Determination of biocompatibility: A review

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Abstract
Toxicity of a material upon placement in patient's oral cavity triggers the immune system to elucidate an appropriate response. Thus, one of the many fundamental responsibilities bestowed on the dentist is to shield patients from any untoward reaction. A plethora of dental materials is currently available in the market. The material has to advance several tests in order to be safely introduced for clinical practice. This review highlights the spectrum of tests routinely practiced for evaluation of biocompatibility.

Introduction
Biocompatibility (or tissue compatibility) describes the ability of a material to perform with an appropriate host response when applied as intended. The oral environment is complex and varied. A dental material used in such a compound environment might encourage unnecessary disturbance. A strict scrutiny of these dental materials, therefore, is prudent before the commercial influx. The validation of these tests is not entirely dependent on the tested material; it is however influenced by the method employed for testing. This review article highlights the fundamental and routinely practiced methods for determination of the biocompatibility of dental materials.

Standardization
Several organizations, namely the Food and Drug Administration, American National Standards Institute, American Dental Association, International Organization for Standardization (ISO) and certain accessory bodies such as Nordic Institute of Dental Materials and the European Union, diligently work on the materials to be tested. ISO 7405 are exclusively for evaluating dental materials and ISO 10993 evaluate both medical and dental materials.

Testing Hierarchy
Testing follows a characteristic hierarchy [Table 1]. Not until unobjectionable results are obtained in the initial stages, the material can be channelized further to tests with more vigor, simulating a clinical (or near clinical) testing.

This pattern of testing was developed in accordance with the cumulative measure of biocompatibility that was proposed by Autian in 1970 [Figure 1]. It was a linear paradigm from primary through usage tests where the material has to clear the previous tests to be eligible for the next test.

Soon after this concept was put across, Mjör et al. [Figure 2] compared in vitro, animal and usage tests for clinical usage and found the materials that cleared the first two tests were not entirely harmless at the clinical usage level. Thus, in 1977 he modified the linear paradigm to a non-linear pattern, where simultaneously all three tests would be performed on the tested material.

Currently, the initial risk assessment is performed by an expert. Information on the physical, chemical and biological
characteristics from the available literature is reviewed, and studies on the missing dimension are performed. If an iota of change is observed in a material previously in practice, then the material is said to have an acceptable risk after performing certain basic tests. On the contrary, if considerable change or new components are incorporated, tests with heightened magnitude need to be conducted.[2]

Types of Tests

Initial tests
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.

Cell culture test
Increased number (nearly 20) of cell culture techniques are currently available in the literature.[6] Animal or human tissues are used for these tests after growing them under suitable conditions on culture plates.[7] Routinely used cell lines are permanent mouse fibroblasts (L-929, 3T3) or human epithelial cells. These permanent cell lines have a predictable behavior that has been well comprehended and is consistent.[8]

The cells are kept in culture dishes and placed under suitable conditions for reproduction until a mono-layer of cells are formed. The tested material is then added to the culture and allowed to be in place for 3 days. After thorough examination under the microscope, the changes in the cell lines are recorded.[9] To analyze a cell damage, dyes are used wherein the vital cells take up the stain in contrast to the cells exhibiting membrane rupture.[2]

One of the innate disadvantages of these cell counts in the inability to distinguish between living and dead cells if no morphological alterations have occurred. The viable cell count can be extremely subjective, and any artifact further compounds the problem.

Membrane integrity assay
Membrane integrity assay is a colorimetric or florescent assay wherein the membrane integrity is tested for its permeability. These tests substantially diminish the artifact based errors. The precision to distinguish between viable and dead cells is also of high standard. Trypan blue staining,[10] red vital staining,[11] and neutral red staining combined with amido black staining[12] have been used to study the toxicity of some endodontic materials for the routine determination of cell viability. However, a problem posed by this assay is the inability to detect sub-lethal changes that occur prior to the changes in the membrane permeability.[13]

Barrier screening test
This concept is based on the assumption that a layer of dentin would be normally present between the material to be used and the pulp tissue.[14] In this technique, a barrier of dentin disc is positioned between the tested material and the cell culture. A positive cytotoxic response occurs if the material (or its by-products) penetrate the disc.[4]

Agar diffusion test
This test, also known as agar overlay or tissue culture overlay test,[15,16] uses a layer of agar overlaid on a monoculture cell layer. The material to be tested is placed upon the agar layer. This technique can be used for ubiquitous cytotoxicity imparted from the leachable components. Dye used in this study is usually red vital stain.[3] Identification of the toxic substance is evident as the presence of dye on the cell line indicates leaching.[11]
Filter diffusion testing method
This method bears close resemblance to the agar diffusion tests. A millipore (cellulose acetate) filter is interspersed between the primary cell line and tested material. Presence of any leachable component disseminates through a pore size of 0.45 μm filter pore. The filter paper is examined at the tested material side and graded based on a criterion by ISO.[17]

Dentin barrier test
Though an in vitro test, this method closely mimics the oral environment. It is also known as model cavity method. The original idea was proposed by Outhwaite et al.[18] These tests factor the diffusion of the cytotoxic materials through the dentinal tubules.[19-21]

Tooth slice culture assay
This assay preserves the vitality of the tooth and hence a possible extrapolation of a clinical scenario is permissible. The method is a satisfactory surrogate for animal and human testing.[21] In comparison to the above methodologies, a cytotoxic impact on the pulpal tissue, growth factors, stem cell and gene therapy could be precisely evaluated.[22,23] This method largely inhibits the confounding factors that would otherwise have a bias. The cost factor is glaringly reduced.[24]

Ame’s test
It was developed in the 1970 by Prof. Bruce Ames. It is one of the popular in vitro tests. His work extensively involved in ‘Identifying Environmental Chemicals Causing Mutations and Cancer’. His pioneering work used bacteria to identify potential carcinogens without involving mammals. Mutant strains of the bacteria Salmonella typhimurium were used. These bacteria contain mutations in the enzyme that synthesize histidine. Histidine in-turn is responsible for further synthesis of proteins. Bacteria cannot grow and form colonies on a special culture agar, which is histidine-deficient. If any mutagenic substance is present, the growth of the bacteria is evident suggesting mutagenicity.[25]

Style’s test
It is a modification of Ame’s test that uses normal fibroblasts cultured from baby hamster kidney. The presence of a carcinogen can be identified upon the addition of the substance, if carcinogenic, turns the material into quasi-cancerous state.[27]

It is arduous to derive conclusions merely based on the cytotoxicity tests. These tests simply categorize the materials based on cytotoxicity under a given condition. Generally, vast variations are encountered in a clinical scenario. This necessitates the demand for more exacting tests.

Usage Tests
Testing on animals simulates a near clinical scenario especially on the systemic and cytotoxic properties.[28] Animals such as ferrets,[29] dogs[30] and even non-human primates[31] are among the tested specimen. The ISO 7405 guidelines classify the duration for testing as short term (7 ± 2 days) and long-term (70 ± 5 days). Investigators prefer to split lap the duration between varied time-intervals from 3 to 60 days or more.[30,31] The cost can be a major limitation for adopting a short-time period. However, longer periods give a clue on the healing and regenerative responses.

Inhalation test
The tested material is dispensed as a spray or aerosol. It is sprayed near the mouth and chest of animals for 30 s. The testing continues there-on where for every half hour interval, the material is sprayed for 10 times. If animal dies within 3 min, the material is considered extremely toxic. If not, a long-term follow-up is essential for the material to be described safe.[27]

Implantation test
The material is subcutaneously, intramuscularly or intraosseously implanted in rats, rabbits, etc. Implantation periods can range from 1 week to many months. Following a short implantation test, about 1-2 weeks, inflammation adjoining the implanted material is evaluated. Extended periods of implantation, lead to the formation of a connective tissue encapsulation that can be evaluated as well. Intra-osseous implanted materials might use primates, dogs, pigs, guinea pigs, etc. Histological assessment of the tissue around the tested material can be performed.[2]

Maximization test
For this test, a component called the Complete Freund’s Adjuvant (FCA) is used. It is one of the several used chemicals in research. The primary form of this chemical is called the Freund’s adjuvant. Addition of killed Mycobacterium tuberculosis to the primary form produces the FCA. Absence of the bacteria produces the incomplete form. FCA has a tendency to release antigens steadily.[33,34] The experimental material is intra-dermally injected into the animal with FCA and evaluated after 7 days. On the 7th day, the same substance is topically applied for the next 2 days. This enhances the sensitivity of the tests. After 14 days, the skin reactions are appraised. Care should be taken while performing this test, as the antigen, if applied in excessive quantities might lead to an irritating skin reaction and can be a confounding factor.[35]

Buehler’s test
This test is very similar to the maximization test. The major advantage of this test is the elimination of the FCA component. The experimental animals suffer reduced damage due to the extirpation of the antigen.[37]

Pulp-dentin test for restorative materials
Pulp compatibility is of utmost significance while discussing about restorative materials. This test is either conducted in experimental animals or on human teeth that are to be orthodontically extracted. After preparation of a Class V cavity, the material to be tested is filled and kept under observation for weeks to months following which histological examination of the
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pulp is microscopically evaluated for the signs of inflammation and odontoblast reaction. Pulpal insult might occur if the materials release toxic products or if microleakage is present between the material and the tooth tissue.[2]

**Pulp-capping and pulpotomy material test**
It is a minute modification of the pulp-dentin test. If a portion of the pulp is exposed or extirpated following which the material is applied, the assessment of pulp cappings or pulpotomies can be done.[2]

**Mucosal damage and mucosa usage test**
The inflammatory reactions to mucous membranes or abraded skin are tested. Hamsters are used for the test where the test material is placed in the cheek-pouch and observed for any reactions after several weeks of contact. The pre- and post-operative photographs are compared and biopsy specimens are analyzed for inflammatory changes.[38]

**Periapical tissue damage and endodontic usage test**
This test simulates a reaction that would be elicited following a conventional endodontic treatment. This animal model uses large animals such as primates or dogs. The evaluation is done by histological sections of the periapical region. Intentional necrosis of the pulp to replicate a clinical scenario is also occasionally performed.[39] Due to the enigmatic ethical issues, limited literature is available on these tests. Instead, other implantation tests can be performed to elucidate a near-equal response.

**Gingival usage test**
Preparation of the cavity with subgingival extensions to ensure direct contact between the tested material and the gingiva. An evaluation period of around 7-30 days exists. The confounding variables of this study would be the accumulation of bacterial plaque that might lead to inflammation and hinder the actual estimation of material toxicity. Another important variable is the under- or over-contoured margins. Rugged margins further exacerbate the condition.[40]

**Teratogenic effects and influence on reproduction**
These damages are to assess if a material can possibly elucidate any inimical effect of fetuses or newborns. The material is attached to the rodents prior to mating to both sexes or after mating to only females. The fetuses and newborns are evaluated microscopically and macroscopically for any aberration. This can be continued to the next generation as well to further survey any malformations.[2]

**Diagnostic Test on Patients**
A direct corroboration of the clinical performance and diagnostic tests on patients can be drawn. It is imperative that the material clear the initial and the usage tests.[34] Considerable legal and ethical considerations exist while performing these tests. The clinical testing of restorative materials are evaluated according to the United States Public Health Service[45] or Ryge criteria[42] prior to commercial sales. The materials should be monitored for a prolonged period, i.e. 1 year, and also obtain a 90% success rate. In case of a deviation from either of the criteria, the test material should be withdrawn from sale. The scoring range from Alpha (perfect), Beta (not perfect, but clinically acceptable), Charlie (restoration requires placement) to Delta (failure).[43]

**Allergy tests**
These tests are further divided as:

- **Patch test**
  - Delayed hypersensitivity reactions
  - Prick test
  - Immediate hypersensitivity reaction
  - Radioallergosorbent test
  - Alternative to prick test

**Patch test**
Jadassohn described the patch test.[44] Allergens are loaded on to adhesive tapes at concentrations mildly high that would initiate a positive response attached to patient’s back. These reactions are mild, non-irritating and essentially harmless. The allergens are packed in a standard series when commercially available. Certain precautions from the patient are obligatory such as warding off from a sun exposure, avoid increased sweating or scratching the target area. The classical signs include redness, itching, blisters. Skin is evaluated followed by the removal of the patch after 2-3 days. A second evaluation after 7-10 days should be conducted to rule out delayed hypersensitivity responses. The immunocompetent T lymphocytes require a few days before a detectable change is evident.[2]

Patch test is to detect delayed hypersensitivity (Type IV) reactions. Skin and oral mucosa are comparable. When the same test is performed at the actual site, in the oral cavity, it is termed as epidermal test. However, due to factors such as saliva and a mild discrepancy in the immunological reaction, the allergic response is not always elicited. A higher concentration may be necessary to overcome this problem. Thus, an ideal, reliable and an accurate place would be the patient’s back.[45]

Patch tests are not randomly indicated to every patient since the procedure itself may sensitize the patients, and a subsequent exposure might trigger the allergic response. Hence, only when suspicions compel the test, should it be performed. When a general patch testing is performed although no previous allergic response noted, it is termed as a prophetic test and should be avoided. If any intra-oral sign demonstrates inflammation, the allergic response could be substantiated upon removal (elimination test) of the suspected allergen (e.g., Denture) and re-entry (provocation test) of the same allergen to elucidate the earlier noted response.[46]

**Prick test**
This test can be employed to detect an immediate-type allergic reaction or the Type I hypersensitivity reactions. The allergen is
placed as a drop on the skin, and the skin is pierced through the drop. Presence of any positive reaction is detected after a period of 5-30 min. In contrast to the patch test, the risk of sensitization is fairly low with prick test.[2]

Radioallergosorbent test (RAST)
This test was described in 1967 by Wide, Bennich, and Johansson as an in vitro diagnostic test for allergen antibodies. It is an in vitro substitute of prick test. It is used to detect immediate hypersensitivity. The allergen is linked to a material that is insoluble and the serum taken from the patient is added. The allergen evokes a response by binding to the antibody if the added serum has antibodies to it. Further on radiolabelled IgE antibody is added, and a complex reaction occurs with the already linked insoluble material. IgE antibodies that did not react and form a complex are washed away. It is a quantitative test in the sense, the linked radioactivity determines the amount of serum IgE released for a given allergen.[47]

Intraoral voltage
A patient can occasionally experience a metallic taste or a galvanic shock when two dissimilar metals come in contact with each other. This can be attributed to the voltage difference between the metals. This electrical phenomenon can be measured using intraoral voltage devices. This particularly becomes important owing to the perimeter of the permissible tolerance of a given material for a patient.[48]

Intraoral alloy analysis
To perform a test on a removal of prosthesis is relatively straightforward using analytical methods such as polished metallic micrograph sections in conjunction with energy dispersive X-ray analysis. The technique gets cumbersome if the same has to be extrapolated to a fixed prosthesis.[49] Chip test can be used in such conditions, where using a silicon carbide stone or a tungsten carbide bur, the extra/intracoronal surface is brushed and the resultant particles are gathered on a self-adhesive graphite plate. Electrical conductivity is accomplished by the self-adhesive carrier. The accuracy is up to ±1% for individual alloy component. Thus, a patient having those metals need not be subjected to an allergic test if no previous allergic response has been documented.[49]

Advantages and Disadvantages of the Test Categories
Provided below is a summary of the advantages and disadvantages of the test categories for an improved understanding.

In vitro tests

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
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<tbody>
<tr>
<td>Quick to perform</td>
<td>Relevance to use of material – questionable</td>
</tr>
<tr>
<td>Least expensive</td>
<td>Expensive</td>
</tr>
<tr>
<td>Can be standardized</td>
<td>Time consuming</td>
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<tr>
<td>Large scale screening</td>
<td>Legal/ethical concerns</td>
</tr>
<tr>
<td>Good experimental control</td>
<td>Difficult to control</td>
</tr>
<tr>
<td>Excellent: Mechanism of interaction</td>
<td>Difficult to interpret and quantify</td>
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In vivo tests

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allows complex systemic interactions</td>
<td>Relevance to use of material – questionable</td>
</tr>
<tr>
<td>More comprehensive response than in vitro</td>
<td>Expensive</td>
</tr>
<tr>
<td>More relevant than in vitro</td>
<td>Time consuming</td>
</tr>
<tr>
<td></td>
<td>Legal/ethical concerns</td>
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<tr>
<td></td>
<td>Difficult to control</td>
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<td></td>
<td>Difficult to interpret and quantify</td>
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Usage tests

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relevance to use of material is assured</td>
<td>Very expensive</td>
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<tr>
<td></td>
<td>Very time-consuming</td>
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<tr>
<td></td>
<td>Major legal/ethical concerns</td>
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<tr>
<td></td>
<td>Can be difficult to control</td>
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<td>Difficult to interpret and quantify</td>
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Conclusion
It is essential to achieve genuine dental products to shield patients from the trivial form of danger. To accomplish this, an amalgamation of multi-spectral tests currently available to us should be used before declaring any material safe. However, having said that, a reduced screening duration in the animal screening and clinical usage test can be implemented due to ethical and legal concerns. To compensate for void thus created, stringent preliminary tests can be conducted.

References