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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 ($2.5 \times 10^3$ μM, $3.3 \times 10^3$ μM, $4.1 \times 10^3$ μM and $8.2 \times 10^3$ 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 $5.0 \times 10^5$ 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