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EXPERIMENTAL / LABORATORY STUDIES

Influence of Dental Alloys and an All-Ceramic Material on Cell Viability and Interleukin-1beta Release in a Three-Dimensional Cell Culture Model

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Abstract: The purpose of this study was to determine the influence of various types of dental casting alloys and ceramic upon cell viability and the synthesis of IL-1beta (β) in a three-dimensional cell culture system consisting of human gingival fibroblast, and to determine their effect in gingival inflammation. Au-Pt-In alloy (Pontostar), Ni-Cr-Mo alloy (Remanium-CS), a titanium alloy (Ti-6Al-4V), copper (Cu), and an all ceramic (In-Ceram) were used as test materials. The materials were exposed to a three dimensional cell culture in order to determine their effect on cell viability and IL-1β secretion level. Cell viability was measured by MTT test after exposure for 24h and 48h. For IL-1β measurement, assay aliquots were taken from exposed media after 1h, 3h, 7h, 24h and 48h. Assays for IL-1β were carried out by ELISA. In-Ceram, Pontostar and Ti-6Al-4V alloy did not influence cell viability. Copper (52-64%), and Remanium CS (17-20%) were found to be cytotoxic, compared to control cultures. In-Ceram, Pontostar and Ti-6Al-4V had no significant influence on IL-1β secretion. Remanium-CS increased IL-1β levels two fold compared to untreated control cultures. Our findings suggested that Ti-6Al-4V, In-Ceram and Pontostar did not cause elevated IL-1β release from cells at non-toxic levels. On the other hand, Remanium-CS showed moderate toxicity and caused increase in IL-1β levels.

Key Words: Cell viability, MTT, IL-1β, Dental alloys, Three-dimensional cell culture

Introduction

Many alloys are used in fixed and removable prosthodontics. When selecting a dental casting alloy for a clinical situation, the dentist's decision may be influenced by the physical properties of the alloys, cost and biocompatibility (1). High noble alloys have successful clinical use but as the price of gold increased, alternative cheaper based alloys (Co-Cr, Ni-Cr) were developed (2). However, nickel, cobalt, and chromium may sometimes cause sensivity (3). In contrast, titanium and its alloys have high biocompatibility in the mouth (4).

Despite the long-standing use of alloys and ceramic as fixed and removable restoration materials, there still are open questions about the behaviors in the biological environment (5). To investigate this possibility, prosthodontic research must involve cell and molecular biological approaches to assess the host's immune, non-immune, and chronic inflammatory responses to materials in contact with the oral tissues (5,6).

Cell culture studies are useful tools for dental material investigations. Cell culture methods, are better standardized and reproducible. They are rapid and easy to perform at relatively low costs (7). Special cell culture methods have been developed. A three-dimensional (3 D) cell culture system has been described for testing the time-dependent irritancy of cosmetic products (8,9). In this cell culture model, fibroblasts attached to the mesh proliferated, and created an environment similar to in vivo situations (8-11). Further studies have demonstrated the possible suitability of this system for toxicity testing of dental materials (12).

The immune system reaction is followed by the production of various soluble substances, called cytokines. These molecules have many biological

functions and they play a physiological role in bone remodeling. Deregulated cytokine and immunoglobulin production at local disease sites have been considered to be major contributors to the development of inflammatory reactions. Among the numerous cytokines involved in the induction and regulation of host responses in inflammation, IL-1β seems to play a central role in the inflammatory reaction. IL-1 has been shown to enhance various immune responses in vitro, including B lymphocyte differentiation (13,14). Masada et al. (15) showed that both IL-1a and IL-1β were produced and released locally in periodontal disease at concentrations sufficient to mediate tissue inflammation and bone resorption. Jandinski et al. (16) also demonstrated that IL-1β producing cells were present in greater numbers in tissue of diseased sites than in normal tissue.

The aim of this study was to evaluate effects of titanium alloy (Ti-6Al-4V), Au-Pt-In alloy (Pontostar), Ni-Cr-Mo alloy (Remanium-CS) and all-ceramic (In-Ceram) on cell viability and IL-1β release in 3D human gingival fibroblast cultures, as an indicator of their biological performance in gingival tissues.

Materials and Methods

Preparation of the test materials

The test materials used in this study were Ti-6Al-4V, Pontostar, Remanium CS, In-Ceram and Cu. The composition of the test materials are given in Table 1. The specimens of the Pontostar, Remanium CS and Ti-6Al-4V alloy (10mmx10mmx1mm) were prepared by the loss-wax technique. Ti-6Al-4V patterns were invested (Rematitan Plus, Dentaurum) and the invested specimens were heated on a slow heat cycle and casting proceeded using a 31 g ingot in a titanium casting machine (Rematitan, Dentaurum) set to 0.95 bar argon pressure. The wax patterns for Palladium and Remanium CS alloy were invested (Deguvest Impact, Degussa) and the castings were produced in a centrifugal casting machine with an electric crucible (Multicast Compact, Degussa). The same size of In-Ceram, an aluminum oxide ceramic system, was prepared according to the manufacturers' instructions (VITA, Germany). The test materials were ground and polished to simulate clinical conditions (17). The polished test materials were subjected to 95% ethanol for 5 minutes, rinsed in sterile water, cleaned in acetone for 10 minutes, and dried.

Cell culture

All participants in the study gave informed consent to the experimental procedures. Local ethic committee approva was obtained for this study. Healthy human gingival tissue was obtained from six volunteer subjects undergoing extraction of the premolar region for orthodontic reasons. Patients were recruited from non smokers aged between 17 and 25. Patients suffering from any chronic inflammatory or immunological condition and systemic infections were not included in the study. Removed gingival tissue was immediately placed in Hanks' balanced salt solution (HBSS) (Biological Industries, Kibbutz Beit Haemek, Israel) containing penicillin (100 units/ml) (Sigma, St Louis, MO, USA)/ streptomycin (100 microgram/ml) (Sigma, St Louis, MO, USA). Thereafter biopsies were stored at 4 °C for a maximum of 6 hours. Specimens were minced into small pieces (3x3x3 mm) and the epithelium was separated from the lamina propria by using thermolysin treatment (500 µg/ml). Gingival fibroblasts were extracted from the lamina propria by using 0.125U/ml of collagenase-P (Boehringer Mannheim, Laval, QC, Canada) (18). Fibroblasts were cultivated with 5% CO_2 at 37 °C in Dulbecco's modified Eagles medium (Biological Industries, Kibbutz Beit Haemek, Israel) and 10% fetal calf serum

Table 1. Composition of test materials

MANUFACTURER	TEST MATERIAL	COMPOSITION (MASS %)
Bego, Germany	Pontostar	85.6 Au, 11.4 Pt, 2.3 In
Dentaurium, Germany	Remanium CS	61 Ni, 26 Cr, 11 Mo, 1.5 Si < 1 Fe <1 Ce, <1 Al
Oslo, Germany	Titanium-6Aluminium-4Vanadium[a]	Ti 6.15, Al 4.08, V 0.026C, 0.21 Fe, 0.009 N
Vita, Germany	In-Ceram	Al_2O_3
Aldrich,Germany	Copper	99,9%

[a]Ti-6Al-4V grade 5, according to ASTM Designation B. 265-279

(FCS) (Biological Industries, Kibbutz Beit Haemek, Israel) containing penicillin/streptomycin and amphotericin B (100 mg/ml) (Sigma, St Louis, MO, USA). When the cultures reached 100% confluence, the cells were detached and used for the three-dimensional cell culture (11,17-19).

Before use, medical-grade nylon meshes (Tetko, Minneapolis, MN, USA) were pretreated with 0.1M acetic acid, thoroughly washed with phosphate buffered saline (PBS) (Biological Industries, Kibbutz Beit Haemek, Israel) and sterilized. They were then placed in 6-well-plates and coated with fibronectin (RK028A, DiaPharma, US) (0.1% in distilled water). The third passage of human gingival fibroblasts were seeded to medical-grade nylon mesh (100µ, 10 x 10 mm) 5 x 10^4 cells/cm^2 in conventional growth medium. The carrier grids were then placed into 24 well plates and incubated at 37 °C in 5% CO_2. Next, one test specimen was placed on each cell culture. Contamination of cell cultures was excluded by visual control of the cultures under the light microscope. At different time points (1, 3, 7, 24 and 48 hours) the media were collected for IL-1β assay and stored (-80 °C) until required. Each material was tested 3 times; untreated cultures were used as negative controls. Specimens of copper were included in each experiment as a positive reference material.

MTT assay

The succinic dehydrogenase (SDH) activity of the cells is representative of the mitochondrial function of the cells. The SDH activity was measured by the 3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-SH-tetrazolium bromide (MTT) (Sigma, St.Louis, MO, USA) histochemical method as previously described (20). To measure SDH activity, the test materials were removed from the cell-culture wells after 24h and 48h exposure then cells were detached with 0.25% trypsin containing 1mm ethylene diamine tetra acetic acid (EDTA) for 5 min at 37 °C, and subsequently the cells were resuspended in the medium at 1x10^5 cells/µL. After verification of cell viability by tryptan blue dye exclusion assay, 200 µL of cell suspension were distributed into each well of 96-well microtiter plates (Costar, Chambridge, MA, USA), and each plate was incubated for 24 hours. Wells containing 200 µL medium alone without cells and reagents were used as negative controls. After treatment for the stated incubation times, 20 µL of MTT solution (5mg/mL) was added to each well, and the microplates were further incubated at 37 °C for 4 hours. The unreactive supernatants in the well were discarded, and 100 µL of acidified isopropanol (0.04 N HCI in isopropanol) was added to the cultures and mixed thoroughly to dissolve the dark blue crystals of formazan. The absorbance values (A) of each well were determined with a microplate enzyme-linked immuno-assay (ELISA) reader (EL312, Bio-Tek, , Vermont, USA) equipped with a 570-nm filter. The negative control well was used for baseline zero absorbance. Results were presented as percentage cell viability determined as (100-A of experimental well/A of positive control well) X 100. Each experiment was repeated 3 times with representive data presented.

IL-1β assay

The amount of IL-1β in the assay aliquots was assayed by ELISA kit (Cytelisa Human IL-1β, Cytimmune Sciences Inc., College Park, MD, USA) with recombinant IL-1β monoclonal antibody as a standard. All assay procedures were carried out according to the manufacturer's instructions. Before the study, assay aliquots were cooled to room temperature. The amount of IL-1β in each assay aliquots was determined from IL-1β standard calibration curves (8 to 500 pg/ml). The peroxidase-substrate color reaction was read on a plate reader (EL312, Bio-Tek, , Vermont, USA) set to a wavelength of 490nm. The calibration curve was plotted by regression analysis and optical density of each assay aliquot used to estimate the concentration of IL-1β in pg/ml. IL-1β secretion of the control tissues was set to 100%. Results of the other test materials were expressed as a percentage of the untreated control to yield comparable data.

Statistical analysis

Statistical analysis was performed by using the non-parametric Mann-Whitney pair-wise test in SPSS software for Windows. A P value <0.05 was considered as statistically significant.

Results

Cell viability

Cell growth of the three dimensional fibroblast cultures was monitored by mitochondrial dehydrogenase activity (MTT-assay) after 24 and 48 h exposure periods (Figure 1). Copper was the most toxic material tested. Copper was ordinarily included as a positive control material due to the demonstrated high toxicity. Copper

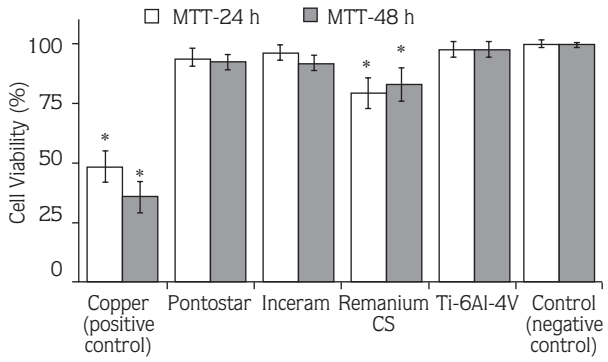

Figure 1. Percentage of cell viability (mean + standard deviation) of the casting alloys and ceramic relative to the control (control = 100% cell viability). The indicated values are medians, minima and maxima from triplicates.

exposed cultures, caused a time dependent decrease in cell viability to levels of 48% and 36% compared to untreated control cells in repeated experiments ($P < 0.05$).

In-Ceram, Pontostar and Ti-6Al-4V alloy did not influence cell viability after 24 h and 48 h ($P > 0.05$) (Figure 1). Remanium CS cell survival rates were about 80% of control cultures ($P < 0.05$).

IL-1β assay

The time-dependent increase of IL-1β levels and summary of IL-1β release from exposed cultures were illustrated in Figure 2. Total amounts of IL-1β released from cell cultures exposed to test materials and from control culture steadily increased during the exposure period. The highest amounts of IL-1β released were determined in cell cultures exposed to copper. Remanium CS induced IL-1β levels about 2-fold higher than those released from control cultures ($P < 0.05$).

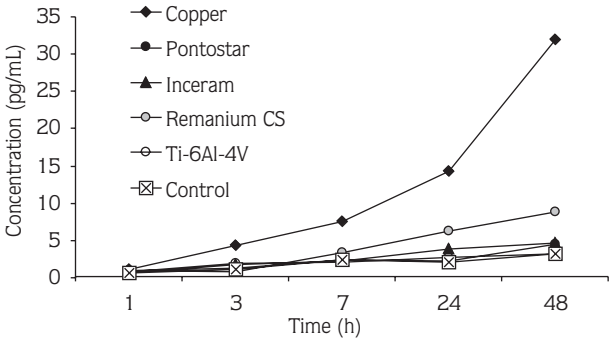

Figure 2. Time course of IL-1β release from three-dimensional cell culture. Significant differences ($P < 0.05$) are indicated by*.

Titanium alloy, In-ceram and Pontostar which were not toxic in MTT assay produced minimal increase in IL-1β released after 24 h and 48 h. This indicated that cells exposed to a Ti-6Al-4V alloy, In-Ceram and Pontostar did not cause elevated IL-1β release from cells as compared to negative controls.

Figure 3 demonstrates that cell cytotoxicity is well correlated to the IL-1β levels released from control cultures and treated cultures ($R^2 = 0.9947$, $P < 0.001$).

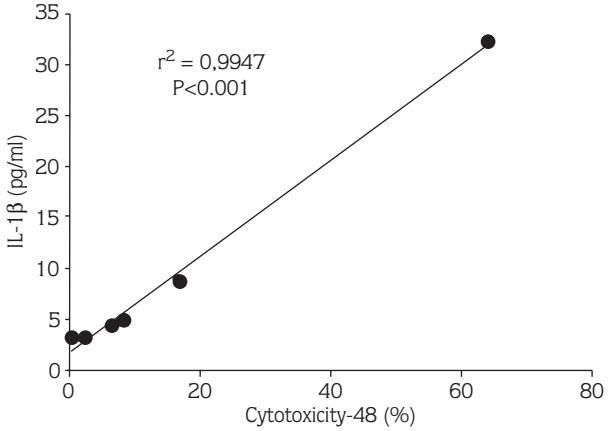

Figure 3. Correlation between the total amount of IL-1β and cytotoxicity.

Discussion

It is well established that IL-1β plays the central role in inflammatory reactions and also enhances various immune responses in vitro (13-15). However, there is at present no direct evidence that an increased IL-1β release after exposure to dental alloys and ceramic specifically indicates inflammatory reactions in vivo. The results of this study support the hypothesis that alloys and ceramic affect the activity of cells and IL-1β secretion from gingival fibroblasts.

Three-dimensional culture systems have been developed to mimic natural interactions between cells. Furthermore, in the three dimensional cultures, primary cells are used. Fibroblasts do not proliferate under normal in vivo conditions. Therefore, the relatively low proliferate response of fibroblasts in the three-dimensional culture system described here may be a more realistic estimate of the in vivo behavior of the cells than monolayer cultures (19). The fibroblasts in a three-dimensional cell culture demonstrate a profound

difference in proliferation and biosynthetic capacities compared to monolayer cultures (8-11). What makes such a three dimensional system unique and preferable to monolayer system is that the fibroblasts shape more closely resembled that of the cells in tissue biopsies (12,21).

The cleavage of MTT has several desirable properties for assaying cell survival and proliferation. The tetrazolium salts, such as MTT, are reduced into colored formazon compounds by all living, metabolically active cells that we have tested. The biochemical procedure is based on the activity of mitochondrial enzymes which are inactivated shortly after cell death. The main advantage of the colorimetric assay is the speed with which samples can be processed. The assay can be read a few minutes after the addition of acid-isopropanol, for dissolving the non-soluble formazon compound, and the color is stable for a few hours at room temperature. The results are also apparent visually, which is very useful if rapid qualitative results are required (19,22,23).

In this study, the toxicity of copper was the highest. Exposure to copper IL-1β secretion was observed 10 fold in comparison to control groups. This effect of copper is in accordance with data from other in vitro and in vivo studies (24-26). Therefore, we carried Cu as the positive group.

In our study, Ti-6Al-4V alloy induced similar IL-1β secretion and cytotoxic effects to the control groups. This may be related to the high content of the titanium and thus have good biocompatibility (4). Spyrou et. al (27) evaluated cytokine release by osteoblast cells cultured on implant discs of varying alloy compositions. The authors concluded that Ti-6Al-4V alloy produced the least biological response (IL-1, IL-6, IL-18). These findings are in accordance with our study findings. Furthermore, the dental ceramic (In-ceram) and Au-based alloy (Pontostar), known non-toxic material (12,22,25) produced no more IL-1β release than Ti-6Al-4V alloys. These findings indicated that dental ceramic, Pontostar and Ti-6Al-4V may not cause inflammatory reaction.

The greater cytotoxic effect and IL-1β release were observed with Ni-Cr alloy (Remanium CS) in comparison to the high-noble alloy, ceramic and titanium alloy. These findings agree with other reports, which showed that Ni-Cr alloys and their products adversely affect the activity of cells (26).

In conclusion, our findings suggest that test material cytotoxicity levels are in parallel to their IL-1β levels. Moreover, ceramic, Pontostar and Ti-6Al-4V alloys may not be involved in proinflammatory activity at non-toxic levels. IL-1β and MTT parameters may provide a better result than a single endpoint about the biological response of the test materials.

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