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Interference of magnesium corrosion with tetrazolium based cytotoxicity assays

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Abstract
Magnesium (Mg) alloys are promising materials for the development of biodegradable implants. However, the current *in vitro* test procedures for cytotoxicity, cell viability and proliferation are in some cases not suitable for this class of materials. In this paper we show that tetrazolium salt-based assays which are a widely used in practice are influenced by the corrosion products of Mg based alloys. Corroded Mg converts tetrazolium salts to formazan leading to a higher background and falsifying the results of cell viability. Tetrazolium-based assays are therefore not an useful tool to test the cytotoxicity of Mg in static *in vitro* assays.

Introduction
Degradable magnesium (Mg) based implants have been shown to be a promising material for orthopaedic and trauma surgery [1,2]. For these special applications they are much more suitable than other metals, polymers or ceramics because of their mechanical properties similar to bone [3,4]. Polymers like polylactid acid (PLA) or polyglycolic acid (PGA) and ceramics (hydroxyapatite) are not suitable for load bearing applications. In contrast, currently used metallic implant materials (titanium, cobalt-chromium-based alloys and stainless steels) have higher elastic moduli than bone [5]. New metallic biomaterials are designed with improved load transfer to bone. Implant materials with mechanical properties close those of bone may involve the implant surrounding bone tissue in load bearing processes and reduce thereby the incident of implant loosening [5,6]. Mg in itself has the advantage that it may be osteoconductive. Mg materials can stimulate the generation of bone tissue and enable the ingrowth into a biodegrading implant [2,7] leading to a higher stability of the implant and finally in the replacement of the degrading implant. Because Mg degrades completely, no second operation for the removal of plates, nails or screws is needed, which lowers health costs and improves the quality of life for the patient. Mg is an essentially needed element for the human

body and naturally occurring in bone tissue. Excess Mg as it might result from the corrosion process is excreted via the urine.

A significant drawback is the low corrosion resistance of Mg. Mg materials degrade too fast for orthopaedic application [3,8,9]. To overcome this problem Mg alloys with improving alloying elements are produced. There are several alloying elements available which are known to enhance the corrosion resistance of Mg (aluminium, manganese, zinc, rare earth elements and yttrium) [10] which are used to tailor the mechanical properties of the implant. Great endeavour is done to find non-cytotoxic alloying elements which can improve mechanical properties and corrosion resistance.

In order to minimize the number of necessary animal tests, a suitable in vitro test system is needed to preselect appropriate implant candidates. The EN ISO 10993-5:1999 [11] requires that the cytotoxicity is measured via cell death, inhibition of cell growth, cell proliferation or colony forming ability. Tetrazolium salt-based assays are widely used in order to measure cytotoxicity or cell proliferation [12]. The principle of the tetrazolium-based tests MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) and XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-((phenylamino)carbonyl)-2H-tetrazolium hydroxide) is the conversion of the yellow tetrazolium salt by metabolically active cells into a blue (MTT) or orange (XTT) formazan dye. MTT was first described by Beyer [13] and utilized by Mosmann for cellular bioassays to quantify cellular growth and cytotoxicity [14]. This first generation tetrazolium salt MTT is reduced to formazan intracellularly by NAD(P)H-dependent enzymes of the endoplasmatic reticulum and to a small amount by succinate dehydrogenase within the mitochondrial complex II [12]. Solubilisation solution is necessary in order to dissolve the insoluble MTT formazan. The second generation tetrazolium salt XTT uses intermediate electron acceptors and produces soluble formazan end products. XTT is reduced by dehydrogenase enzymes [15].

These assays are easy, fast, non-radioactive and work specifically with living and metabolically active cells, not with dead cells or erythrocytes [14]. A significant drawback of the tetrazolium-based tests is that the difference between cytotoxic (cell death) and cytostatic (reduced growth rate) effects can not be distinguished [16]. Furthermore, the tests can be influenced by a variety of parameters and chemicals. But although MTT and XTT are both tetrazolium salts, they are influenced by different parameters and chemicals. XTT can be reduced by NAD(P)H in the absence of cells while MTT can only be reduced by NAD(P)H when cells are present [12]. XTT can also be reduced by dithiothreitol (DTT), mercaptoethanol, reduced glutathione, L-cysteine and L-ascorbic acid in the absence of cells [12]. Human serum albumin can reduce both MTT and XTT tetrazolium salts [17]. MTT reduction can be influenced by even more parameters: vitamin C was described as reducing agent and vitamin D even enhances this effect by catalysis [18]. Flavonoids with hydroxyl groups such as quercetin, rutin or luteolin are influencing the test results [19,20]. Without cells, thiol-containing antioxidant compounds like beta-mercaptoethanol, DTT, pyrrolidine-dithiocarbamate, and N-acetyl-L-cysteine (acetylcysteine) reduce MTT tetrazolium salt [21]. Further substances which exert influences on the test are D-glucose content of medium [22], Glutathione S-transferase [23] or nanoparticles [24]. Also the pH of medium can adulterate the results of an MTT or XTT test.

The aim of our study was to determine the corrosion environment during Mg corrosion to identify possible factors influencing formazan based viability tests and the comparison of these tests with a luminescence based assay (BrdU). Furthermore different assays for the analysis of cellular reactions to *in vitro* Mg corrosion were tested for their applicability.

Materials and Methods

Material production

The materials used in this study were chosen due to their promising properties (increased corrosion resistance and mechanical stability) for the use as biodegradable implant material. Two techniques were used in order to obtain the specimens – permanent mould casting and sintering. The resulting alloys show a different degradation behavior due to their composition and the kind of production technique. Two sample sizes were fabricated for each material.

The bigger specimens are used for the extract tests in order to get sufficient amounts of extracts. The smaller specimens were fabricated for the direct tests. The sintered specimens have a different size because the shape was defined by the machine.

Permanent mould casting

Magnesium (Mg_{cast}) and Mg4Y (4 wt% yttrium) were prepared by permanent mould casting. All materials have been molten under protective atmosphere (Ar+2% SF6) at a temperature of 750°C. After adding the pure alloying elements the melt has been stirred for 30 min with 200 rpm prior to casting the materials into preheated moulds (550°C) made out of mild steel. To assure cleanliness of the cast ingots a filter (Foseco SIVEX FC, Foseco GmbH, Borken, Germany) has been used. Cylindrical specimens with a diameter of 5 mm and 10 mm and a height of 1.5 mm were cut from the cast blocks via electrical discharge machining.

Sintering

Magnesium ($Mg_{sintered}$) and MgCa1eu (1 wt% calcium, eutectic) specimens were obtained via dry pressing and sintering. Cylindrical test specimens were directly obtained by dry pressing in a glove box system (Unilab, MBraun, Garching, Germany) under argon in a manual mode press (Enerpac RC55, Enerpac, Düsseldorf, Germany) at 75 (further indicated as $Mg_{sintered}$ 75 MPa) and 100 MPa (further indicated as $Mg_{sintered}$ 100 MPa). MgCa1eu specimens were only pressed with 100 MPa. The resulting specimen sizes were 8 mm in diameter and 12 mm in height for the big specimens and 4 mm in diameter and height for the smaller specimens. The heat treatment of the specimens was performed in a hot wall furnace (XRetort, Xerion, Freiberg, Germany) under Ar 6.0 at 605°C for 64 h.

In total five materials were produced from pure magnesium and two different alloys with two sizes each (table 1).

Specimen sterilization

The samples were sonificated for 20 min in dry isopropanol and gamma-sterilized at the In Core Irradiation (ICI) facility of the Geesthacht neutron facility with a total dosage of 29 kGy.

Cytotoxicity test assays

MTT test

The MTT test was performed according to Mosmann [14]. MTT (Thiazolyl Blue Tetrazolium Bromide (Sigma-Aldrich Chemie, Taufkirchen, Germany) was dissolved in Phosphate Buffered Saline (PBS) at 5 mg/mL. This MTT solution was added into the wells with a resulting dilution factor of 1:11. Then the plates were incubated for 4 h under cell culture conditions (37°C, 5% CO_2, 20% O_2, 95% rH). 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 24 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.

XTT test

The Cell Proliferation Kit II (XTT) (Roche Diagnostics, Mannheim, Germany) was used according to the manufacturer's instructions. In brief, XTT labelling reagent was mixed with electron-coupling reagent (5 mL + 100 µL) and added into the wells in a resulting dilution factor of 1:3 and incubated for 24 h in the incubator. The formed orange formazan is soluble in aqueous solutions and can directly be quantified by an ELISA plate reader at a wavelength of 492 nm and a reference wavelength of 655 nm.

BrdU test

The "Cell Proliferation ELISA, BrdU (chemoluminescent) Kit" (Roche Diagnostics, Mannheim, Germany) was performed using the manufacturers protocol. Cells were incubated for 2 h under cell culture conditions with 5-bromo-2'-desoxyuridine (BrdU)-labeling solution. After removal of labeling solution, the cells were fixed and the DNA was denaturated with FixDenat for 30 min at RT. Antibody conjugate anti-BrdU-POD-solution was added to the cells and

incubated with the cells for 30 min at RT. Several washing steps with PBS were performed (minimum three times for 5 min each) to remove the unbound antibodies and to prevent high background luminescence due to unspecific binding. A substrate mixture with luminol, 4-iodophenol and H_2O_2 was added to the cells and the measurement of the plate started immediately. The luminescence was measured via a microplate luminometer (Victor^3, PerkinElmer, Jügesheim, Germany). Values are expressed as relative light units / second (rlu/s).

Cell Culture

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 5th passage were used.

Determination of changes in pH and osmolality during magnesium corrosion

Online pH measurement

The dynamic pH measurement was performed with the SDR SensorDish Reader system (PreSens GmbH, Regensburg, Germany) on special 24-well-plates with an integrated pH sensor (multidish "HydroDish", PreSens GmbH, Regensburg, Germany). A sensor spot is fixed at the bottom of each well of the multidish. The sensor spot contains a luminescent dye. It is excited by the SensorDish Reader placed below the multidish, and its luminescence lifetime is detected non-invasively through the transparent bottom. The measurement range of the system is limited from pH 4 to pH 9. Two corrosion media were used: (i) Hank's balanced salt solution (HBSS; Invitrogen Corporation, Karlsruhe, Germany) as unbuffered physiological salt solution and (ii) Dulbecco's modified eagle medium (DMEM) Glutamax-I as CO_2 buffered cell culture medium (Sigma.Aldrich Chemie, Taufkirchen, Germany). Both

media were supplemented with 10% fetal bovine serum (FBS; PAA Laboratories, Linz, Austria).

Mg_{cast}, Mg4Y, $Mg_{sintered}$ 100 MPa and MgCa1eu were analyzed. The smaller Mg materials (4x4 mm sintered specimens and 5x1.5 mm cast specimens) were placed in the multidish and covered by 1.5 mL of the respective corrosion medium. Pure corrosion media without specimens served as control. The medium was changed every 2-3 days. The pH was measured every 5 min in all wells at the same time and recorded by the systems software.

Osmolality measurement

Five different Mg materials (Mg_{cast}, Mg4Y, $Mg_{sintered}$ 75 MPa, $Mg_{sintered}$ 100 MPa, MgCa1eu) were incubated in DMEM Glutamax-I supplemented with 10% FBS in 6-well-plates (Greiner Bio One, Frickenhausen, Germany). The specimens were incubated for 24, 48 and 72 h under cell culture conditions. The osmolality was measured using an osmometer (Osmomat 030, Gonotec, Berlin, Germany). Furthermore, extracts of the specimens incubated for one day were diluted with medium to final concentrations of 25%, 50% and 75% were measured. Medium supplemented with 5 mM, 10 mM, 15 mM, 20 mM and 25 mM $MgCl_2$ was analysed as reference.

Measurement of pH influence on MTT and XTT test

In order to measure the influence of pH on the test assays, the pH of the cell culture medium was measured as control. PH was additionally adjusted with NaOH (Merck, Darmstadt, Germany) to five different pH values (8.0, 8.5, 9.0, 9.5 and 10.0) with a pH meter. These six test solutions were then incubated on 96-well-plates (Nunc, Wiesbaden, Germany) for 72 h under cell culture conditions. Afterwards a MTT and XTT test was performed.

Measurement of $MgCl_2$ influence on the MTT and XTT test

The influence of $MgCl_2$ (Sigma-Aldrich Chemie, Taufkirchen, Germany) on the test assays was analysed using concentrations from 0 to 50 mM. A 1 M stock solution of $MgCl_2$ dissolved in distilled water was prepared and sterile filtered. The solution was delivered on 96-well-plates (Nunc, Wiesbaden, Germany) in 11 different concentrations (0-50 mM, varied in steps

of 5 mM), incubated for 72 h under cell culture conditions and then measured with a MTT and XTT test.

Measurement of different Mg materials influence on the MTT and XTT test

In order to measure the influence of different magnesium materials, eight different materials were tested. The specimens were put with 1.5 mL cell culture medium supplemented with 10% FBS on 24-well-plates (Greiner Bio-One, Frickenhausen, Germany), one specimen per well. The amount of medium put on the cells was relative to the specimen surface. The specimens were incubated for 72 h under cell culture conditions and then measured with a MTT and XTT test.

Cytotoxicity assays

Extract assay

Extract preparation

The extract preparation for the extract assay was performed according to EN ISO 10993-12:2003 [11]. Three days prior to the cell seeding, the specimens were incubated in cell culture medium for 72 h under cell culture conditions in 6-well-plates (Greiner Bio-One, Frickenhausen, Germany). The relation between sample weight and extract medium was 0.2 g/mL. For the extract assays, the 10x1.5 mm (permanent mould cast specimens) and 8x12 mm (sintered specimens) were used. The obtained extracts were applied to the cells in four different concentrations (100%, 75%, 50% and 25%). The dilution of the extracts was made with cell culture medium.

Extract assay

The extract assay was performed in 96-well-plates (Nunc, Wiesbaden, Germany) (35 mm^2 growth diameter). MG-63 cells were seeded with a cell density of 15,000 cells/cm^2 (for the tests with cast specimens) and 5,000 cells/cm^2 (for the tests with sintered specimens) and allowed to settle for 24 h. The extract was added to the cells by replacement of the initial cell culture medium and incubated for 72 hours. This type of assay is further referenced as “with cells”.

Direct assay

For the direct assay, the 5x1.5 mm (permanent mould cast specimens) and 4x4 mm (sintered specimens) were put directly onto the cells not regarding the different sizes of surface areas of the different materials. The direct assay was performed in 24-well-plates (Greiner Bio-One, Frickenhausen, Germany) (1.9 cm^2 growth area). MG-63 cells were seeded with a cell density of 15,000 cells/cm^2 (for the tests with cast specimens) and 5,000 cells/cm^2 (for the tests with sintered specimens) and allowed to settle for 24 h.

Both assays were incubated for 72 h under cell culture conditions followed by a MTT, XTT or BrdU test. Pure cell culture medium and cells with cell culture medium served as controls. The two test assays (extract and direct assay) were furthermore used in order to investigate the influence of corroding magnesium alloys themselves on the test system. For such experiments, the specimens and the different concentrations of extracts were incubated the same way but without cells (further on referenced as “without cells”).

A summary of the applied tests and materials can be found in table 2.

Microscopy

While tetrazolium salt is converted into formazan, needles are formed which are visible under the microscope [20,25]. This needle formation was also investigated in this study with a Nikon ECLIPSE Ti-S/L100 microscope (Nikon GmbH, Düsseldorf, Germany). Furthermore, microscopy was used in order to show existing viable cells in the direct assay with 5 mm Mg_{cast}.

Statistical analysis

Statistics were performed using the SigmaStat package (Systat software GmbH, Erkrath, Germany). For the analysis of concentration dependent effects linear 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.

Quantum chemical calculations

For all calculations on the density functional theory level, the Program RIDFT was used [26]. Energies and geometries were developed on the nonlocal level of theory. For geometry optimization the energies were corrected for nonlocal exchange according to Becke [27,28] and for nonlocal correlation according to Perdew (BP-86) [29] in the selfconsistent procedure. The SVP-split valence base set was used for all atoms supplied by the program [30].

Results

Analysis of the corrosion environment

Online pH-measurements

The pH measurement was performed for Mg_{cast}, Mg4Y, $Mg_{sintered}$ 100 MPa and MgCa1eu. Two test solutions were used, HBSS as unbuffered physiological salt solution and DMEM Glutamax-I as CO_2-buffer cell culture medium. Both media were supplemented with 10% FBS. The peaks indicate the refreshing of the test solutions.

All tests showed that actively corroding Mg materials lead to a dramatic increase in pH to basic pH values (figure 1). This effect was more pronounced in case of the physiologic salt solution (HBSS), which increased from pH 6.7 to pH 9 and above during all experiments. In contrast, cell culture medium with FBS had a basic pH of about 7.7 due to its buffering capacity and this was increased differently for the different alloys. Therefore pH is an important parameter that needs to be taken into account as possible influencing effect on the MTT and XTT test.

Osmolality and Osmolarity

The osmolality is linearly rising with increasing concentrations of $MgCl_2$. The regression equation (r^2=0.9954) was determined as (eq. 1) Osmolality = 0.3368 + 0.00236 * $Concentration_{MgCl2}$ (data not shown). This linear behaviour was used to extrapolate the osmolalities measured for the extracts by the simplifying assumption, that most of the changes in osmolality are induced by Mg.

To determine time-dependent changes corroding samples were probed every 24 h. A gradually increase of osmolality with increasing incubation time was only detectable for Mg4Y and MgCa1eu. The calculated molarities were highest for MgCa1eu and lowest for Mg_{cast} (table 3).

After extract preparation the pure extract and the dilution series was measured to determine linearity of the dilution. The measured and calculated values are depicted in figure 2. Relying on the regression analysis the linearity of osmolality of the extracts could be proven for both sintered materials ($Mg_{sintered}$ 75MPa: R^2=0.989, p<0.01 and MgCa1eu: R^2=0.995, p<0.01). However, the cast materials showed a slight deviation from linearity concerning the pure extract (Mg_{cast}: R^2=0.932, p<0.05 and Mg4Y: R^2=0.957, p<0.05) leading to a lower significance level. For both assays the osmolalilty and the calculated molarity are significantly higher than measured in medium with FBS (osmolality: 0.35, molarity 5 mM). Therefore, osmolality was identified as possible factor affecting the formazan based tests.

Influence of the change in pH and molarity on the MTT and XTT assay

Due to the presented results an affection of MTT or XTT by pH and molarity seems reasonable. Therefore both factors were tested to determine the amount of influence.

Influence of pH

Five different pH values (including the control) were analysed in order to examine the influence on the MTT and XTT test. The pH had a strong influence on the XTT test and even more on the MTT test (figure 3). Culture medium with a pH of 10.0 showed for the MTT test an almost two times higher OD value than the control culture medium with a pH of 7.35. For both tests the increase in absorption was significant starting from pH 9 (MTT: ANOVA on ranks, $H=45.199$, degrees of freedom (d. f.)=5, $p<0.001$; XTT: $H=44.916$, d. f.=5, $p<0.001$). This shows that the pH is an important parameter for the MTT and XTT test and needs to be taken into account. In order to derive reasonable results, the pH influence needs to be studied further and has to be included in the array test development.

Influence of molarity and magnesium salt

$MgCl_2$ concentrations up to 50 mM were used to investigate the influence of molarity on the MTT and XTT test. The results showed that $MgCl_2$ is not reducing the MTT and XTT to formazan and thereby increasing the absorption. In contrast, $MgCl_2$ lowered the absorption values significantly in the XTT test. Only at 5 and 15 mM $MgCl_2$ no significant deviation from the control was determined, all other values were significantly lower (ANOVA: $F=7.497$, $p<0.001$; Holm-Sidak Post-Hoc test, $p<0.05$). Statistically, the MTT test was also affected, but to a lesser extend. Absorption values at 25, 35, 40 and 45 mM were significantly lower than the control (ANOVA on ranks, $H=27.550$, $p<0.01$). The calculated differences (figure 4) seemed bigger for the MTT, but this was due to the generally lower absorption of MTT in medium. Therefore the molarity can be excluded as factor leading to false positive results.

Cytotoxicity assays for the test of different magnesium alloys

Following the characterisation of the corrosion environment and the identification of factors affecting the test system, two different assays to examine the cytotoxicity were performed: (i) the extract and (ii) the direct assay.

Extract assay

In this assay, three magnesium materials were analysed: pure sintered $Mg_{sintered}$100 MPa, sintered MgCa1eu and permanent mould cast Mg4Y. The extract test was performed with four different extract concentrations (25%, 50%, 75% and 100%) with and without cells (figure 5).

In the extract test without cells (figure 5a) an increase of absorption could be observed for nearly all extract concentrations and for all materials. This effect was most abundant for MgCa1eu, resulting in about 3.5 times higher OD values for the MTT at 75% and 100% extract amount compared to the control. Significant differences compared to the control were observed for MTT at extract concentrations of 50, 75 and 100 % (ANOVA on Ranks, $p<0.05$). The effect was even more pronounced for XTT were all extract concentrations led to a significant increase in absorption, although the values were lower compared to MTT. The same trend can be seen for Mg4Y, significant differences were observed for 100% (MTT) and 75 and 100% (XTT). $Mg_{sintered}$ induced this effect only for MTT (significant increase at 75 and 100% extract), but not for XTT. However, all XTT values were elevated compared to the control and reached significance for 50% extract.

In the presence of cells the absorption values show an opposite trend. All values for $Mg_{sintered}$ were lower than the control and decreased with increasing extract concentrations. The decrease was significant for all MTT values (ANOVA, Holm-Sidak post hoc test, $p<0.05$), for XTT at 75 and 100% extract (ANOVA on ranks, $p<0.05$). This was also observable for Mg4Y for extract concentrations higher than 50%. At 25% extract the value for MTT was increased, while XTT was comparable to the control. The trend for XTT and MTT measurements for the aforementioned materials was comparable. In contrast, the alloy MgCa1eu showed clear differences between both assays. MTT values increased significantly at 25 and 50% followed by a decrease, which was significant at 100% extract. XTT values were all significantly lower than the control (ANOVA on ranks, $p<0.05$), but no clear trend was observable (figure 5b).

Direct assay

In the direct assay, the specimens are actively corroding during incubation with or without cells. In the absence of cells, the increase in MTT values for all materials was at least three times higher than the control, in the case of Mg4Y even an increase by a factor of 16 was observed. XTT was not influenced by the $Mg_{sintered}$ materials, all other materials also led to an increase in absorbance. In the presence of cells MTT was comparable or higher than the control, with Mg4Y having the highest absorption (200% of control). In contrast, XTT values were all lower than the control, the highest value (57% of control) was observed for Mg_{cast} (figure 6). As optical control micrographs were taken which showed a drastically decreased amount of adhered cells accompanied by many rounded (presumably dying) cells (figure 7).

Needle formation

As a first attempt to explain the observed influences, the reduction of formazan was confirmed by optical microscopy. The reduction of tetrazolium in the MTT assay to blue formazan by cells is observable microscopically by the formation of blue crystals or “needles” (figure 8). But also in case of corroding magnesium material specimens needles were detectable in the MTT tests in the absence of cells but not in the XTT tests.

The presented results for both assays raise doubt, that the results “as measured” are representing real determinations of cell viability. Therefore two approaches were used to get reasonable results: (I) as an alternative assay based on luminescence the BrdU was tested preliminarily and (II) the assay procedure for the MTT and XTT was changed..

BrdU

The BrdU test was performed using Mg_{cast} extract in four different concentrations (25%, 50%, 75% and 100%). The test was performed using the extract assay method. Without cells no significant increase in rlu/s values was detectable with increasing amount of extract (figure 9). Cells incubated with extracts showed decreasing viability with increasing extract concentration. The decrease was significant for 75 and 100% extract (ANOVA, $p<0.001$), which was supported by the optical control of the assay. This leads to the assumption that

there is no influence of Mg material extract on the BrdU test. Further experiments with different magnesium alloys are currently performed.

Optimisation of the MTT and XTT tests for reasonable results

Due to the generation of possibly false positive measurements a strategy was developed to get reasonable results out of the MTT and XTT tests. This strategy included the following steps:

1. Measurement of the respective assay with cells (extract or direct) as described
2. additional measurement of the respective assay without cells (extract or direct)
3. Calculation of the absorbance values by subtraction of the results without cells from the measurements with cells for each value separately

This strategy was only applicable in case of the extract assay. In contrast to the previously shown results (figure 5) for all materials, as well as both tests the same trend (an increase in extract concentrations induces decrease in cell viability) was observable (figure 10). Values were comparable to those obtained with the BrdU assay. The calculation for the values of the direct assay by this strategy resulted in negative values for nearly half of the samples; therefore formazan based test should not be used with this assay.

Discussion

One aim of this study was the examination of environmental conditions during *in vitro* Mg corrosion and the influence on formazan based tests.

The corrosion environments were hallmarked by a significant increase in pH and osmolality, but to a different extend with regard to different materials, production route or alloy (figure 1 and 2, table 3). Each material or alloy has a special corrosion profile, which should be determined prior to *in vitro* experiments. This study only aimed at the determination of influencing factors on viability tests; therefore no deeper analysis of such profiles was

performed. One further drawback is the use of static cell culture, which is responsible for the observed corrosion environment. The increase in pH and osmolality are due to this system, and the corrosion environment changes drastically by the utilization of e.g. laminar flow cell culture [31]. However, as a first approach the static system is suitable, as it is recommended by international standards [11,32].

This study shows that the MTT and XTT test can be influenced by a variety of parameters. At first we analysed and quantified the effect of pH on the MTT and XTT test. An increased pH results in an increased amount of reduced tetrazolium salts (figure 3). The pH was 90% higher for the MTT and 35% higher for the XTT test in comparison to fresh normal culture medium with a pH of 7.35. In general, the MTT is more sensitive for pH changes to basic values than the XTT test. It is known that the pH has an influence on MTT due to a shift in absorption maxima at low pH or high cell density. Moreover, the absorption peak at 570 nm is increased with increasing pH [33]. Hence, the results are in good agreement with literature.

As a second factor osmolality was measured and assumed to be another influencing parameter. The measurement of osmolality is by far easier than the determination of element molarities, because the total content of salts in solution is analysed irrespective of the element compounds. We hypothesized during the experiments that the main amount of released elements would account for Mg. Although this is a very simplifying assumption the results showed a highly significant correlation of $MgCl_2$ molarity with the measured osmolalities. Hence, we used this correlation to calculate molarities for the solutions which were in the range of 45 to 82 mM during corrosion (table 3) and between about 40 to 60 mM for the extracts (figure 2). We analyzed the influence of $MgCl_2$ up to 50 mM on the MTT and the XTT assay (figure 4). In contrast to pH a decrease in absorption values was observable. Thus, osmolality / molarity may be excluded as influencing parameter leading to false positive results, but should be kept in mind as possible factor inducing false negative values. Further analyses also in higher concentration ranges are subject of ongoing research.

A further aim of this study was to determine the suitability of two assays – extract assay and direct assay – for cytocompatibility studies. In the state “as measured” both assays were influenced. In case of the extract assay there is no actively corroding Mg material present during the formazan reduction, still the extracts enhance the absorption of both MTT and XTT in a concentration-dependent manner (figure 5). In case of the direct test the complete specimens are actively corroding within the medium during the viability tests. The impact on the direct assay is much stronger than on the extract assay leading to the assumption that besides pH actively corroding magnesium is an influencing parameter (figure 6). The results indicate that the use of both tests as recommended leads to incorrect, mainly false positive results. If this effect is not taken into account even the results of very elaborate studies [34] may be questionable.

The proposal of a possible alternative for the data treatment regarding MTT and XTT tests resulted from this dilemma by introducing additional measurements and calculation of more reasonable results (figure 10). Please note that this alternative evaluation is based on the results presented in figure 5 without additional measurements. In contrast to the simple MTT or XTT assay with just one positive and/or negative experimental control this approach leads to a doubling in the required work for the assays. This is due to the fact, that not only a single control is performed, but the whole assay is performed under the same conditions twice - one preparation without cells and one including the cells. The following data treatment is not very sophisticated, but still another elevation in working time. However, due to the existing influence of pH it is still doubtful whether the obtained results are exact measurements. The direct assay proved not to be useful, because it did not produce experimental data which were reliable, and the described data treatment was not working with this assay. Hence, the BrdU assay as alternative viability test was evaluated, and preliminary results were shown (figure 9). Its measurement principle is based on luminescence instead of absorption and makes use of a luminescent molecule incorporating into DNA [35]. The preliminary results of

this test show that it may not be influenced by corroding Mg, a thorough investigation of this assay regarding also other influencing factors is performed at the moment.

Possible quantitative alternative test assays to replace formazan based assays for the study of corroding magnesium specimens are available. Promising candidates are the lactate dehydrogenase (LDH) assay and the DNA measurement. The LDH test measures the enzyme LDH that can be found in most cells and is released when cells are damaged [36]. The DNA measurement uses the fluorochrome bisbenzimid. By intercalation with DNA the emission wavelength of bisbenzimid changes and can be detected by a fluorometer. The amount of measured DNA can be correlated to the number of living cells if appropriate standard curves are experimentally established [37]. These three test systems may not be influenced by the corrosive environment during Mg corrosion and will be evaluated in further studies.

Beside the practical implications discussed before it would be helpful to understand the underlying mechanisms of the interference of material degradation with formazan-based assays. All Mg material specimens show increased OD values in all tests although no cells were present (figure 6). The increased OD implicates that the tetrazolium salt is converted into formazan. We speculate, when MTT is reduced, the ring form of the tetrazolium salt is opened and the quaternary amine is converted to a tertiary amine. When a cell reduces MTT to formazan, a second tertiary amine that was bound to the quaternary amine binds a hydrogen atom [20]. This leads to the formation of formazan and a change in colour. Mg is a very reactive element. It is conceivable that Mg may be able to open the ring form of the tetrazolium salt and bind to it which could lead to a change in colour similar to the formation of formazan in case of the MTT and XTT tests with cells (figure 11).

Assuming, ring opening of the tetrazolium salt is mediated by Mg, the resulting Mg^{2+} ions might bind to the lewis-basic nitrogen atom of the formazan product. In order to gain deeper insights into the binding of Mg^{2+} to a formazan we performed density functional calculations on a putative XTT formazan Mg and a MTT formazan Mg complex. The Mg^{2+} ions in related

ketiminato complexes are tetrahedral coordinated [38]. In a formazan complex the ligand binds by two nitrogen donor atoms to a Mg-center. To fill the coordination sphere two water molecules were added to the Mg-center in the calculations revealing distorted tetrahedral coordination geometry. The optimized structures are depicted in figure 12.

The formazan functions as a chelate ligand to a Mg^{2+} ion. The main differences in bonding modes between the two complexes depicted in figure 12 are the hydrogen bridges revealing two for the XTT formazan and only one for the MTT derivative. The intramolecular H-bridges can be suppressed by intermolecular packing in but is also strongly dependent on the proton concentration (pH) which might influence crystal growth in media. Experimental studies to determine the actual crystal growth are currently under investigation.

Conclusion

The MTT and XTT test are widely used cytotoxicity tests because they are easy, fast and cheap. In case of Mg materials, the use of these test kits leads to false positive or false negative results. It is possible to obtain approximately real results for the extract assay but a lot of work is needed in order to analyze all influencing parameters and for adjusting the test systems. A bigger variety of controls is needed in order to detect all influencing effects and prevent false positive or false negative results. If all necessary conditions would be made, the MTT and XTT test system would not be easy, fast and cheap any more. In summary, tetrazolium-based tests are not an useful tool in order to investigate cytotoxicity of different Mg materials in static *in vitro* assays. It makes more sense to use another test system that is not or not so much influenced by Mg materials and other parameters. Our preliminary results for the BrdU test showed no influence by corroding magnesium. Therefore, BrdU seems to be a useful tool to test the cytotoxicity of Mg materials.

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Table 1: Production technique and sample dimensions of Mg materials used in this study.

Production technique	Permanent mould casting		Sintering		
Material	Mg_{cast}	Mg4Y	$Mg_{sintered}$ 75 MPa	$Mg_{sintered}$ 100 MPa	MgCa1eu
Size (diameter x height [m])	10 x 1.5 5 x 1.5	10 x 1.5 5 x 1.5	8 x 12 4 x 4	8 x 12 4 x 4	8 x 12 4 x 4

Table 2: Mg materials used for the different test assays and cytotoxicity test systems

Assay	Test	Test material	Sample size
Extract assay	MTT	Mg4Y	10x1.5
		$Mg_{sintered}$ 100 MPa	8x12
		MgCa1eu	8x12
	XTT	Mg4Y	10x1.5
		$Mg_{sintered}$ 100 MPa	8x12
		MgCa1eu	8x12
	BrdU	Mg_{cast}	10x1.5
Direct assay	MTT	Mg_{cast}	5x1.5
		Mg4Y	5x1.5
		$Mg_{sintered}$ 75 MPa	4x4
		$Mg_{sintered}$ 100 MPa	4x4
		MgCa1eu	4x4
	XTT	Mg_{cast}	5x1.5
		Mg4Y	5x1.5
		$Mg_{sintered}$ 75 MPa	4x4
		$Mg_{sintered}$ 100 MPa	4x4
		MgCa1eu	4x4

Table 3: Measured osmolalities and calculated molarities for different time points during corrosion of the specimen.

Sample	Time (h)	Osmolality [Osm/Kg]	Calculated Molarity [mM]
$Mg_{sintered}$ 75 MPa	24	0.474	58.13
	48	0.487	63.64
	72	0.482	57.28
MgCa1eu	24	0.443	45
	48	0.513	74.66
	72	0.529	81.44
Mg_{cast}	24	0.469	56.02
	48	0.473	57.71
	72	0.463	53.47
Mg4Y	24	0.471	56.86
	48	0.488	64.06
	72	0.492	65.76

Figure Captions

Figure 1: Online measurement of pH changes of the analysed alloys in different corrosion media. Pure solutions without specimen were used as control. The peaks indicate medium changes. The range of the SDR system is limited to pH 9, all exceeding values were set as 9.

Figure 2: Calculated molarities and measured osmolalities for the dilution of extracts. The sintered samples show a linear behaviour, cast samples show slight deviations from linearity in the pure extracts. For the sake of clarity regression curves are not plotted. The significance levels of the regression are given in the legend (* = $p<0.05$; ** = $p<0.01$).

Figure 3: Influence of pH on the MTT and XTT test. Absorption values were measured and calculated as percent of control (pH 7.35). The absorption values of the unchanged medium were set to 100%. Significant differences ($p<0.05$) for both tests are indicated by the asterisks.

Figure 4: Influence of increasing MgCl2 concentrations on absorption values expressed as percent of control on the MTT and XTT test. Significant lower absorption values of the MTT are indicated by asterisks (* = $p<0.05$), while for the XTT all absorption values were significantly lower, except at the marked concentrations (n.s.=not significant).

Figure 5: Analysis of formazan reduction in the MTT and XTT test by extracts of magnesium materials without and with cells. The measured absorption values were normalized as percent of control (pure cell culture medium without specimen). The control was set to 100%. Significant differences are indicated by asterisks (ANOVA / ANOVA on ranks, significance level $p<0.05$).

Figure 6: Analysis of the formazan production (MTT and XTT) without and with cells for the direct assay. The ODs of the specimens incubated without or with cells are compared to the ODs of the control (pure culture medium and cells incubated without specimen, respectively). The controls are set to 100%.

Figure 7: Micrographs from the cells in the direct assay with Mgcast. The left image shows the control cells and the right image shows cells after incubation with a 5 x1.5 mm Mgcast specimen for 72 h.

Figure 8: Left: Needle formation in a preparation with cells in medium with MTT. Right: Needle formation in a preparation with a Mgcast specimen without cells.

Figure 9: Analysis of the influence of the BrdU test by Mgcast extract with and without cells. The rlu/s values of the cells incubated with specimens are compared to the values of the control (cells incubated without specimen). The values of the specimens are compared to the values of the control (pure cell culture medium without specimen). The controls are set to 100%. Significant differences are indicated by asterisks ($p<0.05$).

Figure 10: Recalculated values for the MTT and XTT test incubated with extracts of magnesium materials with cells. As specific background the respective measurement without cells is subtracted and compared to the control cells (cells incubated without specimen). The controls are set to 100%.

Figure 11: Assumed formation of formazan by Mg materials.

Figure 12: Optimized structures of [XTT formazan Mg(H2O)2]- (left) and [MTT formazan Mg(H2O)2] (right). The tetrahedral geometries of the Mg-centers are distorted by intramolecular hydrogen bridges to the formazan ligands.

Figure 1a

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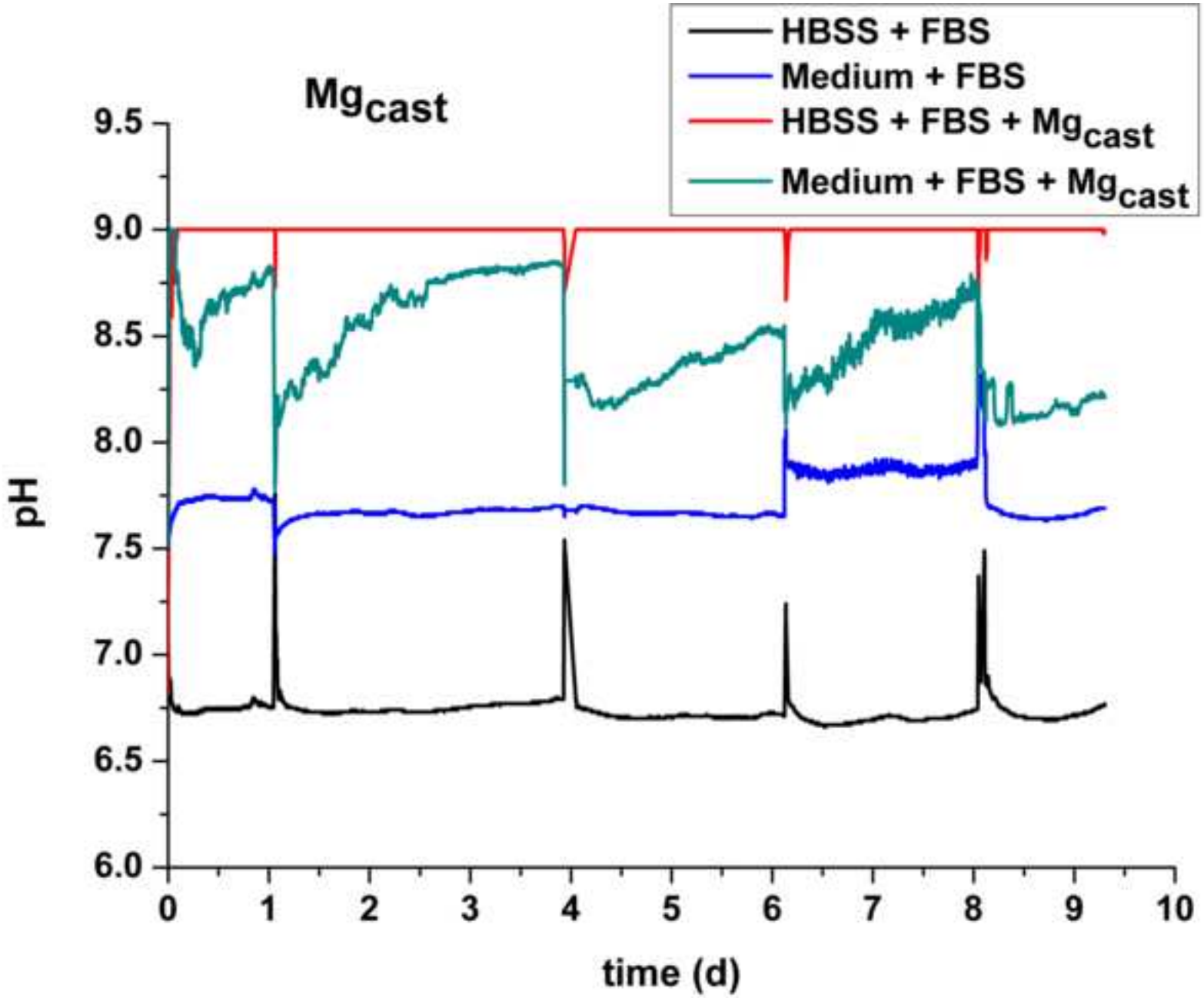

Figure 1b

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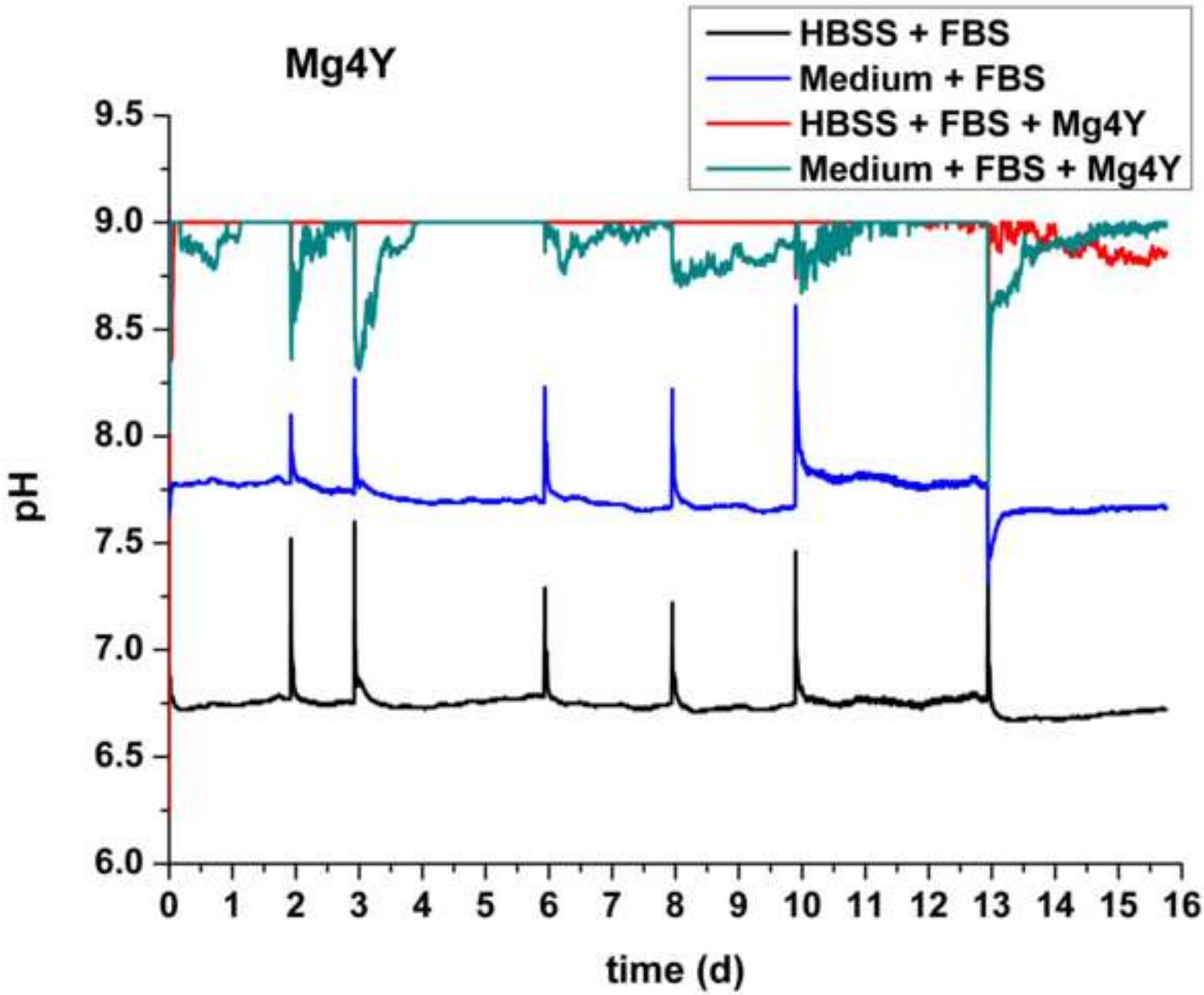

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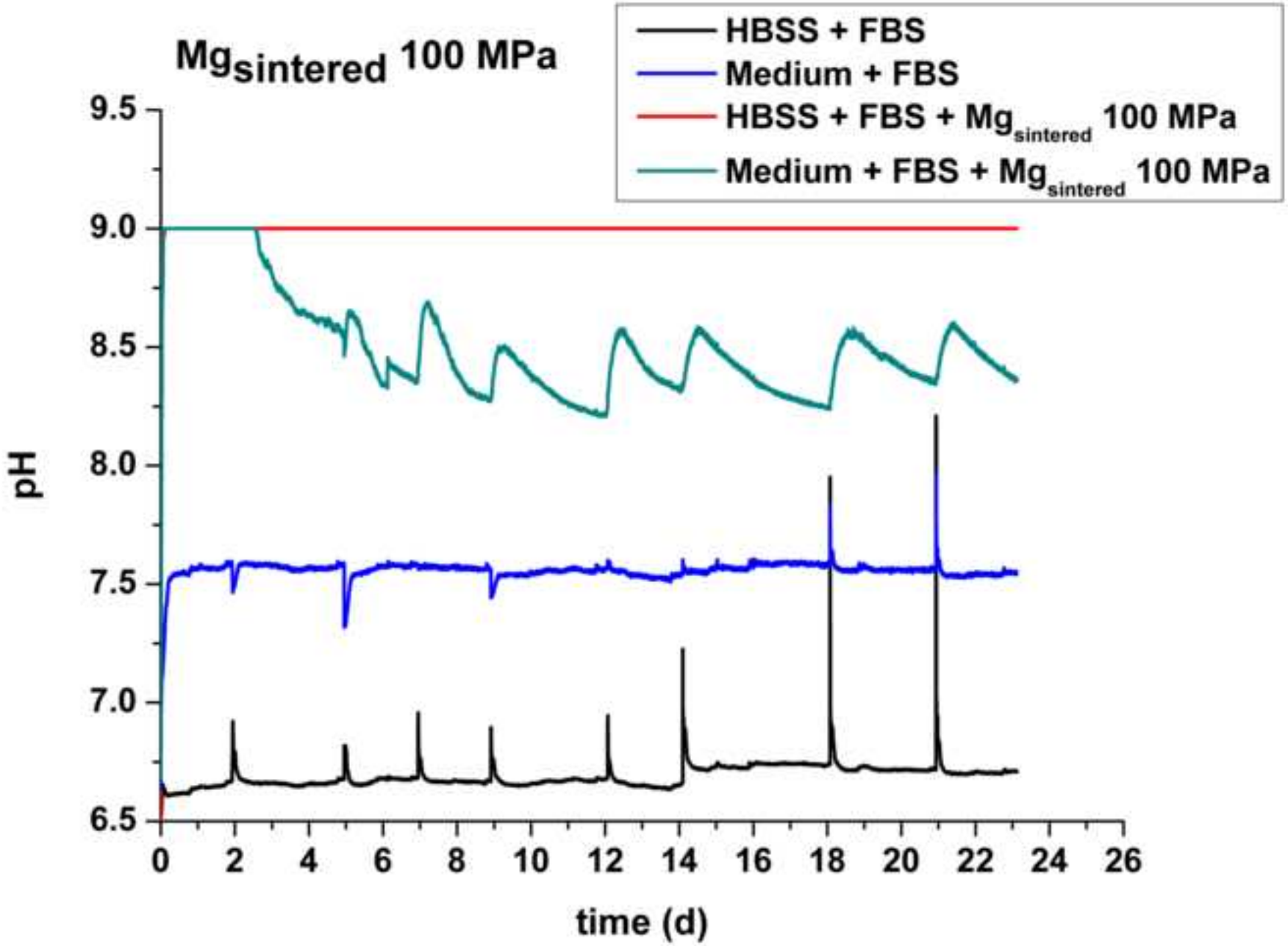

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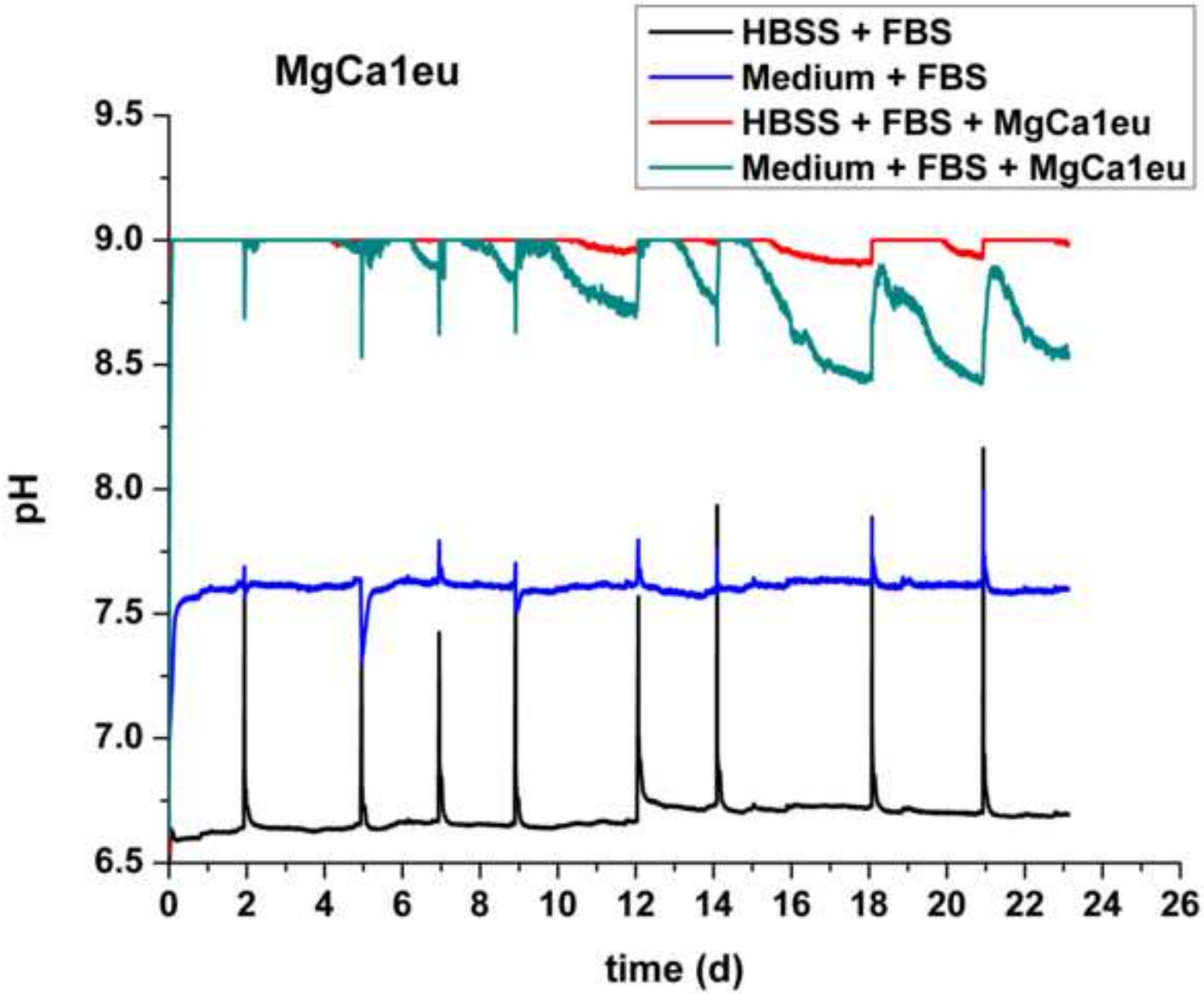

Figure 2

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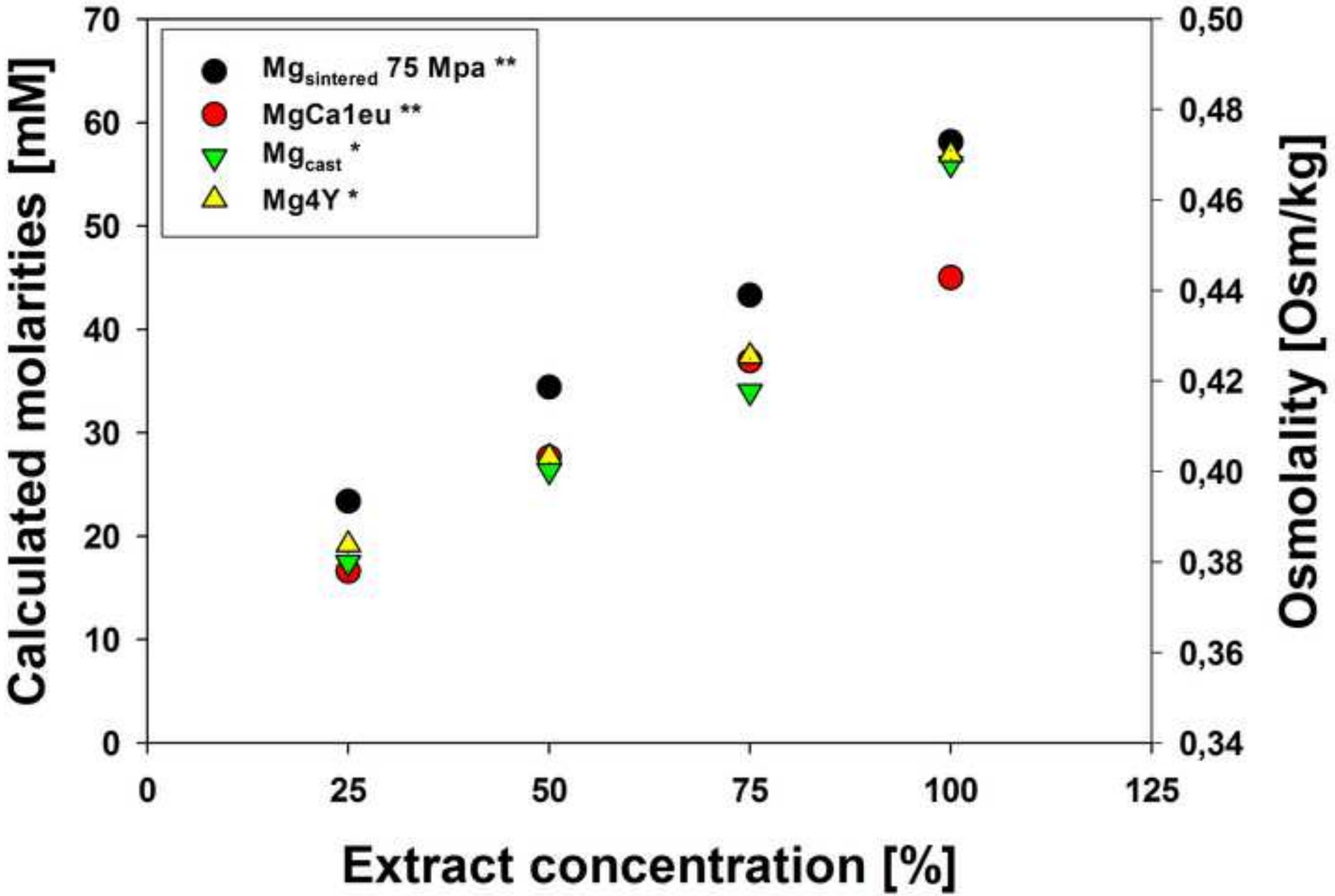

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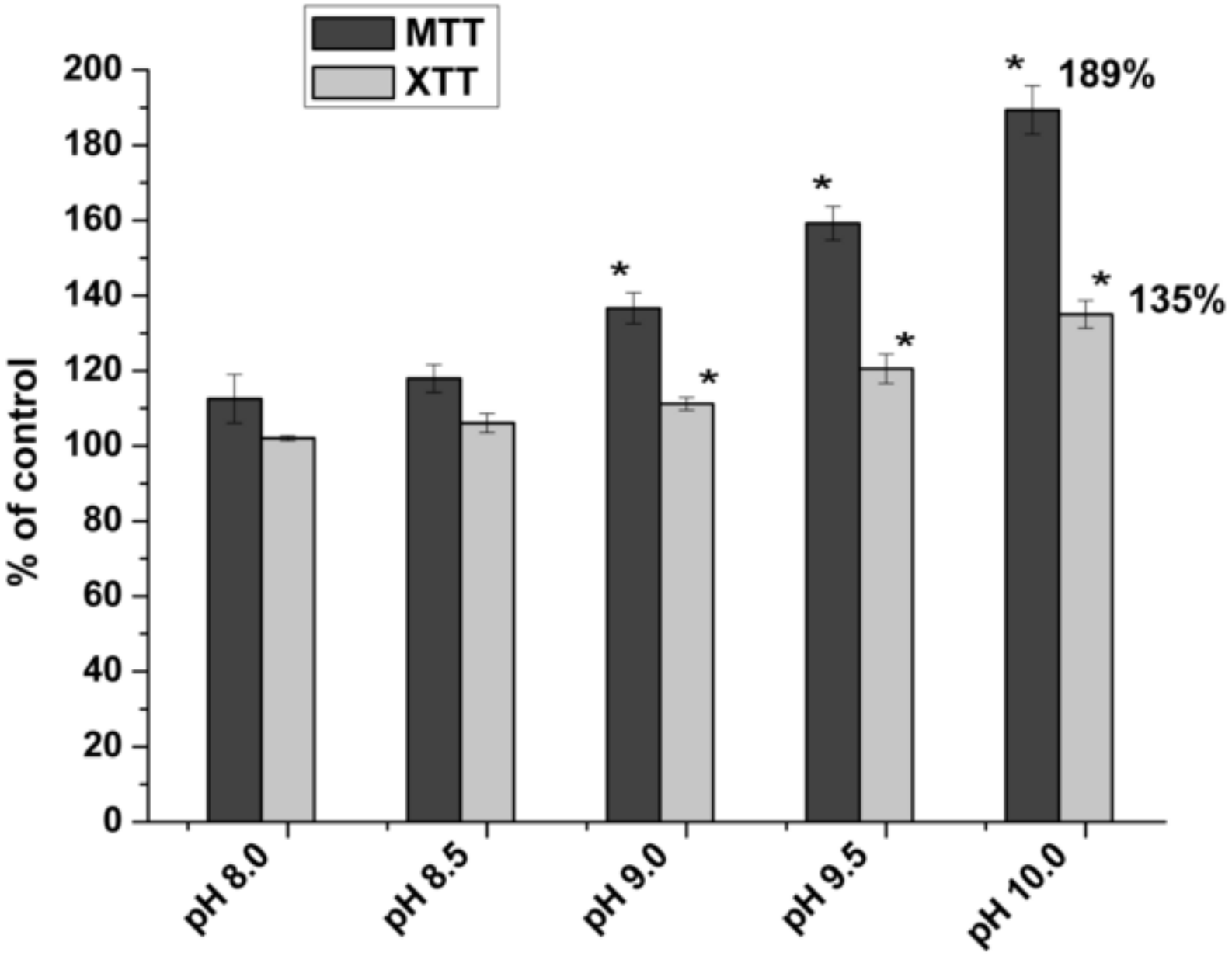

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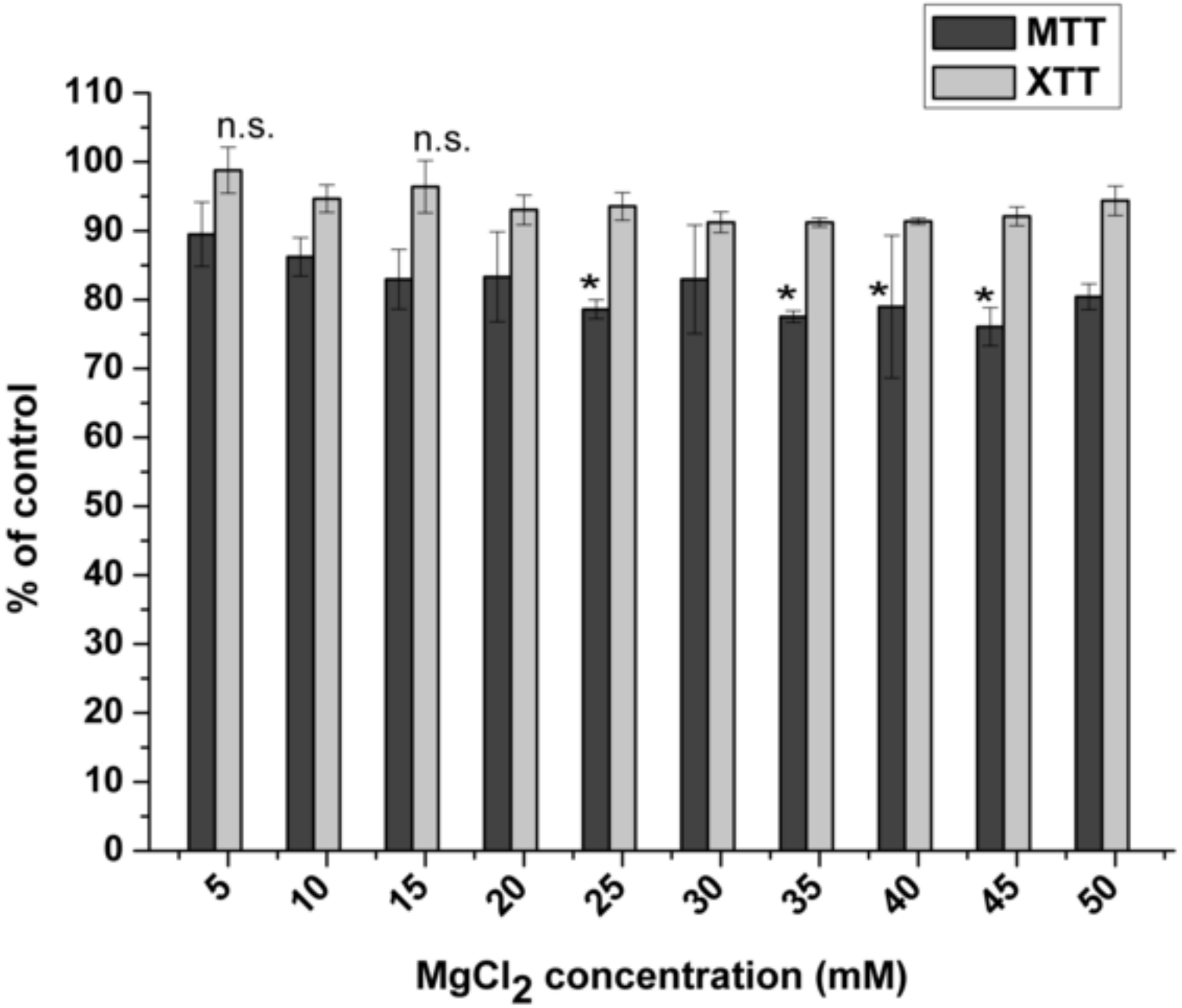

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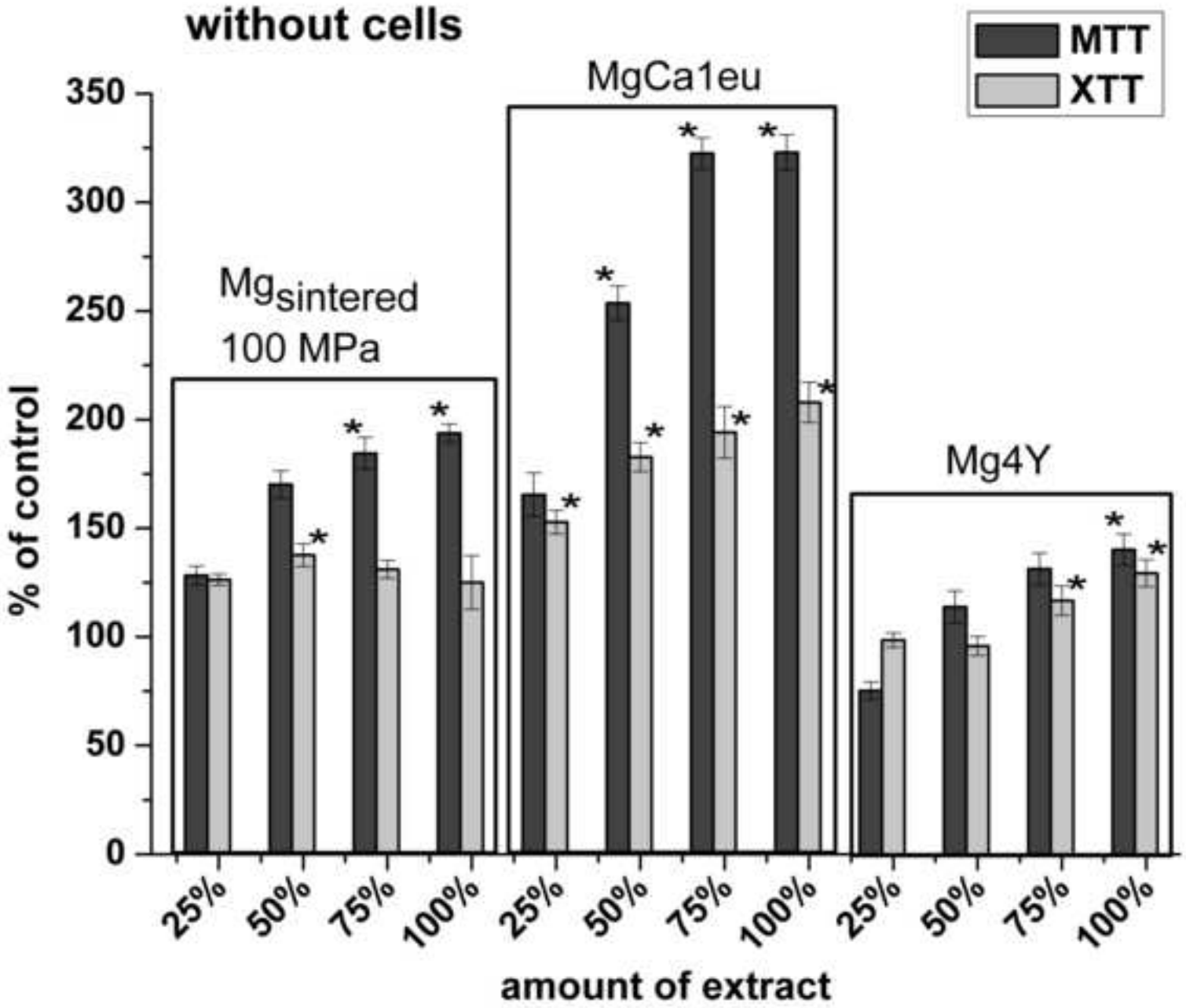

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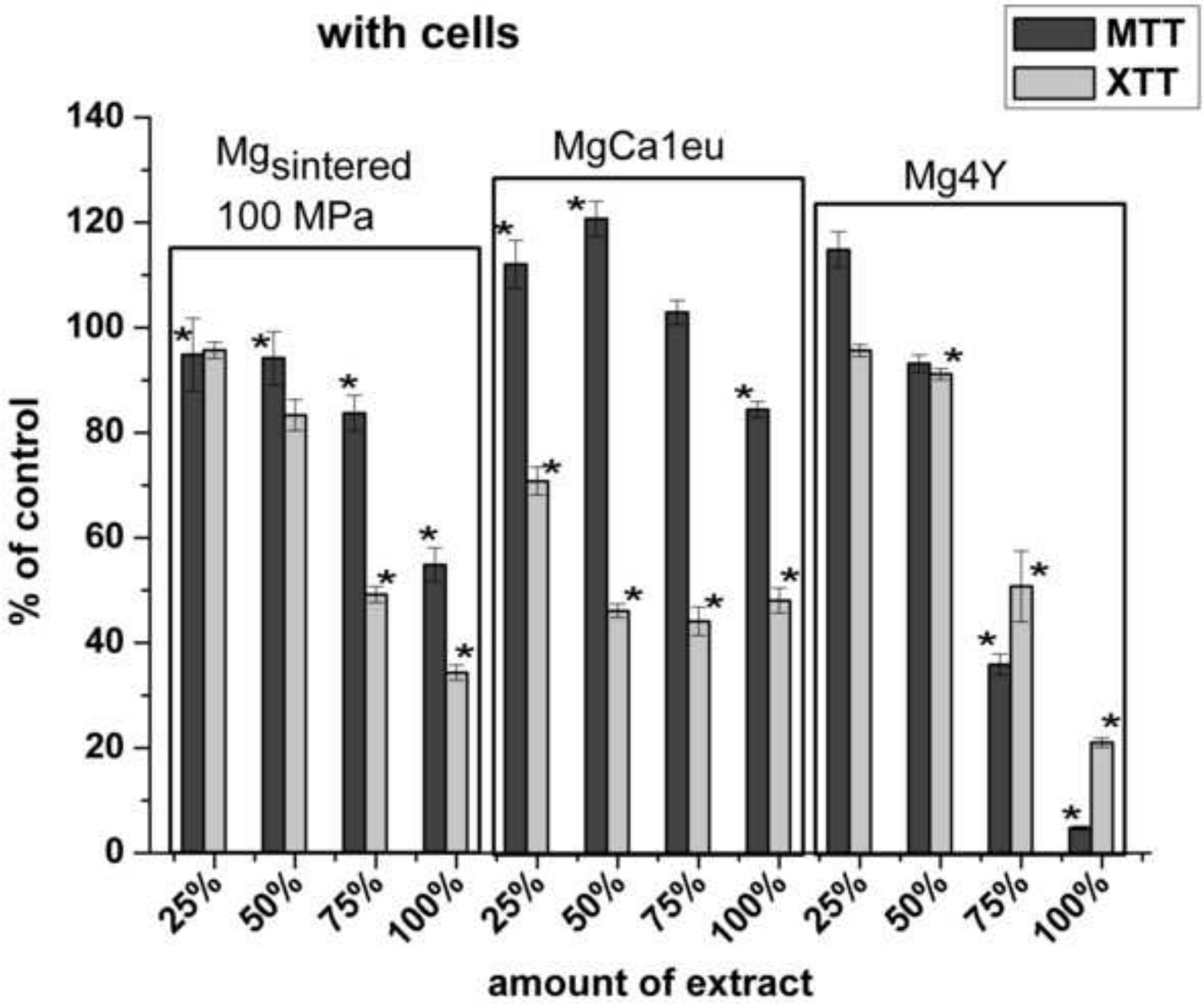

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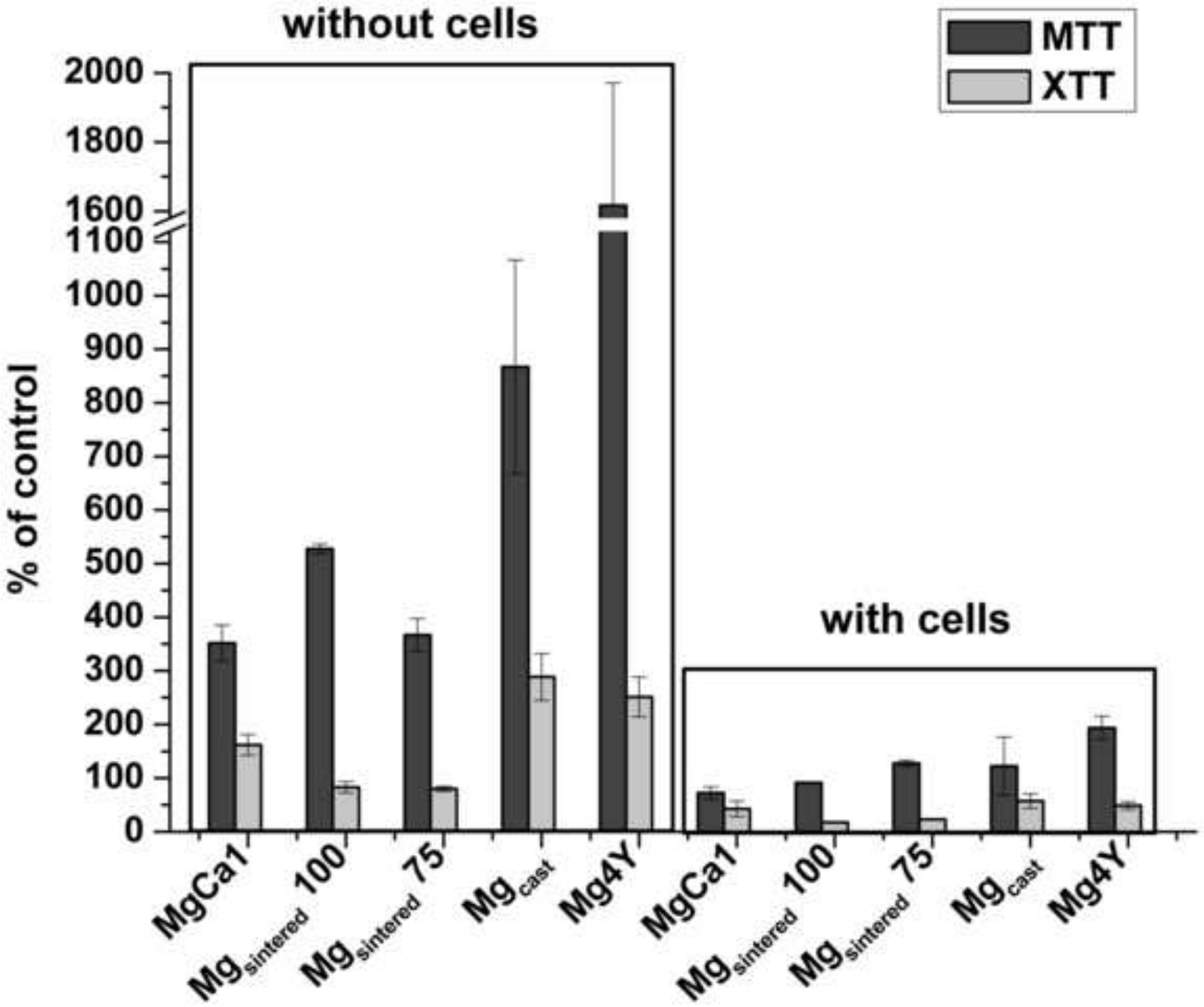

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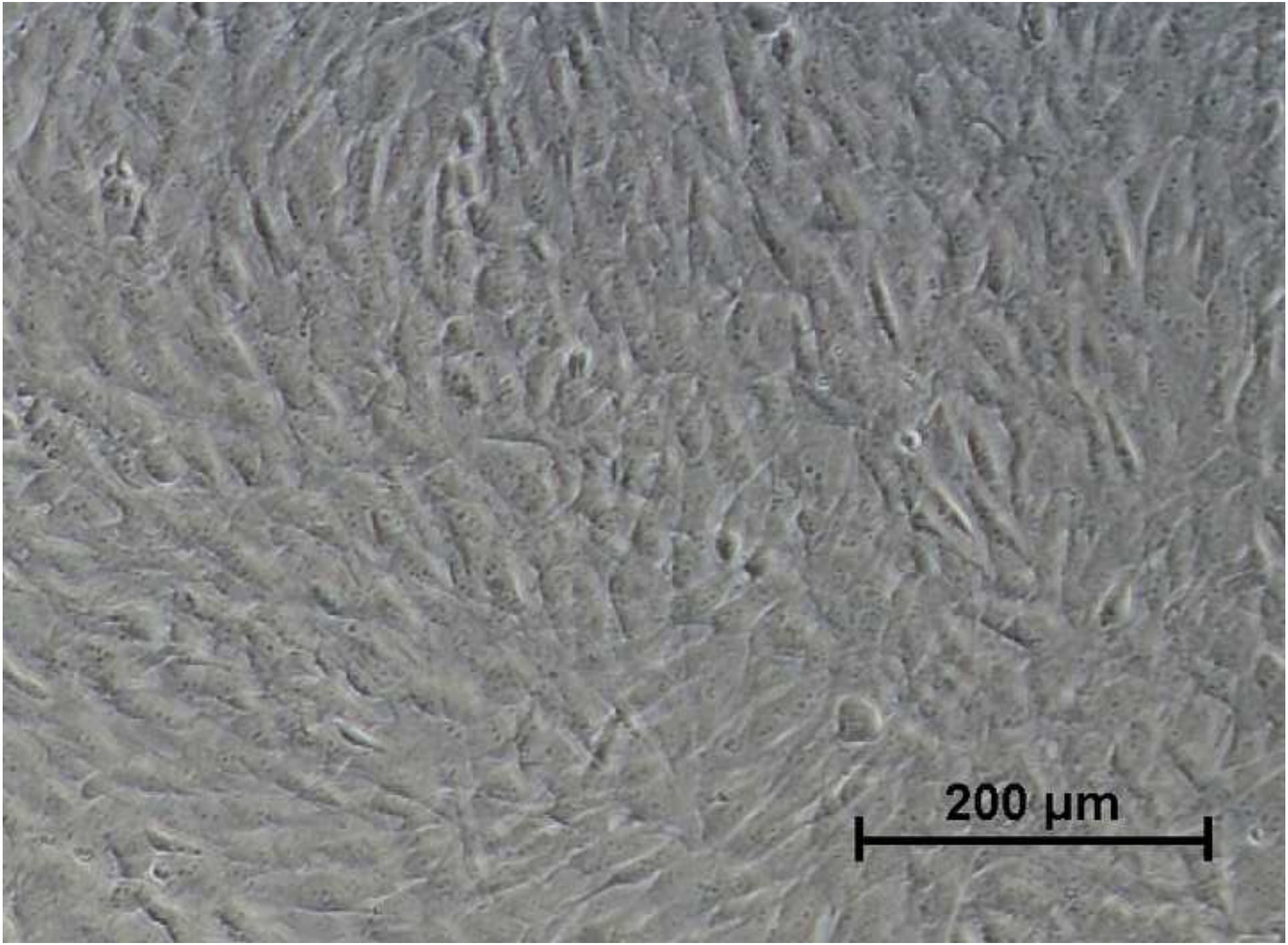

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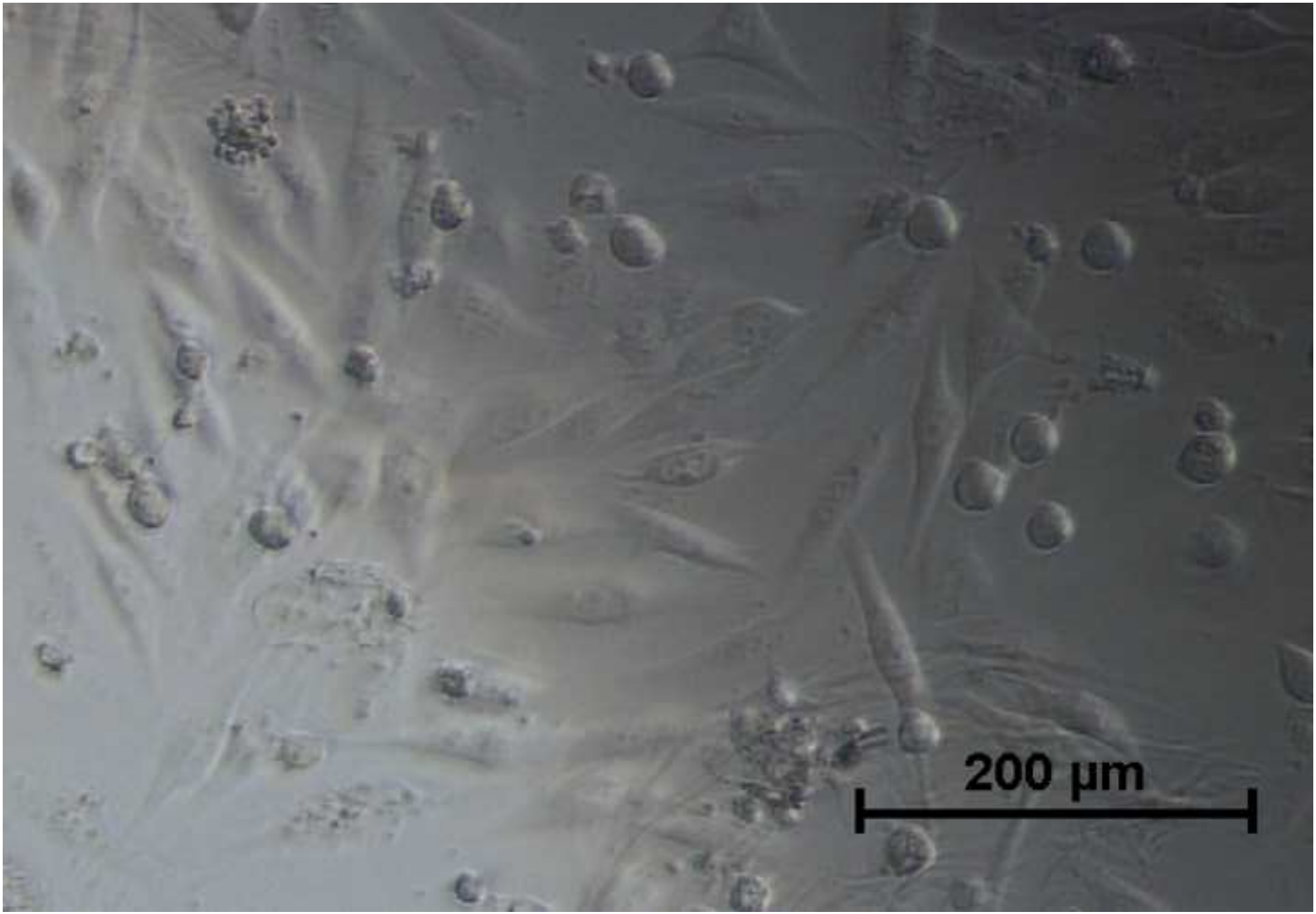

Figure 8a

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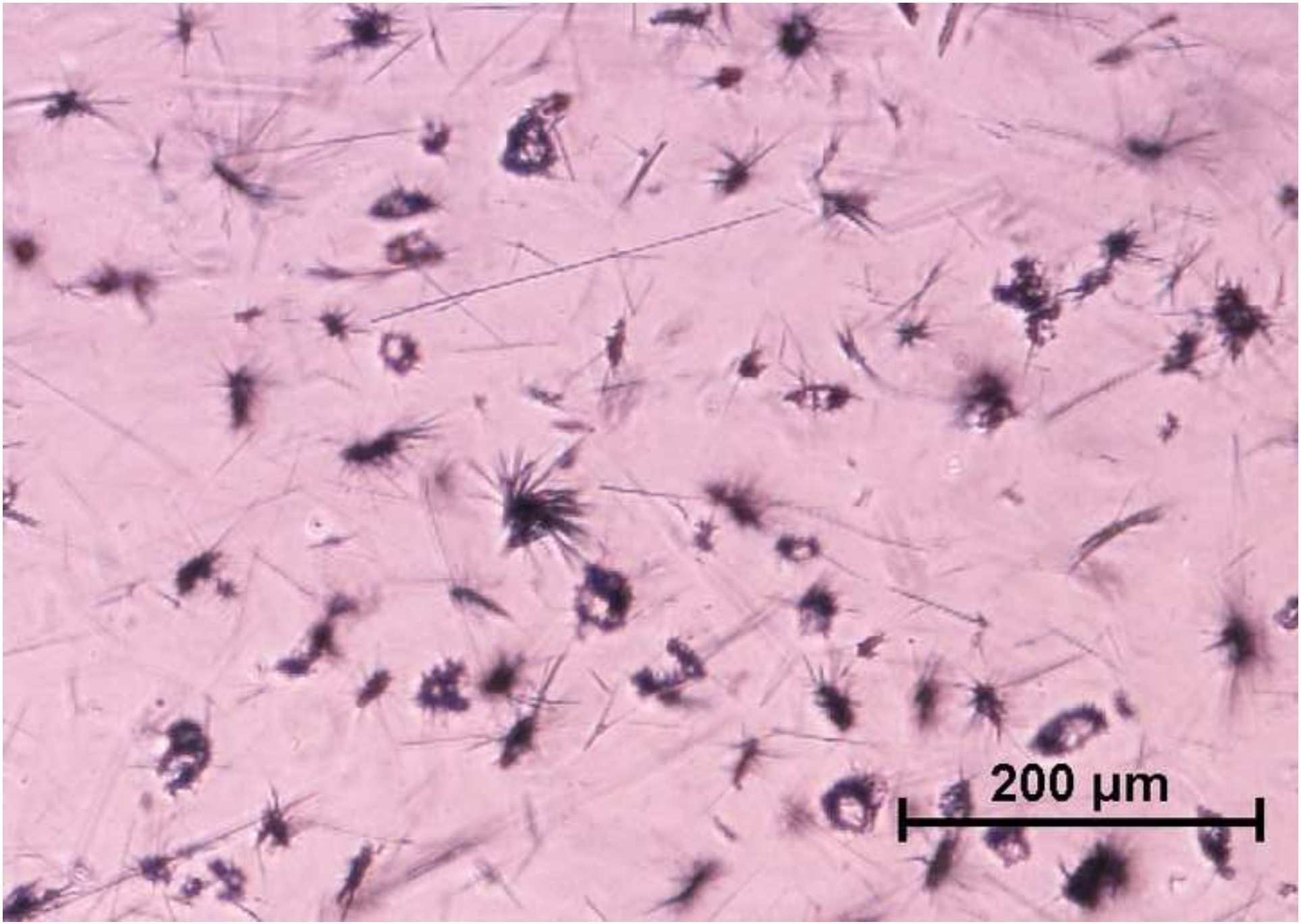

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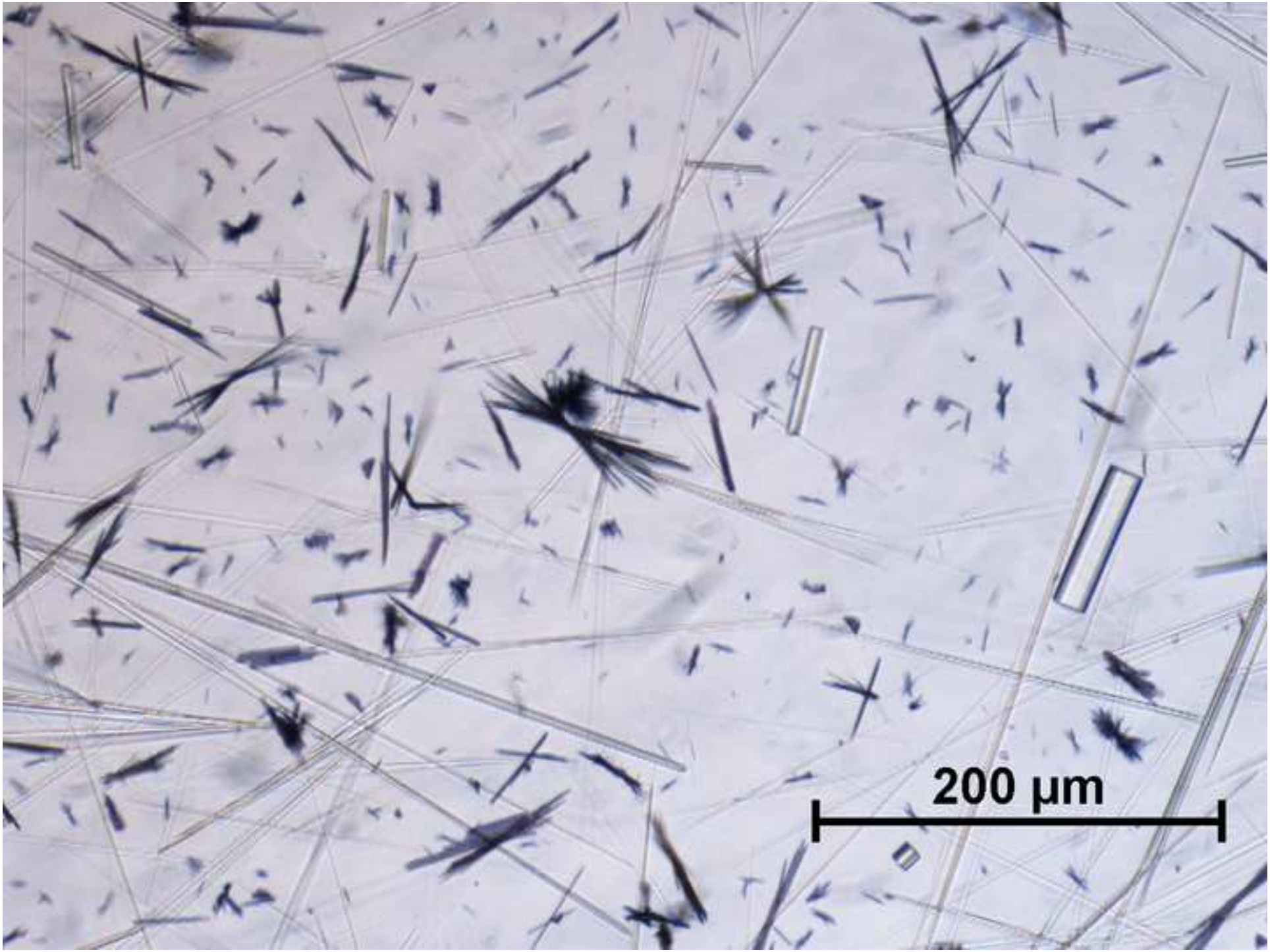

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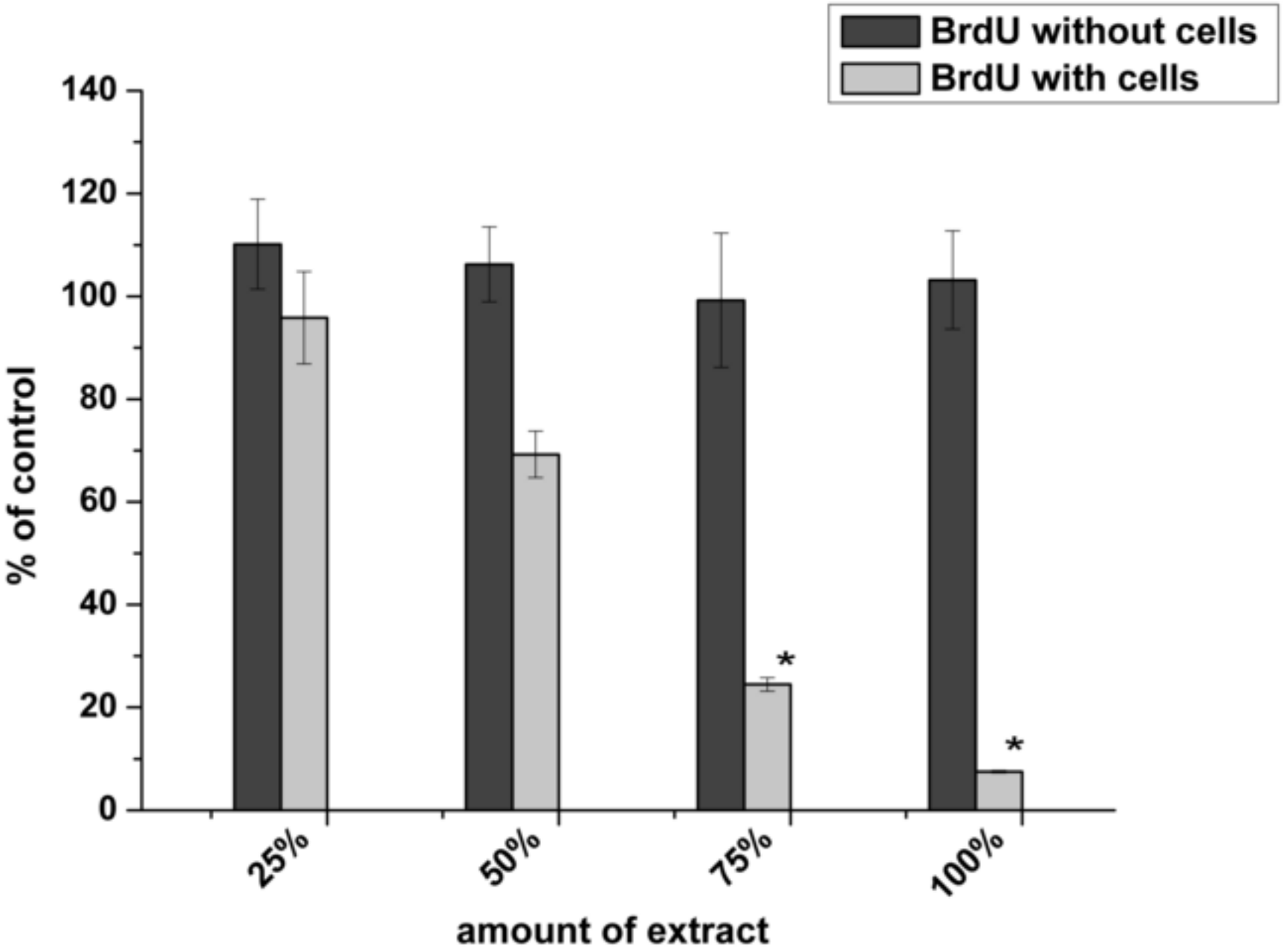

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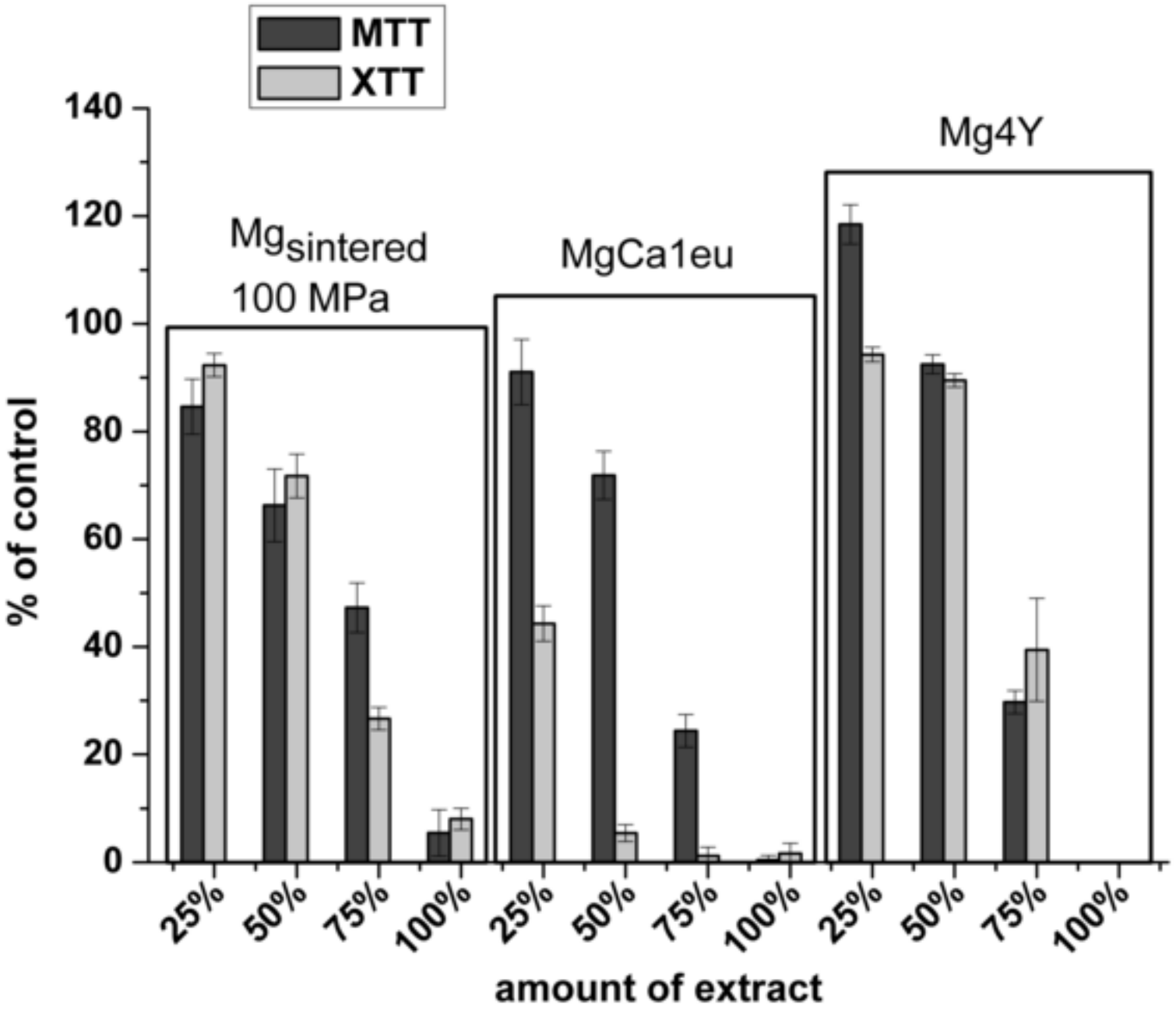

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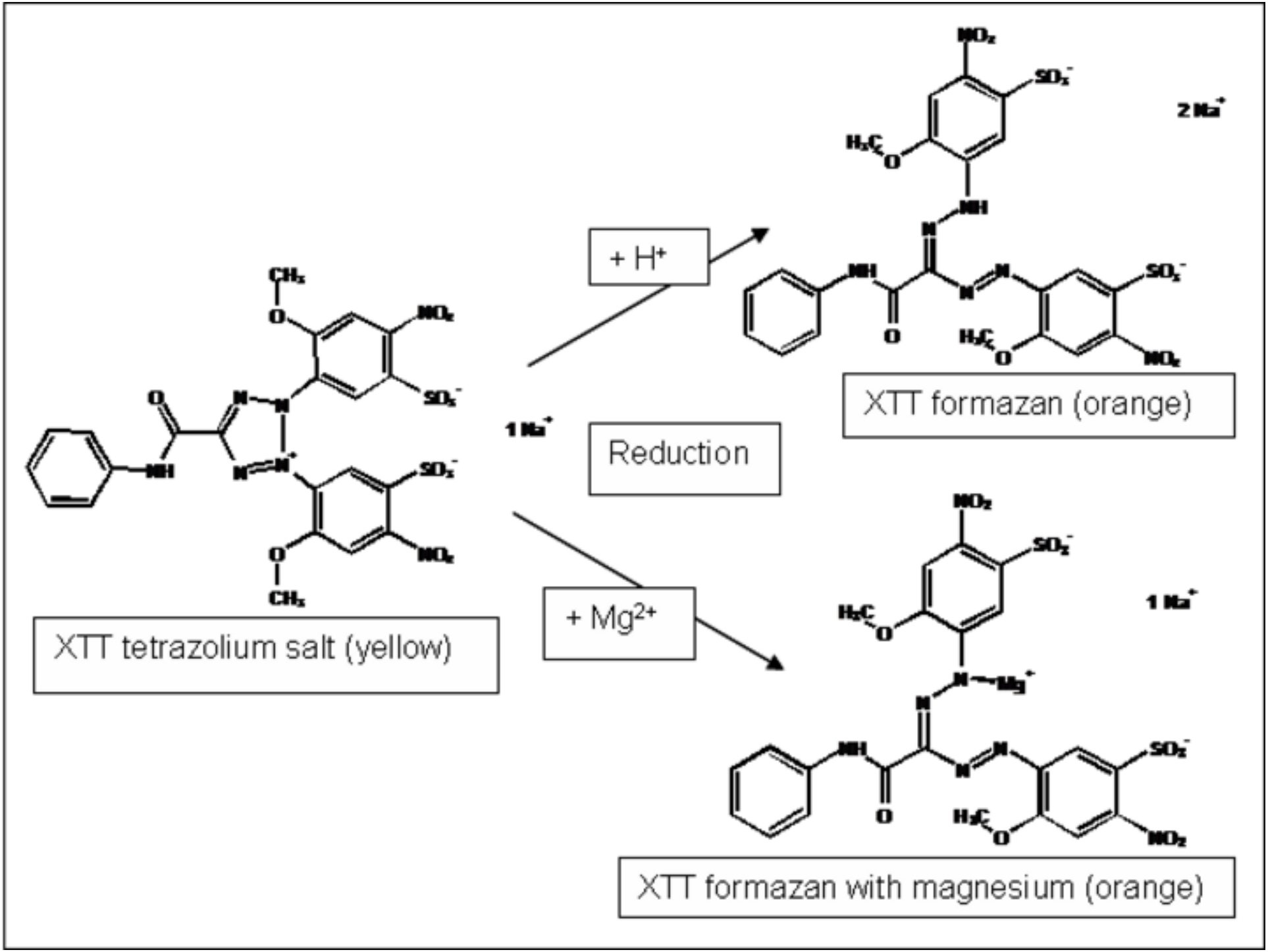

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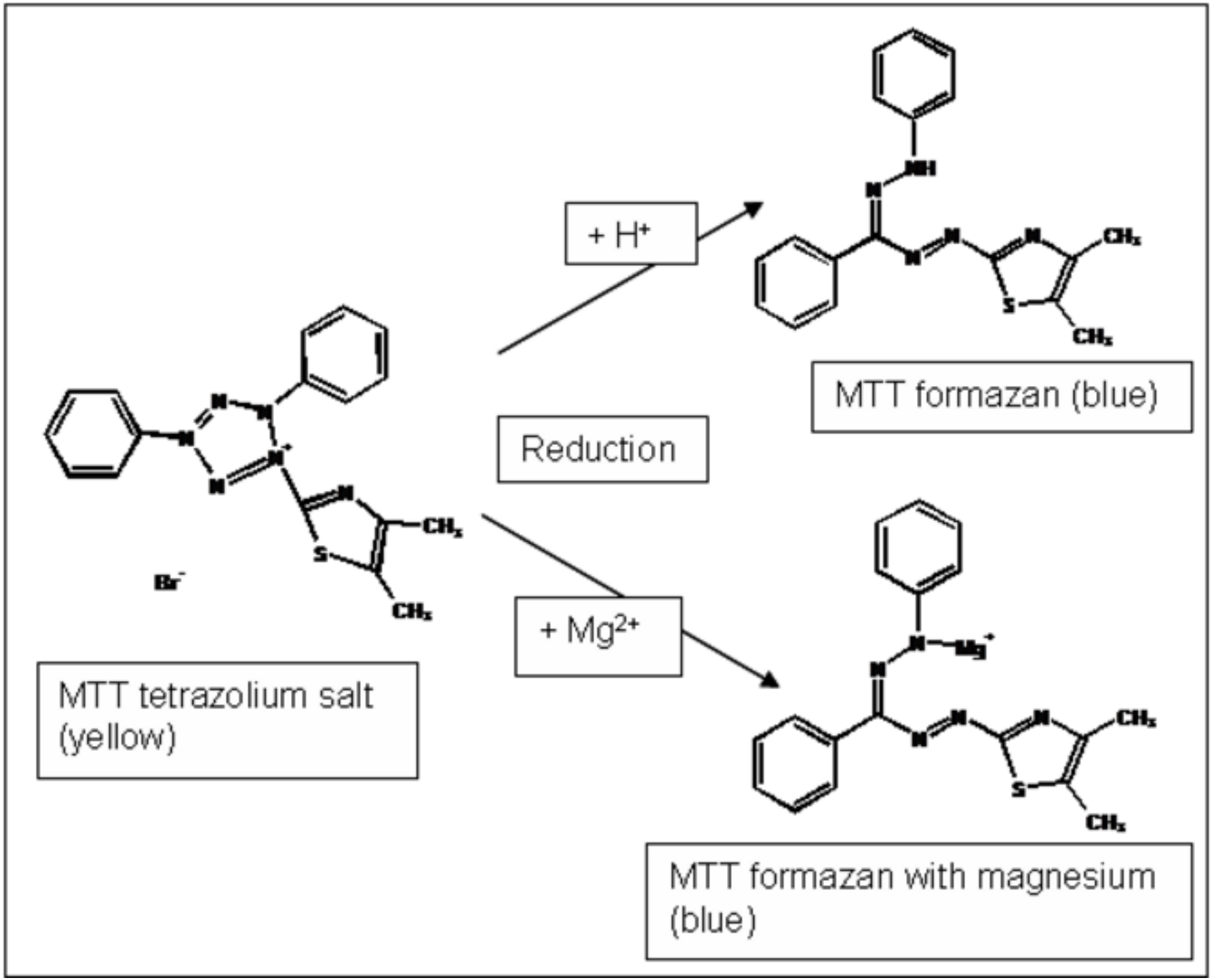

Figure 12

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