

How is the biocompatibility of dental biomaterials evaluated?

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Received: 10-4-2007

Accepted: 16-4-2007

Murray PE, García-Godoy C, García-Godoy F. How is the biocompatibility of dental biomaterials evaluated?. Med Oral Patol Oral Cir Bucal 2007;12:E258-66.

© Medicina Oral S. L. C.I.F. B 96689336 - ISSN 1698-6946

Indexed in:

-Index Medicus / MEDLINE / PubMed
-EMBASE, Excerpta Medica
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-Indice Médico Español
-IBECs

ABSTRACT

All biomaterials used in dentistry must be evaluated for biocompatibility using screening assays to protect patient health and safety. The purpose of this review is to explain the international biocompatibility guidelines, and to explain the structure of a test program. The test program requires the structured assessment of materials into four phases; general toxicity, local tissue irritation, pre-clinical, and clinical evaluation. Different types of screening assays are available, and it is important to understand the advantages and limitations of the various types of assays that are available, so that they can be selected for appropriateness and interpreted accurately. New scientific advances in terms of the chemical properties of dental materials, tissue engineering, stem cell, genetic transfer, biomaterial, and growth factor therapies are under development. These new therapies create improved opportunities to restore and regenerate oral tissues, but they can also present new hazards to patients. Prior to their clinical use, these new technologies must be proven to be safe, and not hazardous to human health. A structured biocompatibility assessment and advice on the selection of assays are outlined to evaluate these new therapies.

Key words: Cytotoxicity, biocompatibility, dental materials, growth factors, gene therapy, tissue engineering, stem cell therapy.

INTRODUCTION

The need for biocompatible materials for use in restorative dentistry and endodontics has generated a requirement for cytotoxicity assays to screen compounds and characterize the potentially harmful effects of a material to oral tissues prior to clinical use. Cytotoxicity screening assays provide a measure of cell death caused by materials or their extracts. There are a vast number of cytotoxicity screening methods available for measuring the biocompatibility of a dental restorative material. The application of different methods of cytotoxicity screening has been shown to produce a spectrum of biocompatibility assessments for the same material (1-4). Evaluating the biocompatibility of a material using an *in vitro* cell culture assay, and from this, attempting to predict *in vivo* oral tissue responses is controversial (5). It has been found that the biocompatibility assessments produced by cell culture assays have not necessarily been in agreement

with animal *in vivo* biocompatibility implantation test (6-9). These interpretational difficulties have provided the impetus for efforts to standardize the use of cytotoxicity assays, and regulate the context of their application at national and supranational levels.

BIOCOMPATIBILITY

The traditional concept of biocompatibility is regarded as a lack of significant adverse reaction between the oral tissues (10). It is now recognized from that there are few materials, if any, which do not create a significant interaction with the host tissues (11). Such reactions may aid the oral healing response following restorative treatment. An updated definition of biocompatibility might be the ability of a restorative material to induce an appropriate and advantageous host response during its intended clinical usage.

CYTOTOXICITY

Dental material biocompatibility has long been described throughout the dental literature, however, information about the factors that determine biocompatibility responses is only just emerging. An obvious determinant of biocompatibility is the effect a material may have on cell survival. The term 'cytotoxicity' is used to describe the cascade of molecular events that interfere with macromolecular synthesis, causing unequivocal cellular and functional and structural damage (12). Cytotoxicity is a difficult process to characterize as there is almost an infinite number of ways to trigger cellular disruption. An understanding of this complexity is compounded by the capabilities of the cellular proteins to aid or extend cell survival (13), and the genetic disposition of cells to activate particular modes of cell death (14). Cytotoxic-mediated reactions can be employed therapeutically, such as in cancer chemotherapy (15). However, as part of dental treatment, it is advantageous to maintain maximal tissue vitality and cytotoxic reactions must be prevented, hence the requirement to screen all dental compounds before they are used clinically.

BIOCOMPATIBILITY TESTING STANDARDS

The International Organization for Standardization (ISO) is a worldwide federation of national standards bodies (ISO member bodies). At present, national standards exist for biocompatibility testing methods (16-18). International standards cover specifically dental materials (ISO 7405) (19) and medical devices (ISO 10993) (20), which also include dental materials. A dental material is defined as a substance or combination of substances specially prepared and/or presented for use by authorized persons in the practice of dentistry and/or its associated procedures (19).

1. International standard ISO 7405: International standard ISO 7405 (19) is entitled the Preclinical evaluation of biocompatibility of medical devices used in Dentistry - Test methods for Dental Materials. This ISO document was prepared in conjunction with the World Dental Federation. It concerns the preclinical testing of materials used in Dentistry, and supplements ISO 10993 (20).

2. International standard ISO 10993: International standard ISO 10993 (20) entitled the Biological evaluation of medical devices is a combination and harmonization of International and National Standards and guidelines. The stated primary goal of ISO 10993 is the protection of humans. This document has been continually updated, and is the overall guidance document for the selection of tests, to be used for the evaluation of biological responses relevant to medical or dental material and device safety.

Guidelines ISO 7405 (19) and ISO 10993 (20) and have recommended standard practices for the biological evaluation of dental materials. In summary these include; (i) It is incumbent upon the dental material manufacturer to select the appropriate tests, based on the intended use of the material, and known and assumed toxicity profile of the material or its components. (ii) A manufacturer may select one of three cytotoxicity tests in preference to another because of cost, experience or other reasons. (iii) Overall, there are four levels of testing. New

materials should be evaluated using initial cytotoxicity and secondary tissue screening tests prior to extensive animal experimentation testing and clinical trials. (iv) The test result should always be evaluated and interpreted with consideration for the manufacturers stated use for the material.

TEST PROGRAM FOR THE BIOLOGICAL TESTING OF DENTAL MATERIALS

The selection and evaluation of any material or device intended for use in humans requires a structured assessment. The test program for the biological testing of dental materials is normally divided into four stages (Table 1). The initial tests (Phases I and II) are of a short duration, simple and cost effective. Only after completing these tests satisfactorily does a material progress through the testing hierarchy to become evaluated in preclinical animal usage studies (Phase III) prior to clinical testing with a limited number of patients (Phase IV).

CYTOTOXICITY SCREENING METHODS

General guidance for *in vitro* cytotoxicity testing is presented in ISO 10993-5. Detailed test protocols for the agar diffusion and filter diffusion methods appropriate to dental materials, are included in ISO 7405. For *in vitro* cytotoxicity screening, the recommended testing methods include; (i) Direct cell culture and culture extract testing, or barrier screening assays. (ii) Agar diffusion testing. (iii) Filter diffusion testing, and (iv) Dentin barrier testing. These methods are described in the following paragraphs:

1a. Direct cell culture and culture extract testing: More than twenty different cell culture techniques have been applied to assess the cytotoxicity of dental materials (21-22). The strategy has been to test the toxicity of individual compound components of a dental material when placed directly onto cells in a mono-layer culture, over a short duration (normally >24 hours). Dose-response curves can then be determined which estimate the cytotoxic potential of compounds within a material. This information is valuable to manufacturers, who are then able to formulate dental materials potentially containing the least quantity of cytotoxic compounds. The practical complication of cell culture cytotoxicity screening is that cell and colony counts are time consuming, tedious, and sensitive to minor variations in morphology. Cell counts enumerate morphologically intact cells but do not distinguish between living and dead cells. Colony counts often require subjective judgments about what constitutes a colony, and are subject to a wide variety of troublesome artifacts that can greatly complicate their interpretation.

1b. Barrier testing methods: Of the four recommended cytotoxicity tests (ISO 7405) (19), three are examples of barrier testing methods. One of the criticisms of cell culture tests with direct material-cell contact is that the testing situation is often not clinically relevant, as most materials used to restore lost tooth substance are not in direct contact with the cells. There is normally a barrier of dentin between the material and the pulp tissue (7). Barrier testing methods are used to mimic the dentin barrier, to test for the ability of a material to dissolve dentin and diffuse through den-

tinal tubules, thereby permitting an estimate of material toxicity related to its diffusional capacity. The ability of this simple strategy to determine the cytotoxic hazard of a material *in vitro* and generalize this to *in vivo* systems has been somewhat successful, mainly because the results are relatively easy to interpret. This explains the adoption of barrier testing, as the method recommended for the bulk of cytotoxicity screening assays for dental materials (ISO 10993-5) (20).

2. *Agar diffusion test*: Probably the longest established cytotoxicity barrier testing method is the Agar Diffusion Test or the Tissue Culture Overlay Test (23,24) where the test material is simply incubated on a layer of agar overlying a monolayer cell culture. This method is used for testing the non-specific cytotoxicity of the leachable components of test substances after diffusion through agar or agarose (ISO 7405) (19) using permanent cell lines. These cells are stained with neutral red vital stain dye, overlaid with an agar layer on which the test material is incubated for 24 hours (25). The presence of leachable toxic substance(s) is manifested by the loss of dye within the cells as they lyse (a type of membrane integrity assay) provided the concentration and cytotoxicity of the diffusing substance(s) are high enough. Although simple and inexpensive to use as a cytotoxicity screening method, this technique has the disadvantage that materials or compounds have to 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.

3. *Filter diffusion testing methods*: The Millipore (cellulose acetate) filter method modifies the oral contact situation in that primary cells are grown on one side of the filter, and the test material is placed in contact with the opposite surface of the filter. Thus, any leachable substance must diffuse through the 0.45µm filter pores to exert any cytotoxic effects on the cells (26). 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) (19). Assay endpoints which 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 among the enzymes tested (7), indicating that the results from all of these endpoints are comparable.

4. *Dentin barrier testing*: Although recommended as cytotoxic screening methods (ISO 7405) (19) the agar overlay test and the filter diffusion test, may not necessarily be the best methods, for mimicking barriers of the oral environment. A recent adjunct to the cytotoxicity tests has been the development of dentin barrier testing, or the model cavity method as it is sometimes known (27). The original idea by Outhwaite et al., (28) has been refined over the years to define the factors which affect diffusion through dentinal tubules. These include the size and concentration of molecules, density of dentinal tubules, length of dentinal

tubules, diameter of tubules, the effect of temperature, and measurement of cytotoxic effects on pulpal cells (29-31). Clearly, adopting the use of a dentin barrier simulates the *in vivo* oral environment more closely, thereby helping to identify specific dental compound components which may be responsible for pulpal effects through dentin, an option not available with other testing methods. The dentin barrier test may also help identify compounds that repress or intensify the cytotoxic effect of a substance, by reducing or increasing dentin permeability (32-35). With the importance of the principle of generalizing *in vitro* cytotoxicity findings to the human *in vivo* clinical situation, this technique is recommended for use in preference to the others (ISO 7405) (19).

ENDPOINTS OF THE CYTOTOXICITY ASSAY

The recommended testing methods (ISO 10993; ISO 7405) use cell counting, dye-binding, metabolic impairment or membrane integrity as end-points of the cytotoxicity test or assay. The direct use of cell and colony counting as an assay endpoint is probably the least reliable method. Ideally, endpoints should conform to strict criteria for classifying results, so that the subjective element when determining a measure of cytotoxicity for a test material is minimized. This makes assays a very reproducible and therefore powerful form of cytotoxicity testing, as it permits a stable basis for comparing results between laboratories. A description of the assay endpoints are in the following paragraphs:

1. *Metabolic impairment assay*: Metabolic impairment assays measure the decay of enzyme activity or metabolite concentration following toxic damage. Cytotoxicity testing by this means has included alterations in lysosomal acid phosphatase (24), cytoplasmic lactate dehydrogenase (36,37), succinate dehydrogenase (38), enzyme activity (39), and the incorporation of labelled precursors (40). These methods are generally more complex and artifact-prone than membrane integrity assays. Their validity requires that very precise conditions be met; deviation from these conditions can lead to extremely serious errors that invalidate the assay. Metabolic impairment assays are nevertheless popular because they distinguish between normal and reduced levels of cellular metabolism, which is a surrogate index of metabolic viability though not necessarily an accurate predictor of cellular proliferative capability. The end point assumption is that the proliferative capability, and metabolic activity of viable cells are preserved.

2. *Membrane integrity assay*: Membrane integrity assays measure the ability of cells to exclude impermeable extracellular molecules. They can be either colorimetric or fluorescent, and require the same instrumentation as dye-binding assays. Assays of this type tend to be less artifact prone than metabolic impairment assays, but have the same ability to estimate 'viability', which in this case is the ability to distinguish between the normal and impaired exclusion of extracellular molecules. Membrane integrity assays are complicated by the fact that living cells slowly accumulate probe molecules, therefore, their protocols must be carefu-

lly optimized. Trypan blue staining (41), red vital staining (25), and neutral red staining combined with amido black staining (42), have all been used to study the toxicity of some endodontic materials for the routine determination of cell viability. Stanford (25) recommends staining cells with neutral red vital stain dye, overlaid with an agar layer on which the test material is incubated for 24 hours. After incubation, the presence of leach-able toxic substance(s) is manifested by the loss of dye within the cells as the membranes lyse. Alternatively, some dyes or isotopes are used which are only taken up by viable cells, diacetyl fluorescein (43), or the Radiochromium (^{51}Cr) Assay (44,45). The use of this assay, allows direct cell-material contact, in dissolved materials as well as semisolid, setting or set materials (25). In general this assay is restricted to measuring membrane permeability, one of the final events of cell death; sub-lethal cellular changes are not measured. This technique has been shown to give similar biocompatibility results to the agar overlay method of cytotoxicity screening (46,47), with the agar overlay method being less expensive and without the complications of radioisotope handling and disposal. Despite improvements in the utilization of the screening assays, surprisingly little attention has been paid to the fact that the toxicity parameters selected for these screening tests should be appropriate to the chemical nature of the components within the test materials. For example, hydrophilic chemicals are likely to change intracellular enzyme activities at lower concentrations rather than influencing membrane permeability. Therefore, a metabolic impairment assay would seem to be the most appropriate test method, rather than a membrane integrity assay. This situation would be reversed for a test material containing lipophilic chemicals. All this evidence suggests that *in vitro* biocompatibility assays can only provide accurate information about test materials if they are used appropriately.

ANIMAL USAGE ASSAY

Biological testing relies heavily on animal experimentation. Before a dental material can be used clinically, it must always be tested to the fullest extent in several species of laboratory animals to establish its systemic and cytotoxic properties (48). The use of animals helps to predict the possible toxic hazards that may be encountered in man. However, there are some notorious exceptions such as thalidomide when this did not happen (49). Sometimes dogs (50) and ferrets (51) among other large animals, are used to evaluate the biological responses of teeth to experimental restorative treatments. The ISO 7405 guidelines recommend preclinical testing in adult non-human primates (19). Every year it is estimated that 57,000 non-human primates are used worldwide for medical experimentation, as well as many types of animals including millions of rodents (48). The precise numbers of animals used for dental material testing are not known, but they must only be a small fraction of the total numbers. The dental materials to be evaluated should be placed using routine restoration techniques, to closely mimic the intended clinical usage of the test materials. Traditional restorative

materials such as zinc oxide eugenol and more commonly calcium hydroxide are often used to restore adjacent teeth at the same time. These traditional restorative materials have a long-established record of clinical success, and are used for comparison with the test materials. The teeth restored with calcium hydroxide are also used as 'control teeth,' to ensure that the pulpal reactions to restorative treatment are representative with previous investigations. The ISO 7405 guidelines recommend that 7 \pm 2 days (short-term) and 70 \pm 5 days (long-term) have elapsed before the restored teeth are carefully extracted after the administration of general anesthetic and venous infiltration perfusion. However, many investigations prefer to show a timed sequence of reactions to test restorative materials and sometimes, 3, 7, 14, 30 and 60 day time-periods are used (52,53). Short-time periods are often used because non-human primate usage testing are expensive investigations to complete. However, the use of short-term studies may not show the full healing and regenerative response of teeth to test materials. Some other investigators prefer longer post-treatment time-periods of between 30 and 730 days (54,55). Increasing the length of the post-treatment extraction period increases the probability of detecting healing problems and complications associated with test materials. Some direct pulp capped teeth appear to be healing early on but can become non-vital several weeks later (56). Another prime example is the leakage of bacteria through restorations. Bacterial leakage is more commonly detected in the longer-term studies (54,55), but not the shorter-term studies (52,53). Bacteria are detected using Brown and Brenn (57) or other histological stains for gram positive microorganisms (58). The presence of bacteria is often a more serious cause of pulpitis and necrosis, than the test-materials in isolation (59,60). Whatever the advantages and limitations of selecting different testing time-periods, adhering to the 70 \pm 5 days time-period with some teeth is important in order to accomplish some commonality and comparability between all the different investigations.

Histological processing and analysis of pulp reactions: Following extraction, the restored teeth are fixed in formalin, dehydrated in alcohol, and demineralized in acid. Teeth are then embedded in paraffin wax or plastic blocks to be sectioned into 5-7 micron thickness sections. The tooth sections are collected on glass-slides and stained with hematoxylin and eosin or other stains, and processed for routine light microscopy histological analysis. Pulpal injury and regeneration is measured and categorized according to standardized ISO histological criteria (19). The inflammatory cell activity of each pulp is categorized from "none," "slight," and "moderate" to "severe", according to published criteria (61). The categories of pulp inflammation are: none: the pulp contains few inflammatory cells, or an absence of inflammatory cells associated with cut tubules of the cavity floor; slight: the pulp has localized inflammatory cell lesions predominated by polymorphonuclear leukocytes or mononuclear lymphocytes; moderate: the pulp has polymorphonuclear leukocytes lesions involving

less than one third of the coronal pulp; and severe: the pulp has polymorphonuclear leukocytes lesions involving less than one third of the coronal pulp. Pulp necrosis following chronic inflammatory cell injury is also noted. It is extremely important to separate the responses of restored teeth to the test materials and all the restorative variables. This involves taking into account whether the material is placed in contact with the exposed pulp, or measuring the cavity remaining dentin thickness (CRDT). Few materials can stimulate pulp reactions if the RDT is more than 2mm, therefore, the CRDT ideally should be standardized between all the restored teeth to 1 mm (62). As mentioned previously, it is important to quantify the presence and penetration of bacteria leakage, to exclude and isolate this effect from pulpal responses to the test restorative materials. While ISO criteria is standardized for pulp inflammatory activity and pulp necrosis; The test materials can stimulate none or slight categories, but must not stimulate moderate or severe categories of inflammation, or pulpal necrosis in order to pass this phase of preclinical testing (19,20). However, there are no precisely defined quantitative criteria for measuring the reactionary and reparative healing responses (63,64). Such as the numbers of pulpal cell survival or dentin regeneration (65). These measures are left to the discretion of individual investigators, but comparisons between the traditional 'control' materials and the test materials are recommended (19). Surprisingly, it is not necessary to evaluate the histological reactions of clinically restored teeth to meet ISO guidelines.

CLINICAL TESTING

The ideal approach for biocompatibility evaluation is to test solely *in vivo* with human subjects, which is problematic because of legal and ethical considerations. To protect human health, clinical testing can only be conducted with test materials and treatments that have successfully passed the first three phases of biocompatibility testing recommended by ISO guidelines (19,20) (Table 1). The clinical testing of restorative materials are evaluated according to the United States Public Health Service (USPHS) (66) or Ryge criteria (67) prior to commercial sales. This criteria requires the placement of test materials in patients following institutional review board approval, and patient informed consent. The restorations must be monitored for at least one year, and a 90% success rate must be achieved. If 90% of the restorations are not successful over this time, the test material must be removed from sale in the United States. The purpose of the USPHS criteria was to standardize the collection and assessment of clinical data, however almost all studies have modified the criteria in some way making direct comparisons very difficult (68,69). The USPHS criteria to evaluate the success of restored teeth is shown in Table 2. These criteria require the use of two independent examiners trained to 80% reproducibility. The system uses a grading system based on subjective observations of such parameters as restoration color, marginal adaptation, recurrent caries, anatomical form, as assessments of clinical

performance. For each parameter the scores range from Alpha (perfect), Beta (not perfect, but clinically acceptable), Charlie (restoration requires placement) to Delta (failure). These parameters are used in many studies as a continuum to judge longevity or failure, a strategy that may be inappropriate (68). This is because often the estimated failure rate according to USPHS evaluations, does not match the surveys of restoration failure and replacement, for the same type of materials (69,70). The reason for this disparity requires further investigation. Particularly for the possible roles of restoration technical quality, patient diet, oral hygiene, and dental treatment monitoring, because this information is scarce.

ALTERNATIVES AND NEW DEVELOPMENTS FOR EVALUATING BIOCOMPATIBILITY

The optimization of in vitro cell culture assays: Much work is underway to further refine and optimize the cell culture based *in vitro* cytotoxicity screening tests currently in use. But in general, what a screening test does, at most, is to rank the test materials in regard to their cytotoxicity under the testing conditions in question. In any form of *in vitro* cell culture test, the test system is so different from the clinical conditions of use, that few conclusions may be drawn as to the possible cytotoxicity of the material when used to clinically restore teeth. Hence the need to develop *in vitro* cytotoxicity testing assays using models relevant to the clinical situation such as the culture of odontoblast cells, the culture of tooth slices and embryonic organ culture for evaluating the general toxicity of dental materials (Table 1). These experimental testing strategies are described in the following paragraphs:

Primary Cell Lines: The choice of cell line for *in vitro* biological tests which assess the cytotoxicity of dental materials is controversial, because the apparent cytotoxicity of a material, can be significantly affected by the cell line selected for the test (71). Permanent cell lines, such as transformed mouse fibroblasts (clone L-929) are generally available and are able to provide a means of good reproducibility of cytotoxicity testing between different laboratories (10). Nevertheless, it could be argued that permanent cell lines are simple replicating systems, lacking the specific metabolic potential that the dental pulp or gingiva cells have *in vivo* (19,20). A primary cell culture of the odontoblasts or pulp fibroblasts could simulate the human *in vivo* tissue responses more faithfully (72-74). Ideally, cultures of odontoblasts could be used for the *in vitro* cytotoxicity screening of dental restorative materials, but these cells cannot be readily grown when separated from their association with the dentin matrix (75,76). Attempts have been made to transform primary oral explanted cells in culture, into permanent cell lines, but their phenotypic resemblance to the primary cell lines are questionable (77,78). In the case of odontoblasts, these are post-mitotic cells, meanwhile the transformed cells can divide readily, for example. These cells also express some different proteins in comparison to the original odontoblasts, and are derived from rodent rather than human teeth.

Table 1. Testing protocols for dental biomaterials.

Test phase	1	Assays under evaluation, not yet recommended by guidelines			2	3	4
Test evaluation	General toxicity	Assays under evaluation, not yet recommended			Local tissue irritation	Preclinical	Clinical
Assay type	Monolayer cell culture	Monolayer Cell culture	Embryonic organ culture	Tooth culture	Animal implantation	Animal usage assays	Patient trials
Tissue source	Permanent cell lines	Primary cell lines	Human or animal	Human or animal	Small animals; guinea-pig	Primates; Monkey	Human
Test type	<i>In vitro</i>	<i>In vitro</i>	<i>In vitro</i>	<i>In vitro</i>	<i>In vivo</i>	<i>In vivo</i>	<i>In vivo</i>
Test element	3T3 cell lines	Odontoblast cell lines	Papilla	Tooth slice	Subcutaneous implant	In situ teeth	In situ teeth
Experimental time (days)	≥ 1 day	≥ 1 day	21-35 days	≥ 14 days	≥ 365 days	70 +/- 5 days	≤ 365 days
Test suitability							
Tissue irritation & inflammation	No	No	No	No	Yes	Yes	Yes
Hypersensitivity	No	No	No	No	No	No	Yes
Carcinogenic or mutagenic	No	No	No	No	Yes	Yes	Yes
Cytotoxicity and dentinal injury	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Bacterial leakage	No	No	No	No	No	Yes	Yes
Genetic engineering	No	No	No	Yes	No	Yes	Yes
Growth factor therapy	No	No	No	Yes	No	Yes	Yes
Stem cell therapy	No	No	No	Yes	No	Yes	Yes

Table 2. Modified United States Public Health Service Rating System.

Criteria	Assessment
Success (Alpha)	Restoration is clinically acceptable
Secondary caries	Caries lesion is visible adjacent to existing restoration
Bulk fracture	Isthmus fracture or any fracture through the main part of the restoration
Marginal fracture	Restorations appearing well adapted to tooth tissues, but with marginal fractures or defective margins with no evidence of caries.
Tooth fracture	The fracture of a tooth adjacent to a restoration, such as fracture of a cusp or small enamel fractures.
Poor anatomic form	Loss of substance due to material degradation and wear, sufficient to result in loss of restoration form and possibly function.
Allergic sensitivity	Any kind of pain/ sensitivity requiring restoration replacement is listed under this category.
Loss of tooth	The tooth was lost.
Other failure	The restoration has been wholly or partially lost

In common with other relatively differentiated cell lines, pulp fibroblasts can be quite readily cultured, but their growth characteristics and sensitivity to cytotoxins can vary greatly from culture to culture (79-81). Regardless of the fact that permanent cell lines have an improbable relevance to the clinical situation. The philosophy of guideline ISO 10993 on the standardization of cell culture experiments is to advocate the use of permanent cell lines, over primary cell lines, to achieve a good reproducibility in the standard assays as part of biocompatibility screening.

Tooth slice culture assay: The *in vitro* tooth culture of teeth maintains pulp tissue vitality and allows experimental conditions to be precisely controlled. The use of cultured slices of teeth for some experiments preserves a close link to the clinical situation without the need for animal or human experimentation. This testing method was developed in response to ISO 7405 (19) guidelines that recommended alternative non-patient/non-animal testing strategies be evaluated. The benefit of using the tooth slice culture assay is that it permits the cytotoxicity screening of test materials on pulpal tissues (82), allows the evaluation of restorative therapies (83), and growth factor (84,85), and probably stem cell and gene therapy (Table 1), without presenting a risk to animal or human health (86). Another advantage is the reproducibility of this method, this is because the teeth are maintained in identical experimental conditions and are not subject to animal or patient confounding variables, such as oral hygiene standards, treatment history and diet. The culture of tooth slices also provides an economical approach for investigating a high number of potential treatments using a near physiological and pathologically identical tooth population. The preservation of tooth structure allows the role of cavity preparation and restoration variables to be measured (83); until recently the effects of these variables was only able to be measured in animals and human clinical trials. Therefore, the increased utilization of tooth slices as part of biocompatibility screening assays may be useful for reducing the numbers of animal and clinical experimentation because it can reduce the number of variables to be measured in the latter phases of testing. This assay method requires further development, and may be useful for evaluating the early stages of genetic engineering, growth factor, and stem cell therapy (Table 1).

The standards for evaluating the biocompatibility, cytotoxicity and clinical success of restorative materials are continually updated or modified, according to new scientific advances or to prevent the recurrence of problems that have arisen in the past. While there is often a strong impetus to periodically modify the criteria. The need to preserve a comparison with previous investigations provides an incentive to preserve the status quo. However, in the last update, the ISO 7405 guidelines recommended the evaluation of alternative non-patient/non-animal testing strategies. This was in response to political and public pressure to reduce animal usage testing. Some European countries are considering the introduction of legislation to ban animal usage assays (48). However, it must be recognized that not all types of

preclinical testing can be simulated or replaced by other means, such as *in vitro* modeling. Examples of the type of investigations that cannot be replaced by *in vitro* assays are essentially investigations of whole body systemic effects, such as inflammation and carcinogenicity, or where the integrity of the blood and nerve supply is required. Thus for the foreseeable future, the continued application of animal biocompatibility experiments is a vital safeguard to minimize possible hazards to human health. Animal usage assays are the only testing methods, apart from clinical testing, that are suitable for evaluating tissue irritation/inflammation, hypersensitivity, carcinogenic or mutagenic potentials and bacterial leakage (Table 1). However, there is a continuing requirement to develop alternative *in vitro* biological testing strategies to reduce the numbers of *in vivo* animals used in experimentation.

CONCLUSIONS

This review has concluded that animal and clinical screening are necessary to protect patients from possible hazards presented by dental materials and new biological restorative treatments. This is because there are few realistic models to replace these forms of biocompatibility screening. However, the numbers of animal and clinical screening tests can be minimized in order to make this form of testing more legally and ethically acceptable. This can be achieved by not duplicating previous experimentation, and by the more rigorous screening of materials and restorative treatments using existing and experimental screening assays. The continued utilization of some basic cytotoxicity screening assays is questionable because of a limited usefulness to the structure of teeth. This problem is most acute for evaluating the safety of genetic engineering, growth factors and stem cell therapies, where some initial biocompatibility tests are required that allow these therapies to be evaluated without the need for a complete and total reliance on *in vitro* animal or clinical testing.

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