ts JOURNAL OF BASIC AND APPLIED GENETICS

Journal of Basic & Applied Genetics, (2008) **19** (1): **43-48**

ISSN: BAG 1666-0390

CYTOCOMPATIBILITY, CYTOTOXICITY AND GENOTOXICITY ANALYSES OF DENTAL IMPLANTS

REIGOSA M.[1-2 1], LABARTA V.[1], MOLINARI G.[2] AND BERNALES D.

1-Instituto Multidisciplinario de Biología Celular, (IMBICE)
CCT - La Plata - CONICET-CIC. C.C. 403 (1900) La Plata. Argentina.
2- Cátedra de Citología. Facultad de Ciencias Naturales y Museo. UNLP
(Footnotes)
1

E-mail address: miguelreigosa@hotmail.com

SUMMARY

Different materials are used for prostheses in dental medicine, though titanium is the most frequent. In the present report, several commercial dental implants and their potential capacity to integrate to bone tissues have been analysed by means of cytotoxicity, genotoxicity and cytocompatibility techniques. Cultures from osteosarcoma-derived UMR 106 cell line were used for bioassays since they showed several osteoblast properties. Samples of dental implants provided by B&M national company (ASTM titanium 8348 GR2, acid-etched surface) were compared with some others commercialized by foreign firms in Argentina (Nobel: anodic-oxidized surface, and 3i: sandblasted surface). Using the neutral red uptake test, lysosome and alkaline phosphatase enzymatic activities were measured in order to assess cytotoxicity level. Acridine ethidium/ orange bromide technique was used to determine cell viability, and genotoxicity was analysed using the comet assay. Results from bioassays showed high biocompatibility and no significant differences among dental samples evaluated. Techniques employed for the present study proved to be a useful tool for the analysis of other materials used in dental medicine.

RESUMEN

Diferentes materiales son utilizados en la constitución de prótesis empleadas en medicina dental, el titanio es el más frecuente. En el presente trabajo distintas muestras de implantes dentales fueron estudiadas en cuanto a su capacidad potencial de integrarse al tejido óseo mediante técnicas de citotoxicidad, genotoxicidad y citocompatibilidad. Cultivos celulares de la línea establecida derivada de un osteosarcoma UMR-106 fueron empleados en los bioensayos por poseer propiedades de células osteoblásticas. Las muestras de implantes dentales fueron provistas por la empresa nacional B&W, el implante de su producción (ASTM titanio 8348 GR, con tratamiento ácido en su superficie) fue comparado con otras marcas comercializadas en Argentina (Nobel: con oxidación anódica y 3i con arenado en su superficie). Por medio de la técnica de Rojo Neutro y la actividad enzimática de la fosfatasa alcalina se estudiaron los niveles de citotoxicidad. Además, a través de la técnica de Bromuro de etidio-naranja de acridina se determinó la viabilidad celular y el uso del «ensayo cometa» se empleó para determinar la genotoxicidad. Los resultados de los bioensayos mostraron una alta biocompatibilidad y no evidenciaron diferencias significativas entre las diferentes muestras de implantes evaluadas. Las técnicas empleadas en el presente estudio pueden constituirse en herramientas de análisis para otros materiales de uso odontológico.

Keywords: Cytotoxicity- Cytocompatibility- Single cell electrophoresis- Osteoblast

INTRODUCTION

Biomaterials employed in dental medicine may release different cytotoxic compounds to the oral environment due to corrosive processes (Mockers et al., 2002). In the particular case of implants, several changes to their metal surface have been developed in order to favor osteointegration (Ramirez et al., 2001). Surface topography has been modified by different techniques, such as sandblasting, or acid- etching (Cochran et al., 1996; Ramirez et al. 2001; Jayaraman at al., 2004). Also, chemical modifications, such as organic monolayers (Sukenik et al., 1990) or adhesive peptides (Dee et al., 1996) have been implemented since chemical factors associated with the surface composition usually cause adverse effects on the surrounding tissue, such as toxic reaction, allergy, mutagenic or inflammatory effects.

The study of the interactions of osteoblast-like cells with the implant interface through *in vitro* systems can provide further information on the oseointegration efficiency of *in vivo* implants (Grill et al., 1997; Schedle at al., 1998). However, cytotoxicity and genotoxicity analyses of the corrosive products following ISO 10993-5 norm (1999) are quite relevant for the study of biocompatibility (Wataha et al., 1995; Shaini et al., 2000).

Several metal ions diffused through hard and soft tissues are known to induce clinical symptoms, such as pain and tissue necrosis. Recent reports have focused on the effects of different metal ions released from dental metal alloys to cells *in vitro* (Wataha et al. 1995; Messer et al., 1999; Shaini et al., 2000). Furthermore, cell proliferation assays have demonstrated a positive correlation between the toxic effect and the level of ions released into the medium (Wataha and Hanks, 1996; Grill et al., 1997; Reigosa et al., 2005).

Three different implants commercially available were tested in order to assess biocompatibility level. The presence of toxic and/ or genotoxic elements released *in vitro* was evaluated in extracts prepared from the implants under standardized conditions (ISO 10993-5 norm, 1999). The analyses were performed in osteosarcoma-derived UMR 106 cell line using the acridine ethidium/orange bromide technique (Gonzalez et al., 2003), the neutral red uptake test (Beufrend and Puerner, 1984), the measurement of alkaline phosphatase enzyme activity (Cortizo et al,1995), and the single cell gel electrophoresis assay (Singh et al., 1996) as cytotoxic and genotoxic end-points.

MATERIALS AND METHODS

1.1. Cell cultures

Established UMR-106 rat cell line from the American Type Culture Collection (ATCC, CRL 1661, Rockville, MD, USA) was used for bioassays. The cell line was grown in D-MEM culture medium (Gibco, Grand Island, NY) supplemented with 10% bovine fetal serum (Natocor, Córdoba, Argentina), 100 U/ml Penicillin, and 100 µml Streptomycin (Gibco) in 95% humid 5% CO_2 atmosphere at 37°C. Cells were sub-cultured using 0.25% trypsin in PBS without calcium or magnesium.

1.2. Dental implants

Samples from three different dental implants commercially available were tested (B&W, Nobel, and 3i). Providers apply different technologies to the titanium surface of implants: B&W = acid etching, Nobel = anodic oxidation, 3i = sandblasting.

1.3. Toxic vehicles extraction

Culture medium was prepared following ISO10993-5 norm (1999), and each implant was incubated with serum during 120 h at 37ºC (2cm^2 /ml). For cytotoxicity experiments, 1x10^5 cells were sown in 35x10 mm Petri dishes and incubated in these extraction media for 24h.

2. Cytotoxicity and genotoxicity studies

2. 1. Cell viability

Cell viability was measured by means of the ethidium bromide / acridine orange technique described by Gonzalez et al. (2003). Briefly, cells grown in the extraction medium were stained with a mixture of 1:1 ethidium bromide (100 µ/ml, Sigma Chemical Co., Missouri, USA) and acridine orange (100 µg/ml, Sigma Chemical Co.) and immediately counted in a Zeiss epifluorescence inverted microscope, with the corresponding filters (viable cells showed fluorescent green color while dead cells were orange).

2.2. Cytotoxicity assay

Neutral red (NR) uptake technique was performed as described by Borenfreund and Puerner (1984). Cells were grown in 96 multi-well plates with the extraction medium, and then incubated with NR for 3 h. After incubation, cells were lysed and absorbance at 540 nm was measured as indicator of NR uptake. Untreated medium was considered as negative control and medium added with formol as positive.

2. 3. Alkaline phosphatase assay

Alkaline phosphatase enzymatic activity was quantified by measuring the transformation of p-nitrophenylphosphate (pNPP) into p-nitrophenol (pNP), as the absorption at 405 nm in 20 mM buffer HEPES, pH 8, 20 mM potassium chloride and 30 mM magnesium chloride, at 37ºC during 15 min (Cortizo et al., 1995). After grown in the extraction medium, 10% aliquots of the cell extract were used for quantifying total proteins (Bio Rad Bradford technique), and 10-20% of the remaining populations were used for the assessment of the enzyme activity. Results were expressed as alkaline phosphatase specific activity (nmoles/min/mg protein).

2.4. Single cell gel electrophoresis (SCGE) assay

The SCGE assay was performed following the alkaline procedure described by Singh et al. (1996) with minor modifications, as follows: 200 µl 0.5% normal melting agarose (NMA) in Ca^{2+}-Mg^{2+} -free PBS were transferred onto a roughened slide, pre-cleaned with 100% ethanol, spread evenly, and left at 37°C for agarose to solidify. Afterwards, 2.5 x 10^4 cells suspended in 25 µl Ca^{2+}-Mg^{2+}-free PBS were mixed with 75 µl 0.5% low melting agarose (LMA) in Ca^{2+}-Mg^{2+}-free PBS, covered with a coverslip, and placed at 4°C for 15 min. A third layer of 100 µl 0.5% LMA was then added, and slides were left at 4°C for 15 min. Immediately after, slides were immersed in ice-cold freshly prepared lysis solution (1% sodium sarcocinate, 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris pH 10.0, 1% Triton X-100, 10% DMSO) and incubated at 4°C for 2 h in the dark Afterwards, slides were placed in a horizontal electrophoresis device with freshly prepared electrophoresis buffer (1 mM Na_2EDTA, 300 mM Na OH), incubated for 20 min at 4°C for DNA denaturation, and electrophoresed (25 V) in the same buffer at 4°C for 30 min. Slides were neutralized with 0.4M Tris-HCl, (pH 7.5) and stained with 4´6-diamidino-2-phenylindole (DAPI) (Vectashield mounting medium H1200; Vector Laboratories, Burlingame, CA, USA). Olympus BX50 fluorescence photomicroscope with appropriate filter combination was used for the analysis. The cellular nucleus diameter and the comet length, determined as the diameter of the nucleus plus migrated DNA, were individually measured using a calibration scale with X100 fluorescence objective in 50 randomly selected cells per experimental point (two duplicates). Cells were classified into four categories depending on DNA damage level: undamaged, slightly damaged, damaged, and highly damaged, as suggested by Lebailly et al. (1997).

3. Direct contact assay

This assay allows evaluating cytotoxicity and cytocompatibility. Implants were fixed to the surface of 35 mm x 10mm Petri dishes with silicone grease, and afterwards inoculated with a cell suspension (3 ml with 3x10^5 cells). Then, samples were incubated in humid atmosphere during 120 h with 5% carbon dioxide at 37ºC.

RESULTS

None of the three implants analysed seemed to release cytotoxic compounds to the media, as shown by the ethidium bromide/acridine orange test. No toxic effect on the exposed cell population was observed (see Figure 1).

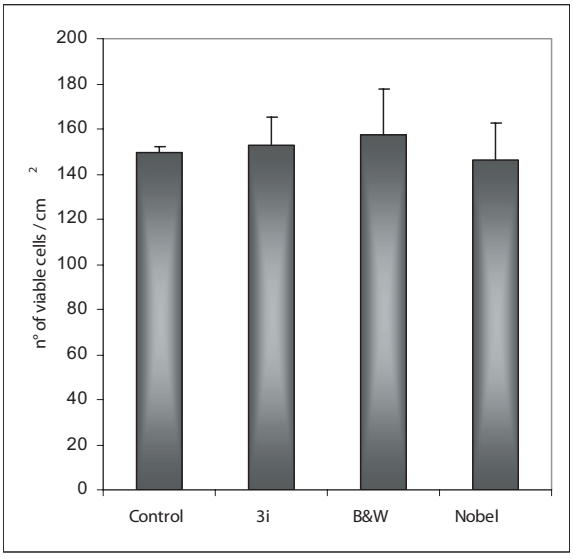

Figure 1: Evaluation of cytotoxicity
Cytotoxicity of the medium prepared following ISO10993-5 norm for toxic vehicles extraction was quantified as the number of viable cells/ cm^2 by means of ethidium bromide / acridine orange differential staining

As shown in Figure 2, the neutral red uptake of cells cultured in the implants treated media exhibited values comparable to those found for negative controls (cells cultured in untreated medium). Highly significant differences were observed in absorbance values when compared with those from phenol treated cells (Figure 2).

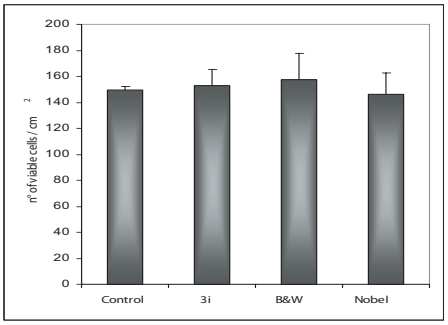

Figure 2: Absorption values are proportional to the number of viable cells. The negative control (-) is represented by the non-prepared culture medium, and the positive control (+) corresponds to the culture medium with 0.01% phenol.

Alkaline phosphatase activity in the extract from cells exposed to the extraction media showed no significant differences with that of controls (Figure 3)

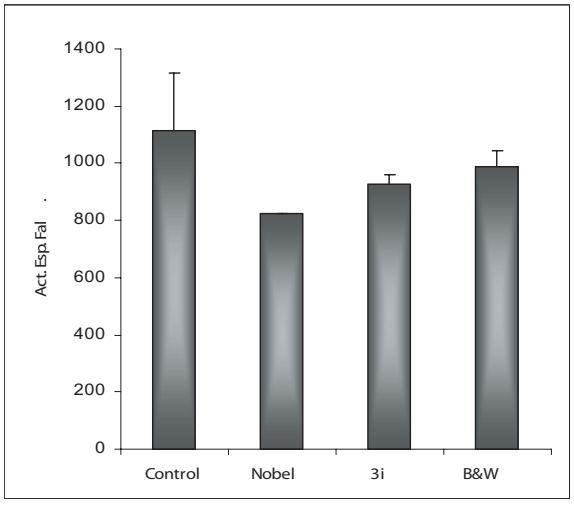

Figure 3: Alkaline phosphatase enzymatic activity
The enzymatic activity was measured in cells cultured in media prepared with the samples. Cells cultured in unprepared medium were included as negative controls.

In the single cell electrophoresis test, cells grown in the extraction media behaved as those cultured in untreated medium (Figure 4).

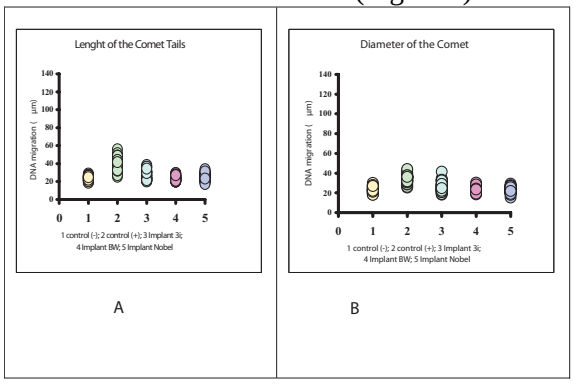

Figure 4: Single cell gel electrophoresis
Effect of the extraction media on the length of comet tails (Panel A) and on diameter (panel B) at the estimated trailing edge pf comet tails in UMR-106 cells. DNA migration (µm) was determined in 50 cells for each experimental point.

These results suggest that none of the prostheses studied released genotoxic compounds to the medium during incubation. Furthermore, when UMR 106 cells were seeded directly onto implants for 120 h, no morphological changes and high compatibility with substrates were observed (data not shown).

DISCUSSION

The culture medium used as extraction vehicle might resemble corrosion on the metal surface in the oral environment which could prompt the release of unwanted products to the surrounding tissue (Wataha et al. 1995; Messer et al., 1999; Shaini et al., 2000; Mockers et al., 2002). Results shown in this report suggest that the corrosive effect on the dental implants studied does not generate the release of toxic compounds that could affect UMR 106 osteablastic cells viability (Figures 1 and 2). Alkaline phosphatase activity of osteoblasts in the prepared media was comparable to that observed for controls, indicating that the exposed cells preserved their osteoblast biochemical marker (see Figure 3). Moreover, findings from the single cell electrophoresis test suggest that none of the prostheses studied release genotoxic compounds to the medium during incubation (Figure 4).

Results shown in the present report suggest that the implants samples studied are biocompatible and that neither potentially toxic nor genotoxic compounds are generated due to the corrosive effect acting on the metal surface.

ACKNOWLEDGEMENTS

We wish to thank B&W S.R.L. for providing the samples and Mr. Daniel Castrogiovanni for technical assistance.

REFERENCES

Benfreund, E. Puerner, J. A simple quantitative procedure using monolayer culture for toxicity assays. Journal of Tissue Culture Methods. 9, 7-9. 1984.

Cochran, D. Nummikoski, F. Higgibotton, J. Hermann, S. Makins, S. Buser, D. Evaluation of an endosseous titanium implant with a sandblasted and acid-etched surface in the canine mandible: radiographic results. Clin Oral implants Res.7, 3.240-252. 1996.

Cortizo, A. Etcheverry, S. Vanadium derivatives act as growth factor- mimetic compounds upon differentiation and proliferation of osteoblast-like UMR 106 cells. Molecular and Cellular Biochemistry. 145: 97-102.1995.

Dee, K. Rueger, D. Andersen, T. Bizios, R. Conditionswhoch promote mineralization at the bone-implant interface: model in vitro study. Biomaterials. 17: 209-215. 1996.

Grill, V. Sandrucci, M. A. Basa, M. di Lenarda, R. Dorigo, E. Martelli, A. M. Barreggi, R. Narducci, P. The influence of dental alloy on cell proliferation and fibronectin arrangement in human fibroblast cultures. Arch Oral Biol 42. 641-647. 1997.

González, M. Soloneski, S; Reigosa, M. Larramendy, M. Effect of dithiocarbamate pesticide zineb and its commercial formulation, azzurro IV. DNA damage and repair kinetics assessed by single cell gel electrophoresis (SCGE) assay on Chinese hamster ovary (CHO) cells. Mutation Research. Vol. 534. 145-154. 2003.

International Standard. Biological evaluation of medical devices. Part. 5 .Test for in vitro cytoxicity. ISO. 10993-5. Second edition. 1999.

Jayaraman, M. Meyer, U. Buhner, M. Joos, U. Wiesmann, H. Influence of titanium surfaces on attachment of osteoblast-like cells in vitro. Biomaterials. 25. 625- 631. 2004.

Lebailly, P. Vigreux, C. Godard, T. Sichel, F. Bar, E. Letalaër, J. Henry-Amar, M. Gauduchon, P. Assessment of DNA damage induced in vitro by etoposide and two fungicides (carbendazim and Chlorothalonil) in human lymphocytes with the comet assay, Mutat. Res. 375 . 205-217. 1997.

Messer, M. Bishop, S. Lucas, L. Effects of metallic ion toxicity on human gingival fibroblast morphology. Biomaterials. 20. 1647-1657. 1999.

Mockers, O. Deroza, D. Camps, J. Cytotoxicity of orthodontic band, brackets and archwires in vitro. Dental Materials. 18. 311-317. 2002.

Ramírez, P. Romito, A. Cosentino, F. Milella, E. The XXXXX of titania/hydroxyapatite composite coatings on in vitro osteoblasts behaviour. Biomateriales. 22. 1467-1474. 2001

Reigosa, M. Labarta, V. Goméz de Saravia, S.

Fernández Lorenzo de Mele, M. Comparación del efecto citotóxico del cobre sobre líneas celulares de ovario y osteoblastos. XV Congreso Argentino de Bioingeniería. Paraná, Entre Ríos, Argentina, CD 4 pp ISBN; 950-698-155-8. 2005.

Schedle, A. Samorapoompichit, P. Fureder, W. Rausch-Fan, X. Franz, A. Sperr W. Slavicek, R. Simak, S. Klepetko, W. Ellinger, A. ghannadan, M. basghestanian, M. Valent, P. Metal ion-induced toxic histamine release from human basophils and mast cells. J. Biomed Mater Res. 39: 560-567. 1998.

Shaini, F. Shelton, R. Marquis, P. Sortall, A. In vitro evaluation of the effect of mixed amalgam and gallium-based alloy on viability of primarity periosteal and osteoblast cell culture. Biomaterials. 21. 113-119. 2000.

Singh, N. Microgel electrophoresis of DNA from individual cells; principles and methodology. Technologies for detection of DNA Damage and Mutations, Plenum Press, New York, pp. 3-24.1996.

Sukenik, C. Balachander, N. Culp, L. Lewandowska, K. Merrit, K. Modulation of cell adhesion by modification of titanium surface with covalently attached self-assembled monolayers. J. Biomed Mater Res. 24: 1307-1323. 1990.

Wataha, J. Malcolm, C. Hanks, C. Correlation between cytotocity and the elements released by dental casting alloy. Int. J. Prosthodont. 8. 9-14. 1995.

Wataha, J. Hanks, C. Biological effects of palladium and risk of using palladium in dental casting alloys. J. Oral Rehabil. 23. 309-320.1996.