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Evaluation of short term effects of rare earth and other elements used in magnesium alloys on primary cells and cell lines

Frank Feyerabend[a], Janine Fischer[a], Jakob Holtz[a], Frank Witte[c,d], Regine Willumeit[a], Heiko Drücker[e], Carla Vogt[e], Norbert Hort[b]

[a] GKSS Research Centre, Institute for Materials Research, Department of Structural Research on Macromolecules, Max-Planck-Str. 1, D - 21502 Geesthacht, Germany

[b] GKSS Research Centre, Institute for Materials Research, MagIC - Magnesium Innovation Center, Max-Planck-Str. 1, D - 21502 Geesthacht, Germany

[c] Laboratory for Biomechanics and Biomaterials, Hannover Medical School, Anna-von Borries-Str. 1-7, 30625 Hannover, Germany

[d] Department of Biotechnology and Bioengineering, Ben-Gurion University of the Negev, Beer Sheva, Israel

[e] Institute for Inorganic Chemistry, Leibniz University of Hannover, Callinstr. 9, 30167 Hannover, Germany

Correspondence:
Dr. Frank Feyerabend, GKSS Research Centre, Institute for Material Research, Department of Macromolecular Structure Research, Max-Planck-Str. 1, 21502 Geesthacht, Germany.
Phone: 0049 4152 87 1291/1259, Fax: 0049 4152 87 1356
email: frank.feyerabend@gkss.de

Abstract

Degradable magnesium alloys for biomedical application are on the verge of being used clinically. Rare earth elements (REE) are used to improve the mechanical properties of the alloys, however in more or less undefined mixtures. Therefore in this study the *in vitro* cytotoxicity of the elements yttrium (Y), neodymium (Nd), dysprosium (Dy), praseodymium (Pr), gadolinium (Gd), lanthanum (La), cerium (Ce), europium (Eu), lithium (Li), zirconium (Zr), was evaluated by incubation with the chlorides (10-2000 µM), magnesium (Mg) and calcium (Ca) were tested at higher concentrations (200 and 50 mM, respectively). The influence on viability of human osteosarcoma cell line MG-63, human umbilical cord perivascular (HUCPV) cells and mouse macrophages (RAW 264.7) was determined, as well as the induction of apoptosis and the expression of inflammatory factors (TNF-α, IL-1α). Significant differences between the applied cells could be observed. RAW exhibited the highest and HUCPV the lowest sensitivity. La and Ce showed the highest cytotoxicity of the analysed elements. From the elements with high solubility in magnesium alloys Gd and Dy seem to be more suitable than Y. The focus of magnesium alloy development for biomedical applications should include most defined alloy compositions with well known tissue specific and systemic effects.

Keywords

Magnesium; Rare earth elements; in vitro; toxicity; inflammatory response

Introduction

For biomedical applications there is a focus on tailored magnesium alloys with adjustable corrosion rates and suitable mechanical properties [1-3]. The most advanced clinical applications are biodegradable cardiovascular magnesium stents. They were successfully tested in animals [4] and first clinical human trials have been conducted [5-8]. Magnesium alloys were also investigated for orthopaedic applications. They can be applied in various designs e.g. as screws, plates or other fixture devices. Open-porous scaffolds made of magnesium alloys were introduced as load bearing biomaterials for tissue engineering [9,10]. Moreover, elevated extracellular magnesium concentrations have been shown to be beneficial for cartilage tissue engineering [11].

Most of the successful reports on *in vivo* application of magnesium implants are investigating magnesium alloys that contain rare earth elements. Especially, magnesium alloys for magnesium stents (WE43; W=Yttrium, E=rare earths), are containing rare earth element (REE) mixtures based on neodymium or cerium as hardeners. However, commercially available hardeners are not very well defined mixtures of almost any rare earth element which can also contain traces of impurities such as iron or manganese [12]. The contents of different elements of a neodymium based hardener are depicted in table 1.

Other interesting elements besides the REE are lithium (Li), zirconium (Zr) and calcium (Ca). Lithium enhances ductility and formability of magnesium alloys by changing the lattice structure from hexagonal close-packed to body-centered cubic. It is used in the aluminium-containing alloy LAE442 [13,14]. Zirconium is an effective grain refining agent in Al free magnesium alloys. It contributes to strengthening due to the formation of fine grains by grain boundary strengthening. Calcium improves the alloy's strength and creep resistance and acts as grain refining agent [12]. Moreover, it

decreases the corrosion rate in trace amounts and is well tolerated in the human body since it is an essential cation. Therefore the addition of Ca should not lead to adverse reactions during material degradation.

REE are defined as a group of seventeen elements [15]. These elements are chemically classified by their ionic radii in three groups: (I) light REE from La to Pr, (II) medium REE from Nd to Gd, and (III) heavy REE from Tb to Lu. Their ionic radii are close to that of calcium, therefore all of these elements can act as calcium anatagonist [16-18]. REE in magnesium alloying are predominantly used for strengthening and to improve corrosion resistance [19]. There are two groups: elements with (I) high solid solubilities (Y, Gd, Tb, Dy, Ho, Er, Tm, Yb and Lu) and (II) limited solubilities (Nd, La, Ce, Pr, Sm and Eu) [20]. During alloy production some amount of the REE is kept in solid solution and therefore can strengthen the material by solid solution strengthening. Additionally, all REE can form complex intermetallic phases with e.g. Al or Mg. These intermetallic phases are pinning grain boundaries, can act as obstacles for the dislocation movement at elevated temperatures and cause precipitation strengthening. REE with limited solubility are forming intermetallic phases during early solidification, arresting grain boundaries at elevated temperatures and contribute to strength. The original field of REE application is in transportation industries where they are used to improve creep and corrosion resistance and to increase the materials service temperature. Nearly all of the investigated magnesium alloys were developed for transportation application and not for biomedical use [12]. For some REE there are already clinical applications: neodymium is used in magnetic prosthetic devices [21], lanthanum is administered as a phosphate binder (“Fosrenol”) in chronic renal failure [22] and gadolinium based contrast agents are widely used in Magnetic resonance imaging (MRI) [23]. Additionally, evidence is rising that many REE exhibit anti-cancerogenic properties [24-

[27]. Therefore, magnesium alloys containing REE could be of particular interest for bone cancer treatments. Magnesium implants containing REE could provide multiple functions in one material: It could stabilize the bony defect after cancer surgery temporarily until the bony defect is healed and simultaneously it could prevent growth of remaining cancer cells.

However, during corrosion of implanted magnesium alloys there will be a release of the alloying elements which may induce toxic reactions dependent on the locally released element concentration or on systemic accumulation. Still for some of these REE such as dysprosium the available cytotoxicity data are sparse. Therefore, the aim of our study was to determine the effects of rare earth and other elements used in magnesium alloys for application in the skeletal system on a human bone cell line, mesenchymal stem cells derived from the umbilical cord and macrophages. Another aim was to compare the reaction of cell lines and primary cells.

Material and Methods

Analysis of element composition of WE43 alloy

WE43, a commercially available magnesium alloy (Magnesium Electron, Manchester, UK) was used to establish a precise method for the analysis of its elemental composition. About 1.5 mm thick discs of the alloy with an edge length of 1 cm were cut off cubes, their surfaces were polished with SiC abrasive paper followed by 3 minutes treatment with an ultrasonic bath in ultrapure water. The samples with a mass of about 0.25 g were then dissolved completely in a solution of about 5 ml ultrapure water to which ten times the sample weight of subboiled HNO_3 (65 wt%) was added dropwise within a day to avoid the strong reaction normally taking place with concentrated HNO_3. These solutions were diluted prior to the analysis with ICP-OES (Spectroflame and Spectro Arcos, Spectro Analytical Instruments, Kleve,

Germany) by a factor of 2000. For each element signals from two lines were measured and the results were averaged from background corrected intensities. Criteria for the chosen lines were freedom of interferences from other emission lines besides a good fitting sensitivity at the analysed concentration. The measurements were performed using a modified bracketing procedure, although the drift of the instrument did not exceed 1.5 % during the day and therefore a drift correction was not necessarily required. Recovery rates determined from standards containing all elements under investigation were found to be between 99.2 and 100.4 wt%, which demonstrates the high precision obtained with the described method.

Preparation of solutions of tested elements

The chlorides of the elements yttrium (Y), neodymium (Nd), dysprosium (Dy), praseodymium (Pr), gadolinium (Gd), lanthanum (La), cerium (Ce), europium (Eu), lithium (Li), zirconium (Zr), magnesium (Mg) and calcium (Ca) were obtained from Sigma-Aldrich-Chemie, Taufkirchen, Germany. Due to solvability reasons Zr was obtained as zirconium oxychloride. All chlorides were dissolved in distilled water at a concentration of 1 M and sterile filtered. By dilution the final concentrations from 10 µM to 2 mM (in case of calcium and magnesium up to 50 and 200 mM, respectively) were obtained.

Isolation and culture of cells

MG-63 cells

The human osteosarcoma cell line MG-63 was obtained from the European collection of cell cultures (ECACC, Salisbury, UK). The cells were cultured under standard cell culture conditions (37°C, 5% CO_2, 95% rH) in Dulbecco's modified eagle medium (DMEM) Glutamax-I (Invitrogen Corporation, Karlsruhe, Germany) with 10% fetal bovine serum (FBS, PAA Laboratories, Linz, Austria). Cells were passaged at

subconfluency (70 – 80%) and reseeded in a density of 2 x 10^4 cells/cm^2. For cell culture experiments cells after the 5th passage were used.

RAW 264.7 Cells

The tumour-derived mouse macrophage cell line (RAW 264.7, ECACC, Salisbury, UK) is capable of producing cytokines like nitric oxide (NO) and tumor necrosis factor α (TNF-α) as an *in vitro* inflammatory response. Cells were cultured in DMEM low glucose with 2 mM glutamine and 10% FBS and passaged at 60-70% confluence. Cells starting from the 5th passage were used for experiments.

Human umbilical cord perivascular cells (HUCPV)

Mesenchymal stem cells derived from Wharton`s jelly of the umbilical cord were isolated by a modified isolation protocol from Sarugaser et al. [28]. Isolation was approved by the local ethical committee. In brief, umbilical cords from consenting full-term caesarean section patients were cut into pieces of about 5 cm. The vessels from the cord pieces were isolated and tied together at the ends with sutures, leading to a vessel loop. These were placed in T-175 cell culture flasks and cultured for 10 days without medium change in α-MEM (Invitrogen Corporation, Karlsruhe, Germany) with 10% FBS for mesenchymal stem cells (Stem Cell Technologies, Vancouver, Canada). After visible outgrowth from the loops medium was changed every 2-3 days. At about 60% confluency the cells were harvested with a cell scraper and subcultivated in a density of 1000 cells/cm^2. For the experiments cells of the third to fifth passage were used.

Biochemical analysis

MTT-assay for metabolic activity

Cell viability was analysed by MTT (methylthiazolyldiphenyl-tetrazolium bromide) assay. In brief, cells were cultured as described in 96-well plates in 100 µL of the cell-type specific medium. 10 µL of the MTT-solution (5 mg/mL MTT in phosphate

buffered saline PBS) was added. After an incubation period of 5 hours the cells were lysed and the formazan crystals solubilized by adding 1 mL solubilization solution (10% SDS in 0.01M HCl; Merck, Darmstadt, Germany), followed by an incubation overnight in a humidified atmosphere (37° C, 5% CO_2). The solubilized formazan product was photometrically quantified using an ELISA reader (Tecan Sunrise, TECAN Deutschland GmbH, Crailsheim, Germany) at 570 nm with a reference wavelength of 655 nm. The same experiments were performed without cells to exclude an influence of the salt concentrations on the MTT-assay.

Tumour necrosis factor α (TNF-α) determination

Levels of TNF-α were measured with a sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturers' instructions (R&D Systems, Wiesbaden, Germany). Briefly, 50 µL of standard, control, or sample were added per well in a 96-well plate. The samples were incubated for 2 h at room temperature, followed by two rinses in washing buffer to remove unbound substances. Then, 100 µL of an enzyme-linked polyclonal antibody were added to each well and incubated for 2 h at room temperature. After a second washing step, 100 µL of substrate solution was added to each well and then incubated in the dark for 30 min. The optical density of each well was determined using an ELISA reader at 450 nm with a reference wavelength of 570 nm. The sample concentrations were determined by comparison with a standard curve. All samples were assayed in triplicate.

Quantitative analysis of Interleukin 1 alpha (IL-1α)

Concentrations of IL-1α were determined by ELISA (R&D Systems, Wiesbaden, Germany). In brief, 200 µL of standard, control, or sample were added per well in a 96-well plate and incubated for 2 h at room temperature, followed by three rinses in washing buffer to remove unbound substances. Then, 200 µL of an enzyme-linked polyclonal antibody were added to each well and incubated for 1 h at room

temperature. After a second washing step, 200 µL of substrate solution was added to each well and then incubated in the dark for 20 min. The optical density of each well was determined using an ELISA reader at 450 nm with a reference wavelength of 570 nm. The sample concentrations were determined by comparison with a standard curve. All samples were assayed in triplicate.

Cytotoxicity assay

To evaluate possible cytotoxic effect of the tested elements, a cytotoxicity assay was performed. RAW 264.7, MG-63 or HUCPV cells were seeded in 96-well plates (Nunc, Wiesbaden, Germany) in a density of 2 x 10^3 per well/100 µL and incubated at 37°C for 24 hours to allow cell adhesion. Thereafter, concentrations in the given ranges of the different elements were added to the wells. After another 48 hours incubation time, cell viability was measured by MTT. For each concentration 16 replicates were performed. LPS (10 ng/mL) served as negative control for the RAW cell line, tissue culture plastic (TCP) was used as positive control. Experiments were repeated at least three times.

To determine significant influences of the added elements on the different cell types two markers were used. On one hand the half-lethal dose (LD_{50}) was used as a measure for the concentration capable of killing half of the seeded cells. On the other hand the initial inhibitory concentration (IIC) was calculated, which is derived from the statistical analysis (ANOVA vs. control group, Dunn's or Dunnet's post hoc test, see section Statistics). This value indicates the first significant negative deviation from the viability data obtained from the untreated control group.

In vitro inflammatory response of RAW 264.7 macrophages

The determination of inflammatory reaction was analysed by the cytokine secretion of RAW 264.7 mouse macrophages. Therefore, 5 x 10^4 cells were seeded on 24-well plates (about 26.000 cells/cm^2) and allowed to adhere for 12 hours. Then the

chlorides of the elements were added at a concentration of 500 and 1000 µM and incubated for 48 h at 37°C. After this incubation period the medium was completely removed and used for the analysis of tumour necrosis alpha and interleukin 1 alpha (see below). The medium was replaced and the MTT-test was performed according to the protocol to obtain cell viability data. LPS was used as positive control, cells on TCP served as negative control or standard.

Apoptosis assay

Apoptosis of MG63 cells was measured by the cell death detection ELISA (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Untreated cells served as negative control to allow calculation of the enrichment factor of mono- and oligonucleosomes released into the cytoplasm. 2 x 10^5 MG63 cells were seeded in 6-well plates and allowed to adhere for 24 h. Then different concentrations (500, 1000, 1500 µM) of the elements Ce, Gd, Nd, Pr and Eu were added. La was administered at 250, 500 and 1000 µM, while Mg was supplemented at 10, 25 and 50 mM.

Statistical analysis

Statistics were performed using the SigmaStat package (Systat software GmbH, Erkrath, Germany). For the analysis of dose effects stepwise regression analysis was performed. Standard analysis comparing more than two treatments was done by using the one-way ANOVA. Depending on the data distribution either a one-way ANOVA or an ANOVA on ranks was performed. Post-hoc tests were Holm-Sidak or Dunn's versus the control group, respectively. Statistical values are indicated at the relevant experiments.

Results

Alloy composition of WE43 alloy

The elemental composition of a commercially available WE43 (Magnesium Electron, Manchester, UK) measured by ICP-OES is given in table 2. The rare earth compound (E, 3 wt%) of this alloy is mainly made up of Nd, Gd and Dy. Traces of other rare earths were observable to a minor extend below 0.1 wt%. The amount of all REE sums up to about 3.4 wt%. The measured concentration of Ca was an unexpected finding, and can not be regarded as impurity.

Cell reaction to elements

Magnesium and Calcium

As a first attempt to examine differences in reaction of the different cell types magnesium chloride was used. Concentrations from 1 mM up to 200 mM were tested and viability of the cells was calculated as percent of control (n=16 for each concentration). Different cell behaviour was observable: LD_{50} were determined to be at 50 mM for MG63 and between 50 and 60 mM for RAW cells, while the stem cells exhibited the highest tolerance with a LD_{50} value at 80 mM (figure 1**Error! Reference source not found.**).

Moreover, the dose response was different with regard to the deployed cells. RAW showed a typical sigmoidal curve progression (R^2=0.9722, p<0.001), whereas MG63 followed a nearly linear dependency up to complete cell death at 100 mM $MgCl_2$ (R^2=0.9877, p<0.001). HUCPV showed a plateau after an initial drop in viability and the decrease in viability started at higher concentrations than observed at the other cell types (figure 2).

Differences in cellular reactions were also observed for calcium, which was tested in the concentration range up to 50 mM. RAW showed a decrease in viability starting at 10 mM, at 50 mM less than half of the cells survived (43.587±4.096). The same trend was observed for MG63 with a higher viability at the end concentration (50 mM: 62.287±6.14), while HUCPV levelled at about 80% viability over the whole concentration range (50 mM: 78.713±3.45; figure 3). Regarding these results, RAW seems to be the most sensitive cell type when subjected to higher salt concentrations.

Zirconium and Lithium

HUCPV exposed to zirconium showed a slightly reduced viability over the whole concentration range (mean viability between 88.1 and 98.8%). The reduction was significant at the concentrations 20, 40, 70-90 and 800-2000 µM (ANOVA, $p<0.05$). Such a reduction was not observed for the other cell types. In the tested concentration range lithium did not have an influence on cell viability in any cell type. The analysis of the inflammatory response showed an intermediate production of IL-1α (figure 5) and no increase in TNF- α (figure 6).

Rare earth elements

The analysed rare earth elements (REE) except Y belonged to three different groups: (I) light REE containing La, Ce and Pr, (II) medium REE containing the elements Nd, Eu, Gd and (III) heavy REE containing Dy.

The viability data for the different cell types are summarized in table 3. As an overall finding it can be summarized:

1. the sensitivity of the different cell types was the same as observed for the other elements with RAW macrophages being the most and stem cells the least sensitive and

2. light rare earth elements showed cytotoxic effects already at lower concentrations than medium or heavy rare earths.

Lanthanum and cerium showed the earliest onset of toxic effects. One remarkable effect in MG63 was the steep decrease in viability in the millimolar range. While for all elements except cerium and lanthanum the viability at 1 mM was statistically still in the range of the control, viability at 2 mM was dramatically decreased to values below 25 % (figure 4). This clear cut between 1 and 2 mM was not observable in the other cell types. RAW showed in most cases typical dose response curves with a sigmoidal slope, whereas HUCPV cells did not even reach the LD_{50} dose for most of the elements. This effect is shown exemplarily in figure 5, where the differences in viability of cells exposed to neodymium are illustrated.

In vitro inflammatory response

The selected assay for TNF-α showed already in the control on TCP a basic level of TNF-alpha production around 400 pg/mL. Levels of the analysed elements differed to both directions. An increase was observed for La (1 mM), Pr and Nd, while Ce, Dy, Eu and Zr showed decreased levels compared to the control (figure 5a). Due to different viabilities the data were normalized by calculating the relation between the TNF-alpha production and the viability data. Compared to the control these data showed that at a concentration of 500 µM elevated levels were observed for La, Pr, Nd and Y; Gd showed elevated levels at a concentration of 1 mM. The lowest influence was measured for Ce, Eu and Dy. However, it should be noted that the normalized TNF-alpha production of La at 1 mM even exceeded the observed production induced by LPS (figure 5b).

Based on the TNF-alpha results a higher probability of Interleukin 1-alpha secretion as late inflammatory marker was determined to be at higher concentrations (1 mM), except for La with its higher impact on cell viability (tested concentration: 500 µM). The secretion of interleukin 1-alpha was low for all analysed elements compared to LPS (80 pg/mL). The highest concentrations were observed for Y and La, followed by Zr, Li, Dy and Gd. Ce and Eu induced nearly no secretion of IL-1α. Lanthanum therefore has to be classified as critical element, followed by praseodymium and neodymium.

Induction of Apoptosis

For the measurement of apoptosis MG63 cells were used. For magnesium there was a decrease in the enrichment factor measurable compared to the untreated cell control at 10 and 25 mM, while the addition of 50 mM Mg led to an enrichment factor comparable to the control (data not shown).

Due to its higher toxicity lanthanum was measured at reduced concentrations (250, 500 and 1000 µM). From the tested rare earth elements Ce, Gd, Nd and Eu showed a concentration dependent increase in apoptosis, while La and Pr showed a decrease in the enrichment factor. The most pronounced induction of apoptosis was induced by Gadolinium followed by neodymium, while the other elements only showed a moderate increase (figure 7).

Discussion

The presented work was performed to clarify two important questions in *in vitro* analyses:

I. are there profound differences between the use of cell lines of different origin and primary cells and

II. are *in vitro* analyses suitable to allow recommendations for targeted magnesium alloy development.

The experimental results shows that the first point is crucial for *in vitro* testing. The used cell types in this study were of different species origin (RAW: mouse, MG-63 and HUCPV: human) and of different cellular state – MG-63 as a cancer derived cell line and HUCPV as primary mesenchymal cell type with high osteogenic differentiation potential [28,29]. The use of the latter is intended to avoid time consuming isolation of primary bone cells [30] which moreover does not yield sufficient cell numbers for the applied test system. The species origin for *in vitro* experiments is limited by the availability of suitable cell lines regarding the culture conditions, in this case adherent cell types. The test system should be designed to simulate the *in vivo* environment of the actual implant as good as possible. However, also in *in vivo* studies surprising results can occur, as the i.v. administration of Eu in lower doses in rabbits leads to spontaneous death with in a short time (pers. Comm. Witte). All available toxicity data on Eu after intravenously administration were obtained from experiments with rats. Our experiments have shown that rats can cope a ten-times higher concentration of dissolved europium chloride than rabbits, which are dying on cardiatic arhythms and general bleeding.

From the analyses of Mg and Ca it can be stated that RAW 264.7 are the most sensitive cells for the influence of salts and HUCPV tended to be the most robust cellular assay system. Similar results were obtained in a previous study, where primary osteoblasts showed a lower susceptibility to La compared to MG63 cells [31]. However, in the long run magnesium and its alloys suitable for orthopaedic applications should be tested in a non-static co-culture of primary osteoblasts and osteoclasts.

Magnesium concentrations in the applied concentration range seem to be exaggerated, as the normal serum content of magnesium levels at about 1.5 to 1.7 mM - but this only accounts for ionic Mg^{2+} [32]. The total Mg^{2+} content of a single cell can accumulate up to 20 mM [33]. Moreover one key element of magnesium regulation is bone tissue and in contrast to stent applications, where a continuous blood flow is prevalent, the bony environment enhances the possibility of ion accumulation. The critical range of extracellular magnesium concentrations in this study starts between 10 and 20 mM (figure 1). Thus, this concentration range of released magnesium ions may be a threshold for the effects of degrading magnesium alloys. Further studies examining the influence of such concentrations on the intracellular magnesium are currently performed.

Besides the REE as additional elements calcium, lithium and zirconium were included in the analyses. Calcium was not only chosen due to its appearance in the WE43 analysis (table 2), but also as important and essential cation with even higher involvement in cellular processes than magnesium. In magnesium alloying Ca belongs to the elements with low solid solubility and therefore the amount of possible Ca addition is limited to 0.82 wt% [20]. The observed calcium amount in the WE43 alloy (0.3 wt.%) can not be regarded as accidentally at hand or as impurity. In this amount the Ca can stay in solid solution and can improve mechanical properties at room temperature [19]. It also may influence the degradation in a positive manner, but this assumption needs further detailed investigations. The tested concentrations are exceeding the possible release by far and were taken more with the intention to serve as a second comparison of the cellular reaction of the different cell types. It can also

be regarded as a validation of the test systems, as the viability data indicate a similar trend for magnesium and calcium in the applied concentrations.

Lithium and zirconium were included in this study because of their importance for magnesium alloy development [12]. However, in the analysed concentration ranges, there were no apparent influences. Both elements have a long term clinical use and are therefore not discussed in detail.

This study did not have the aim to completely cover all REE. The analysis of WE43 (table 2) was used as a guideline for element selection. The threshold for inclusion to analyses was set to 0.1 wt%. La and Eu were additionally included because of paralleling studies. The calculation of the salts in the molar ratio was chosen to ensure comparable equimolar addition of the investigated cations to the assays. For instance, if anhydrous $PrCl_3$ (molecular weight 247.24 g/mol) was used to produce a stock solution, this would result in a molarity of 404.5 µM for 100 µg/mL, while the heptahydrate (MW 373.77 g/mol) addition sums up to 267.5 µM. Therefore the applied method should be suitable for the comparison of cation amounts of different elements. However, there is a major drawback of the test system: at higher concentrations (mostly starting at 800 µM) precipitation of the salts as mainly insoluble phosphates occurs [34], therefore the theoretically applied concentrations could be different from the actual values. However, the transition from the micromolar to the millimolar concentration range was only crucial for MG63 cells subjected to REE except Ce and La (table 3). While the viability at 1 mM was still high, at 2 mM it was drastically decreased (> 25 % viability, figure 4). In contrast, HUCPV cells tested with the same elements did not even reach the LD_{50} at the 2 mM concentrations and RAW cells showed more typical sigmoidal dose responses (figure 5). This may be

attributed to the fact that the precipitating REE products have a direct effect on cells of cancerous origin. Different studies have shown such effects of La and Ce [24] and Ce, La, Nd, Gd, Sm and Dy [26,35] on leukemic cell lines. General effects of Motefaxin gadolinium on cancer cells [27] were observed as well as the activity of a lanthanum containing compound against more than 60 tumor models [36].

REE with high solid solubility included in this study were Y, Gd and Dy. Yttrium exhibited effects on viability of macrophages and an accompanying influence on the inflammatory reaction, whereas the cellular reaction to Gd and Dy was comparably low, with a slight advantage for Dy. Own studies which compared the cell reaction to WE43 and AZ91D (9 wt% aluminium, 1 wt% zinc) already showed, that in terms of cell viability WE43 is favourable to aluminium containing alloys such as AZ or AM [37]. WE43 as stent material is already in clinical application, but there are hints that the expected effects are not as prominent as anticipated [38]. From the material aspect, the combination of zirconium and yttrium as zirconia ceramic for femoral heads led to catastrophic failure of several hundred implants in 2001 and induced a discussion about the future of such biomaterials [39]. However, later studies with modified materials have revealed positive effects [40,41].

Gadolinium showed better tolerability for RAW and HUCPV, and the production of inflammatory markers was lower than observed for yttrium. In a study applying intraperitoneal administration of $GdCl_3$ the LD_{50} for rats was 550 mg/kg. For the oral administration no acute toxicity was observed and only the liver of male rats was affected [42]. The toxicity can be drastically reduced by chelating agents such as EDTA, and therefore Gd-Chelates as contrast agent for magnetic resonance imaging (MRI) were introduced in the 1980's. In recent years there is rising evidence that these substances induce contrast agent induced nephropathy [23,43]. However, other *in*

vitro studies with different cell types such as smooth muscle cells [38] or a different type of administration (gadolinium containing phosphatidylserine lipososmes tested on RAW macrophages [44]) have shown consistent results regarding the influence of gadolinium on cell viability.

From the analysed elements with high solubility dysprosium was tolerated best, but the least studies on toxicity are available. LD_{50} in mice was determined at 585 mg/kg [45]. Dy exhibits antimicrobial effects at a concentration of 300 µM [46]. Parallel to Gd Dy is also used as contrast medium for MRI [47]. The effects of Dy and Y *in vivo* are similar: with increasing doses the distribution of the elements in spleen and lungs increases, while the concentration in kidneys and blood decreases, hepatotoxicity was not observed [17].

REE with low solid solubility included in this study were Nd, La, Ce, Pr and Eu. Except Eu all elements showed higher influences on the cell reaction than elements with high solid solubility. The results showed the largest influence of Ce and La on viability and inflammatory response, followed by Pr and Nd. Especially the light REE La, Ce and Pr are known to be hepatotoxic by inducing fatty liver [17,48]. Pr is the most toxic element leading to animal death in comparable concentrations used for Ce, probably due to its low clearing rate [17]. However, a pretreatment with low doses can prevent the lethal effect nearly complete [49]. All these elements have been shown to induce chromosome aberrations in the mouse *in vivo* [50]. The *in vitro* study of Palmer [51] where pulmonary macrophages were exposed to La, Ce and Nd showed similar results: Ce was highly cytotoxic (LD_{50}: 29 µM), followed by La (LD_{50}: 52 µM), whereas Nd showed only a slight reduction of viability. Eu was well tolerated in all analysed cells and its toxic effects are comparable to that of Y and Dy. The LD_{50} measured in rats *in vivo* was reached at 550 mg/kg [52]. Eu is used as fluorescent

marker designed for the cellular uptake and was tested in different cell types for this purpose without inducing major alterations [53-55].

For all elements with low solubility there is the indispensable need for further insights into the release by a degrading alloy and accumulation kinetics in the body. Strict precautions should be taken when such elements are supposed to be added to magnesium alloys. However, due to the low solubility the amount of the elements is restricted and therefore the concentrations needed to induce cytotoxic effects may not be reached during degradation. Further investigations of the degradation kinetics are needed to determine the amount of element release and more important the product characterization. There might be a huge difference in toxicity or systemic reactions between ionic release and precipitation as e.g. phosphates or hydroxides.

Taking all results into account, a “matrix” for the tested REE for high concentrations could be developed, which is summarized in table 4. At such high concentrations, La should be avoided and only Eu and Dy would be suitable. Due to the solid solubility only Dy could eventually reach the tested concentrations. *In vitro* analysis therefore seems to be suitable as an advisory tool for the development of alloy compositions for degradable magnesium alloys. However, further understanding and improvement of methods to correlate *in vitro* to *in vivo* results is urgently needed.

An improvement of research on magnesium alloys for biomedical applications could be reached by the avoidance of undefined mixtures of mainly REE with unspecified local or systemic influences. The scientific road should lead to most defined alloy compositions with well known tissue specific and systemic effects. The presented study therefore can be seen as a further building block for the generation of a

knowledge base for further development and understanding in the area of degradable magnesium alloys.

Conclusion

This study shows that the choice of the applied cells is crucial for the *in vitro* analysis of cellular reactions. The use of different cell types in parallel increases the informative value of the results. The effects of the analysed rare earth elements seem to related to their ionic radii. For magnesium alloy development the highly soluble Dy and Gd seem to be more suitable than Y. Suitable elements with low solid solubility could be Eu, Nd and Pr. If not avoidable La and Ce, should be used cautiously. In general, alloy development should target on most defined alloy compositions.

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Table 1: Contents of different rare earth elements and impurities (in italics) in different hardeners (Unit: mg/Kg).

Element	Nd-Hardener
Calcium (Ca)	**<200**
Cerium (Ce)	**<20**
Copper (Cu)	**<20**
Erbium (Er)	**<20**
Europium (Eu)	**<20**
Iron (Fe)	**790**
Gadolinium (Gd)	**<20**
Lanthanum (La)	**<20**
Manganese (Mn)	**23**
Neodymium (Nd)	**103000**
Nickel (Ni)	**<20**
Praseodymium (Pr)	**1250**
Sulphur (S)	**<10000**
Silicium (Si)	**<1000**
Samarium (Sm)	**<20**
Tin (Sn)	**<20**
Yttrium (Y)	**<20**
Ytterbium (Yb)	**<20**
Zinc (Zn)	**<20**
Zirkonium (Zr)	**<20**

Table 2: Concentrations of selected elements present in a commercially available WE43 magnesium alloy. Values are means of three independent measurements by ICP-OES. Elements analysed in this study are highlighted in grey.

Element	Mean concentration (wt %)
Magnesium (Mg)	92,458
Aluminium (Al)	0,031
Calcium (Ca)	0,301
Zinc (Zn)	0,007
Yttrium (Y)	3,690
Neodymium (Nd)	2,319
Lanthanum (La)	0,073
Samarium (Sm)	0,045
Gadolinium (Gd)	0,453
Terbium (Tb)	0,038
Dysprosium (Dy)	0,441
Holmium (Ho)	0,032
Erbium (Er)	0,080
Thulium (Tm)	0,007
Ytterbium (Yb)	0,007
Lutetium (Lu)	0,001

Table 3: Viability of macrophages, osteoblast-like cells and stem cells exposed to different rare earth elements up to concentrations of 2 mM. Given are the initial inhibitory concentration (IIC), which is described as the first statistically significant drop in viability compared to the control and the lethal dose 50 (LD_{50}). n/a = no effect in the measured concentration range.

	RAW264.7		**MG63**		**HUCPV**	
Element	**IIC / µM**	**LD_{50} / µM**	**IIC / µM**	**LD_{50} / µM**	**IIC / µM**	**LD_{50} / µM**
Yttrium	200	n/a	2000	>1000	2000	n/a
Light rare earths						
Lanthanum	50	>400	400	>500	500	>1000
Cerium	30	>1000	60	>1000	90	>1000
Praseodymium	600	>1000	2000	>1000	2000	n/a
Medium rare earths						
Neodymium	700	>1000	2000	>1000	2000	n/a
Europium	500	>1000	2000	>1000	n/a	n/a
Gadolinium	2000	>1000	2000	>1000	2000	n/a
Heavy rare earths						
Dysprosium	2000	>1000	2000	>1000	2000	n/a

Table 4: Summarized features of the tested rare earth elements. Positive influences (+), intermediate influences (+-), negative influences (-) and significant effects (--) on the applied test systems are indicated (n/a – no results for the apoptosis test). Additionally the LD_{50} values derived from literature are given. Elements with high solid solubility are highlighted in grey.

Element	Viability	Inflammatory response		Apoptosis	LD_{50} *in vivo* (Ref.)
		TNF-α	IL-1α		
La	--	--	-	+	150 mg/kg [34]
Ce	--	+	+	+-	10 mg/kg [56]
Gd	+	+-	+-	-	550 mg/kg [42]
Nd	+-	-	+	-	600 mg/kg [57]
Pr	+-	-	+	+-	600 mg/kg [57]
Eu	+	+	+	+-	550 mg/kg [52]
Dy	+	+	+-	n/a	585 mg/kg [45]
Y	+	-	-	n/a	n/a [58]

Figure captions

Figure 1: Dose response and viability determined by MTT of macrophages (RAW), osteosarcoma cells (MG63) and stem cells (HUCPV) subjected to increasing concentrations of magnesium chloride. Values are given as percent of control (tissue culture plastic, TCP). The concentration where half of the cells are dead (LD50) is indicated by the black line.

Figure 2: Regression analysis of the dose response of a) RAW, b) MG63 and c) HUCPV cells.

Figure 3: Viability of the different cell types subjected to increasing concentrations of calcium chloride. Data are represented as percent of control (TCP).

Figure 4: Viability calculated as percent of untreated cells of MG63 subjected to REE (except Ce and La) in concentrations up to 2000 µM. A steep decrease can be observed from 1000 to 2000 µM for all analysed elements. Shown are the means, standard deviations were omitted for the sake of clarity.

Figure 5: Concentration dependent dose response to exposure to $NdCl_3$ of RAW 264.7, MG63 and HUCPV. RAW show a sigmoid decrease in viability, whereas MG63 and HUCPV viabilities are fluctuating around 100 % up to concentrations of 1000 µM. The decrease at 2000 µM is significantly higher for MG63 cells.

Figure 6: Production of TNF-α by RAW 264.7 cells after incubation with different elements. In the upper graph the measured absolute concentrations are plotted, while in the lower graph the concentrations are normalized to the viability data determined by MTT. C = control.

Figure 7: Secretion of IL-1α by RAW 264.7 cells after incubation with different elements. Data were obtained from triplicate measurements; the control on TCP was below the detection limit and therefore set to zero. The values represent means and standard deviations.

Figure 8: Apoptotic enrichment factors of mono- and oligonucleosomes of MG63 cells after incubation with different elements. Data were obtained from triplicate measurements; data are normalized to the values of the control on TCP (factor 1).

Figure 1

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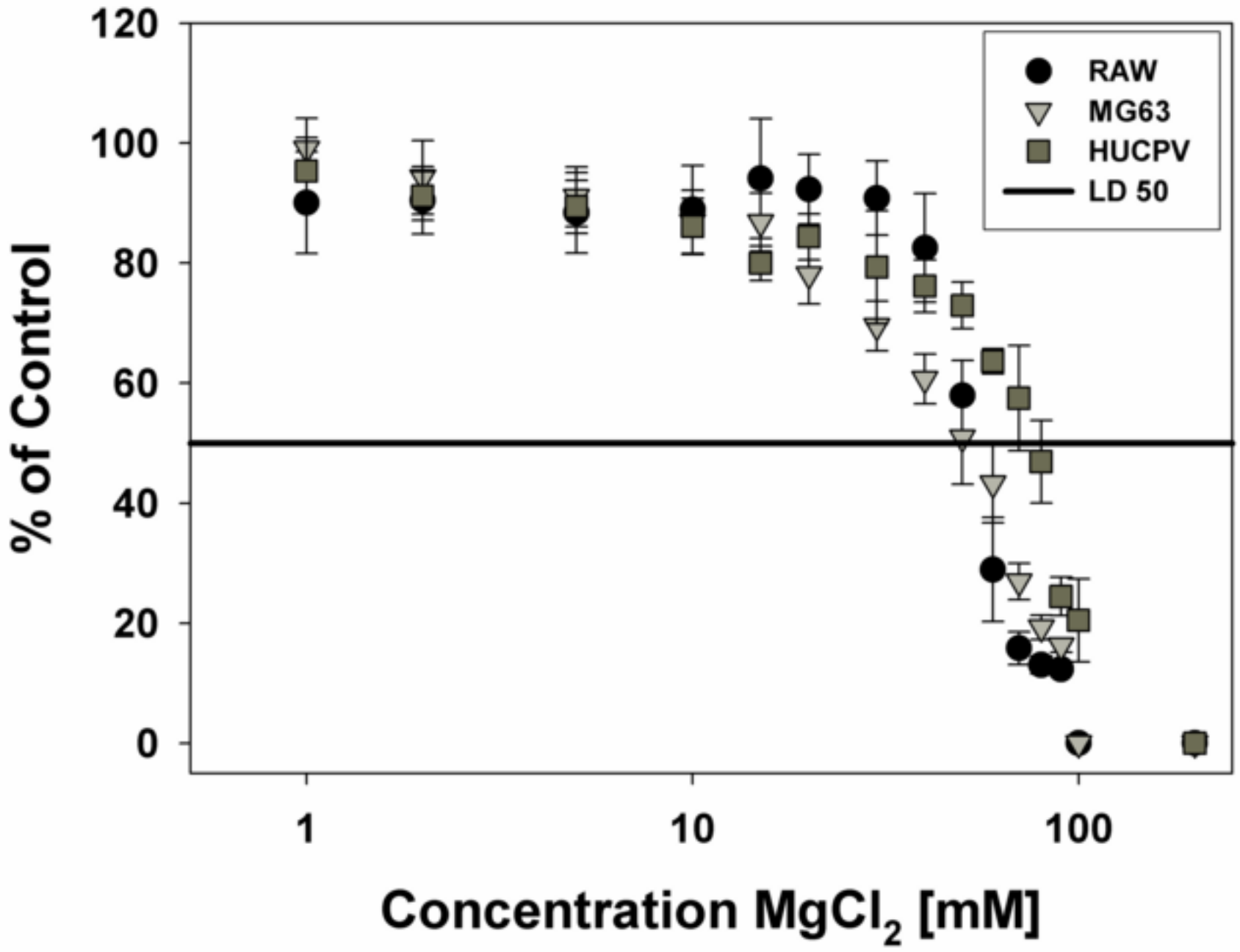

Figure 2a

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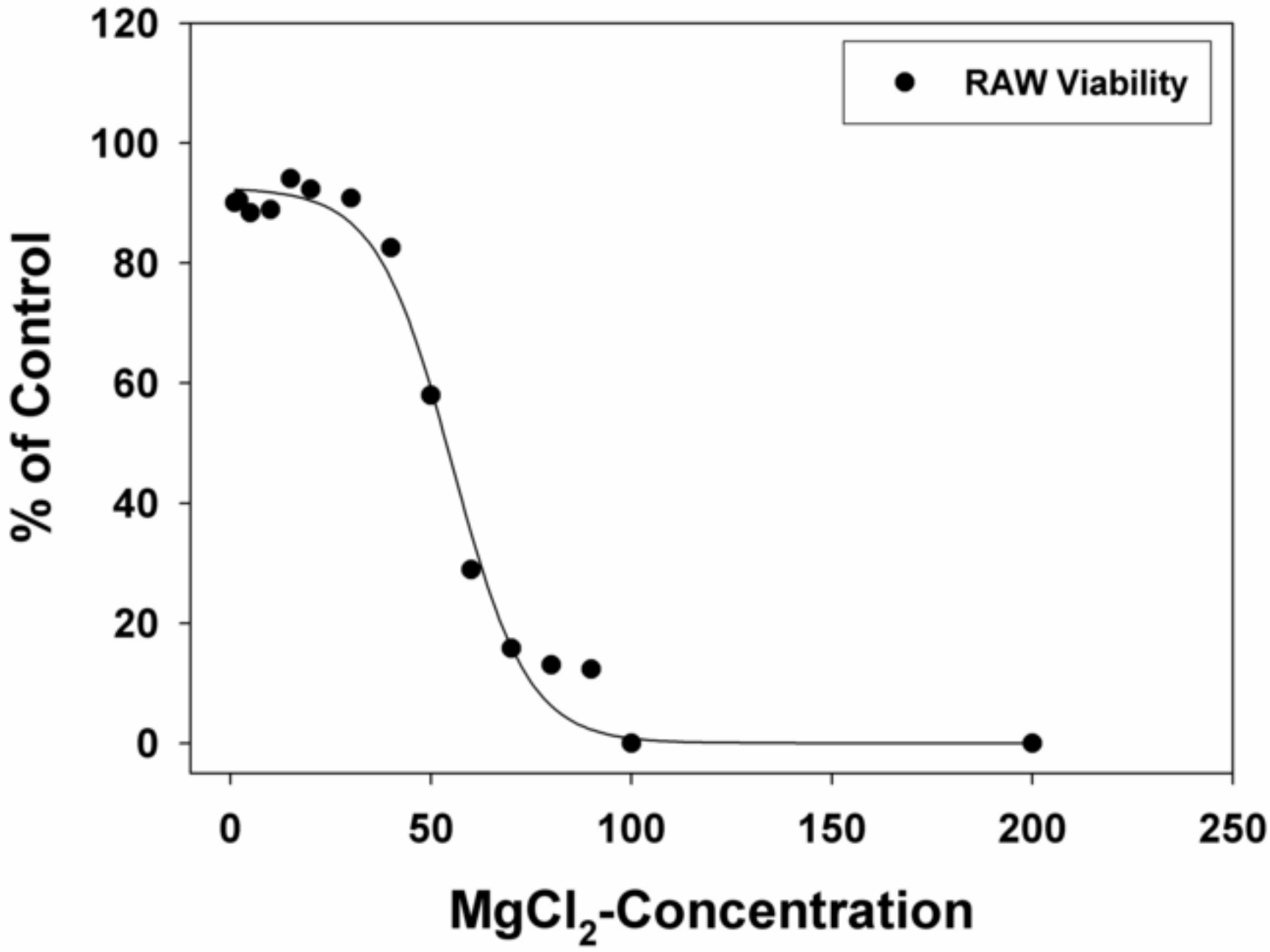

Figure 2b

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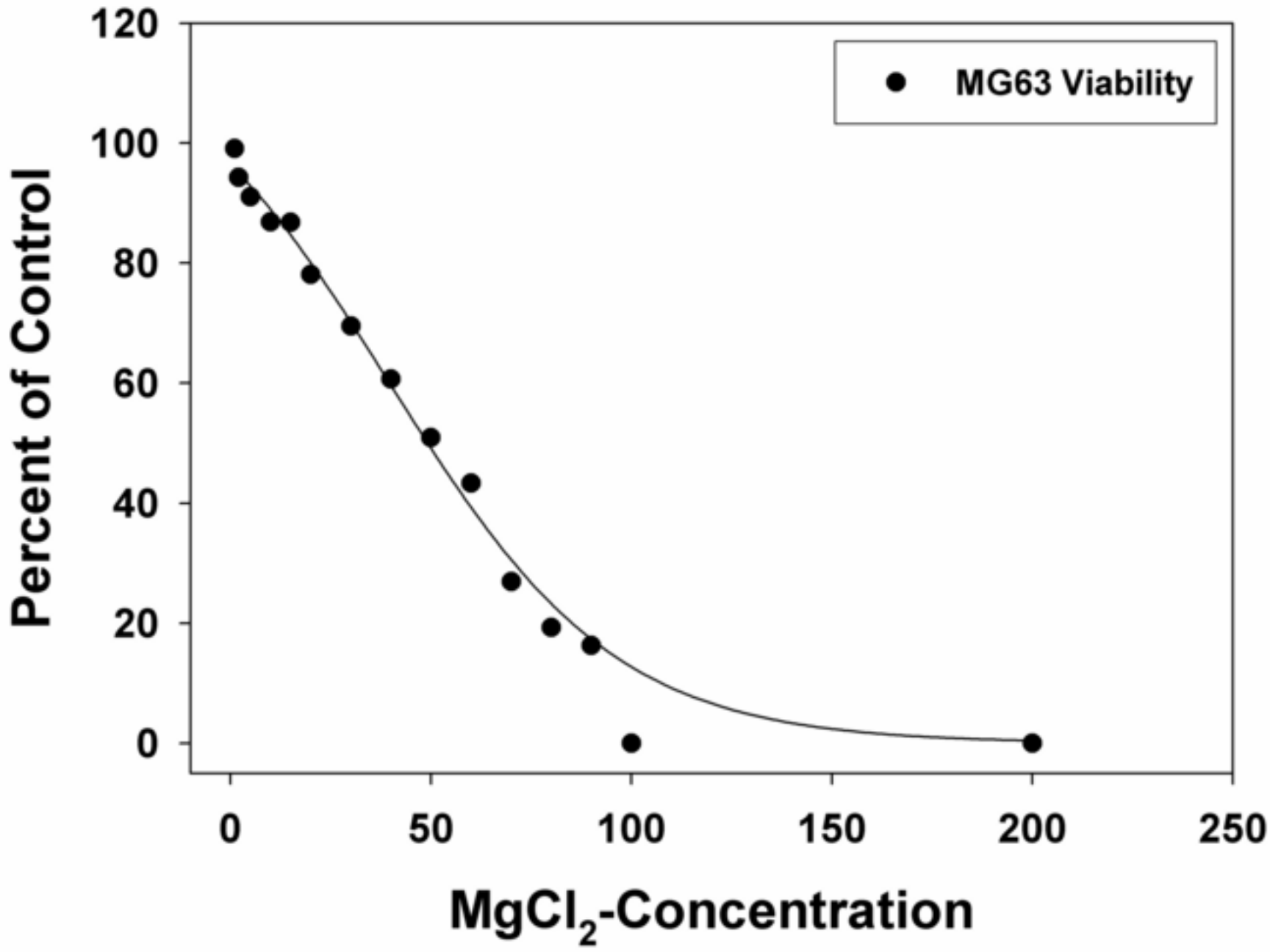

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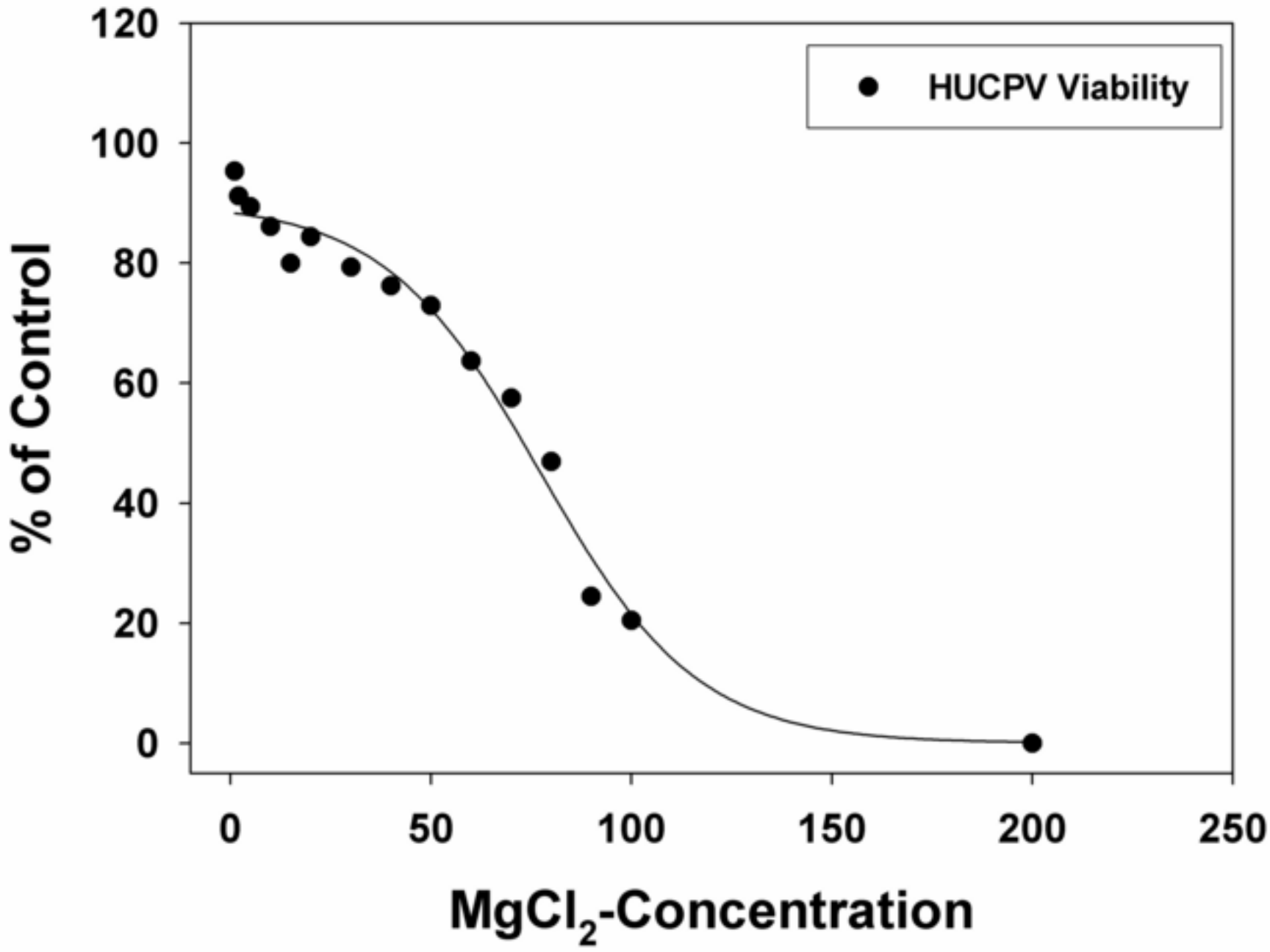

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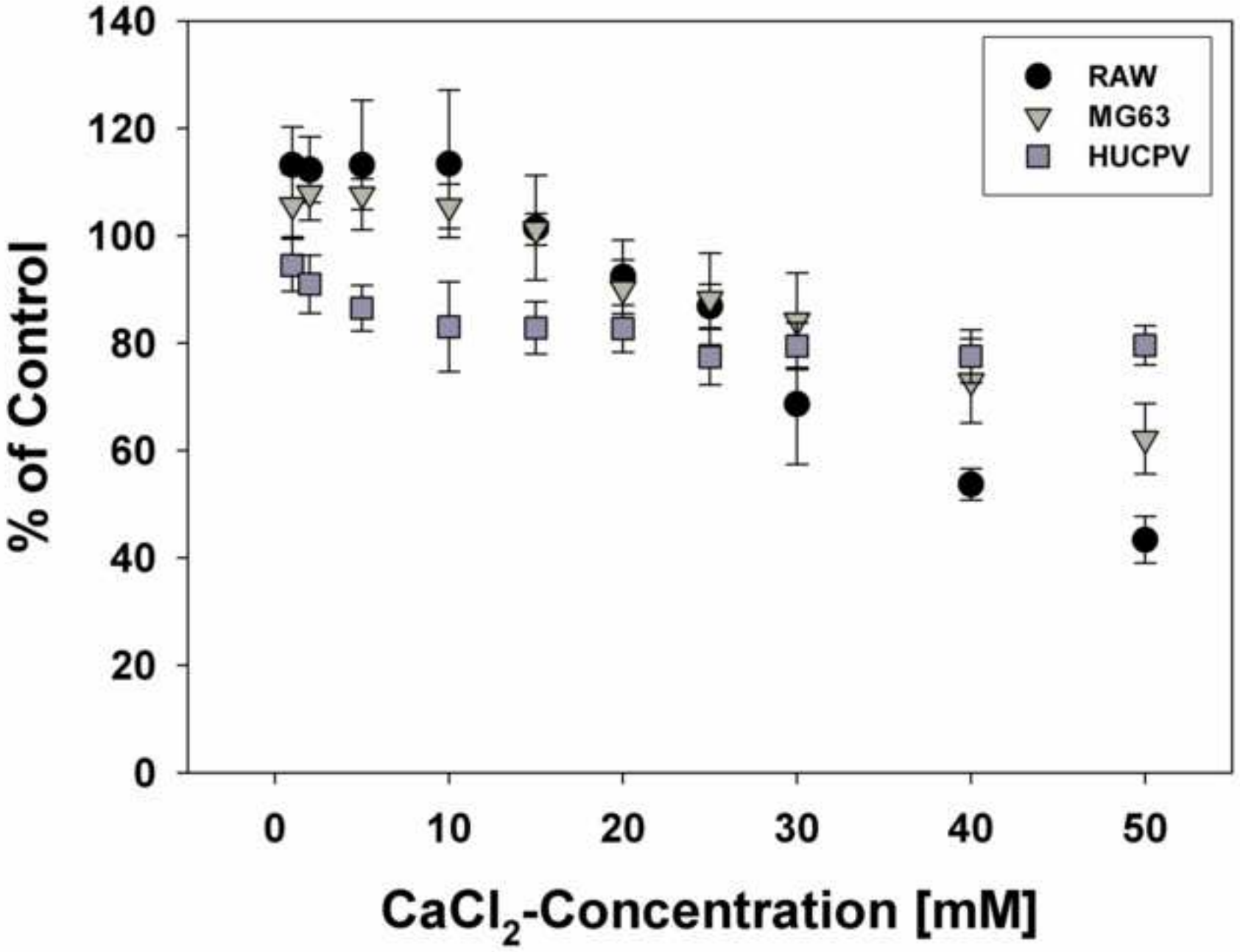

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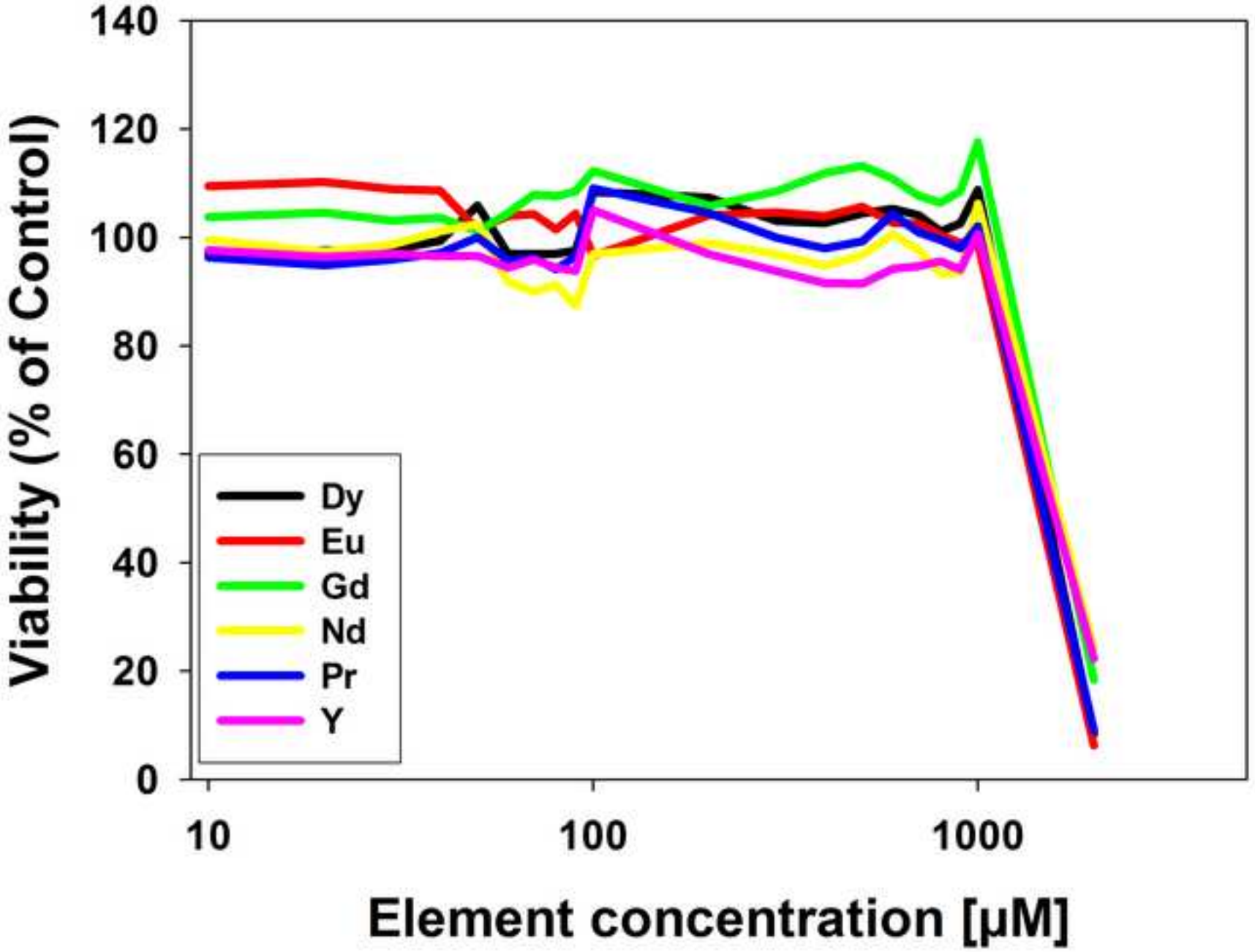

Figure 5a

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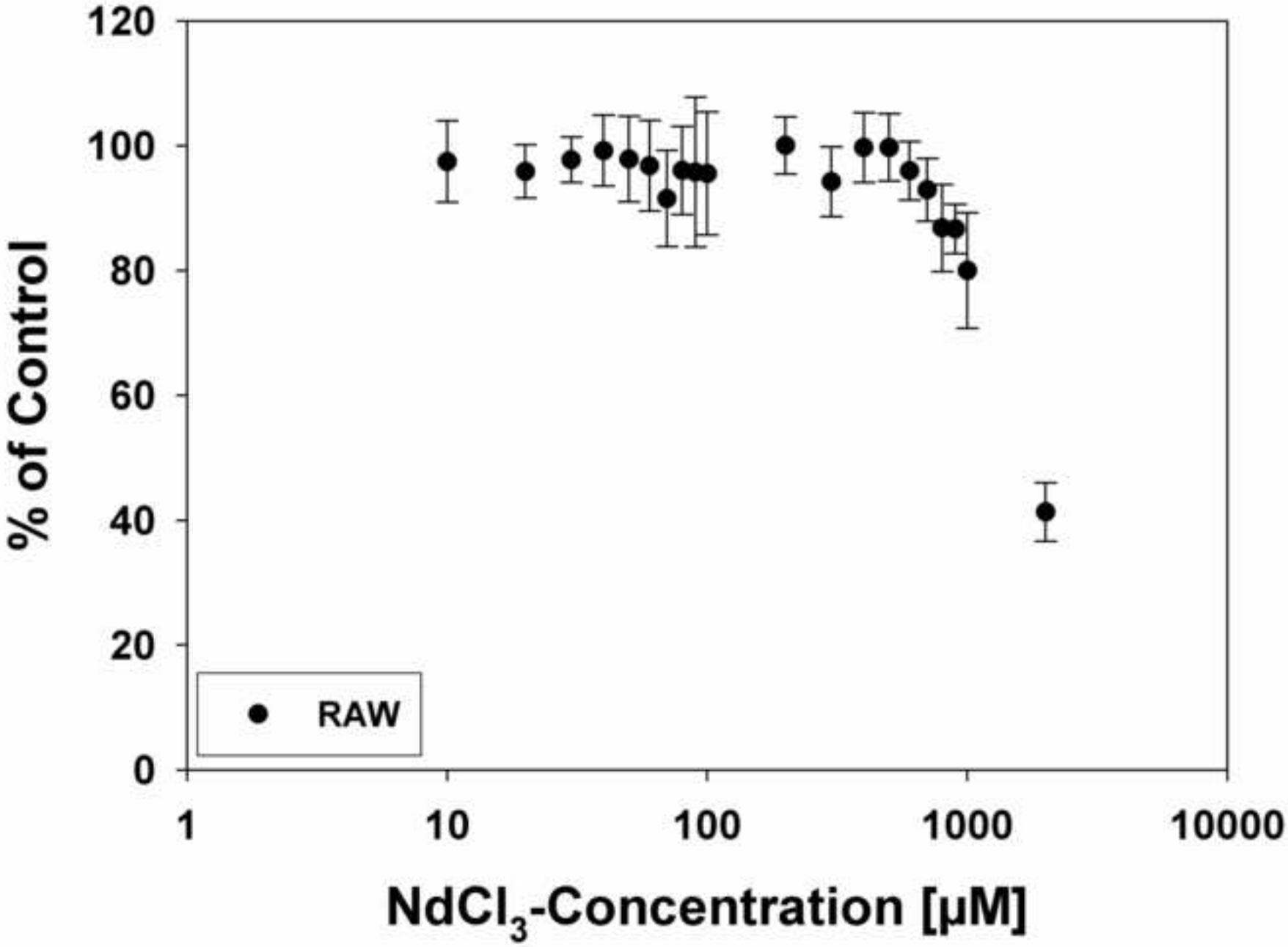

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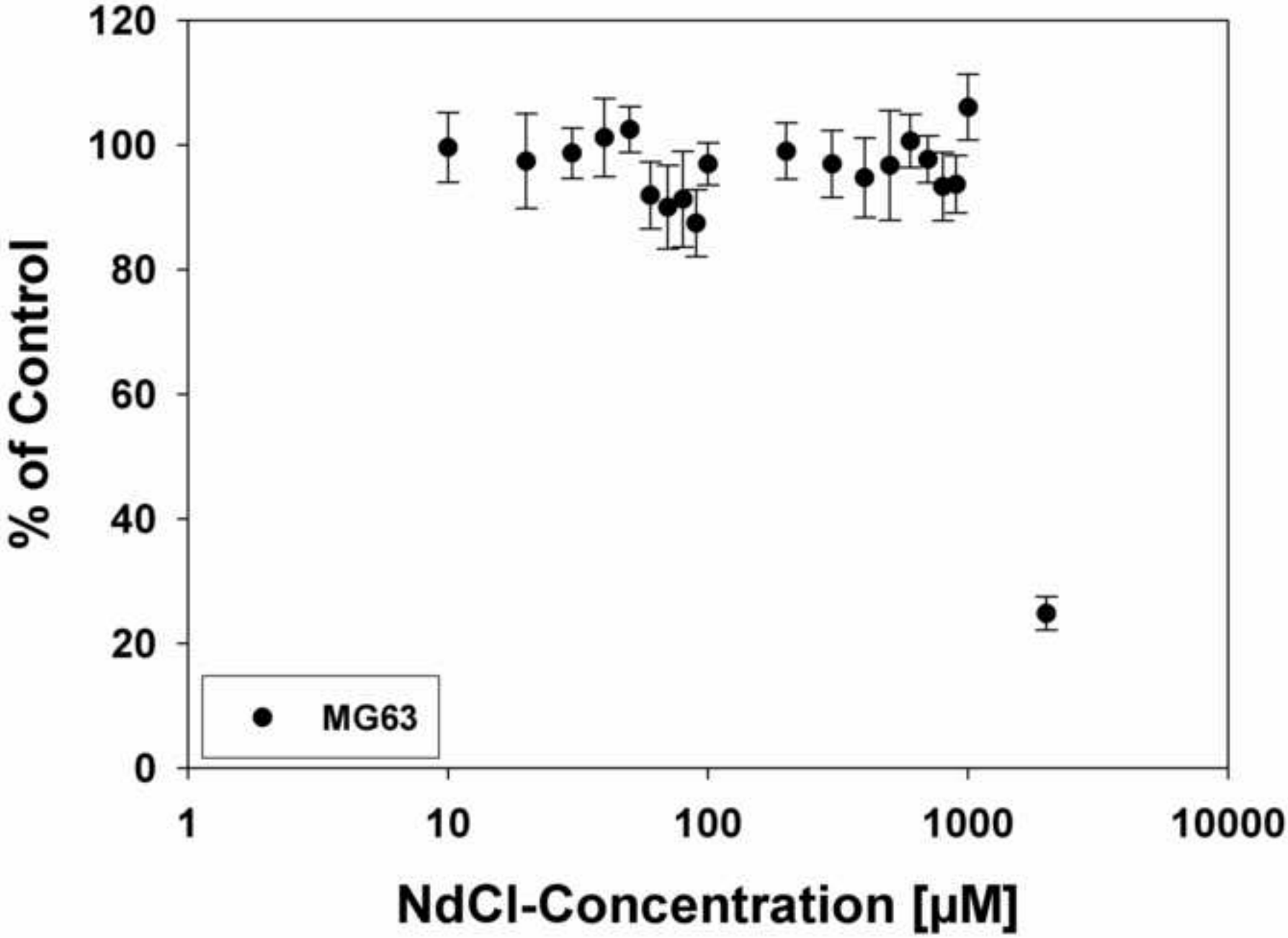

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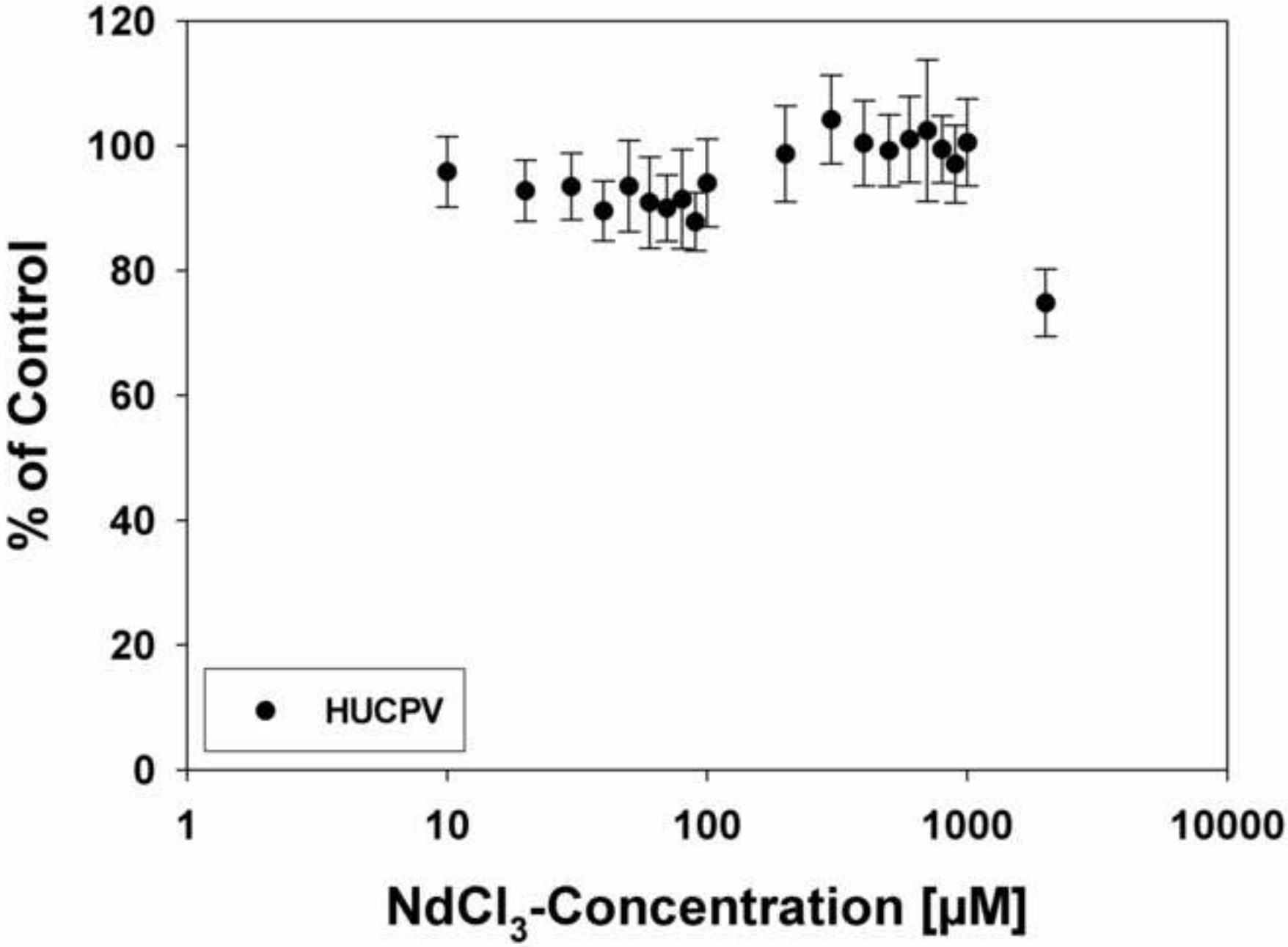

Figure 6a

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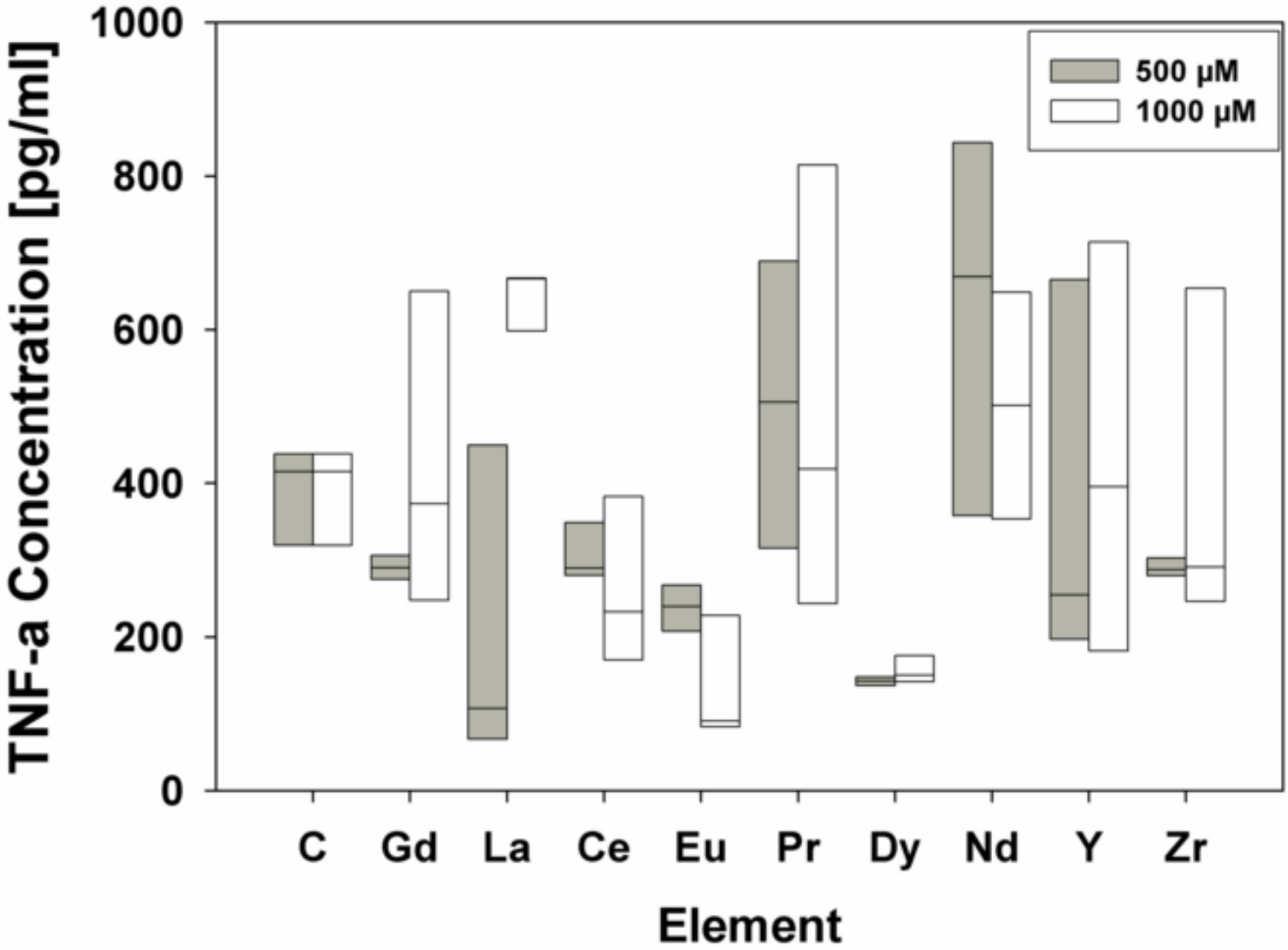

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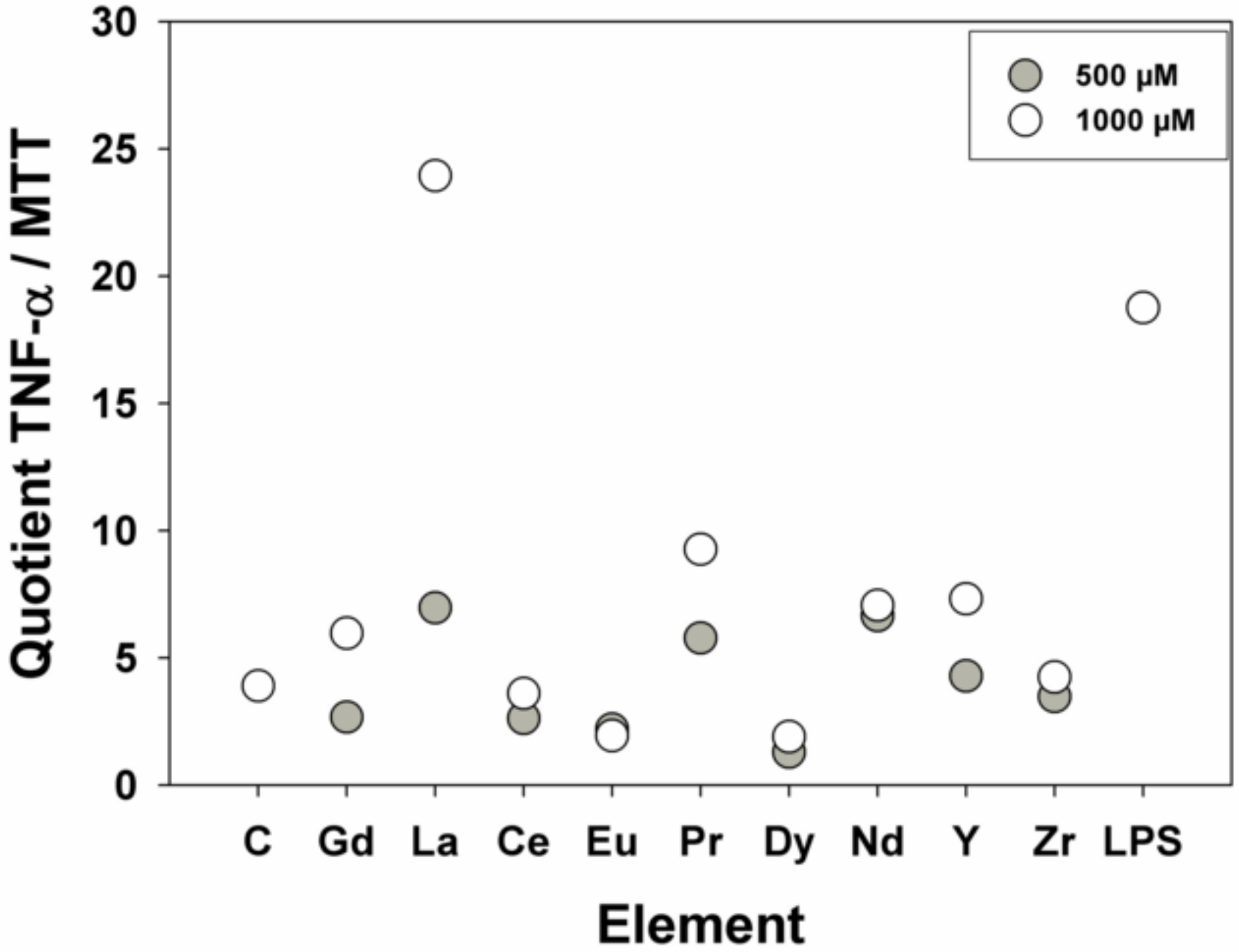

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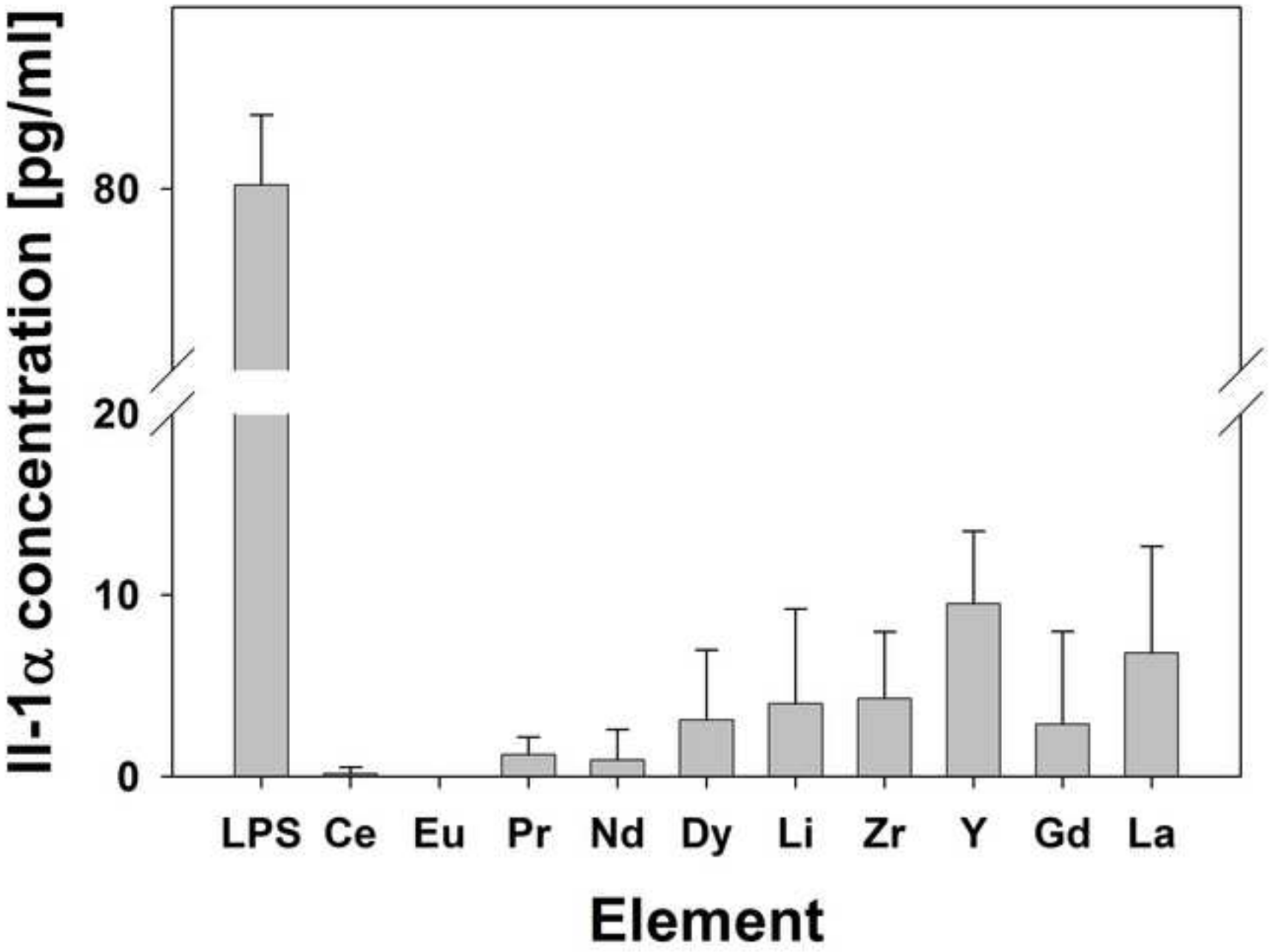

Figure 8

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