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Relationship between radial diffusion of copper ions released from a metal disk and cytotoxic effects. Comparison with results obtained using extracts

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Abstract

The extended use of metallic biomaterials yields to increasing sources of metal ions within the human body and may result in inflammation of the surrounding tissues, cell damage, and cancer. The aim of this study was to investigate the relationship between the radial diffusion of metal ions released from a metal disk by the corrosion process and the toxic effect on a cell line that grew around it. Results obtained with the metal disks (direct contact) were compared with assays made with extracts obtained from the dissolution of a metallic sample ex situ and then added to the cell culture to elucidate the cause of apparent inconsistencies in previous reports.

The change of copper concentration due to corrosion and transient diffusion of copper ions from the copper disks into the cell line was evaluated according to Fick's 2nd law. Surviving cells distribution was interpreted considering the radial and time-dependence of copper concentration. We concluded that the toxic effect on those cells close to metallic biomaterials may be underestimated when only the extract methodology is employed for cytotoxic tests or when during the experiments with disks the presence of concentration gradients and the non-homogeneous distribution of dead cells are disregarded.

Keywords: Copper ion; Cytotoxicity; Extracts; Diffusion; Corrosion

1. Introduction

Metal ions have complex effects on mammals. Different forms of biological reactions can be distinguished depending on concentration, exposure time and source. The use of metallic biomaterials for dental, orthopaedic and cardiovascular devices in human body yields to increasing sources of metal ions. They can be released through liberation of wear particles, corrosion, fretting, and disruption of the oxide layers, among other processes [1–5]. Metal ions can be spread over the human organism by circulation, both locally and systemically. They can penetrate cell plasma membrane, bind cellular proteins and enzymes, or modulate cytokine expression [6–8]. Even though metal ions are required in trace amounts for the normal function of living organisms, extensive exposure to certain metals has been associated to inflammation, cell damage, and cancer. The physiologic effects of these ions are poorly understood and their potential toxicity remains a cause for concern [8–10].

Ions from corroding metals and alloys diffuse to adjacent soft and hard tissues. Cytotoxic effects, which may ultimately lead to cell death, can cause marked morphological changes, damage to proteins, and modified protein expression [8,11,12]. Previous studies have demonstrated that copper released from copper-containing samples produces an increase in the inflammatory response, destabilization of cell membranes via superoxide and hydrogen peroxide production, affinity to RNA, DNA and

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inhibition of transcription [13,14], and induction of apoptosis [11,15], among other toxicity responses [16,17]. Like bacterial toxins, corrosion elicits bioreactions with clinical symptoms such as pain, swelling, inflammation, lyses and necrosis. Recent in vivo studies have reported high levels of cell death 48 h after copper implant [12]. Considering that copper is one of the main components in dental alloys [5,6,11,18] and intrauterine devices (IUD) used as contraceptives [19–22], copper ions released from biomaterials deserve particular attention. In view of clinical and in vitro results, further investigations about copper release and its effect on the surrounding cells have turned significantly necessary [12]. With respect to IUD, the possible accumulation of copper ions in the surrounding environment of the device justifies further studies on the possible reduction of its effectiveness as well as the eventual cause of pain.

Results from some investigations about toxicity levels of metallic ions seem to be inconsistent [23,24]. A careful investigation of the materials and methods employed in the studies showed that materials and methods of in vitro assays are diverse. There are at least three sources of metallic ions used in the experiments: a) the extracts obtained from the dissolution of a metallic sample *ex situ* and then added to the cell culture (extract contact: ExtC) [25–28]; b) the dissolution of metal samples *in situ* (direct contact: DC) [24,29–34]; c) the metal salts (ES) [7,8,23,35–38]. The concentration of metallic ions in ExtC and ES is closely uniform, whereas in DC the concentrations may be time-dependent and different throughout the Petri dish because of diffusion gradients.

The aim of this study was to investigate the influence of experimental methodology on the evaluation of metal ions cytotoxicity in an attempt to elucidate the apparent inconsistencies of previous results. Qualitative (morphological studies) and quantitative (cell count) methods were used to assess and compare the effect of copper ions. The sources of the ions employed in the research experiments are: (a) the extracts (ExtC) at constant copper ions concentration, (b) the copper disks (DC) (which release ions due to the corrosion process) immersed in the cell cultures. The change of copper ions concentration due to the dissolution and the transient diffusion of copper ions into the cell line was evaluated according to Fick's 2nd law for the last case. Results about surviving cells distribution during DC were interpreted for the first time considering the radial and time-dependence of copper concentration.

2. Materials and methods

2.1. Cell culture and incubations

UMR 106 rat osteosarcome derived cells were obtained from the American Type Culture Collection (ATCC) (Rockville, Mi, USA). Cells were grown in TC 75 cm^2 flasks at 37 °C in humidified 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM, GIBCO; Grand Island, NY) with 10% fetal bovine serum (SBS, GIBCO; Grand Island, NY) supplemented with 100 U/ml Penicillin and100 μg/ml Streptomicin. At confluence, adhered cells were detached with 0.25% trypsin/0.02% EDTA (SIGMA-Aldrich), in Ca/Mg phosphate buffered saline solution (PBS). Cells were seeded in 30×10 mm Petri dishes at 1×10^5 cells/ml (total volume of medium added to each Petri dish was 3 ml). Petri dishes were divided into three regions so that the difference between the inner radius and the outer radius was the same for all the regions. Thus, Region A, B and C were determined by marking circles at the bottom of the dishes according to Fig. 1 [Region A (inner radius=2.5 mm, outer radius=11.66 mm, area=130.6 mm^2); Region B (inner radius=11.66 mm, outer radius=20.82 mm, area=299.9 mm^2); Region C (inner radius=20.82 mm, outer radius=29.98 mm, area=457 mm^2)]. UMR106 osteoblast-like cell cultures without copper were used as negative controls. With the aim of comparison the enumeration of cells in the different regions was referred to the unit area (microscopic field area).

2.2. Copper samples and extracts

Cooper disks (99.7%) 5 mm diameter and 0.1 mm thick were washed in sterile double-distilled water and sterilized in autoclave before being transferred to the culture medium without cells (ExtC) or with cells (DC). Silicone grease was used in order to center the metal piece on the plate. Copper ions concentration was measured in the supernatant solutions after the different exposure periods by flame atomic absorption spectrophotometry (sensibility: 0.1 μg/mL for copper).

2.3. Experiments with extracts

Extracts for ExtC were obtained by the immersion of the copper disks in Petri dishes "PExt" with sterile culture medium (DMEM) during the following immersion periods: 12 h (Ext12), 24 h (Ext24), 48 h (Ext48), and 72 h (Ext72). In parallel the cells were seeded in other Petri dishes "Pcell". The supernatant culture media of the cells grown in Pcell were replaced by the same volumes of the extracts obtained from "PExt". Cell cultures with the extracts were then incubated for 3, 6, 9, and 24 h. After these periods the number of cells was evaluated as surface density of cells (number of cells/microscopic field area). The concentration of copper ions was measured.

2.4. Experiments with disks

Cells were seeded in Petri dishes with a copper disk in each center. They were incubated with the disk during different

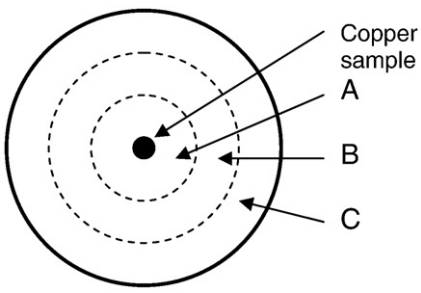

Fig. 1. Petri dish Regions. Regions A, B, C of the Petri dish, and the copper sample (disk) in the center.

exposure periods (12 h, 24 h, 32 h, and 72 h). Subsequently, cell viability was evaluated as surface density of cells (number of cells/microscopic field area) in each region (A, B, or C, according to Fig. 1). The concentration of copper ions was measured.

2.5. Cell viability

After incubation, the medium was removed by aspiration, washed once with Hank's Buffered Saline Solution Ca/Mg free (HBSS), and cells were detached by addition of 0.25% trypsin/0.02% EDTA. DMEM supplemented with 10% FBS was added to stop the reaction. A sample of each dish was mixed with trypan blue 0.4% solution and cells were counted using a hemocytometer.

Cell viability was also assessed by means of the Ethidium Bromide/Acridine orange assay, as described by González et al. [39]. Briefly, one aliquote of 5 µl 1:1 freshly prepared mixture of Ethidium Bromide (100 µg/ml) and Acridine Orange (100 µg/ml) was mixed with 50 µl of the cell suspension. Afterwards, cells were analyzed using an Olympus BX50 Fluorescence Photomicroscope with adequate filter combination. Viable cells appear green fluorescent whereas orange-stained nuclei indicate dead cells.

2.6. 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 Student's *t*-test.

3. Results

Fig. 2 shows the variation of copper ions concentration with time during extracts preparation. Each value corresponds to the average value of copper ions accumulated in the whole Petri dish during the selected period. An almost linear relationship was found for the first 48 h in absence of cells. A similar relationship with slightly lower values was found in presence of cells (DC).

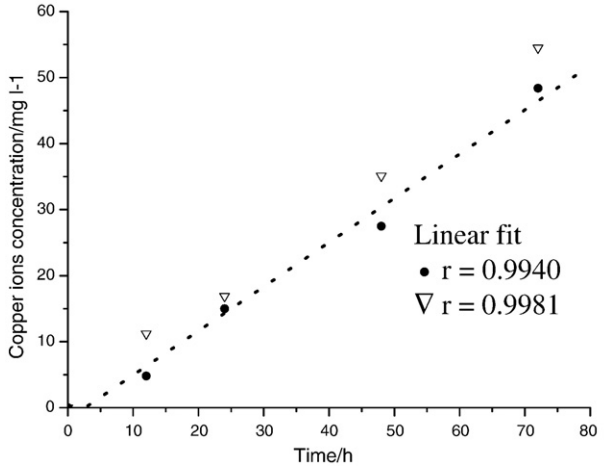

Fig. 2. Copper ions concentration vs. time plot. Disk-containing culture medium (▽) without cells, and (●) with cells.

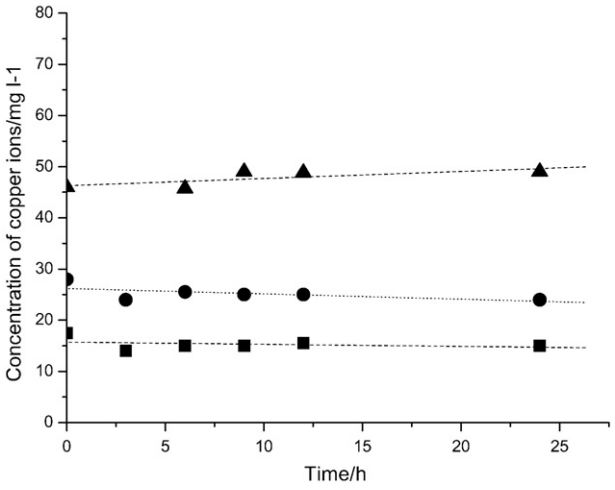

Fig. 3. Copper ions concentration of in the culture media, after the addition of extracts. (■)Ext 24 h, (●)Ext 48 h, (▲)Ext 72 h, vs. time.

3.1. Experiments with extracts

Cell counting was performed in order to quantify the cytotoxic effect of copper ions after different exposure periods. After these periods, concentration of the supernatant solution was measured and the surviving cells were counted.

Fig. 3 shows that the concentration of ions in the supernatant solutions in contact with the cells maintains almost stable values (there are some changes in the cell cycles), with average values similar to the original extracts (Fig. 2). In these cases, the distribution of live and dead cells was random all over the Petri dish.

The number of live cells in contact with Ext12 (not shown) and Ext24 was almost constant during 24 h of exposure to these extracts (Fig. 4). However, an increase in the number of cells was detected in the control which reached 479 cells/field after 72 h. Consequently, proliferation of cells was inhibited in presence of copper ions. Fig. 4 also shows that Ext48 and Ext72 produced

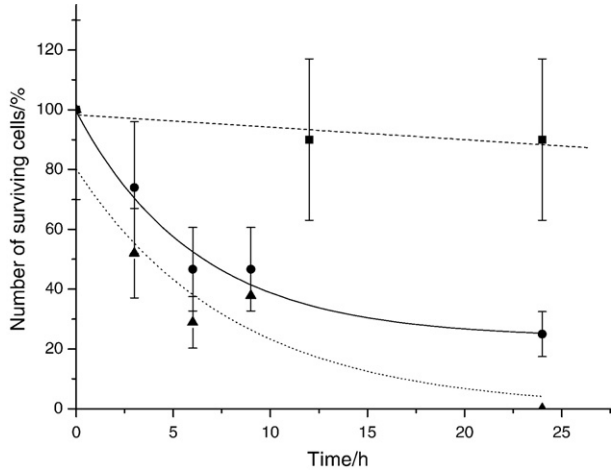

Fig. 4. Average number of surviving cells within the well (% of the initial value) after different exposure periods with the extracts. (■) Ext 24 h, (●) Ext 48 h, (▲) Ext 72 h).

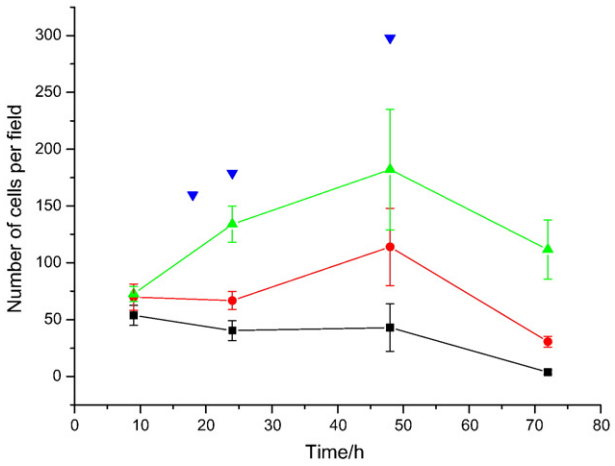

Fig. 5. Number of surviving cells vs. time after different exposure periods in a copper-disk-containing well. (■) Region A, (●) Region B, (▲) Region C, (▼) Control values. Control value for 72 h=497 cell/field (not shown, out of scale limits).

26% and 48% reduction in the number of live cells (100% was assigned to the initial value), respectively, after only 3 h exposure. The number of live cells decreased with time to reach 46.7% and 28.9% after 6 h, and 25% and 0% respectively after 24 h exposure. Accordingly, 16.9 mg/L (Ext24) were not able to decrease the number of cells after 24 h exposure, while 54.5 mg/L (Ext72) killed the cells completely after the same period.

The remaining live cells of experiments with Ext48 and Ext72 showed loss of cell attachment, picnotic nuclei with distorted shape, and bubbling of the plasma membrane, in agreement with characteristics previously reported [11]. No mitotic pictures were observed in the few cells that remained alive after 48 h.

3.2. Experiments with copper disks in cell cultures

Fig. 5 shows the number of the attached live cells in each region (A, B, C) of the Petri dish and those corresponding to the control after different exposure periods. An increase in the number of cells can be observed in the order Region A<Region B<Region C which is probably inversely correlated with the concentration of copper ions, higher in Region A decreasing towards the border of the dish. The curves corresponding to regions B and C reached maximum at 48 h and then the number of cells decreased. After 24 h exposure, growth rate was higher than death rate in Region C (far from the disk), but it was lower for Region A (close to the disk). The cytotoxic effect of copper ions was very strong in this region. Conversely, proliferation was not inhibited in the control assay and a continuous increase in the number of cells was observed.

For the case of Region C, there were 182 surviving cells per field at the maximum (Fig. 5) but after 72 h the amount of live and dead attached cells was lower (141 cells) than that of the maximum. This allows to conclude that an important detachment of cells occurred.

Results showed that the effect of copper ions on osteoblast cells is time and distance-dependent. Copper ions reduced the number of live cells and their proliferation, and favoured their detachment.

4. Discussion

ExtCs showed that the number of surviving cells could be correlated with the concentration of copper ions in a time and dose-dependent manner (Fig. 4) with a homogeneous distribution of live and dead cells. Conversely, DCs showed that the surface density of surviving cells was dependent on their distance from the copper disk. Thus, the rate of the death process that decreased towards the border of the Petri dish may be governed by the transient diffusion of copper ions into the cell line, in response to the concentration step. Therefore, when ExtC and DC are compared, it must be considered that during ExtC the concentration of ions is nearly constant all over the Petri dish (however there are some changes due to the cell cycles) and during the whole period of the experiment, whereas in DC, the concentration of ions may change from place to place and from time to time.

It is worthy to mention that there was a threshold value of concentration (16.9 mg/L) below which no decrease in the cell number was observed. However, in agreement with results from a previous report [11], a decrease in the mitotic figures under this condition was noticed, which could be correlated with the decrease in the proliferation rate with respect to the control.

4.1. Distribution of copper ion concentration levels

In order to describe and simulate the variation of ions concentration with time and location, Fick's 2nd law equation can be applied [40,41] for DC. Due to the complexity of the system, we assumed that it could be simulated considering that copper ion was a non-interacting species which diffused into the cell line. Convective transport within the biofilm was assumed to be negligible. Under these conditions, the transient diffusion of copper ions released by the copper disk into the biofilm in response to the concentration step (between the surrounding of the disk and the bulk), can be described using radial coordinates, as follows:

$$\frac{\partial C}{\partial t} = \frac{1}{r}\frac{\partial}{\partial r}\left(rD_e\frac{\partial C}{\partial r}\right) \quad (1)$$

It was also assumed that copper ion concentration was constant on the copper surface and that the axial coordinate was negligible in relation to the radial one. The term on the left side represents the local accumulation of ions and the term on the right represents the net change in concentration due to diffusion.

Parameter D_e is the effective diffusion coefficient in the biofilm. Value D_e will be reduced compared to the diffusion coefficient in water, D_{aq}, due to the presence of cells, extracellular polymeric substances, inorganic particles, gas bubbles, etc. The relationship D_e/D_{aq} depends on the size and charge of the diffusing solution (solute) (copper ions) and the biomass density in the biofilm [42].

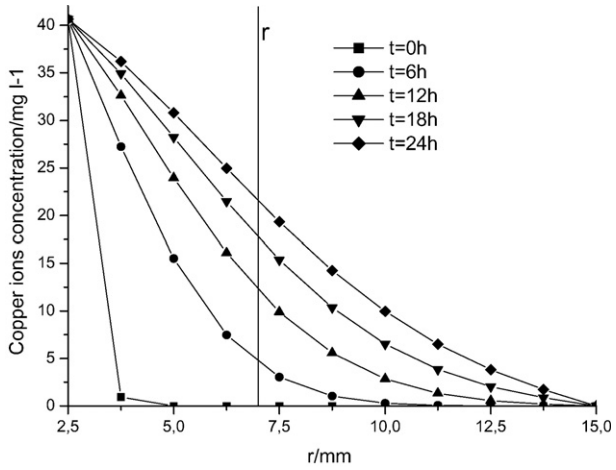

Fig. 6. Calculated concentration of ions ($C(t,r)$) obtained according to Eq. (4). The concentration gradients within the Petri dish for each exposure period (6 h, 12 h, 18 h, 24 h) are shown. The vertical line shows the intersections with the curves indicating the concentration of copper ions associated to different exposure times for $r=7$ mm.

The following equations were selected as boundary conditions:

$$C = 0 \quad r = 2a, \quad t \geq 0,$$
$$C = f(r), \quad 0 \leq r \leq 2a, \quad t = 0$$
$$f(r) = \begin{cases} C_0 & \text{if} \quad 0 \leq r \leq r_0 \\ C_0 \dfrac{\exp(r_0^2)}{\exp(r^2)} & \text{if} \quad r_0 \leq r \leq 2a \end{cases} \tag{2}$$

where r_0 is the radius of the copper disk, and a is the radius of the Petri dish.

$C(r,t)$ solution to Eq. (1) is:

$$C(r,t) = \frac{2}{(2a)^2} \sum_{n=1}^{\infty} \exp\left(-D_e \alpha_n^2 t\right) \frac{J_0(r\alpha_n)}{J_1^2(2a\alpha_n)} \int_0^{2a} r f(r) J_0(r\alpha_n) dr \tag{3}$$

α_n are the roots of

$$J_0(2a\alpha_n) = 0,$$

and α_n defined by:

$$\int_0^{2a} \{J_0(\alpha r)\}^2 dr = \frac{1}{2}(2a)^2 J_1^2(2a\alpha_n),$$

and

$$f(r) = \sum_{n=1}^{\infty} A_n J_0(r\alpha_n),$$

where $J_0(x)$ is the 1st class, zero order Bessel's function, $J_1(x)$ is the 1st order Bessel's function, and α_n are the roots for $J_0(x)$.

The final expression was obtained by using $f(r)$ (Eq. (2)):

$$\frac{C(r,t)}{C_0} = \frac{2}{a^2} \sum_{n=1}^{\infty} \exp\left(-D_e \alpha_n^2 t\right) \frac{J_0(r\alpha_n)}{J_1^2(a\alpha_n)} \left[\int_0^{r_0} r J_0(\alpha_n r) dr + \int_{r_0}^{a} \frac{\exp(r_0^2)}{\exp(r^2)} r J_0(\alpha_n r) dr \right] \tag{4}$$

Fig. 6 reproduces graphically the last equation. This representation shows the most rapid diffusive delivery of ions into the biofilm as the analysis incorporates neither reaction nor absorption [40], and C_0 is supposed to be constant. According to this figure, the concentration of copper ions accumulated during t, decreases from the surface of the metal sample towards the wall of the Petri dish. In order to facilitate the interpretation of this graph, let us consider the following example: after $t=6$ h the concentration of ions is 5 mg/L at $r=7$ mm (see vertical line, Fig. 6) and decreases at higher r values. Subsequently, the concentration of ions changes from 5 to 12.5, 17, and 20 mg/L after exposure periods of 12, 18, and 24 h.

Consequently, the cells placed at radial distance $r=7$ mm should have been exposed to different concentrations of ions from 0 to 20 mg/L during 24 h. On the other hand, after 24 h the cells near the copper disk ($r=2.5$ mm) are exposed to 40 mg/L, those near the middle of the Petri dish ($r=.7.5$ mm) to 20 mg/L, and those close to the border ($r=15$ mm) to 0 mg/L. Accordingly, C depends on time (t) and distance, i.e. $C=C(t,r)$. The situation would be even more complex if we consider changes in the corrosion rate with time (in this case C_0 varies with time).

Concentration values usually reported for cytotoxicity DC assays correspond to the average concentration of the supernatant solution. Fig. 7 shows the comparison between one of the curves in Fig. 6 ($t=12$ h) and the average value (11.25 mg/L = measured value) that results from the distribution of concentration values in the Petri dish for the 12 h exposure

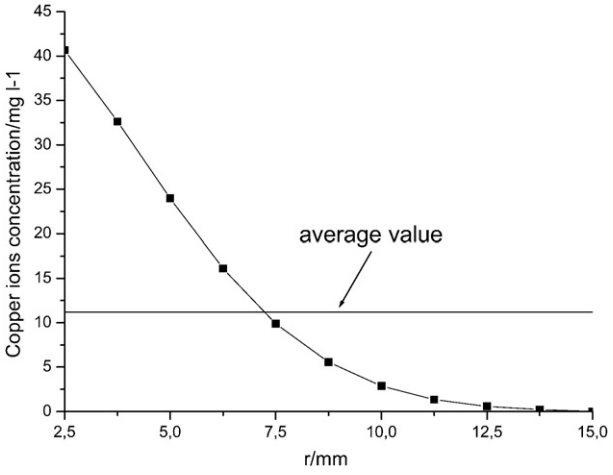

Fig. 7. Comparison of the concentration of ions vs. r values (from Fig. 6) with the average value. The average value for 12 h exposure, corresponding to the supernatant solution after mixing is indicated.

period. It can be noticed that the concentration of copper ions near the disk could be more than twice the average value. However, the existence of concentration gradients, which implies that the cells close to the disk are in contact with high copper ions levels, is disregarded when average concentration values or extracts are used.

Importantly, for exposure periods longer than 24 h and according to Eq. (4), the concentration of copper ions will be different from zero at the border of the Petri dish. As these ions are not able to diffuse out of the Petri dish, they will accumulate at the border and diffuse in opposite direction, that is, towards the centre of the Petri dish. Under these circumstances (exposure periods longer than 24 h), Eq. (4) is not valid. Consequently, for exposure periods longer than 24 h, the distribution of concentrations cannot be described with the mathematic analysis developed above.

4.2. Distribution of surviving cells

Present results (Fig. 4) show that the surface density of cells for ExtC depends exclusively on exposure time because the concentration of copper ions in the extract is almost constant (there are slight changes due to cell cycles). Figs. 5 and 6 show that during DC the density of dead cells and probably the concentration of copper ions are radial-dependent. Thus, we can infer that the distribution of cells is related to the copper ion concentration gradients in a dose and time-dependent manner. However, the number of dead cells or the changes in other parameters (cell proliferation, maturation, differentiation) during DC are usually reported as average values [29,31–34], though it does not actually reflect the effect of high ions concentration on the metal surroundings [37].

Data in Fig. 4 (ExtCs) show that after 24 h, the average value of live cells for the whole Petri dish is close to 100%. However, the number of cells in Region A during DC experiments after 24 h is very low with respect to the control (Fig. 5). When results from ExtC and DC for 24 h exposure are compared, it can be noticed that for Ext24 the number of surviving cells after 24 h was similar to the initial value whereas for DC the average number of cells (average number for the whole Petri dish) was only 40% (Fig. 4). Results seem to be contradictory; however it must be taken into account that the concentration of extracts in ExtC is nearly constant and close to 16.9 mg/L. For the case of DC, and according to the model of diffusion (Fig. 6, t=24 h), the concentration of ions could be two-times close to the copper disk (Region A) and a half when near the border of the Petri dish (Region C). The apparently inconsistent results can now be explained considering that: i) during ExtC the average concentration of extracts (16.9 mg/L for Ext24) was below the toxic limit, ii) during DC the level of copper ions was lethal in Region A and sub-lethal in Region C. Fig. 8 shows the comparison of the number of surviving cells and the concentration values obtained from Figs. 5 and 6 for the different regions after 24 h exposure with the disk. The average value of the concentration and number of surviving cells for the whole Petri dish is also indicated in order to compare results from different methodologies with important clinical implications. It can be noticed that the high effect of copper ions on the cells near the disk (Region A) is underestimated when the average values of surviving cells are reported. They may induce significant errors when applied to clinical conditions in which Region A represents the surroundings of the implant. Consequently, it is important to focus on the experimental conditions corresponding to data reported on toxic levels in relation to their application in vivo.

4.3. Threshold values: time and concentration

Exposure to copper ions during short periods (2 h) did not cause cell death even at high ion concentration values (Ext72). Besides, concentrations lower than 16.9 mg/L (Ext24) even after 24 h exposure, did not reduce the number of surviving cells significantly. Consequently, there are threshold values for time and concentration below which the effect of copper ions could not be detected through the decrease in the number of cells in relation to the initial value. However, reduction was observed in the mitotic process which results in the reduction of the final number of surviving cells in relation to those of the control.

4.4. Some consequences for IUD performance

The radial- and time-dependence of copper ions concentration (Fig. 6) has important implications in IUD performance. Reports on corrosion experiments with copper in simulated uterine fluid for IUD applications [4,20,43] usually show the kinetics of copper release and the release of ions in μg/day (that is, copper ions accumulated during 24 h) as an average value for the whole vessel. Experimental conditions used for these corrosion data reported are dissimilar to those corresponding to in vitro assays with cell lines and to those of clinical situations. For the latter case, diffusion concentration gradients are established leading to high concentrations close to the metal. These concentration gradients are particularly important during the early stages of implantation due to the high corrosion rate of IUDs, characterized by large active areas. Thus, the pain that women suffer during the first days after implantation may be in part related to this high concentration

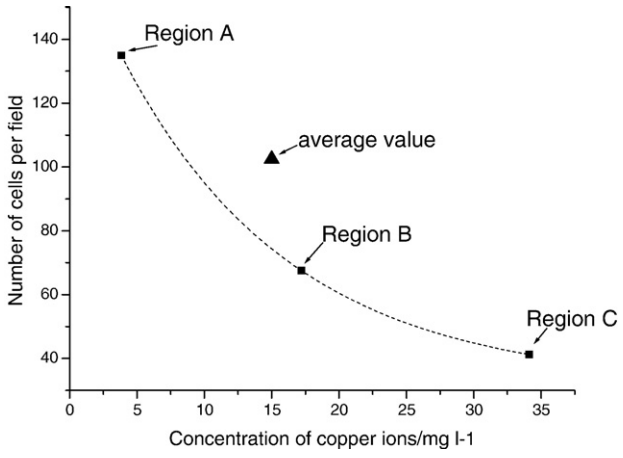

Fig. 8. Surviving cells in each region of the well (A, B, C) vs. calculated copper ions concentration (from Eq. (4)). The average number of live cells in the whole Petri dish and the corresponding average concentration of copper ions (measured value) are indicated for comparison.

of ions around the metal, that affects the surrounding tissues. Additionally, it should be considered that the efficiency of the device may be time-and distance-dependent.

5. Concluding remarks

Knowledge on the effects of copper or other ions released by metals on biocompatibility is strongly dependent on the understanding of the events at the interface biomaterial-tissue. Definitely, clinical situations are very complex, mainly in the neighboring tissues where the concentration of ions is high. Nevertheless, in vitro assays are useful to determine the toxic levels for each particular cell line as a first biological approximation to reduce the number of in vivo assays. However, when comparisons are made between different in vitro assays it must be considered that the experimental methodology may be significantly different. Thus, concentration gradients are not considered in evaluations such as LC50 or TC50 and experiments with extracts or salts that use ion-containing solutions in the absence of the metal sample. In these cases, conditions are very different from the clinical situation of the implant surroundings. Additionally, even when metal samples are used in some cytotoxicity assays, the non-homogeneous distribution of dead cells within the Petri dish (high number of dead cells near the metal sample) and the changes in proliferation and differentiation (which may be radial-dependent) should be also taken into account. They have significant consequences in in vivo processes involving metals employed for osteointegration, contraception, dental restoration and metallic stents.

In our opinion, ExtC and DC provide different but complementary information. However, DC assays seem to be closer to the clinical situation. The toxic effect of metals may be underestimated when exclusively ExtC methodology is employed or when the non-homogeneous distribution of dead cells and the changes in proliferation, maturation and differentiation, related with the presence of concentration gradients, are disregarded in DC.

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