The cytotoxicity of dental alloys studied on cell culture

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Abstract
The metal alloys used in dental practice are the subject of numerous discussions referring to the effect they have on oral cavity tissues. Numerous scientists established that the organic molecules did not exert a significant influence on the degradation of metallic implants.

This paper is to study the cytotoxic potential of the most used dental alloys, the Ni–Cr alloy and the Co–Cr alloy. The tests were made on cell culture of pure cell line dermal fibroblasts and of those obtained from skin biopsies, for both, dental alloys and their eluates. The results were compared with control samples. At seven days after inoculation, we observed the relative similarity between the Ni–Cr alloy and the Co–Cr alloy, where the cells did not detach from the plate and they grow to the edge of the material. In case of the eluates, there were no fragments detached, the cells having a relatively high confluence. Therefore, the cytotoxic effects of the two alloys are similar, even if there are speculations in the literature according to which Ni–Cr alloys would have a more pronounced effect. In conclusion, our study revealed non-cytotoxicity of these two dental alloys, and we believe so they can be used successfully in dental practice.

Keywords: dental alloys, biocompatibility, cytotoxicity, cell culture.

Introduction

The alloys along with the entire arsenal of materials used in dentistry are the subject of numerous discussions referring to the effect they have on oral cavity tissues.

Typically, the human body contains cations of sodium, potassium, calcium and magnesium, and anions of chlorides, bicarbonate, phosphate and organic acids, generally in concentrations between 2×10⁻³ moles and 150×10⁻³ moles. A number of organic molecules such as proteins, enzymes and lipoproteins are also present, their concentrations being possibly situated in a very wide range of values. Most of the previous investigators established that the organic molecules did not exert a significant influence on the degradation of metallic implants [1].

Nowadays studies indicate that the interactions between implants material and oral cavity proteins should be taken into account. Depending on the particularities of the implant material or type of the dental application used, the alteration of pH in the oral cavity biological environment could also be considered. In such special conditions, the metallic materials used in dental practice may present certain grade of degradation resulting in the formation of different degradation products that can interact with the biological system in various ways [2].

Therefore, the identification and quantification of these degradation products [3] are important steps in evaluating the biological performances [4] of the medical equipment or supplies [5].

The cytotoxic potential of dental materials is the reflection of their composition. The published data about the alloys used in dental practice, but also their projection in the law are closely related to the specificity of each country regarding the use of these materials. For example, Ni–Cr alloys are widely used in the U.S., but banned in Sweden.

The aim of this paper was to study the response of oral mucosa connective tissue fibroblasts to the alloys used in our country in dental practice, but also to the residues resulted after their use, according to ISO 10993 and ISO 7405 [6, 7].

Materials and Methods

This study was composed from three groups of samples: first for the metal alloys, second for alloys eluates and third for control samples. Each group contained cell culture with two different lines of fibroblasts: a pure cell line purchased from a specialized company and a primary culture obtain by us in the laboratory.
In this paper, we studied two dental metal alloys. First material tested, referred to as M1, is the Nickel–Chromium (Ni–Cr) alloy. The second material, called as M2, is the Cobalt–Chromium (Co–Cr) alloy.

For the study, from each material, there were prepared 96 small cylindrical samples with a diameter of 5 mm and a height of 2 mm, the number of the chosen samples being large enough so that all the measurements to be performed in triplicate.

To obtain eluate probe, 48 samples of each material, M1 and M2, were placed for seven days in a culture medium specific to the cellular types chosen for testing. The culture plates were placed in an incubator at 37°C. After this period, the materials were removed, and the remaining culture medium, considered as an eluate, was added over the cells cultures according to the protocol procedures described below. In the next lines, the eluate obtain in contact with M1 material is called eluate EM1 and that obtain from M2 material is referred to as eluate EM2.

For the pure cellular line culture of fibroblasts (L), we chose Adult Human Dermal Fibroblist cell line (HDFa), purchased from Life Technologies Company and kept in laboratory in liquid nitrogen until the moment of performing the experimental procedures.

For the primary culture of cutaneous tissues fibroblasts (F), we used skin fibroblasts, which had been previously isolated in our laboratory from cutaneous biopsies and kept by cryopreservation.

To obtain cultured fibroblasts, the expanded cells were seeded in 24-well plates at a density of 5000 cells/cm², in a culture medium specific to each cellular type:

- For L: DMEM/F-12 (1:1) (Dulbecco’s Modified Eagle’s Medium provided by Gibco Life Technologies, Grand Island, New York, USA) + 10% fetal bovine serum (Sigma-Aldrich, Milwaukee, WI, USA) + 10 ng/mL EGF (Epidermal Growth Factor from R&D Systems, Minneapolis, MN, USA) + 3 ng/mL bFGF (basic Fibroblast Growth Factor from R&D Systems, Minneapolis, MN, USA) + 10 µg/mL Heparin (Biochemie GmbH, Kundl, Austria) + 1 µg/mL Hydrocortisone (Stem Cell Technologies Inc., Vancouver, Canada) + 1% Penicillin/Streptomycin (Sigma-Aldrich, Milwaukee, WI, USA);
- For F: DMEM/F-12 (1:1) (Dulbecco’s Modified Eagle’s Medium provided by Gibco Life Technologies, Grand Island, New York, USA) + 20% fetal bovine serum (Sigma-Aldrich, Milwaukee, WI, USA) + 1% Penicillin/Streptomycin (Sigma-Aldrich, Milwaukee, WI, USA) + 3 ng/mL bFGF (basic Fibroblast Growth Factor from R&D Systems, Minneapolis, MN, USA) + 0.1% streptomycin (Sigma-Aldrich, Milwaukee, WI, USA) + 0.1% penicillin (Sigma-Aldrich, Milwaukee, WI, USA) + 10 µg/mL Heparin (Biochemie GmbH, Kundl, Austria) + 1% Penicillin/Streptomycin (Sigma-Aldrich, Milwaukee, WI, USA).

In order to obtain the final probes of dental alloy, for both types of fibroblast cultures, L and F, the cylindrical samples of M1 and M2 were placed in the center of each well.

In order to obtain the final probes of eluate, 1 mL of it was added in each well.

The visualization of the cells was performed daily with a Nikon Eclipse E600 microscope with reversed light, in phase contrast. We carefully watched and noted the time elapsed from exposure. The images were acquired using a digital camera connected to the microscope.

The results obtained on the eluate and alloys samples, for both materials M1 and M2, were compared with those on the control sample represented by the seeded fibroblasts, HDFa and dermal type, respectively, in specific culture medium with no addition of alloy or eluate.

### Results

The fibroblasts from control samples were viable in both cell culture obtained, with cell line HDFa and with fibroblasts from skin biopsies. In both cell culture, there can be noticed the normal proliferative aspect and retained their fibroblastic features. As we expect, in the HDFa cell culture, the fibroblasts were closely packed forming bundles of fusiform cells compared with the cell culture obtained from skin biopsies were the fibroblasts were disposed isolated or in small groups of two or three spindle cells (Figures 1 and 2).

At seven days after inoculation, in samples resulted after culture of pure cells HDFa in medium with addition of eluate EM1, we noted a proliferation index similar with that obtained in simple proliferation medium. The new formed cells kept their fibroblastic features, with small, thin cytoplasmic prolongations (Figure 3).

At samples resulted between skin fibroblasts and medium with the addition of eluate EM1, at seven days after inoculation, we observed a relatively normal proliferation; however, there can be identified a percentage of cells that have lost their fibroblastic characteristics, becoming small and round (Figure 4).

If we added the eluate EM2 at the simple medium, after seven days, the new formed fibroblasts from pure cell HDFa had similar pattern of growth and morphologic characteristics as those for eluate EM1 or for simple proliferation medium with no material added (Figure 5).

In the primary culture of skin fibroblasts in the medium with addition of eluate EM2, after seven days from inoculation, it is encountered a relatively normal proliferation. Like in case of eluate EM1, some cells have lost their fibroblastic appearance and have tendency to detach from the plate (Figure 6).

For both alloys materials, M1 and M2, and for both types of fibroblasts, pure cell HDFa or skin fibroblasts, in case of the samples in contact with the alloys materials, we noticed a normal proliferative aspect. The cells grew to the edges of the materials, without tendency to colonize them (Figures 9–12).

At the edges of the samples in contact with alloys materials, it is not affected neither the number nor the cell shape and we are dealing with a homogeneous area without chattering tendency (Figures 9–12).

Even if at the demarcation area between material M2 and pure cell line HDFa we can distinguish a slight decreasing of the fibroblasts number and the presence of round cells with pyknotic nuclei, which are not features of the cell line used, however, this effect is moderate (Figure 9).

### Discussion

The oral cavity is covered by buccal mucosa composed of non-keratinizing squamous stratified epithelium and underlying loose or dense connective tissues. These two structures are connected by basal membrane, an acellular layer that forms numerous papillae between epithelium...
and connective tissue. Even if the oral mucosa has different zonal particularities, the main structure remains the same. The dental alloys materials could affect primary the cells of the oral mucosa tissues, the most important of them being the connective tissues fibroblasts. Because of the similarity between the histology of buccal mucosa and that of the skin, we chose to work with dermal fibroblasts. More, in the decision of chose dermal fibroblasts instead of mucosal fibroblasts the cost reasons were very important also, the HDFa line being already purchased for other studies. In the same time, we considered useful to investigate not only the behavior in the culture, in addition of the alloys and eluates, of the dermal fibroblast as a pure cellular line, but also the behavior of a primary culture, obtained in our laboratory by processing of some cutaneous fragments. That was the third pros for dermal fibroblasts, the skin biopsies being more easily to obtain that those of oral mucosa.

The most important properties of the dental alloys are biocompatibility, corrosion resistance, mechanical properties and ease of handling, all of these being determined by the components of alloy [1, 8].

The microstructure and chemical composition of the alloys are those that influence the corrosion behavior, which can be evidenced by the chemical and electrochemical tests [1, 9, 10].

![Figure 1 - Cell line HDFa in simple proliferation medium (ob. 10×).](image1)

![Figure 2 - Primary culture of skin fibroblasts in simple proliferation medium (ob. 10×).](image2)

![Figure 3 - Cell line HDFa in medium with addition of eluate EM1, seven days (ob. 10×).](image3)

![Figure 4 - Primary culture of skin fibroblasts in medium with addition of eluate EM1, seven days (ob. 10×).](image4)

![Figure 5 - Cell line HDFa in medium with addition of eluate EM2, seven days (ob. 10×).](image5)

![Figure 6 - Primary culture of skin fibroblasts in medium with addition of eluate EM2, seven days (ob. 10×).](image6)
The corrosion properties of the alloys depend on its composition, potential variations, electrode potential, surface roughness, oxidation degree, pH, environment temperature, alloying ability, the presence of inhibitors [11–13]. Moreover, the biocompatibility of the alloys is affected in a large extent by its corrosion properties [14, 15].

Relying on the main toxic substances recognized by the European standards, we analyzed representatives of two dental alloy classes commonly used in dental practice in our country, the Ni–Cr alloy, used for crowns and the Co–Cr alloy, used for framed prostheses.

Not only can the alloys material itself produce cytotoxic effect on oral mucosa tissues. Because of the biochemical properties of the connective tissue ground substance, the liquid solution created after the degradation and the corrosion of the dental alloy material in this special environment can have cytotoxic impact. According to ISO 10993-5, 12, we considered useful to check not only the response of the fibroblasts to the alloys materials themselves, but also to the eluate samples corresponding to each material.

In the etiology of the oral cavity diseases, the cytotoxic effect of the alloys was often incriminated, but only rarely documented probably because of the local conditions [16]. For example, saliva by its acid pH and permanent brushing reduce the cytotoxic action of metal alloys.

The rich vasculature of oral mucosa contributes to rapid dispersion of the allergens followed by their absorption in the blood. It is well known that oral mucosa contains a large number of lymphocytes and plasma cell, responsible in the genesis of immune response. The quick decrease of the number of allergens may be an advantage because it diminishes the contact between immune cells of oral mucosa and the allergens, followed by a reduced immune response. In the same time, from the cytotoxic perspective, the rapid absorption of the active elements resulted from alloys degradation can constitute a disadvantage.

The clinical manifestations of alloys cytotoxic effect are polymorphic. The symptoms can be divided into local and systemic.

The local symptomatology appears first and initially is usually subjective, being characterized by pain, paresthesias and burning mouth syndrome [17, 18]. The important cytotoxic changes occur after a long period (five years) of contact between alloys and oral mucosa tissues. Most of them are objective symptoms and may be represented by stomatitis, glossitis, gingivitis, depapillated tongue, dry desquamative cheilitis, perleche or even peri-oral skin rash [19–21].

The lesions may be described as plaques, erosions and ulcers with erythematous, atrophic or reticular aspect. They appear especially on the buccal mucosa and tongue edge [22, 23], and are due to cations released from alloys corrosion [4, 24].

Systemic manifestations can have different localizations.
It is not very well documented if they appear only because of the alloy cytotoxic action. There are different studies that suggested a cumulative effect of two or more alloys with different localization (oral cavity and other part of the body). As in our study, they highlighted that the cytotoxic effect is probably the same, having no importance if the alloys are made from the same metal or different ones [25–28]. In our paper, we observed the same cytotoxic effect for both alloys studied.

Moreover, we emphasized the relative similarity between the material M1 and M2, where the cells do not detach from the plate and they grow to the edge of the material. In case of the eluates, there are no fragments detach from the plate and they grow to the edge of the alloys can be successfully used in the dental practice fibroblasts from cell culture. We believe that the tested was similar and had minimal in vitro effects on their initial characteristics. The cytotoxicity of the alloys results are similar with other published data [25–28].

Therefore, the cytotoxic effects of the two alloys are similar, even if there are speculations in the literature according to which Ni–Cr alloys would have a more pronounced effect.

Even if there are few articles in the literature that highlight the cytotoxicity of elements Ni, Cr, Co, our results are similar with other published data [25–28].

Conclusions

All the samples we used during the study maintained their initial characteristics. The cytotoxicity of the alloys tested was similar and had minimal in vitro effects on fibroblasts from cell culture. We believe that the tested alloys can be successfully used in the dental practice despite of the tendency to give up metal in this medical field.

References


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