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Improved Cytotoxicity Testing of Magnesium Materials

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Abstract

Metallic Magnesium (Mg) and its alloys are highly suitable for medical applications as biocompatible and biodegradable implant materials. Magnesium has mechanical properties similar to bone, stimulates bone regeneration, is an essential non-toxic element for the human body and degrades completely within the body environment. In consequence, magnesium is a promising candidate as implant material for orthopaedic applications. Protocols using the guideline of current ISO standards should be carefully evaluated when applying them for the characterization of the cytotoxic potential of degradable magnesium materials. We recommend using 10 times more extraction medium than recommended by the ISO standards to obtain reasonable results for reliable cytotoxicity rankings of degradable materials *in vitro.* In addition primary isolated human osteoblasts or mesenchymal stem cells should be used to test magnesium materials.

Keywords
magnesium, implants, cytotoxicity tests, *in vitro*, ISO standards, osmolality

Introduction

Biodegradable materials are new promising materials for implant production. Magnesium- or iron-based materials and degradable polymers (PGA, PLA) have the potential to give implant stability for a needed healing period of time and degrade and dissolve within the human body after the target tissue is healed and the implant is not needed any more. Therefore a second surgery for the removal of the implant is not necessary and therefore no material is left in the body which could lead to possible inflammation or long term allergic reactions. Our aim was to develop an implant material for orthopaedic applications (e.g. screws, nails). Magnesium is most appropriate for orthopaedic application because it stimulates the bone regeneration and has similar mechanical properties as natural bone, which commonly used non-

degradable implant materials (e.g. stainless steel or titanium-based alloys) or degradable iron- and polymer-based materials do not have **[1]**. The often unpredictably corrosion rate of degradable magnesium materials represents a severe drawback of such implant materials, which requires the development of new alloy materials and processing routes to tailor the corrosion behaviour in an application specific manner **[2,3,4].** In order to test newly designed magnesium materials, the EN ISO standards 10993:5 and 10993:12 were applied **[5]**. These testing standards were originally developed for non-degradable materials. Therefore the application of these standards on degradable magnesium materials is problematic in several ways. A central point is the preparation of extracts from the materials leading to very high osmolalities and pH-values which kill the cells *in vitro* by osmotic shock. In addition the test system is static which means that it can not adopt to changes in the environment of a degradable implant material as the human body does. Some research groups avoid these challenges of *in vitro* tests by directly performing animal trials after materials development and mechanical characterisation of new magnesium materials. Due to ethical reasons and the need for a fast *in vitro* pre-screening for the different varieties of newly developed magnesium materials, establishment and optimization of reliable test systems is needed. In this study, the extract preparation and cytotoxicity tests of *in vitro* studies were done under physiological conditions to more accurately reflect the anticipated *in vivo* conditions. This means a temperature of 37°C, a relative humidity of 95 %, a CO_2 level of 5% and an O_2 level of 21%. In addition we used cell culture medium supplemented with serum to prepare the extracts. This is more realistic than salt solutions which are not suitable because they increase the corrosion process of magnesium based materials dramatically **[6,7,8]**. The aim of this study was to define the optimal extract preparation along the ISO standard. Furthermore we investigated the influence of magnesium extracts on an osteosarcoma cell line and on primary isolated human osteoblasts to evaluate and to underline the importance of using primary cells. This was crucial since cell lines are currently preferably used by many research groups because they are easier to obtain than primary cells from human patients.

Materials and Methods

Specimen preparation

Pure magnesium (99.95%), Magnesium with 0.6wt.-% Calcium (MgCa0.6) and Magnesium with 1w.t-% Calcium (MgCa1) alloys were prepared by permanent mould direct chill casting. Pure Mg and Mg0.6Ca have been molten under protective atmosphere (Ar+2% SF_6, 1 l/min) at a temperature of 750°C, the melting temperature of MgCa1 was 725 °C. . After adding the pure alloying elements the melt has been stirred for 15 min with 145 rpm prior to casting the materials into preheated moulds (600°C) made out of mild steel. All materials have been hold at a temperature of 690 °C for 30 min. The melts were subsequently solidified by dipping the mould into running water (15 °C) to obtain castings with the dimensions of 55 mm x 110 mm x 200 mm. Cylindrical specimens with a diameter of 10 mm and a height of 1.5 mm were cut from the cast blocks via electrical discharge machining.

Specimen sterilization

The specimens were sonificated for 20 min in 100% isopropanol (2-propanol, Merck; Darmstadt, Germany) and gamma-sterilized at the In Core Irradiation (ICI) facility of the Geesthacht neutron facility with a total dosage of 29 kGy.

Extract preparation

Magnesium alloy specimens were incubated in Dulbecco's modified eagle medium (DMEM) Glutamax-I (Invitrogen Corporation, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (FBS, PAA Laboratories GmbH, Linz, Austria) for 72 h under physiological conditions (5% CO_2, 21% O_2, 95% humidity, 37°C). The extract prepared according to the EN ISO standards 10993:5 and 10993:12 (relation of specimen weight to extraction medium is 0.2 g/L) is here called 1x. Less concentrated extracts were prepared using the same procedure but more extraction medium, e.g. for the preparation of the 2.5x extract 2.5 times more medium was used. The same procedure was applied for the other extracts (5x, 10x, 15x, 20x and 25x). For cytotoxicity testing, the extracts were sterile filtered using a 0.2 µm syringe filter.

Osmolality and magnesium concentration measurement

The osmolality of the extract was measured using a Gonotec 030-D cryoscopic osmometer (Gonotec, Berlin, Germany). The analyzed extract volume was 50 µL. For the quantitative determination of the magnesium concentration of the different extracts an Agilent 7500c collision cell inductively coupled plasma mass spectrometer (CC-ICP-MS, Agilent Technologies, Tokyo, Japan) was used. It features an octopole ion guide operated in an RF-only mode, which is used as collision/reaction cell with respect to reduce the abundance of interfering polyatomic ions such as $^{12}C^{12}C^{+}$ or $^{12}C^{14}N^{+}$. To reduce the background, especially on the main isotope ^{24}Mg, H_2 with an optimum flow-rate of 5 mL min^{-1} has been used during all experiments. In addition kinetic energy discrimination obtained by the settings of the octopole and quadrupole bias has been applied to further reduce the background on the main Mg isotope. Argon 5.0 (99.999 % purity) was used as plasma gas. Hydrogen 5.0 (99.999 % purity) was used as cell gas inside the octopole reaction system during all experiments. Both gases were obtained from Air Liquide (Air Liquide, Lübeck, Germany). A CETAC ASX 500 (CETAC, Omaha, Nebraska, USA) and a micro concentric nebuliser (PFA 100, Elemental Scientific, Omaha, Nebraska, USA) combined with the standard quartz double pass Scott spray chamber supplied with the ICP-MS instrumentation have been used for sample introduction. Solutions, standards and samples were prepared under clean room conditions (class 10000) inside a clean bench (class 100) to avoid contamination. Prior to the ICP-MS analysis, three aliquots of every sample were incubated with an internal standard (50 µg Rhodium) over night in 65% high purity subboiled nitric acid (Suprapur, Merck, Darmstadt, Germany) with respect to solubilise all sample constituents. Before the measurement all samples have been diluted 1/1000 using ultra pure water (18 MΩ cm) obtained from a Millipore Elix 3/Milli-Q element water purification system (Millipore, Milford, MA, USA). To obtain a good counting statistic, every sample was measured 8 times. The isotope ^{24}Mg and ^{25}Mg beside other elements were quantified using an external 6 point calibration based on a Merck ICP IV Multielement Standard (Merck, Darmstadt, Germany) which contains 23 different elements.

Cell culture

MG63

The human osteosarcoma cell line MG-63 was obtained from the European collection of cell cultures (ECACC, Salisbury, UK). The cells were cultured in Dulbecco's modified eagle medium (DMEM) Glutamax-I (Invitrogen Corporation, Karlsruhe, Germany) with 10% fetal bovine serum (FBS, PAA Laboratories, Linz, Austria) at 37°C, 21% O_2, 5% CO_2 and 95% humidity (hereafter referenced as cell culture conditions). Cells were passaged at about 80% confluency. For the experiments cells after the 5^{th} passage were used.

Isolation and culture of primary human osteoblasts

Osteoblasts were grown out of bone chips obtained from two patients (named OB 1 and OB 2 in this study) undergoing total hip arthroplasty following the protocol of Gallagher **[9]**. The isolation protocol was approved by the local ethic committee. In brief, cancellous bone was cut into pieces of about 5 mm. After removal of bone marrow and non-bone components and the bone chips were cultured in Dulbecco's modified eagle medium (DMEM) Glutamax-I (Invitrogen Corporation, Karlsruhe, Germany) with 10% fetal bovine serum (FBS, PAA Laboratories GmbH, Linz, Austria), 1% penicillin, and 100 lg/mL streptomycin (Invitrogen Corporation, Karlsruhe, Germany) for about 10 days without medium change. At visibility of outgrowing HBDC, medium was changed every three days. Passaging was done at 70–80% confluence. Experiments were performed with cells in the 2^{nd} passage.

Cytotoxicity test assay

MTT assay

Osteoblasts were seeded on 96-well-plates in a density of 5000 cells per well. MG63 cells were seeded with 2500 cell per well because they proliferate much faster than the osteoblasts. The cells were precultivated for 24 h under cell culture conditions to obtain optimal adherence. The cell culture medium was replaced with extracts and incubated for 72 h under cell culture conditions. The cytoxicity of the extracts was tested via MTT. Prior to the addition of the MTT substrate tetrazolium, all media and extracts were replaced with fresh cell culture medium in order to prevent any interference of the magnesium extract with the tetrazolium salt **[8]**. The MTT test was performed following a protocol published by Mosmann **[10]**. MTT (Thiazolyl Blue Tetrazolium Bromide (Sigma-Aldrich Chemie, Taufkirchen, Germany) was dissolved in Phosphate Buffered Saline (PBS) at 5 mg mL^{-1}. This MTT solution was added into the wells with a resulting dilution factor of 1:11. The cells were incubated for 4 h under cell culture conditions. In order to dissolve the formazan crystals, solubilisation solution (50 g sodiumdodecyl sulphate (Sigma-Aldrich Chemie, Taufkirchen, Germany) in 500 mL 0.01 M HCL) was added resulting in a dilution factor of 1:2 followed by incubation for 12 h. The acid in the solubilisation solution was used to change the colour of the indicator phenol red of the medium from red to white in order to prevent an interference with the measurement of the blue formazan. The formation of the blue formazan was quantified by an ELISA plate reader (Tecan Sunrise, TECAN Deutschland GmbH, Crailsheim, Germany) at a wavelength of 570 nm and a reference wavelength of 655 nm.

Statistical Analysis

The statistical correlation of the magnesium concentration to the osmolality of the different concentrated magnesium extracts was done using the SigmaPlot and SigmaStat package (Systat software GmbH, Erkrath, Germany). For the analysis of the correlation, a linear regression analysis was performed.

Results

Magnesium osmolality and concentration

During the extract preparation, the tested magnesium materials showed different corrosion behaviour. After the specimens were delivered into the extraction medium, for pure magnesium specimens immediately gas evolution was visible, whereas for both calcium containing alloys MgCa1 and MgCa0.6 this was not observed. This immediate and strong corrosion may have led locally to a very alkaline pH because the normally red medium was colourless after the extract preparation of the highest concentrated pure magnesium extract 1x (**Table 1**). This may be caused by the deprotonization of the indicator phenol red which becomes colourless at pH >14. All pink coloured and colourless extracts showed a pH higher than 8.00.

The magnesium concentration of pure (1x) and slightly diluted (2.5x) extracts were 84 times higher than that of cell culture medium (0.017 g L^{-1}) and the osmolality was 1.45 times higher. The magnesium content of the 5x extract was 34x higher and that of 10x extract 19 times higher than that of cell culture medium. The 15x, 20x and 25x extracts were comparable to cell culture medium in osmolality and the magnesium content was maximum 9 times higher (**Figure 1**).

The increase of osmolality was correlated to the increase of magnesium concentration in a linear order. A linear regression analysis was applied (regression equation $y = 9.7455\ x - 0.0393$; $R^2 = 0.9613$; $p<0.0001$) to evaluate the correlation between the increase of osmolality and the increase of magnesium concentration of different extracts (**Figure 2**). The increase was calculated relative to the osmolality and magnesium concentration of pure cell culture medium supplemented with 10% serum (Osmolality = 0.3385 Osmol/kg; magnesium concentration = 0.017169 g/L). Using this correlation the magnesium contents of the pure magnesium, MgCa1 and MgCa0.6 extracts used for the viability tests could be calculated by this equation from their measured osmolality (**Figure 3 a-c**).

Cell culture results

The cytotoxicity testing showed a higher tolerance of osteoblasts towards magnesium extracts compared to osteosarcoma cells MG63 (**Figure 3 a-c**). Despite of possible donor differences both primary osteoblast isolations exhibited a similar pattern. All three cell types showed a similar tolerance to the extracts regarding the type of magnesium alloy. Pure magnesium was the most toxic material. There were no cells detectable in case of the 1x and 2.5x extract. The alloys supplemented with calcium showed better results, however still no cells were alive when incubated with the MgCa1 1x extract. The MgCa0.6 1x was only tolerated by osteoblasts from one donor. Osteoblasts exposed to both 2.5x extracts showed metabolic activity compared to the control. MG63 showed a better tolerance for the 2.5x extract than for pure magnesium but the metabolic activity was still less than 25% compared to the control cells. The osteoblasts showed a metabolic activity of more than 90% in comparison with control cells for the MgCa1 and MgCa0.6 extract dilutions higher than 2.5x and for the pure magnesium extracts higher than 5x. In addition the MG63 cells showed good tolerance (more than 80%) for the MgCa1 and MgCa0.6 extract dilutions higher than 10x but they never exceeded 80% in the pure magnesium

extracts. As expected, the results of the cytotoxicity test were reversely correlated to the osmolality. The higher the osmolality of the extract, the higher was the cytotoxic potential. Due to the linear correlation of the increased magnesium concentration to the increased osmolality, the reverse correlation applied also to the magnesium concentration. The dotted lines show the lethal dosage 50 (LD50) which is the concentration needed to kill 50% of a used cell population. The arrow is pointing on the corresponding extract concentration. The LD50 of pure magnesium was between the 2.5x and the 5x extract dilution. In case of the MgCa1 extract the LD50 for MG63 cells was between 2.5x and 5x. The osteoblasts tolerated higher concentrated extracts. Here the LD50 was between the 1x and the 2.5x extract dilution. For the MgCa0.6 extracts the osteoblasts derived from donor OB 1 did not exceed an LD50 for the tested extract dilutions. Even in the highest concentrated extract, the cells showed a viability of 77%. The cytotoxicity of the MgCa0.6 extracts was similar to the MgCa1 extracts for the osteoblasts of donor OB 2 and the MG63 cells.

Discussion

The current ISO standards are developed for the testing of permanent implant materials and need to be carefully evaluated in their present form for the testing of degradable magnesium materials. In this study we analyzed the extract prepared according the ISO standards. The degradation process of magnesium materials led to a highly concentrated extract with a very high osmolality and magnesium concentration. In addition severe changes in pH, strong hydrogen production and the formation of several corrosion products depending on the surrounding medium can be observed. *In vivo* the human body regulates these changes in the local environment by active transport processes. Therefore *in vitro* a dynamic flow system such as a bioreactor would be best simulating the situation in the human body. However, if a flow system is not available, a dilution of the extract prepared according to ISO standards is recommended. In this study we analyzed the effect of different concentrated extracts to find a suitable concentration range which would show the influence of the extract on cells without killing them by osmotic shock. The osmolality, the magnesium concentration and the colour of the extract (shows the pH during extract preparation, indicated by the indicator phenol red of the extraction medium) can be used to characterize the different extract dilutions and to obtain parameters that can be used to distinguish the suitable extract dilution and to finally determine the cytotoxic potential of the extracts.

The cytotoxicity of seven different dilutions (1x, 2.5x, 5x, 10x, 15x, 20x, 25x) of extracts from three different magnesium materials (pure magnesium, MgCa1, MgCa0.6) was evaluated for primary isolated human osteoblasts and a human osteosarcoma cell line. Generally, the metabolic activity of cells exposed to magnesium extracts was higher for osteoblasts compared to MG63. This better tolerance for magnesium can also be seen in the osteoconductivity which was found in animal trails **[11, 12]**. Since MG63 cells are not capable to produce bone matrix, they are most likely not an adequate tool to be used for cytotoxicity testing of magnesium materials **[13]**. Therefore, we suggest to use human bone derived osteoblasts or mesenchymal stem cells from different sources as the most appropriate *in vitro* test system **[14].**

Using the extract prepared according to the EN ISO standards (1x) would lead to the result that the tested magnesium materials are highly cytotoxic (**Table 2**). Therefore diluted solutions should be used. To determine the right concentration one ideally takes into account the osmolality. From our measurements we can deduce that values below 0.4 Osmol/kg are tolerable. We therefore suggest – as a rule of thumb - to use a 10-fold diluted extract to still have a measurable effect of the extract which is not caused by osmotic pressure. We are aware that this rule of thumb is deduced from analysing only three materials. However as a starting point for further measurements it gives better results than the extract prepared according the ISO standard.

Finally it should be pointed out that coming closer to *in vivo* conditions some observations should be considered: Witte **[15]** showed that the *in vitro* corrosion of the tested magnesium material AZ91D and LAE441 were 4 orders of magnitude higher *in vitro* compared to *in vivo* by using a salt based solution. In our approach cell culture conditions were applied which change the corrosion environment dramatically **[6]**. The addition of serum to the extraction medium slows down the corrosion rate of magnesium materials **[7, 16]**. Therefore the whole ensemble of parameters: the right corrosion medium which includes proteins, the right concentration range, cell culture conditions and the use of primary cells should be combined for the elucidation of the biocompatibility of newly designed magnesium alloys.

Conclusion

On the base of the presented results we propose the following improvement of cytotoxicity testing of magnesium: Tests should be done with cell culture medium supplemented with 10% fetal bovine serum (containing albumin and globulin) under cell culture conditions. Protocols using the guideline of current ISO standards should be carefully evaluated by applying them for the characterization of the cytotoxic potential of degradable magnesium materials. We recommend using 10 times more extraction medium than recommended by the ISO standards to obtain reasonable results for reliable cytotoxicity rankings of degradable materials *in vitro.* MG63 or other cell lines may be used for screening purposes. Primary isolated human osteoblasts or mesenchymal stem cells should be used to finally compare magnesium materials due to their ability to produce bone matrix which is an important factor for the evaluation of suitable orthopaedic implant materials.

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Figure Captions:

Figure 1: Magnesium concentration and osmolality from magnesium material extracts.

Figure 2: Increase of osmolality of pure magnesium material extracts plotted against the increase of magnesium concentration. A linear regression was performed and showed a significant correlation between the increase of osmolality and the increase of magnesium concentration.

Figure 3 a-c: Osmolality and calculated magnesium concentration of the extracts. Cell viability of osteoblasts (OB 1, OB 2) and MG63 cells cultivated with extracts and then measured by MTT. Data are normalized to the control: cells cultivated with pure cell culture medium. The dotted lines show the lethal dosage 50 (LD50), the arrows point at the corresponding extract concentrations.

Figure 1

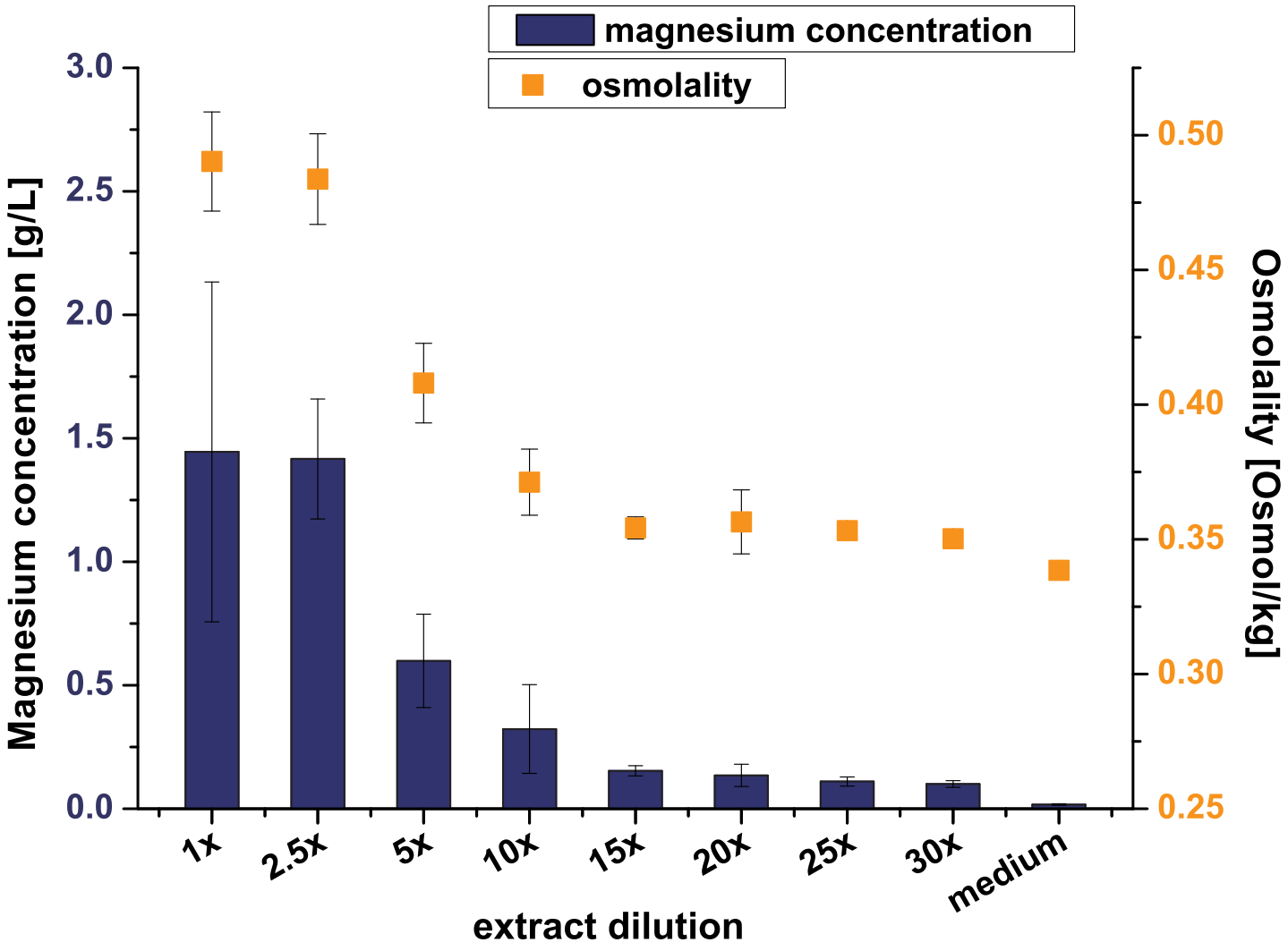
magnesium concentration
osmolality
Magnesium concentration [g/L]
Osmolality [Osmol/kg]
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.50
0.45
0.40
0.35
0.30
0.25
1x
2.5x
5x
10x
15x
20x
25x
30x
medium
extract dilution

Figure 2

Click here to download high resolution image

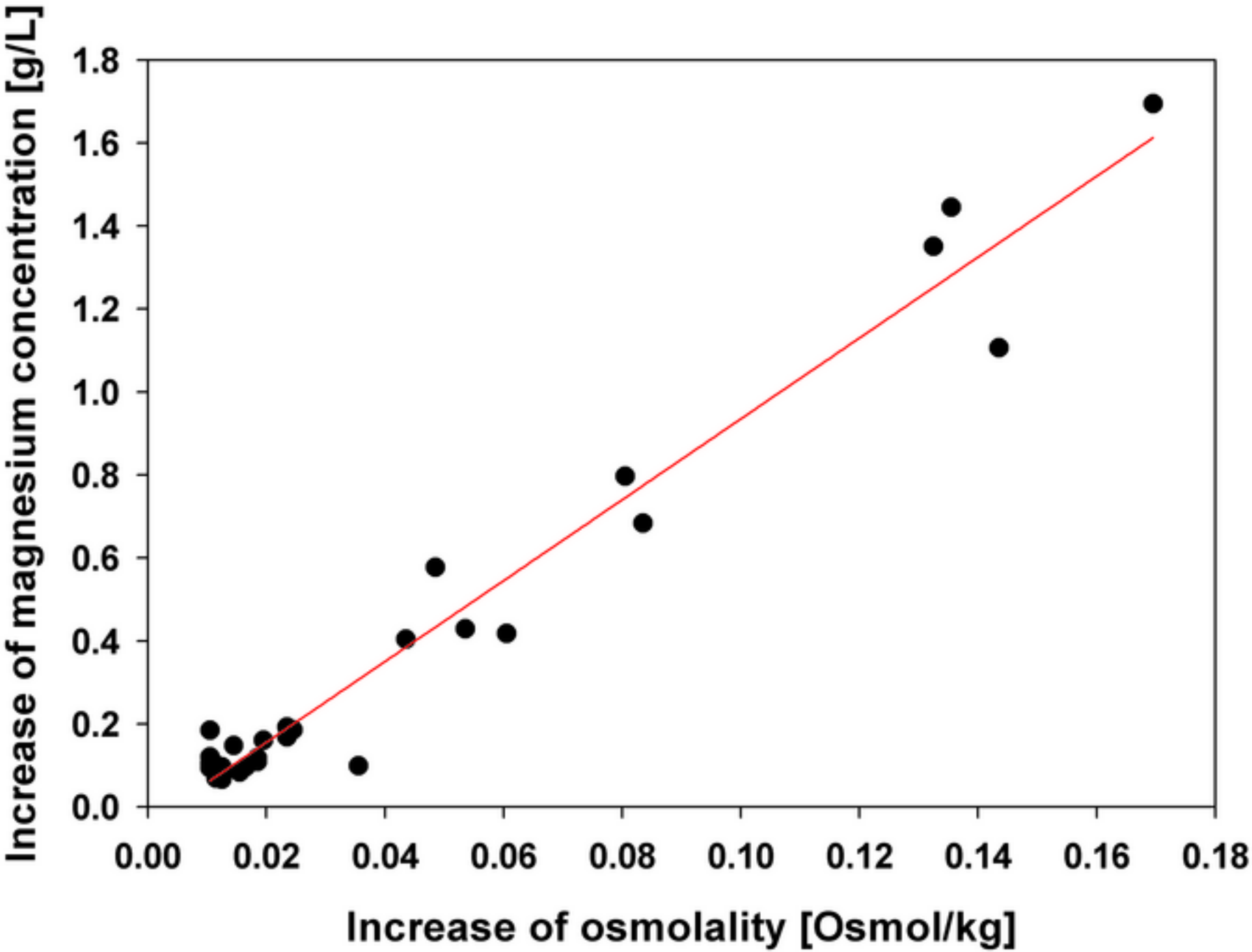

Figure 3a

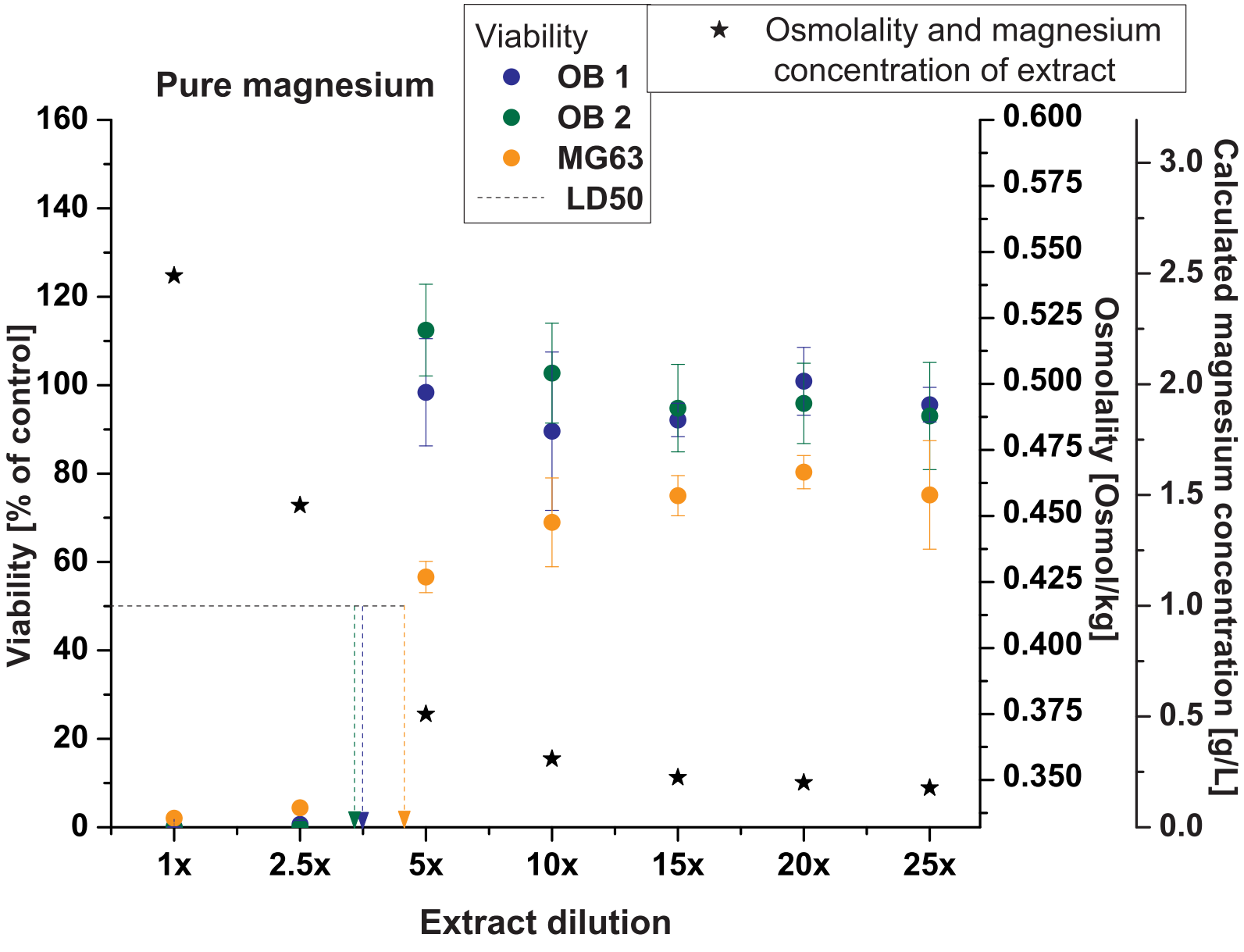
Pure magnesium
Viability
OB 1
OB 2
MG63
LD50
Osmolality and magnesium concentration of extract
Viability [% of control]
Osmolality [Osmol/kg]
Calculated magnesium concentration [g/L]
Extract dilution
1x
2.5x
5x
10x
15x
20x
25x
0
20
40
60
80
100
120
140
160
0.350
0.375
0.400
0.425
0.450
0.475
0.500
0.525
0.550
0.575
0.600
0.0
0.5
1.0
1.5
2.0
2.5
3.0

Figure 3b

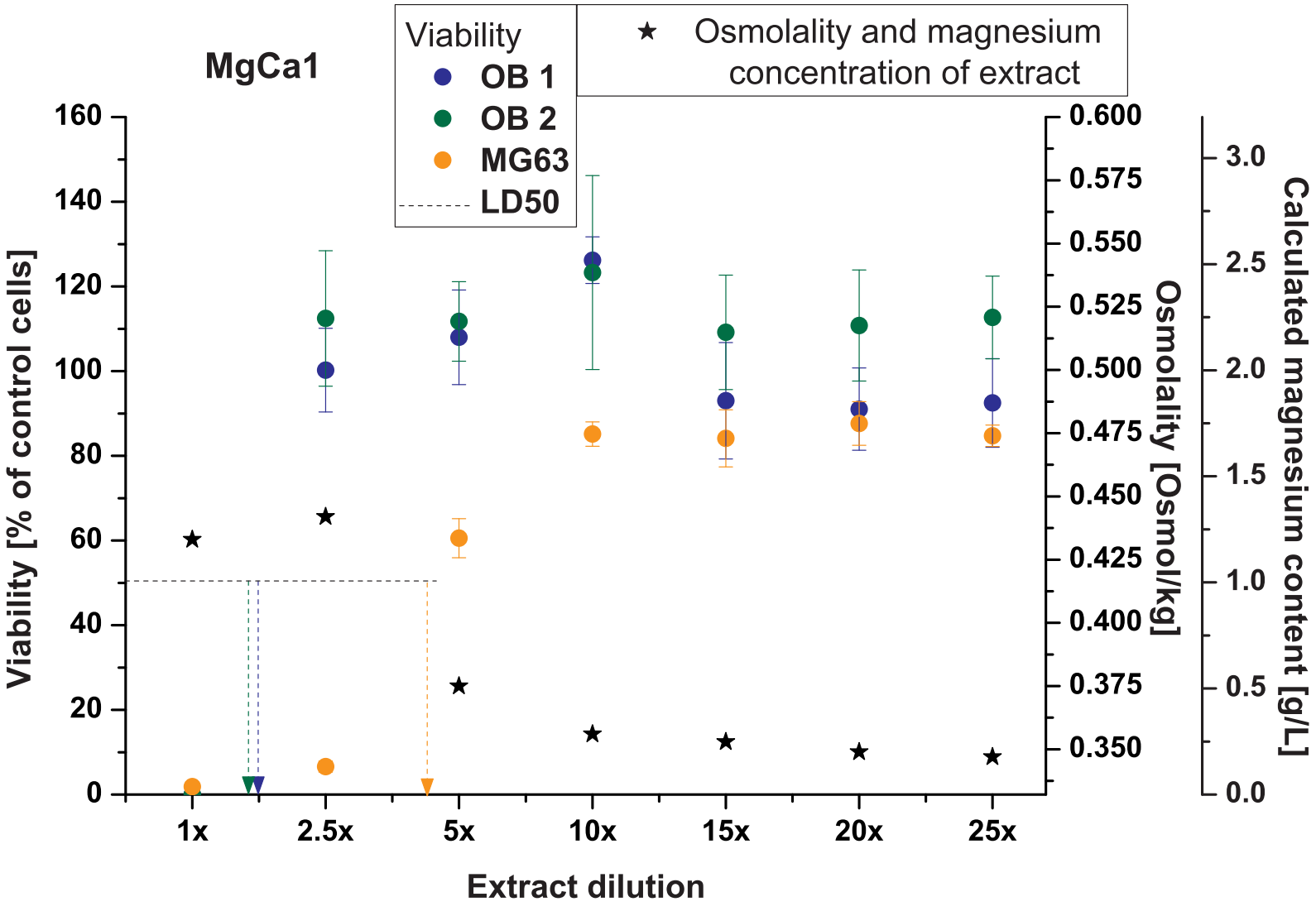
MgCa1
Viability
OB 1
OB 2
MG63
LD50
Osmolality and magnesium concentration of extract
Viability [% of control cells]
Osmolality [Osmol/kg]
Calculated magnesium content [g/L]
Extract dilution
1x
2.5x
5x
10x
15x
20x
25x
0
20
40
60
80
100
120
140
160
0.350
0.375
0.400
0.425
0.450
0.475
0.500
0.525
0.550
0.575
0.600
0.0
0.5
1.0
1.5
2.0
2.5
3.0

Figure 3c

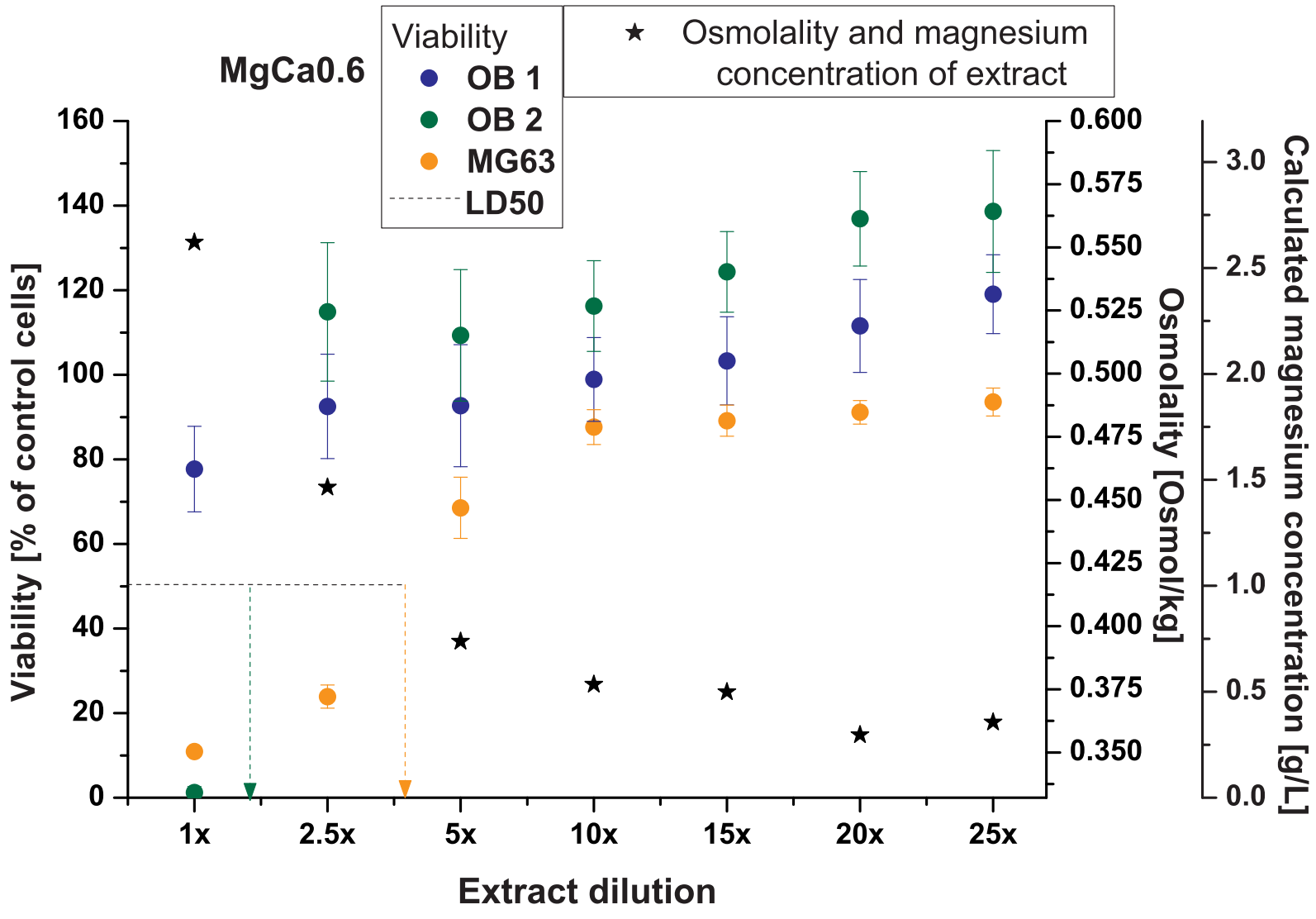
MgCa0.6
Viability
OB 1
OB 2
MG63
LD50
Osmolality and magnesium concentration of extract
Viability [% of control cells]
Osmolality [Osmol/kg]
Calculated magnesium concentration [g/L]
Extract dilution
1x
2.5x
5x
10x
15x
20x
25x
0
20
40
60
80
100
120
140
160
0.350
0.375
0.400
0.425
0.450
0.475
0.500
0.525
0.550
0.575
0.600
0.0
0.5
1.0
1.5
2.0
2.5
3.0

Table 1

Table 1: Osmolality of the extracts and the colour of the indicator phenol red of the extraction medium after the extract preparation indicating the pH value that was reached due to the corrosion of the magnesium materials during the extract preparation.

	Pure magnesium		MgCa0.6		MgCa1	
	Osmolality [Osmol/kg]	Colour of the extract (indicator phenol red)	Osmolality [Osmol/kg]	Colour of the extract (indicator phenol red)	Osmolality [Osmol/kg]	Colour of the extract (indicator phenol red)
1x	0.541	Colourless	0.552	Pink	0.433	Pink
2.5x	0.454	Pink	0.455		0.442	
5x	0.375		0.394		0.375	
10x	0.358	Red	0.377	Red	0.356	Red
15x	0.351		0.374		0.353	
20x	0.349		0.357		0.349	
25x	0.347		0.362		0.347	
Medium	0.344		0.344		0.344	

Table 2: Assessment of the grade of cytotoxicity according to **[5]**

Grade of Cytotoxicity	Interpretation	Growth inhibition compared to control (%)
0	No cytotoxicity	< 25
1	Low cytotoxicity	26-40
2	Moderate cytotoxicity	41-60
3	High cytotoxicity	> 60