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Metallic Dental Material Biocompatibility in Osteoblastlike Cells

Correlation with Metal Ion Release

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ABSTRACT

Ions released from metallic dental materials used in orthodontic appliances could induce undesirable effects on cells and tissues. This study evaluates the biocompatibility of two of the most labile components of metallic dental alloys on osteoblastlike cells. The influence of protein and ions on metal dissolution properties is also investigated using different electrolyte solutions. Morphological alterations, cell growth, and differentiation of osteoblasts were assessed after exposure to pure metals (Ag, Cu, Pd, Au) and Ni–Ti alloy and correlated with the kinetic of elements released into the culture media. Results showed that Cu and Ag were the most cytotoxic elements and the other metals were biocompatible with the osteoblasts. The parameters of biocompatibility were correlated with the levels of ions detected into the culture media. Metal ions induced cell death through early mitosis arrest, apoptotic phenomena, and necrotic processes. Voltamograms showed that anions and proteins interfered in the corrosion process. Fetal bovine serum (FBS) strongly affected the electrochemical process, increasing the oxidation rate of the metals. In conclusion, copper and silver ions showed a time-dependent low biocompatibility, which correlated with the concentration of released ions. The dissolution of the metallic materials was dependent on the composition of the simulated biological media.

Index Entries: Biocompatibility; metallic dental materials; osteoblasts; cytotoxicity; atomic spectroscopy; corrosion; metal ion release.

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INTRODUCTION

Metallic dental alloys are widely used in orthodontic appliances like molar bands, brackets, and wires as well as in restorative materials, prostheses, and implants. These materials are in long-term intimate contact with epithelial cells and connective and bone tissues *(1).* Some of the dental alloy components of clinical relevance are gold, palladium, silver, nickel, cobalt, or titanium *(2)* and, eventually, their metallic ions could be released from the biomaterials. These ions can produce several undesirable effects such as toxic, allergic, inflammatory, and mutagenic reactions. It has been reported that the local toxicity will depend on the corrosion of the alloy, on the lability of the elemental species *(3),* on the concentration of released ions, on the exposure time of the metal to the cells, and on the sensibility of the specific cells or tissues *(4).* In addition, it is important to consider that there is a relatively high local concentration of the elements in "microenvironments" formed between the alloys and the tissues *(1).*

To identify a material as biocompatible with human tissues, attention should be paid to its effects on specific cellular functions. Different methods and models have been used to evaluate the biocompatibility of dental biomaterials—in particular, in vitro studies using fibroblast or osteoblast cell cultures. UMR106 is a clonal osteosarcoma cell line of rat, which conserves certain characteristics of differentiated osteoblastic phenotype; for instance, they express type I collagen, high levels of alkaline phosphatase, and parathyroid hormone (PTH) receptors *(5).* Likewise, the MC3T3E1 preosteoblastic line *(6)* has been proved to be a good model for biocompatibility studies. The direct effect of adding to the cell culture pure metals that were components of dental alloys could be analyzed and significant information could be obtained on their effects, such as their particular toxicity *(7–10).*

It is worth mention that in the case of dental alloys, the lability of an element can be altered by the other elements in the alloy. For example, palladium has been shown to reduce the lability of copper from gold-based alloys. Moreover, the mass release of a particular element is generally not related with its atomic percent in the alloy. Notwithstanding this, assays made with pure metals are useful to study the effect of each component on the behavior of cells. In this way, the particular concentration of metal ions that suppresses cell activity could be evaluated.

The effects of released metal elements could cause adverse tissue reactions close to dental cast alloys. In general, the cytotoxicity correlates to some extent with elemental release into the media *(4);* however, this correlation is not a linear function of the concentration *(11,12).* Wataha et al. *(4)* developed a model to measure the toxicity of alloy extracts, consisting in exposing fibroblasts for 48 h to these ion-containing alloy extracts. The latter were obtained from a media with 3% serum where metallic alloys were preincubated for 6–96 h in the absence of cells. This approach provides very important information on metallic toxicity levels, although it does not allow one to follow the slow kinetic of metal release from the alloys during the cell

growth. In real situations, the concentration of the metal cations varies with time and with the distance from the metal sample. The effect of possible high local ion concentration in the vicinity of the metal can be investigated when the metal is present during the period of cell culture. In this case both, cell growth and ions release from the metal occur simultaneously.

Among 14 metals analyzed, Ag+ was found to cause the highest toxic effect *(13).* Among silver-containing gold alloys, the alloy with the highest atomic percent of silver was defined as the least biocompatible, considering both the cellular proliferation rates and the expression of an extracellular matrix protein *(9).* Moreover, copper and silver are the most likely agents depressing cellular mitocondrial activity, as evaluated by the succinic dehydrogenase activity *(11).* Copper and silver are also present in dental amalgams and orthodontic appliances. Copper and zinc have been classified as the most cytotoxic elements in dental amalgams, followed by mercury and silver *(4,11,14).* Brazing alloys in orthodontic devices generally consist of silver and copper and induce a large reduction in cell proliferation.

The adsorption of proteins on copper and silver is very high *(15).* Some metals adsorb considerably greater amounts of proteins than might be expected from surface energy values *(16).* It has been reported that metal ion release depends on the content of proteins *(17).* Through protein binding of the metals, corrosion products can be disseminated from the site of implant *(18).* In spite of the influence of proteins in the dissolution processes *(19),* many of the in vitro corrosion studies on biomaterials were made using saline solutions. Thus, metal ions release from the metal sample immersed in the culture medium might be different from that of the metal sample immersed in a saline abiotic solution (like saline solutions of international standards). Even in the case of saline solutions, different compositions are used in the electrochemical assays, making it difficult to compare results obtained by different authors *(20–24).*

The aim of our research was to evaluate the biocompatibility of two of the most labile components of dental alloys by using well-characterized osteoblastlike cell lines. Morphological alterations, proliferations, and differentiation of osteoblasts were assessed and correlated with the kinetics of the ions released from pure metals to investigate the individual effect of each one. The influence of protein and ions on the metal dissolution properties was also investigated in vitro by using solutions of increasing complexity in composition.

MATERIALS AND METHODS

Metal Samples

Clinical relevant metal components of several dental alloys were used to test their biocompatibility. Five-millimeter-long wires of pure Ag, Au, Pt, Pd (circular section, 0.05 cm diameter, exposed area = 0.157 cm^2),

Cu (circular section, 0.1 cm diameter, exposed area = 0.314 cm^2), and Ni/Ti alloy (nitinol, square section, exposed area = 0.2 cm^2) were tested. The wires were polished with alumina powder (1 μm). After being rinsed in 96% ethanol solution, they were washed in sterile double-distilled water and sterilized before being transferred into the cell culture. The total volume of the media added to the well was 1 mL. The surface area-to-volume ratio was in the range of 0.6–6 cm^2/mL, as recommended by the International Standards Organization for assessment of biomaterials *(25).*

Cell Culture and Incubations

UMR106 rat osteosarcoma-derived cells were obtained from American Type Culture Collection (ATCC) (Rockville, MD, USA) and MC3T3E1 cells were from the Cancer Center of the University of North Caroline at Chapel Hill (Chapel Hill, NC, USA). Cells were grown in 75-cm^2 flasks at 37°C in a humidified 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Gaithersburg, MD, USA), supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (FBS) (Gibco, Gaithersburg, MD, USA). These cell lines have been shown to conserve certain characteristics of differentiated osteoblastic phenotype and have been used in our laboratory to investigate the effect of vanadate and other agents that regulate osteoblastic growth and differentiation *(26,27).* When 70–80% confluence was reached, cells were subcultured using 0.1% trypsin and 1 m*M* EDTA in Ca^{2+} – Mg^{2+}-free phosphate-buffered saline (PBS). For experiments presented in this study, cells were seeded in six-well plates at a density of 2.5×10^4 cells/well, the medium was replaced by 0.5% FBS–DMEM, and cells were incubated with different metallic wires or medium alone (basal control), under the conditions described in the legends of the figures. At the end of the experiments, the conditioned media was saved and frozen to –20°C until the analysis of elements was made. The rationale for selecting 0.5% FBS was both to avoid the masking effect of higher serum concentrations on the metal effects and to reach a serum concentration needed for cell survival. Under this condition, control cells (without metal wires) continued proliferating and did not show any apparent alterations after being serum starved (0.5% FBS) for 48 h.

Studies of Cell Growth and Morphology

In order to determine cell growth, the monolayers were washed twice with PBS, fixed with methanol for 5 min at room temperature, and stained with a solution of Giemsa for 10 min *(28).* Then, the plates were washed with water and the cell number was evaluated by counting in a light microscope (total magnification ×400) the stained nuclei in six fields per well. Mitotic index was defined as the number of mitotic figures per field over the total number of cells per field.

Cell Differentiation Assay

Alkaline phosphatase specific activity (ALP) has been used as a marker of osteoblast phenotype *(27–29).* The cell layer was washed with PBS and solubilized in 0.1% Triton X-100. Aliquots of the total cell extract were used for protein determination by the Bradford technique *(30).* Measurement of ALP was carried out by spectrophotometric determination of initial rates of hydrolysis of p-NPP to *p*-nitrophenol (*p*-NP) at 37°C for 10 min. The formation of the product was assessed by the absorbance at 405 nm.

AU: Pls spell out "NPP" at first use.

Electrolyte Media

With the aim of studying the influence of the DMEM components of the culture medium, different electrolyte solutions were assayed: (a) NaCl (9 g/L), (b) NaCl (6.5 g/L) + KCl (0.2 g/L) with the same chloride concentration as DMEM; (a) solution (b) + $CaCl_2$ (0.2 g/L) + $NaH_2PO_4 \cdot H_2O$ (0.125 g/L), $MgSO_4$ (0.097 g/L) with the same ion concentrations as DMEM. In order to study the effect of proteins (FBS) on the metal dissolution, experiments either with 9 g/L NaCl + 10% FBS (solution d) or DMEM + 10% FBS (solution e) were also made.

AU: "+" before $MgSO^4$

Electrochemical Cell

A conventional double-wall Pyrex glass cell was employed in the experiments. A platinum sheet was employed as a counterelectrode and a saturated calomel electrode was used as the reference to which the potentials in the text are referred. Pure Ag or Cu wires were used as working electrodes (0.157 and 0.314 cm^2, respectively).

Electrochemical Experiments

The electrodes were polished with alumina, 1 μm, and then successively rinsed with ethyl alcohol and distilled water. Solutions were deaerated with pure nitrogen in order to avoid the interference of oxygen in the electrochemical spectrum. They were thermostatized at 37°C. Potentiodynamic polarization curves were performed at 0.02 V/min with different anodic and cathodic limits between –1.0 V and 0.6 V potential range. At the end of the electrochemical experiments, the electrodes were observed through an optical microscopy method previously developed *(31).*

Measurements of the Concentration of the Released Ions

The ion concentration in the culture medium was measured by flame atomic absorption spectrophotometry (sensibility: 0.1 μg/mL for copper and 0.5 mg/mL for silver). In the case of silver, the solutions were concentrated in order to permit the concentration measurements within the detection limit.

Statistical Analysis

For each experimental condition, at least three separate experiments were performed. Data were expressed as the mean ± SEM. Statistical differences were analyzed using a Student's *t*-test. The correlation among different parameters was analyzed using the Pearson's correlation coefficient.

RESULTS

Effect of Metal Dental Materials on Osteoblastlike Cell Cultures

We evaluated the effect of different metallic components of dental materials on specific osteoblastic properties like cell morphology, growth, and differentiation. The control UM106 cells exhibited normal polygonal morphology with cytoplasmic processes connecting neighboring cells (*see* Fig. 1a). Nuclei were well stained and characteristically heterogeneous in size and showed several mitotic figures. After 24 h in culture, cells exposed to Cu showed important morphological changes. No osteoblasts were found around the wire, the area of cell death being approx 50% of the dish surface. The remaining osteoblasts showed loss of processes, picnotic nuclei with distorted shapes (*see* Fig. 1b), and blebbing of the plasma membrane (*see* Fig. 1c). A longer incubation (48 h) with the Cu sample induced almost complete cellular death of the culture. Exposure to Ag for 48 h also caused a slight reduction in the number of healthy cells in the culture. Osteoblasts were relatively small, with loss of processes, although some mitotic figures and apoptotic bodies were still observed (*see* Fig. 1d). The monolayer of MC3T3E1 control cells shows the aspect of the typical fibroblastlike culture (*see* Fig. 1e). Cells were stellate in shape and exhibited slender lamellar expansions. The nuclei contained moderately thick chromatin granules and the cytoplasm showed numerous inclusions and vacuoles. Copper-exposure cells of 24-h culture became smaller, with a denser cytoplasm (*see* Fig. 1f). Loss of plasma membrane integrity, shrinkage, as well as membrane blebs were also observed. Nuclei showed condensation and picnosis. These alterations suggest that necrotic–apoptotic processes were involved in the Cu-induced cell death. Cells cultured with Au, Pt, Pd, or Ni/Ti for 48 h appeared similar to the control cells (data not shown).

The metallic component of biomaterials differentially affected UMR106 cell growth after 48 h of incubation, as it was evaluated by counting the number of cell surviving and the mitotic index. As can be seen in Fig. 2, Cu was the most toxic element, inducing a significant reduction ($p<0.001$) in the number of viable cells in the culture. No mitotic figures were observed in the few cells that remained under this condition. Cells

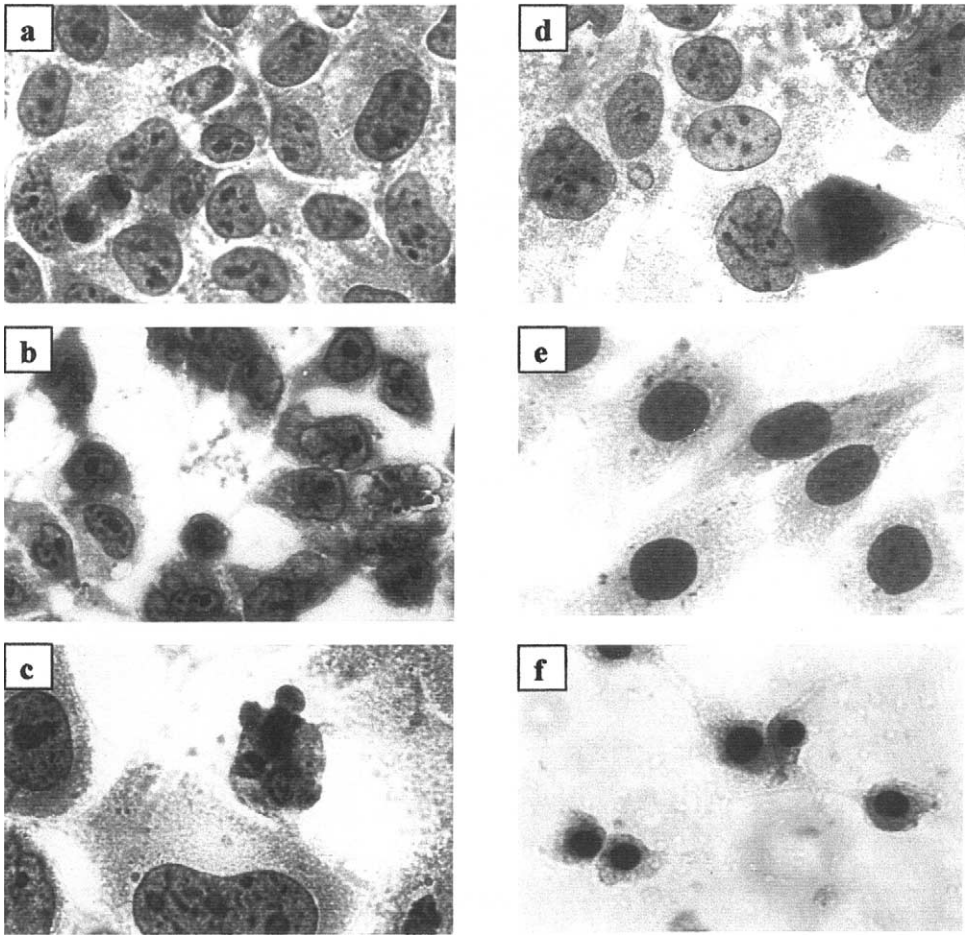


Fig. 1. UMR106 osteoblastlike cells incubated either with 0.5% FBS–DMEM alone (control) **(a),** media plus the Cu wire for 24 h, **(b,c)** or medium plus the Ag wire for 48 h **(d)** MC3T3E1 cells incubated with 0.5% FBS–DMEM alone **(e)** or medium plus Cu-wire for 24 h **(f)** Magnification: ×40 (**a, b, e,** and **f**); ×63 (**c** and **d**).

exposed to Ag also showed a small but not statistically significant inhibition of cell growth. However, the mitotic index was significantly reduced ($p<0.001$), suggesting an arrest of the proliferation induced by the silver on the osteoblastic culture.

In addition, the effect of different metals on the UMR106 osteoblastic differentiation was also evaluated as a parameter of biocompatibility. ALP, a specific marker of the osteoblastic phenotype, was statistically inhibited by a 48-h incubation with Cu and Ag materials (*see* Fig. 3). Conversely, the noble elements as well as the Ni/Ti alloy were biocompatible with the osteoblastic phenotype.

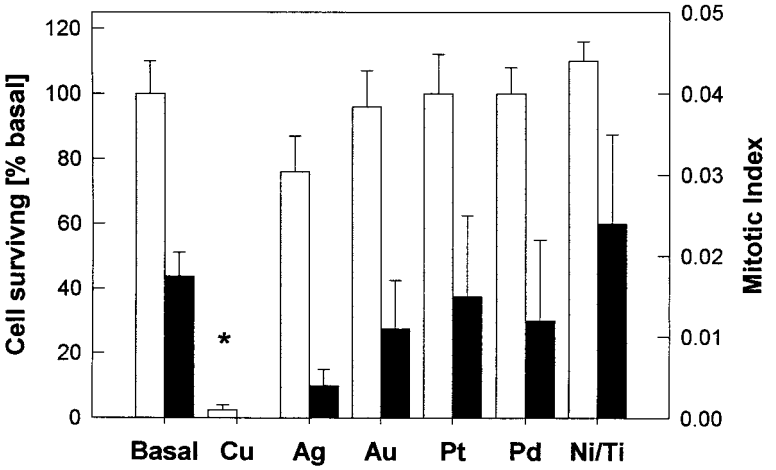


Fig. 2. Effect of metallic materials on UMR106 cell growth. Cells were incubated in 0.5% FBS–DMEM alone (basal) or in the presence of different metallic wires for 48 h. Cell growth was evaluated by the number of surviving cells (white bars) and the mitotic index (black bars). The mitotic index was zero in the case of copper wire. Results are expressed as means ± SEM (n = 18). Basal values are 136 ± 13 cells/field and 0.017 ± 0.003 mitotic figures/total cell number. Differences with the basal are $p<0.001$, indicated by an asterisk.

AU: Pls check. "white bars".

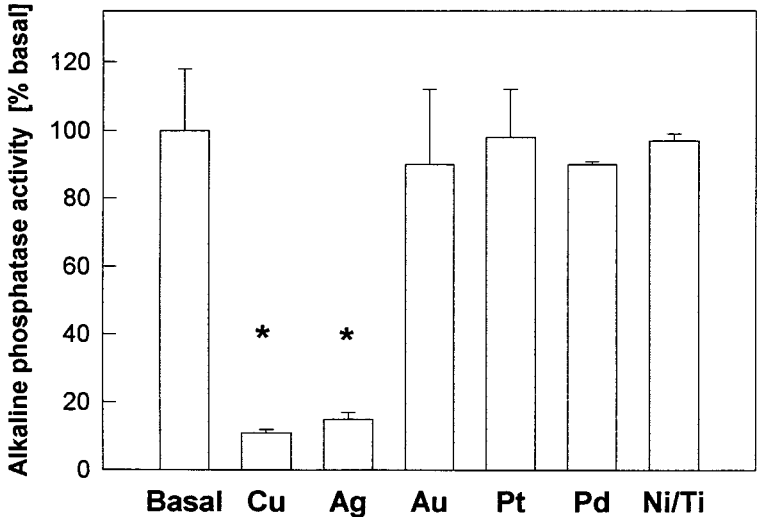


Fig. 3. Effect of metal materials on UMR106 cell differentiation. Cells were incubated in 0.5% FBS–DMEM alone (basal) or in the presence of different metallic wires for 48 h. ALP was assessed as an osteoblastic marker. Results are expressed as means ± SEM (n = 6). The basal value is 414 ± 73 nmol p-NP/min/mg protein. Differences with the basal are $p<0.001$, indicated by an asterisk.

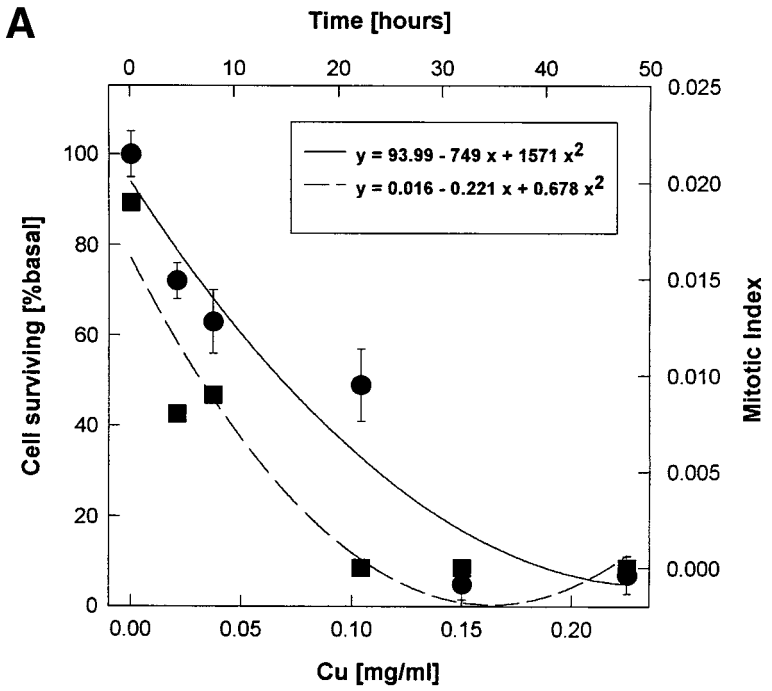
Kinetics of Metal Release and Their Correlation with Osteoblatic Growth

We next analyzed the kinetics of ion-induced growth inhibition by culturing the osteoblasts with a Cu or Ag wire for different incubation periods. The release of metals was evaluated by atomic absorption spectroscopy in the culture media. Figure 4a shows that the effect of Cu on the inhibition of UMR106 cell growth was time dependent, starting as early as 4 h of incubation. This effect was in parallel with the release of Cu from the dental material (*see* Table 1). Both cell surviving and the mitotic index (MI), significantly correlated with the Cu levels in the media (*see* Fig. 4a), although this correlation was stronger when the number of cell surviving was analyzed ($r = –0.936$, $p<0.01$) in comparison with the MI ($r = –0.823$, $p<0.05$). In the MC3T3E1, the copper release affected mainly the cell surviving (*see* Fig. 4b) without changes in the mitotic index (data not shown). After 24 h of culture, copper ions significantly reduced cells surviving in the range of 40–60% of basal condition.

The kinetics of Ag release into the culture media was also evaluated. Table 1 shows that Ag dissolution is lower than that of copper. It was detected in the media after 4 h of cell incubation with the silver wires. The UMR106 cell growth, evaluated either by the number of cell surviving or the MI, showed a trend to decrease as a function of time (*see* Fig. 5). However, these correlations were not statistically significant, as there was not a relationship between cell growth and the levels of Ag released in the media (data not shown). Finally, metal ions released after 48 h of cell incubation, from Au, Pt, Pd, and Ni/Ti alloy, were below the detectable limits.

Electrochemical Experiments

Figure 6a shows the voltamograms corresponding to silver electrodes immersed in different saline solutions (a, b, and c). They show similar shapes with only small differences in the associated charges. It can be observed that the dissolution of silver begins at approx 0.06 V, with a sharp anodic current increase. During the cathodic scan, two negative current contributions can be distinguished: a peak at –0.03 V and a shoulder at 0.02 V. They correspond to the reduction of silver ions yielding to the formation of a dark porous layer (not shown), as we have previously described *(32).* Voltamograms show that copper (not shown) and silver dissolutions (*see* Fig. 6a) are mainly governed by the chloride concentration. It can be observed that the height of the cathodic peak depends on the chloride concentration (solutions a and b). The addition of the other ions (solution c) did not influence markedly the dissolution process. Conversely, the addition of FBS strongly affected the electrochemical process (*see* Fig. 6b). The cathodic current contribution changed their shape and the potential of the peaks were shifted in the cathodic direction when FBS was added (compare with the voltammograms of Fig. 6a). Two high negative current peaks at –0.04 V and –0.11 V


A
Time [hours]
0
10
20
30
40
50
0.025
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0.015
0.010
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Mitotic Index
y = 93.99 - 749 x + 1571 x2
y = 0.016 - 0.221 x + 0.678 x2
100
80
60
40
20
0
Cell surviving [%basal]
0.00
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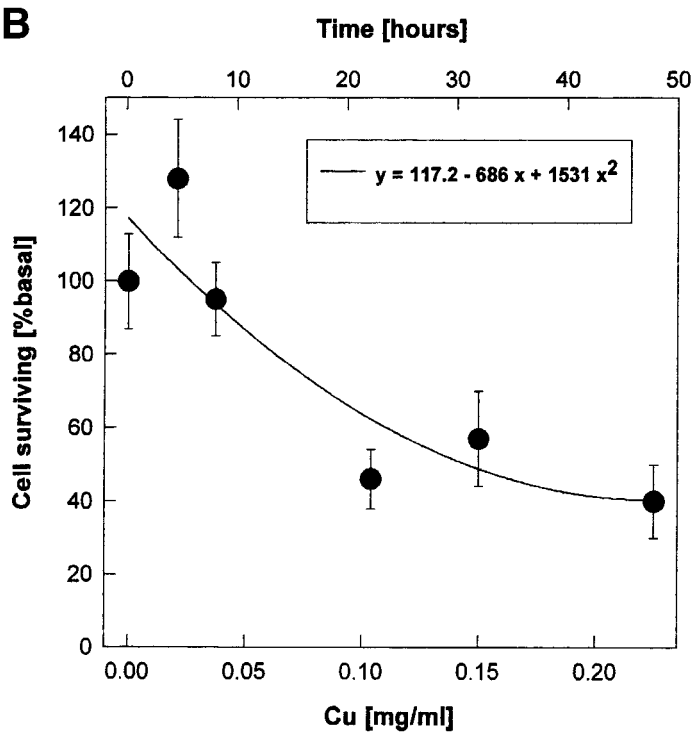
0.15
0.20
Cu [mg/ml]


B
Time [hours]
0
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140
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80
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40
20
0
Cell surviving [%basal]
0.00
0.05
0.10
0.15
0.20
Cu [mg/ml]

Table 1
Kinetics of Ion Release from Wires into the UMR106 Osteoblastic Media

Wire	Time [hours]					
	0	4	8	24	32	48
Cu [mg/ml]	0	0.021	0.037	0.104	0.150	0.225
Ag [μg/ml]	0	0.25	0.25	0.50	0.75	1.00

are recorded in the case of solution e. When successive cyclic scans were performed, a marked decrease in the current values was noticed, which could be related to the progressive inhibition of the oxidation process. This effect was not observed in the plain saline solution. The electrochemical behavior of silver in solutions d and e was very different during the cathodic scan. The height of the two peaks decreased when the anodic limit was decreased. Microscopic observation (not shown) of the layers formed after the cyclic potential scan in solutions d and e showed that they present different surface characteristics. In addition, the dark film formed in solution e after the reduction process seemed to be more firmly adhered to the metal. Consequently, some of DMEM components, such as amino acids, vitamins, and glucose could also affect the reduction process.

Voltamograms of copper in solutions a, d, and e show higher cathodic values for solution a (*see* Fig. 6c). The addition of FBS to the NaCl solution d increased the dissolution rate of copper (*see* Table 2). In agreement with

Fig. 4. Kinetics of Cu release and the correlation with UM106 **(a)** or MC3T3E1 **(b)** cell growth. Cells were cultured in media with Cu wires for the incubation periods indicated for the top *x*-axis. Cell growth was evaluated by the number of surviving cells (●) and the mitotic index (■). Results are expressed as means ± SEM (n = 6). The release of Cu into the media (bottom *x*-axis) was assessed by atomic absorption spectroscopy. Basal values are 126 ± 7 UMR106 cells/field, 0.019 ± 0.004 mitotic figures/total cell number, and 42 ± 6 MC3T3E1 cells/field. The inset shows the equation of second-order regression for cell surviving versus Cu and the MI versus Cu, respectively.

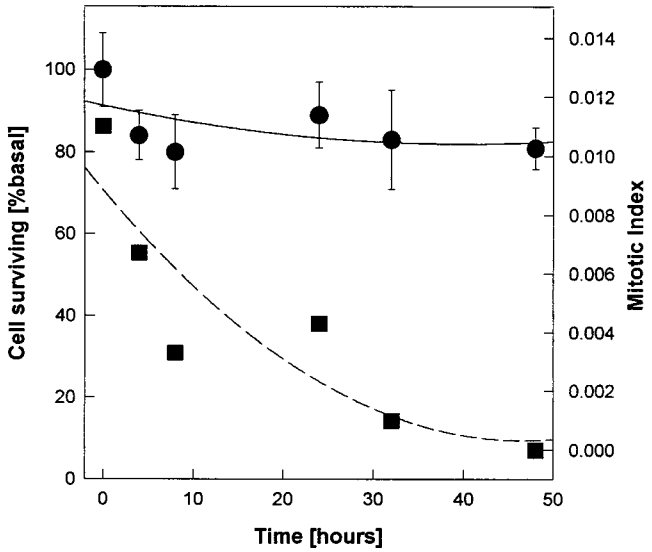

Fig. 5. Kinetics of Ag effect on UMR106 cell growth. Cells were cultured in media with Ag wires for the different incubation periods. Cell growth was evaluated by the number of surviving cells (●) and the mitotic index (■). Results are expressed as means ± SEM (n = 6). Basal values are 152 ± 14 cells/field and 0.011 ± 0.004 mitotic figures/total cell number.

these results, the anodic charges are higher than cathodic charges in the case of solutions d and e, indicating that some of the cations could have remained in the solution and were not reduced. After the immersion in solution d, the copper wires were more brilliant than those immersed in solution a.

DISCUSSION

In this study, we have investigated the biocompatibility of two of the most labile metallic components of dental materials on the specific func-

Fig. 6. **(a)** Voltamperograms of Ag at a 0.02-mV/min scan rate in different saline solutions (a) NaCl (9 g/L), (b) NaCl (6.5 g/L) + KCl (0.2 g/L) with the same chloride concentration as DMEM, (c) solution (b) + $CaCl_2$ (0.2 g/L) + $NaH_2PO_4{\cdot}H_2O$ (0.125 g/L), $MgSO_4$ (0.097 g/L). **(b)** Voltamperograms of Ag at a 0.02-mV/min scan rate in different solutions (d) 9 g/L NaCl + 10% FBS and (e) DMEM + 10% FBS. Successive cycles (first and second) are shown. **(c)** Voltamperograms of Cu at a 0.02-mV/min scan rate in different solutions (a, d, and e).

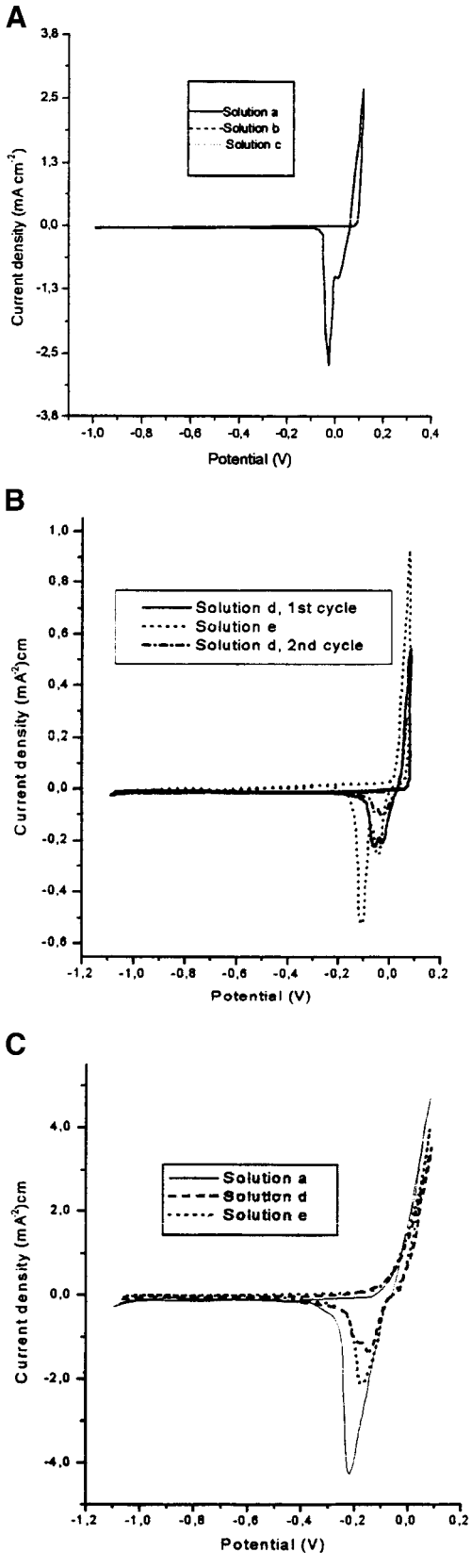
A
Solution a
Solution b
Solution c
Current density (mA cm-2)
Potential (V)
B
Solution d, 1st cycle
Solution e
Solution d, 2nd cycle
Current density (mA2)cm
Potential (V)
C
Solution a
Solution d
Solution e
Current density (mA2)cm
Potential (V)

Table 2
Copper Ion Release into Solutions into Electrolyte Solutions

	Cu [μg/ml. cm^2]			
Exposure period	0	4h	8h	24h
NaCl 9 g/L	0	5.4	10.5	15.9
NaCl 9 g/L + 10% FBS	0	10.5	15.9	21.3
DMEM + 10% FBS	0	15.9	26.4	58.3

tion of osteoblastlike cells. The UMR106 and MC3T3E1 cell lines have been successfully used in our laboratory for different studies on cytotoxicity *(33,34).* Several methods of biocompatibility testing were adopted in the current study: the cell morphology, the osteoblastic proliferation, and the differentiation. Results suggest that the noble elements (Au, Pt, Pd) and the Ni/Ti alloy were very compatible with the culture system. Osteoblasts maintained their cellular morphology, rate of growth, and osteoblastic phenotype in the presence of these noble elements. This is in agreement with previous results obtained with Au, Pt, and Pd alloys, which show low labilities and are unlikely to be released at high levels, even though their atomic percent in the alloy is high. Thus, our present observations with noble elements were considered as controls made in the presence of inert biocompatible metals. On the contrary, Cu and Ag were cytotoxic for these cells. The morphological studies showed that osteoblastlike cells exposed to Cu and, to a lesser extent, to Ag progressively died after arresting cell division. Usually, eukariotic cells die in two distinct modes: necrosis and apoptosis *(35);* both processes seem to occur in the cytotoxicity induced by metal released from dental implants *(36).* Nevertheless, there is increasing evidence that classic apoptosis and necrosis represent only the extreme ends of a wide range of possible deaths. These modes of demise can occur simultaneously in tissues or cell cultures exposed to the same stimulus *(37).* Because necrosis and apoptosis have common features, the distinction between the processes could be difficult *(38).* Cells undergoing apoptosis display characteristic structural changes in the nucleus and cytoplasm, including blebbing of the plasma membrane. Our present morphological studies suggest that the apoptosis occurs early in the cytotoxicity induced by Cu and Ag, evidenced by the observation of membrane blebs and apoptotic bodies. However, after a longer metal exposure, signs

of necrosis became more prevalent.

Studies on cell proliferation showed that Cu induced an arrest of the osteoblastic growth in a dose- and time-dependent manner. It also decreased the ALP, a specific marker of osteoblastic phenotype. Importantly, the kinetic study showed a strong, although nonlinear, correlation between the inhibition of cell growth and the levels of Cu released into the culture media. Experiments carried out by Wataha also demonstrate a correlation between the Cu and Ag levels and the succinic dehydrogenase activity of cultured fibroblasts *(4).* They also suggest that cellular toxicity is not generally a linear function of metal concentration. In addition, it was reported that the use of dental alloys does not allow one to evaluate the toxicity of individual metal components. Through the analysis of the results of several authors, it could be inferred that a simple test should not be enough to assess biocompatibility and that the results will also depend on the sensitivity of the cell under study. Using three different methods (morphological alterations, cell growth, and osteoblastic differentiation), our results suggest that Cu and Ag were the most cytotoxic elements for the osteoblastlike cells in our culture model. These data are in agreement with those obtained by other authors, who have shown a low biocompatibility for Cu and Ag in fibroblasts *(4,11,13).*

The concentration of the released ions depends on the dissolution rate of the metal. In the case of osteoblastic culture medium, the measured concentrations (*see* Table 1) are an average value and do not reflect the gradient concentration that should exist within the well. The dissolution rate depends on the effective diffusion coefficient (D_e). We know that the average value of the ion concentration changes with time according to Table 1 data. The value of D_e is lower than that of water because of the presence of the cells, extracellular matrix proteins, organic molecules, and abiotic particles. The relationship D_e/D_{aq} (D_{aq} = diffusion coefficient of water) can be estimated according to the charge and size of the cation *(39).* Because of the existence of concentration gradients, the concentration of cations will be in the vicinity of the wire, higher than the average measured value, and lower at longer distances. This explains the lower number of survival osteoblasts observed by light microscopy near the wire, after Cu and Ag treatments.

Voltamperograms of copper showed that the oxidation process was higher than the cathodic one when FBS was added. In agreement, spectrophotometric analysis showed that the dissolution is increased in the presence of serum proteins. It has been reported that proteins can adsorb on copper and bind to the metal ions *(14,16–19).* It seems that this process could facilitate the transport of the cations out of the neighborhood of the wire *(40,41)* and, consequently, also favors the dissolution process. Further studies are needed to elucidate the particular action of each component of the biological media on the corrosion process.

In conclusion, the current study shows that the assays made with pure metals are useful for studying the individual cytotoxic effect of each ion on the osteoblastic progression. Cytotoxic processes are strongly

related to silver ion release, which, in turn, is dependent on the composition of the biological media, on the exposure time, and on the position of the cells (distance from the metal sample). Copper and silver ions release in saline media is mainly governed by the concentration of chloride. However, in biological media, the electrochemical processes are also influenced by the composition of the medium, particularly by the presence of proteins. A strong correlation between the ion release from biomaterials and their biocompatibility is suggestive of phenomena that, if it occurs in vivo as a consequence of a long contact with the oral cavity, could induce important alterations in the human tissues located in the vicinity of the biomaterial. Although noble elements as well as the Ni/Ti alloy were biocompatible with osteoblastlike cells, further investigations into the long-term effect of these materials are needed and they are in progress in our laboratory.

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