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Degradation of bioabsorbable Mg-based alloys: Assessment of the effects of insoluble corrosion products and joint effects of alloying components on mammalian cells

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ABSTRACT

This work is focused on the processes occurring at the bioabsorbable metallic biomaterial/cell interfaces that may lead to toxicity. A critical analysis of the results obtained when degradable metal disks (pure Mg and rare earth-containing alloys (ZEK100 alloys)) are in direct contact with cell culture and those obtained with indirect methods such as the use of metal salts and extracts was made. Viability was assessed by Acridine Orange dye, neutral red and clonogenic assays. The effects of concentration of corrosion products and possible joint effects of the binary and ternary combinations of La, Zn and Mg ions, as constituents of ZEK alloys, were evaluated on a mammalian cell culture. In all cases more detrimental effects were found for pure Mg than for the alloys. Experiments with disks showed that gradual alterations in pH and in the amount of corrosion products were better tolerated by cells and resulted in higher viability than abrupt changes. In addition, viability was dependent on the distance from the source of ions. Experiments with extracts showed that the effect of insoluble degradation products was highly detrimental. Indirect tests with Zn ions revealed that harmful effects may be found at concentrations ≥150 μM and at ≥100 μM in mixtures with Mg. These mixtures lead to more deleterious effects than single ions. Results highlight the need to develop a battery of tests to evaluate the biocompatibility of bioabsorbable biomaterials.

1. Introduction

In recent years, an important progress has been made on the development and application of biodegradable Mg-based alloys for several applications [1–5]. Due to their low density and mechanical properties close to those of cortical bone, they are considered to be suitable for fracture repair of weight bearing bone [6–8]. Researchers are attracted by the prospect of avoiding a second surgery for implant removal, which is necessary with other permanent implant biomaterials. Moreover, they are promising for dental and cardiovascular and airway stent applications [9–14]. However, Mg has the disadvantage of undergoing, in human body fluids and blood plasma with high Cl^- concentrations, a high corrosion rate. It must be taken into account that during degradation Mg-based micro-debris and ions are released to the biological medium. Additionally, H_2 burbles are evolved, and the medium turns more alkaline and $Mg(OH)_2$ precipitates. These processes occur according to the following simplified overall reactions [15]:

$$Mg + 2H_2O \leftrightarrow Mg^{+2} + 2OH^- + H_2 \quad (1)$$

$$Mg^{+2} + 2OH^- \leftrightarrow Mg(OH)_2. \quad (2)$$

In order to reduce corrosion problems different combinations such as Mg–Zn, Mg–Mn, and Mg–Al, among other alloys, have been investigated [16–19]. The degradation rate of Mg-alloys depends, among other variables, on the alloy composition [20–21]. Studies by Arrabal et al. have suggested that alloying metals such as Zn and very small quantities of rare earth (RE) elements could be tolerated in the human body and could also increase corrosion resistance [22–24] leading to different results according to the alloy composition; although RE innocuousness is not definitively proven. Therefore, there is a definite requirement to carefully select alloying elements that are non-toxic to the human body. Several reports informed that RE- or Zn-containing alloys show good biocompatibility [3,20,25–32]. A novel Mg alloy, ZEK100 with a reduced content of RE has revealed promising biomechanical properties *in vitro* [23,33]. ZEK100-plates have already been evaluated in a Hanks' balanced salt solution *in vitro* [34] and ZEK100-pins

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intramedullary in rabbit tibia *in vivo* [6,35] were found as potential biodegradable implant materials for osteosynthesis due to their good mechanical characteristics. Huehnerschulte et al. [35] reported that this alloy displays degradation characteristics which are favorable from an engineering point of view. Unfortunately, the authors also informed that degradation of ZEK100 implants may cause adverse host reactions by inducing osteoclastogenic resorption of bone and a rushed formation of new bone periosteally. On the other hand it has been informed that Ca addition improve the Mg–Zn properties in different ways: better mechanical properties and creep resistance due to the formation of fine Mg_2Ca precipitates and enhancement of the oxidation resistance and reduction of the grain size of the alloys when adding in the 0.5–1.0% range [36].

In order to reduce the number of *in vivo* assays to evaluate Mg alloys as biomedical materials, their degradation behavior must be investigated *in vitro* in a physiological environment. Under this situation, variations of the pH and interactions between proteins and the implant surfaces occur and may change the corrosion process [37–38]. It is generally believed that proteins interact and alter corrosion behavior in two ways: adsorption and chelation [39]. However, most of reports informed the evaluation of the corrosion in simulated body fluid with different ion concentrations but, in the absence of proteins and cells [40–42].In this sense, several authors investigated the corrosion susceptibility of Mg alloys in solutions containing bovine serum albumin [27, 43–45], as well as in the presence or absence of cells [46–47] and demonstrated the marked influence of the proteins and cell metabolism on the corrosion process. Consequently, it is very important to select the appropriate methodology, to conduct *in vitro* biocompatibility studies devoted to evaluate the fitness of a device for *in vivo* applications, as well as its potentially harmful impact.

Typically, the biological *in vitro* testing of medical devices is designed to determine the reactivity of mammalian cell cultures that are in contact with the biomaterial and to evaluate the effect of the extracts (Exs) obtained after the immersion of the biomaterial in an appropriate medium. Some *in vitro* studies on bioabsorbable metals reported in literature have focused on the methodology employed in the evaluation of the cytotoxicity of solid materials [48–50]. Other authors determined cellular effects (morphology, viability, metabolic activity, inflammatory cytokine expression) after exposures to the extracts (Exs) of the alloy. Alternately toxicity analyses through the evaluation of the cellular response to simple salts of one of the components of the alloy [51–52] or different mixtures of metal ions were made to detect joint effects [53–58]. However, *in vitro* results are difficult to compare due to the different experimental design and sometimes seem to reveal dissimilar behaviors.

After examining the reported data, we conclude that an important fact that is not frequently addressed is the set of processes occurring at the biomaterial/cell interface due to the simultaneous action of the corrosion process and cell metabolism. In this sense, the aim of this study was to compare the results obtained when metal disks are in contact with the cell culture (direct methods) with indirect methods such as the use of metal salts and Exs that are obtained in culture media without cells and then are added to the cell culture. Pure Mg and Mg-based alloys (ZEK100) were used as bioabsorbable materials that were tested by direct and indirect methods. Effects of exposure time, concentration and pH gradients, hydrogen burble formation, presence of insoluble corrosion products and possible joint effects of the binary and ternary combinations of La, Zn and Mg ions, as constituents of ZEK100, on a mammalian cell culture were evaluated. With the aim of comparison, experiments with ZEK100 alloys with Ca addition (0.2%, 0.4% and 0.6%) and LAE442, a commercially available rare earth-containing alloy were also included.

2. Materials and methods

2.1. Cells culture

The CHO-K1 cell line frequently used in toxicity evaluations [59–60] was originally obtained from American Type Culture Collection (ATCC) (Rockville, MD, USA). Cells were grown as monolayer in Falcon T-25 flasks containing 10 ml Ham-F12 medium (GIBCO-BRL, LA, USA) supplemented with 10% inactivated fetal calf serum (Natocor, Carlos Paz, Córdoba, Argentina), 50 IU/ml penicillin and 50 μg/ml streptomycin sulfate (CCM: complete culture medium) at 37 °C in a 5% CO_2 humid atmosphere. Cells were counted in an improved Neubauer hemocytometer, and viability was determined by the Trypan Blue (Sigma, St. Louis, MO, USA) exclusion method; in all cases viability was higher than 95%.

2.2. Materials

Metal disks (1 cm in diameter and 0.3 cm in thickness, area = 0.785 cm^2) of pure Mg (99.7%) and Mg alloy ZEK100 (0.96 wt.% zinc, 0.21 wt.% zirconium, 0.3 wt.% RE) were employed for cytotoxicity analyses by clonogenic and viability assays. With the aim of comparison ZEK100 alloys with the addition of 0.2%, 0.4% and 0.6% Ca and LAE442 alloy (89.6 wt.% Mg, 4.0 wt.% Li, 3.9 wt.% Al, 2.2 wt.% RE, 0.2 wt.% Mn) as a commercially available alloy, were also tested.

Indirect methods were used to evaluate cellular effects of metal ions released by the Mg and ZEK100 alloy. For these assays, Exs were obtained after the immersion of pure Mg and alloy disks in 10 ml CCM at 37 °C for 24 h. The total volume was divided into two fractions (with and without filtering, pore: 0.22 μm) in order to compare results with and without insoluble corrosion products. An aliquot of this filtered Ex was used to measure the release of Mg ions. CCM without a metal disk was incubated under the same conditions and was used as control.

Several sets of experiments were designed to assess the effect of combined treatments between Mg, Zn and La ions on CHO-K1 cells by the neutral red (NR) assay. For these assays the corresponding salts (2.5×10^3 μM, 3.3×10^3 μM, 4.1×10^3 μM and 8.2×10^3 μM $MgSO_4$; 200 μM $LaCl_3 \cdot 7H_20$; and 50 and 100 μM $ZnCl_2$, Sigma-Aldrich, St. Louis, MO, USA) dissolved in the CCM were employed. The selection of RE/Mg concentration relationships for these assays was based on the results of our previous report [61]. For the selection of Zn ion concentrations, a dose–response analysis considering the 50–250 μM dose range was performed. Taking into account these results, 50 and 100 μM doses were used for combined treatment.

2.3. Experimental design

Three sets of experiments were designed to assess: 1) the cellular effect of Mg and Mg alloy disks by direct contact with the cells, using viability with Acridine Orange (AO) staining, and clonogenic assays; 2) the cellular effect of Exs obtained from metal disks by indirect methods employing the NR assay; and 3) the possible joint effects of combined treatments using Zn and La salts with and without Mg ions in binary and ternary mixtures by NR and clonogenic assays.

2.4. Cellular effect of Mg and Mg alloy disks by contact direct assays

2.4.1. Viability with Acridine Orange staining

For this set of experiments 5.0×10^5 cells were cultured in a Petri dish and grown at 37 °C in a 5% CO_2 humid atmosphere in CCM for 24 h. Then, the medium was removed, Mg and Mg alloy (ZEK100 with and without Ca, LAE442) disks were placed in the center of each Petri dish, and immediately fresh CCM was incorporated. Cells were grown under these conditions during 24 h. The CCM was removed and stored at −20 °C to measure the Mg ion content. Surface compositions of the metal disks after exposure to the culture medium were obtained by EDS analysis of the disk (see Section 2.7). A CHO-K1 cell culture without the metallic disk was used as negative control. To facilitate the analysis of cytotoxic effects as a function of the distance from the source of ions, the area with cells was divided into regions A and B with limiting radii: A = 2.0 cm and B = 4.0 cm (Fig. 1). After the exposure period, adherent cells were stained with AO dye (Sigma, St. Louis, MO, USA) and subsequently, 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. Surface densities of cells were obtained from digital images using the Image-Pro® 6.2 software. Three experiments were performed in independent trials to assess reproducibility.

2.4.2. Clonogenic assay

The colony formation (CF) or clonogenic assay is an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony [62]. The analysis was performed considering the regions showed in Fig. 1. Colonies were stained with AO and the images were taken under fluorescence microscopy with a 10× objective (Olympus BX51, Olympus Corp., Tokyo, Japan). The diameters of the colonies were measured using the Image-Pro® 6.2 software. Three experiments were performed in independent trials to assess reproducibility.

2.5. Cellular effect of metal ions released by metal disks. Indirect methods through the NR assay

The NR assay was performed according to Borenfreund and Puerner [63]. This test measures cellular transport based on dye uptake of living cells. Absorbance change is assumed to be directly proportional to lysosomal activity of cells. For this analysis, 2.0×10^4 cells/well (96 multiwell plate) were grown in CCM for 24 h at 37 °C in a 5% CO_2 humid atmosphere. Then, the CCM was replaced by Exs containing metal ions released from Mg and ZEK100 disks. After 24 h, the medium was removed and replaced by a fresh medium containing 40 μg/ml NR dye (Sigma, St. Louis, MO, USA). After 3 h incubation, cells were washed with phosphate buffer solution (PBS). Afterward, 0.1 ml of 1% acetic acid in 50% ethanol solution was added, subsequently the die was released and the red color was observed. The plate was shaken for 10 min and the absorbance was measured at 540 nm using an automatic ELISA plate reader (μQuant BioTek, USA). Negative controls (untreated cells) were run simultaneously in cultures with CCM. Cytotoxicity percentage was calculated as $[(A - B)/A] \times 100$, where A and B correspond to the absorbance of control and treated cells, respectively. Each experiment was repeated in three independent sets of 16 wells, that is a total of 48 wells for each concentration tested. Data were analyzed using the one-way ANOVA test and multiple comparisons were made using p values corrected according to the Bonferroni method.

2.6. Measurement of Mg ions released from Mg and ZEK100 Mg alloy disks

Measurement of Mg ions in CCM was performed i) after the viability assay (Section 2.4.1, exposure period: 24 h) and ii) after CF (Section 2.4.2, exposure period: 6 days) and iii) using Exs obtained after 24 h incubation period. The soluble Mg concentration of these samples was analyzed by flame atomic absorption spectrophotometry (Shimadzu AA6650, Shimadzu Corporation Kyoto, Japan), obtained using internal quality control, according to standard procedures.

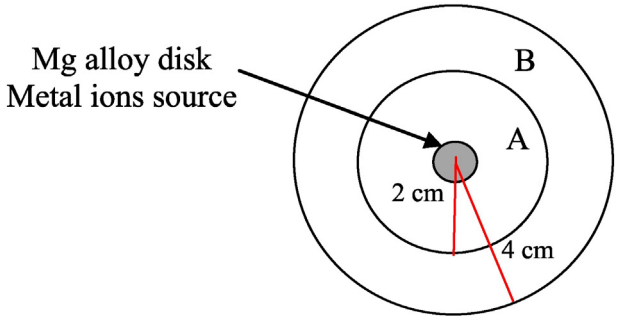

Fig. 1. Scheme of the regions selected for contact direct assays with Mg and ZEK100 Mg alloy disks. The radii of the regions are: A = 2 cm and B = 4 cm.

2.7. Surface analyses of Mg and ZEK100 Mg alloy disks

An environmental scanning electron microscope (ESEM, FEIC Quanta 200) was used to observe the surface morphology and alterations caused by corrosion of Mg and ZEK100 disks. The energy-dispersive spectrometer for X-ray (EDS, Apollo 40 Silicon Drift Detector) analysis was employed to detect the constitutional elemental surface compositions of the disks after 24 h immersion in CCM in the presence of cells. Samples of Mg and ZEK100 from the viability assay (Section 2.4.1) were used for both analyses.

2.8. Cellular effect after treatment with binary or ternary combination between Zn, La and Mg salts

The possible joint effects were analyzed using Zn, Mg and La combinations. For binary combinations between Zn and Mg doses of 50 μM and 100 μM of Zn ions and 2.5×10^3 μM, 3.3×10^3 μM, and 4.1×10^3 μM of Mg ions were used and the effects were analyzed through NR assays. For ternary mixtures, 200 μM of La, 50 or 100 μM of Zn and 8.2×10^3 μM of Mg ions were employed for both, NR and CF tests.

3. Results

3.1. Cellular effects of corrosion products released by Mg and ZEK100 Mg alloy disks (direct methods)

Direct effects of the metallic disks on the number of living cells as a function of the distance from the metal were measured after AO staining (Fig. 2). Results showed that after 24 h exposure those cells which were close to the Mg surface (Region A) were severely altered ($p < 0.001$) showing low cell density values. The number of cells grown in the presence of ZEK100 with and without Ca and LAE442 showed significantly higher values than in the presence of Mg. For longer distances (Regions B) cell coverage increased in all cases, indicating that they were less affected by the products of the degradation process (ions released, H_2 burbles, insoluble corrosion products and micro-debris) that had gradually increased with time.

CF showed cellular effects of the corrosion products released by the metal disks on the diameter of colonies formed after 6 days of exposure (Fig. 3). Microscopic measurements of the colony diameters revealed that the colonies formed in the presence of both, Mg, ZEK100 (with

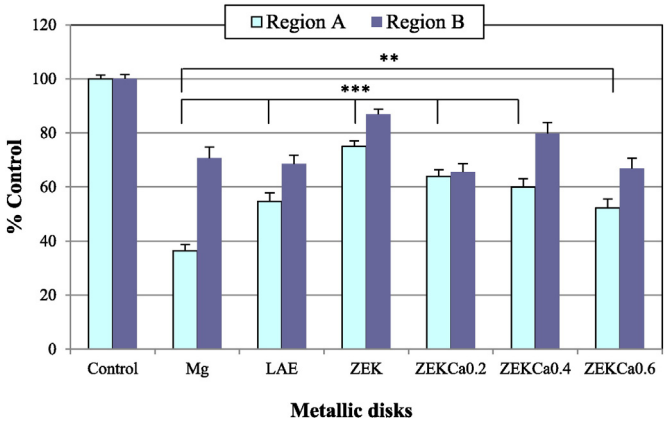

Fig. 2. Viability of cells in Regions A and B (Fig. 1) by Acridine Orange staining after 24 h in presence of metallic disks (Mg, LAE442 (LAE), ZEK100 (ZEK with different Ca contents: 0%, 0.2%, 0.4% and 0.6%)). Error bars indicate standard error of the mean values.***Significance of the difference at $p < 0.001$. **Significance of the difference at $p < 0.01$.

and without Ca) and LAE442 disks were smaller than the control colonies in both regions ($p < 0.001$). However, the average diameter was shorter in the case of Mg disks than in the case of alloy disks (Figs. 3 and 4); among them, diameters corresponding to LAE442 and ZEK100 + 0.6% Ca are slightly shorter than those of ZEK100 without Ca. A similar tendency in the reduction of viability was observed when the numbers of colonies were compared. In the case of Mg disks (Region A) these values were *ca.* 30% of the control while in the case of Mg alloys they were higher than 65% (Fig. 4).

3.2. Cellular effect of metal ions released by Mg and ZEK100 Mg alloy disks evaluated by the NR assay (indirect methods)

The extracts obtained after the 24 h immersion period of the metallic samples in the CCM showed a change in color due to the pH shift and the presence of insoluble products, more noticeable in the case of Mg. Fig. 5 shows the results of the NR assay after 24 h CHO-K1 culture exposure to the Exs (filtered and non-filtered). A dramatic decrease in lysosomal activity to 20% of the control value was observed with Mg-Exs ($p < 0.001$) which contained a great amount of corrosion products (ions, insoluble products and change in pH) released from Mg disks. Remarkably, no deleterious effect was observed in the case of filtered Mg-Exs (without insoluble corrosion products). Alternatively, ZEK100-Exs did not show any effect with both filtered and non-filtered Exs.

3.3. Measurement of Mg ions released from Mg and ZEK100 Mg alloy disks

The results from the measurement of soluble Mg ions released in CCM after AO viability (Section 2.4.1) and CF (Section 2.4.2) assays as well as measurements made with filtered Exs (Table 1) revealed that the concentration of soluble Mg ions is markedly greater in the sample obtained with Mg than with ZEK100 disks after 24 h and 6 days of incubation. The relationship between Mg ions in the 24 h extracts obtained from pure Mg and ZEK100 in the absence of cells was 15.82 mg/l / 108.26 mg/l = 0.146, revealing the higher degradation of pure Mg than ZEK100. On the other hand, in the 24 h AO viability assay, made with the metallic disks in the presence of cells, both values were higher (51.98 and 127.6 mg/l, respectively) showing that cell metabolism and the corrosion process are interdependent. After 6 days in the presence of cells the release of ions increased to more than 4.8 times and 6.8 times larger than those corresponding to 24 h AO viability assays (249.32 and 868.79 mg/l for ZEK100 and Mg, respectively).

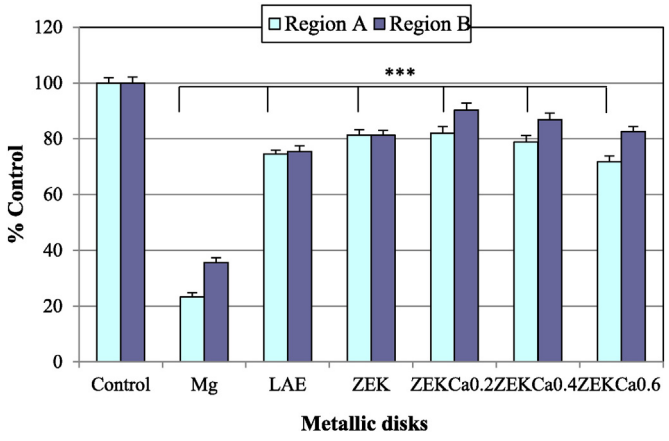

Fig. 3. Average diameters of CHO-K1 colonies formed in Regions A and B (Fig. 1) in presence of metallic disks (Mg, LAE442 (LAE), ZEK100 (ZEK with different Ca content: 0%, 0.2%, 0.4% and 0.6%)) after 6 days of exposure. Error bars indicate standard error of the mean values. ***Significance of the difference at $p < 0.001$.

Table 1
Mg ions (mg/l) released from Mg and ZEK100 in Exs, and after AO viability and clonogenic assays.

	24 h Exs	24 h AO viability assay	6 days clonogenic assay
Mg disk	108.26	127.60	868.79
ZEK100 disk	15.82	51.98	249.32

3.4. Surface analysis of Mg and ZEK100 Mg alloy disks

The ESEM and EDS analysis of Mg and ZEK100 disks that had been immersed in CCM showed a heterogeneous surface with bare areas and ditches (circles A1, B1 in Fig. 6(I)) in which EDS showed a Mg peak with only minor contributions of other elements (Fig. 6(II), A1, B1). Other regions (circles A2, B2 in Fig. 6(I)) depicted important O signals (Fig. 6(II), A2, B2). In the case of Mg the relationship O/Mg is compatible with the presence of $Mg(OH)_2$. The ZEK100 alloy spectrum of the covering layer (Fig. 6(I) and (II), B2) shows a very low Zn signal and higher peaks of P and Ca than on pure Mg indicating the presence of a greater amount of calcium- and phosphate-containing precipitates than on pure Mg.

3.5. Cellular effect after treatments with binary and ternary combinations of Zn, La and Mg salts

Effects of different doses of Zn ions (50–250 μM) on the viability of CHO-K1 cells after 24 h of exposure evaluated by the NR assay are shown in Fig. 7. Considering these results, 50 and 100 μM Zn doses (free of cytotoxic effect) were selected for binary combined treatment. Fig. 8A and B shows that no significant effects in NR assays were found for Zn treatments (50 μM, and 100 μM) combined with several Mg doses (2.5×10^3 μM, 3.3×10^3 μM and 4.1×10^3 μM) after 24 h exposure. Similarly, no significant effects on CHO-K1 cell lysosomal activity of ternary (Zn + La + Mg) combined treatments (Fig. 9) between Zn (100 μM), La (200 μM) and Mg (8.2×10^3 μM) salts, after 24 h of exposure, were found. Interestingly, when 8.2×10^3 μM Mg was mixed with 100 μM Zn lower viabilities with statistically significant differences were found when compared with mixtures of Zn with lower Mg concentration (comparison between results of Fig. 8 (2.5×10^3 μM, 3.3×10^3 μM and 4.1×10^3 μM Mg) and Fig. 9 (8.2×10^3 μM), $p < 0.001$). These results show a detrimental effect of high Mg concentration in Zn + Mg mixtures.

Fig. 10 summarized the effect of combined treatments between Zn (100 μM), La (200 μM) and Mg (8.2×10^3 μM) salts on CHO-K1 cells after 6 days of exposure, evaluated by CA. Results revealed that the number of colonies is *ca.* 20% lower than the control in all cases. Additionally, the average diameter of colonies was even lower ($p < 0.001$) when Zn was present in the mixtures (Zn + La, Zn + Mg, Zn + La + Mg) with respect to the diameter of the colonies with the mixture without Zn (for example Mg–La). The Zn + Mg mixture is significantly worse than single Zn or Mg, and an additive effect was found, confirming the effect detected after 24 h NR tests at the concentration values assayed. Likewise, La + Zn + Mg is significantly worse ($p < 0.001$) than La + Mg due to the addition of Zn. Conversely, the La + Zn + Mg effect is significantly less dangerous than Zn + Mg due to the presence of La ($p < 0.001$).

4. Discussion

4.1. 24 h assays: NR, AO

Different direct and indirect assays have been proposed to evaluate the biocompatibility of implantable metals [48,57]; however, results of different tests showed dissimilar responses and cannot be compared. In order to find similarities and dissimilarities between the different

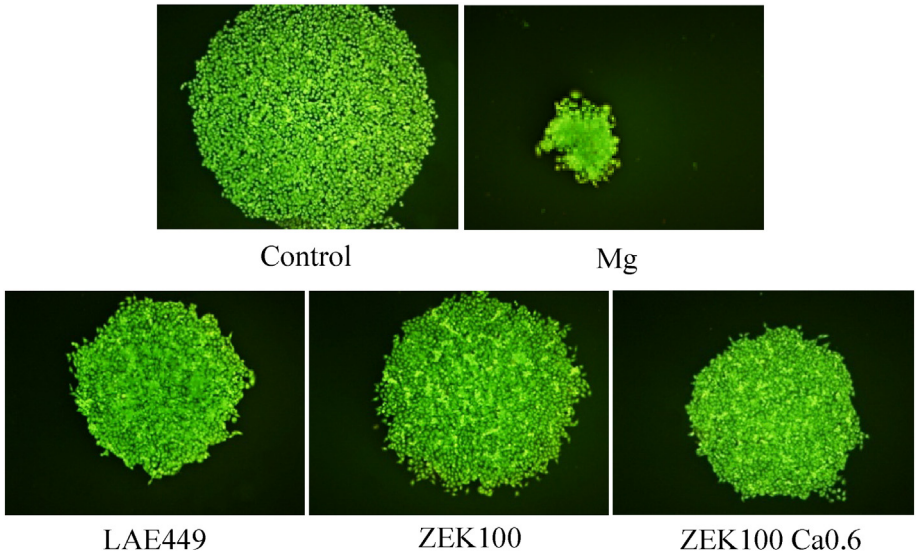

Fig. 4. Microphotographs of colonies grown during 6 days in the absence (Control) and in the presence of Mg and Mg alloys (LAE442, ZEK100 and ZEK100 + 0.6% Ca) disks. The diameters of the colonies formed in the presence of ZEK100 + 0.2% Ca or ZEK100 + 0.4% Ca are similar to those obtained with ZEK100 disks. Magnification: 100×.

assays a critical analysis of several techniques applied to Mg and ZEK100 is made here. In doing so, three questions arose when the comparison of 24 h assays was made: i) What are the environmental differences when cells are exposed to direct or indirect exposures?; ii) why do the 24 h direct (with disks) and indirect (with Ex) assays showed so dissimilar results?; and iii) why the NR results with Exs of pure Mg showed so low viability?

When Mg and Mg alloys are immersed in an aqueous chloride-containing solution like a CCM for 24 h a high corrosion rate develops which can be visualized by the hydrogen evolution on the metal surface. However, the degradation rate of Mg was significantly higher than that of ZEK100. During degradation, the pH of the CCM changed and insoluble products were formed on the metal surface. Thus, the Ex is a medium with metal ions and insoluble products accumulated during 24 h, with higher pH than that of the CCM. Due to its higher degradability the accumulation of corrosion products is significantly greater in the case of pure Mg.

CCM environmental changes (hydrogen evolution and pH^{shift}) were observed when the disk is in the presence of cells. However, in this case, parameters such as pH, concentration of ions and amount of insoluble products of the medium, change gradually with time and distance from the metallic source. Gradual alterations seem to be better tolerated by cells than drastic changes caused when Exs are added during indirect assays. Importantly, those cells which are close to the disk experience more notable changes than those which are far from the source of ions revealing that cells are very sensitive to concentration changes.

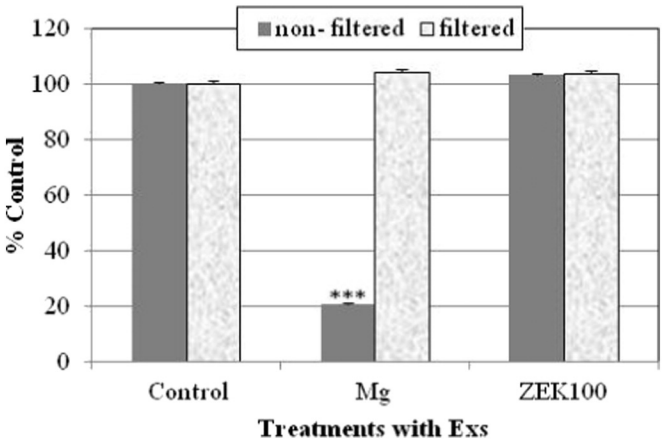

Fig. 5. Effect of Mg-Exs and ZEK100-Exs (filtered and non-filtered) on CHO-K1 cells after 24 h exposure, evaluated by neutral red assay. Error bars indicate standard error of the mean values. ***Significance of the difference at $p < 0.001$.

Remarkable differences were detected when results of 24 h assays by NR (with Exs) and AO (with disks) are compared. Thus, experimental conditions related to the environment close to the cells should be compared in detail in order to detect differences. During indirect assays once the Ex without filtering (*i.e.* with insoluble products) is added to the cell culture, cells suffer an abrupt change in concentration, pH and amount of insoluble products that resulted in a drastic decrease of viability in the case of Mg assays. However, experiments with filtered Exs, and those with ZEK100 Exs did not show any decrease revealing that insoluble products seriously affect the metabolism of cells.

Accordingly, two of the previous questions can be responded: The environment of cells in direct assays changes gradually and they are space- and time-dependent while in indirect assays alterations are abrupt but they are not space-dependent; in the latter case the initial concentration change is radical but changes with time are minor. Thus, for direct assays viability depends on the distance between cells and the metal. However, results are usually reported as average values without taking into account the higher deleterious effects on cells that are close to the metal, which are the main participants in the biomaterial/cell interactions [64]. Consequently, it is expected that results after 24 h exposure show differences in direct and indirect assays.

Additionally, it is noteworthy to highlight that viability in assays with Mg Exs without filtering is very low after indirect NR assays. The change was less relevant in the case of ZEK100 because, according to the measurements of the Mg ion content in the solution, the corrosion rate during the first 24 h was more than 6 times lower than that of pure Mg. In order to evaluate the effect of insoluble products, the lysosomal activity after the exposure to Exs with and without filtering was assessed. The marked decrease in cell viability after exposures to Exs without filtering indicates that insoluble products clearly affect cell metabolism (Fig. 5). Accordingly, viability in the experiments performed with Mg salt at different Mg ion concentrations was high and similar to that of filtered extracts (without insoluble products).

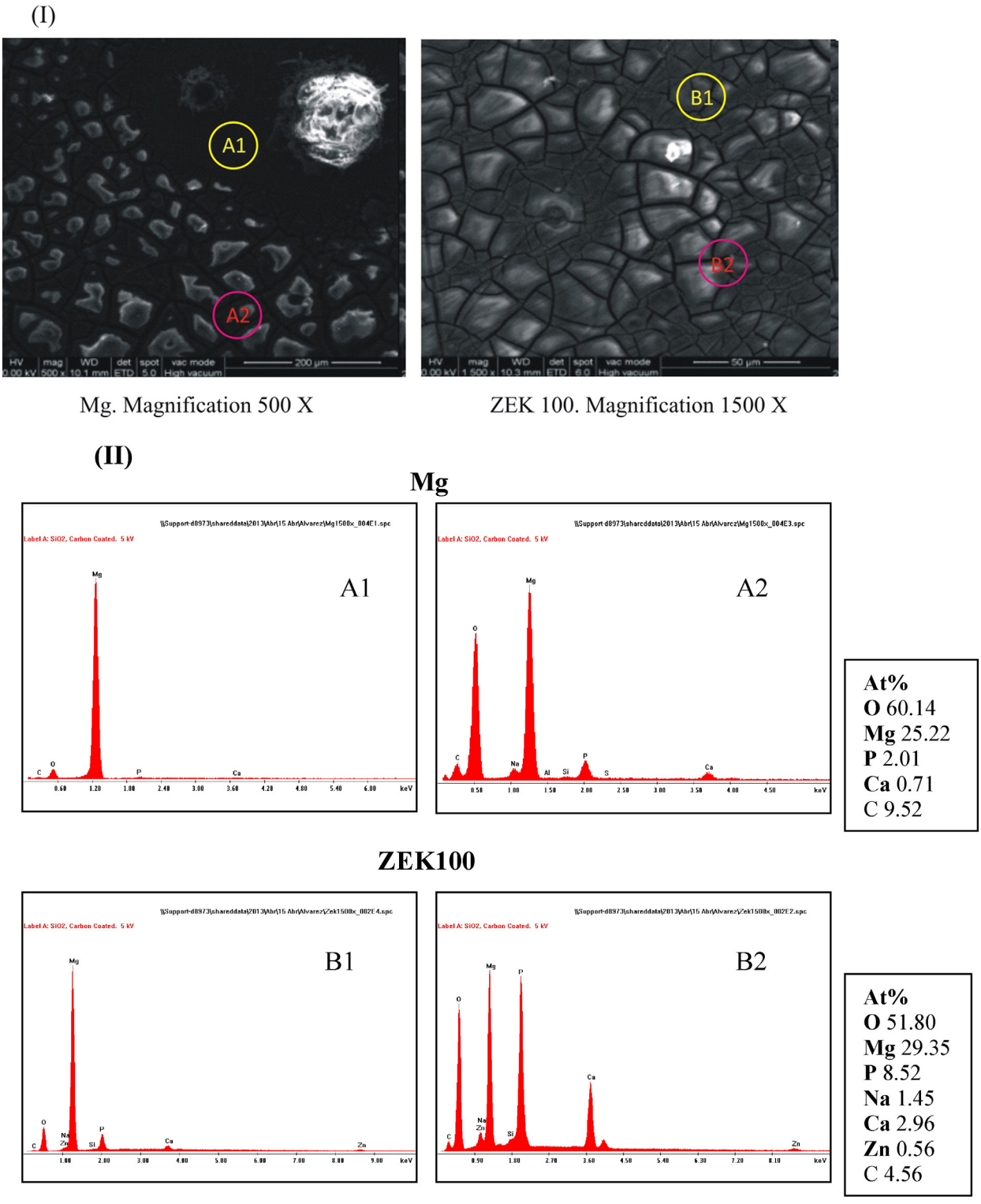

Fig. 6. Surface analysis of metallic disks after immersion in the cell culture. (I) ESEM microphotographs of pure-Mg and ZEK100 alloy surface. (II) EDS spectrum of pure Mg and ZEK100 alloy disks. Surface compositions of Regions A2 and B2 are shown on the left.

4.2. 6 days of assays: CF

The CF assay evidenced the effects of degradation products after long exposures (six days). In these direct assays, with the disks immersed in the cell culture, it could be noticed that the amount of corrosion products was higher in Mg experiments than in assays with ZEK100 alloys with and without Ca. After 6 days (Table 1) the mass of Mg ions was several times the amount found after 24 h for Mg and ZE100. Besides, in ZEK100 experiments the concentration of Mg ions (249.32 mg/l) was markedly lower than that found in the case of pure Mg (868.79 mg/l). The alloy components as well as phosphate precipitation detected by EDS seem to reduce the ZEK100 corrosion rate and consequently the

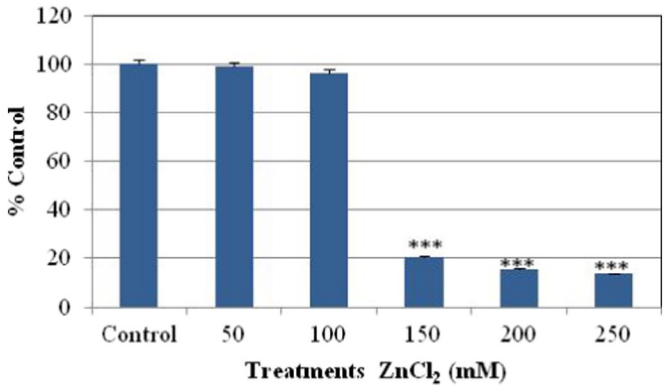

Fig. 7. Effect of Zn Cl_2 on CHO-K1 cells after 24 h of exposure, evaluated by neutral red assay. Error bars indicate standard error of the mean values. ***Significance of the difference at $p < 0.001$.

amount of corrosion products. Accordingly, deleterious effects such as reduction of the number and diameter of colonies decreased in the case of alloys. Thus, the number and diameter of colonies grown in the presence of alloy disks in Region A only decreased to *ca.* 70%–80% of the control value and those exposed to pure Mg to *ca.* 20% of the control value. Conversely, cells were particularly affected in the case of pure Mg assays showing the diameter of colonies being markedly shorter (*ca.* 20% of the control value).

4.3. Effect of single ions and mixtures of ions

Notwithstanding that results of Mg alloys show a less cytotoxic effect than Mg, significant detrimental effects of the alloys with respect

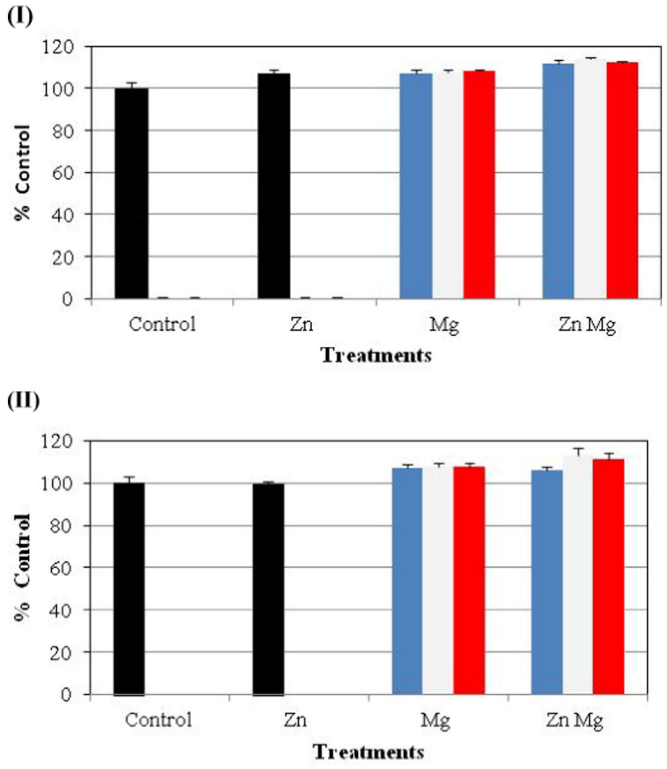

Fig. 8. Effect of binary combined treatments between Zn (50 μM) and Mg salts (I) and Zn (100 μM) and Mg salts (II) on CHO-K1 cells after 24 h of exposure, evaluated by neutral red assay. Error bars indicate standard error of the mean values. ■ Mg: 2.5×10^3 μM; □ Mg: 3.3×10^3 μM; ■ Mg: 4.1×10^3 μM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

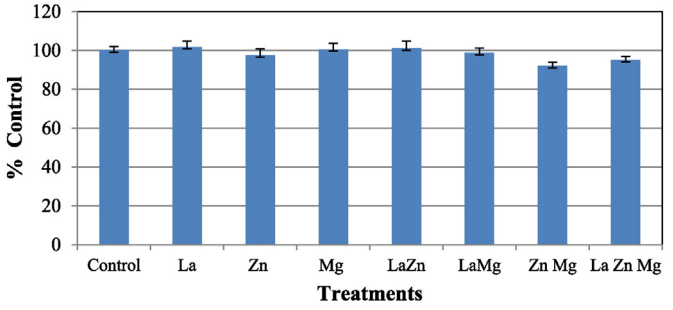

Fig. 9. Effect of ternary combined treatments between Zn (100 μM), La (200 μM) and Mg (8.2×10^3 μM) salts on CHO-K1 cells after 24 h exposure, evaluated by neutral red assay. Error bars indicate standard error of the mean values.

to the control were found (Fig. 3). Thus, it is interesting to investigate which of the components may be the most dangerous and the possibility of joint effects. Beneficial and detrimental cellular effects were informed for La, Mg and Zn [56,61,65–67]. It has been reported that high levels of intracellular Zn induce apoptosis in different cell types and tissues. If Zn is accumulated as a consequence of an exogenous source, it activates pro-apoptotic molecules leading to cell death [68–69]. Additionally, cell death may also be induced by inhibition of the energy metabolism by increased intracellular Zn levels [70–71]. In agreement, the present CF results show that the addition of Zn to La or Mg (Fig. 10) leads to a decrease in viability. Similarly, the addition of Zn to a La + Mg mixture showed a significantly more dangerous effect than the binary mixture.

Conversely, a favorable effect was found for La since the addition of La to Zn + Mg resulted in a significantly less dangerous situation, while a harmful effect of Mg in Zn + Mg mixtures was observed at the highest concentration assayed. Due to their positive effects La and Ca were considered promising alloy components [36,72].

To identify the more critical situations between binary and ternary combinations, several mixtures of Zn, Mg and La were assayed employing the metal salts, *i.e.* without the interference of pH changes and insoluble corrosion products. These mixtures are only some examples of the numerous combinations that may be obtained when the concentration of one of the metal ions is changed. The influence of exposure time and concentration was remarkable. Thus, the addition of 100 μM Zn to Mg, harmless at low Mg concentrations (Fig. 8), is deleterious at 8.1×10^3 μM Mg (concentration of released Mg for 6 d, Table 1) and resulted in a more negative effect than individual Zn. This effect was weak but significant after 24 h exposure (NR assay, Figs. 8 and 9) and important after 6 days of CF assay (Fig. 10). Accordingly, it has been reported that Mg ions may produce a concentration dependent deleterious effect

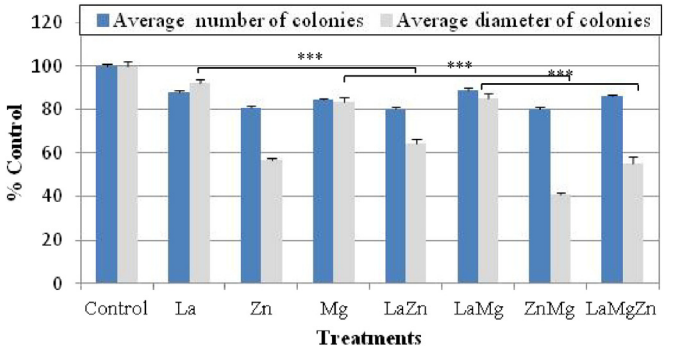

Fig. 10. Effect of ternary combined treatments between Zn (100 μM), La (200 μM) and Mg (8.2×10^3 μM) salts on CHO-K1 cells, evaluated by clonogenic assay after 6 days of exposure on the average number of colonies and on the average colony diameter. Error bars indicate standard error of the mean values. ***Significance of the difference at $p < 0.001$.

decreasing the viability of cells after exceeding certain concentration values [46,73].

Xu et al. [57] explored the toxic mechanism of the joint action of metal compounds and proposed a model that uses a bio-concentration factor (BCF) to evaluate the combined toxic effects of metal ions. Zn is a metal with a relatively high BCF value (2.73) and consequently could be more easily accumulated and thus be more toxic than other ions. The authors found a detrimental joint effect when Zn was added to a solution of other single metal ions. In agreement, our 6 day clonogenic results show that the colonies that grew with the Zn + Mg mixture were smaller than the control colonies and smaller than those corresponding to Mg as a single metal ion. Conversely, the average diameters of those cells that were in contact with Zn + La mixtures were longer than those that were exposed to single Zn ions although no statistically significant difference was found.

Additionally, it was predicted [57] that ternary mixtures lead to an average behavior of the whole components of the mixture. In this respect, our La + Mg + Zn assays revealed viability values which are close to the average value between La + Zn, Zn + Mg and Mg + La but are lower than the average of the effects of single La, Mg and Zn.

Finally, after comparing the effects of mixtures of ions of different osmolarities, the resulting effect does not seem to be directly dependent on the osmolarity of the solution.

5. Conclusions

The analysis and comparison of the results obtained with pure Mg and rare earth-containing alloys (ZEK100 with and without Ca, LAE442) by different assays (direct or indirect) lead to the following conclusions:

- A decrease in cell viability was dependent on exposure time, nature (composition and solubility) and concentration of corrosion products and pH changes.
- The addition of Ca did not cause important changes in the biocompatibility of the ZEK100 alloy.
- Gradual alterations in pH and in the amount of corrosion products (direct assays) were better tolerated by cells and resulted in higher viability than severe changes (indirect assays).
- A marked decrease in the diameter of colonies formed in the vicinity of the disk was observed confirming that the decrease in viability was dependent on the distance of the source of ions, *i.e.* the concentration of corrosion products.
- The corrosion process was altered in the presence of cells and, likewise, cell metabolism was affected by metal degradation.
- In the case of alloys, synergistic or antagonist joint effects may be found according to the nature and concentration of the released ions, particularly in the case of Zn.
- Direct assays with metal disks (CF assays after 6 day-exposures) are better to simulate *in vivo* situations than indirect assays (24 h NR and AO viability assays). However, indirect assays provide complementary data and help to understand the processes that occur in direct assays with disks. This highlights the need of using a battery of tests to evaluate the biocompatibility of bioabsorbable biomaterials.

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