



BIOCOMPATIBILITY TESTS OF DENTAL MATERIALS

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ABSTRACT

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Dental materials may result in damage to various tissues. Therefore, a great variety of different test methods are applied to evaluate the risk of such damage to ensure material compatibility. However, the results of such evaluations are dependent not only on the tested material but also on the test method used. The findings of these studies and the results should be critically challenged by the dentist, so dentists need to be familiar with the principles and, in particular, with the problems of these test methods. Evaluation of the biocompatibility of dental materials is a complex and comprehensive area because unwanted

tissue reactions may occur in a great variety of types. Moreover, individual test methods are usually adequate only to describe or document a single aspect of a certain type of unwanted reactions. Scientists have recognized that the most accurate and cost-effective means to assess biocompatibility of a new material is a combination of in vitro, animal, and usage tests. The ways in which these tests are used together, however, are controversial and have evolved over many years as knowledge has increased and new technologies were developed. This review article emphasizes on the biocompatibility tests and its importance in evaluating dental materials.

KEYWORDS: Biocompatibility, cell cultur, diagnostic tests, allergy.

INTRODUCTION

During the past few years, the biocompatibility of dental materials has evolved into a comprehensive, complex and independent discipline of dental material science^[1]. A new era in dentistry is rapidly emerging as a result of the process of intelligent evolution. The transition between the purely mechanical phases to the highly evolved biologic phases of dentistry has occurred slowly, over a period of 150 years. As dentistry moved into the 21st century, it provided a coupling of high technology materials, integration of techniques, and diagnostics with scientifically based research^[2]. Biocompatibility of a material depends upon the biological interaction between the location of material in the body, the duration of material in the body, properties and composition of the material and health of the host^[3]. The primary purpose of biocompatibility tests is to protect the dental patients who will be treated with the materials and the office staff and lab technicians who will be handling these materials¹. Since no dental biomaterial is absolutely free from the potential risk of adverse reactions, the testing of biocompatibility is related to risk assessment. Thus, tests of biocompatibility are important to manufacturers, practitioners, technicians, scientists and patients.

CLASSIFICATION OF BIOCOMPATIBILITY TESTS

BY CRAIG²:

- In vitro tests
- Animal tests
- Usage tests

IN VITRO TESTS

It has been long recognized that in vivo, direct contact does not exist between the cells and materials. Separation of cells may occur from keratinized epithelium, dentin or extracellular matrix. Hence in vitro barrier tests were developed to mimic in vivo conditions^[1,6]. In vitro tests can be roughly subdivided into those that measure; cytotoxicity or cell growth, metabolic or other cell function and the effect on the genetic material in a cell-mutagenesis assays^[7]. *Primary cells* are those cells taken directly from an animal and cultured. These cells will grow for only a limited time in culture but usually retain many of the characteristics of cells in vivo^[8]. *Continuously grown cell lines* are cells that have been transformed previously to allow them to grow more or less indefinitely in culture. However, the genetic

and metabolic stability of continuous cell lines contributes significantly toward standardizing assay methods.

CYTOTOXICITY TESTS^[8]: Cytotoxicity tests assess cell death caused by a material by measuring cell number or growth before and after exposure to that material^[2]. For in vitro cytotoxicity screening, the recommended testing methods include the following methods.

Direct cell culture - Cells are plated in a well of cell culture where they attach and the material is placed in the test system. If the material is not cytotoxic, cells will remain attached to the well and proliferate with time^[9]. If the material is cytotoxic, cells may stop growing, exhibit cytotoxic features or detach from the well. The practical complication of cell is that it is 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.^[10]

Agar overlay method- A monolayer of cultured cells is established before adding 1% agar at a low melting temperature plus a vital stain to freshen the culture media. The agar forms a barrier between the cells and the material which is placed on top of the agar^[11]. Nutrients, gas and soluble toxic substances can diffuse through agar. However the disadvantage with this test is due to the variability of the agar's diffusion properties it is difficult to correlate the intensity of the colour or the width of the zone around the material with the concentration of leachable toxic products^[12].

Filter diffusion testing methods - The Millipore 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 filter¹³. Thus, any leachable substance must diffuse through the 0.45µm filter pores to exert any cytotoxic effects on the cells. After exposure to the test samples the filter is removed and the effect of sample on a cellular metabolic activity is determined^[14]. 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^[15].

Dentin barrier tests: Dentin-barrier tests simulate the tooth conditions by placing a dentin disc between target cells and test material. The material is placed on one side of the dentin disk in the device used to hold the dentin disk. Collection fluid is on the other side of the disk. Cells

can also be grown on the collection side. To assess the rate of diffusion, the collection fluid can be circulated into and out of the collection chamber.^[16]

Membrane permeability: This test is based on the basis that the membrane permeability is equivalent or nearly equivalent to cell death. Two types of dyes are used; vital dyes which are actively transported to viable cells where they are released unless the cytotoxic effects increase the permeability of the membrane and non vital dyes which are not actively transported and are taken up only if the membrane permeability has been compromised by cytotoxicity.^[17,18]

MTT assay: *The* method determines the activity of mitochondrial enzymes photometrically via a color change reaction. It measures the activity of cellular dehydrogenases which convert a chemical MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] via several cellular reducing agents to a blue insoluble formazan compound. If the dehydrogenases are not active because of cytotoxic effects the formazan compound will not form. The production of formazan can be either quantified by dissolving it and measuring the optical density of the resulting solution. Other formazan generating chemicals are been used such as : NBT (nitroblue tetrazolium), XTT [2,3-Bis-(2-methoxy-4-nitro-5 sulfophenyl)-2H- tetrazolium-5-carboxanilide salt], WST (a water-soluble tetrazolium).^[19]

MUTAGENESIS ASSAY- Mutagenesis assays assess the effect of a biomaterial on a cell's genetic material^[2]. The tests done to measure mutagenicity are.

AMES Test - Most widely used short-term mutagenesis test that is considered thoroughly validated. Basically, the influence of a material on the genome (DNA) of bacteria or of mammalian cells is investigated. Genetically altered bacteria are used as test organisms. These bacteria cannot grow and form colonies on a special culture agar, which is histidine-deficient. But as soon as they come into contact with a mutagenic substance, they begin to grow. The number of forming colonies is a criterion for the mutagenicity. The result indicates that the genome has been changed and was passed on to the next generation of bacteria. It uses mutant stocks of salmonella typhimurum that require exogenous histidine.^[20,21]

Styles' cell transformation test. - This test on mammalian cells offers an alternative to bacterial tests. This assay quantifies the ability of potential carcinogens to transform standardized cell lines so they will grow in soft agar. Four different continuous cell lines have

been used, namely; Chang liver cell line which is derived from the cervix of humans, Baby hamster kidney cell line, HeLa [human epithelial carcinoma cell line] WI-38 [Lungs, foetal from human].^[22]

Micronucleus test - It is a mutagenic test system for the detection of chemicals which induce the formation of small membrane bound DNA fragments i.e. micronuclei in the cytoplasm of interphase cells. The purpose of the micronucleus assay is to detect those agents which modify chromosome structure and segregation in such a way as to lead to induction of micronuclei in interphase cells. If a substance induces a concentration-related increase or a reproducible increase in the number of cells containing micronuclei, it is classified as a positive result.^[23,24]

HPRT tests -The HPRT [hypoxanthine phosphoribosyltransferase] assay is an in vitro mammalian cell gene mutation test. V79 Chinese hamster cells are used as they have one functional copy of the gene which codes for the HPRT enzyme. HPRT enzyme activity is important for DNA synthesis. Those cells that are able to form colonies are assumed to be mutant cells resulting from either a spontaneous mutation or from an induced mutation caused by a chemical agent. This assay is used to evaluate the potential of a chemical, formulation or extract to induce mutations.^[24, 25, 26]

Barometer test -The new method, developed by the National Institute of Standards and Technology represents a novel application of existing bench-top scientific instruments. It is a two-step process. The first step involves using a device called a polymerase chain reaction instrument to measure the levels of an organism's cytokines when exposed to a given material. The second step involves testing exposed cells for a specific protein in the cell membrane, the presence of which indicates cells are dying. Cytokines are signaling molecules released by white blood cells to protect the body from foreign materials. Higher levels of cytokine production generally indicate non-biocompatible materials have caused inflammation.^[27]

ANIMAL EXPERIMENTS

Animal tests involving mammals such as mice, rats, hamsters, or guinea pigs, are distinct from usage tests; which are also often done in animals in that the material is not placed in the animal with regard to its final use.^[28] The biological responses in animal tests are more

comprehensive and considered more relevant than *in vitro* tests. A variety of animal tests have been used to assess biocompatibility.

Test to evaluate acute and chronic lethal dose- LIMIT TEST^[29] involves administration of a fixed dose, e.g., 2,000 mg/kg body weight. If this concentration is not high enough to reach the LD₅₀, then generally no further tests will be done. The chronic systemic toxicity will be determined by administering the material or extract over several months.^[30] At the end of these studies, survival rates of the animals and patho-histological alterations of the main organs will be determined. However, dental materials are characterized by a low acute systemic toxicity in general.

Implantation tests-Implantation tests are used to evaluate materials that will contact subcutaneous tissue or bone. Although amalgam and alloys are tested because the margins of the restorative materials contact the gingiva, most subcutaneous tests are used for materials that will directly contact soft tissue during implantation, as well as endodontic and periodontal treatment materials^[30]. Materials are implanted, subcutaneously, intramuscularly, or in the bone of an experimental animal. After different periods of implantation of the material in the tissues which is between 1 week and several months, the adjacent tissue is investigated macroscopically and microscopically.^[31]

Intraosseous implant test-Materials used for dental implants are inserted into the jaw of test animals. Penetration of the epithelial barrier, equivalent to the treatment of patients, is simulated on experimental animals. Tissue reaction is assessed histologically with the tissue in contact with the implant being of particular interest. Animal studies by Donath *et al* show that implants based on titanium or ceramics are generally well tolerated by the surrounding tissue and a good correlation of these findings with patients' situations can be expected. When testing alloys by means of implantation, an extended implantation period of tissue contact of more than 4 weeks is necessary.^[2,32]

USAGE TESTS

Usage tests employ larger animals that have similar oral environments to humans, such as dogs, mini-swine or monkeys. When humans are used, the usage test is termed a clinical trial^[33]. The overwhelming advantage for usage tests is their relevance these tests are the gold standard. However the significant disadvantages of the usage test are; extremely expensive, last for long periods, involve many ethical and often legal concerns, exceptionally difficult to

control and interpret accurately and may harm the test participants. In dentistry, the main targets of usage tests are.

Pulp-dentin test - Pulp compatibility of a material is investigated on teeth of experimental animals or on human teeth that have to be extracted for orthodontic reasons. In both cases, class V cavities are prepared as atraumatically as possible and are then filled with the test material. After a period of days to several months, the teeth are removed and histologically prepared, and the pulps are microscopically evaluated for signs of acute or chronic inflammation and odontoblast reaction. In addition, the space between test material and the cavity wall is investigated for bacterial penetration.^[34,35]

Mucosal damage and mucosa usage tests- Tissue response to materials with direct contact of gingival and mucosal tissues is assessed by placement in cavity preparations with sub gingival extensions. The material's effect on gingival tissues are observed and responses are categorized as Slight, Moderate and Severe depending on the number of mononuclear inflammatory cells mainly lymphocytes and neutrophils in the epithelium and adjacent connective tissues. A difficulty with this type of study is the frequent presence of some degree of preexisting inflammation in gingival tissue due to the presence of bacterial plaque, surface roughness of the restorative material, open or overhanging margins, and over- or under-contouring of the restoration.^[35,36]

Periapical and endodontic usage test-Animal models (e.g., primates, dogs) that allow the application of a given material into the root canal according to endodontic techniques after a usual root canal preparation have been used in this test. Compatibility is assessed by histologic evaluation of the periapical tissues. It is also possible to induce pulp gangrene as a disease model in the experimental animal and to perform an appropriate treatment. Classic endodontic usage test is very elaborate and includes the same technical and ethical problems as the pulp/dentin test using large experimental animals.^[37,38]

ALLERGENIC PROPERTIES

In general, allergenic properties (type IV reaction) of dental materials are currently preclinically tested on experimental animals. According to OECD [Organization for Economic Co-operation and Development] Guideline 406, two test methods are recommended using guinea pigs.

Maximization test^[39] - The investigated substance is at first injected intradermally into the experimental animal, together with Freud's Complete Adjuvans (FCA). Seven days later, the same substance is applied topically at the same site for 2 days. It is intended to amplify the immunological effect by FCA and, thus, to increase the sensitivity of the test. Fourteen days after this induction period, the test substance is applied on a different area of the skin. Subsequently, the skin reaction is assessed after an appropriate exposure time. Injections of FCA should be subcutaneous or intraperitoneal, because intradermal injections may cause skin ulceration and necrosis; intramuscular injections may lead to temporary or permanent muscle lesion, and intravenous injections may produce pulmonary lipid embolism.

Buehler test^[40] -Buehler test is similarly executed on guinea pigs but without the application of FCA [Freud's Complete Adjuvans]. Therefore, the Buehler test is considered to be more protective for the animals than the maximization test. However a study done by Seren Frankild et al indicated that the Buehler test is less sensitive than the maximization test and higher induction concentrations were needed to show allergenicity in the Buehler test and for some allergens the Buehler test protocol was not sensitive enough to demonstrate allergenic potential.

Teratogenic effects and influence on reproduction^[41] -To assess these types of damage, the test substance will be applied to animals, such as rodents, before mating (both males and females) or after mating (only females). At the end of the study, female animals and fetuses/newborns are macroscopically and microscopically evaluated for malformations. This trial may possibly be extended to the next generation. The indication of these extensive studies is considered with great reservation in relevant standards regarding dental materials (ISO 10993-3).

DIAGNOSTIC TESTS ON PATIENTS

Diagnostic tests on patients are used to more deeply analyze claimed or real unwanted side effects in individual subjects. This branch of biocompatibility studies has become very important since many materials do not cause clinically manifest reactions in the vast majority of the population but may generate claimed or real disease symptoms linked to materials in single cases.^[42]

ALLERGY TESTS- The tests used to determine the allergenic properties of dental materials in patients are.

Patch test- The patch test originally developed and described by Jadassohn, is the most important allergy test regarding dental materials. This test can be applied to identify delayed type hypersensitivity as the cause for an allergic contact dermatitis. Adhesive tapes containing the potential allergens at concentrations that are just high enough to trigger the allergic reaction but which are nonirritating are adhered to the clinically sound skin of the patient's back. During the following days, after the tape has been removed; the skin is evaluated for test reactions: redness, itching, blisters.^[43] The patient should avoid excessive sweating or exposure to sun as well as scratching of the back, and should not have a shower or bath. Skin reactions are assessed after 2 and 3 days, but later checks after 5 and 7 days are also necessary to detect late reactions, since immunocompetent T lymphocytes occasionally require several days before they cause a visible allergic reaction.^[44]

Epimucosal test - Skin and oral mucosa react similarly in the case of an allergy; therefore the skin is considered an adequate organ for the appropriate allergy tests. The basic requirement for the stimulating effect on T lymphocytes is that the allergen is released from the material in sufficiently high quantities and then penetrates the skin. It was recommended, as an alternative to the patch test, to assess the allergy at the actual tissue of target, the oral mucosa. However this approach is much more difficult to perform, and results are considered to be less meaningful as saliva will dilute the allergens, and the oral mucosa may have a different immunological reaction. Additional information may be obtained by a POSITIVE ELIMINATION TEST and a PROVOCATION TEST^[45]. These tests are possible only with removable restorations, such as dentures. If the dentures are removed (elimination test), complaints may decrease and may reappear after reinsertion (provocation test). Additional manifestations of an allergy are extraoral symptoms, like eczema on hands or face. Purely subjective extra oral complaints, such as itching, that are chronologically linked to a material exposure, represent a limited indication for a patch test.

Prick test- This test is used to detect immediate-type allergies (type I reactions). Although the risk of provoking an immediate allergic reaction by the test itself is very minor, it cannot be completely excluded. Therefore, this test should be executed only by qualified personnel. The risk of sensitization of a patient by the prick test is considered low. The allergen is applied as a drop to the skin, and then the skin is pricked through the drop. After 5–30 min, the skin reaction is assessed for redness or formation of weals.^[46]

Radioallergosorbent test (RAST)- The RAST belongs to the group of in vitro tests for diagnosing an allergy. It is used to diagnose immediate type allergies (IgE mediated) by identifying an allergen-specific IgE in the patient's blood. This test can be used for diagnosing suspected allergies to medication or latex, potentially in combination with the prick test. But indication, execution, and assessment need to be done by an experienced allergologist through an allergy laboratory.^[47]

Immunotoxicological test methods- LYMPHOCYTE TRANSFORMATION TEST (LTT) is the most widely accepted immunotoxicological method. For the LTT, a blood sample is taken from the patient with suspected allergy, and then the proliferation of T-lymphocytes in the presence of the allergen is determined. This test has been claimed to detect sensitization to metals. The MEMORY LYMPHOCYTE IMMUNOSTIMULATION ASSAY (MELISA) is a modification of the LTT. Monocytes derived from the patient's blood are used for the MELISA. Although immunotoxicological testing of materials is a widely accepted procedure, the testing of the specific reactions of patients using the LTT and MELISA tests is still under scientific evaluation.^[48]

Measurement of intraoral voltage- All metals in the oral cavity are exposed to an aqueous environment. They corrode and at the same time release different positively charged ions. The metal surface thereby becomes negatively charged, which will then cause the attachment of positively charged ions from saliva. Voltage differences can be found against a reference electrode or between two metals in the oral cavity. If there is a conductive contact between the two metals e.g., direct contact or through wires, then ionic electricity can circulate in the tissue or saliva. Measurement devices are available that can be used for determining intraoral voltages between different restorations. These devices require a high internal resistance - at least 20 mega ohms. The current discharge is measured by determining the specific internal resistance.^[12,49]

Analysis of intraoral alloys-Clinical evaluation is difficult if restorations such as crowns, inlays, or bridges are fixed in the oral cavity and cannot be removed for identification of the alloy and the structure in the laboratory. In these cases, the composition of an intraoral alloy can be identified using the CHIP TEST⁵⁰. A small amount of alloy particles (chips) is produced intraorally using a silicon carbide stone or a tungsten carbide bur^[51]. The alloy particles are collected on a small, circular, self-adhesive graphite plate. This self-adhesive

carrier conducts electricity. Subsequently, the collected alloy particles can be identified quantitatively and qualitatively by means of EDX analysis.

Analysis of metals in saliva and biopsies-Examination of saliva to diagnose material linked side effects concentrates on the detection of metals, although most recently, resin components were also identified in saliva. A defined amount of “morning saliva” (before any food or drink intake or oral hygiene measures) is collected and, is analyzed, such as by atomic absorption spectrometry (AAS). Biopsies, for instance from the gingiva adjacent to metal restorations, were also used to determine the metal content. Metal concentrations in biopsies are usually analyzed by AAS.^[52]

CONCLUSION

The biocompatibility of dental materials can be only characterized based on a battery of different test methods. Statements about biocompatibility based on one test method only, have to be assessed very critically. Materials release substances specifically before setting and immediately after mixing, which may cause side effects. Therefore, dental personnel represent a risk group for these materials. Clinical studies are decisive for the final assessment of a material. However, regarding the biocompatibility there may be problems, since some damages, e.g., of the pulp, may occur without clinical symptoms. Therefore, clinical studies always need to be evaluated together with pre-clinical tests.

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