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RESEARCH ARTICLE

# Cytotoxicity Testing of Dental Materials

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## Abstract

**Objectives:** In this manuscript, we aim to review and evaluate the cytotoxicity data of 16 dental materials that have been generated in our laboratory over the past five years. **Methods**: Cytotoxicity data of dental materials generated in our laboratory over the past five years were retrieved from the archives. We collected and analyzed detailed information regarding the dental materials, tests conducted, regulatory standards followed, and the results obtained. **Findings:** Our laboratory conducted tests on a total of 16 dental materials over the course of the past five years, which included both metals and polymers. The commonly employed regulatory standard for these tests was ISO 10993-5 and ISO 7405. We utilized various methodologies such as elution, direct contact, agar diffusion, and colony formation assays. Out of the 16 materials tested, 15 were found to be non-cytotoxic. However, during the agar diffusion assay, one specific material, namely Oxyalkylene polymer with synthetic diamond powder, exhibited moderate cytotoxicity. **Novelty:** This study contributes to the existing body of knowledge by providing detailed information on the cytotoxicity profiles of certain dental materials, including both metals and polymers.

**Keywords:** Dental Biomaterials; ISO 7405; ISO 109935; Biocompatibility; Cytotoxicity

## 1 Introduction

Teeth are important sensory organs that support our daily activities. Sadly, there are a variety of causes for tooth loss, including trauma, caries, and periodontal disease. Although dental caries and trauma injuries are more common in younger people, tooth loss due to periodontal disease affects older people more frequently[(1)]. Sometimes it seems like the fashion in the dental implant age is to extract problematic teeth and replace them with dental implants. There are numerous treatment options available to either save teeth or replace missing teeth in the dental implant era of today[(2)]. Dental implants, supportive periodontal therapy, guided bone regeneration,

periodontal therapy, and endodontic therapy are all options [3]. Nearly 12 million implants are being inserted each year in the world, and this number is steadily rising [4]. In line with this, more peri-implantitis cases are anticipated. Peri-implantitis with bone loss affects about 28% of all implants. According to a Swedish study, 4.2% of implant users lost their implant after 9 years [5].

Biomaterials including Metals such as Titanium, stainless steel, metal alloys such as Co-Cr-Mo, Fe-Cr-Ni are commonly used for dental applications; in addition, ceramics and polymers are also used as dental biomaterials based on the application. The safety of these biomaterials needs to be carefully assessed given the rise in their application. Biocompatibility of medical devices including those used for dental applications are evaluated using the ISO 10993 [6] and ISO 7405 [7] standards. Dental materials are tested for *in vitro* cytotoxicity, sensitization potential, irritation potential, systemic toxicity and local effects following implantation.

The American Dental Association has provided certain guidelines for dental implants, one of which is to determine the safety and biocompatibility of the dental biomaterial, including testing for cytotoxicity and tissue interference characteristics [8].

In this manuscript, we have reviewed a range of dental biomaterials that were tested in our laboratory for their potential to cause cytotoxicity. Results from various cytotoxicity testing methods are elaborated.

## 2 Methodology

We identified all cytotoxicity studies conducted in our laboratory over the past five years by referring to the master schedule and study files. From the study files, we collected the following information: materials used, cytotoxicity test methodologies employed, and the corresponding results obtained.

### 2.1 Dental Materials

The following dental materials were tested in our laboratory: Titanium alloy (Ti-6Al-4V ELI), 304 stainless steel, 316L stainless steel, bovine cancellous bone (calcium phosphate), aluminium corundum, titanium grade 4, oxyalkylene polymer with synthetic diamond powder, vinylpolysiloxane, silver fluoride salt solubilized in water with ammonia, peritoneum from porcine origin, co-polyester, ethyl methacrylate polymer, medical grade silicone, zirconia, ethylene/vinylene acetate copolymer, and stainless steel with diamond particles.

### 2.2 Cytotoxicity methodologies

#### *2.2.1 Agar Diffusion*

The ISO 10993–5 (2009) and USP (2017) qualitative agar diffusion/ overlay assay is appropriate for high density and liquid devices. This assay is conducted by adding a thin layer of nutrient-supplemented agar over the near confluent monolayer of L929 cells, and the test material (a solid sample, or an extract of the test material or liquid placed on filter paper) is placed on top of the agar layer. The cushioning effect of an agar layer protects the L929 cells from potential mechanical damage by the test material's movement. Cells are examined after 24 h incubation for signs of toxicity and stained with neutral red dye to differentiate between the viable, stressed or lysed cells. Cytotoxic leachates diffuse into the cell layer through the agar, and toxicity is indicated by a loss of viable cells around the test device as evidenced by area devoid of stain under and around the test material (ISO 10993-5, 2009; USP, 2017).

#### *2.2.2 Filter Diffusion*

Balb/c 3T3 cells cultured in DMEM with 1% penicillin/streptomycin antibiotic and 10% heat inactivated NBCS were used in the study. The cells were approximately 75% confluent. For the filter diffusion assay, 2.5 x $10^5$ cells per mL were seeded in 6-well plates. In a sufficient number of culture dishes, 3 mL of freshly prepared agarose supplemented with culture medium was added and allowed to solidify at room temperature. The cellulose acetate filters with the cell monolayers, were carefully removed, washed with phosphate-buffered saline solution at 37 $\pm$ 1 °C and transferred, cell side down on to a layer of solidified agar. The extracts of the test item, positive (Natural rubber latex) and negative control (HDPE- High Density Polyethylene film) were loaded on the top of the filters (acellular layer) and incubated for 2 h and 24 h at 37 $\pm$ 1 °C in 5% CO2. Evidence of cytotoxicity was evaluated after exposure periods of 2 h $\pm$ 10 min and 24 h $\pm$ 10 min. Following incubation, the filter paper was gently loosened from the agar/agarose. The area of reduced cellular enzyme activity was cytochemically assessed using succinate dehydrogenase method or non-specific hydrolase method using fluorescein diacetate dye. Following staining of the filters, the diameter of the zone formed was measured and graded based on the area of decolorization.

### *2.2.3 Colony Formation*

Colony formation assay involves the use of V79 cells cultured in MEM (Minimum Essential Medium) with 1% penicillin/streptomycin antibiotic and 10% heat inactivated HI-FBS. The cells used in the experiments were approximately 75% confluent. For the colony formation assay, 2.5 x $10^5$ cells per mL were seeded in 6-well plates. After 24 h, the cell confluency and morphology were assessed under a microscope. The media was then carefully removed and replaced with 2 mL of the test item extract prepared in various dilutions, negative control (HDPE- High Density Polyethylene film), positive control (Polyurethane film containing 0.1% zinc diethyldithiocarbamate -ZDEC), extracts at five different concentrations (i.e., 2%, 4%, 6.25%, 50% and 100%), and blank. All the treatments were performed in triplicates. The 6-well plates were then incubated at 37 ± 1 ºC with 5% CO2 for 6 to 7 days. After incubation for minimum 6 days, all the wells were rinsed with PBS. The colonies were then fixed with methanol for 5 min and stained with 5 % Giemsa solution. The number of colonies in each well was counted.

### *2.2.4 Direct contact*

Exponentially growing Balb/c 3T3 cells were seeded in culture flasks at a concentration of 1 x $10^5$ cells per mL and maintained at 37 °C for 24 h in an atmosphere of 5% $CO_2$. On the day of treatment, after confirming confluency (80%) fresh medium was replaced in each culture flask. Then, the test item, Negative control (HDPE- High Density Polyethylene film), and Positive control (natural rubber latex) was placed on the respective culture flasks. After 24 h, both qualitative and quantitative (neutral red uptake) evaluations were performed.

### *2.2.5 Elution methods*

2.2.5.1 MTT assay. Exponentially growing L-929 mouse fibroblasts cells were seeded in 96-well plate at 1 x $10^4$ cells per well. After 24 h, 1x MEM supplemented with 10% heat inactivated fetal bovine serum and 1% penicillin/streptomycin solution (complete growth medium) was removed and replaced with a series of six different dilution of the test item extract (undiluted, 1:2, 1:4, 1:8, 1:16 and 1:32), vehicle control, positive control (HDPE- High Density Polyethylene film) extract and negative control (Polyurethane film containing 0.1% zinc diethyldithiocarbamate -ZDEC) extract. The cell cultures were then incubated with 5% CO2 at 37 ± 1 ºC > 90% humidity for 24 h. After incubation, the cells were subjected to qualitative measurements viz., cytotoxicity was graded based on the morphology of the cells.

2.2.5.2 XTT assay. Exponentially growing L-929 mouse fibroblasts cells were seeded in 96-well plate at 1 x $10^4$ cells per well. After 24 h, 1x MEM supplemented with 10% heat inactivated foetal bovine serum and 1% penicillin/streptomycin solution (complete growth medium) was removed. The cells were treated with positive control, negative control, vehicle control and four different concentrations (12.5%, 25% 50% and 100%) of the test item extract. Six replicate cultures were treated for each concentration. The plates were then incubated at 37 ± 1°C with 5 % CO2 for 24 h. After 24 h of incubation period, the cells were observed to identify systematic cell seeding errors and growth characteristics of the control and treated cells.

2.2.5.3 NRU assay (neutral red uptake). Exponentially growing Balb/c 3T3 cells were seeded in a 96-well plate at a concentration of 1 x 104 cells/well. After 24 h of incubation, the cells were approximately 80% confluent. The complete growth medium was removed from all the wells and six replicates of appropriate concentrations of the test item extract (30, 40, 50, 60, 70, 80, 90 and 100%), neat extract of negative control (100%), vehicle control and positive control (0.07 mg/mL) were added to their respective culture wells. The plate was then incubated at 37 ± 1°C, > 90% humidity with 5 % CO2 for 24 h. After incubation, the cells were evaluated qualitatively (microscopic evaluation) to determine cell morphology and quantitatively (neutral red uptake method) to determine cell viability.

## 3 Results and Discussion

The advent of novel biomaterials has certainly improved the quality of life of patients in terms of preserving aesthetics and functionality. In terms of therapeutic efficacy, biocompatibility is one of the most important properties of dental materials [9,10]. Chemical, mechanical, electrical and surface properties can affect biocompatibility [11,12]. In line with this, the ISO 10993-1 [13] necessitates physical and chemical characterization as the first step in the biological evaluation of a medical device. However, it cannot be used to identify local effects and therefore some basic biocompatibility tests that involve local reactions have to be performed.

The *in vitro* cytotoxicity is the preliminary screening test to determine if the leachables from a given biomaterial or device could be potentially cytotoxic. ISO 10993-5:2009 [7] is the testing standard that describes test methods to assess the *in vitro* cytotoxicity of medical devices. Each test method has its own specification in terms of cell line, exposure time, method of exposure, incubation time and determination of cytotoxicity. While ISO 10993-5 provides guidance for *in vitro* cytotoxicity

**Table 1.** Results of cytotoxicity testing on dental devices

| Biomaterials | Dental device types | Agar diffusion method | Colony forming assay | Filter diffusion method | Direct contact | Elution method |
|---|---|---|---|---|---|---|
| Titanium alloy (Ti-6Al-4V ELI) | Dental implants | Non cytotoxic | | | | Non-cytotoxic |
| 304 stainless steel and 316L stainless steel | Dental space maintainers | Non cytotoxic | | | | |
| Bovine cancellous bone (calcium phosphate) | Dental mineral matrix | Non cytotoxic | | Non cytotoxic | | Non-cytotoxic |
| Aluminium Corundum | Dental fillings | Non cytotoxic | | | | |
| Titanium Grade 4 | Dental implants | Non cytotoxic | | Non cytotoxic | | Non cytotoxic |
| Oxyalkylene polymer with synthetic diamond powder | Dental polish | Moderately cytotoxic | | | | |
| Vinylpolysiloxane | Dental impression material | | Non cytotoxic | | | |
| Silver fluoride salt in water solubilised with ammonia | Dental varnish | | | | Non cytotoxic | |
| Peritoneum from porcine origin | Collagen membrane | | | | | Non cytotoxic |
| Co-polyester | Dental braces | | | | | Non cytotoxic |
| Ethyl Methacrylate Polymer | Dental braces | | | | | Non cytotoxic |
| Medical Grade Silicone | Washer | | | | | Non cytotoxic |
| Zirconia | crown | | | | | Non cytotoxic |
| Ethylene/Vinylene Acetate Copolymer | Mouthguard | | | | | Non cytotoxic |
| Stainless steel with diamond particles | Dental burr | | | | | Non cytotoxic |
| polymer | Root canal filling | | | | | Non cytotoxic |

testing, detailed test protocols for sample preparation for the agar diffusion and filter diffusion methods specific to dental materials are provided in ISO 7405.

This manuscript aimed to assess the results obtained from testing sixteen different biomaterials used for dental applications using various test methods. Out of the 16 materials tested (Table 1), 15 were non-cytotoxic and one of the materials, Oxyalkylene polymer with synthetic diamond powder was moderately cytotoxic. The cytotoxicity observed with Oxyalkylene polymer with synthetic diamond powder could be attributed to the presence of residual monomers and/or manufacturing residues. Polymers, in general are not cytotoxic in their cured forms; residual monomers may be responsible for cytotoxic responses. Curing of polymers also has a great impact on the cytotoxicity of dental materials. Lee et al[14], which reported that uncured materials were the most cytotoxic, followed by light-cured materials and those with the oxygen-inhibition layer removed.

Biocompatible materials are expected to be non-toxic, non-immunogenic, non-mutagenic and non-carcinogenic[15]. Cytotoxicity is a preliminary test in the biocompatibility testing panel and a positive cytotoxic response is considered indicative of a potential *in vivo* toxicity. It is important to note that cytotoxicity involves the use of cells cultured *in vitro*, which makes them vulnerable to cytotoxic leachables. However, as the ISO 10993 standards indicate, positive cytotoxic response does not represent the end of a biomaterial/device and that the biological relevance of the observed cytotoxicity must be studied *in vivo* using animal models. If the *in vivo* tests, usually, the intracutaneous irritation test in rabbits/oral mucosal irritation test in Syrian hamsters and the sensitization test in guinea pigs indicate lack of any irritation or sensitization, then the *in vitro* cytotoxicity observed is not considered to be biologically relevant.

In summary, this study contributes to the existing body of knowledge by providing detailed information on the cytotoxicity profiles of certain dental materials, including both metals and polymers.

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