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Synergistic cytotoxic effects of ions released by zinc–aluminum bronze and the metallic salts on osteoblastic cells

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Abstract: The use of copper-based alloys for fixed dental crowns and bridges is increasingly widespread in several countries. The aim of this work is to study the dissolution of a zinc–aluminum–bronze and the cytotoxic effects of the ions released on UMR-106 osteoblastic cell line. Two sources of ions were used: (1) ions released by the metal alloy immersed in the cell culture and (2) salts of the metal ions. Conventional electrochemical techniques, atomic absorption spectroscopy [to obtain the average concentration of ions (AC) in solution], and energy dispersive X-ray (EDX) spectroscopy analysis were used to study the corrosion process. Corrosion tests revealed a strong influence of the composition of the electrolyte medium and the immersion time on the electrochemical response. The cytotoxicity was evaluated with (a) individual ions, (b) combinations of two ions, and (c) the mixture of all the ions released by a metal disc of the alloy. Importantly, synergistic cytotoxic effects were found when Al–Zn ion combinations were used at concentration levels lower than the cytotoxic threshold values of the individual ions. Cytotoxic effects in cells in the vicinity of the metal disc were also found. These results were interpreted considering synergistic effects and a diffusion controlled mechanism that yields to concentration levels, in the metal surroundings, several times higher than the measured AC value. © 2013 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00A:000–000, 2013.

Key Words: cytotoxicity, metal ion, bronze, dental alloy, metallic salt

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INTRODUCTION

In the last years, several hundreds of alloys for prosthodontic restorations have been developed. Many factors affect alloy selection, among others: economic reasons, physical and mechanical properties, casting technique, corrosion, and biocompatibility.[1] In parallel, there has been increasing demand for safety evaluation and control of dental materials due to the toxic/irritative or allergic reactions, both local and systemic, that were detected in patients.[2] Such reactions are most frequently located in the contact zone with the biomaterial.[3]

Biocompatibility of dental alloys is strongly related to their metal ions release.[4] The biological effects of these metal ions are significantly different and depend of several factors such as oral environment, exposure time, nature and amount of release metal cations, and so on.[5,6]

Cellular interactions with metal ions show differences in rates of uptake,[7] in concentrations levels which affect cell response, and in the cellular functions affected.[8–12] Different sources of ions have been used in cytotoxic studies: (a) the extracts obtained from the dissolution of a metallic sample *ex situ* and then added to the cell culture;[13–15] (b) the dissolution of metal samples *in situ*;[16–21] and (c) the metal salts added to the cell culture.[22–28] Data of the amount of metal ions released by several dental casting alloys were obtained in different assays.[13] The cytotoxic effects of several ions were also evaluated.[3] However, nonuniform distribution of the ions with higher local concentration of metallic elements in microenvironments formed between the alloys and the tissues was found.[29–31] It is worth noting that data found in the literature rarely include copper-based alloys, because it is frequently assumed that they are not used as dental casting alloys.

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TABLE I. Concentrations of the Metal Ions Released by CuBA Immersed in Synthetic Saliva

Metal Ion	Concentration of the Metal Ion Released by CuBA in Synthetic Saliva[a] AC24h[b] mg/L	AC24h[b] μg/cm^2	AC8h[c] mg/L	Composition of CuBA % w/w
Copper	0.041 ± 0.014	0.175 ± 0.068	0.167	81.5
Aluminum	2.384 ± 1.958	9.797 ± 7.923	9.731	7.0
Nickel	0.088 ± 0.015	0.376 ± 0.078	0.359	4.5
Iron	0.113 ± 0.076	0.493 ± 0.341	0.461	3.0
Manganese	0.071 ± 0.015	0.306 ± 0.078	0.290	2.0
Zinc	0.102 ± 0.022	0.439 ± 0.112	0.416	2.0

[a] Detection limits (mg/L): Cu = 0.005; Al = 0.025; Ni = 0.006; Fe = 0.006; Mn = 0.008; Zn = 0.007.
[b] AC24h are ion concentration values measured by atomic absorption spectrophotometry released from 49 cm^2 CuBA samples immersed in 200 mL SS after 24 h exposure period, at 37°C.
[c] AC8h are concentration values referred to 3 cm^2 dental alloy, exposed to 1 mL of SS for 8 h, calculated from the AC24h data.

Despite expressed doubts as to their suitability,[32] copper-based alloys have been used as dental casting alloys for fixed prosthesis in the US, Japan, South America, and some countries of Eastern Europe for more than 25 years.[33,34] According to dental technicians and dental suppliers, their popularity is due to their good mechanical strength, high elastic modulus, low density, its bright yellow colour with strong resemblance gold and its low-cost relative to noble metal alloys.[35–37]

Notwithstanding its good mechanical properties, aluminum–bronze dental alloy shows low corrosion resistance when it is exposed to saline or artificial saliva.[33] Tibballs and Erimescu[33] reported a marked galvanic action that caused pit-etching. They found pits with depths and widths in the range 0.5–1 μm. In agreement, Ardlin et al.[35] reported high corrosion rate for this copper alloy due to its inability to create a protective surface layer. Additionally, they found high levels of toxic ions after static immersion testing, with adverse effect on the biological activity in the surroundings.

Clinical studies have shown that corrosion of crowns and posts of such alloys causes staining and inflammatory processes in the surrounding gum tissue and blue–green pigment in the roots of teeth.[36,37] Thus, the widespread use of copper-based alloys in several countries is a cause of great concern due to the undesirable effects found on surrounding cells by metals ions released from these alloys (Cu, Ni, Zn, Al ions).[33,35,36,38]

One of the copper-based alloys widely used is an aluminum–bronze commercially available as Ventura Orcast PLUS®. It is featured to be used for fixed dental prosthesis like crowns and bridges covered with resins but it is also used in endodontical posts.

The aim of this work is to study the cytotoxic effects of the ions released by Orcast PLUS® casting alloy as a response to the increasing demand of control of dental materials. The highly toxic environment provoked by the release of ions is also a suitable area of study to assess an additional purpose: to elucidate the cause of the different behavior cells in relation to (a) ions released by a metal alloy immersed in the cell culture (*in situ*) and (b) the presence of equivalent amounts of salts of the metal ions. For this reason, the effect of a disc of the copper-based dental casting alloy on the surrounding cells and the variation of the cytotoxic effect with distance between cells and the source of ions were assessed in this work. Conventional electrochemical techniques and energy dispersive X ray (EDX) spectroscopy analysis were used to study the corrosion process and the concentration of the ions released was measured by atomic absorption spectroscopy. The cytotoxic effect of individual ions, some combinations of two ions and the mixtures of all the ions of the alloy components on osteoblastic cells were also analyzed to investigate possible synergic effects.

MATERIALS AND METHODS

Cells culture

Rat osteosarcoma derived cells (UMR-106 line) was originally obtained from American Type Culture Collection (ATCC) (Rockville, MD). Cells were grown as monolayer in Falcon T-25 flasks with 10 mL D-MEM cell culture medium (CCM) (GIBCO-BRL, Los Angeles) supplemented with 10% inactivated fetal calf serum (Natocor, Carlos Paz, Córdoba, Argentina), 50 IU/mL penicillin, and 50 μg/mL streptomycin sulfate complete culture medium (CCM) at 37°C in a 5% CO_2 humid atmosphere. Cells were counted in an improved Neubauer hemocytometer and viability was determined by the exclusion Trypan Blue (Sigma, St. Louis, MO) method; in all cases viability was higher than 95%.

Copper-based alloy and metal ions released

The copper-based alloy (CuBA, see Appendix for acronyms) used in the assays was Orcast PLUS® (Madespa S.A., Toledo, Spain) which composition is shown in Table I.

Cylindrical casting copper and CuBA electrodes, casting CuBA discs for experiments with cells, for optical microscopic observations, and EDX analyses, and casting CuBA samples for atomic absorption spectroscopy were made by lost-wax casting process.[39] With this objective, a wax pattern with the suitable shapes for each purpose was formed.

To assess the metal ions release square shape sheets of the casting copper alloy of about 49 cm^2 of geometrical area were immersed for 24 h, at 37°C in 200 mL of acid synthetic saliva (SS) pH = 4.77 to assay the aggressive condition of low pH related to fermentable dietary sugars, metabolic products of dental plaque,[35] and so on. After this 24 h period of static immersion, the metal ion concentrations in the SS medium was measured (AC24h) by atomic absorption spectrophotometry using a VARIAN Spectrophotometer model SpectrAA55 (serial number EL99043438). The experimental conditions (large area and long exposure period) were selected to detect the concentrations of ions that were close to the detection limit. Each measurement was repeated three times in independent experiments. The composition of SS used in all the experiments was: NaCl 0.40 gr/L, KCl 0.40 gr/L, $CaCl_2$ 0.80 gr/L, NaH_2PO_4 0.16 g/L, urea 1.00 g/L, and KSCN 0.16 g/L.

Prior to each assay, the square shape samples of CuBA were prepared according to the following polishing and cleaning procedure, and then were directly immersed in the quiet test SS solution. They were initially abraded using successively SiC 80, 220, 800, and 1200 mesh emery paper. A final polishing and cleaning sequence was performed as follows: polishing with diamond paste (6 μm) and alumina (1.0 μm and 0.3 μm). All the abraded and polishing procedures were followed by water rinsing and sonicated with water in each step. Finally, the sheet of CuBA was degreased using dichloromethane and ethanol, sonicated with water and dried with nitrogen gas.

Corrosion tests

To analyze the dissolution process of CuBA, electrochemical assays were carried out. These experiments were performed in a conventional undivided gas-tight glass cell with dry nitrogen gas inlet and outlet. The working electrode was an Orcast PLUS® bar encapsulated in Teflon, with an exposed geometrical circular area of 0.1256 cm^2, the counter-electrode was a 2 cm^2 Pt foil and as reference a saturated calomel electrode (sce) (to which all potentials reported are referred) was used.

A computer controlled PAR 273A potentiostat was used for experiments. The potentiodynamic measures were performed in nitrogen deareated SS (20 mL) and in nitrogen deareated CCM (pH = 7) (20 mL).

Prior to each electrochemical measurement, the working electrodes were prepared according to the following procedure, and then were directly immersed in the test solution. They were initially abraded using successively SiC 600, 1000, and 2500 mesh emery paper. A final polishing and cleaning sequence was performed: polishing with alumina (1.0 μm, 0.5 μm, and 0.3 μm) and diamond past (0.3 μm) followed by water rinsing in each step. Finally, the electrode was degreased using dichloromethane, sonicated with water, and dried with nitrogen gas.

To analyze the dissolution process of CuBA under simulated conditions close to those of oral environment, potential region close to the open circuit potential (ca. ±300 mV) was investigated. Potentiodynamic polarization curves of CuBA and copper electrodes exposed to SS and CCM were obtained in the conventional way. Potentiodynamic scans were made at 5 mVs^{-1} sweep rate (v) and started at −0.580 V in the anodic direction to +0.050 V. Prior to each measurement, the electrode was subjected to a cathodic pretreatment by holding it potentiostatically at −0.580 V for 60 s, to reduce oxide film possibly formed in air; then, polarization was started after a constant immersion time (5 min) of the alloy in the electrolyte. The selected cathodic limit was also suitable to detect the possible effect of sulfur containing species.[40] Occasionally, to investigate the effect of the immersion time on the dissolution process, prior to the potentiodynamic experiments the electrodes remained immersed in the quiet electrolyte (CCM or SS) during periods between 30 and 1440 min. The assays were repeated at least three times to check the reproducibility and the polarization curves were repetitive.

All the chemicals used in the experiments were of analytical grade. Milli Pore-MilliQ water was used to prepare the solutions.

Microscopic observations (microscope used: Olympus BX51 connected to an Olympus DP71 color video camera, Olympus Corp., Tokyo, Japan) of freshly polished CuBA surface discs (diameter: 0.5 cm; height: 0.1 cm; exposed area: 0.35 cm^2) before and after an exposure time of 10 days in SS, al 37°C were performed. Prior to each experiment, the discs of CuBA were prepared according to the polishing and cleaning procedure described above (see section Copper-based alloy and metal ions released), and then were directly immersed in the test SS solution maintained at the constant temperature. After the selected time, the discs were washed briefly with water, thoroughly dried with nitrogen gas, and mounted into the microscope without any further treatment. The surface composition of the discs before and after the 10 days of immersion in SS, at 37°C was analyzed by EDX (spectrometer Philips 505 SEM; Soft Imaging Sistem ADDA II, EDAX detecting unity, UTW). The discs washed and dried as above described were mounted into the spectrometer without any additional treatment.

Metal ion solutions for cytotoxicity assays

Cytotoxicity tests with metal ions were made using the corresponding salts dissolved in the culture medium. The concentration values of the ions released by salts named as AC8h values were obtained from the atomic absorption spectroscopy analysis data (AC24h, 24 h, 49 cm^2, 200 mL SS) previously mentioned. AC8h values were calculated assuming that a sample of 3 cm^2 of geometric area was exposed to 1 mL of SS during 8 h period (sleep time period), and that there are linear relationships between concentration and immersion time and concentration and exposed area. Concentrations corresponding to 30 (AC8h × 30), and 60 (AC8h × 60) times higher than AC8h level were also assayed.

The evaluation of cytotoxicity of metal ions was made by exposures to solutions of different concentrations of the corresponding metal salts obtained from Merck Chemical Co. (Darmstadt, Germany). The metal salts included, copper

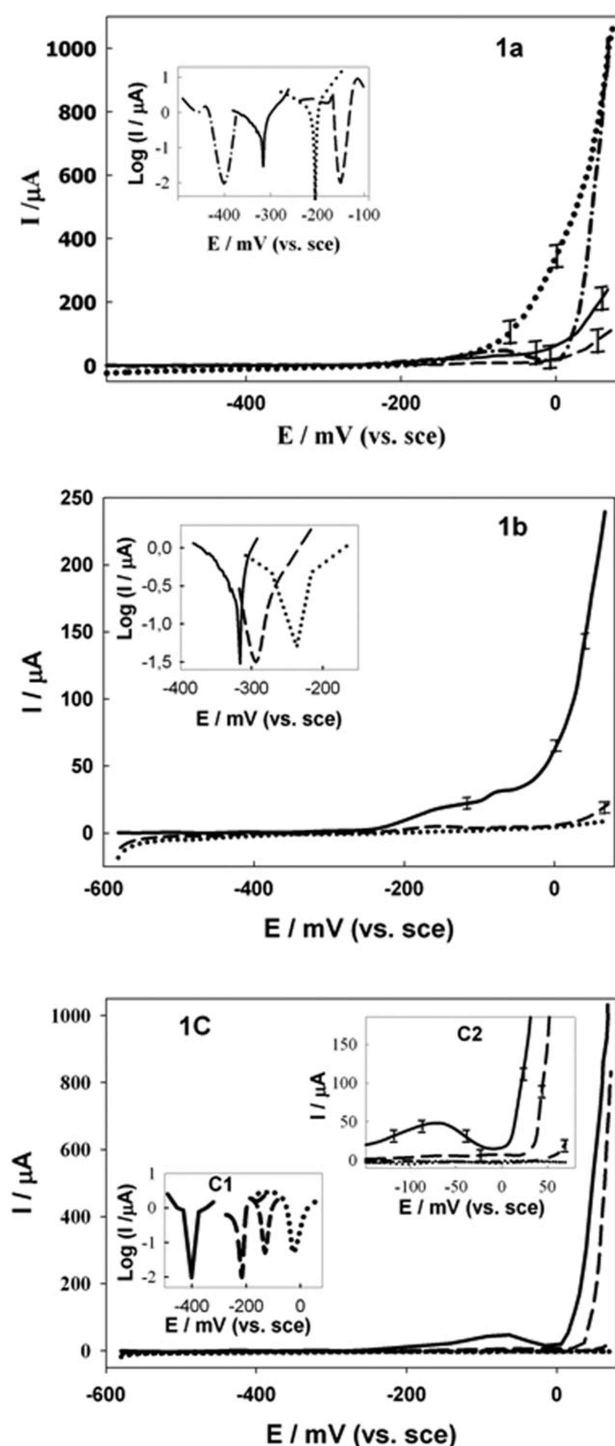

FIGURE 1. (a) Typical potentiodynamic polarization curves for Orcast PLUS® copper-based alloy immediately after immersion in deaerated (——) synthetic saliva solution (SS, pH = 4.77) and (— •) culture medium (CCM, pH = 7.0), at 37°C. Potentiodynamic polarization curve for copper, the metal base of alloy, is included for comparison: (- - -): SS, pH = 4.77, (• • •): CCM, pH = 7.0. Inset: Log I vs. E plots. (b) Typical potentiodynamic polarization curves for Orcast PLUS® copper-based alloy measured (— —) immediately, after (- - -) 30 min, and (•••) 60 and 1080 min of immersion in deareated and quiet artificial saliva, at 37°C. Inset: Log I vs. E plots. (c) Typical potentiodynamic polarization curves for Orcast PLUS® copper-based alloy measured (——) immediately, after (— —) 60 min, (- - - -) 180 min, and (•••••••) 1440 min of immersion in deaerated and quiet culture medium, at 37°C. Insets: (C1) Log I vs. E plots; (C2) Detail of the −150 to 75 mV potential region with the error bars.

($CuCl_2.6H_2O$), aluminum ($AlCl_3$), nickel ($NiCl_2.6H_2O$), iron ($FeCl_2.\ 6H_2O$), manganese ($MnCl_2.4H_2O$), and zinc ($ZnCl_2$) salts.

Evaluation of the effect of metal ions release by Acridine Orange staining

For this set of experiments, 4.5×10^4 cells were seeded in Petri dish (100 mm diameter) and grown at 37°C in 5% CO_2 humid atmosphere in CCM (pH = 7), for 24 h. Then, the medium was removed, a 4-mm diameter CuBA alloy disc was added in the center of each Petri dish, and immediately fresh medium was incorporated. Cells were grown under these conditions during different periods: 3 h, 24 h, and 48 h. UMR-106 cell culture without the alloy disc were used as negative controls. To facilitate the analysis of cytotoxic effects as a function of the distance from the source of ions, the area with cells was divided in concentric regions around the metal disc (according to the inset of Fig. 7): Region A (divided in subregions A1, A2) close to the metal disc, B and C (the farthest). After exposure periods, adherent cells were stained with Acridine Orange dye (Sigma, St Louis, MO) and immediately after, they were examined by fluorescence microscopy (Olympus BX51, Olympus Corp., Tokyo, Japan) equipped with appropriated filter, connected to an Olympus DP71 (Olympus Corp., Tokyo, Japan) color video camera. The images were taken immediately after opening the microscope shutter to the computer monitor.

Evaluation of the effect of metal ion release on the formation of cell colonies

To investigate the effect of the release of ions by CuBA on the distribution, number and size of the colonies, colony formation, or clonogenic assays were performed. This experiment is an *in vitro* cell survival test based on the ability of a single cell to grow into a colony.[41] For this analysis, 50 cells /Petri dish were grown at 37°C in 5% CO_2 humid atmosphere in CCM in presence of an alloy disc in the center of each place. An additional cell culture without alloy disc was used as negative control. After incubation for 7 days, the colonies of acceptable size were taken into account for scoring. They were fixed with methanol:acetic acid (3:1) and stained with Acridine Orange. The enumeration and classification of colonies (cell clusters) was made under fluorescence microscopy with a 40× objective (Olympus BX51, Olympus Corp., Tokyo, Japan). Two experiments were performed in independent trials to assess reproducibility.

Determination of cytotoxicity of metal ions by Neutral Red assay

Metals ions cytotoxicity was estimated in UMR-106 cells using the Neutral Red (NR, 3-amino-7-dimethylamino-2-methylphenazine hydrochloride) assay according to Borenfreud and Puerner.[42] This assay measures cellular transport based on the dye uptake by living cells. Absorbance change is directly proportional to the number of viable cells.

For this analysis, 2.7×10^3 cells/well were cultured in 96 multiwell plate and grown at 37°C in 5% CO_2 humid

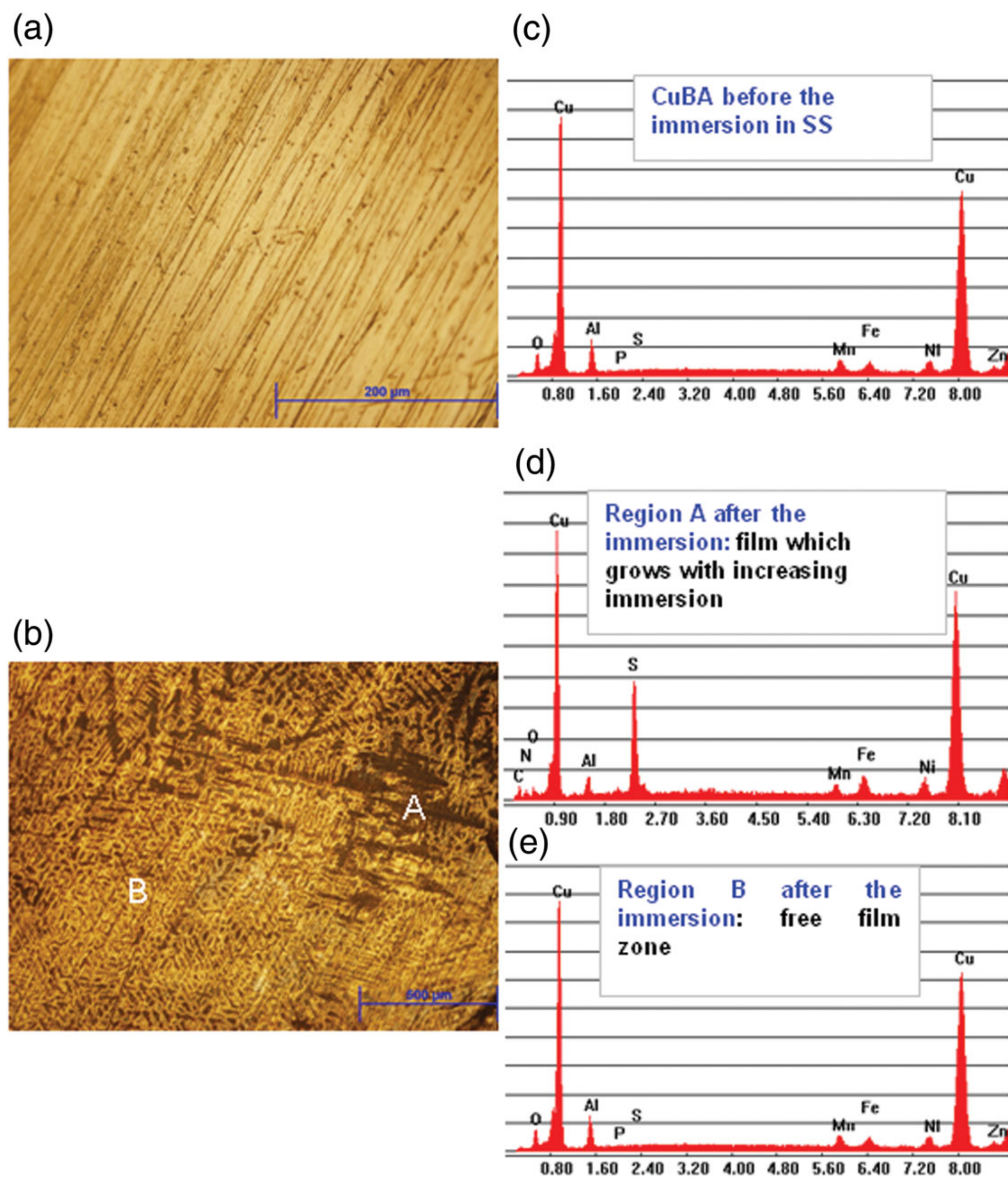

FIGURE 2. Optical microphotographs (magnification ×40) of freshly polished CuBA surface before (a) and after (b) the immersion in synthetic saliva for 10 days, at 37°C. EDX profile analyzes of freshly polished CuBA surface discs before the immersion (c) and after 10 days immersion period (d, e), on different zones of the surface [A, B according to Fig. 2(b)]. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

atmosphere in CCM for 4 h. Then, the medium was replaced by other with different metal ion concentrations (MIC). After 24 h, the liquid medium was removed and fresh medium containing 40 μg/mL RN dye (Sigma, St. Louis, MO) was added. After 3 h incubation, cells were washed with a phosphate buffer. Color was developed by the addition of 0.1 mL 1% acetic acid in 50% ethanol. The plate was shaken for 10 min and the absorbance was measured at 540 nm using an automatic ELISA plate reader (μQuant Bio-Tek). Ethanol (7%) was used as positive control. Cytotoxicity percentage was calculated as $[(A - B)/A] \times 100$, where A and B are the absorbance of control and treated cells, respectively. Each experiment was repeated in two independent assays every one including 16 wells, that is 32 wells for each concentration tested. Data were analyzed using one-way ANOVA test and multiple comparisons were made using *p*-values corrected using the Bonferroni method. The evaluation of the threshold value for cytotoxicity of individual ions and mixture of ions was made by exposures to the solutions of the corresponding metal/s salt/s of different concentrations in the AC8h to AC8h × 60 range. The concentration of each salt used was that necessary to obtain the required amount of metal ions, assuming total dissociation of this salt.

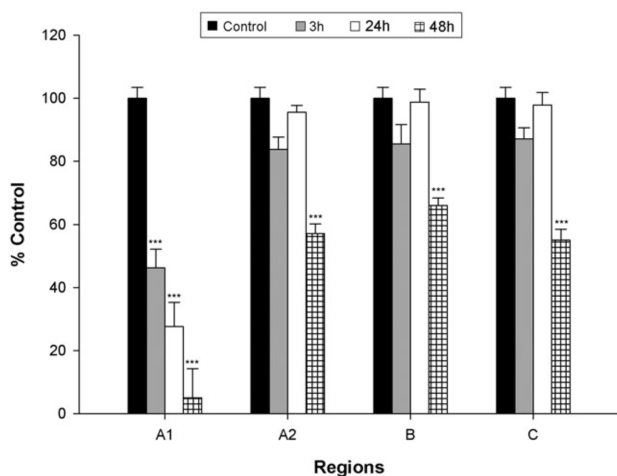

FIGURE 3. Viability of cells by of the Acridine Orange staining after 3, 24, and 48 h exposure to CuBA. Variation with the distance from CuBA disc. Regions A1, A2, B, and C according to the inset of Figure 7. The outer radii (r) of the regions are: rA1 = 0.9 cm; rA2 = 1.4 cm; rB = 2.4 cm; and rC = 3.4 cm.

RESULTS

Corrosion test

Potentiodynamic polarization curves for CuBA immersed in SS solution (pH = 4.77) and in CCM (pH = 7.0) [Fig. 1(a)] showed similar current intensity values in the potential zone that includes the corrosion potentials [−0.400/−0.150 V, see Fig. 1(a) inset] and up to about −0.050 V, immediately after the immersion. With the aim of comparison, polarization curves measured with pure copper were also included in Figure 1(a). The log I versus E curves [Fig. 1(a) inset] show lower corrosion potentials in CCM as electrolyte. Besides, the current intensity values corresponding to both metals immersed in SS (pH = 4.77) are lower than those of CCM (pH = 7.0) for potentials far away from the corrosion potentials, that is, higher than about +0.025 V.

When the electrode had previously been immersed in SS [Fig. 1(b)] and in CCM [Fig. 1(c)], and then the polarization curves were recorded, current intensity markedly diminished in both media and the corrosion potential shifted in the anodic direction.

Microscopic observations of freshly polished CuBA surface discs before and after an exposure time of 10 days in SS, at 37°C [Fig. 2(a,b)] show important changes on the surface characteristics due to the nonuniform surface corrosion process. The results of EDX analysis of the surface composition was also included in this Figure [see Fig. 2(c,d,e)]. The EDX profile analysis of freshly polished CuBA surface before the immersion shows the following composition (% w/w): Al: 8; Mn: 2.04; Fe: 3.15; Ni: 4.41; Cu: 79.4; and Zn: 3. After the immersion in SS Figure 2(b–d) show that zones with different surface characteristics and composition can be detected: dark regions “A” coated with a film which grows with the increasing immersion time, and light zones “B” without film (Zone A: C: 4.54; N: 4.14; O: 2.35; Al: 3.86; S: 12.05; Mn: 1.45; Fe: 3.05; Ni: 4.07; Cu: 64.48; Zone B: O: 5.52; Al: 8.63; P: 0.12; S: 0.33; Mn: 2.24; Fe: 1.8; Ni: 3.74; Cu: 74; Zn: 3.62).

To quantify the metal ions released by the copper alloy, atomic absorption measurements were performed (Table I) after 24 h exposure of the metal samples (AC24h). AC8h ion concentrations calculated from the AC24h data measured by atomic absorption spectrophotometry (see Experimental, section Metal ion solutions for cytotoxicity assays) are also included in Table I.

Effect of metal ions released by CuBA on the number of living cells as a function of the distance from the metal and exposure time

The influence of the distance from the source of metal ions on cell viability was measured by epifluorescence microscopy after Acridine Orange staining. These assays showed (gray bars, Fig. 3) that after 3 h exposure those cells which were close to the metal surface (Region A1) were severely altered ($p < 0.001$). For longer distances (Regions A2, B, C), a slight decrease in the number of living cells to 85% of the control value was detected indicating that they were less affected by the metal ions released by the copper alloy.

The effect of possible high local concentration of ions in the vicinity of the metallic alloy was also investigated after exposures to the metal for complete growth periods. In this case, both cell duplication and the accumulation of ions released by the biomaterial occur simultaneously.

After 24 h and 48 h, epifluorescence microscopy images revealed that the deleterious effect on the growing cells is again more notorious in Region A1 (Fig. 3), where strong reductions ($p < 0.001$) to 30 and 5% in the number of cells related to the control value were detected, respectively. Interestingly, higher number of cells than in the case of 3 h assay was found in the A2, B, C zones after 24 h (Fig. 3 white bars), indicating that cells were able to duplicate during this period. Conversely, new young cells were severely altered by the metal ions during the following growth

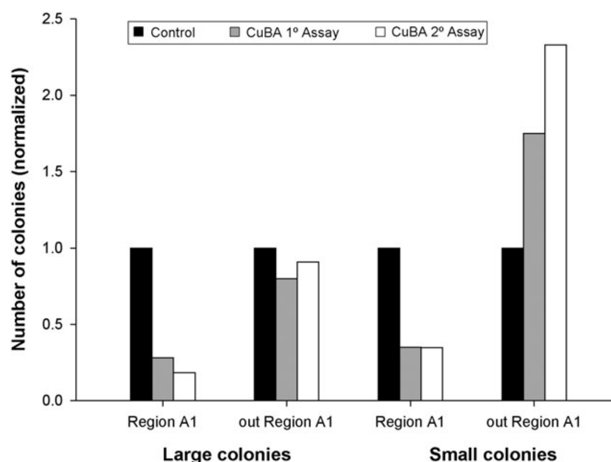

FIGURE 4. Number of small (diameter < 95 μm) and large (diameter > 95 μm) colonies in Region A1 and out of Region A1 corresponding to two assays. The values are referred to the control value without the CuBA sample. Inner and outer radii of Region A1 are 0.5 (CuBA disc border) and 0.9 cm, respectively.

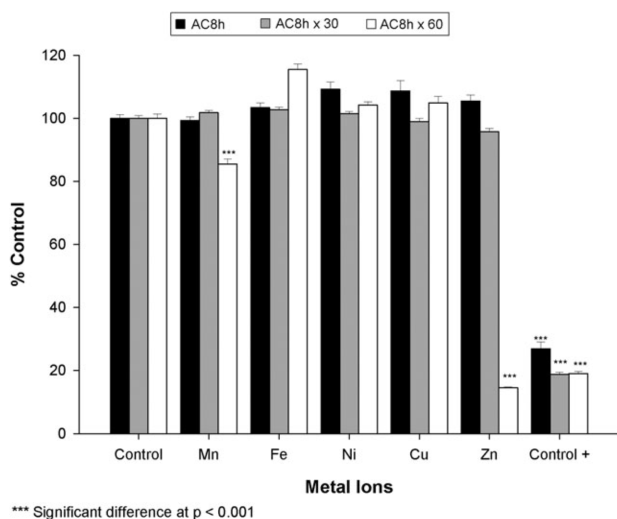

FIGURE 5. Cytotoxicity of metal ions by Neutral Red assay at different concentrations: AC8h, 30 × AC8h, and 60 × AC8h.

period (48 h data, Fig. 3). This resulted in a marked decrease ($p < 0.001$; close to 60% of the control value) of the number of cells after a 48 h period in Regions A2, B, C.

Effect of metal ions released by CuBA on colony forming units

Microscopic observations revealed that the average diameters of all the colonies corresponding to the control and those grown with CuBA were significantly different 112.21 ± 5.45 μm and 80.83 ± 4.95 μm, respectively. In Figure 4, the number of small (<95 μm) and large colonies (>95 μm), of two assays are reported. In the vicinity of the alloy (Region A1, Fig. 3), after 7 days of exposure to the metal ions released by the alloy disc, a drastic decrease in the number of small and large colonies with respect to the control experiment without the metal can be observed (Fig. 4). Interestingly, a marked increase of small colonies out of Region A1 with respect to the control value can also be noticed.

Cytotoxicity of metal ions by NR assays: Effect of the ions from metal salts

Lisosomal activity (NR Assay) in UMR-106 cell line after treatments with the ions of each component of the alloy was tested. Assays with AC8h concentration with single ions did not show any decrease in the lisosomal activity but a slight increase in this activity was observed in some cases (Fig. 5). Similarly, the mixture with all the metal ions at AC8h did not show any reduction in cellular activity (data not shown).

Figure 5 shows that no significant effects were found for single Mn, Fe, Ni, Cu, and Zn ions, when the ion concentrations used were 30 and 60 times higher than the average (AC8h × 30; AC8h × 60). In the case of Al, the solubility limit impeded the use of concentrations higher than AC8h × 9. Only Mn and Zn showed weak and strong cytotoxic effects, respectively ($p < 0.001$). A slight increase in lisosomal activity was found for Fe ions. Concentrations ≥ AC8h × 36 of Zn ions reduced the lysosomal activity of cells to about 30% (not shown).

In the case of Al ions (Fig. 6), a reduction to about 40% was observed for AC8h × 9. When Al–Zn combinations were used (Fig. 6), metabolic activity was reduced to 90% in case of Al–Zn of AC8h × 6 and close to 50% in case of AC8h × 9. Interestingly, the effect was more relevant when the total mixture (TM) of all the components of the alloy was used (TM × 9 = AC8h × 9 = 20.69%, not shown).

DISCUSSION

Aluminum–bronzes have appeared as economical substitutes of conventional gold rich alloys to fabricate crowns and bridges.[34,43] However, previous reports have shown a very low corrosion resistance and possible cytotoxic effects on surrounding cells.[35] Consequently, the widespread use of these dental alloys is a cause of great concern.

Several questions arise when the corrosion of aluminum–bronze in oral environment and the cytotoxic effects of the released ions are analyzed. Among them: (1) is the concentration of metal ions released related to the composition of the metal alloy?; (2) are the concentrations of the ions uniform in the metal surroundings; (3) are the concentrations measured higher than the cytotoxic threshold values; (4) is the response of the cells of the metal surroundings similar to that observed by exposing the cells to ion concentrations equal to the amount of released ions that was measured; (5) if not, how can the cell behaviour be interpreted?; (6) is there any synergistic cytotoxic effect when mixtures of two ions are used?; and (7) is the cytotoxic effect of the mixture of all ions higher than that of the mixture of two ions?. The analysis of the electrochemical and cytotoxic assays may yield to the answers of these questions.

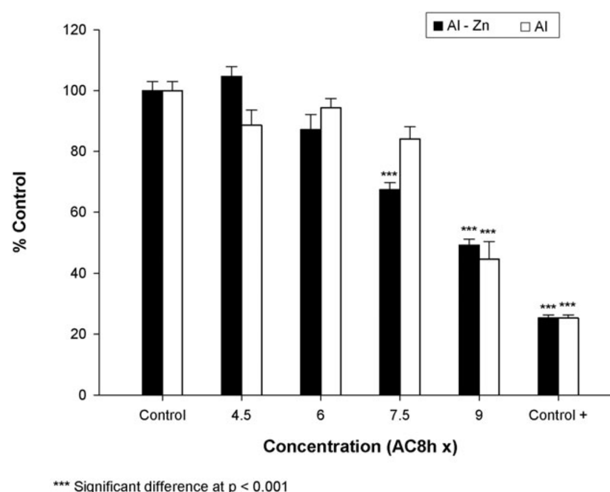

FIGURE 6. Effect of Al and Al + Zn mixtures of ions on the lysosomal activity of cells at concentrations in the 4.5 × AC8h to 9 × AC8h range.

Electrochemical results

As it was expected, our electrochemical data showed that the dissolution rate of CuBA is different from that of pure copper, with more cathodic corrosion potential values that are dependent on the pH and composition of the electrolyte (SS or CCM). The effect of the composition and pH of the medium (SS or CCM) can be clearly observed through the shift of the corrosion potential value for the alloy in CCM towards more cathodic values than in SS [inset of Fig. 1(a)], measured immediately after immersion in the electrolytes. This potential shifts may be related to the complexing action of some of the organic compounds present in the culture medium.[44,45] Conversely, the shifts of the corrosion potential to a more anodic direction and the decrease of the current densities for longer immersion times in the electrolytes may be associated to the more protective layer of oxygen-containing species formed during this period.[46] In agreement, previous results showed that the polarization resistance (*R*p) increased with time during the first six days and reached relative stability after eight days of exposure.[47] The action of other organic components of the culture medium, that can be adsorbed on the active sites of CuBA and probably inhibits the dissolution reaction (by the formation of a protective film which grows with increasing exposure time) must not be disregarded.[48] In the case of SS, the lower corrosion potential values [Fig. 1(b)] than those measured in CCM [Fig. 1(c)] for increasing immersion times, may be related to the adsorption of sulfur-containing species detected by EDX (Fig. 2).[40] Our study also confirms the claims of other authors in relation to the importance of defining exactly the composition (including pH values) of biological solutions used to assess *in vitro* corrosion and biocompatibility of dental casting alloys.[13,35]

Metal ions release and surface composition after the immersion period

In agreement with previous results,[1,33,34] AC24h ion concentration values measured by atomic absorption spectroscopy showed the high corrosion susceptibility of CuBA. The comparison of Table I data show that the concentrations of the released ions are not proportional to the composition of the metal alloy. Thus, contrary to expectations higher ion release was found for aluminum than for copper. Besides, surface corrosion was not uniform, etching corrosion of the interdendritic region of the alloy can be observed in Figure 2 in accordance with previous reports.[33] The EDX profile analysis of freshly polished CuBA surface disc before and after the immersion in SS showed that the composition of the surface changes with the immersion time in SS. After the immersion in SS [Fig. 2(b–d)], two zones with different surface characteristics and composition were detected: Regions "A" coated with a film which grows with the increasing immersion time, and zones "B" without film (bare). Zones "A" show a high quantity of sulfur and the presence of traces of nitrogen and carbon that were not identified before the immersion [Fig. 2(c)]. Oxygen and traces of sulfur and phosphorus were detected in Region B (they were absent before the immersion).

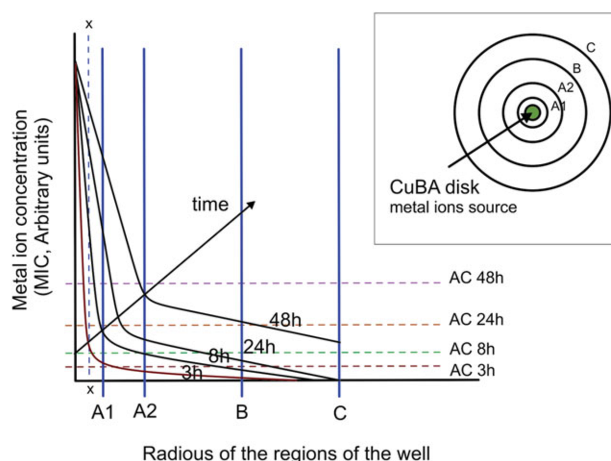

FIGURE 7. Concentration gradients as a function of radial distances from the disc alloy, after different times (3, 8, 24, and 48 h), in arbitrary units. Inset: Scheme of the regions of the Petri dish and the disc of Orcast PLUS® copper-based alloy in the center. The inner radius of Region A1 is 0.5 cm (CuBA disc border). The outer radii (*r*) of the regions are: $r_{A1} = 0.9$ cm; $r_{A2} = 1.4$ cm; $r_B = 2.4$ cm; and $r_C = 3.4$ cm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The dissolution rate of aluminum–bronzes seems to be highly dependent on the Fe content. Tibballs and Erimescu[33] found strong differences in the electrochemical behavior of alloys with 1.4 wt % Fe and 4 wt % Fe. In acidic solutions, exposed interdendritic volumes of the alloy with higher Fe content showed dissolution of both Ni-enriched precipitated species and the copper-rich matrix. Results of Figure 2 show that the Ni content of Region A, more corroded than B, is higher.

Apparent inconsistencies of the results of concentration measurements and cytotoxic effects

In agreement with clinical tests,[36,38] the high initial release rate of metal ions from CuBA resulted in cytotoxic effects on the surrounding cells (Fig. 3). These results seem to disagree with data that show no cytotoxic effect when the cells were exposed to single ions (except for Al) or the TM at concentrations in the AC8h to AC8h $\times$ 30 range. Probably, a diffusion controlled process governs the mass transfer of ions yielding to a concentration gradient around the metal disc. Under these restricted conditions, the concentration of ions in the Region A1 may be several times higher than that of the bulk.[29] Changes in pH values may also occur. Thus, data may be interpreted on the basis of a process controlled by the diffusion of ions.

Distribution of metal ion concentrations in the different zones

To describe and simulate qualitatively, the space/time variation of ion concentrations Fick's second-law equation can be applied.[49] Due to the complexity of the system, we assumed that it could be simulated considering that metal ions are noninteracting species which diffused into the cell line. Consistent with a previous work[29] convective transport within the cell monolayer was assumed to be negligible. According

to this model, concentrations gradients yielding to very high concentration levels close to the metal surface and decreasing towards the border of the Petri dish should be found for each ion. Figure 7 reproduces qualitatively the results of the temporal/space analysis of the diffusion of the metal ions released by the alloy. The average ion concentrations (AC3h, AC8h, AC24h, AC48h) calculated for each profile are also included in Figure 7. It can be noticed that in the Region A1, concentration values of each profile are several times higher than the calculated average values. According to this figure, the MIC accumulated during the times t (3, 8, 24, and 48 h), decreases sharply from the border of the disc (Region A1) towards the wall of the Petri dish. Results of simulations reveal that MIC values, several times higher than those of the bulk, can be found close to the metal surface.[29] To facilitate the interpretation of the graph, let us consider the following example: at a distance x (very close to the metal), MIC increases with time (MIC 3 h $<$ 8 h $<$ 24 h $<$ 48 h) and reaches values several times higher than that of the average concentration of ions (AC). Under these conditions, the cytotoxic threshold value is reached after only 3 h in this region. In agreement, it was reported that there is a relatively high local concentration of the metallic elements in microenvironments formed between the alloys and the tissues.[27] This also explains the marked decrease of the number of the colonies (small and large) growing close to the metal disc (Region A1) (Fig. 4).

After longer exposures (48 h), the threshold concentration levels of cytotoxicity can be reached in Regions B and C (Fig. 3). Accordingly, Figure 3 shows the decrease of the number of cells after 48 h exposure period. It is worth mentioning that this decrease may also be related to the arrest of cell division yielding to the decrease in cell proliferation. According to Figure 7, the highest AC values would be found after the longest exposure period (AC3h $<$ AC8h $<$ AC24h $<$ AC48h). The cytotoxicity out of Region A1 can also be inferred from Figure 4, where higher number of small colonies out of Region A1 than the control and reduction in the average size of colonies from 121 $\pm$ 5.45 μm to 80.83 $\pm$ 4.95 μm can be observed when CuBA is present. Thus, an important effect of the concentration of ions released by CuBa was observed in the distribution, size, and number of the colonies (Fig. 4).

Effect of individual ions and synergistic cytotoxic effects of the combination of metal ions

Results of cytotoxic effect by NR assays are in line with those previously reported related to MTT assays.[35] In both cases, a dose-response correlation and threshold value for cytotoxic effect were observed confirming toxic mechanism. Besides, our findings are in accordance with previous results where metal ions (Cu, Fe, Ni, and Zn ions) released by alloys also induced marked cytotoxicity.[26,50] The ranking of metal toxicity based on TC_{50} values reported by Riley et al.[50] was Zn $>$ Cu $>$ Ni $>$ Fe. They also observed interactions for exposures to solutions containing two metal ions.

The comparison of the cytotoxic effect of single ions showed a high cytotoxic effect for Al cations at AC8h $\times$ 9 concentration level (Fig. 6). A similar effect was shown in case of Zn when AC8h $\times$ 36 were used (not shown). Cytotoxic effects of Al ions have been previously reported.[51–54] However, to the best of our knowledge, possible synergistic effect of some combinations of the ions released by aluminum–bronze has not been analyzed. The assays with Al–Zn combinations, when concentrations between AC8h $\times$ 6 and AC8h $\times$ 9 were used, showed synergistic effects. Accordingly, the uptake of each ion (Al or Zn) may be interferred by the other yielding to a significant higher cytotoxic effect (synergism). Urania et al.[55] showed that when results obtained with Zn ions used singly or in combination (Zn–Cu) were compared they observed that Zn was accumulated in cells at a significant higher concentration when used in combination with Cu. Additionally, a marked decrease in cell viability and protein content was found for this mixture. Fe and Zn can also interfer in other ions uptake processes.[56,57] It was suggested that the synergism of the mixture Cu + Zn was evident as the redox-active metal Cu could enhance the Zn absorption in a living system.[58,59] Furthermore, this combination of Cu + Zn might also play a main role in the mixtures with other ions. In addition, synergic effects of mixtures of some metal ions (Cu, Fe, Ni, Cr ions) on oxidative DNA damage mediated by a Fenton-type reduction have been previously identified.[60] Xu et al.[61] made toxicological assays with individual, binary, ternary, and quaternary mixtures of ions and showed that in most of the binary combinations, the interactions were synergistic. Accordingly, our results revealed that when the osteoblastic cells were exposed to the overall mixture of ions at AC8h $\times$ 9 a higher cytotoxic effect (20% of the lisosomal activity of the control) than in the case of Zn–Al combination (close to 50%) was found. Considering that each individual ion (except Al) did not show any cytotoxic effect up to AC8h $\times$ 30 concentration value, these results clearly indicate the dramatic influence of the simultaneous presence of several ions at concentrations close to AC8h $\times$ 9 on osteoblastic cell viability.

Consequently, our results on cytotoxic effects in the vicinity of metal discs at AC values lower than toxic level can be interpreted considering that the cells in this region are exposed, as a result of concentration gradients, to mixtures of ions that, even nontoxic as single ions, are cytotoxic in mixtures, due to synergistic effects. The difference in response to ions released from the alloys and salt solutions representing the ions released from the alloys reported by other authors[11] may also be interpreted following this scheme.

Used appropriately, *in vitro* tests could play a significant role in risk assessment.[62] These tests cannot fully replicate clinical conditions, but valuable information related to clinical risks may be obtained. It is known that, as a result of corrosion, ions from the metal implants can enter the biological environment. *In vitro* tests reported in literature have been made by using a salt of the corresponding metal or extracts obtained from the dissolution of the metallic sample *ex situ* [these extracts contain the metal ions released by the biomaterial (pure metal or alloy)]. However, to the best of our knowledge, the analysis of possible

synergistic effects resulting from the comparison of the effect of solutions with single metal ions with that of mixtures of two or more of the released ions has not been reported. Our results show that valuable insights related to clinical risks of some synergistic combinations of ions can be obtained from this comparison.

Cellular response to metal discs within the cell culture and culture media with metallic salts

At this point, it is interesting to complement the interpretation of some apparent inconsistencies previously reported by other authors[11,63–65] in the results of biocompatibility assays. They found that cellular functions were not similarly altered in response to ions released from the alloys and to their salts. They highlighted that salt solutions cannot be easily used to represent alloy cytotoxicity because ionic release from alloys is a complex process with a dose-time dependence. We suggested that when the concentration of the ions released is evaluated by atomic absorption spectroscopy, the concentration of the samples measured for this analysis is the corresponding average concentration value, AC of the original concentration gradient (variation of concentration with distance, Fig. 7). When salts or extracts are used to simulate the effect of ion release in cultures, the concentration is uniform and similar to the corresponding AC (no concentration gradients). Thus, Schmalz et al.[26] demonstrated that the results of experiences with salts and extracts were only slightly different. However, in experiments with discs, concentrations close to the discs are high and time dependent, reaching values markedly higher than the AC value, and exciding citotoxic threshold levels, mainly in the case of mixtures with synergistic effects. Thus, although the AC value measured by atomic absorption spectrophotometry is below the toxic threshold value, cytotoxic effects may be found near the metal disc (where the level of ions is high, Fig. 7) leading to the decrease in cell viability.

In the oral environment, changes in quantity and quality of saliva, diet, oral hygiene, polishing of the alloy, distribution of occlusal forces, and burshing can also influence on the rate of ions release. Although the release of copper, aluminum, nickel, manganese, and iron from the CuBA remains far below the upper tolerable human intake levels[1] they may cause cytotoxic effects and inflamation. Recently, several *in vitro* and *in vivo* studies reported the action of macrophages in the implant neighboring, where important amounts of degradation products (ions and debris) may be present.[66–70] Particularly, Lee et al.[71] studied the migration and activation of live macrophages close to titanium biomaterials and their degradation products. They found that macrophages were more abundant in the vicinity of metallic surfaces than in more remote areas, suggesting that the concentration of the degradation products would be inversely proportional to the distance from the biomaterial. Accordingly, our results showed a higher cytotoxic effect in this area.

Overall, different criteria have been applied when the cytotoxicity of metal ions is assessed. Some authors have suggested that complete materials should be used to evaluate the cytotoxity of dental materials. Others consider that even such assays can assess the total cytotoxicity of a dental alloy, the evaluation of the toxicity of individual components is impeded. The existence of concentration gradients may contribute to the interpretation of apparent discrepancies reported by other authors when two different sources of metal ions are used: multiple ion salt solutions and samples of the dental alloys. Importantly, present study demonstrated that experiments with dental alloys and the use of mixture of ions is highly applicable to evaluate possible synergic effects of ions and also space/time variation in cytotoxicity.

The clinical relevance of the results is related to the possible existence of diffusionally controlled concentration gradients reaching levels higher than the cytotoxic threshold values close to the metal. These high ion concentrations may explain the significant cytotoxic effect in this region, in agreement with clinical tests.[3,30,36,38]

CONCLUSIONS

It could be concluded that:

i. The concentrations of the ions released by the CuBA are not proportional to the composition of the metal alloy.
ii. No cytotoxic effect of individual and mixtures of ions was found when exposures to the average AC8h concentration were made. The cytotoxic threshold values were higher than the AC8h of the ions (Al $=$ AC8h $\times$ 9; Zn $=$ AC8h $\times$ 36; Mn $=$ AC8h $\times$ 60; Fe, Ni, and Cu $>$ AC8h $\times$ 60).
iii. The concentration of each ion and the pH value is probably not uniform around the metal. The existence of diffusional controlled concentration gradients with values higher than the cytotoxic threshold levels close to the metal was postulated.
iv. The high concentration values close to the metal may explain the significant cytotoxic effect in this region (lower cell viability, smaller cell colonies) and the results of clinical tests.
v. Synergistic cytotoxic effect was found when mixtures of Al–Zn ions were used. The effect was more relevant when the TM of all the components of the alloy was used.

APPENDIX

Acronyms

AC: Average concentration of ions.

AC24h: Ion concentrations of ions released by the Orcast PLUS® (49 cm^2) measured by atomic absorption spectroscopy after 24 h exposure to SS (200 mL).

AC8h: Concentration of ions based on AC24h value and assuming that a sample of 3 cm^2 of geometric area was exposed to 1 mL of SS for 8 h (sleep time period).

AC8h $\times$ 30, AC8h $\times$ 60: Ion concentrations corresponding to 30 and 60 times AC8h level.

CCM: Cell culture medium.

CuBA: Orcast PLUS® copper-based alloy (zinc–aluminum–bronze).

MIC: Metal ion concentration.

NR: Neutral Red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) assay.

SS: Synthetic saliva.

TM: Total mixture of ions, solution of the salts of the metal ions which produce AC8h ion concentrations or several times higher.

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