tests in primates); and 3. clinical investigations in humans. Steps 1 and 2 do not reflect the clinical situation of applying a material, and therefore, Step 3 is currently needed to adequately describe material toxicity.

Immunotoxicity of material shows the adverse effects on the function and/or structure of the immune system that in turn can impair the host response and cause inflammation (Syed et al., 2015). Cytotoxicity of immune cells such as monocytes is the first step to assess immunotoxicity; however, due to the complexity of immunity, further *in vivo* assessments are necessary for an accurate interpretation. Substances released from dental materials may generate an inflammatory response or apoptosis as local reactions. Moreover, these substances can trigger an allergic reaction in the host, particularly if the organism was previously sensitized to the compound. These allergic reactions consist of four distinctive categories (types I, II and III are mediated by antibodies and type IV is primarily conveyed by cells), which can affect not only patients but also dental personnel (Syed et al., 2015).

Tissue reactions to microbes

The potential interactions between endodontic materials and their individual components with microorganisms should be considered when discussing biocompatibility. Microorganisms may persist within the pulp chamber or root canal after endodontic treatment, re-infect the canals through microleakage or proliferate in extraradicular tissues (Oguntebi, 1994; Torabinejad et al., 1990). These microorganisms can influence the biocompatibility findings of endodontic materials and intensify adverse effects. Therefore, it would be of great benefit if these materials have antibacterial capacity in addition to biocompatibility. However, endodontic materials with strong antimicrobial activity commonly demonstrate higher toxicity and even mutagenic effects, and therefore, biocompatibility of such materials is scrutinized (Heil et al., 1996; Ørstavik & Hongslo, 1985). The antimicrobial activity of endodontic materials to pathogens is usually examined by simple *in vitro* tests such as agar diffusion tests and the direct contact tests.

Biocompatibility testing standards

The biocompatibility of endodontic materials is characterized by numerous variables, and it is virtually impossible to be assessed by a single test method. The first structured approach for biocompatibility testing for dental materials was presented in 1970 (Autian, 1970). This approach encompasses three levels:

(i) Nonspecific toxicity tests including cell culture *in vitro* tests;
(ii) Specific toxicity such as usage tests in animal models; and
(iii) Clinical assessments.

In this approach, nonspecific test means the test system cannot reflect the clinical condition, whereas specific testing applies to the use of appropriate clinically relevant models. The following sequence was adopted by the ISO (ISO 10993 series, ISO 7405, and ISO 14155-1) and is summarized in Table 1:

Group 1: *in vitro tests* of cytotoxicity
Group 2: tests following the ISO 10993 series of standards (local toxicity, hypersensitivity and genotoxicity)

Group 3: tests, specific for medical devices used in dentistry, not referred to in the ISO 10993 series of standards (pulp capping test and usages tests)

This standardized system not only consists of guidelines for the selection of tests but also contains descriptions of methods for various test designs. Table 2 summarizes the method standards according to ISO protocols.

The norm ISO 10993 (2018 edition) requires assessing the chemical and physical properties of a medical device including endodontic materials. Under this name, a series of standards are summarized, which are mostly issued by ISO and the European Committee for Standardization (CEN). This series contains guidelines for selecting appropriate test methods for evaluating various aspects of biocompatibility. Nine biological tests are listed in ISO 10993 for biological assessment and risk evaluation of implanted materials. Based on this standardization system, comprehensive implantation evaluations may supplement systemic toxicity assessments (acute, subacute and chronic), and if sufficient animals and time-points are included, such separate studies for acute, subacute and chronic toxicity are not always mandatory (1997).

Bioactivity as one of the aspects of biocompatibility is defined in ISO 22317 (implants for surgery). This standard describes *in vitro* tests for materials that induce the formation of calcium phosphate and apatite in synthetic body fluids, which can be applicable for materials utilizing in regenerative endodontics (Schmalz & Fan, 2009).

The ISO 7405 is a biocompatibility standard associated with ISO 10993–1, particularly for dental materials

TABLE 2 Status of relevant standards for testing the biological properties of dental restorative material including endodontic materials

International standard/European standard/ADA	Title	Methods
ISO 10993 series:	Biological evaluation of medical devices	Physical and chemical information
ISO 10993-1: 2003	Evaluation and testing	Cytotoxicity
ISO 10993-3: 2003	Tests for genotoxicity, carcinogenicity, and reproductive toxicity	Irritation or intracutaneous reactivity
ISO 10993-4: 2002	Selection of tests for interactions with blood	Pyrogenicity
ISO 10993-5: 1999	Tests for *in vitro* cytotoxicity	Acute systemic toxicity
ISO 10993-6: 2007	Tests for local effects after implantation	Subchronic toxicity
ISO 10993-10: 2002	Tests for irritation and delayed-type hypersensitivity	Chronic toxicity
ISO 10993-11: 2006	Tests for systemic toxicity	Implantation effects Genotoxicity
ISO 10993-16: 1997	Toxicokinetic study design for degradation products and leachables	Carcinogenicity
ISO 7405	Dentistry—evaluation of biocompatibility of medical devices used in dentistry	Cytotoxicity (2 methods are noted) Delayed-type hypersensitivity Irritation or intracutaneous reactivity Acute systemic toxicity Subchronic (subacute) toxicity Genotoxicity Chronic toxicity Implantation Pulp capping Endodontic usage Endosseous implant usage
ISO 14971	Medical devices—risk management. Part 1: Application on risk analysis	Safety and risk management Establish objective criteria for risk acceptability
ISO 14155-1	Clinical Investigation of Medical Devices for Human Subjects—Part 1	Systemic evaluation on test subjects Evaluate the safety and performance of a certain medical device
ISO 23317	Implants for surgery—*in vitro* evaluation for apatite-forming ability of implant materials	Apatite formation on the surface after exposure to simulated body fluid

including endodontic materials (1997). Several tests are similar in these two standards. In addition, ISO 7405 defines dental bioactive endodontic materials as capable of stimulating apical hard tissue formation applied in various methods (retrograde or orthograde therapies). An endodontic usage test and pulp capping also should be considered for endodontic materials to evaluate the biocompatibility with remaining pulpal tissue and/or periapical tissues (Primus et al., 2019).

The norm ISO 14155–1 conceives the clinical investigations of medical devices for human subjects for systemic and safety considerations. This standard depicts the responsibilities, competencies and principal process of a clinical evaluation. A clinical study is essentially based on the prerequisites of the Declaration of Helsinki for the protection of test subjects.

In summary, the endodontic material researcher/manufacturer must select adequate tests, according to the standards, use of the material and current evidence regarding the toxicity profile of the material. A researcher/manufacturer may select a set of tests in preference to others due to the cost, experience or other reasons. Overall, three critical steps are starting from *in vitro* to animal evaluation, and from pre-clinical to clinical usage testing on human beings. For the interpretation of the findings, the clinical relevance should also be considered. The advantages of standards are mainly improved reproducibility resulting in a better basis for comparing data from various studies. However, standardization also means the selection of tests and protocols for setting up a standard. Standardization can be time-consuming and usually does not include the very specific methods or recently developed tests. It is important to consider standard tests whenever suitable. Nonstandard test administration should be limited to address more specific issues.

Endodontic materials as a subcategory of dental materials and devices are also subject to legal regulation worldwide. The global harmonization task force as an international group of medical device regulators from Europe, the United States, Australia, Canada and Japan is currently included in an international regulatory system to be implemented by jurisdictions in individual countries. The main aspect of these regulations is to set a frame with specific requirements including concerns regarding the safety of a biomedical product. Table 3 summarizes these regulations amongst some countries, which address safety (such as biocompatibility) and efficiency of medical devices and materials. Dentists and manufacturers should know about the regulations and their responsibilities in the country they are working and marketing.

Another important aspect according to Medical Device Directive (MDD) is the fact that safety is not only associated with patients but also influences the users (whole dental team). Previous reports demonstrated that dental personnel are amongst a high-risk group due to the frequency and close contact with many materials in clinical or laboratory settings (Jacobsen et al., 1991; Jolanki et al., 1995; Schmalz et al., 1994a, 1994b). Ultimately, the basis for the safety evaluation of a biomedical product is risk management, which is necessary to be understood and applied in clinics by dentists.

The degree or extent of possible health damage is described by the term 'risk'. Risk analysis and assessment must be taken into consideration all available evidence including physical, chemical and biological data of a material and its impact on living tissues. During risk analysis, insufficient data may be found; thus, additional tests must be performed to provide the necessary level of evidence. Guidelines for such a selection of tests and performing them are provided by standards such as ISO 10993 and ISO 14971.

TABLE 3 Status of regulations regarding safety and efficacy of dental materials in various countries/regions

Country/region	Regulation
European Union (EU)	Medical Device Directive (MDD) European Chemical Regulation for Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) Drugs (01/83/EEC) *In vitro* diagnostics (98/79/EEC)
United States	Federal Food, Drug, and Cosmetic (FD&C) Act All marketed medical devices divided into one of three classes (I, II and III) in consideration of safety (including biocompatibility) and efficiency.
Australia	A classification system of dental products has been designed since 2002 by the Australian Therapeutic Goods (TGA) based on their safety and performance.
Japan	A category of medical devices has been designed since 2005 and is regulated by the Pharmaceutical Affairs Law (PAL).

The ISO 14971 describes the guidelines for the general procedure of risk management. In this context, risk evaluation must be distinguished from risk analysis (Figure 3). Risk assessment tries to answer the question of whether a risk can be accepted or not by measuring the estimated risks and comparing them with the

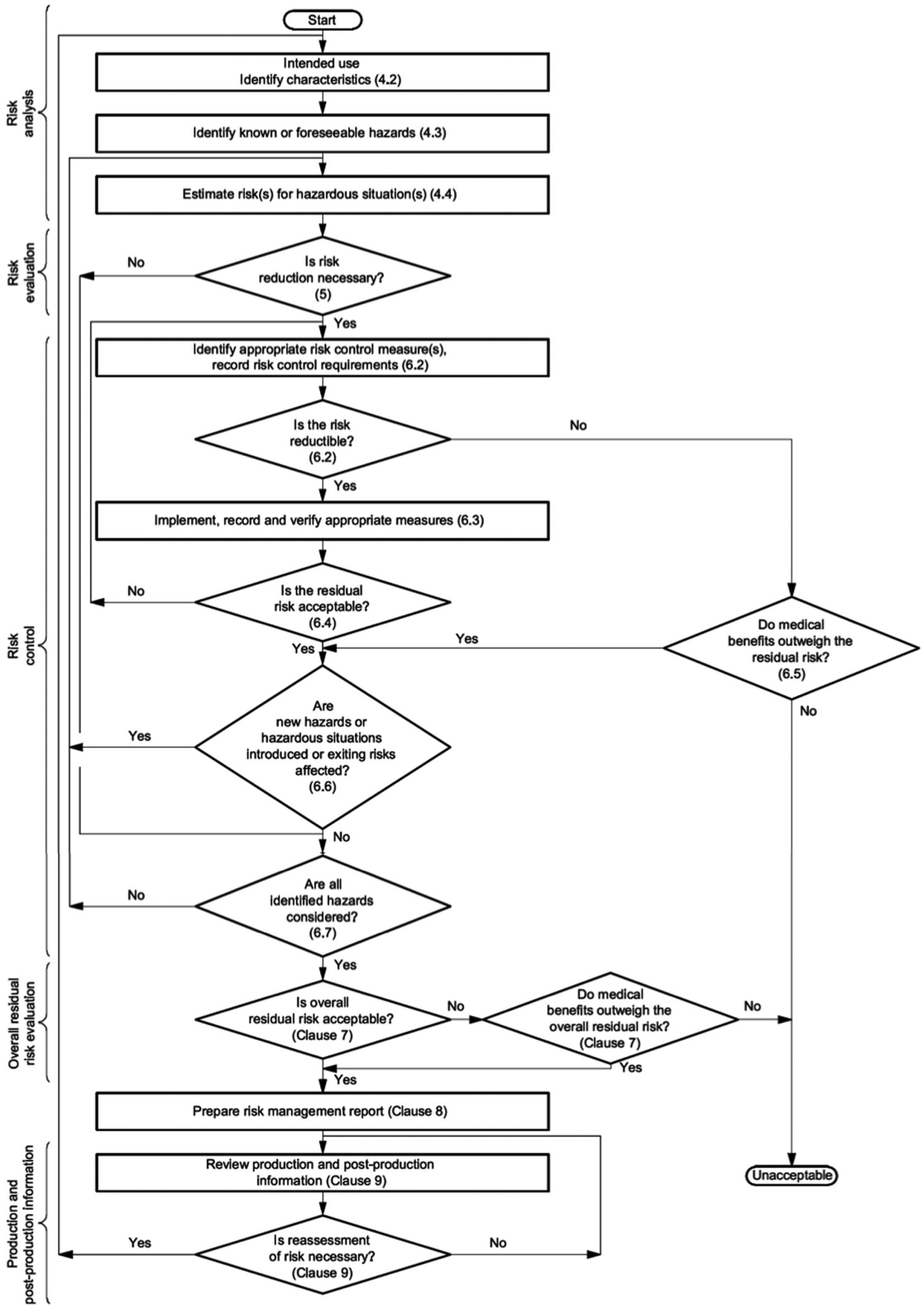

FIGURE 3 Risk management flowchart provided in ISO14971 (edition 2012) to give the user an overview of the risk management process for medical devices

risk criteria. However, risk analysis aims to determine whether it is necessary to perform more investigations, or whether the current evidence is conclusive. Both can be evaluated by the methods recommended by standard guidelines such as ISO 14971 (2019). As it is suggested in ISO 14971 (Figure 3), the first step is risk analysis and then risk evaluation.

Biological testing of endodontic materials

Local toxicity and local tissue compatibility must be differentiated. Local toxicity depends on the chemical interactions between biological molecules and toxic substances. However, local tissue compatibility can be based on causes other than just material toxicity such as bacterial accumulation, lack of seal or temperature alterations due to the settings. Generally, biocompatibility tests can be categorized into *in vitro* assessments, *in vivo* evaluations and clinical investigations. Relatively simple tests such as *in vitro* methods or implantation tests represent the local toxicity. These tests are only unspecific toxicity assessments and will not represent target tissues after usage of the endodontic materials in the oral cavity. On the contrary, usage studies in animal models and humans are equivalent to the subsequent administration on patients.

In vitro tests

The use of animals generally faces ethical problems, is under public discussion and is limited by regulations (Association EAR, 2013). Animal tests are expensive and time-consuming. There is, thus, a demand for developing low-cost nonanimal test methods, which simulate *in vivo* conditions and are rapid (Svendsen et al., 1996). Cell culture models have been developed to meet these requirements for *in vitro* tests. Most of the current data available on the biocompatibility of products are based on these cell culture studies. Isolated cells derived from human or animal tissues are grown in culture plates and then are used for these tests (Kawahara, 1955; Maizumi, 1962). Permanent cell lines are the most popular growing cells for this purpose due to the ease of culturing and amplifying, known behaviour and relatively consistency during passages (Schmalz, 1981). Permanent mouse fibroblasts (3T3 and L-929) and human epithelial cells (HeLa cells) are two of the most commonly used cell lines for this purpose. In addition, other cells such as primary cells cultured from tissue biopsies such as gingival or pulpal fibroblasts are utilized to simulate the target tissue application. Cells can be also grown *in vitro* three-dimensionally, which improves the *in vivo* simulation (Schuster et al., 2001).

The cells during *in vitro* tests are incubated with the materials or their extracts for a certain time. Then, various parameters will be measured via a series of tests based on the aims of the study, including a percentage of viable cells, protein synthesis, gene expression, enzyme activity and production/secretion of inflammatory mediators (Schmalz, 1994).

Although cell culture studies are relatively simple and quick to conduct, and are easily reproducible, their findings are highly dependent on the selected test conditions. Thus, it is always necessary to assess and compare test material with a similar material whose clinical impact is well known (Schmalz, 1994). The major problem associated with cell cultures is the interpretation of the data and extrapolation of the findings to clinical conditions. There are situations in which this extrapolation is feasible. For example, some endodontic materials damage cells immediately after setting but not in the set state (Gociu et al., 2013). Similarly, pulpal changes can be found after the application of pulp capping agents, and these alterations usually disappear after a few weeks in a healthy pulp (Al-Saudi et al., 2019). However, discrepancies between cell culture (*in vitro*) findings and clinical data are also documented (Schmalz et al., 1994b). For instance, although zinc oxide eugenol cement and calcium hydroxide are highly toxic in cell culture, in the presence of a dentine barrier, both have low toxicity when appropriately used in patients (Farhad & Mohammadi, 2005; Schmalz et al., 1994b). Hence, an assessment of this cement based on *in vitro* cytotoxicity tests would result in an incorrect evaluation of the material's biocompatibility. These discrepancies can be solved by a more appropriate simulation of a clinical condition such as dentine barrier tests (Schmalz et al., 1999), developing new methods and performing further *in vivo* studies.

Although the influence of material on the genome is mainly assessed by *in vitro* techniques (Maron & Ames, 1983), genotoxicity/mutagenicity/carcinogenicity can be also documented on small animals, which are commonly used for environmental toxicology (Heil et al., 1996). The number of studies addressing these aspects of endodontic materials is comparatively low and mostly limited to *in vitro* findings (Heil et al., 1996; Schweikl & Schmalz, 1999; Schweikl et al., 1995). For some materials, for instance some epoxy-based root canal sealers, potential mutagenic effects have been reported (Heil et al., 1996; Schweikl et al., 1995, 1998).

The assessment of the findings of mutagenicity tests is difficult. It has been shown that out of 300 chemicals assessed by the Ames test, 90% of carcinogenic chemicals were mutagenic and 87% of the noncarcinogenic substances were nonmutagenic (McCann et al., 1975).

Moreover, a long exposure time is necessary for the emergence of a malignancy; such an outcome is extremely rare in clinical settings. Hence, it is only feasible to conclude other fields such as occupational exposure to substances to a potential carcinogenic effect. However, it is imperative to consider that results from a single mutation test do not draw conclusions regarding a potential carcinogenic or mutagenic effect of material in humans. At least three different test systems with two in mammalian cells followed by an animal experiment are necessary to allow conclusions (2017).

Testing for the teratogenic impacts of a material may use small animals (e.g. rodents) before/after mating in females. These animals and newborns/foetuses are macroscopically and microscopically evaluated for malformations. The ISO 10993-3 explains these extensive studies in more detail (2017). As yet, no clinical case with a suspicion of such effects caused by exposure to dental material has been reported.

Types of exposure

Appropriate contact between test material and cells is imperative in biological assessments. This contact can occur in three ways: direct, indirect and contact via extracts (Polyzois, 1994). These three types of exposure to the test material also are stated in the ISO 10993-5 for cytotoxicity tests.

In a test based on direct contact, a test material is in physical contact with the culture medium or the cells. In this regard, water-soluble materials can be easily dissolved in the culture medium to provide an adequate cell–material contact (Polyzois, 1994). For non-water-soluble material, there are several ways to achieve direct contact. The test material can be either placed on the top of an established monolayer of culture cells or placed on the bottom of the cell culture plate (Franz et al., 2003; Kasten et al., 1989; Schedle et al., 1998). For the latter, the cells can either be added to the plate in a suspension culture form or directly cultured on the specimens (Leirskar & Helgeland, 1972; Spangberg, 1973).

Although growing the cells directly on the test material provides a good cell–material contact, the surface characteristics of the material, in this case, would be influential. For instance, if the surface charge of the material is low, the cells will not adhere to it, and consequently, the growth rate would be low (Polyzois, 1994). Another factor that may affect the results is the culture media, which can mitigate the toxic impact by diluting the leachable components or binding with toxic agents (Moharamzadeh et al., 2007). It has been shown that using a three-dimensional (3D) oral mucosal model can minimize the effect of the culture medium by direct exposure of test material to the mucosal layer (Moharamzadeh et al., 2009). In these models, the tissue model is fed only from the connective tissue beneath the mucosal layer and the arrangement is similar to the clinical situation.

In a test system based on indirect contact, there is a permeable intermediate between cells and test material. Thus, this method is independent of the physical state of the material (solid, semi-solid or liquid), and since the test specimen is not covered by culture medium, even unset materials can be tested (Polyzois, 1994). Guess et al., (1965) introduced the first indirect cell–material contact test named the agar overlay technique. This method is designed to assess the cytotoxic impacts of diffused components of the test material through an agar layer located between specimen and monolayer cell culture and commonly administered in biocompatibility testing of dental materials (Kostoryz et al., 1999; Schmalz et al., 1994b). More recent techniques use dentine slice, synthetic filter or Millipore filters to test indirect contact (Tyas, 1977; Wennberg et al., 1979).

Extracting leachable components or emulsifying agents by a solvent is a method to assess the effects of insoluble materials on the cells. This extraction technique has been used commonly in cytotoxicity assessment of different dental materials (Moharamzadeh et al., 2009), including dental cement (Hanks et al., 1981) and adhesives (Szep et al., 2002). Various extraction media have been utilized for this purpose including culture media (Bouillaguet et al., 2002; Lefebvre et al., 1994; Szep et al., 2002), saline (Hanks et al., 1981), dimethyl sulphoxide (DMSO) (Issa et al., 2004; Kostoryz et al., 2003), ethanol (Eick et al., 2002) and distilled water (Geurtsen et al., 1999; Pelka et al., 2000). Only a few studies have compared various extraction techniques (Hanks et al., 1981; Moharamzadeh et al., 2007). It has been shown that type of extraction media, time of assessment and chemical properties of the solvent can significantly affect the results of the cytotoxicity test (Hanks et al., 1981; Moharamzadeh et al., 2007).

Types of experimental designs for *in vitro* pulpal toxicity tests

Monolayer cultures

Monolayer cultures of cells such as dental pulp stem cells and fibroblasts are suitable biological systems for the screening cytotoxicity test of the endodontic materials since the pulp tissue is usually the first target for the toxic components of these materials. Various cells from different sources such as animal/human pulp cells, human THP-1 monocytes and mouse odontoblast cell line

(MDPC-23) (Chen et al., 2003; Heil et al., 2002; Huang & Chang, 2002; Noda et al., 2003; Schmalz et al., 1994b; Souza et al., 1999) have been utilized for this purpose. It has been shown that cell type can affect the results of cytotoxicity assessments (Thonemann et al., 2002). Having done these tests, dose–response curves can be then determined, which is invaluable to manufacturers and dentists. Although monolayer cultures are rapid, relatively low-cost and informative, in a clinical situation, the pulp is mostly protected by a layer of dentine. To simulate the *in vivo* condition, other advanced culturing techniques have been described.

Barrier and diffusion systems

The dentine barrier system was first introduced by Schmalz et al., (1999) by modifying a commercially available cell culture perfusion chamber. They replaced the original membrane with a dentine disc to serve as a substrate for cell culture. Since the cell and test material were placed on the two sides of the dentine slice, only leachable components from the specimen could reach the other side and pass through the dentine barrier. Then, the effect of these transdentinal diffusions and subsequent cytotoxicity can be assessed by various methods.

In addition to the dentine barrier, different diffusion tests using agar or specific filters were also established as cytotoxicity barrier testing (Grasso et al., 1973; Guess et al., 1965; Wennberg et al., 1979). The overlay agar diffusion test is probably the longest established method in this category (Grasso et al., 1973; Guess et al., 1965). This method uses an agar overlay on a monolayer cell culture to test the nonspecific cytotoxicity of the leachable components and is included in ISO 7405 (1997).

Although simple and inexpensive to use as a cytotoxicity screening method, this technique has the disadvantage that materials or compounds must diffuse through the agar overlaying the monolayer of cells. Therefore, materials that do not dissolve in or diffuse through agar will not cause cellular damage, although they could nevertheless be cytotoxic when employed clinically.

Filter diffusion testing methods using cellulose acetate Millipore (0.45 μm pore size) filters are another technique for cytotoxicity assessments of the leachable components of test materials with barriers. The appearance of the test filters at the material cell contact areas is registered according to a scoring system to classify the cytotoxic response to a test material (ISO 7405) (1997). Assay end-points that have been used with this testing method include lactate dehydrogenase, glucose-6-phosphate dehydrogenase and cytochrome oxidase (examples of metabolic impairment assays). No differences in enzyme activity patterns have been observed amongst the enzymes tested (Hensten-Pettersen, 1988), indicating that the results from all of these end-points are comparable.

3D culture tooth models

The main shortcoming of using two-dimensional cell cultures monolayers is that the cells to matrix interactions do not form properly (Mazzoleni et al., 2009), decreasing the relevance to the clinical situations. In this regard, a 3D model was described that can mimic *in vivo* conditions (Gaudin et al., 2020; Silva et al., 2017), for example a 3D cell model consisting of a tooth suspended in a bioscaffold, which supports cell growth and function and allows for an evaluation of cell morphology, metabolism and cell-to-cell interactions that are more similar to the *in vivo* condition (Carletti et al., 2011).

Organ culture is another way to maintain the *in vivo* structure and function in the culture medium (Browne & Tyas, 1979; Laurent et al., 2012). The lack of circulation and excessive tissue damage during sample preparation are the main challenges in this approach. Mandibular first molar explants from chick embryo or mouse embryos (Beele et al., 1992; Hetem et al., 1989; Hikage et al., 1989) have been used for the evaluation of the biocompatibility of dental materials. Successful experiments on the rat tooth slice organ culture for the assessment of dental materials on pulp tissue (Murray et al., 2000; Saw et al., 2005) revealed the potential of *in vitro* setups to mimic *in vivo* pulp tests, which might replace some of the animal studies if it overcomes practical obstacles. Moreover, recently advanced tissue engineering techniques developed several 3D dentine/pulp reconstructions, such as customized cell perfusion chambers, tooth bud models and 3D cultured dentine–pulp complexes (Hadjichristou et al., 2021). Although some of these techniques provided consistent findings and demonstrated a complete *de novo* approach (Hadjichristou et al., 2021), there are still limitations regarding duration of the *in vitro* culture (Pedano et al., 2019), clearance of cellular wastes (Téclès et al., 2008) and simulation of inflammatory reactions (Laurent et al., 2012).

Tooth-on-chip (microfluidic) models

To perform real-time analysis of the responses of the dental pulp cells to dental materials particularly at the material–pulp or material–dentine–pulp interface,

recently developed tooth-on-a-chip models have been presented (França et al., 2020; Rodrigues et al., 2021). Although it has been shown that these models can successfully investigate the biological effects of endodontic materials on pulp cells (Rodrigues et al., 2021) and they have opened a new window for biocompatibility assessments, these models are still too primitive and they cannot entirely simulate the structure of the dentine–pulp complex.

Biological end-points

Cytotoxicity tests, as the primary *in vitro* screening tests for biocompatibility of endodontic materials, basically assess the cell reaction that can be described qualitatively (morphological assessments) or quantitatively (cell viability, proliferation and function). The norms ISO 10993 and 7405 recommended cell counting, dye binding and metabolic impairment as end-points of the cytotoxicity assays. Table 4 summarizes some of the most widely used assays in biocompatibility assessments.

Morphological assessment is based on pathological alterations including abnormal cellular shapes, abnormal organelles, and nuclear enlargement and abnormalities. Nuclear disintegration and pyknosis can be indicative of cell death. In a clinical situation, damage in tissues usually appears with lower metabolic rate and proliferation because of a lack of viable cells. Thus, several *in vitro* assays have been presented to assess the viability and proliferation status of the cells in the presence of dental materials (Table 4).

Metabolic impairment as another indicator of cytotoxicity can be measured by the decay of enzyme activity or metabolite concentration following the toxic effect of the material. Metabolic impairment assays measure these alterations in cytoplasmic lactate dehydrogenase (Rae, 1975; Ratner, 2015), succinate dehydrogenase (Barker & Farnes, 1967), lysosomal acid phosphatase or the incorporation of labelled precursors (Hynes et al., 2006; Rai et al., 2018).

In vivo tissue compatibility tests

In vivo biocompatibility tests are performed inside a living organism. Animal tests are the most common type of these tests and commonly include implantation of test materials into the animal body to assess local reactions; indeed, animal experimentation is essential for biological testing. Before an endodontic material can be utilized clinically, it must be tested in several species to establish its cytotoxic and systemic properties (Rowan, 1997). Animal studies help to anticipate the possible toxic hazards that may be encountered in human beings, but there were some notorious exceptions including thalidomide experiments (Stanley & Pameijer, 1985). Thus, it is worth noting for the researchers that no model could entirely replicate the complex human reaction and animal studies should be just performed to provide significant data and direction towards further clinical investigations (Zhan et al., 2016). *In vivo* tests allow many complex interactions to be examined between test material and biological system, which is more relevant compared with *in vitro* assays. Nevertheless, these tests are time-consuming and expensive. Moreover, controlling numerous variables and ethical considerations is also challenging. Appropriateness of an animal species to represent the human body reactions is also a question that researchers should ask before any research design (Schmalz et al., 1996; Wataha, 2001). Rodents are the most widely used animals for biocompatibility testing due to the low cost, high genetic homogeneity and ease of handling. However, larger animal models are more similar to the human body and their teeth have a higher degree of similarity to human teeth particularly when operating in the root canal space (Nakashima et al., 2019). Moreover, the inherent heterogeneity of animals could lead to inconsistent findings (Robinson et al., 2019). Dogs (Tziafas et al., 1996) and ferrets (Smith et al., 1994) are amongst large animals, which are used to assess biological reactions of teeth to dental materials. For pre-clinical testing, nonhuman primates are recommended by the ISO 7405 (1997); however, only a few studies have ever been performed on these animal models (Nagendrababu et al., 2019).

In vivo biocompatibility tests can be generally categorized into three major types: implantation tests, usage tests and assessments of systemic effect.

Implantation tests

In these studies, test materials are either directly injected or implanted into the muscle (Schmalz & Schmalz, 1981), connective tissue (Steinbrunner et al., 1991; Zmener, 2004) or bony tissue (Tassery et al., 1997) of an animal model. Commonly inert silicone material serves as the negative control for these assessments (Moharamzadeh et al., 2009). Biological end-points after a certain amount of time (≥365 days recommended by the ISO 7405) include necrosis, inflammation, infiltration of cells, tissue function and organization of the cells in the tissue (Ellender et al., 1990).

In vivo nonspecific tissue reactions to endodontic materials are generally assessed by histological

TABLE 4 *In vitro* biological tests for biocompatibility assessments of endodontic materials

Biological end-point(s)	Detection test	Advantage(s)	Disadvantage(s)
Morphological changes	Light microscopy Inverted phase-contrast microscopy	Inexpensive Rapid	Processing of the samples Difficult comparison (only qualitative)
	Confocal laser scanning microscopy	High accuracy Rapid	Expensive equipment Special training Processing of the samples Difficult comparison (only qualitative)
	Electron microscopy	High accuracy Rapid	Expensive equipment Special training Processing of the samples Difficult comparison (only qualitative)
DNA damage (genotoxicity)	Ames test HPRT enzyme test	Relatively simple Inexpensive	Difficult to interpret the results Low reliability (short-term follow-ups)
Cell viability and proliferation	Colorimetric cytotoxicity assay MTT assay Alamar blue assay Neutral red assay Propidium iodide assay	Most common method Relatively simple Inexpensive Rapid Measure membrane integrity	Toxic for the cells Depending on culturing condition and cell type Technique sensitive
	Protein content measurement LDH assay	Demonstrate membrane damage or cytolysis	Poor dynamic range Lack of sensitivity
	DNA content measurement 3H-thymidine incorporation assay Bromodeoxyuridine incorporation assay	Determine the DNA content Rapid inexpensive	Questionable sensitivity Radioactivity
	Apoptosis assay The comet assay Annexin V assay Protease activity assay Esterase substrate assay	Sensitive and specific	Expensive Requires specific equipment
Metabolic impairment (cell function)	Gene expression analysis Microarray test Polymerase chain reaction	Clinically relevant Sensitivity	Technique sensitive
	Protein content measurement Inflammatory mediators measurement Glutathione determination Heat-shock protein assay	Clinically relevant Sensitivity	Relatively costly Technique sensitive
Cell migration (cell function)	Cell migration assay Tenascin expression assay	Provide detailed information on biological interactions between the cells and test materials	Difficult to translate to clinical situation

Abbreviations: HPRT, hypoxanthine-guanine phosphoribosyltransferase;LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

investigations following the implantation of material into the tissue of the animal. Subcutaneous implantation has been used in various animal models including dogs, ferrets, rabbits, guinea pigs, rats and hamsters (Altaii et al., 2017; Binnie & Rowe, 1973; Kolokouris et al., 1998; Maher et al., 1992; Olsson et al., 1981; Safavi et al., 1983; Spangberg et al., 1973; Thomas et al., 1985; Torabinejad et al., 2011; Torneck, 1961). Different periods of implantation are required for histological studies. After a short implantation time for one or two weeks, inflammation around the implant is discernible, whereas after a longer time, the nature and quantity of the connective tissue

encapsulation can be assessed (Schmalz & Arenholt-Bindslev, 2009).

The implantation of dental materials into the jawbones is another way to investigate local tissue reactions. Appropriate animals are dogs, primates, guinea pigs and rats. Tissue reaction surrounding the implant is assessed histologically (Donath, 1985).

Finally, local interactions of materials with oral mucosa are essential to be understood. Various animal models were also described for oral mucosa tests (Klötzer & Langeland, 1973; Schmalz et al., 2000; Wirthlin et al., 1997). However, based on the experience of the cosmetic industry, *in vitro*-cultured mucosa and skin equivalents may offer a similar condition to *in vivo* tests with a highly controlled environment, lower cost and rapid procedures (Schmalz & Arenholt-Bindslev, 2009). These equivalents have an interesting perspective, but experiences with dental materials remain very limited (Carvalho et al., 2018; Klausner et al., 2021; Tabatabaei et al., 2020).

Usage tests

In vivo toxicity tests usually consist of the use of test material for root canal treatment in animal models, mainly dogs (Hauman & Love, 2003; Sonat et al., 1990; Torabinejad et al., 1995) and monkeys (Torabinejad et al., 1997). Root is either filled to the apical foramen or intentionally overextended to assess the reaction of periapical tissues (Sonat et al., 1990). Conventional materials with a long-established clinical record such as calcium hydroxide are recommended to be used on the adjacent tooth at the same time to facilitate the comparison and interpretation of the findings. The ISO 7405 guidelines recommended 7 ± 2 days as short-term and 70 ± 5 days as a long-term period between root canal treatment and extraction of samples for histological evaluations. Nevertheless, many researchers prefer to observe the effects of materials in a timed sequence of reactions (e.g. 3, 7, 14, 30 and 60 days) (Kitasako et al., 2000a, 2000b). Although short-term follow-ups are often used to reduce the cost especially for nonhuman primate usage testing, these studies may not show the full healing or reaction of the tissues. Increasing the length of the follow-up period increases the probability of detecting potential complications. In fact, some direct pulp capped teeth demonstrate healing at early stages but can lose their vitality several weeks later (Stanley, 1998). Another consideration is bacterial leakage, which is more commonly detectable in the long term (Cox, 1992; Cox et al., 1987). Adhering to a standard follow-up span (e.g. 70 ± 5 days) improves comparability between various studies.

Pulp/dentine tests

Pulp tissue compatibility of endodontic materials is naturally of great importance for endodontists and dentists. Conventionally, pulp compatibility of a dental material is investigated on animal models or extracted human teeth (1997) using a Class V cavity preparation and filling with test materials. After the designated follow-up period, the teeth are histologically prepared and microscopically evaluated for signs of infection, necrosis, tissue reaction and bacterial leakage (Klötzer & Langeland, 1973). Figure 4 shows the histological evaluation of pulpal inflammation after application of pulp capping materials (Nair et al., 2008). For endodontic materials to mimic the clinical condition, the pulp is exposed, or part of the pulp tissue is removed before the test material is applied. In this way, materials utilized for direct pulp caps or pulpotomies can be tested. The major causes of pulp damage resulting from these materials are 1. toxic substances released from them, 2. bacteria/endotoxin leakage, 3. inflammation and 4. tertiary dentine formation and obliteration of the pulp. Nevertheless, the other aspects of bioactivity of the material for instance hard tissue formation (dentine barrier) can be clearly observed in histological assessments (Figure 4).

For histological analysis of pulpal injury and reactions, after histological processing of the specimens, standardized ISO histological criteria can be helpful (1997). The inflammatory cell's activity of pulp can be categorized as none, slight, moderate and severe (Mjör & Tronstad, 1972). 'None' means the pulp contains no or few inflammatory cells. In slight inflammation, only localized inflammatory cells predominated by polymorphonuclear leucocytes are detectable, whereas moderate inflammation indicates less than one-third of the coronal pulp is involved. Finally, when the inflammation affects more than one-third of the coronal pulp, it is categorized as severe. Chronic inflammation may lead to pulp necrosis. It is important to differentiate pulp responses to test material and other restorative variables, for instance whether the material is placed in contact with the exposed tissue, or there is a cavity with remaining dentine thickness (CRDT). The CRDT commonly uses 1 mm for assessing restorative materials (Stanley, 1968). Moreover, as aforementioned, the effect of bacterial leakage and penetration should be excluded, and teeth must be isolated if biocompatibility testing is the only aim of the experiment.

Although the pulp/dentine usage testing in animal models provides remarkable evidence regarding the biocompatibility of endodontic materials, there are some limitations regarding these experiments. The pulp tissue in animals, particularly primates, seems to be more resistant to chemical substances and less resistant to bacteria/endotoxins than human pulp (Aubeux et al., 2021). These

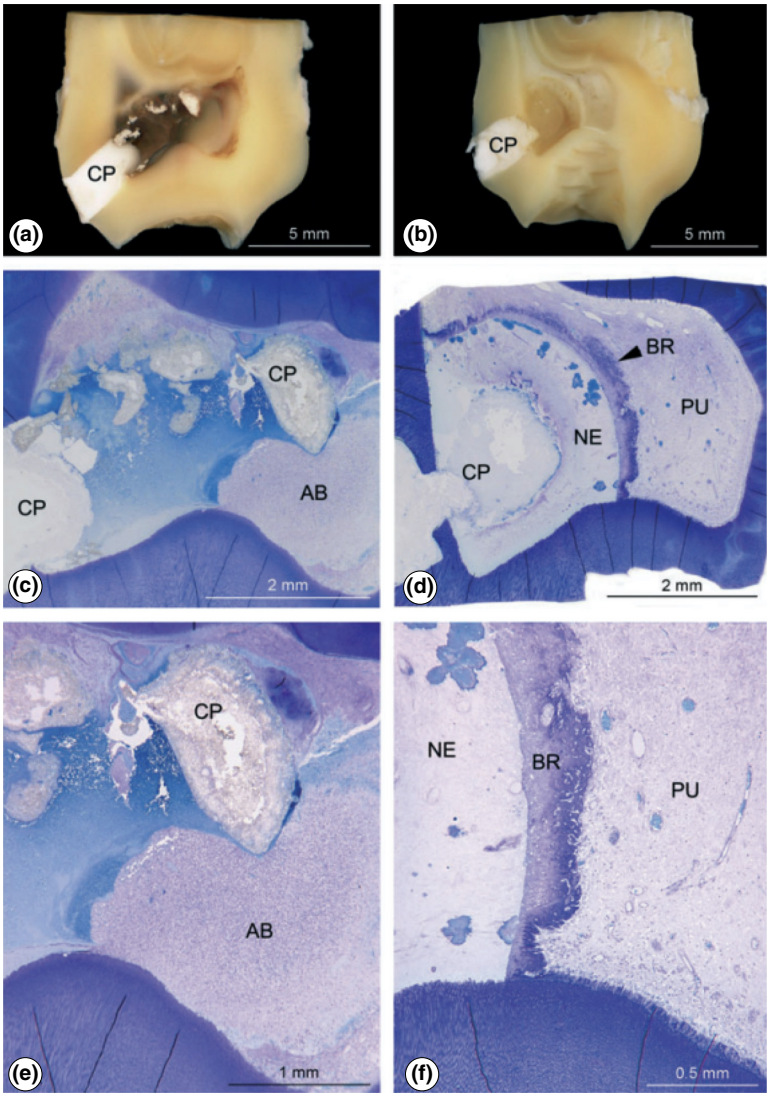

FIGURE 4 Severe inflammatory (a, c, e) and healing (b, d, f) pulpal responses to Dycal capping after 1 month observation in human third molars. The capping material as a white plug in the cavity preparation and pulp chamber can be seen in the photographs. The acute pulpal response and abscess (AB) formation can be seen in c and e. On the right-hand column, a healing pulpal (PU) response can be observed (d, f). A hard tissue barrier (BR) stretching across the full length of the exposed pulp that is devoid of any signs of inflammation has been formed after 3 months. Reproduced with permission from Nair, P. N. R., Duncan, H. F., Pitt Ford, T. R., & Luder, H. U. (2008). Histological, ultrastructural, and quantitative investigations on the response of healthy human pulps to experimental capping with mineral trioxide aggregate: a randomized controlled trial. International endodontic journal 41(2), 128-150

experiments are costly and increasingly questioned by the public regarding ethical considerations.

Periradicular tissue damage test

The classic endodontic usage test is intricate and involves the same ethical considerations as the pulp/dentine test using large animal models. Although relatively few studies used this test method, the presented data have revealed a good correlation with clinical observation (Fouad et al., 1993; Tagger & Tagger, 1989). Evaluating the effects of the test material on specific periradicular cells (e.g. cementoblasts) is not the major concern; implantation tests using inert tubes filled with the test material may be used as alternatives (Schmalz & Arenholt-Bindslev, 2009).

Regenerative endodontics, endodontic complication management and biocompatibility considerations

Accidental root perforation during root canal treatment, vital pulp therapy and management of immature teeth with necrotic pulps require advanced interventions to decrease inflammatory responses and promote tissue regeneration

(Peters et al., 2021). Therefore, ideal endodontic materials not only should be safe to apply but also need to be bioactive. In animal studies, histological evaluations of the repair tissue are the main tool to measure this concept of tissue compatibility. Whilst ISO standards explain detailed criteria for pulp inflammation and necrosis, there are no precisely defined quantitative criteria for assessing the reparative, reactionary, or regenerative responses (Mjör et al., 2001) including dentine regeneration or dental pulp cell survival rates (Murray et al., 2001). These measures are left to the judgement of the individual researcher; however, comparing the test materials with the conventional control materials are recommended (1997).

Systemic effects

These reactions include allergy and hypersensitivity, systemic toxicity, mutagenicity and teratogenic effects.

Allergic reaction

Allergic properties of dental materials are currently tested on animal models (pre-clinical evaluations). Based on the 'Organisation for Economic Co-operation and Development' (OECD) Guideline 406, maximization test and the Buehler test are recommended to be performed on guinea pigs (Basketter et al., 1993; Frankild et al., 2000; Magnusson, 1969). For the maximization test, the test material is injected intradermally into animal models, and seven days later, the same substance is applied topically, and the application is refreshed in two weeks. Subsequently, the immunological effects (e.g. irritating skin) are assessed. The Buehler test is similarly performed but without the application of Freud's complete adjuvant. The Buehler test is less sensitive than the maximization test and more protective for the animals (Frankild et al., 2000).

Systemic toxicity

Systemic toxicity generally is acute and/or chronic. In a common method for testing acute systemic toxicity, animals receive a series of injections (either intraperitoneally or intravenously) of the test material or extract. Then, histopathological analysis is performed after two hours. Studies on these adverse reactions are rare, and histopathologic criteria to evaluate them are still not standardized. The test for assessing the chronic systemic toxicity is designed to measure detrimental effects from multiple exposures to test materials or extracts during 10% of the entire life of the animal model. Following implantation of various dental material, some ingredients release and circulate via the blood. For instance, (Pan et al., 2020) found that trace metals released from nickel–chromium (Ni-Cr) alloy, cobalt–chromium (Co-Cr) alloy and commercially pure titanium (CP-Ti) were accumulated transiently in the blood, liver and kidney of Syrian hamsters after 8 weeks but had no effect on the histopathology of the liver or kidney. Moreover, the systemic toxic effect of DiaRoot BioAggregate (Diadent Group International) and grey ProRoot MTA (Dentsply, Tulsa Dental) on the liver and kidney was investigated after 7 and 30 days in albino rats (Khalil & Eid, 2013). It has been reported that subcutaneously implanted materials can significantly increase liver function and the number of inflammatory cells (Figure 5). Although the amount of release and consequent effects can vary depending on the location of usage, these findings indicate the potential systemic cytotoxicity, which requires to assess.

Clinical testing

Ultimately, controlled clinical studies in human subjects are the most reliable source of evidence. However, due to legal and ethical considerations and to protect human health, clinical usage testing can only be performed with test materials that have already successfully passed the first ISO recommended steps of biocompatibility testing (Table 2) (ISO10993 and ISO7405). Consequently, this reflects the number of clinical studies compared with *in vitro* and animal testing (Figure 2). Most clinical studies focus on the efficiency of new material (e.g. treatment outcome, sealing, longevity), and generally, the assessment of the biocompatibility of endodontic materials is not their focus. For instance, if a pulp capping material is investigated, clinical variables such as pulp sensitivity and postoperative pain are usually examined, but more detailed histological investigations are rarely performed. These studies must be approved by an ethical committee (mainly based on the Helsinki Declaration).

Blinding is a common method for clinical studies, particularly for drug assessment. A study design can be either single-blinded where patients are not informed whether they receive placebo or the tested drug or double-blinded where both patients and dentists are not informed of the administrations. For dental material evaluations, a split-mouth design is more common. In this strategy, both test and control materials are applied in the same patient preferably on similar teeth in different quadrants at the same time. The allocation of the groups in both methods should be entirely randomized (Moher et al., 2001). Such an approach is not feasible for all endodontic material testing and should be precisely selected based on the purposes of the study and the limitations of the therapeutic strategies.

Clinical biocompatibility testing data are clearly of special interest for dentists, because the examination applies to the real target group, patients. Although controlled clinical

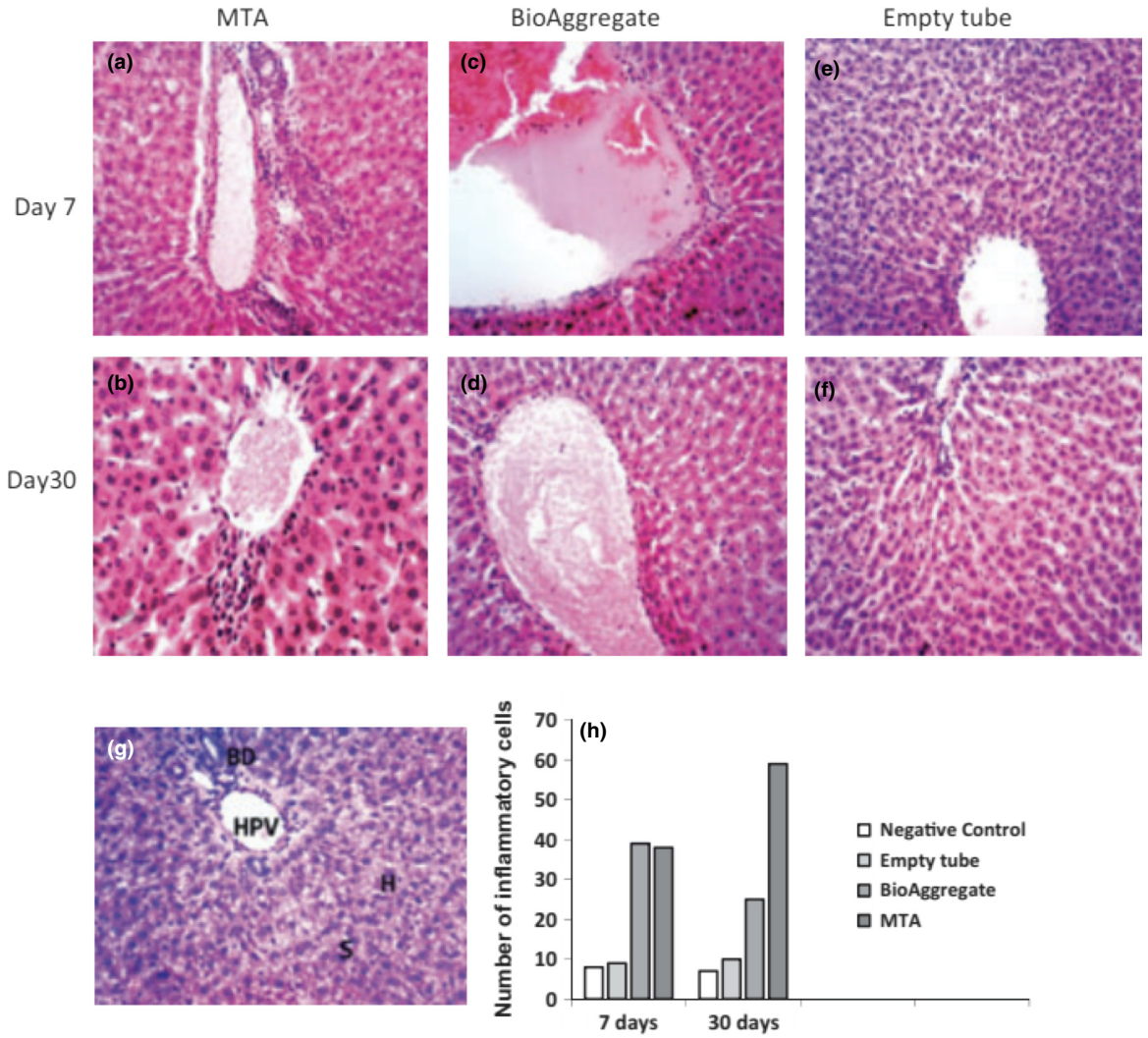

FIGURE 5 Haematoxylin-and-eosin-stained histograms of rat liver after 7 and 30 days of subcutaneous implantation of DiaRoot BioAggregate and grey ProRoot MTA (a–d). Panel g shows the negative control. The mean number of inflammatory cells infiltrating the liver after 7 and 30 days significantly increased in the ProRoot MTA group (h) ($p < .05$). However, the severity decreased after 30 days. Reproduced with permission from Khalil and Eid, (2013). Biocompatibility of BioAggregate and mineral trioxide aggregate on the liver and kidney. *International Endodontic Journal* 46, 730–737

trials possess a greater level of evidence compared with *in vitro* and animal usage studies, this should not conceal the fact that clinical studies also have several limitations. Therefore, an uncritical transfer of the findings of any clinical investigation to patients may result in complications. Moreover, clinical diagnosis is not always definitive.

For instance, it has been reported that the clinical diagnosis of pulp conditions does not necessarily align with histological conditions (Galicia & Peters, 2021). Pathological processes in pulpal tissue may proceed without any clinical symptoms (Trowbridge, 1986), for example as observed in the past with silicate cement restorations (Klötzer & Langeland, 1973). Another limitation of clinical studies is that usually a short period is considered for the observation. However, many undesirable reactions appear only after prolonged exposure to the materials. Small sample sizes and strictly selected individuals are other limitations of a clinical investigation particularly when the rate of side effects is very low. For instance, for a side effect with the frequency of 0.1%, a sample size of 3750 subjects is needed to be able to document such side effects (Garhammer et al., 2001; Kallus & Mjor, 1991). Thus, practically such large sample sizes are not included in clinical studies and the potential side effects may not be identified. In these situations, monitoring the market and observation that relies on practising dentists become essential.

Allergy tests

The patch test is the most-often used allergy test for dental materials. This test identifies type IV (delayed)

hypersensitivity reactions caused by allergic contact dermatitis (Cohen, 2004). Type 1 (immediate) allergic reactions can be diagnosed by the prick test. Further immunological tests such as the radioallergosorbent (RAS) test also have been used to some extent but should not be used for routine diagnosis yet (Schmalz, 2009).

Although many attempts have been made, cell cultures are still not accepted for the diagnosis of type IV hypersensitivity reactions (Galbiati et al., 2016). Oral mucosa and skin react similarly in case of allergy similar to many other diseases. Thus, the skin is an appropriate organ for adequate allergy tests. For this assessment, the test material (allergen) should be released in sufficiently high quantities to penetrate the skin and then potentially stimulates T lymphocytes. Although for endodontic materials, performing an allergy test on oral mucosa seems more reasonable at first look, this approach is much more difficult to conduct, and the findings also can be misleading. Since saliva dilutes the test material (allergen), and there is a higher tolerance of oral mucosa to allergen exposure compared with the skin (Okamura et al., 2003), a higher concentration of allergen is required to trigger a positive response. Therefore, the patch test (skin of the back) is recommended by several international and national allergy associations for diagnosis of type IV hypersensitivity reactions (Beltrani et al., 2006; Bourke et al., 2001; Bruze et al., 1999).

A prick test is another allergy evaluation performed on the skin (Korting, 1997). The test material or extract is applied on the skin, and after 5–10 min, the reaction of skin including redness, wheal formation is assessed. It is highly unlikely to initiate an immediate allergic reaction or future hypersensitivity during/after the test, but it is theoretically possible (Korting, 1997). It is important for the dentist as the patient's primary contact person to know when these allergic tests are indicated. It is important to realize that patch tests should be done only if there is well-founded susceptibility to a type IV allergic reaction. Since even a negative result of patch test cannot exclude the possibility of allergic reaction in future, a general patch testing of patients with no clinical symptoms is not adequate. In addition, undiluted test material itself may increase the chance of hypersensitivity.

Clinical and radiographic assessments

Finally, clinical and radiographic evaluations of pulpal and periapical tissues reflect the outcome of vital pulp therapy, root canal treatment and surgery, which commonly includes postoperative pain, inflammation, soft tissue and hard tissue effects. Although these findings might not directly focus on the biocompatibility of the endodontic materials, indirectly they can demonstrate the interaction between host and materials particularly in terms of bioactivity in regenerative approaches and hard tissue formation. Pulp sensibility testing also may reveal nerve functionality, which is mainly based on the application of cold and electric current (Cooley et al., 1984; Fuss et al., 1986). Although these tests are commonly used in biocompatibility tests in terms of material-associated pulp damages in clinical investigations, a decisive limitation of sensibility tests is that they only indicate the existence of at least several functional nerves, which are responsible for the positive results. However, these tests cannot show the specific inflammatory condition of the pulp or prove vitality (blood supply). For instance, a histological control revealed that despite positive sensibility responses, almost 40% of the evaluated cases had some amount of pulp necrosis (Hyman & Doblecki, 1983). This can occur since the neural structures are more resistant (Montgomery & Ferguson, 1986), and a negative response is usually achieved once >90% of the pulp is nonvital. Overall, sensibility tests tend to overestimate the biocompatibility of a material in contact with the pulp (Hyman & Doblecki, 1983). Sometimes, histopathologic assessments display healing, but no significant radiographic difference between study groups could be observed (Primus et al., 2019). This contradiction might be because of the limitation of radiographic evaluation. New advanced radiographic equipment such as cone-beam computed tomography can overcome these issues (Chen et al., 2015).

CONCLUSIONS

Modern concepts of biocompatibility evaluation of endodontic materials are concerned with regulatory and technical aspects. Regulatory aspects depend on expert assessment of physical, chemical and available biological evidence during risk assessment. The first step for the selections of essential testing is standard assays and, if necessary, more novel nonstandard alternatives. Much work is ongoing to further optimize and refine *in vitro* testing, particularly current *in vitro* cytotoxicity screening tests. Generally, these tests are to rank the test materials in terms of their cytotoxicity within the limitations of the testing conditions. In any form of *in vitro* cell culture test, the test system is enormously different from the clinical environment, in which few conclusions may be drawn as the potential adverse effects (mainly cytotoxicity) of the endodontic material when applied in a clinical situation. Moreover, it is imperative to consider the difference between biocompatibility and cytotoxicity despite being in the same domain as discussed before. Thus, there is

a need to develop *in vitro* cytotoxicity assays using clinically relevant models such as embryonic organ culture, using differentiated cells, culture on the tooth structures and real-time cell analysis systems. Although alternative approaches such as usage of suitable barrier systems, appropriate target cells and application of clinically relevant markers are trying to simulate *in vitro* conditions, animal and clinical screening is necessary to assess the biocompatibility of endodontic materials. *In vitro* testing methods also provide the basis for a more mechanistic approach in describing the biocompatibility of endodontic materials with trying to understand the actions, mechanisms and molecular alterations. On the contrary, clinical experience with endodontic materials over a long period is a phenomenological approach that provides essential information regarding the biocompatibility and safety of the materials.

The standards for biocompatibility testing, toxicity assessments and clinical success of endodontic materials are perpetually modified or updated based on recent scientific advances or to avoid the recurrence of the issues such as allergies and toxic reactions that have arisen in the past (Hensten-Pettersen & Jacobsen, 1991; Syed et al., 2015). Although there is often a robust motivation to regularly modify the criteria, the need to sustain a comparison with previous data provides an incentive to conserve the status quo. In the ISO 7405 guidelines, the evaluation of alternative nonanimal/nonpatient testing methods has been recommended, which is in response to the public and political pressure to decrease animal usage wherever possible. It is also necessary to consider that not all types of pre-clinical testing can be mimicked or replaced by *in vitro* models. For instance, investigations of systemic effects such as carcinogenicity, inflammation and hemocompatibility are not currently feasible outside the body. Hence, at least for the foreseeable future, the continued utilization of animal models for biocompatibility testing is a crucial safeguard to minimize potential hazards to the patients. These tests are still the only methods apart from clinical evaluations that are appropriate for assessing tissue inflammation, pulp sensitivity, carcinogenic effects and bacterial leakage. Finally, it must be emphasized that all evaluations, including well-designed clinical trials, only yield a statistical approximation of the biocompatibility of an endodontic material and even a material rated with high biocompatibility might cause an adverse reaction in several individuals.

ACKNOWLEDGEMENT

Open access publishing facilitated by The University of Queensland, as part of the Wiley - The University of Queensland agreement via the Council of Australian University Librarians. [Correction added on 21st May 2022, after first online publication: CAUL funding statement has been added.]

CONFLICT OF INTEREST

Drs. Hosseinpour and Gaudin have stated explicitly that there are no conflicts of interest in connection with this article. Dr. Peters has served as consultant for Dentsply Sirona outside the scope of the present work.

AUTHOR CONTRIBUTION

OP and SH involved in conceptualization, datacuration and writing - review and editing. SH in-volved in formal analysis, investigation, methodology and writing the original draft. OP and AG reviewed and supervised the project.

ETHICAL APPROVAL

No ethics statement is needed because of the nature of this research.

ORCID

Sepanta Hosseinpour https://orcid.org/0000-0002-6503-4223
Alexis Gaudin https://orcid.org/0000-0003-4139-8927
Ove A. Peters https://orcid.org/0000-0001-5222-8718

REFERENCES

Al-Saudi, K.W., Nabih, S.M., Farghaly, A.M. & AboHager, E.-A.-A. (2019) Pulpal repair after direct pulp capping with new bioceramic materials: a comparative histological study. *The Saudi Dental Journal*, 31, 469–475.

Altaii, M., Richards, L. & Rossi-Fedele, G. (2017) Histological assessment of regenerative endodontic treatment in animal studies with different scaffolds: a systematic review. *Dental Traumatology*, 33, 235–244.

Association EAR. (2013) *EU regulations on animal research*.

Aubeux, D., Renard, E., Pérez, F., Tessier, S., Geoffroy, V. & Gaudin, A. (2021) Review of animal models to study pulp inflammation. *Frontiers in Dental Medicine*, 2, 673552.

Autian, J. (1970) The use of rabbit implants and tissue culture tests for the evaluation of dental materials. *International Dental Journal*, 20, 481–490.

Autian, J. & Dillingham, E. (1975) Overview of general toxicity testing with emphasis on special tissue culture tests. *In Vitro Toxicity Testing*, 1976, 23–49.

Barker, B.E. & Farnes, P. (1967) Histochemistry of blood cells treated with pokeweed mitogen. *Nature*, 214, 787–789.

Basketter, D., Selbie, E., Scholes, E., Lees, D., Kimber, I. & Botham, P. (1993) Results with OECD recommended positive control sensitizers in the maximization, Buehler and local lymph node assays. *Food and Chemical Toxicology*, 31, 63–67.

Beele, H., Thierens, H., Deveux, R., Goethals, E. & De Ridder, L. (1992) Skin organ culture model to test the toxicity of polyoxyethylene networks. *Biomaterials*, 13, 1031–1037.

Beltrani, V.S., Bernstein, I.L., Cohen, D.E. & Fonacier, L. (2006) Contact dermatitis: a practice parameter. *Annals of Allergy, Asthma & Immunology*, 97, S1–S38.

Binnie, W.H. & Rowe, A. (1973) A histological study of the periapical tissues of incompletely formed pulpless teeth filled with calcium hydroxide. *Journal of Dental Research*, 52, 1110–1116.

Black, J. (2005) *Biological performance of materials: fundamentals of biocompatibility*. Boca Raton, FL, USA: CRC Press.

Bouillaguet, S., Shaw, L., Gonzalez, L., Wataha, J. & Krejci, I. (2002) Long-term cytotoxicity of resin-based dental restorative materials. *Journal of Oral Rehabilitation*, 29, 7–13.

Bourke, J., Coulson, I. & English, J. (2001) Guidelines for care of contact dermatitis. *British Journal of Dermatology*, 145, 877–885.

Browne, R. (1988) The in vitro assessment of the cytotoxicity of dental materials–does it have a role? *International Endodontic Journal*, 21, 50–58.

Browne, R. & Tyas, M.J. (1979) Biological testing of dental restorative materials in vitro—a review. *Journal of Oral Rehabilitation*, 6, 365–374.

Bruze, M., Conde-Salazar, L., Goossens, A., Kanerva, L. & White, I.R. (1999) Thoughts on sensitizers in a standard patch test series. *Contact Dermatitis*, 41, 241–250.

Carletti, E., Motta, A. & Migliaresi, C. (2011) Scaffolds for tissue engineering and 3D cell culture. In: Haycock, J. (Ed.) *3D cell culture. Methods in molecular biology (methods and protocols)*, Vol 695, Humana Press. https://doi.org/10.1007/978-1-60761-984-0_2

de Carvalho, D.K., de Sousa, D.L., Barbugli, P.A., Cerri, P.S., Salih, V.M. & Vergani, C.E. (2018) Development and characterization of a 3D oral mucosa model as a tool for host-pathogen interactions. *Journal of Microbiology Methods*, 152, 52–60.

Chen, I., Karabucak, B., Wang, C., Koyama, E., Kohli, M.R., Nah, H.D. et al. (2015) Healing after root-end microsurgery by using mineral trioxide aggregate and a new calcium silicate-based bioceramic material as root-end filling materials in dogs. *Journal of Endodontics*, 41(3), 389–399.

Chen, R.-S., Liu, C.-C., Tseng, W.-Y., Jeng, J.-H. & Lin, C.-P. (2003) Cytotoxicity of three dentin bonding agents on human dental pulp cells. *Journal of Dentistry*, 31, 223–229.

Cheng, X. & Liu, X. (2020) Antimicrobial endodontic materials. In: Li, B., Moriarty, T.F., Webster, T. & Xing, M. (Eds.) *Racing for the surface: pathogenesis of implant infection and advanced antimicrobial strategies*. Cham: Springer International Publishing, pp. 247–266.

Cohen, D.E. (2004) Contact dermatitis: a quarter century perspective. *Journal of the American Academy of Dermatology*, 51, 60–63.

Cooley, R.L., Stilley, J. & Lubow, R.M. (1984) Evaluation of a digital pulp tester. *Oral Surgery, Oral Medicine, Oral Pathology, and Oral Radiology*, 58, 437–442.

Cox, C. (1992) Microleakage related to restorative procedures. *Proceedings of the Finnish Dental Society. Suomen Hammaslaakariseuran Toimituksia*, 88, 83–93.

Cox, C.F., Keall, C.L., Keall, H.J., Ostro, E. & Bergenholtz, G. (1987) Biocompatibility of surface-sealed dental materials against exposed pulps. *The Journal of Prosthetic Dentistry*, 57, 1–8.

Donaruma, L.G. (1988) Definitions in biomaterials, D. F. Williams, Ed., Elsevier, Amsterdam, 1987, 72 pp. *Journal of Polymer Science: Polymer Letters*, 26, 414.

Donath, K. (1985) The diagnostic value of the new method for the study of undecalcified bones and teeth with attached soft tissue, (Säge-Schliff, (Sawing and Grinding) Technique). *Pathology-Research and Practice*, 179, 631–633.

Eick, J., Kostoryz, E., Rozzi, S., Jacobs, D., Oxman, J.D., Chappelow, C. et al. (2002) In vitro biocompatibility of oxirane/polyol dental composites with promising physical properties. *Dental Materials*, 18, 413–421.

Ellender, G., Feik, S.A. & Gaviria, C. (1990) The biocompatibility testing of some dental amalgams in vivo. *Australian Dental Journal*, 35, 497–504.

Farhad, A. & Mohammadi, Z. (2005) Calcium hydroxide: a review. *International Dental Journal*, 55, 293–301.

Fouad, A.F., Walton, R.E. & Rittman, B.R. (1993) Healing of induced periapical lesions in ferret canines. *Journal of Endodontics*, 19, 123–129.

França, C.M., Tahayeri, A., Rodrigues, N.S., Ferdosian, S., Puppin Rontani, R.M., Sereda, G. et al. (2020) The tooth on-a-chip: a microphysiologic model system mimicking the biologic interface of the tooth with biomaterials. *Lab on a Chip*, 20, 405–413.

Frankild, S., Vølund, A., Wahlberg, J.E. & Andersen, K.E. (2000) Comparison of the sensitivities of the Buehler test and the guinea pig maximization test for predictive testing of contact allergy. *Acta Dermato-Venereologica*, 80, 256–262.

Franz, A., König, F., Anglmayer, M., Rausch-Fan, X., Gille, G., Rausch, W.D. et al. (2003) Cytotoxic effects of packable and nonpackable dental composites. *Dental Materials*, 19, 382–392.

Fuss, Z., Trowbridge, H., Bender, I., Rickoff, B. & Sorin, S. (1986) Assessment of reliability of electrical and thermal pulp testing agents. *Journal of Endodontics*, 12, 301–305.

Galbiati, V., Papale, A., Kummer, E. & Corsini, E. (2016) In vitro models to evaluate drug-induced hypersensitivity: potential test based on activation of dendritic cells. *Frontiers in Pharmacology*, 7, 204.

Galicia, J.C. & Peters, O.A. (2021) Proposal for a new diagnostic terminology to describe the status of the dental pulp. *International Endodontic Journal*, 54, 1415–1416.

Garhammer, P., Schmalz, G., Hiller, K.-A., Reitinger, T. & Stolz, W. (2001) Patients with local adverse effects from dental alloys: frequency, complaints, symptoms, allergy. *Clinical Oral Investigations*, 5, 240–249.

Gaudin, A., Tolar, M. & Peters, O.A. (2020) Cytokine Production and Cytotoxicity of Calcium Silicate–based Sealers in 2-and 3-dimensional Cell Culture Models. *Journal of Endodontics*, 46, 818–826.

Geurtsen, W., Spahl, W., Müller, K. & Leyhausen, G. (1999) Aqueous extracts from dentin adhesives contain cytotoxic chemicals. *Journal of Biomedical Materials Research*, 48, 772–777.

Gociu, M., Pătroi, D., Prejmerean, C., Păstrăv, O., Boboia, S., Prodan, D. et al. (2013) Biology and cytotoxicity of dental materials: an in vitro study. *Romanian Journal of Morphology and Embryology*, 54, 261–265.

Grasso, P., Gaydon, J. & Hendy, R. (1973) The safety testing of medical plastics. II. An assessment of lysosomal changes as an index of toxicity in cell cultures. *Food and Cosmetics Toxicology*, 11, 255–263.

Guess, W.L., Rosenbluth, S.A., Schmidt, B. & Autian, J. (1965) Agar diffusion method for toxicity screening of plastics on cultured cell monolayers. *Journal of Pharmaceutical Sciences*, 54, 1545–1547.

Hadjichristou, C., About, I., Koidis, P. & Bakopoulou, A. (2021) Advanced in vitro experimental models for tissue engineering-based reconstruction of a 3D Dentin/pulp complex: a literature review. *Stem Cell Reviews and Reports*, 17, 785–802.

Hanks, C.T., Anderson, M. & Craig, R.G. (1981) Cytotoxic effects of dental cements on two cell culture systems. *Journal of Oral Pathology & Medicine*, 10, 101–112.

Hauman, C. & Love, R. (2003) Biocompatibility of dental materials used in contemporary endodontic therapy: a review. Part 2. Root-canal-filling materials. *International Endodontic Journal*, 36, 147–160.

Heil, J., Reifferscheid, G., Waldmann, P., Leyhausen, G. & Geurtsen, W. (1996) Genotoxicity of dental materials. *Mutation Research/Genetic Toxicology*, 368, 181–194.

Heil, T., Volkmann, K., Wataha, J. & Lockwood, P. (2002) Human peripheral blood monocytes versus THP-1 monocytes for in vitro biocompatibility testing of dental material components. *Journal of Oral Rehabilitation*, 29, 401–407.

Hensten-Pettersen, A. & Helgeland, K. (1977) Evaluation of biologic effects of dental materials using four different cell culture techniques. *European Journal of Oral Sciences*, 85, 291–296.

Hensten-Pettersen, A. (1988) Comparison of the methods available for assessing cytotoxicity. *International Endodontic Journal*, 21, 89–99.

Hensten-Pettersen, A. & Jacobsen, N. (1991) Toxic effects of dental materials. *International Dental Journal*, 41(5), 265–273.

Hetem, S., Jowett, A. & Ferguson, M. (1989) Biocompatibility testing of a posterior composite and dental cements using a new organ culture model. *Journal of Dentistry*, 17, 155–161.

Hikage, S., Atsuta, M. & Sato, A. (1989) Biological evaluation of biomaterials using cultured chick embryo femurs (3). *Journal of the Japanese Society for Dental Materials and Devices*, 8, 642–647.

Huang, F.-M. & Chang, Y.-C. (2002) Cytotoxicity of resin-based restorative materials on human pulp cell cultures. *Oral Surgery, Oral Medicine, Oral Pathology, and Oral Radiology*, 94, 361–365.

Hyman, J.J. & Doblecki, W. (1983) Computerized endodontic diagnosis. *The Journal of the American Dental Association*, 107, 755–758.

Hynes, J., Hill, R. & Papkovsky, D.B. (2006) The use of a fluorescence-based oxygen uptake assay in the analysis of cytotoxicity. *Toxicology in Vitro*, 20, 785–792.

(2017) *ISO 10993: biological evaluation of medical devices*. Geneva: International Organization for Standardization. Available at: https://www.iso.org/standard/68936.html [Accessed on August 2017]

(2019) *ISO 14971: medical devices-application of risk management to medical devices*. Geneva: International Organization for Standardization. Available at: https://www.iso.org/standard/72704.html [Accessed on December 2019]

(1997) *ISO 7405: preclinical evaluation of biocompatibility of medical devices used in dentistry-Tests methods for dental materials*. Geneva: International Organization for Standardization. Available at: https://www.iso.org/standard/14134.html [Accessed on November 1997]

Issa, Y., Watts, D., Brunton, P., Waters, C. & Duxbury, A. (2004) Resin composite monomers alter MTT and LDH activity of human gingival fibroblasts in vitro. *Dental Materials*, 20, 12–20.

Jacobsen, N., Aasenden, R. & Hensten-Pettersen, A. (1991) Occupational health complaints and adverse patient reactions as perceived by personnel in public dentistry. *Community Dentistry and Oral Epidemiology*, 19, 155–159.

Jolanki, R., Kanerva, L. & Estlander, T. (1995) Occupational allergic contact dermatitis caused by epoxy diacrylate in ultraviolet-light-cured paint, and bisphenol A in dental composite resin. *Contact Dermatitis*, 33, 94–99.

Kallus, T. & Mjor, I.A. (1991) Incidence of adverse effects of dental materials. *European Journal of Oral Sciences*, 99, 236–240.

Kasten, F.H., Pineda, L.F.R., Schneider, P.E., Rawls, H.R. & Foster, T.A. (1989) Biocompatibility testing of an experimental fluoride releasing resin using human gingival epithelial cells in vitro. *In Vitro Cellular & Developmental Biology*, 25, 57–62.

Kawahara, H. (1955) Studies on the effects of dental metals upon the mesenchymal cells in tissue culture. *Journal of Osaka Odontology Society*, 18, 348.

Khalil, W.A. & Eid, N.F. (2013) Biocompatibility of BioAggregate and mineral trioxide aggregate on the liver and kidney. *International Endodontic Journal*, 46(8), 730–737.

Kitasako, Y., Inokoshi, S. & Tagami, J. (2000) Effects of direct resin pulp capping techniques on short-term response of mechanically exposed pulps. *Journal of Esthetic and Restorative Dentistry*, 12, 172.

Kitasako, Y., Shibata, S., Pereira, P. & Tagami, J. (2000) Short-term dentin bridging of mechanically-exposed pulps capped with adhesive resin systems. *Operative Dentistry*, 25, 155–162.

Klausner, M., Handa, Y. & Aizawa, S. (2021) In vitro three-dimensional organotypic culture models of the oral mucosa. *In Vitro Cellular & Developmental Biology – Animal*, 57, 148–159.

Klötzer, W. & Langeland, K. (1973) Testing of materials and methods for crown and bridge prosthesis on animals. *Schweizerische Monatsschrift Fur Zahnheilkunde = Revue Mensuelle Suisse d'odonto-stomatologie*, 83, 163–244.

Kolokouris, I., Economides, N., Beltes, P. & Vlemmas, I. (1998) In vivo comparison of the biocompatibility of two root canal sealers implanted into the subcutaneous connective tissue of rats. *Journal of Endodontics*, 24, 82–85.

Korting, H.C. (1997) *Diagnostische Verfahren in der Dermatologie: mit Tabellen*. Berlin: Blackwell.

Kostoryz, E., Eick, J., Glaros, A., Judy, B.M., Welshons, W.V., Burmaster, S. et al. (2003) Biocompatibility of hydroxylated metabolites of BISGMA and BFDGE. *Journal of Dental Research*, 82, 367–371.

Kostoryz, E., Tong, P., Chappelow, C., Eick, J., Glaros, A. & Yourtee, D. (1999) In vitro cytotoxicity of solid epoxy-based dental resins and their components. *Dental Materials*, 15, 363–373.

Laurent, P., Camps, J. & About, I. (2012) Biodentine(TM) induces TGF-β1 release from human pulp cells and early dental pulp mineralization. *International Endodontic Journal*, 45, 439–448.

Lefebvre, C.A., Knoernschild, K.L. & Schuster, G.S. (1994) Cytotoxicity of eluates from light-polymerized denture base resins. *The Journal of Prosthetic Dentistry*, 72, 644–650.

Leirskar, J. & Helgeland, K. (1972) A methodologic study of the effect of dental materials on growth and adhesion of animal cells in vitro. *European Journal of Oral Sciences*, 80, 120–133.

Leyhausen, G. (1995) The genotoxic potential of composite components. *Dtsch Zahnarztl Z*, 50, 134–136.

Magnusson, B. (1969) The identification of contact allergens by animal assay. The guinea pig maximization test. *Journal of Investigative Dermatology*, 52, 268–276.

Maher, W.P., Johnson, R.L., Hess, J. & Steiman, H.R. (1992) Biocompatibility of retrograde filling materials in the ferret canine Amalgam and IRM. *Oral Surgery, Oral Medicine, Oral Pathology, and Oral Radiology*, 73, 738–745.

Maizumi, H. (1962) Die Wirkung verschiedener Vitalerhaltungs-und Wurzelkanalfullmittel auf Gewebekulturen. *Dtsch Zahnarztl Z*, 17, 1628–1635.

Maron, D.M. & Ames, B.N. (1983) Revised methods for the Salmonella mutagenicity test. *Mutation Research/Environmental Mutagenesis and Related Subjects*, 113, 173–215.

Mazzoleni, G., Di Lorenzo, D. & Steimberg, N. (2009) Modelling tissues in 3D: the next future of pharmaco-toxicology and food research? *Genes & Nutrition*, 4, 13.

McCann, J., Choi, E., Yamasaki, E. & Ames, B.N. (1975) Detection of carcinogens as mutagens in the Salmonella/microsome test: assay of 300 chemicals. *Proceedings of the National Academy of Sciences of the United States of America*, 72, 5135–5139.

Mittal, M., Chandra, S. & Chandra, S. (1995) Comparative tissue toxicity evaluation of four endodontic sealers. *Journal of Endodontics*, 21, 622–624.

Mjör, I. (1980) A comparison of in vivo and in vitro methods for toxicity testing of dental materials 1. *International Endodontic Journal*, 13, 139–142.

Mjör, I.A. (1992) Problems and benefits associated with restorative materials: side-effects and long-term cost. *Advances in Dental Research*, 6, 7–16.

Mjör, I., Hensten-Pettersen, A. & Skogedal, O. (1977) Biologic evaluation of filling materials. A comparison of results using cell culture techniques, implantation tests and pulp studies. *International Dental Journal*, 27(2), 124–129.

Mjör, I.A., Sveen, O.B. & Heyeraas, K.J. (2001) Pulp-dentin biology in restorative dentistry. Part 1: normal structure and physiology. *Quintessence International*, 32, 427–446.

Mjör, I.A. & Tronstad, L. (1972) Experimentally induced pulpitis. *Oral Surgery, Oral Medicine, Oral Pathology, and Oral Radiology*, 34, 102–108.

Moharamzadeh, K., Brook, I.M. & Van Noort, R. (2009) Biocompatibility of resin-based dental materials. *Materials*, 2(2), 514–548.

Moharamzadeh, K., Van Noort, R., Brook, I. & Scutt, A. (2007) HPLC analysis of components released from dental composites with different resin compositions using different extraction media. *Journal of Materials Science: Materials in Medicine*, 18, 133–137.

Moher, D., Schulz, K.F. & Altman, D.G. (2001) The CONSORT statement: revised recommendations for improving the quality of reports of parallel-group randomised trials. *Lancet*, 357, 1191–1194.

Montgomery, S. & Ferguson, C. (1986) Endodontics. Diagnostic, treatment planning, and prognostic considerations. *Dental Clinics of North America*, 30, 533–548.

Murray, P.E., About, I., Franquin, J.-C., Remusat, M. & Smith, A.J. (2001) Restorative pulpal and repair responses. *The Journal of the American Dental Association*, 132, 482–491.

Murray, P.E., Lumley, P.J., Ross, H.F. & Smith, A.J. (2000) Tooth slice organ culture for cytotoxicity assessment of dental materials. *Biomaterials*, 21, 1711–1721.

Nagendrababu, V., Kishen, A., Murray, P.E., Nekoofar, M.H., Figueiredo, J.A.P., Priya, E. et al. (2019) Preferred reporting items for animal studies in Endodontology: a development protocol. *International Endodontic Journal*, 52, 1290–1296.

Nair, P.N., Duncan, H.F., Pitt Ford, T.R. & Luder, H.U. (2008) Histological, ultrastructural and quantitative investigations on the response of healthy human pulps to experimental capping with mineral trioxide aggregate: a randomized controlled trial. *International Endodontic Journal*, 41(2), 128–150.

Nakashima, M., Iohara, K., Bottino, M.C., Fouad, A.F., Nör, J.E. & Huang, G.-T.-J. (2019) Animal models for stem cell-based pulp regeneration: foundation for human clinical applications. *Tissue Engineering Part B: Reviews*, 25, 100–113.

Noda, M., Wataha, J.C., Lockwood, P.E., Volkmann, K.R., Kaga, M. & Sano, H. (2003) Sublethal, 2-week exposures of dental material components alter TNF-α secretion of THP-1 monocytes. *Dental Materials*, 19, 101–105.

Oda, Y., Nakamura, S.-I., Oki, I., Kato, T. & Shinagawa, H. (1985) Evaluation of the new system (umu-test) for the detection of environmental mutagens and carcinogens. *Mutation Research/Environmental Mutagenesis and Related Subjects*, 147, 219–229.

Oguntebi, B. (1994) Dentine tubule infection and endodontic therapy implications. *International Endodontic Journal*, 27, 218–222.

Okamura, T., Morimoto, M., Yamane, G. & Takahashi, S. (2003) Langerhans' cells in the murine oral mucosa in the inductive phase of delayed type hypersensitivity with 1-chloro-2, 4-dinitrobenzene. *Clinical & Experimental Immunology*, 134, 188–194.

Olsson, B., Sliwkowski, A. & Langeland, K. (1981) Intraosseous implantation for biological evaluation of endodontic materials. *Journal of Endodontics*, 7, 253–265.

Ørstavik, D. & Hongslo, J. (1985) Mutagenicity of endodontic sealers. *Biomaterials*, 6, 129–132.

Pan, Y., Lin, Y., Jiang, L., Lin, H., Xu, C., Lin, D. et al. (2020) Removal of dental alloys and titanium attenuates trace metals and biological effects on liver and kidney. *Chemosphere*, 243, 125205.

Pedano, M.S., Li, X., Jeanneau, C., Ghosh, M., Yoshihara, K., Van Landuyt, K. et al. (2019) Survival of human dental pulp cells after 4-week culture in human tooth model. *Journal of Dentistry*, 86, 33–40.

Pelka, M., Danzl, C., Distler, W. & Petschelt, A. (2000) A new screening test for toxicity testing of dental materials. *Journal of Dentistry*, 28, 341–345.

Peters, O.A., Paranjpe, A. & Gaudin, A. (2021) Dentine-pulp complex regeneration. In: Hosseinpour, S., Walsh, L.J. & Moharamzadeh, K. (Eds.) *Regenerative approaches in dentistry: an evidence-based perspective*. Cham: Springer International Publishing, pp. 35–62.

Polyzois, G. (1994) In vitro evaluation of dental materials. *Clinical Materials*, 16, 21–60.

Primus, C.M., Tay, F.R. & Niu, L.-N. (2019) Bioactive tri/dicalcium silicate cements for treatment of pulpal and periapical tissues. *Acta Biomaterialia*, 96, 35–54.

Rae, T. (1975) A study on the effects of particulate metals of orthopaedic interest on murine macrophages in vitro. *The Journal of Bone and Joint Surgery*, 57, 444–450.

Rai, Y., Pathak, R., Kumari, N., Sah, D.K., Pandey, S., Kalra, N. et al. (2018) Mitochondrial biogenesis and metabolic hyperactivation limits the application of MTT assay in the estimation of radiation induced growth inhibition. *Scientific Reports*, 8, 1531.

Ratner, B.D. (2015) The biocompatibility of implant materials. *Host response to biomaterials*. Philadelphia, PA, USA: Elsevier, pp. 37–51.

Robinson, N.B., Krieger, K., Khan, F.M., Huffman, W., Chang, M., Naik, A. et al. (2019) The current state of animal models in research: a review. *International Journal of Surgery*, 72, 9–13.

Rodrigues, N.S., França, C.M., Tahayeri, A., Ren, Z., Saboia, V.P.A. et al. (2021) Biomaterial and biofilm interactions with the pulp-dentin complex-on-a-chip. *Journal of Dental Research*, 100, 1136–1143.

Rowan, A.N. (1997) The benefits and ethics of animal research. *Scientific American*, 276, 79.

Sadoh, D., Sharief, M. & Howard, R. (1999) Occupational exposure to methyl methacrylate monomer induces generalised neuropathy in a dental technician. *British Dental Journal*, 186, 380–381.

Safavi, K.E., Pascon, E.A. & Langeland, K. (1983) Evaluation of tissue reaction to endodontic materials. *Journal of Endodontics*, 9, 421–429.

Saw, T.Y., Cao, T., Yap, A.U.J. & Ng, M.M.L. (2005) Tooth slice organ culture and established cell line culture models for cytotoxicity assessment of dental materials. *Toxicology in Vitro*, 19, 145–154.

Schedle, A., Franz, A., Rausch-Fan, X., Spittler, A., Lucas, T., Samorapoompichit, P. et al. (1998) Cytotoxic effects of dental composites, adhesive substances, compomers and cements. *Dental Materials*, 14, 429–440.

Schmalz, G. (1981) *Die Gewebeverträglichkeit zahnärztlicher Materialien-Möglichkeiten einer standardisierten Prüfung in d. Zellkultur*. Germany: Thieme.

Schmalz, G. (1994) Use of cell cultures for toxicity testing of dental materials—advantages and limitations. *Journal of Dentistry*, 22, S6–S11.

Schmalz, G. (1998) Concepts in biocompatibility testing of dental restorative materials. *Clinical Oral Investigations*, 1, 154–162.

Schmalz, G. (2009) Determination of biocompatibility. *Biocompatibility of dental materials* pp. 13–43. Germany: Springer.

Schmalz, G. & Arenholt-Bindslev, D. (2009) *Biocompatibility of dental materials*. Springer.

Schmalz, G. & Fan, P. (2009) Regulations and standards. *Biocompatibility of dental materials*. Springer, pp. 45–58.

Schmalz, G., Hiller, K.-A. & Dörter-Aslan, F. (1994) New developments in the filter test system for cytotoxicity testing. *Journal of Materials Science: Materials in Medicine*, 5, 43–51.

Schmalz, G. & Schmalz, C. (1981) Toxicity tests on dental filling materials. *International Dental Journal*, 31, 185–192.

Schmalz, G., Schuster, U., Nuetzel, K. & Schweikl, H. (1999) An in vitro pulp chamber with three-dimensional cell cultures. *Journal of Endodontics*, 25, 24–29.

Schmalz, G., Schweikl, H., Esch, J. & Hiller, K.-A. (1996) Evaluation of a dentin barrier test by cytotoxicity testing of various dental cements. *Journal of Endodontics*, 22, 112–115.

Schmalz, G., Schweikl, H. & Hiller, K.A. (2000) Release of prostaglandin E2, IL-6 and IL-8 from human oral epithelial culture models after exposure to compounds of dental materials. *European Journal of Oral Sciences*, 108, 442–448.

Schmalz, G., Thonemann, B., Riedel, M. & Elderton, R.J. (1994) Biological and clinical investigations of a glass ionomer base material. *Dental Materials*, 10, 304–313.

Schuster, U., Schmalz, G., Thonemann, B., Mendel, N. & Metzl, C. (2001) Cytotoxicity testing with three-dimensional cultures of transfected pulp-derived cells. *Journal of Endodontics*, 27, 259–265.

Schweikl, H., Schmalz, G., Stimmelmayr, H. & Bey, B. (1995) Mutagenicity of AH26 in an in vitro mammalian cell mutation assay. *Journal of Endodontics*, 21, 407–410.

Schweikl, H., Schmalz, G. & Federlin, M. (1998) Mutagenicity of the root canal sealer AHPlus in the Ames test. *Clinical Oral Investigations*, 2, 125–129.

Schweikl, H. & Schmalz, G. (1999) Triethylene glycol dimethacrylate induces large deletions in the hprt gene of V79 cells. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 438, 71–78.

Scott, A., Egner, W., Gawkrodger, D., Hatton, P.V., Sherriff, M., van Noort, R. et al. (2004) The national survey of adverse reactions to dental materials in the UK: a preliminary study by the UK Adverse Reactions Reporting Project. *British Dental Journal*, 196, 471–477.

da Silva, E.J.N.L., Zaia, A.A. & Peters, O.A. (2017) Cytocompatibility of calcium silicate-based sealers in a three-dimensional cell culture model. *Clinical Oral Investigations*, 21, 1531–1536.

Smith, A., Tobias, R., Cassidy, N., Plant, C.G., Browne, R.M., Begue-Kirn, C. et al. (1994) Odontoblast stimulation in ferrets by dentine matrix components. *Archives of Oral Biology*, 39, 13–22.

Sonat, B., Dalat, D. & Günhan, O. (1990) Periapical tissue reaction to root fillings with Sealapex. *International Endodontic Journal*, 23, 46–52.

de Souza, C.C., Vaerten, M., Edwards, C. & Hanks, C. (1999) Cytotoxic effects of current dental adhesive systems on immortalized odontoblast cell line MDPC-23. *Dental Materials*, 15, 434–441.

Spangberg, L. (1973) Kinetic and quantitative evaluation of material cytotoxicity in vitro. *Oral Surgery, Oral Medicine, Oral Pathology, and Oral Radiology*, 35, 389–401.

Spangberg, L., Engström, B. & Langeland, K. (1973) Biologic effects of dental materials: 3. Toxicity and antimicrobial effect of endodontic antiseptics in vitro. *Oral Surgery, Oral Medicine, Oral Pathology, and Oral Radiology*, 36, 856–871.

Stanley, H.R. (1968) Design for a human pulp study: Part I. *Oral Surgery, Oral Medicine, Oral Pathology, and Oral Radiology*, 25, 633–647.

Stanley, H. (1998) Criteria for standardizing and increasing credibility of direct pulp capping studies. *American Journal of Dentistry*, 11, S17–34.

Stanley, H.R. & Pameijer, C.H. (1985) Pulp capping with a new visible-light-curing calcium hydroxide composition (Prisma VLC Dycal). *Operative Dentistry*, 10, 156–163.

Stea, S., Savarino, L., Ciapetti, G., Cenni, E., Stea, S., Trotta, F. et al. (1994) Mutagenic potential of root canal sealers: evaluation through Ames testing. *Journal of Biomedical Materials Research*, 28, 319–328.

Steinbrunner, R., Setcos, J. & Kafrawy, A. (1991) Connective tissue reactions to glass ionomer cements and resin composites. *American Journal of Dentistry*, 4, 281–284.

Svendsen, O., Garthoff, B., Spielmann, H., Hensten-Pettersen, A., Jensen, J.C., Kuijpers, M.R. et al. (1996) Alternatives to the animal testing of medical devices: the report and recommendations of ECVAM workshop 17. *Alternatives to Laboratory Animals*, 24, 659–669.

Syed, M., Chopra, R. & Sachdev, V. (2015) Allergic reactions to dental materials-a systematic review. *Journal of Clinical and Diagnostic Research*, 9(10), ZE04–ZE09.

Szep, S., Kunkel, A., Ronge, K. & Heidemann, D. (2002) Cytotoxicity of modern dentin adhesives—in vitro testing on gingival fibroblasts. *Journal of Biomedical Materials Research*, 63, 53–60.

Tabatabaei, F., Moharamzadeh, K. & Tayebi, L. (2020) Three-dimensional in vitro oral mucosa models of fungal and bacterial infections. *Tissue Engineering Part B: Reviews*, 26, 443–460.

Tagger, M. & Tagger, E. (1989) Periapical reactions to calcium hydroxide-containing sealers and AH 26® in monkeys. *Dental Traumatology*, 5, 139–146.

Tassery, H., Remusat, M., Koubi, G. & Pertot, W.-J. (1997) Comparison of the intraosseous biocompatibility of Vitremer

and super EBA by implantation into the mandible of rabbits. *Oral Surgery, Oral Medicine, Oral Pathology, and Oral Radiology*, 83, 602–608.

Téclès, O., Laurent, P., Aubut, V. & About, I. (2008) Human tooth culture: a study model for reparative dentinogenesis and direct pulp capping materials biocompatibility. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, 85, 180–187.

Thomas, G.P., Adrian, J.C., Banks, K.E., Robinson, J.A. & Peagler, F.D. (1985) Biocompatibility evaluation of resins in hamsters. *The Journal of Prosthetic Dentistry*, 53, 428–430.

Thonemann, B., Schmalz, G., Hiller, K.-A. & Schweikl, H. (2002) Responses of L929 mouse fibroblasts, primary and immortalized bovine dental papilla-derived cell lines to dental resin components. *Dental Materials*, 18, 318–323.

Torabinejad, M., Corr, R., Buhrley, M., Wright, K. & Shabahang, S. (2011) An animal model to study regenerative endodontics. *Journal of Endodontics*, 37, 197–202.

Torabinejad, M., Ford, T.R.P., McKendry, D.J., Abedi, H.R., Miller, D.A. & Kariyawasam, S.P. (1997) Histologic assessment of mineral trioxide aggregate as a root-end filling in monkeys. *Journal of Endodontics*, 23, 225–228.

Torabinejad, M., Hong, C.-U., Lee, S.-J., Monsef, M. & Ford, T.R.P. (1995) Investigation of mineral trioxide aggregate for root-end filling in dogs. *Journal of Endodontics*, 21, 603–608.

Torabinejad, M., Ung, B. & Kettering, J.D. (1990) In vitro bacterial penetration of coronally unsealed endodontically treated teeth. *Journal of Endodontics*, 16, 566–569.

Torneck, C.D. (1961) Reaction of hamster tissue to drugs used in sterilization of the root canal. *Oral Surgery, Oral Medicine, Oral Pathology, and Oral Radiology*, 14, 730–747.

Trowbridge, H.O. (1986) Review of dental pain—histology and physiology. *Journal of Endodontics*, 12, 445–452.

Tyas, M.J. (1977) A method for the in vitro toxicity testing of dental restorative materials. *Journal of Dental Research*, 56, 1285–1290.

Tziafas, D., Veis, A. & Alvanou, A. (1996) Inability of calcium hydroxide to induce reparative dentinogenesis at non-peripheral sites of dog dental pulp. *European Journal of Oral Sciences*, 104, 623–626.

Wataha, J.C. (2001) Principles of biocompatibility for dental practitioners. *The Journal of Prosthetic Dentistry*, 86, 203–209.

Wennberg, A., Hasselgren, G. & Tronstad, L. (1979) A method for toxicity screening of biomaterials using cells cultured on millipore filters. *Journal of Biomedical Materials Research*, 13, 109–120.

Wennberg, A., Mjör, I. & Hensten-Pettersen, A. (1983) Biological evaluation of dental restorative materials—a comparison of different test methods. *Journal of Biomedical Materials Research*, 17, 23–36.

Wirthlin, M.R., Armitage, G.C., Rao, S., Fritzinger, B., Phillips, S. & Heller, J. (1997) A mucosal irritancy test device for intraoral use in dogs. *Journal of Periodontology*, 68, 746–749.

Witte, I., Jacobi, H. & Juhl-Strauss, U. (1996) Suitability of different cytotoxicity assays for screening combination effects of environmental chemicals in human fibroblasts. *Toxicology Letters*, 87, 39–45.

Zhan, X., Wang, F., Bi, Y. & Ji, B. (2016) Animal models of gastrointestinal and liver diseases. Animal models of acute and chronic pancreatitis. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 311, G343–G355.

Zmener, O. (2004) Tissue response to a new methacrylate-based root canal sealer: preliminary observations in the subcutaneous connective tissue of rats. *Journal of Endodontics*, 30, 348–351.

How to cite this article: Hosseinpour, S., Gaudin, A. & Peters, O.A. (2022) A critical analysis of research methods and experimental models to study biocompatibility of endodontic materials. *International Endodontic Journal*, 55(Suppl. 2), 346–369. Available from: https://doi.org/10.1111/iej.13701