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Cytotoxicity of Copper Ions Released from Metal

Variation with the Exposure Period and Concentration Gradients

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ABSTRACT

The aim of this work is to contribute to the elucidation of the cytotoxic process caused by the copper ions released from the biomaterials. Clonal cell lines UMR106 were used in the experiments. Copper ions were obtained from two different sources: copper salts and metal dissolution. Experiments carried out with constant ion concentrations (copper salts) were compared with those with concentrations that vary with time and location (dissolution of the metal). Present results and others previously reported could be interpreted through mathematical models that describe: (1) the variation of concentration of copper ions with time and location within a biofilm and (2) the variation of the killing rate with the concentration of the toxic ion and time. The large number of dead cells found near the copper sample with an average ion concentration below the toxic limit could be interpreted bearing in mind that these cells should be exposed to a local concentration higher than this limit. A logarithmic dependence between the number of cells and exposure time was found for nearly constant ion concentrations. Apparent discrepancies, observed when these results and those of different researchers were contrasted, could be explained considering the dissimilar experimental conditions such as the source of the ions and their local concentration at real time.

Index Entries: Biocompatibility; cytotoxicity; copper; biomaterial; released ions; exposure period.

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INTRODUCTION

Metal ions are released from dental biomaterials during tarnishing and corrosion processes *(1,2)* and can cause toxic, inflammatory, and allergic or mutagenic reactions *(3–6).* Copper is a common component of dental alloys. The study of the release of copper ions in biological media is of clinical relevance *(5–8)* because of the toxicity of these metal ions *(9–12).* Cytotoxicity assays made with pure metals have the advantage of avoiding the interferences of other released ions that occur when alloys are used *(7,12).* The tests frequently consist in studying the effect of the toxic ion on specific cellular functions *(11–17).* Clonal cell lines are often used in "in vitro" cytotoxicity assays *(3,4,7,10–16).*

The determination of mathematic correlations to relate cytotoxicity with the concentration of the released ions is important to establish compatibility levels. The transport of ions is limited by diffusional resistance inflicted by the biological film *(18,19).* Thus, the concentration of copper ions released from the metal and, in turn, the toxic effect on cells are possibly variable both with exposure time and along the whole culture monolayer or tissue. Notwithstanding this, biocompatibility assays are often made by adding to the cell culture either salts of the metal ions or extracts (obtained by exposing the metal sample in a solution without cells) *(5,11).* In these tests, there are neither concentration gradients of copper ions nor variations with time. There are apparent discrepancies between these assays and those made with cultures grown in the presence of the metal sample *(6,10,11,20,21)* where the concentration is not constant.

The aim of this work is to study the toxic action of copper ions against osteoblastlike cells and to analyze its variation with the exposure time and distance from the metal. Mathematical models were used to interpret the results. The influence of experimental conditions is discussed to elucidate the cause of the apparent disagreements among conclusions reported by different authors.

MATERIALS AND METHODS

Metal Samples

Pure Cu (area = 0.314 cm^2) samples were tested. They were polished with alumina powder (1 μm). After being rinsed in acetone, they were washed in sterilized water and then 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 0.6–6 cm^2/mL, as recommended by the International Standards Organization for assessment of biomaterials *(22).* The concentration of the ions in the culture media was measured by flame atomic absorption spectrophotometry. The well was divided in six regions in order to investigate the influence of the location

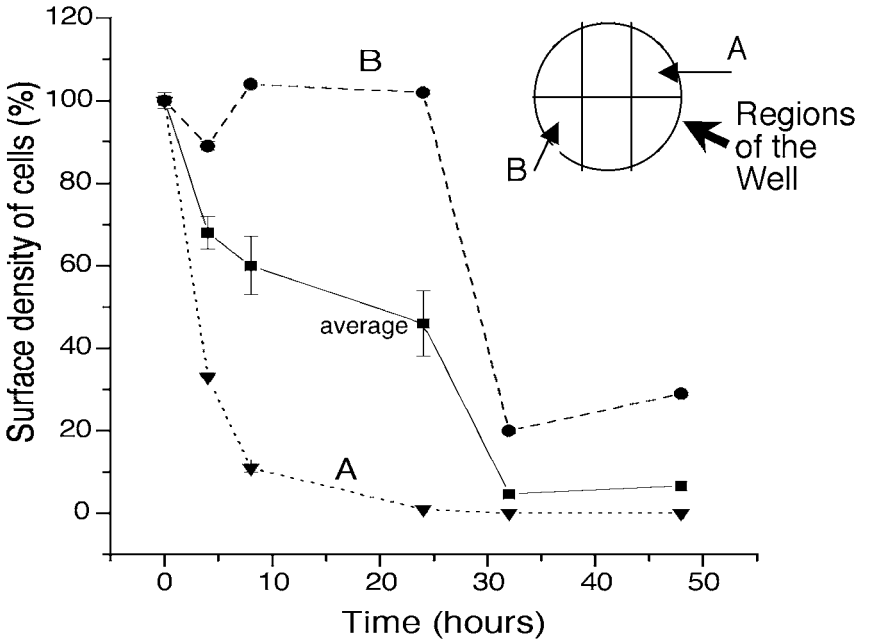

Fig. 1. Number of surviving cells versus exposure time plot (●) in region A (close to the metal sample), (■) in region B (far from the metal sample), and (▼) the average value of the six regions. The release of Cu into the media was assessed by atomic absorption spectroscopy. Results are expressed as means ± SEM (n = 6). Basal values are 126 ± 7 cells/field. The inset shows the representation of the well with the six regions. Regions A (where the copper sample is placed) and B is identified.

on the number of surviving cells. The metal sample was placed in the middle of region A (Fig. 1).

Cell Culture and Incubations

UMR106 rat osteosarcoma-derived cells were obtained from American Type Culture Collection (ATCC) (Rockville, MD, USA). The cells were grown as indicated previously *(12)* 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). 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 copper wires or medium alone (basal control), under the conditions described in the legends of the figures.

Studies of Cell Growth and Morphology

In order to determine cell growth, the monolayers were washed twice with a phosphate buffer solution, as indicated previously *(12),* fixed with methanol for 5 min at room temperature, and stained with a 1/10 solution of Giemsa for 10 min. Then, the plates were washed with water and the cell number was evaluated by counting the stained nuclei in six fields per well.

Statistical Analysis

For each experimental condition, at least three separate experiments were performed. Data are expressed as mean ± SEM. Statistical differences were analyzed using a paired Student's *t*-test.

RESULTS

The wells were divided in six regions in order to investigate the influence of the location on the number of surviving cells (x). The results coming from the region containing the copper sample (region A) and the farthest region (region B) were compared (Fig. 1). The average value of surviving cells (axv) of the six regions was also plotted.

Results showed that after 4 h in culture the number of osteoblasts near the copper sample (region A) was low (<40% of the control; Fig. 1) and the cells showed important morphological changes. Four hours later (8 h), the number of cells of region A decreased to 12%, and to 2% after 24 h. The remaining osteoblasts showed loss of processes, picnotic nuclei with distorted shape, and blebbing of the plasma membrane, in agreement with the characteristics previously reported *(12,23–26).* No mitotic figures were observed in the few cells that remained alive. Exposures of 32 and 48 h in the presence of copper induced almost a complete cellular death in region A. To the contrary, in region B, the number of cells was close to that of the control, during the first 24 h. However, In region B, an abrupt decrease in the surviving cells was observed after 32 h in this region.

The number of surviving cells was also evaluated in solutions containing $CuSO_4$ after 4 h exposure time. In this case, the concentration was uniform throughout the well. The assays showed that even though copper concentration was high (up to 15 mg/mL), the cells were only slightly affected (less than 15% were killed; Fig. 2).

DISCUSSION

Simulation of the Dependence of the Copper Ions Concentration Values with Time and Position

Previous studies *(12)* showed that the average concentration of copper ions (acv) varies nearly linearly with time and that there is a strong and

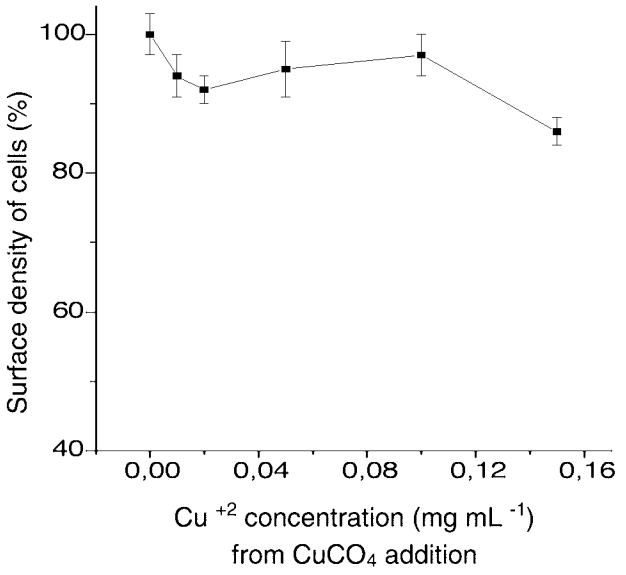

Fig. 2. Number of surviving cells versus copper ions concentration obtained by the addition of $CuSO_4$) after a 4-h exposure time. The basal value is 104 ± 7.

nonlinear (second order) relationship with the number of the total surviving cells (axv) (Fig. 1). However, it must be kept in mind that the acv is the result of the accumulation of copper ions released during the preset time. The instantaneous concentration of copper probably varied both with location (high, near to the metal sample; low, in region B of the well) and during the exposure time.

To simulate the variation of the copper ion concentration with time and position in a cell culture, diffusional equations can be used *(27).* As a first approach, the transient diffusion of copper into the cell culture can be simulated by a one-dimensional form of the diffusion equation for homogeneous medium, assuming a step increase of copper ion concentration. With the aim of simplicity, it can also be assumed that copper ions are noninteracting particles (i.e., neither reaction no sorption processes occur) and that convective transport is negligible. Thus, the nonsteady mass balance for copper ions in a biological film can be represented by *(18,28)*

$$\frac{\partial C}{\partial t} = D_e \frac{\partial^2 C}{\partial z^2} \tag{1}$$

$$\text{at } z=L_f \quad C=C_0 \quad \text{for all } t > 0 \tag{1a}$$

$$\text{at } z=0 \quad \frac{\partial C}{\partial z} = 0 \quad \text{for all } t > 0 \tag{1b}$$

$$\text{at } t \leq 0, \quad C = 0 \quad \text{for all } \; 0 \leq z \leq Lf \tag{1c}$$

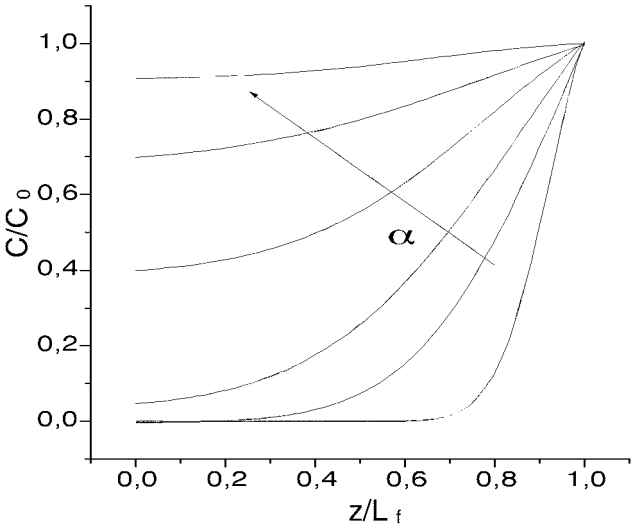

Fig. 3. Transient diffusion of a noninteracting solute into a biofilm. (Adapted from ref. *27*.)

D_e is the effective diffusion coefficient in the cell layer, z is a spatial coordinate indicating the distance from the copper sample (z varies between 0 and a characteristic length L_f=$d/2$, d = diameter of the well), t is time (s), and C is the copper ion concentration. Low values of z, close to 0, correspond to region B and high values (close to 1) correspond to region A. Equation (1) constitutes an unsteady mass balance for the copper ions. The local accumulation of the ions, is represented by the left-hand-side term and the term on the right-hand side represents the net change in concentration caused by diffusion. With the aim of simplicity, the boundary conditions (1a) and (1b) impose a constant concentration C_0 at the metal/cell culture interface (z=L_f) and a concentration gradient $\partial C/\partial z = 0$ at long distances from the metal (z=0), respectively.

The solution of Equation (1) is *(27)*

$$\frac{C}{C_0} = 1 - 2\sum_{n=o}^{\infty} \frac{(-1)^n}{(n+1/2)\pi} \exp\left[-(n+1/2)^2 \pi^2 \alpha\right] \cos\left[(n+1/2)\frac{\pi z}{L_f}\right] \qquad (2)$$

According to Eq. (2), C/C_0 depends on α and is reproduced graphically in Fig. 3 *(27)*. The C/C_0 distribution in the well can be qualitatively interpreted using this figure. It shows that the concentration of copper ions varies with the position (z/L_f) and with time [α=α(*t*)]. The parameter α is given by

$$\alpha = \frac{tD_e}{L^2_f}$$

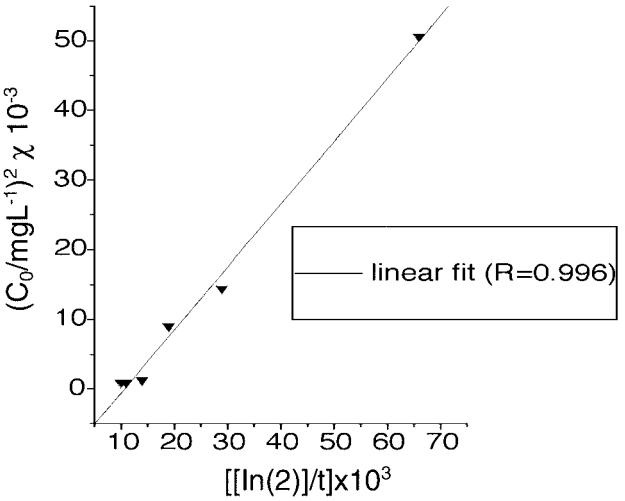

Fig. 4. Ln(50%/100%)/t versus C^2 relationship, set of TC50 data from ref. *5*. The linear regression fits with a 95% confidence; correlation coefficient r=0.996.

Application of the C/C_0 versus z/L_f and α Relationship

Figure 3 shows that C depends both on the distance z and on time [$C=C(z, \alpha)$, $\alpha=f(t)$]. The value of D_e is lower than that of water because of the presence of the cells, extracellular polymeric substances, organic molecules, and abiotic particles. The relationship D_e/D_{aq} can be estimated according to the charge and size of the toxic ion. Assuming that D_e/D_{aq}=0.5 is similar to that of diffusion of cations in a mixed biofilm *(18,19)* and with $D_{aq} = 6 \times 10^{-6}$ cm^2/s (the diffusion coefficient of the cupric cation in dilute aqueous solution), the D_e value can be obtained:

$$D_e = (0.5) \times 6 \times 10^{-6} \text{ cm}^2/\text{s} = 3 \times 10^{-6} \text{ cm}^2/\text{s}$$

The value of α for an exposure period of 2 h, with L_f=1.5 cm (radius of the well), can be calculated as

$$\alpha = \frac{tD_e}{L^2_f} = \frac{7200\,s \times 3 \times 10^{-6}\ cm^2 s^{-1}}{(1.5\,cm)^2} \approx 0.01.$$

Under these conditions, at 0.5 cm from the copper sample (z/L_f=0.67, α=0.01) from Fig. 4, the relationship C/C_0 is approx 0.02 and in region B (z/L_f=0), $C/C_0 \approx 0$. However, 6 h later, z/L_f=0.67, t=28800 s, and α=0.040 yield to $C/C_0 \approx 0.2$. After 24 h exposure, α=0.12 and $C/C_0 \approx 0.42$. Thus, the simulation shows that the concentration is strongly dependent on time, on the position and on the D_e value, which in turn depends on the type of the cell culture.

Each curve of Fig. 3 represents the variation of the concentration with the location for a preset time determined by the α value. For a particular location (z/L_f), the variation of concentration with time can be analyzed by moving vertically on the graph. However, the actual situation is even more complex because C_0 is not constant in a corrosion process and also there must be effects of the borders because the diameter of the well is not very long. Moreover, the diffusion coefficient might not be constant throughout the cell line because it is an inhomogeneous medium and, additionally, some of copper ions might not be free but might be a metal–protein complex with D_e values different from those of the free ions *(28).*

According to Fig. 3, the situation of the cells near the biomaterial is dangerous because notwithstanding that the average value acv was lower than the toxic limit, these cells might be exposed to concentration levels higher than the toxic limits if they are near the metallic biomaterial. In addition, there is an important influence of exposure time on the toxic action.

Comparisons Between Assays Made with Constant and Variable Concentrations

Figure 2 shows that after 4 h exposure, even with 0.15 mg/mL $CuSO_4$, the number of surviving cells was high (approx 85%). However, a higher number of dead cells were found after the same exposure period with very low average copper ion concentration (approx 0.02 mg/mL, from ref. *12).* The high number of dead cells can be explained considering that the concentration of copper ions must be very high near the metal, as is shown in Fig. 3, and yields to an important killing action, even though the average concentration is low.

Dependence of the Killing Rate on the Concentration of Ions and Time

According to the previous discussion, the local concentration that varies with the position and the duration of exposure influences the toxicity of released metal ions. The Chick–Watson mathematical relationship simulates the kinetics of killing action of toxic agents and takes into account both the concentration of the toxic agent (C_0) and the time of exposure *(t) (27).* It could be used as a simple way to interpret the current problem with cell cultures. The dependence of the cell-killing rate *(dX/dt)* with the toxic agent concentration (C_0) could be the following:

$$\frac{dX}{dt} = -k_{tox} C^n X \tag{3}$$

where X accounts for viable cells density, t is time, and k_{tox} is a killing rate constant of the toxic agent.

The exponent n on the concentration captures the concentration dependence of killing. When the concentration C is constant (C_0), the solution of the model after integration yields.

$$\ln\left(\frac{X}{X_o}\right) = -k_{tox}C_0{}^n t \tag{4}$$

where X_0, and C_0 account for cell density and concentration at the initial time. Equation (4) could be useful for analyzing the assays made with copper salts or extracts added to the culture medium in which concentration varies neither with time nor with position.

Application of the Correlations Number of Cells Versus Concentration of Ions and Time

Equation (4) can be used to simulate data obtained under the constant ion concentration reported previously *(5)*. An exponential decay of the number of surviving cells with copper ions concentration with time was found. If C_0 represents the toxic concentration to depress cellular activity by 50% of the original value (100%), Eq. (4) yields

$$\frac{\ln\left(\frac{50\%}{100\%}\right)}{t} = -k_{tox}C_o{}^n$$

Figure 4 shows that the $\ln(2)/t$ versus C_0 set of data fits with a linear regression (r=0.996, 95% confidence, $\ln(2)/t$). =–$\ln(1/2)/t$, n=2).

In the case of present results, the concentration of ions is not constant, $C=C(z, t)$ and $X=X(z, t)$, and for in vivo conditions, the situation seems to be similar; consequently, from Eq. (3) *(27)*

$$\frac{dX}{dt} = -k_{tox}C(z,t)^n X(z,t) \tag{5a}$$

For a particular small region (e.g., region A), Eq. (5a) can be written assuming that the concentration of ions is uniform in this small area but varies with time. Thus, Eq. (5a) becomes

$$\frac{dX_A}{dt} = -k_A C_A(t)^n X_A(t) \tag{5b}$$

$$\frac{dX_A}{X_A} = -k_A C_A(t)^n dt \tag{5c}$$

In the cases of regions A and B, and also for the average data of the six regions (axv), $\ln(X/X_0)$ versus t is linear (r=0.967, r=0.998, and r=0.968, respectively) during the first 24 h. Thus, for region A,

$$\ln\left(\frac{X_A}{X_0}\right) = K_A t$$

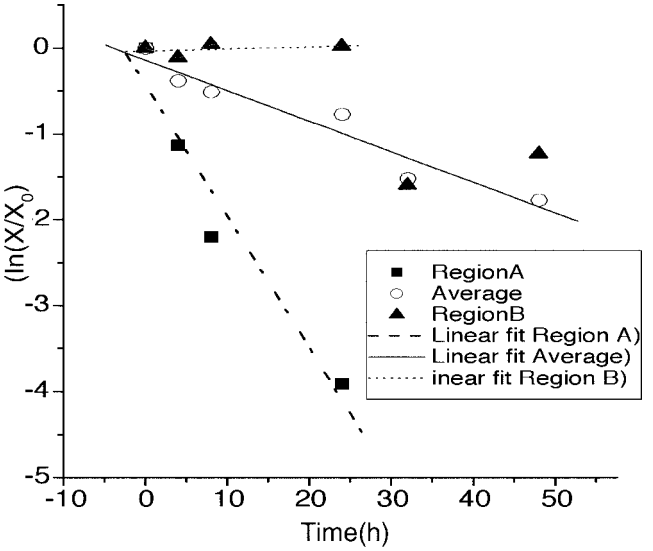

Fig. 5. Ln (X/X_0) versus t plot from data of Fig. 3. X=survival cells at time t, X_0 = survival cells at time t=0 of region A (■), B (▲), and the average value (axv) (○). The linear regression fits with a 95% confidence (r_A=0.968, r_B=0.998, r=0.967).

K_A is a global coefficient for region A. The slopes of the curve of region B and that of axv are lower than that of region A because the corresponding concentrations should be lower according to Fig. 3. Conditions of Fig. 4 are different from that of Fig. 5 because the concentrations of ions during the assays of Fig. 5 increased with time (they were measured at the end of the exposure period), whereas those of Fig. 4 are constant.

In the case of region B, the number of surviving cells seems to be time independent during the first 24 h (Fig. 5), probably because the concentration level of copper ions is below the toxic level for this exposure time. For exposure periods longer than 24 h in region B, there was an abrupt decrease of the surviving cells (Figs. 1 and 5), which is not associated to a sudden increase in the concentration of copper ions. A similar effect was observed with silver ions *(8).* Thus, the killing action of both copper and silver ions is dependent on the exposure time. However, it seems that for a low concentration of toxic ions, there is a threshold value of exposure time that could not be predicted by the mathematical model (Fig. 5). Therefore, it can be deduced that the number of dead cells in clinical situations should also be related not only to the copper ion concentration, which varies with time and position, but to the exposure period as well.

The inferences drawn from the application of the mathematical models are in agreement with the key concepts previously reported by Wataha *(5):* (1) There is a microenvironment around metallic biomaterials; (2) metal ions can cause local toxicity; (3) the toxicity increases with exposure time.

It is well known that the rate of dissolution of pure metals and alloys depends on their composition and previous treatments. Results showed that in the case of copper, cytotoxicity was related to the local concentration of the released ions and exposure time. However, the local biologic effects of ions is still a cause of debate. When pure metals or alloys are used as biomaterials inside the body, synergistic and antagonistic effects were found between these ions and the ions and molecules present in the organic fluids (e.g., the effects of the pairs Cu–Ni, Cu–Ag, Cu–Zn, and Cu–Cd were analyzed) *(29).* Consequently, studies with the particular alloy of interest must be made to determine the antagonistic and synergistic effects of the ions coming from the corrosion process.

Even though the simplest situations were considered, assuming several restrictions in the application of the models with the aim of facilitating the analysis, it can be remarked that the study of the cytotoxic effects of the release of ions from metals in vitro and in vivo is complex including a complicated functionality of the variables involved.

It is helpful to consider previous reflections when assays made with metal samples within the culture are contrasted with those performed with constant ion concentrations (coming from the addition of a salt of the metal ion or from an extract of ions).

CONCLUSIONS

Current results show that the in vitro studies of the kinetic of the killing process caused by toxic ions released from metals is strongly dependent on the location of the cells and on the exposure time. They also show that when toxicity assays made by different researchers are contrasted, the source of the ions (released from the metal [*in situ* or *ex situ* using extracts] or from salts) and how concentration values are measured must be considered. The possible concentration values could be (1) final average values after the accumulation during the exposure time (extracts), (2) variable values, with time and location (metallic biomaterial within the cell culture), or (3) constant values (addition of salts). It is important to bear in mind that in clinical situations, there are concentration gradients close to the metallic biomaterial that change with time.

Some of the apparent discrepancies between results obtained with constant or variable concentration of cations could be explained applying the mathematical models previously proposed.

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