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In vitro evaluation of cytotoxicity of permanent prosthetic materials

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SUMMARY

Objectives. To assess qualitative and quantitative cytotoxicity effect on permanent prosthetic materials to human gingival fibroblasts.

Methods. Human gingival tissues were collected (with informed consent) from patients undergoing periodontal surgical procedures and fibroblasts were cultured in vitro. Cell type was determined by performing proteomic analysis. Selected prosthetic materials including titanium, feldspathic ceramic, gold and chrome-cobalt alloy specimens (5×2 mm) were fabricated. The toxicity of prepared specimens was tested by exposing them to cell culture medium for 48, 72, 96 and 120 hours at 37°C under sterile conditions. Cell viability was estimated using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. The data concerning cell viability were statistically analyzed using two-way ANOVA test and Tukey multiple comparison test.

Results. Results obtained after 48 hours showed no toxic effect of titanium compared to control group. Cytotoxic effect was observed in gold alloy and feldspathic ceramic, however, it was not significant compared to control group. Chrome-cobalt alloy significantly reduced cell viability compared to control group ($p \leq 0.001$). Cytotoxicity diminished with increasing incubation time of specimens.

After 120 hours of incubation all tested materials, except chrome-cobalt alloy, had no cytotoxicity.

Conclusions. Titanium proved to be non-toxic. Gold alloy and feldspathic ceramic had short-term cytotoxic effect. Chrome-cobalt alloy had highest cytotoxic effect on fibroblast cells.

Key words: cytotoxicity, prosthetic materials, gold alloy, feldspathic ceramic, titanium, chrome-cobalt alloy, human gingival cells, fibroblasts.

INTRODUCTION

A great variety of different types of alloys and ceramic materials used in prosthodontics is currently available in the market. Those most widely used are titanium, cast gold and chrome-cobalt alloy as well as all-ceramic materials. When used for treatment purposes permanent prosthetic materials remain for an extended period of time in close contact with the surrounding soft tissues. When placed subgingivally the biocompatibility of such materials is absolutely crucial as having a direct effect upon the condition of marginal gingiva surrounding teeth and implants. Moreover, dental materials should contain no diffusible, leachable or toxic substances that could be absorbed into the circulatory system, causing toxic responses or interrupting the healing process of surrounding tissues. Therefore, it is absolutely necessary to have sufficient knowledge about the biocompatibility of dental materials in relation to the surrounding oral tissues [1].

The concept of biocompatibility should be understood as an ability of a material to perform its intended physical as well as mechanical functions without eliciting any undesired response from the surrounding living tissues [2]. Cytotoxicity term

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is used to define series of molecular events that interfere with macromolecular synthesis leading to indubitable cellular, functional as well as structural damage [3]. The cytotoxicity of dental materials can be assessed directly or by releasing the constituting components from the specimen of the material [4]. Permanent cell lines as well as primary cells of oral fibroblasts have been found to be suitable indicators of an early cytotoxic effects of dental materials [4]. Findings of some studies suggested that primary cells have high degree of differentiation so their reaction pattern might be more similar to reactions in vivo [5].

The present research exercise included an evaluation of the cytotoxic effects on oral fibroblasts produced by the most commonly used prosthetic dental materials.

Objective

The objective of the present research exercise was to assess qualitative and quantitative cytotoxicity effect on permanent prosthetic materials to human gingival fibroblasts following ISO 10993-5:1999 protocol.

METHODS AND MATERIALS

Tissue collection and cell culture of primary human gingival fibroblasts

The protocol for the present research was approved by Vilnius regional biomedical research ethics committee at Vilnius University (No. 158200-11-116-28). Informed consent was obtained from the patient surveyed. Human gingival subepithelial tissues were obtained from a healthy patient undergoing a gingivectomy procedure in the premolar region. Immediately after the biopsy, the tissue (2-3 mm^3 in size) was placed in Dulbecco's modified Eagles Medium (DMEM) enriched with 250 U/mL penicillin, 0.25 mg/mL streptomycin, 0.05 mg/mL gentamycin and 200 U/mL Nystatin for transportation. Then, the subepithelial tissue specimen was minced under sterile conditions and placed into the wells of 96-well plate with Iscove's modified DMEM (IDMEM), supplemented with 20% of foetal calf serum including the antibiotics as referred to above. The plates were incubated at 37°C in humidified atmosphere 95% air and 5% CO_2.

The process of the cell movement from the explants commenced at the 7th to 10th day. After the monolayer was completely formed, the cells were subcultured by using trypsin/EDTA mixture and maintained in IDMEM supplemented with 10% FCS and antibiotics. Cells between passages 5 to 8 were used in experimental work.

Table. Effects of material type and incubation time on gingival cell cytotoxic effect.

Source	F=value	p
Material type + Incubation time	12.282	.000
Incubation time	231.952	.000
Material type	120.736	.000

Cytometric analysis

Primary cell line was characterized by a BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA). Antibodies were used to target CD34, CD29 stem-cell markers as well as Sca-1 fibroblast progenitor cell marker. The surveyed cell culture expressed Sca-1 protein excluding CD34 or CD29 molecules. It was determined that the cell culture mostly included fibroblast progenitor cells.

Manufacture of specimens

Material samples included commercially pure titanium (Grade 2, Everest® T-Blank, KaVo, Biberach, Germany), dental gold alloy (86.2% Au, 11.5% Pt, 1.5% Zn; Bio Heragold B, Heraeus Kulzer, Hanau, Germany), chrome-cobalt alloy (61% Co, 25% Cr, W 9%, Nb 2%, Mo 1.5%, Si 1%, Fe 0.1%, C<0.01%; Ceralloy C, Eukamed, Essen, Germany), feldspathic ceramics (Vita VMK 95, Vita Zahnfabrik, Bad Sackingen, Germany). Titanium specimens were fabricated by Everest CAD/CAM system using a cylinder wax stick (∅=5.2 mm). Gold and chromium-cobalt alloy specimens were moulded using a plaster model. Feldspathic ceramic specimens were fabricated using conventional methods. All specimens were equal in their diameter and height (5.2 mm × 2 mm). Fabricated specimens were divided into four groups (n=5): polished titanium (Everest® T-Blank), gold alloy (Bio Heragold B), chrome-Cobalt alloy (Ceralloy), feldspathic ceramics (Vita VMK 95).

Cytotoxicity assay

Toxic effect of permanent prosthetic materials was evaluated on human gingival cells using a direct-contact format according to the specifications of ISO-10993-5:1999. Specimens of each material (n=5) were sterilized in 96% ethanol with subsequent exposure of both surfaces to UV light for 15 minutes. For cytotoxicity test, the cell suspension of 40 000 cell/ ml was prepared from the primary gingival cell culture. Material specimens were transferred to a 96-well plate and placed in a direct contact with the cell suspension. 5 empty wells with growing gingival cells were used for control purposes.

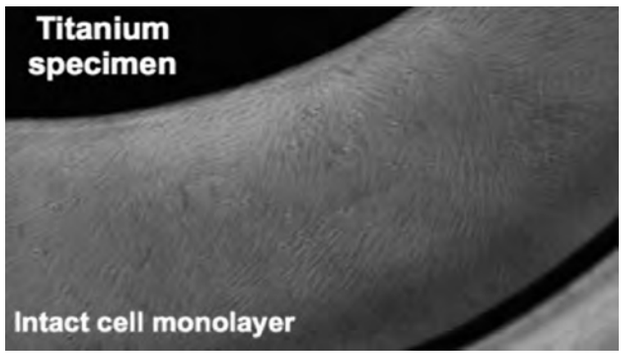

Fig. 1. Intact cell monolayer on titanium visualized by an inverted microscope after first 24 hours

Fig. 2. Disrupted cell monolayer on a chrome-cobalt specimen after first 24 hours

The intensity of cell growth including toxicity of trial materials was observed visually with an inverted microscope by assessing the quality of cell monolayer around the specimen (Fig. 1 and 2) and by using MTT colorimetric test. The duration of the cell-specimen contact was 48, 72, 96 and 120 hours. Following each test material specimens were repolished and sterilized as mentioned earlier. Optical profilometer based on the confocal principle (PLμ 2300, Sensofar, Terrassa, Spain) was used for the visualization of the cell growth (after 120 hours of incubation).

MTT colorimetric assay

The cell viability was determined using the MTT test that is based on mitochondrial succinate dehydrogenase (SDH) ability to reduce the yellow MTT dye (the solvent Tetrazolium salt) into the insoluble blue Formazan. The amount of formazan is directly proportional to the number of viable cells [6]. After adding solution, optical densities (OD) of the resulting solutions were measured with a microplate reader (TECAN Infinite 200) at 570 nm. Optical density was converted to a percentage of the controls for each cell-culture plate.

Statistical analysis

The effects of material type and incubation time on cell viability were evaluated using the two-way ANOVA with statistical software (SPSS 16.0.1 for Windows, SPSS Inc., Chicago, IL, USA). Tukey's HSD post hoc analysis was used to define statistically significant differences among the groups. Statistical significance was set at $p<0.05$.

RESULTS

According to the two-way ANOVA, a statistically significant ($p<0.05$) difference was found between the groups when cell viability was considered. Further the process included the evaluation of the influence of material type and incubation time on the viability or cells. The most significant effect on the cell viability was produced by the incubation time (F-value=231), whereas material type had less effect (F-value=120) (Table).

Qualitative evaluation

Optical profilometry (PLμ 2300, Sensofar) performed on fibroblast-seeded samples after 120 hours showed typically mosaic-shaped confluent cell layer that formed on all specimen surfaces (Fig. 3-6). Fibroblasts appeared to be intimately attached to the surfaces and aligned in rows. Abundant fibroblast cells were visualized on titanium, gold alloy and feldspathic ceramic specimens. A disrupted cell layer was observed on chrome-cobalt specimens.

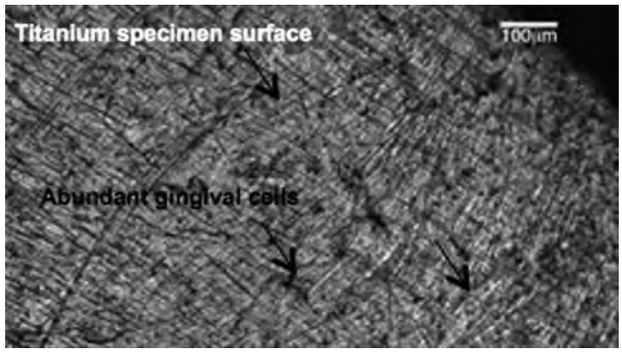

Fig. 3. Optical profilometry image of a titanium specimen after 120 hours of incubation

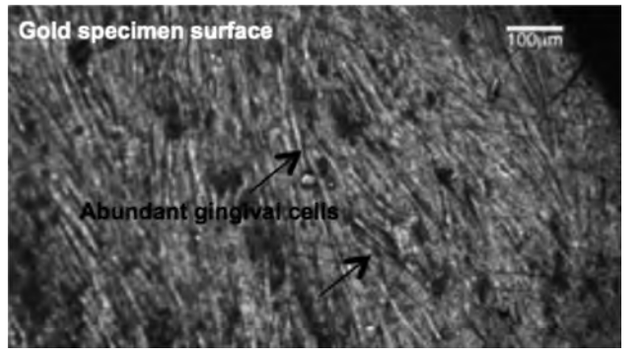

Fig. 4. Optical profilometry image of a gold alloy specimen after 120 hours of incubation

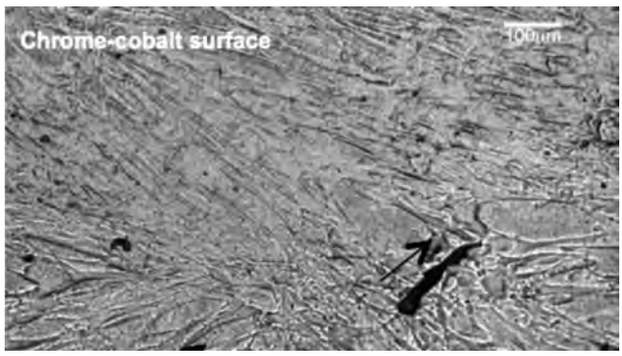

Fig. 5. Optical profilometry image of a chrome-cobalt specimen after 120 hours of incubation

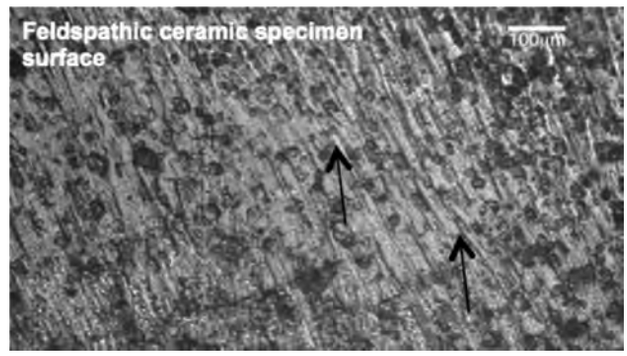

Fig. 6. Optical profilometry image of a feldspathic ceramic specimen after 120 hours of incubation

Quantitative evaluation

Results obtained after 48 hours showed no toxic effect of titanium compared to the control group. Cytotoxic effect was observed in gold alloy (p=0,07) and feldspathic ceramic (p=1.00), however, it was not significant compared to the control group. The chrome-cobalt alloy significantly reduced the cell viability compared to the control group ($p \leq 0{,}001$). Cytotoxicity was diminishing with increasing incubation time of specimens (see Fig. 7).

After 120 hours of incubation the materials subject to the test showed a significant increase in cellular viability, except for chrome-cobalt alloy, in which case the increase was insignificant.

DISCUSSION

An objective of the present research was to evaluate the cytotoxic effects of commercially pure titanium, gold alloy, chrome-cobalt alloy and feldspathic ceramic on human gingival fibroblasts.

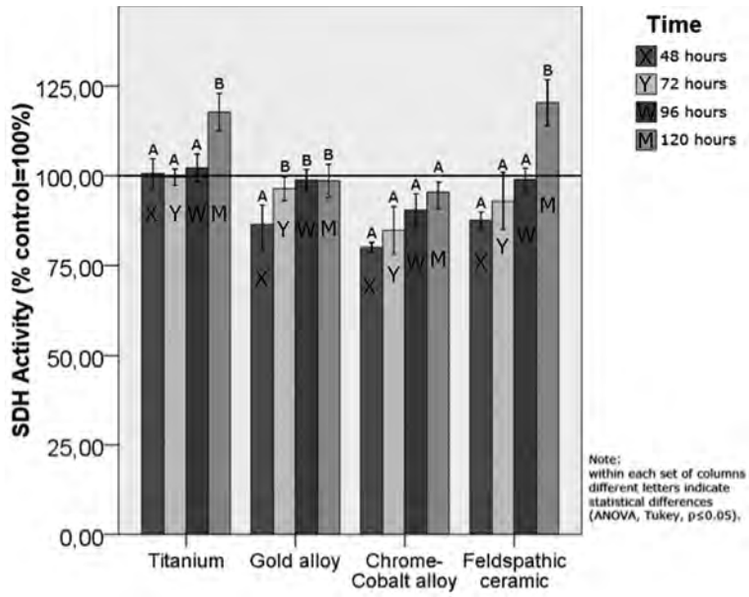

Fig. 7. Graphical representation of fibroblast SDH activity on different types of material and varied incubation time

Human gingival fibroblasts have been widely used to assess cytotoxic effects of various prosthetic materials [7-8].

The present study showed that cytotoxicity levels of all tested materials, except the commercially pure titanium, were decreasing with the extension of the incubation time. The results obtained indicated that the materials were not inert and could provoke certain biological responses [9]. A list of *in vitro* studies [7, 10-12] report reduction of cellular viability due to corrosion products or surface properties of ceramics and metals.

Corrosion products also have an ability to increase cytotoxicity in cells [13]. Corrosion can be minimized by physical limitation of the rate at which reduction-oxidation processes take place. It is known that passivation or the formation of a metal oxide passive layer on a metal surface is a kinetic limitation to corrosion. This passive layer must be without pores, fully cover metal surface, and resist abrasion and mechanical stressing [14]. Defect or removal of such passive oxide layer may renew the process of corrosion of an alloy [15].

Some elements, for example, copper have a higher probability to be released from dental alloys compared to gold alloys that to a lesser extent tend to be released at higher concentrations [16]. Evidence was obtained to conclude that high gold alloys usually contain some amounts of Zn that is responsible for scavenging oxygen and as a rule lowers corrosion [17]. It is a common knowledge that noble metals have better corrosion resistance compared to base metals. Different type of dental alloys may have significant influence on the amounts of elements released. Reported cases indicate that element release into cell culture medium may continue for up to 10 months [18].

The findings of the present study concluded that chrome-cobalt showed the highest levels of cytotoxicity (<20%). Nevertheless, it can be considered as little or mild suppression of SDH activity as most researchers in biocompatibility agree that it is clinically acceptable [18]. Still, the existing level of cytotoxicity may be related to the ions released from chrome-cobalt alloy in culture medium. It has been observed that the entry of Cr^{+3} ions into cells encounters certain difficulties [20], while the findings also showed a decrease in proliferation as well as RNA synthesis in fibroblasts [7, 21].

Messer [12] found that in the first 24 hours of Cr^{+3} cytotoxic experiment fibroblast mitochondria was smaller, with lower visible numbers of polyribosimes and non-dilated rough endoplasmic reticulum (rER). Nevertheless, the rER was dilated in the 72 hours exposure time that may indicate increased metabolic activity and the cell proliferation recovery mechanism.

The research data on cytotoxicity of traditional and contemporary dental ceramics provided evidence that feldspathic ceramics tend to have low initial toxicity effect on mouse fibroblasts [10]. This finding is similar to the results obtained within the present study in the sense that feldspathic ceramic caused only mild (<10% of controls) mitochondrial suppression. Initial cytotoxicity may be related to material surface roughness as well as its composition. A number of studies in the field reported that different volume of mass loss occurs from different ceramics [9, 22].

A major finding under the present research that commercially pure titanium showed no toxic effects on human gingival cells is in agreement with those obtained in other similar studies [23, 24].

TiO_2 can almost be considered as stoichiometric oxide that does not have many ionic defects in its layer, thus only lower level of the transport of the substance into the external environment may be expected [25].

The results of the present study show that the biocompatibility of the tested materials can be enhanced following a prolonged exposure to an organic environment. Cells must attach to a surface in order to function and divide in a normal fashion. Keeping this in mind, cell growth rates may seem decreased and show possible toxic effect of the trial materials. However, it is known that cell surface has a negative charge whereas artificial materials may have either a negative or a positive charge. It is logical to assume that positively charged surfaces could facilitate initial cell adhesion. In addition, cells secrete ECM (extra cellular matrix) and form focal adhesion sites to facilitate adhesion as well as proliferation processes [25].

It must be stressed that different recipient's gingival cells may produce different cytotoxicity results [26], moreover, variations may be observed even within the same cell line [19]. Latter differences may be associated with varying experimental techniques such as deviation in the passage number of the cell as well as the cell density. Therefore any forthcoming studies on the subject matter could include an additional analysis of element release into medium, and the chemical and physical characterization of the surface to facilitate the development of biologically improved dental ceramics or alloys.

CONCLUSIONS

Taking into consideration the limitations related to the present study it can be concluded, that titanium alloy was found to be highly biocompatible. Gold alloy and feldspathic ceramic produced only a minor effect on the reduction of cell viability. Chrome-cobalt alloy had highest cytotoxic effect on fibroblast cells. Significant decrease in cytotoxicity over time of the selected prosthetic materials was observed except chrome-cobalt alloy, in which case however, the cytotoxic effect was within the clinically acceptable limits.

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