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Cellular response to rare earth mixtures (La and Gd) as components of degradable Mg alloys for medical applications

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Cellular response to rare earth mixtures (La and Gd) as components of degradable Mg alloys for medical applications

1.1.1 Abstract

Rare earth (RE) elements have been proposed to improve the corrosion resistance of degradable Mg alloys for medical applications. However, good biocompatibility of the elements released by Mg alloys during degradation is essential for their use in implants. Most studies are focused on material science and engineering aspects, but the effects of ions released at the biological interface are

not frequently addressed. The aim of this study was to contribute to the knowledge of *in vitro* toxicological effects of two RE Mg-alloying elements, La and Gd, as individual ions and in mixtures with and without Mg ions. Different combinations (Mg+Gd, Mg+La, and Mg+Gd+La) were used to evaluate their possible synergistic effects on CHO-K1 cells. Two sets of experiments were designed to assess 1) the cyto-genotoxic effect of La and Gd ions by Neutral red (NR) technique, Reduction of tetrazolium salt (MTT), Viability with Acridine Orange staining, Clonogenic test, and Comet assay; and, 2) the possible synergistic toxicological effect of La and Gd ions in mixtures, and the influence of osmolarity increase on cellular response. Cytotoxic effects of RE were found at concentrations $\geq 200 \mu\text{M}$ RE while DNA damage was detected for doses $\geq 1500 \mu\text{M}$ and $\geq 1600 \mu\text{M}$ for La and Gd, respectively. When mixtures of ions were evaluated, neither synergistic cytotoxic effects nor biological damage related to osmolarity increase were detected

1.1.2 Keywords: cytotoxicity; rare earth; lanthanum; gadolinium; magnesium; DNA damage

1.1.3

1.1.4 1. Introduction

Ideally, a biodegradable material should degrade in the biological system at a desired rate and not be toxic or adversely affect macromolecules and cellular components under physiological conditions. [1-3] Mg and its alloys are degradable and would therefore make surgery for the removal of temporary implants unnecessary. They are promising for orthopedic and dental applications due to their mechanical properties similar to those of bone. [4-7] However, the use of Mg as biomaterial is seriously limited due to its fast dissolution rate in the physiological environment. [8-15] Consequently, different alloying elements have been introduced for specific reasons and, in most of the cases, corrosion resistance has been improved. [12, 14 16-17]. During degradation, Mg and all alloying elements are released over time to the neighbouring environment by various mechanisms, including corrosion, wear, and mechanically accelerated electrochemical processes such as stress corrosion, corrosion fatigue, and fretting corrosion. [18]

Toxic effects were detected at the implantation site of biodegradable materials. [19] The degradation process that leads to the release of metal ions may be closely associated to cytotoxic problems detected at the implantation site. Importantly, this process is neither uniform in space nor constant in time. Thus, the corrosion rate is low in some regions of the Mg alloys and very fast in others. Song and Atrens [20] reported effects that caused the enrichment of certain alloy components and a depletion of magnesium on the surface layer of corroding alloys. In fact, the whole composition of magnesium alloys changes locally during degradation because some ions are eliminated faster than others. A schematic representation of the complex process occurring at the metal alloy/biological medium interface is shown in Figure 1. It can be noticed that localized corrosion, release of metal ions, and accumulation of corrosion products may occur simultaneously at different sites. In this context, the

amount of metal ions released by the alloy are space- and time-dependent and may consequently induce toxic reactions into surrounding cells close to particularly susceptible implant regions or due to systemic accumulation. In this sense, several articles have been published about cytotoxic effects of some Mg-alloying elements such as aluminum, zinc, and manganese [21-23], and others related to Mg-alloy corrosion products. [24-26]

It is well known that mechanical properties of Mg alloys may be improved by the addition of rare earth (RE) elements. This improvement has generally been associated to the formation of metastable RE-containing phases along the grain boundaries. [27] Interestingly, different corrosion mechanisms have been observed for RE-Mg alloy in chloride solutions: general corrosion, pitting, and intergranular corrosion. General corrosion was found under the uniform layer of $Mg(OH)_2$. Pitting corrosion was located within the grain and resulted from the preferential localized dissolution induced by microgalvanic cells originated by precipitates. Intergranular corrosion started at intermetallics in the eutectic region. [28-29] Consequently, the surrounding cells will be exposed to dissimilar amounts of RE ions, according to their location.

Among rare earth elements, La and Gd have been used to improve the properties of Mg alloys for biomedical applications [30] with different results according to the alloy composition. Levels of Gd higher than 10% improve strength and are probably related to a more noble behavior of Mg5Gd found in grain boundaries. However, for 15% Gd-containing alloys with smaller grain size and larger grain boundaries than other alloys, a drastic increase in corrosion was observed. In the case of Mg-xGd-3Y-0.4Zr, the corrosion products were enriched with Gd, but the compactness of the layer decreased with the increase of Gd content. [31] Conversely, in the case of some La-containing alloys, the passive film improved the corrosion resistance of the alloys by the formation of a more compact and thin corrosion product layer. [27] If these electrolyte layers are thin, which is probably the case for some sites of the implant, high pH values are reached as a consequence of the corrosion reaction occurring at the implant surface facilitating the formation of insoluble corrosion products, and locally inducing different corrosion rates. Thus, these environmental changes associated to corrosion processes could affect the functions of neighboring cells.

As La and Gd are also extensively used in different medical fields, specific information about the effect of these ions is available. They are used in the diagnosis (Gd) and in therapeutics (La) of patients with severe kidney pathologies. The possible association between nephrogenic systemic fibrosis and exposure to Gd-containing contrast agents used for magnetic resonance angiography in patients with advanced kidney failure or in hemodialysis-treated patients has been reported. [32] It is known that the free form of Gd^{3+} may be toxic for biological tissues. Moreover, other ions such as Zn^{2+} , Cu^{+2} , or Fe^{+3} have been suggested to destabilize Gd-chelating by transmetallation with the release of Gd^{3+} from its

ligand. It should also be taken into account that Gd ions are scarcely soluble and form phosphate and carbonate salts that may accumulate in tissues. Although LaCl_3 shows antibacterial and anticoagulation effects, the ions may also inhibit the binding of lipopolysaccharide with monocytes in burns. [33]

Several corrosion mechanisms detected in RE-Mg alloys could induce time-dependent concentration gradients of the alloying elements that may differentially contribute to the induction of adverse cellular effects. In this sense, dissimilar criteria have been applied when the cytotoxicity of metal ions is assessed. Complete solid materials have been used by some researchers for direct exposure to cells or to obtain extracts [34-36] while others have evaluated the toxicity of the individual components of the alloy. [37] The extract of ions obtained after the exposure of the culture medium to the metal alloy corresponds to the average concentration of the ion mixture. Consequently, the use of this ion mixture does not allow to evaluate the effect of the presence of different ion mixtures that are found locally and affect neighboring cells. Moreover, the effect of each ion cannot be inferred when an extract is used.

An important issue that is not frequently addressed when ion mixtures are assessed is the effect of increased osmolarity in the biomaterial surroundings, related to the metal dissolution. Decrease in adhesion capacity of cells and increase in apoptosis have been associated to high osmolarity and ionic strength. [38-39] Accordingly, it has been reported that the biological response may be affected by ionic dissolution products of biodegradable biomaterials. [40] These aspects have been recently analyzed in relation to bioactive glasses, where ion release is also relevant. [41] For this reason, the effect of the release of Mg (base component of the alloy) ions, increasing osmolarity, and the possibility of synergistic effects have been analyzed in the present study.

Several reports have revealed cytotoxic effects not only for individual metal salts but also for different mixtures of metal ions, detecting synergistic, antagonistic, or additive effects. [42-45] Xu et al., [46] developed toxicological assays with individual, binary, ternary, and quaternary ion mixtures and showed that in most of the binary combinations, interactions were synergistic. In agreement with these authors, synergistic cytotoxic effects were found in our previous work when Al-Zn ions combinations were used. [26]

Schonnen et al., [47] studied the cytotoxic effects of 100 reference compounds on four cell lines and concluded that the processes underlying basal cytotoxicity may account for the similarity in the effects of toxins on different cell types. In agreement, Barile *et al.*, [48] also reported good predictability of in vitro tests in human and other mammalian cell cultures for human toxicity dosing. Among the cell lines most frequently employed for in vitro tests the Chinese hamster ovary (CHO) cell line is commonly used as model for in vitro genotoxicity studies. [49-50] Nonetheless, toxicity results are sometimes dependent on the cell line tested. [51]

In fact, results from MTT assays showed that RE cytotoxic effects depend on the type of cell line evaluated. They also revealed that adverse effects of La are attained at lower concentrations of Gd ($LD_{50} >400 \mu\text{M}$ and $>1000 \mu\text{M}$, for La and Gd, respectively, in RAW264.7 cell line). Fayerabend *et al* and Liu *et al* [52-53] also reported dose-dependent results. LaCl_3 affected proliferation, osteogenic differentiation, and mineralization of MC3T3-E1 cells. Alternatively, other *in vitro* results from primary cultured rat cortical neurons showed that Gd ions (GdCl_3) induced neural cell death followed by a rapid accumulation of intracellular reactive oxygen species. [54] After examining data reported, Yuen *et al.*, [55] concluded that toxicological information is still insufficient. They considered that further toxicological studies on RE would have to be developed and eventual biological consequences caused by chemical mixtures evaluated in order to ensure their safe use in degradable biomedical implants.

The aim of this study was to contribute to the elucidation of RE (single ions and Mg+Gd, Mg+La and Mg+Gd+La mixtures) effects on CHO-K1 cell cultures. To that end we attempted to answer the following questions:- Are cyto- and genotoxic effects of Gd and La similar? Are cyto- and genotoxicity threshold values dependent on the end point and exposure time assayed? Are there synergistic effects when RE is used in mixtures? In presence of high Mg ions levels, i.e. higher osmolarity and ionic strength conditions, are the effects of RE more deleterious? Notwithstanding its implications for biocompatibility of degradable materials, the effect of the combination RE-Mg on cells *in vitro* has not been previously evaluated. Several endpoints were used in the present study to identify possible cyto- and genotoxic effects of these ions and their combinations.

2. Materials and Methods

2.1. Cell cultures

CHO-K1 cell line 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 $\mu\text{g}/\text{ml}$ streptomycin sulfate (complete culture medium) at 37 °C in a 5% CO_2 humid atmosphere. Cells were counted in an improved Neubauer haemocytometer, and viability was determined by the exclusion Trypan Blue (Sigma, St. Louis, MO, USA) method; in all cases viability was higher than 95%.

2.2. Experimental design

Two sets of experiments were designed to assess 1) the effect of La and Gd as individual ions, as individual ions, on CHO-K1 cells by Neutral red (NR) technique, Reduction of tetrazolium salt (MTT), Viability with Acridine Orange staining, Clonogenic assays, and Comet assay; and, 2) possible

synergistic effects of La and Gd ions and the influence of osmolarity increase with combined treatments using La and Gd salts with and without Mg ions.

2.3. Chemicals

RE toxicity was evaluated by exposure to solutions of different concentrations (1000 μM - 2000 μM concentration range) of La ($\text{LaCl}_3 \cdot 7\text{H}_2\text{O}$) and Gd (GdCl_3) chlorides (Sigma-Aldrich, St. Louis, MO, USA). Clonogenic assays were made using 200, 600, 800, 1000, 1200, 1400, 1600, 1800, and 2000 μM concentrations.

Gentoxicity was assessed (24-h test) by comet assay. Each RE concentration was selected considering the results from cytotoxicity tests with the same exposure time. Thus, the minimum dose selected for La and Gd corresponded to the lowest cytotoxic effect detected in at least one type of trial (1400 μM for La and 1500 μM for Gd) while the maximum was 2000 μM for both ions.

Cytotoxic assays with ion mixtures were also made. RE/Mg relationships were selected based on Krause et al., [56] results about a RE-containing Mg alloy. They reported that the elemental composition of LAE442 surface layer included Mg, O, RE. After 6 months implantation, only 18.28% Mg was detected at the LAE442 outside layer but it was enriched with RE with respect to the base metal (initial = 2% and 2.71% after 6 months). Thus, the surface RE/Mg relationship was $2.71\% / 18.28\% = 0.148$. To select the suitable RE/Mg relationships required to evaluate possible synergistic effects, we assumed that: a) 0.148 ratio was close to the average value found under *in vivo* conditions; and b) cells can tolerate Mg ion concentrations within 300 - 500 $\mu\text{g}/\text{mL}$ range (2.5×10^3 - 4.1×10^3 μM) according to our previous reports. [26] Thus, RE/Mg relationships within 0.10-0.16 range (i.e. including 0.148 value) were selected.

2.4. Neutral red assay (NR)

The NR uptake assay was performed according to Borenfreund and Puerner. [57] 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, 2.5×10^3 cells/well (96 multi-well plate) were grown in complete culture medium for 24 h at 37°C in 5% CO_2 humid atmosphere. Then, the medium was replaced by another one with different RE and RE-Mg concentrations. After 24 h, the medium was removed and replaced by fresh medium containing 40 $\mu\text{g}/\text{mL}$ NR dye (Sigma, St. Louis, MO, USA). After 3 h incubation, cells were washed with phosphate buffer solution (PBS). Afterward, 0.1 mL 1% acetic acid in 50% ethanol solution was added. Subsequently the dye taken by live cells 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 without RE. 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.

2.5. Reduction of tetrazolium salt assay (MTT)

Cytotoxicity of RE-containing media in CHO-K1 cells was also estimated using metabolic competence by the colorimetric method of Mosmann [58] as modified by Twentyman and Luscombe. [59] This assay measures the reduction of tetrazolium salt (MTT) (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to formazan by dehydrogenase enzymes of intact mitochondria in 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 a 96-multiwell plate and grown at 37°C in 5% CO₂ humid atmosphere in complete culture medium for 4 h. This medium was then replaced by different RE-containing media. After 24 h, medium was removed, cells were washed with phosphate buffered saline solution (PBS) and fresh medium containing MTT reagent (1 mg/mL final concentration) (Sigma, St. Louis, MO, USA) was added. After 3 h incubation, cells were washed again with PBS. Color was developed by the addition of 100 µL dimethylsulfoxide (DMSO) (Merck, Química Argentina SAIC, Argentina) to each well for cell lysis and formazan crystals dissolution. The plate was shaken for 10 min and the absorbance was measured at 540 nm using an automatic ELISA plate reader (µQuant BioTek, USA). 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 independently repeated three times. Data were analyzed using one-way ANOVA test and multiple comparisons were made using p values corrected by the Bonferroni method.

2.6. Cell viability by Acridine Orange staining

For this set of experiments, 2.5×10^3 cells/well were cultured in a 12 multi-well plate and grown at 37° C in 5% CO₂ humid atmosphere in complete culture medium for 24 h. Then, the culture medium was replaced by another one with different RE-content. After 24-h exposure, adherent cells were stained with Acridine Orange dye (Sigma, St Louis, MO, USA) and immediately after, they were examined using a fluorescence microscope (Olympus BX51, equipped with appropriate filter) (Olympus Corp., Tokyo, Japan) 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.

2.7 Evaluation of colony formation

Colony formation or Clonogenic assay is an *in vitro* cell survival test based on the ability of a single cell to grow into a colony. [60] It is used to determine cell reproductive death after treatment with cytotoxic agents. For this analysis CHO-K1 cells were grown at 37° C in 5% CO₂ humid atmosphere in complete culture medium. Two sets of experiments were performed with i) different RE concentrations (range detailed in Section 2.3); and ii) combined treatments with RE-Mg ions concentrations (200 μM and 4.1 x 10³ μM, respectively) in each Petri dish. An additional cell culture, grown in complete culture medium, was used as negative control. After 6-day incubation the colonies were scored. They were fixed with methanol:acetic acid (3:1) and stained with Acridine Orange. The colonies were counted and then the diameters were measured under fluorescence microscopy with a 40X objective (Olympus BX51, equipped with appropriate filter) (Olympus Corp., Tokyo, Japan) 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. Three independent experiments were performed in independent trials to assess reproducibility.

2.8 Evaluation of DNA damage by Comet assay

Genotoxicity of RE-containing media in CHO-K1 cells was estimated using single-cell gel electrophoresis (Comet assay, alkaline version) according to the method of Singh et al., [61] with some modifications. [62] Subcultures for experiments were set up the day before treatment. Approximately 1.5 × 10⁵ cells at logarithmic growth phase were treated with different RE concentrations for 24 h. Briefly, conventional slides were covered with a first 180 μL layer of 0.5% normal agarose (GIBCO-BRL, Los Angeles, CA, USA). Then, a mix of 75 μL 0.5% low melting point agarose (GIBCO-BRL, Los Angeles, CA, USA) and 15 μL cell suspension with approximately 1.5 × 10⁴ cells was layered onto the slides, which were immediately covered with coverslips. After agarose solidification at 4°C for 5 min, coverslips were removed and slides were immersed overnight at 4°C in fresh lysing solution [(2.5 M NaCl (JT Baker, Phillipsburg, NJ, USA), 100 mM sodium ethylene diamine tetracetic (Na₂EDTA) (JT Baker, Phillipsburg, NJ, USA), 10 mM hydroxymethyl aminomethane tris (Tris, pH 10) (JT Baker, Phillipsburg, NJ, USA)] containing 1% Triton X-100 (Sigma, St Louis, MO, USA) and 10% DMSO (Merck Química Argentina SAIC, Argentina) added just before use. Two slides from control and from each treated group were prepared under dim light conditions. After lysis, slides were placed on a horizontal gel electrophoresis unit with fresh electrophoretic buffer [300 mM NaOH (Farmitalia Carlo Erba SpA, Milano, Italy), 1 mM Na₂EDTA, pH > 13], left for DNA unwinding for 20 min, and then electrophoresed for 30 min at 1.25 V/cm (300 mA). This procedure was carried out at 4°C under dim light. After electrophoresis, slides were neutralized by washing three times with buffer (0.4M Tris, pH 7.5) every 5 min and then with distilled water. Slides were stained with SYBR Green I (Molecular Probes, Eugene, OR, USA) at the recommended dilution. [63]

Observations were made at 400X magnification using a fluorescence microscope (Olympus BX51, equipped with a 515 to 560-nm excitation filter) (Olympus Corp., Tokyo, Japan) connected to an Olympus DP71 (Olympus Corp., Tokyo, Japan) color video camera. The image from each individual cell was taken immediately after opening the microscope shutter to the computer monitor. Three separate experiments were performed for each experimental condition. A total of 300 images per treatment were scored.

For qualitative analyses, based on the degree of DNA breakage, cells were classified according to their tail length into five categories, ranging from 0 (no visible tail) to 4 (detectable head of the Comet but most of the DNA in the tail). Apoptotic cells [64-65] were assigned to a sixth group. The effects of different concentrations of La and Gd on the frequency of damaged cells were analyzed using the χ^2 test. Undamaged cells (0 degree) were compared with those with low damage (1-2 degree), severe damage (3-4 degree), and apoptosis.

For quantitative analyses, tail moment (TM) parameter [64] was calculated using the CASP software (Comet assay software project). The Mann-Whitney test was employed to evaluate differences between each treatment.

3. Results

3.1. Effects of La and Gd salts on CHO-K1 cells

Cytotoxic effects

The effects of La and Gd salt treatments on CHO-K1 cells evaluated by MTT assay are summarized in Figure 2. An increase in the mitochondrial activity was found for cells treated with 1000 and 1200 μM Gd while cultures with concentrations ≥ 1600 μM showed an important decrease ($p < 0.001$) in the reduction of tetrazolium salt (MTT) to formazan with respect to the control. Distinctly, La assays with concentrations ≥ 1400 μM showed significant decrease with respect to untreated control ($p < 0.001$). The difference between the impact of La and Gd on mitochondrial activity was more relevant for 1400 and 1500 μM assays ($p < 0.001$) with more deleterious effect in the case of La.

Figure 3 shows the results of NR assay after 24-h La and Gd salt treatments with respect to the control. A decrease in lysosomal activity was observed at concentrations ≥ 1500 and ≥ 1600 μM for Gd and La, respectively. Significant differences in the cell behaviour were observed after Gd and La treatments for 1600 and 1700 μM ; again, a more deleterious effect was observed for La. However, for higher concentrations Gd seemed to have worse impact while La reached a steady state, though differences were not significant.

Results from microscopic observations of cell viability by Acridine Orange staining are depicted in Figure 4. Doses ≥ 1400 μM La revealed a concentration-dependent decrease in the number of living

cells ($p < 0.001$). In the case of Gd, this effect was evidenced for 1600 μM ($p < 0.001$). According to previous results (Figures 2 and 3) assays with higher RE concentrations showed lower viability for La than for Gd treatment, but differences between the effects of each ion were significant only for 1400 μM ($p < 0.001$) and 2000 μM ($p < 0.01$).

After 6-day exposure to RE, clonogenic assay showed a non-linear decrease in the size of colonies with respect to the control for both La and Gd, for the whole concentration range tested (200 μM -2000 μM , not shown). Microscopic measurement of colonies diameter (Fig. 5) revealed a weak but significant ($p < 0.001$) decrease (ca. 20%) with respect to the control after exposure to 200 μM RE as well as to 600 μM and 800 μM (not shown). For 1400 μM , a reduction close to 80% of the control value was found in La assays, while no colony (diameter=0) was detected for Gd-treatments. Within the 1600-2000 μM range, no colonies were found for La or Gd. The number of colonies also decreased significantly after RE treatments (82% and 22% after exposure to 1200 μM and 1400 μM La respectively) (not shown).

Figure 6 shows DNA damage percentage evaluated by the alkaline Comet assay. Results revealed that treatments within 1500-1700 μM dose range of La and doses of 1600 and 1700 μM Gd increased DNA migration in relation to untreated cells. Higher frequency of cells with low-level damage (1–2 degree) was observed in qualitative analyses when compared with controls ($p < 0.001$). In addition, a significant increase of severe damage (3-4 degree) was found for both RE treatments at 1700 μM concentration ($p < 0.001$). Apoptosis was detected only after 1700 μM Gd treatment (2.59 %); similar values (2.88 %) were observed when 1500 μM of La was assayed, reaching 5.59 % at the highest dose tested (1700 μM). Results from quantitative analyses (TM parameter) are in agreement with qualitative analyses. However, significant differences in TM values (when compared with the control group ($p < 0.001$)) were only found when CHO-K1 cells were treated with 1700 μM La or Gd.

3.2. Effects of La and Gd salts plus Mg salt on CHO-K1 cells

We investigated whether La and Gd may synergistically affect the cell activity and whether the addition of Mg ions could induce any effect due to the increase in osmolarity/ionic strength of the solution. Treatments with RE mixtures were evaluated by NR and clonogenic assays. MTT assay was not performed in order to avoid any possible interference of Mg with the tetrazolium reaction. [66]

Lisosomal activity (NR assay) in CHO-K1 cell line was tested after 24-h treatments with single La, Gd, and Mg cations, single or in binary and ternary mixtures (Figure 7). Treatments with single Gd and La cations at 200 μM and binary and ternary mixtures with and without Mg ions ($2.5 \times 10^3 \mu\text{M}$, $3.3 \times 10^3 \mu\text{M}$ and $4.1 \times 10^3 \mu\text{M}$) did not show any significant decrease in the lisosomal activity.

Clonogenic assays revealed that the number of colonies was lower than that of the control ($p < 0.001$) in all cases tested. However, the analysis of colonies diameter showed a significant

difference only for La ($p < 0.001$), Gd ($p < 0.001$), and Mg ($p < 0.01$) as simple cations, and in ternary combined treatments ($p < 0.001$) (Figure 8).

4. Discussion

Although good biocompatibility of biomaterials is essential for degradable Mg alloys, most studies focus on materials science and engineering aspects. However despite favorable mechanical properties, Mg alloys can only be considered suitable as biomaterials if elements released during the degradation of a magnesium implant are biocompatible. [67]

The release of metal ions in the body is expected to be non-linear, non-homogeneous, and multifactorial, as shown in Figure 1. It is affected by material properties such as grain size, manufacturing processes, shape, size, surface area, surface roughness, and biological environmental properties such as pH, fluid flow, ion and biological molecule concentrations, and evolved gas bubbles, among others. [55] Some RE elements such as La and Gd have proved to be beneficial as alloying elements or as components of conversion coatings in terms of mechanical and/or corrosion properties. [31, 52, 68] After being released due to the degradation process, they have also shown acceptable biocompatibility when cytotoxicity was evaluated by MTT assays. [52] However, it must be considered that localized corrosion has been detected on the alloy surface and high concentration of ions may be found at these sites affecting cells in the vicinity. In this sense, Yuen and Ip, [55] consider that there is still scarce cellular toxicological data. LD 50 dose has been previously reported for several RE, [52] but this information should be complemented with further cytotoxicity assays because LD 50 uses lethality as end point, thereby ignoring non-lethal health effects occurring at much lower exposure levels. [55].

In vivo environments with biodegradable metals cannot be properly simulated in *in vitro* experiments due to the complexity of living organisms. Thus, sometimes *in vivo* and *in vitro* results cannot be expected to be the same. [69] However, when *in vivo* assays reveal biocompatibility problems related to the metallic biomaterial tested, the cause of this problem remains quite difficult to elucidate due to the great number of variables involved. In this sense, *in vitro* assays provide several advantages. They can be a good complement to investigate the impact of individual variables on surrounding cells (effect of single ion and mixture of ions, variation with time and concentration, changes in osmolarity, kind of cell damage, etc). Moreover, the number of *in vivo* experiments may be drastically reduced in view of *in vitro* results.

In order to select the experimental conditions to be assayed for cytotoxicity tests we have taken into account previous reports on *in vivo* studies with Mg alloys including RE-containing alloys such as WE43, LAE442, WE43, and ZEK100. [56, 67, 70-71] They have shown that RE can be detected in the corrosion layers and that percentage increased with the implantation time. Krause et al., [56] found 58%

increase of the RE content in the outer layer of the implant surfaces between the 3rd and 6th month after implantation. Recently, Dziuba et al., [70] reported long term *in vivo* degradation behavior of ZEK100. During the last 3 months of one-year implantation period, the corrosion rate was higher than before. The weight loss throughout the first 9 months was 52% while 40.5% was lost in the last 3 months. Other studies with ZEK100 implants also showed an increased time-dependent weight loss and a non uniform distribution of corrosion products. Precipitates of RE can be found as elevated streaks, aligned in the extrusion direction. [72] Thus, at the end of the degradation process, corrosion products (debris and ions) with greater RE/Mg relationship than the base metal may be released to the surrounding tissues and induce cytotoxic reactions.

4.1 Threshold values of cyto-genotoxicity values of individual ions

It has been reported that the growth of HL-60 cells after 48-h exposure to 1mM LaCl₃ is inhibited while after 2mM treatments apoptosis was found. However other concentrations within 1 mM - 2 mM range have not been assayed. [73] Present results show a detailed evaluation of cytotoxicity within this range after the assay of nine concentrations and several end points.

Some lanthanides have been reported to promote proliferation of cells and be scavengers of free radicals. [74] In fact, our results showed a weak but significant increase in mitochondrial activity (24-h MTT assay) for 1000 and 1200 μ M Gd (Fig. 2). However, cytotoxic effect is not only dependent on the concentration but on exposure time as well. For certain cells a low ROS level and short exposure have been found to promote cell growth, whereas prolonged exposure to lanthanides and higher concentration resulted in cell death. [75] In agreement, after longer exposure periods (6 d), our results showed a deleterious effect of Gd (Fig. 5 and 6), even at concentrations as low as 200 μ M.

A marked cytotoxic effect of La (60% of the control value) was found at 1400 and 1500 μ M by MTT assay. Accordingly, it has been reported that some lanthanides induce mitochondrial dysfunction that could be the cause of this cytotoxic effect. [74, 76-77] It should be noticed that Gd did not show any toxic effect at these concentrations. Likewise, for 1400 μ M an important difference between La vs Gd results was also observed in viability with Acridine Orange dye. La³⁺ has been reported to alter Ca²⁺ levels and reduce the spread of cells. [78] However, though our results showed that the deleterious effect of La on mitochondrial activity was found at lower concentrations than those of Gd after two different 24-h assays, the clonogenic assay revealed a more toxic result for Gd after 6-d exposure. Thus, both effects are exposure-time dependent but the rate of change is different for each ion.

When results from MTT and NR were compared, significant difference between lisosomal activity (NR) of Gd and La (60% for Gd and 40% for La, $p < 0.001$) was detected at 1700 μ M, higher than in the case of MTT test. Results suggest that Gd is more deleterious than La for the 1800-2000 μ M concentration range, but no significant differences were found between Gd and La data.

Deleterious effect of RE at low concentrations (200 μM) was found in clonogenic assays after RE treatments. Both the number and diameter of the colonies decreased after La and Gd treatments indicating that the replication rate was slower in presence of these ions. This decrease was more significant in the case of Gd at 1400 μM . In agreement, previous results have shown that the most pronounced induction of apoptosis was exerted by Gd while the other elements assayed increased apoptosis moderately. [52] Cytotoxic effects of Gd-containing nanostructures have been also reported by Hemmer et al. [79] They showed that a reduction of Gd^{+3} release by polymeric coatings improved the biocompatibility of the nanostructures when incubated with macrophages. Six-day exposure clonogenic assays presented here are in the line of previous reports. They demonstrate that the effect on cell growth and death rates after longer treatments with Gd was more significant than for La and could be detected at lower concentrations than in the case of 24-h- exposure-RN and -MTT tests. Overall, different threshold concentration values, each one associated to a particular end point, highlight the need of using several assays to test cytotoxicity.

High concentration values of RE, several times higher than that of the metal alloy, could be found at the biomaterial/cell interface, close to grain boundaries where RE precipitates, or near RE-containing debris at the end of the degradation process. Thus, RE ions could eventually reach 200 μM locally and affect the growth rate of cells. Similarly, we found viability decrease after cellular exposure to other ions released by degradable metals. [26, 80]

On the other hand, the Comet assay was used in this study to detect DNA damage induced by RE treatments in CHO-K1 cells. Comet assay is a short-term genotoxicity test able to reveal a broad spectrum of DNA damage. [62] Data showed a dose-dependent increase of genomic damage (revealed by both quantitative and qualitative methods) when cells were cultured at 1500-1700 μM RE concentration range. Significant differences with respect to controls were found for ≥ 1500 and 1600 μM for La and Gd, respectively. Our results are in accordance with previous reports that showed an increase of DNA strand breaks in both, Jurkat cells and human peripheral lymphocytes when exposed to La. [81] Finally, present results show that RE concentrations that reduce cell viability are correlated to DNA fragmentation. In fact, vitamin E has been reported to be able to decrease the DNA strand breaks induced by La, suggesting that oxidative stress may be involved in the detected genotoxicity. [81]

4.2 Analysis of possible synergistic effects of ion mixture and osmolarity increase

The high susceptibility to simultaneous exposure to multiple chemicals in the case of degradable implants requires exhaustive studies to provide a detailed insight into the possible biological consequences caused by chemical mixtures. [82] Xu et al. [46] found synergistic cytotoxic effects when several ions in binary combinations were tested. Oliveira et al., [82] also investigated the role of chemical interaction between La, Ce, and Th in lymphocyte toxicity. In the case of Mg alloys, possible

synergistic effects of RE elements and the effect of the increase in osmolarity of different mixtures (La + Mg, Gd + Mg, La+Gd+Mg) need to be further analyzed. In order to be certain that the concentrations selected are of clinical relevance, a primary approach to *in vivo* conditions was made in this study. Typical *in vivo* assays are usually performed in rabbits with implants between 2.5-3.0 mm diameter and 25-50 mm length (ca. 122-353 mm³, ca. 210-614 mg) with 0.3-2% RE. The total dissolution expected in 12 months (from the beginning to total degradation) implies a total release up to ca. 12280 µg RE during this period, with average values up to 33 µg/day. Thus, we could assume that an average concentration close to 200 µM (27.6 µg/mL La and 31.4 µg/mL Gd) for each RE is a suitable value to be assayed with clinical significance. However, it should be taken into account that RE concentrations higher than this value may be found locally under clinical conditions, after accumulation in small volumes of the surrounding biological fluids.

In a previous article we reported that Zn, a frequent alloying element of Mg alloys, showed cytotoxic effect at 360 µM concentration level. Additionally, synergistic effect was found when Zn-Al mixtures with 45 µM Zn ions were assayed. (Yuen, 2010 #342; Grillo, 2013 #580) Thus, the deleterious effects found *in vivo* for Al- and/or Zn-containing Mg-alloys may be in part assigned to these ions together with the pH change and H₂ evolution at the interface. Present results with RE showed cytotoxic effects at concentration levels ≥ 200 µM after 6-day exposure assays. Mixtures of 200 µM RE for each RE with and without Mg ions showed similar effects to those of individual ions. Neither synergistic nor additional effects due to the increase in osmolarity in one order (from c.a 10⁻⁴ Osm/L to 10⁻³ Osm/L) were detected under these experimental conditions.

5. Conclusions

Significant differences in cell behavior were detected after treatments with La and Gd.

Dissimilar threshold concentration values were found for different 24-h cytotoxicity assays. La showed to be more deleterious than Gd in most cases.

Long exposure assays revealed that after 6-day treatments the adverse effect is detected at lower concentrations (≥ 200 µM RE). A time-dependent deleterious effect was revealed. A lower number of colonies of smaller size than that of the control was found for longer RE treatments.

Cytotoxic effects of RE may be found *in vivo* if concentrations ≥ 200 µM/mL are reached locally (i.e. close to the localized corrosion regions where high amounts of metal ions are released).

Genotoxic analysis showed clastogenic effect after 24-h exposure to ≥ 1500 and ≥ 1600 µM of La and Gd respectively.

Unlike other alloying elements such as Al and Zn, La and Gd mixtures did not show synergistic cytotoxic effects under the conditions assayed. Osmolarity increase in one order, associated to the simultaneous Mg and RE ions release, did not show a significant harmful effect.

Results demonstrate that several end points including those with longer exposure than 24h are needed to test the cytotoxicity of metal ions, both alone and in mixtures, to determine the safe concentration range for biomedical applications.

Complementary studies with other alloying elements of medical interest will be performed in the near future to further contribute to the knowledge on the toxicological effect of ion mixtures released by different degradable Mg alloys.

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Legend for the figures

Graphical abstract. Dose-dependent cellular effect induced by rare earth components of bioabsorbable Magnesium alloys.

Figure 1. Schematic representation of the complex process occurring at the metal alloy/biological medium interface.

Figure 2. Effect of Gadolinium and Lanthanum treatment on CHO-K1 cells after 24 h of exposure, evaluated by MTT assay. Error bars: standard error of the mean value.

Figure 3. Effect of Gadolinium and Lanthanum treatment on CHO-K1 cells after 24 h of exposure, evaluated by Neutral red assay.

Figure 4. Effect of Gadolinium and Lanthanum treatment on CHO-K1 cells after 24 h of exposure, evaluated by Acridine Orange test. Error bars: standard error of the mean value.

Figure 5. Average diameter of CHO-K1 colonies in cultures treated with Gadolinium and Lanthanum after 6 days of exposure to different RE concentrations. Error bars: standard error of the mean value.

Figure 6. Genotoxic effect of Gadolinium and Lanthanum treatment on CHO-K1 cells after 24 h of exposure, evaluated by Comet assay. Error bars: standard error of the mean value.

Figure 7. Effect of Gadolinium (200 μ M), Lanthanum (200 μ M) and Magnesium ions ($2.5 \times 10^3 \mu$ M, $3.3 \times 10^3 \mu$ M and $4.1 \times 10^3 \mu$ M) treatments on CHO-K1 cells after 24 h of exposure, evaluated by Neutral red assay. Error bars: standard error of the mean value.

Figure 8. Number and average diameter of CHO-K1 colonies in cultures treated with Gadolinium (200 μ M), Lanthanum (200 μ M) and Magnesium ($4.1 \times 10^3 \mu$ M) (after 6 days of exposure). Error bars: standard error of the mean value.

Highlights

La and Gd (RE) are components of biodegradable Mg alloys used in implants.

Cytotoxic effects were dependent on type and concentration of RE and exposure time.

Clonogenic effects were found after 6 days exposures to 200 μM La or Gd.

Clastogenic effects to ≥ 1500 μM La and ≥ 1600 μM Gd were found after 24 h exposures.

Several end points are needed to determine the safe concentration range of ions.

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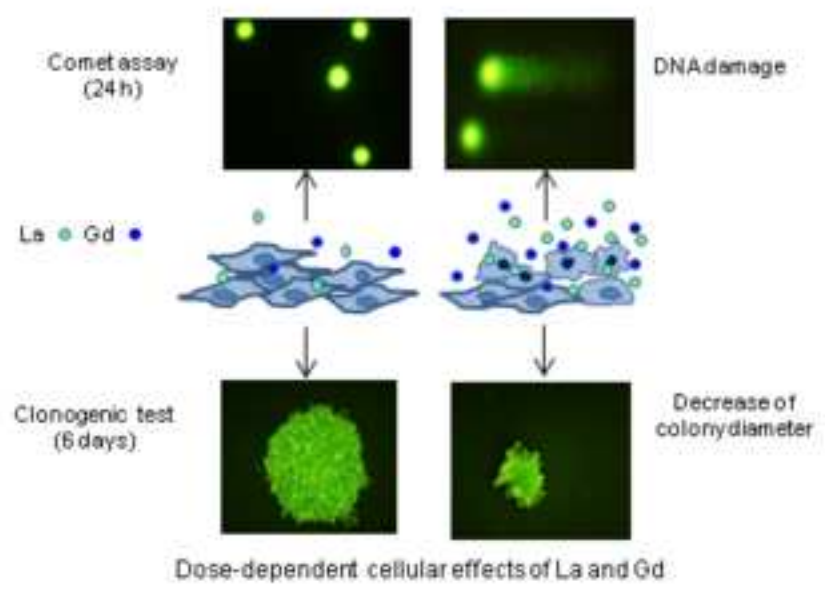


Figure 1

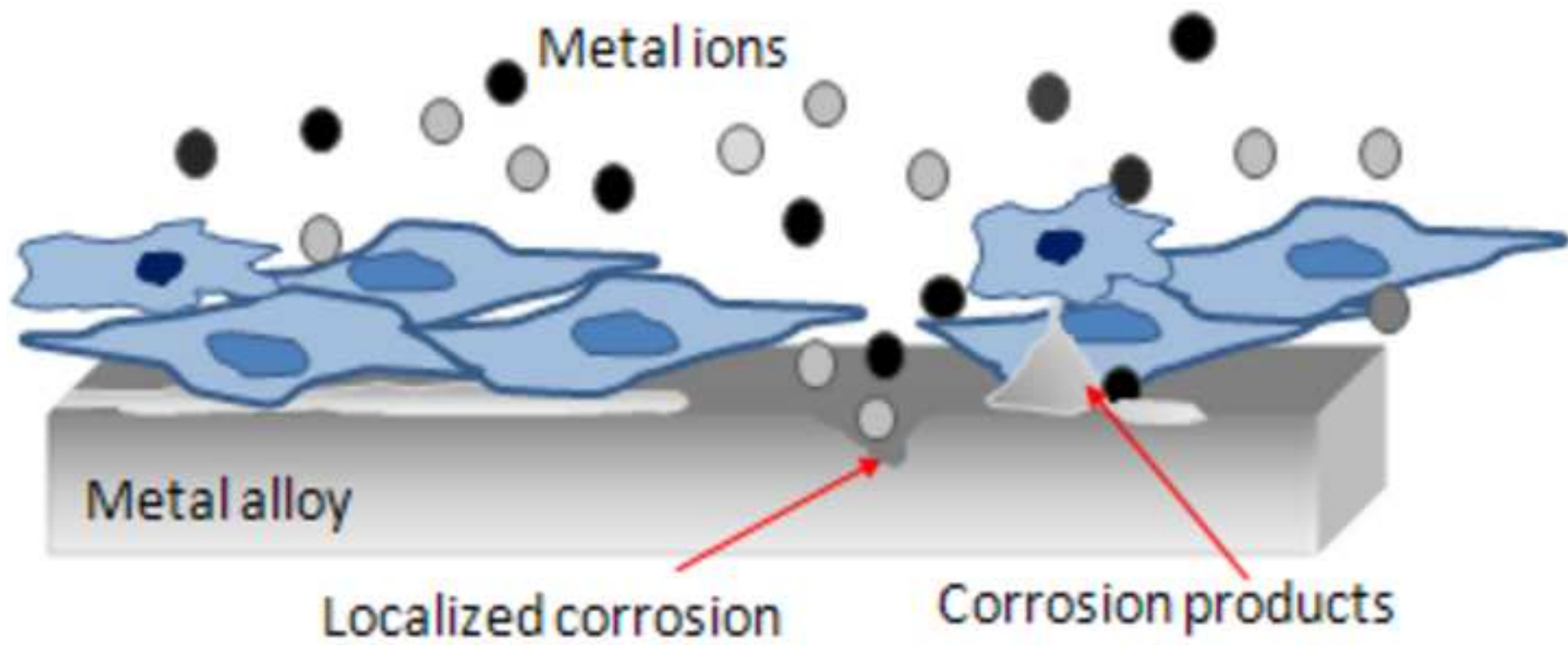


Figure 2

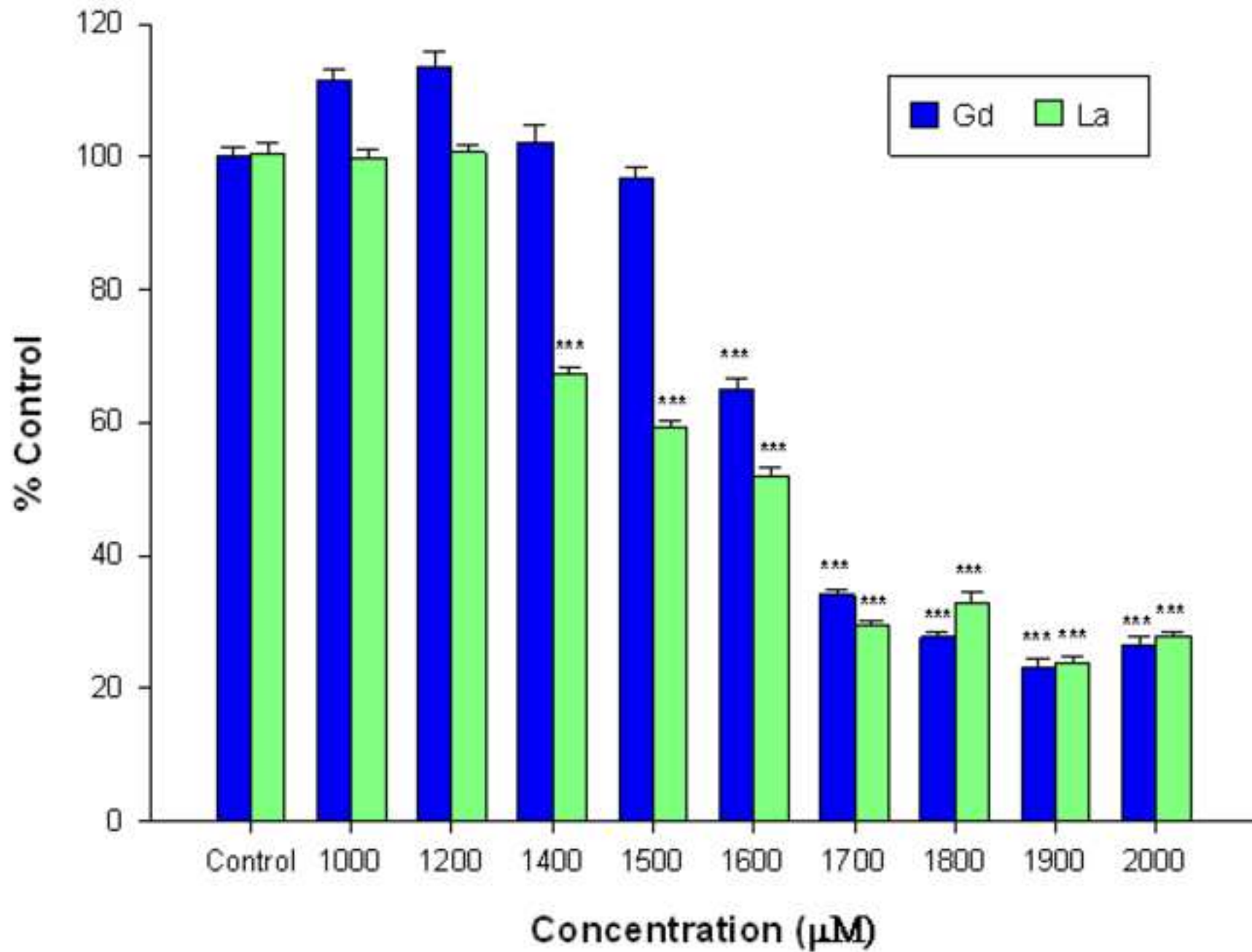


Figure3

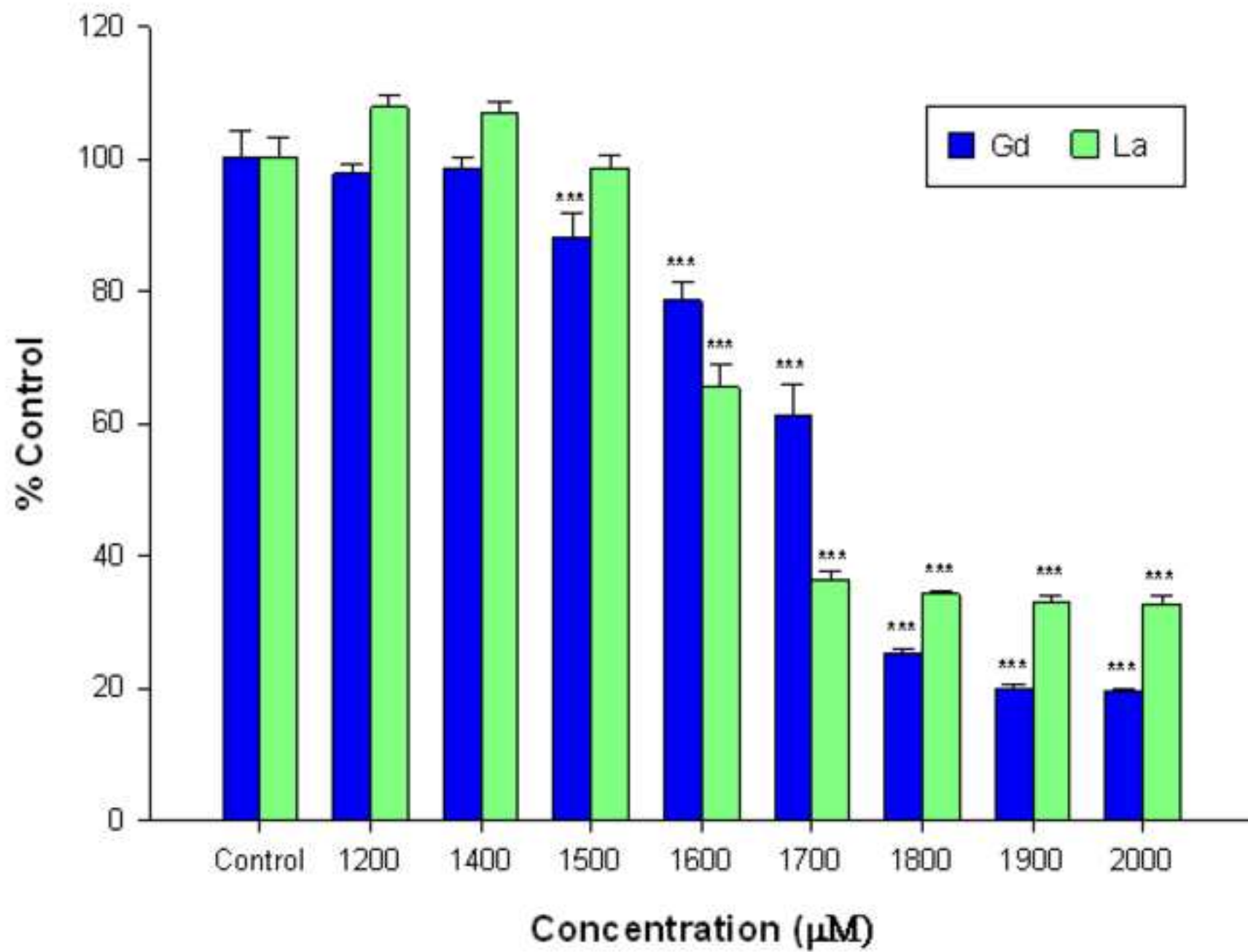


Figure 4

trip

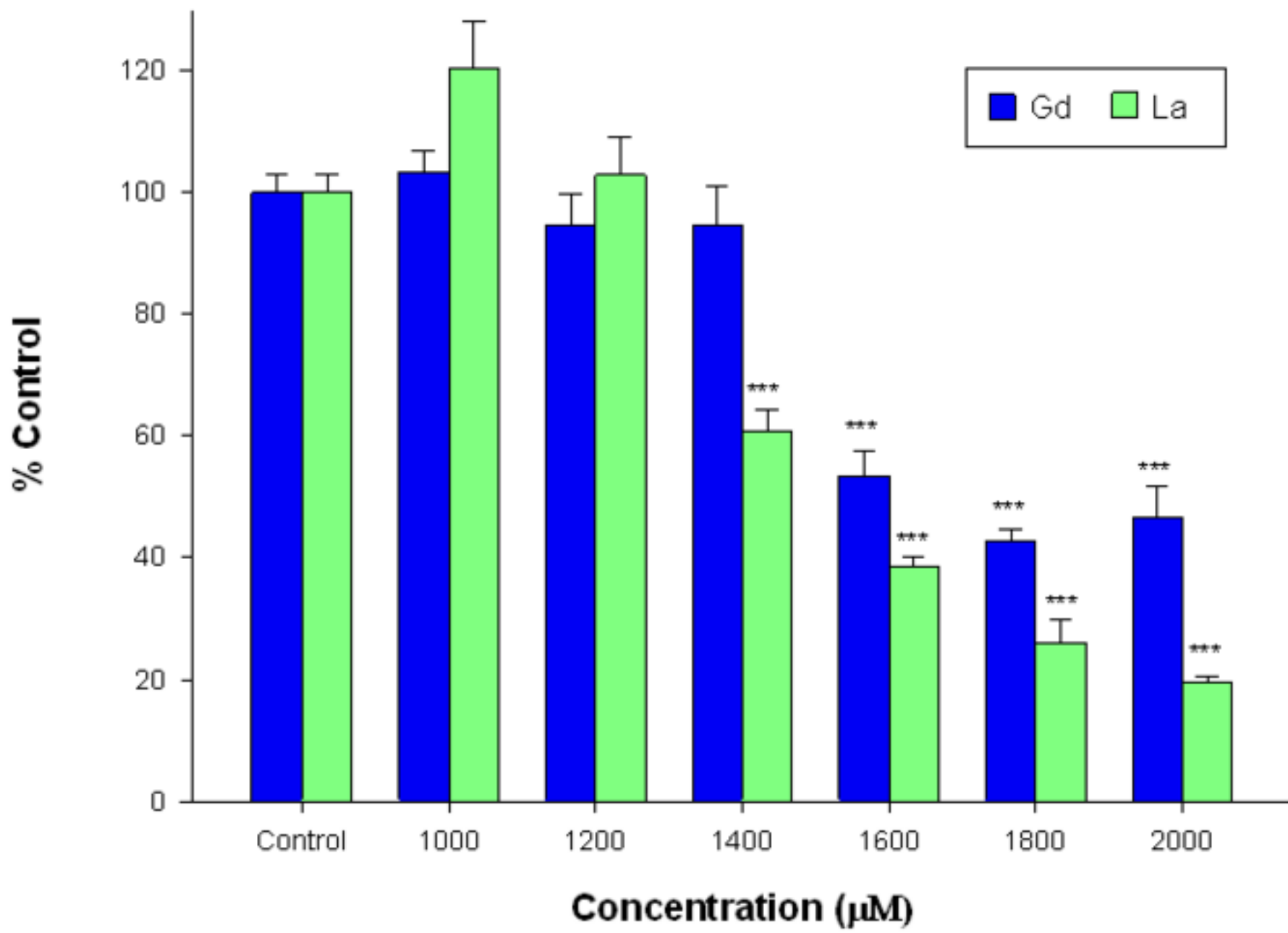


Figure 5

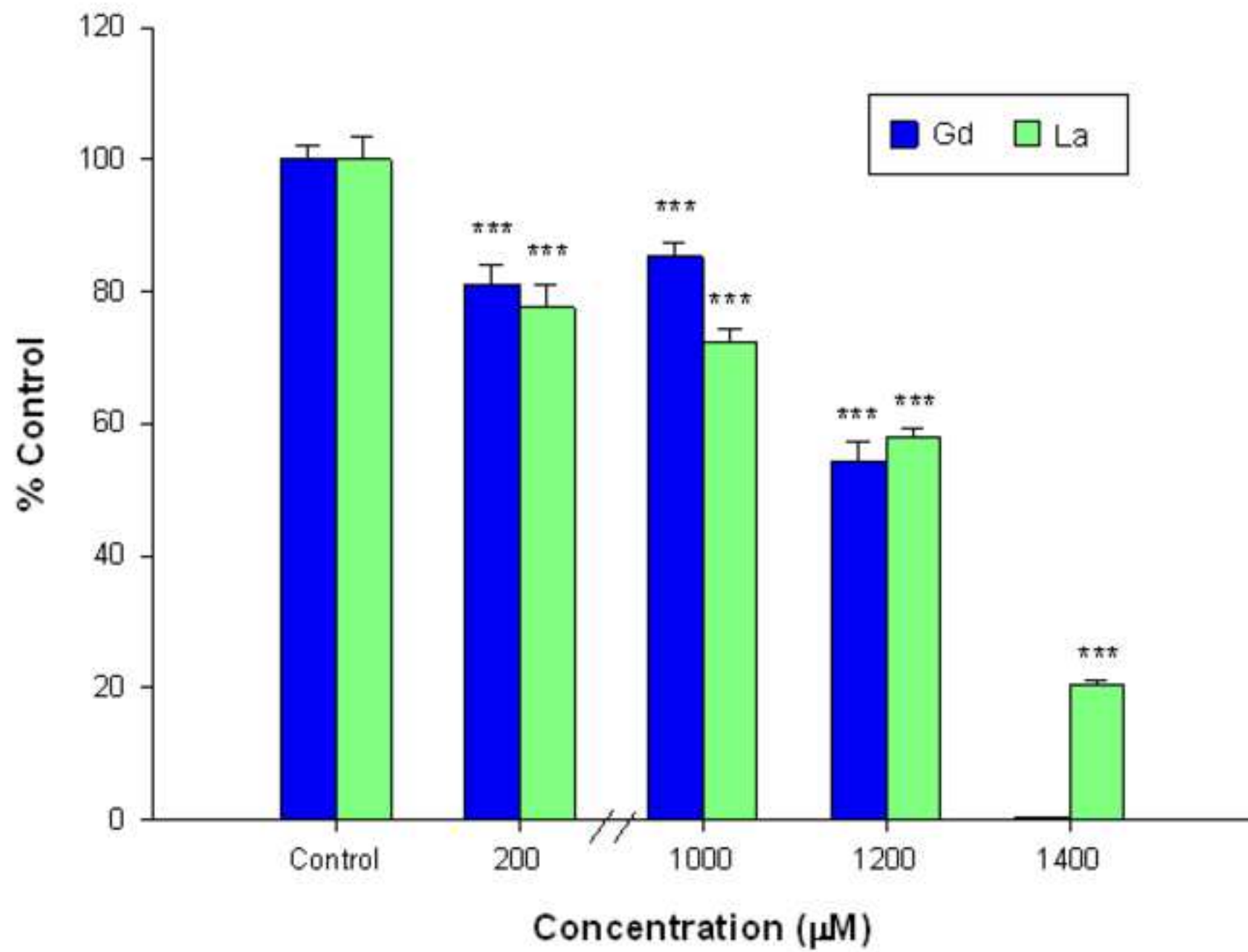


Figure 6

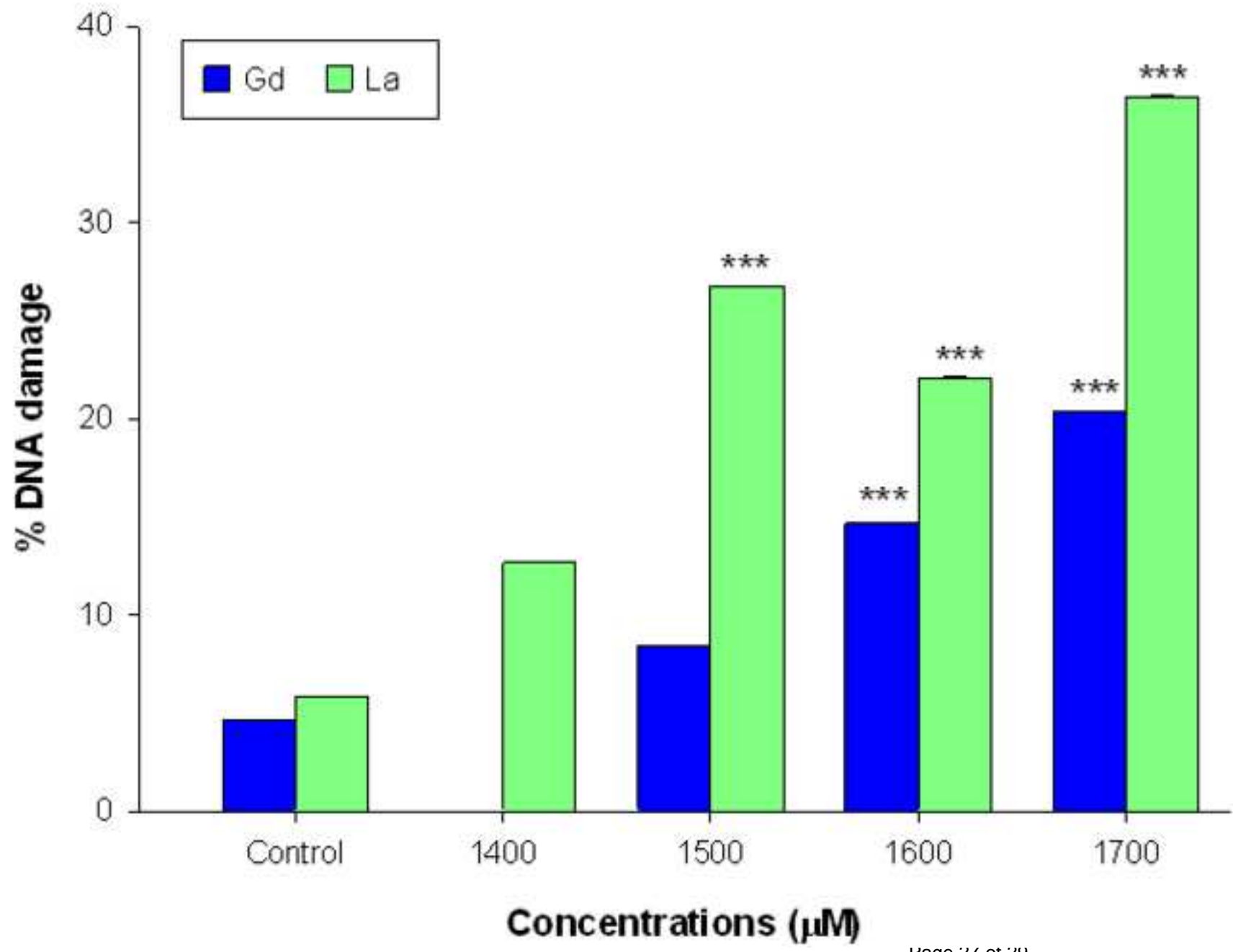


Figure 7

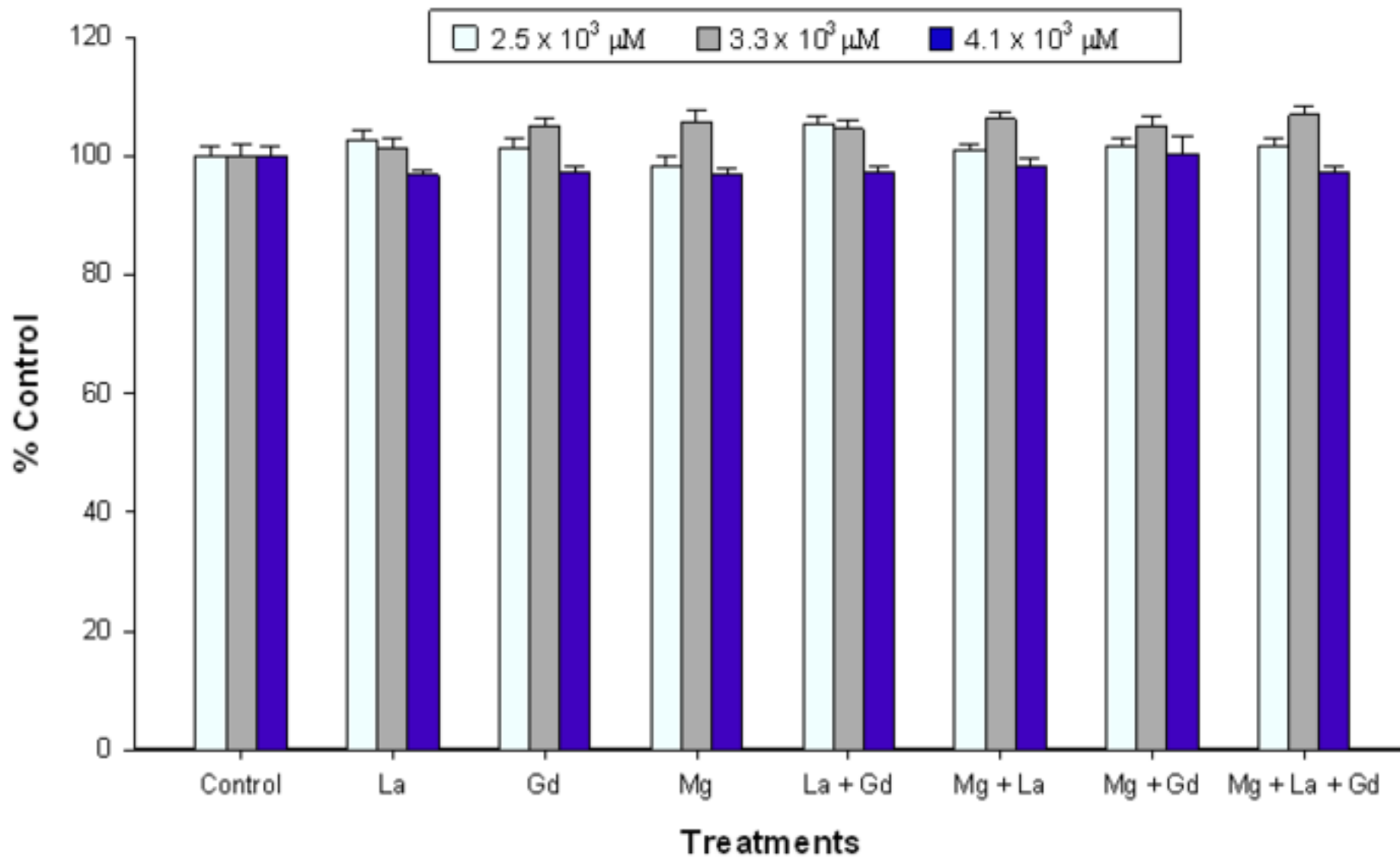


Figure 8

