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Effects of extracellular magnesium on the differentiation and function of human osteoclasts

Lili Wu[1,2,#], Bérengère J.C. Luthringer[1,#,*], Frank Feyerabend[1], Arndt F. Schilling[2], and Regine Willumeit[1]

[1] Institute of Materials Research, Department for Structural Research on Macromolecules, Helmholtz-Zentrum Geesthacht (HZG), Geesthacht, Germany

[2] Department of Plastic Surgery and Hand Surgery, Klinikum Rechts der Isar, Technical University Munich, Munich, Germany

[#] L.W. and B.J.C.L. contributed equally and therefore share first authorship

[*] Please address correspondence to:

Dr. Bérengère Luthringer

E-mail: berengere.luthringer@hzg.de

Telephone: 0049 4152 87 1292,

Fax: 0049 4152 87 2666

Abstract

Magnesium-based implants have been shown to influence the surrounding bone structure. In an attempt to partially reveal the cellular mechanisms involved in the remodelling of magnesium-based implants, the influence of increased extracellular magnesium content on human osteoclasts was studied. Peripheral blood mononuclear cells were driven toward an osteoclastogenesis pathway *via* stimulation with receptor activator of nuclear factor kappa-B

ligand and macrophage colony-stimulating factor for 28 days. Concomitantly, the cultures were exposed to variable magnesium concentrations (from either magnesium chloride or magnesium extracts). Osteoclast proliferation and differentiation were evaluated based on cell metabolic activity, total protein content, tartrate-resistant acid phosphatase activity, cathepsin K and calcitonin receptor immunocytochemistry, and cellular ability to form resorption pits. While magnesium chloride first enhanced and then opposed cell proliferation and differentiation in a concentration-dependent manner (peaking between 10 and 15 mM magnesium chloride), magnesium extracts (with lower magnesium contents) appeared to decrease cell metabolic activity (≈50% decrease at day 28) while increasing osteoclast activity at a lower concentration (twofold higher). Together, the results indicated that (1) variations in the *in vitro* extracellular magnesium concentration affect osteoclast metabolism and (2) magnesium extracts should be used preferentially *in vitro* to more closely mimic the *in vivo* environment. **(199 words)**

Keywords: Osteoclast; Magnesium; Biodegradation; Cell culture

Introduction

Magnesium (Mg) is the second most abundant intracellular divalent cation. Consequently, Mg and its corrosion products (magnesium chloride, oxide, sulphate or phosphate in a saline environment) exhibit excellent biocompatibility (1-3). Most Mg (approximately 67%) is found in bone, 30% of which is exchangeable (present on the crystal surface of the bone). Thus, bone provides a dynamic store for maintaining the intra- and extracellular concentrations of Mg (4, 5). Magnesium is involved in diverse mechanisms (*e.g.*, acting over 300 enzymes as a cofactor or in metabolic pathways) and plays a structural role in the cell membrane and chromosomes (6, 7)). Its clinical importance is often underestimated (as serum Mg levels are not routinely tested). Magnesium is commonly noted as a calcium antagonist (8), *e.g.*, in muscle contraction/relaxation, neurotransmitter release, and action potential

conduction in nodal tissue. Additionally, the role of these elements as a "chronic regulator" (magnesium) or "acute regulator" (calcium) remains a debated question (9). Nevertheless, their fates and roles are interlocked. Under cellular magnesium deficiency (hypomagnesaemia), potassium (K) is affected. As magnesium is needed to activate sodium (Na)/K pumps, Na and K gradients will not be maintained, leading to passive intracellular loss of K and increases in intracellular Na and hydrogen ions (10). Subsequently, intracellular calcium overload will take place, as calcium transport is magnesium dependent, and Na inhibits Na/Ca exchange. This general electrolyte imbalance will lead to decreases in cell activity and vitality as well as electrical instability (*e.g.*, cardiac arrhythmia). Generally, Mg supplementation alone is sufficient to restore electrolyte homeostasis (11). As the kidney can respond rapidly to an elevated serum level of Mg, hypermagnesaemia is rare (mainly due to advanced chronic kidney disease and iatrogenic artefacts, *e.g.*, laxatives and anti-acids (12-14)), and it has therefore not seemed important to study the effects of high Mg concentrations at a cellular level.

Rising life expectancies as well as complications associated with the long-term presence of implants have increased the interest in degradable materials for bone substitution (15). This situation has led to a renaissance of magnesium as a base for alloys for orthopaedic applications. Recent advances in material science and engineering have enabled corrosion rates and mechanical properties to be specifically modulated (16) through the use of alloying elements. Mg-based implants can now provide temporary structural support. Naturally, degradation of these implants leads to a high magnesium concentration around the implant, and it has been shown that this effect can result in a welcome increase in bone mass around the implant (16, 17). While a contribution of bone-forming osteoblasts to effect has been demonstrated, the impact of potentially decreased osteoclastic resorption around the Mg-based implant is still open to discussion.

Therefore, the aim of the present study was to analyse the direct effect of increased magnesium concentrations on the differentiation and function of human osteoclasts.

Materials and Methods

Magnesium-containing solutions

A 1 M $MgCl_2$ (Sigma-Aldrich Chemie GmbH, Munich, Germany) solution was prepared in double-distilled water (ddH_2O) and subsequently sterile filtered through a membrane filter (0.2 µm; Merck KGaA, Darmstadt, Germany). Appropriate dilutions (0 (control), 2, 5, 10, 15, and 25 mM) were prepared by diluting the 1 M $MgCl_2$ solution directly in the cell culture medium.

A magnesium extract (Mg-extract) was prepared according to EN ISO standards 10993:5 (18) and 10993:12 (19) (*i.e.*, the relationship of specimen weight to the extraction medium was 0.2 g/mL). Magnesium specimens were cut from a cast ingot of pure Mg (99.95%; Helmholtz Centre Geesthacht, Geesthacht, Germany) in cuboid form (1 cm x 1 cm x 0.5 cm). After sterilisation via sonication for 20 min in 100% isopropanol (Merck, Darmstadt, Germany), the samples were incubated in extraction medium (Eagle's minimum essential medium, Alpha modification (α-MEM); Life Technologies GmbH, Karlsruhe, Germany) supplemented with 10% foetal bovine serum (FBS; PAA Laboratories GmbH, Linz, Austria) for 72 h under physiological conditions (5% CO_2, 20% O_2, 95% humidity, 37°C). The Mg-extract was diluted 1:5, 1:10, or 1:30 in cell culture medium (referred to as 5x, 10x, and 30x solutions, respectively in this manuscript; pure cell culture medium without the extract was used as a control). The Mg-extract solutions were further characterised by measuring their osmolality, pH and Mg and calcium contents. Osmolality and pH were quantified using a Gonotec 030-D cryoscopic osmometer (Gonotec, Berlin, Germany) and an ArgusX pH Meter, (Sentron Europe BV, Roden, the Netherlands), respectively. Magnesium concentrations were

measured using the metallochromic dye xylidyl blue following a colorimetric method. A 10 µL aliquot of the Mg-extract, extraction medium, or of different concentrations of $MgCl_2$ (standard curve) was mixed with 1.5 mL of a xylidyl blue solution (200 mmol/L trishydroxymethylaminomethane (Tris) buffer, pH 12, containing 0.12 mmol/L xylidyl blue II, 0.05 mmol/L ethylene glycol tetra acetic acid (EGTA, Titriplex), 69 mmol/L potassium carbonate, 2.1 mol/L ethanol, and 0.05% sodium azide (all chemicals supplied by Sigma-Aldrich Chemie GmbH, Munich, Germany). Following a 10 min incubation at room temperature (RT), absorbances of the mixtures were measured at 520 nm with an enzyme-linked immunosorbent assay (ELISA) reader (Tecan Sunrise; TECAN Deutschland GmbH, Crailsheim, Germany). As intensity of the colour is directly proportional to the magnesium content, unknown concentrations were determined using the standard curve. Calcium content was colorimetrically determined using calcium O-cresolphtalein kit (Futura system Srl, Rome, Italy) and a $CaCl_2$ (Sigma-Aldrich Chemie GmbH, Munich, Germany) standard curve. 50 µL of the samples were mixed with 2 mL reagent. After 5 min incubation at RT, absorbances of the mixtures were measured at 580 nm with a Tecan Sunrise microplate reader. Unknown concentrations were determined by the standard curve.

Cell culture

Peripheral blood mononuclear cells (PBMC) were freshly isolated from buffy coat specimens from healthy, consenting donors (purchased from the Institute for Clinical Transfusion Medicine and Immunogenetics Ulm, Ulm, Germany) via a density gradient centrifugation technique using Ficoll Paque Plus (Amersham Biosciences, Uppsala, Sweden). As described in greater detail in (20), heparinised human peripheral blood (approximately 25 mL) was diluted 1:8 in phosphate-buffered saline (PBS; Biochrom AG, Berlin, Germany), carefully layered over Ficoll Paque, and then centrifuged at 350 g for 30 min. The monocyte-enriched PBMC fraction accumulated at the interface between PBS and Ficoll Paque was carefully

collected and washed twice with PBS. PBMCs were then cultured in 48-well plates (with or without dentine chips) at a density of 2 x 10^6 cells/mL in α-MEM (Biochrom AG, Berlin, Germany) supplemented with 10% FBS (Life Technologies GmbH, Karlsruhe, Germany), 2 mM L-glutamine (Life Technologies GmbH, Karlsruhe, Germany), 1% antibiotic/antimitotic solution (PAA Laboratories GmbH, Pasching, Austria) and factors promoting osteoclastic differentiation (*i.e.*, recombinant receptor activator of nuclear factor kappa-B ligand (RANKL; Peprotech Germany, Hamburg, Germany; 40 ng/mL) in combination with a stimulator of hematopoietic precursors (macrophage colony-stimulating factor, M-CSF; Peprotech Germany, Hamburg, Germany; 20 ng/mL) at 37°C and 5% CO_2 and 95% H_2O saturation. Dentine (ivory) was kindly provided by German customs in accordance with the international laws for the protection of species. Non-adherent cells were discarded after 24 h. To evaluate the effects of $MgCl_2$ and the Mg-extract on osteoclastogenesis, different $MgCl_2$ concentrations (0 (control), 2, 5, 10, 15, and 25 mM) and Mg-extract dilutions (0 (control), 5x, 10x, and 30x in culture medium) were added to the media. Human osteoclasts were differentiated for a period of 28 days by changing 50% of the cell culture medium every two days. Biochemical tests (to determine total protein content, viability/proliferation, and tartrate-resistant acid phosphatase (TRAP) activity) were performed on days 3, 7, 14, 21, and 28 to observe the impact of $MgCl_2$ and the Mg-extract on osteoclastogenesis. TRAP staining, immunocytochemistry detection of cathepsin K (CK) and calcitonin receptor (CTR), and resorption assays (testing the cells' ability to form resorption pits) were performed at the end of the cultivation period (d28). For $MgCl_2$, the experiments were performed 3 times with 3 different donors, while for the Mg-extract, they were conducted 2 times with 2 different donors.

Total protein extraction

Total cellular proteins were extracted using radioimmunoprecipitation assay (RIPA) lysis buffer containing 10 mM Tris, pH 7.6 (Carl Roth GmbH, Karlsruhe, Germany), 100 mM NaCl (Carl Roth GmbH, Karlsruhe, Germany), 10 mM ethylenediaminetetraacetic acid (EDTA; Carl Roth GmbH, Karlsruhe, Germany), 0.5% Nonidet P-40 (Sigma-Aldrich Chemie GmbH, Munich, Germany), and 0.5% deoxycholic acid (Sigma-Aldrich Chemie GmbH, Munich, Germany). For this procedure, all solutions and materials used were previously cooled (to avoid any sample degradation). Layers of cells were rinsed twice with PBS and then covered with RIPA lysis buffer (100 µL) that had been freshly supplemented with a protease inhibitor cocktail (Sigma-Aldrich Chemie GmbH, Munich, Germany). The cells were harvested using a plastic cell scraper (TPP, Trasadingen, Switzerland) and subsequently transferred to a 1.5 mL micro-centrifuge tube. Lysis was completed on ice for 30 min, during which the solution was pipetted up and down every 10 min. The lysates were cleared of cell debris via centrifugation (10,000 g for 10 min at 4°C), and the supernatants (60 µL) were collected and either snap-frozen and stored at -80°C or directly used to determine protein contents. The protein contents were quantified using the BCA Protein Assay kit (Thermo Fisher Scientific, Bonn, Germany) according to the microplate procedure recommended by the manufacturer. A 25 µL aliquot of the bovine serum albumin standard (BSA; provided in the kit), blank (lysis buffer), or the unknown samples was mixed with 200 µL of working reagent (50:1, Reagent A:B) and incubated at 37°C for 30 min. Absorbances were then measured with a microplate reader (Berthold Technologies GmbH, Bad Herrenalb, Germany; at 560 nm), and protein concentrations were determined using a standard curve.

Cell metabolism assay

To assess cellular metabolism, water-soluble tetrazolium (WST-1, Roche Diagnostic GmbH, Mannheim, Germany) was used. WST-1 is reduced at the external surface of the plasma membrane by nicotinamide adenine dinucleotide (NADH)-oxidase (mirroring mitochondrial

electron transport, *i.e.*, the metabolic activity of the cell), forming highly coloured formazan, which absorbs at 450 nm. After 3, 7, 14, 21, or 28 days of culture, the cells were incubated with a 10% (v/v) WST-1 dye solution (n=3 wells) under cell culture conditions for 2 h. As blanks, samples of culture media (with corresponding Mg-containing solutions) without cells were treated similarly. After 2 h, 200 µL of the culture supernatant (in triplicate) was pipetted into 96-well plates, which were subsequently read using a microplate reader (Berthold Technologies GmbH, Bad Herrenalb, Germany) at 450 nm with a 620 nm reference wavelength.

TRAP activity assay

After 3, 7, 14, 21, and 28 days of culture, osteoclast differentiation was evaluated based on TRAP activity (3 biological replicates per magnesium concentration). Enzyme activity was assessed extracellularly ("extracellular TRAP", TRAP secreted into the medium, measured in cell culture supernatants). A 150 µL aliquot of the TRAP reaction solution (100 mM sodium acetate (Carl Roth GmbH, Karlsruhe, Germany), 50 mM disodium-tartrate dihydrate (Carl Roth GmbH, Karlsruhe, Germany), and 7.6 mM p-nitrophenyl phosphate disodium hexahydrate (pNPP disodium hexahydrate; Sigma-Aldrich Chemie GmbH, Munich, Germany)) was added to 50 µL of the samples in 96-well plates, followed by incubation for 1 h at 37°C. The enzymatic reaction was then stopped by adding 50 µL of a stop solution (3 M NaOH; Carl Roth GmbH, Karlsruhe, Germany), and the absorbance was measured at 405 nm (reference wavelength, 620 nm) using a microplate reader (Berthold Technologies GmbH, Bad Herrenalb, Germany).

TRAP staining

To confirm the generation of multinucleated osteoclasts, TRAP cytochemical staining was carried out after 28 days (three replicates for each magnesium concentration). Adherent cells

were rinsed with PBS and then fixed/permeabilised in a solution containing 3.7% formaldehyde (Carl Roth GmbH, Karlsruhe, Germany) and 0.2% Triton X-100 (Carl Roth GmbH, Karlsruhe, Germany) in PBS for 5 min at RT. After removal of the fixation/permeabilisation solution, the cells were dyed to detect acid phosphatase for 10-20 min at 37°C (staining solution prepared just prior to use: 0.1 mg/mL naphthol AS-MX phosphate (Sigma-Aldrich Chemie GmbH, Munich, Germany), 0.5 mg/mL fast red violet LB salt (Sigma-Aldrich Chemie GmbH, Munich, Germany), 10 mM disodium-tartrate dihydrate (Carl Roth GmbH, Karlsruhe, Germany), and 40 mM sodium acetate (pH 5.0; Carl Roth GmbH, Karlsruhe, Germany)). The staining solution was then removed and replaced with PBS. Red-stained TRAP-positive cells containing at least three nuclei were scored as osteoclasts. For quantification, the means of at least five representative fields of view per condition were determined (formulated as the number of positive cells per mm^2).

Cathepsin K and calcitonin receptor immunocytochemistry

After 28 days, CK and CTR were detected via immunocytochemistry (two replicates for each magnesium concentration). Cells were washed with PBS, fixed with 3.7% formaldehyde (Carl Roth GmbH, Karlsruhe, Germany) for 10 min at RT, and then rinsed thoroughly with PBS. The fixed cells were subsequently permeabilised (only for CK staining) with 0.1% Triton X-100 (Carl Roth GmbH, Karlsruhe, Germany) and 3% H_2O_2, 1:1 (v/v; Carl Roth GmbH, Karlsruhe, Germany), in PBS for 5 min at RT, followed by two PBS washing steps. Nonspecific binding sites were blocked by immersing the cells in 10% (w/v) BSA (Carl Roth GmbH, Karlsruhe, Germany) in PBS for 1 h at 37°C. Next, the cells were incubated with a polyclonal anti-CK or polyclonal anti-CTR primary IgG antibody (1:450 in PBS containing 1% (w/v) BSA; Santa Cruz Biotechnology, Inc., Heidelberg, Germany) for 1 h at 37°C. After two 5 min PBS washing steps, the samples were incubated with the appropriate secondary antibodies (Alexa Fluor 568 Goat Anti-Rabbit IgG or Alexa Fluor 488 Goat Anti-Rabbit IgG

(Life Technologies GmbH, Karlsruhe, Germany) and goat anti-rabbit IgG-Texas Red (Santa Cruz Biotechnology, Inc., Heidelberg, Germany) for the anti-CK and anti-CTR primary antibodies, respectively; 1:450 in PBS containing 1% (w/v) BSA). Staining omitting the primary antibodies was carried out as a negative control to identify nonspecific staining. To visualise cell nuclei, cells were counterstained with 1.5 µg/mL 4-6-diamidino-2 phenylindole (DAPI; Sigma-Aldrich Chemie GmbH, Munich, Germany) diluted in PBS for 5 min at RT.

Resorption Assay

Osteoclast activity was determined in a lacunar resorption assay using cells cultured on dentine chips (at least 3 chips per condition). At the end of the culture period (28 days), the cells were removed with 30% H_2O_2 (Carl Roth GmbH, Karlsruhe, Germany) and by wiping the surface of the chips with tissue towels. To visualise resorption lacunae, dentine chips were treated with a 1% toluidine blue (Carl Roth GmbH, Karlsruhe, Germany) solution (in ddH_2O) for at least 5 sec at RT. The chips were then rinsed twice with tap water, and the resorbed areas (pits) were detected microscopically. Five representative images were randomly captured from each dentine piece, and the area of pit resorption was quantified using the ImageJ analysis software (21). In an assay to counteract or minimise the intrinsic artefacts that can be introduced by dentine chip variation (*e.g.*, composition), the resorption activity (reported as a percentage of the resorbed area) *per* TRAP-positive cell is presented here.

Statistical analysis

Statistical analyses were performed using the SigmaStat package (Systat software GmbH, Erkrath, Germany; version 11.0). Standard analyses comparing more than two treatments were conducted via one-way repeated measures ANOVA (analysis of variance). Depending on the data distribution, either one-way repeated measures ANOVA or one-way repeated

measures ANOVA on ranks was performed with the Dunn or Holm-Sidak post-hoc test. Statistical significance was accepted if the significance level was $p<0.05$.

Results

Magnesium extract-containing solutions

To characterise the Mg-extract and Mg-extract /medium dilutions, magnesium and calcium contents were measured, as were their pH and osmolalities (Table 1).

Protein content

The PBMC of donors were cultured with media supplemented with factors promoting osteoclastic differentiation and different concentrations of $MgCl_2$ or the Mg-extract. The total protein content was measured for all conditions after 3, 7, 14, 21, and 28 days. For each time point, 0 mM was set as the control condition (100%), to which the protein contents obtained under the other concentrations were normalised. For $MgCl_2$ (Fig. 1A), a bell-shaped distribution could be observed (except for day 14). Increased $MgCl_2$ concentrations were associated with enhanced protein contents (*e.g.*, 10 mM on days 3, 21, and 28), but at higher $MgCl_2$ concentrations (especially 25 mM), this tendency was reversed, and the protein content decreased below that of the controls. For the Mg-extract (Fig. 1B), the results differed compared to those obtained for $MgCl_2$. On day 3, increased Mg-extract contents appeared to enhance protein contents. However, for the subsequent time points, a decreased protein content could be noted under increased Mg-extract concentrations.

Cell metabolism assay

To study the effect of Mg on cell metabolism, WST-1 assays were performed after 3, 7, 14, 21, and 28 days of culture. For $MgCl_2$ (Fig. 2A), on days 3 and 7, increasing WST-1 activity was associated with increased $MgCl_2$ concentrations. This trend changed on day 14, and a

bell-shaped pattern was observable, showing a maximum at 5 or 10 mM. However (as observed in the protein content results), on day 3, the Mg-extract (Fig. 2B) enhanced WST-1 activity (with a statistically significant difference detected between 0 mM and 30x). At the following time points, the Mg-extract dilutions decreased WST-1 activity (*e.g.*, statistically significant differences were detected on days 14, 21, and 28 between the control and 5x Mg-extract dilution; see Fig. 2B, right panel).

TRAP activity

Tartrate-resistant acid phosphatase activity is an important cytochemical marker of osteoclasts. TRAP serum levels are used as a biochemical marker reflecting osteoclast differentiation. Therefore, extracellular TRAP activity was measured over time to monitor the effect of Mg on osteoclast activity. TRAP release was calculated as a percentage of the control release. $MgCl_2$ (Fig. 3A) had almost no influence on TRAP activity (see the statistical analyses, Fig. 3A left panel). However, for the Mg-extract (Fig. 3B), a clearer trend was observed. A constant increase in activity associated with decreased dilutions was observable over days (*e.g.*, a statistically significant difference was detected on day 14 between the control and 5x Mg-extract dilution; see Fig. 3B right panel)

TRAP staining

TRAP is released in the surrounding medium but is also found at high quantities in active osteoclasts. Therefore, TRAP staining was performed on day 28, when cells were expected to display all of the hallmarks of fully differentiated cells. For $MgCl_2$ (Fig. 4A), a bell-shaped distribution was recognisable. An increase in the number of TRAP-positive cells was observed up to the addition of 15 mM $MgCl_2$, followed by a decrease at 25 mM (falling below the control; *e.g.*, a statistically significant difference was detected between the 25 mM and 15 mM treatments; see Fig. 4A5). However, in the Mg-extract treatments, a decrease in the

number of TRAP-positive cells was observed at decreased dilutions (Fig. 4B; statistically significant differences between 0 mM and 10x and between 0 mM and 5x).

Cathepsin K immunocytochemistry

This protease, which is predominantly expressed in active osteoclasts (lysosome), is a well-known osteoclast marker and was assessed *in cyto* at day 28. The number of CK-positive cells initially decreased under treatment with 2 mM $MgCl_2$ (statistically significant differences between 2 mM and 0 mM, 5 mM, 10 mM, 15 mM, and 25 mM; see Fig. 5A5). The number of positive cells then remained stable up to 25 mM, when even fewer positive cells were observed than in the control. For the Mg-extract (Fig. 5B), a continuous decrease in the CK-positive cell count was observed at decreased dilutions of the extract (statistically significant decreases between the control (pure cell culture medium) and 30x, 10x, and 5x treatments; see Fig. 5B5).

Calcitonin receptor immunocytochemistry

The calcitonin receptor is a high-affinity receptor for the peptide hormone calcitonin and contributes to calcium homeostasis and the regulation of osteoclast-mediated bone resorption. As observed for CK staining, under treatment with $MgCl_2$, an increased magnesium content first decreased (2 mM), then increased (up to 15 mM) and, finally, decreased (25 mM) the number of CTR-positive cells (for statistical significances, see Fig. 6A5). For the Mg-extract, no significant variation was observed.

Resorption assay

To study the effect of magnesium on the osteoclast resorption capability, resorption activity was assessed on day 28 on dentine chips. Taking the results (Fig. 7A) together, it can be stated that resorption activity increased up to a certain $MgCl_2$ concentration and then decreased (statistically significant differences between 10 and 25 mM; see Fig. 7A5). For the

magnesium extract, resorption activity surprisingly increased up to a 5x dilution (statistically significant differences; Fig. 7B5).

Discussion

Magnesium chloride and a magnesium extract were chosen to study the *in vitro* effects of high/non-physiological extracellular magnesium contents on the ability PBMC to form fully differentiated and active osteoclasts. PBMC were concomitantly driven along an osteoclastic pathway by supplementing their culture medium with RANKL and M—CSF, which are well-known inducers of osteoclastogenesis (22, 23). Classical osteoclast markers, such as tartrate-resistant acid phosphatase, matrix metalloproteinase cathepsin K, calcitonin receptor, and resorption pits, were either examined over the course of 28 days or assessed at the end of the assay.

The M-CSF and (especially) RANKL cytokines are key players in osteoclastogenesis. Binding of M—CSF to its cell surface receptor (colony-stimulating factor 1 receptor, or c-Fms) induces cell proliferation, survival, and the expression of RANK (the RANKL receptor). Concomitantly, the RANKL/RANK interaction leads to the recruitment of tumour necrosis factor receptor-associated factor 6 (TRAF6), which will in turn, activate the mitogen-activated protein kinase (MAPK) pathway(s). MAPK then participates in signal transduction pathways resulting in downstream activation of transcription factors such as the nuclear factor of kappa light polypeptide gene enhancer in B-cells (NFƙB) and microphthalmia-associated transcription factor (MITF) (24). MITF will then control the expression of genes involved in osteoclast differentiation such as TRAP and CK (25, 26). TRAF6 recruitment also leads to the production of reactive oxygen species (ROS) *via* ras-related C3 botulinum toxin substrate 1 (Rac1) and NADPH oxidase 1 (Nox1). ROS play a positive regulatory role in triggering the MAPK pathway (27). Nox-1 is a membrane-localised NADPH oxidase. Plasma membrane NADPH oxidases are also efficient WST-1 reducers (28). Therefore, WST-1 can be used to

assess cell metabolic activity *via* NADH/NAD^+ (which can be directly linked to cell metabolism/viability). In the experiments involving $MgCl_2$, it was observed for all donors that a burst of formazan production was detected prior to an increase in extracellular TRAP activity (as well as total protein content). Based on the WST-1 results, it can be assumed that $MgCl_2$ does not hinder cell viability, as WST-1/formazan production is always at least in the same range as observed for the control samples.

Considering the results regarding total protein contents, TRAP activity and staining, and CTR and CK immunocytochemistry, it appears that up to a certain concentration, $MgCl_2$ increases the formation of osteoclasts, and further concentration increases then reverse this phenomenon. Additionally, based on analysis of resorption activity divided by the number of TRAP-positive osteoclasts (*i.e*., resorption per osteoclast), it can be stated again that up to a certain $MgCl_2$ concentration, the resorption activity per positive cell is increased, followed by a decrease.

Furthermore, a positive effect of Mg on cell proliferation was reported in the 1970s (29) and more recently in endothelial and epithelial cells (30, 31). Mg is involved in different steps of the cell cycle (*e.g*., initiation of DNA synthesis and modification of the cytoskeleton (32, 33)), and it has been postulated to be a key regulator (in "the membrane, magnesium, mitosis (MMM) model of cell proliferation control" (29, 33)). Up-regulation of cell cycle inhibitors (*e.g.,* p53) has also been detected during Mg deprivation (9). The key role of Mg most likely occurs because (I) Mg is an allosteric modulator of numerous processes, and (II) adenosine triphosphate (ATP) must be bound to Mg to be biologically active (34) (transphosphorylation is a crucial mechanism in numerous processes, such as signal transduction pathways or energy metabolism). However, this positive effect of Mg is not indefinite, as it is dependent on the available pool of ATP.

It has recently been reported that magnesium deficiency increases osteoclast formation but inhibits the activity of these cells (35). Increased osteoclastogenesis was also observed in the present study, but an increased activity was detected as well. How can these results be compatible? The beginning of an explanation can be found by examining TRAP enzyme biochemistry. TRAP contains 1.7 mol of magnesium per mol of enzyme, and it has been found that 1 mM $MgCl_2$ increases the enzymatic activity of TRAP by approximately 20% (36). Moreover, Mg directly increases the number of TRAP-positive cells (chondroclasts and osteoclasts) and positively influences the activity of bone-resorbing cells *in vivo* (37, 38). However, concomitant treatment with calcitonin abolishes the effect of Mg.

Furthermore, after extensive studies on the effect magnesium on cell proliferation, Harry Rubin revealed the central role of mTOR (mechanistic target of rapamycin; (33)). mTOR is a phosphatidylinositol-3-kinase (PI-3K)-related kinase and plays a role as a central regulator of cellular metabolism, growth, and survival (*e.g.*, regulating lipid synthesis, mitochondrial biogenesis, and organisation of the cytoskeleton). An increase in free Mg^{2+} will lead to increased $MgATP^{2-}$ levels and activate mTOR, which displays a high Michaelis constant (K_m) for $MgATP^{2-}$ (*i.e.*, mTOR enzyme kinetics are directly affected by the concentration of the substrate $MgATP^{2-}$). PI-3K is activated downstream the M—CSF/c-Fms pathway (39). mTOR plays an essential role in osteoclast survival (via M—CSF and RANKL signalling, (40, 41)). Increased Mg levels would therefore lead to a higher survival rate of the cells.

Osteoclast adhesion and migration are two mechanisms involved in resorption (42). Adhesion is essential for the initiation of bone resorption (for formation of the sealing zone), mainly *via* αv β3 integrin, which is able to recognise matrix proteins such as osteopontin (43). Osteoclasts express at least two α-integrins (α2 and αv) and three β-integrins (β1, β3, and β5) (44). Integrin-substrate (or cell-to-cell) binding requires divalent cations (45). Several

publications have indicated that Mg^{2+} increases the binding of integrins to a greater extent than Ca^{2+}, which can even be irreversible (46, 47). Therefore, primary adhesion may be facilitated but can be permanent, thereby inhibiting further osteoclast migration and function.

Considering these arguments together, it can be suggested that $MgCl_2$ enhances proliferation and osteoclast function up to a certain concentration, which is exactly what we observed for $MgCl_2$.

However, the biphasic effect of $MgCl_2$ was not found for the Mg-extract. On the contrary, WST-1, protein contents, TRAP staining, and CK staining were consistently decreased under an increased Mg-extract content. However, at a high Mg-extract content, the resorption per osteoclast was increased. Additionally, the number of CTR-positive cells was not significantly affected by the Mg-extract.

Osmolality and pH were both high (Table 1), which are expected to be two negative parameters for osteoclast formation and activity (for the two Mg-extract donor experiments, 1x and 2x dilutions were tested, but the cells had all died on day 3). *In vitro* studies have highlighted the important roles of pH (*i.e.*, acidic conditions can stimulate osteoclast activity *in vitro*) (48, 49). The work of Shibutani and Heersche (50) conducted in osteoclasts from neonatal rabbits shows that osteoclast differentiation and proliferation are optimal at pH 7.0-7.5, while at pH 6.5-7, resorptive activity is enhanced. In the present study, the pH ranged from 8.35 to 7.8. A negative effect on metabolic activity could be seen in the WST-1 results, whereas protein contents and cell differentiation were less affected. It must be stressed that one of the main differences between $MgCl_2$ and the Mg-extract was the calcium depletion in the pure Mg-extract (see Table 1). As noted in the Introduction, the Mg/Ca balance is very important for bones and for homeostasis in general. Circulating Ca^{2+} plays an important role in bone turnover. When Ca^{2+} levels are high, calcitonin (primarily released by the thyroid) will inhibit osteoclast activity in bones. In contrast, when the Ca^{2+} level decreases, calcitriol

(the hormonally active form of vitamin D) and parathyroid hormone (PTH) promote bone resorption *in vivo*. Rubin and co-workers have extensively studied the effects of Mg^{2+} and Ca^{2+} omission, depletion, and excess. Omission of Ca^{2+} from the medium results in a striking increase in cell permeability (51). Mg^{2+} can apparently substitute for Ca^{2+} in maintaining normal permeability, but in a less-efficient manner, as 5-10 mM Mg^{2+} is required to maintain Na^{2+} and K^{+} at normal levels in the absence of Ca^{2+}. Ca^{2+} depletion also causes a shift of Mg^{2+} away from ATP toward other binding sites in the cell (52). Magnesium may therefore not exert the effect observed under $MgCl_2$ supplementation.

It has been reported that transient receptor potential melastin 7 (TRPM7, an inward transporter of Mg) senses osmotic gradients rather than ionic strength, and hypertonic conditions inhibit TRPM7 (53). The positive effects of Mg described above under $MgCl_2$ treatment would then be abolished (resulting in no or a reduced increase in the intracellular Mg content) until the extracellular osmolality was restored. Indeed, in our experiments, we also observed that when osmolality was returned to normal, osteoclast activity was enhanced.

For cells of the nervous system, it has been shown that voltage-dependent Ca channels (VDCCs) can be blocked by external magnesium ions (54). The VDCCs are a group of voltage-gated ion channels (activated by changes in electrical potential near the channel) that are generally found in excitable cells. Long-lasting calcium channels (L-type) are also found in osteoclasts and may promote movement (55). Magnesium blockage would then explain the increased resorption observed at lower magnesium concentrations in the case of the Mg-extract (compared to $MgCl_2$).

With respect to the *in vivo* degradation of magnesium-based implants, magnesium extracts are believed to be the closest *in vitro* model (18, 19). Furthermore, magnesium extracts exhibit different effects than $MgCl_2$, as in the present study, it was found that a lower magnesium content (Table 1) could increase osteoclast function. Such differences have

previously been highlighted in primary human osteoblasts, where extracts have been found to increase the expression of genes involved in bone metabolism (*e.g.*, osteocalcin) in a more effective manner than $MgCl_2$ (56, 57).

Conclusion

$MgCl_2$ and a magnesium extract exhibited different direct effects on osteoclast proliferation and differentiation. $MgCl_2$ was able to enhance proliferation and osteoclast function up to a concentration of approximately 15 mM. The magnesium extract appeared to reduce cell metabolism, while protein contents and cell differentiation were less affected. Both substances activated osteoclastic resorption activity, but the magnesium extract exerted its positive effect at a lower magnesium content.

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Study design: LW, BL, FF, AS, and RW. Study conduct: LW. Data collection: LW. Data analysis: LW and BL. Data interpretation: LW and BL. Drafting manuscript: BL and LW. Revising manuscript content and approving final version of manuscript: LW, BL, FF, AS, and RW. BL takes responsibility for the integrity of the data analysis

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Figure

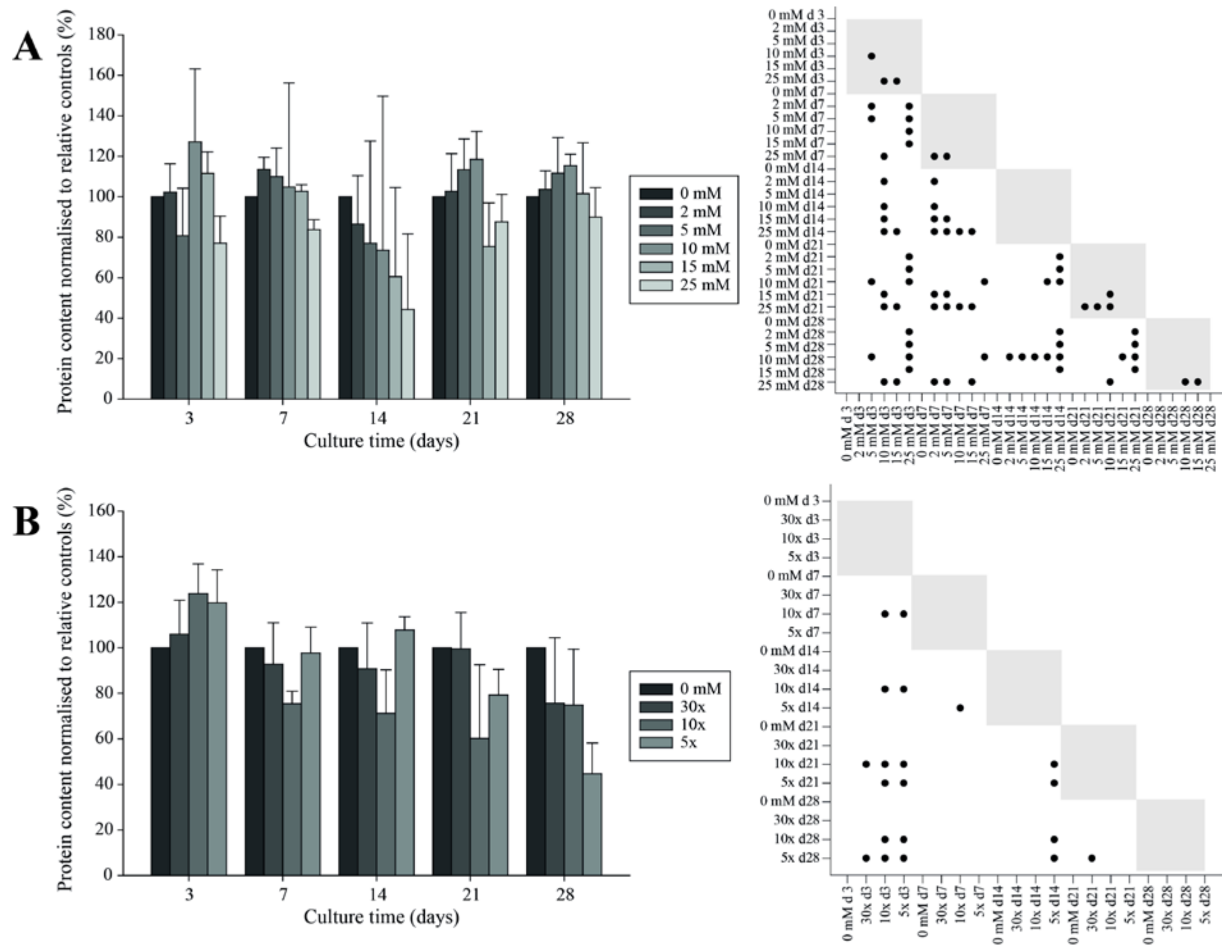


Fig. 1: Protein content. Protein contents of donor cells treated with $MgCl_2$ (A) and donors treated with magnesium extract (B). The respective statistical analyses are presented in multiple comparison graphs (right panel). Symbols indicate significant differences.

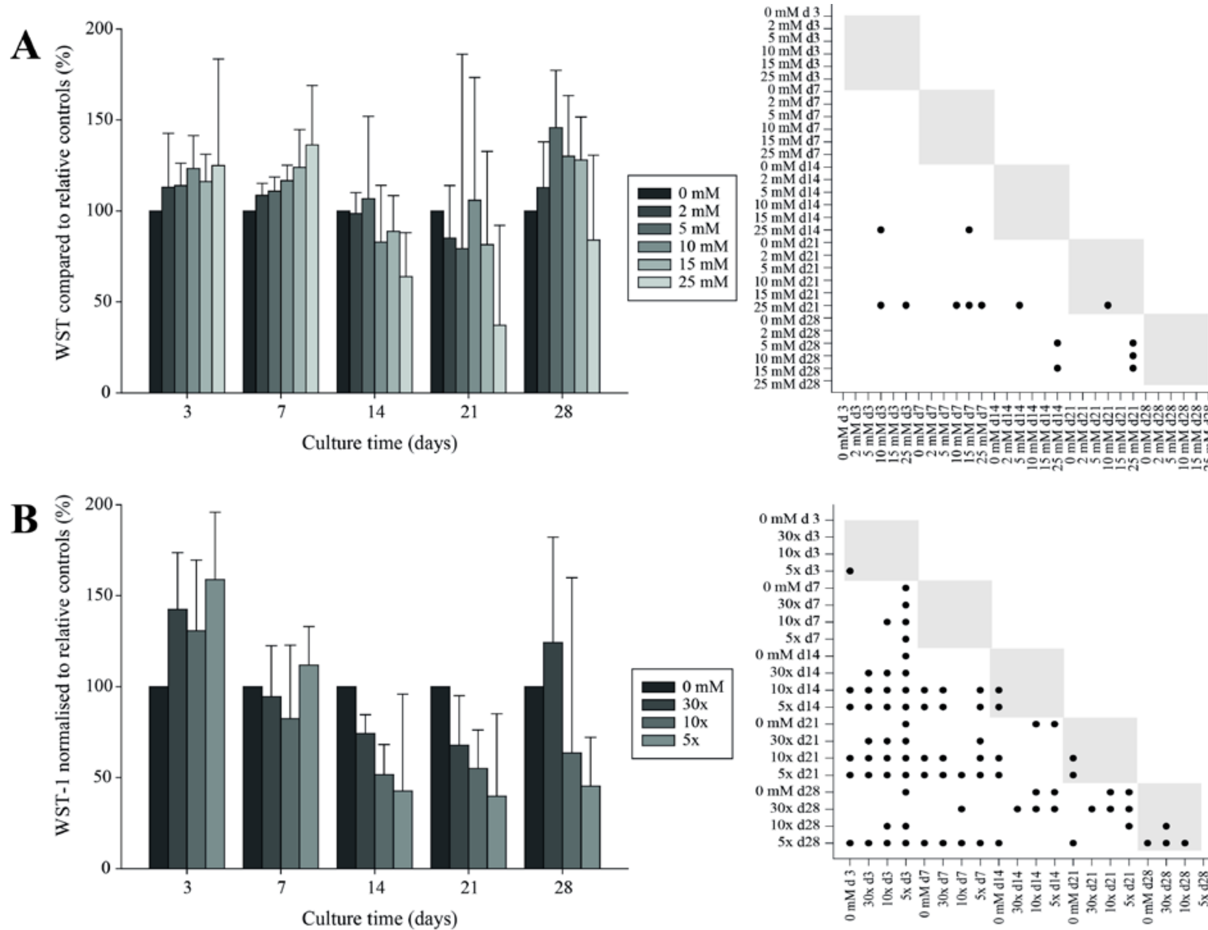


Fig. 2: Cellular metabolic activity. WST-1 measurements from donors treated with $MgCl_2$ (A) and donors treated with magnesium extract (B). For each time point, 0 mM was set as the control (100%), to which the WST-1 activities for the other concentrations were normalised. Respective statistical analyses are presented in multiple comparison graphs (right panel). Symbols indicate significant differences.

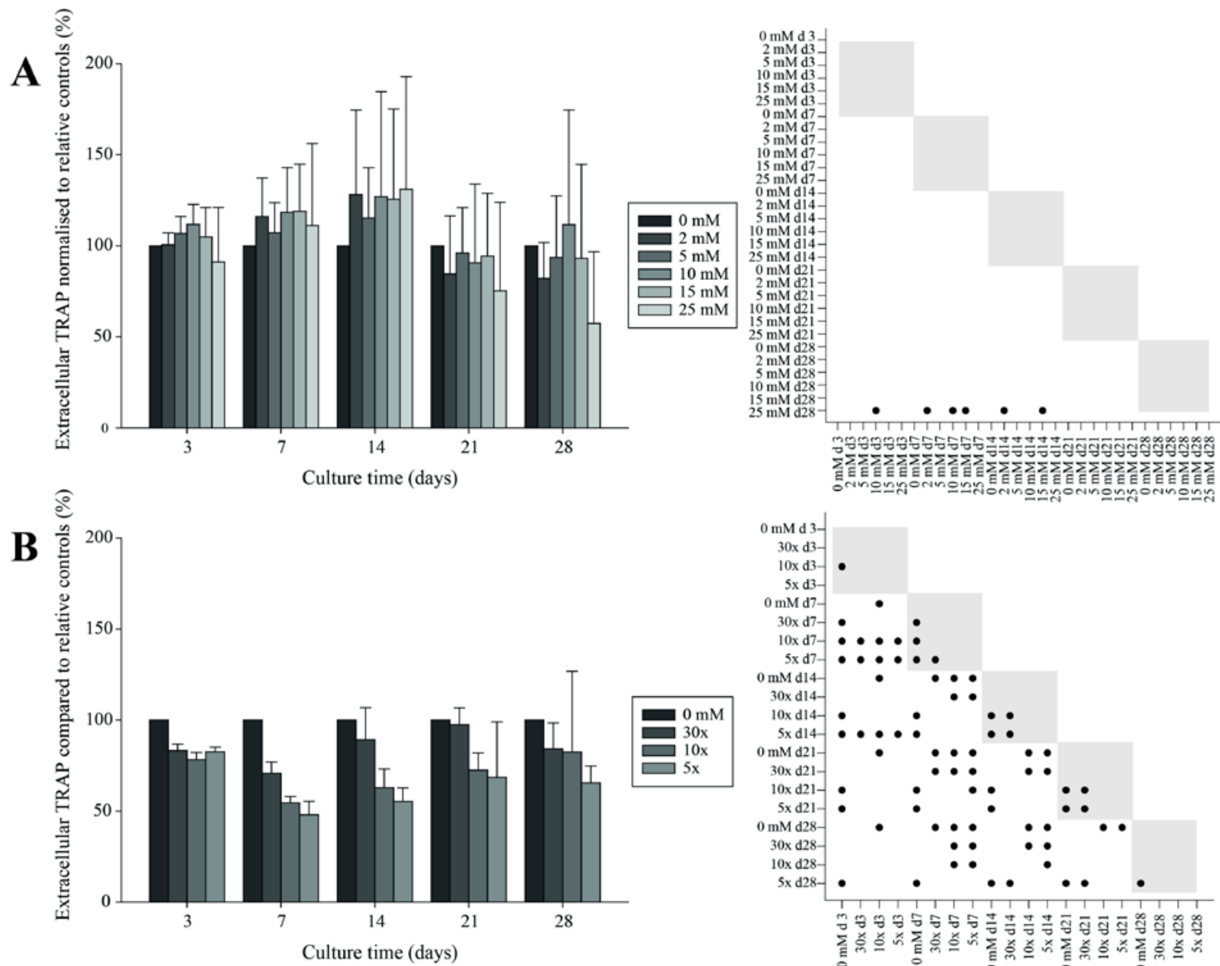
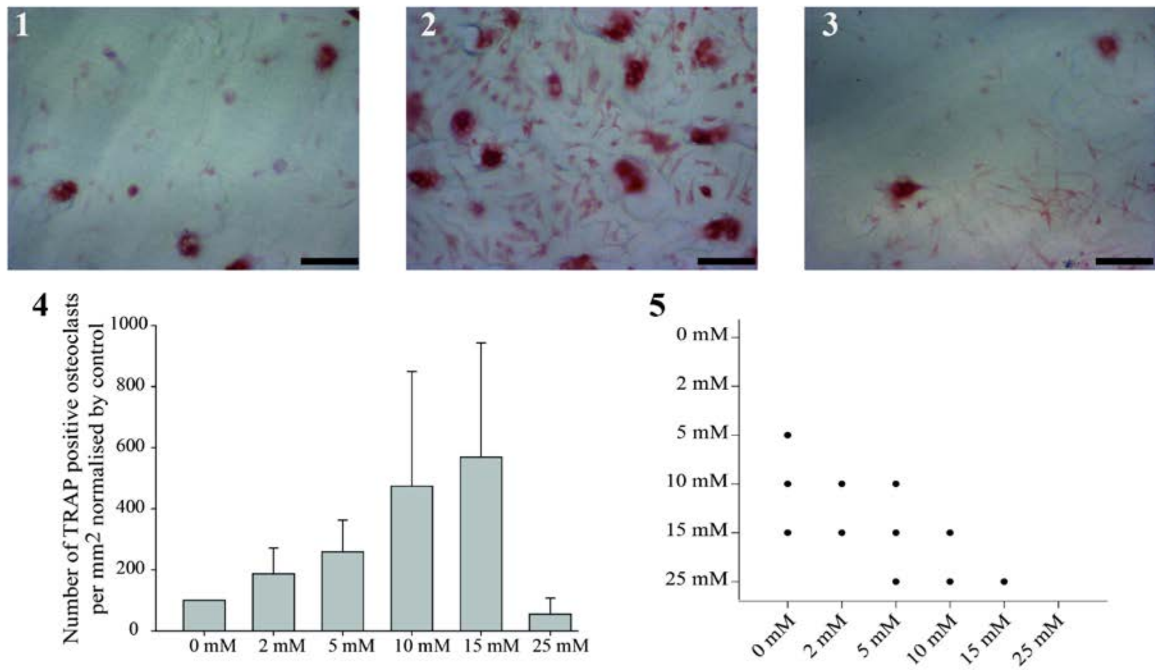


Fig. 3: Extracellular TRAP release. Measurements TRAP release for donors treated with $MgCl_2$ (A) and donors treated with magnesium extract (B). Symbols indicate significant differences. For each time point, 0 mM was set as the control (100%), to which the WST-1 activities for other concentrations were normalised. The respective statistical analyses are presented in multiple comparison graphs (right panel).



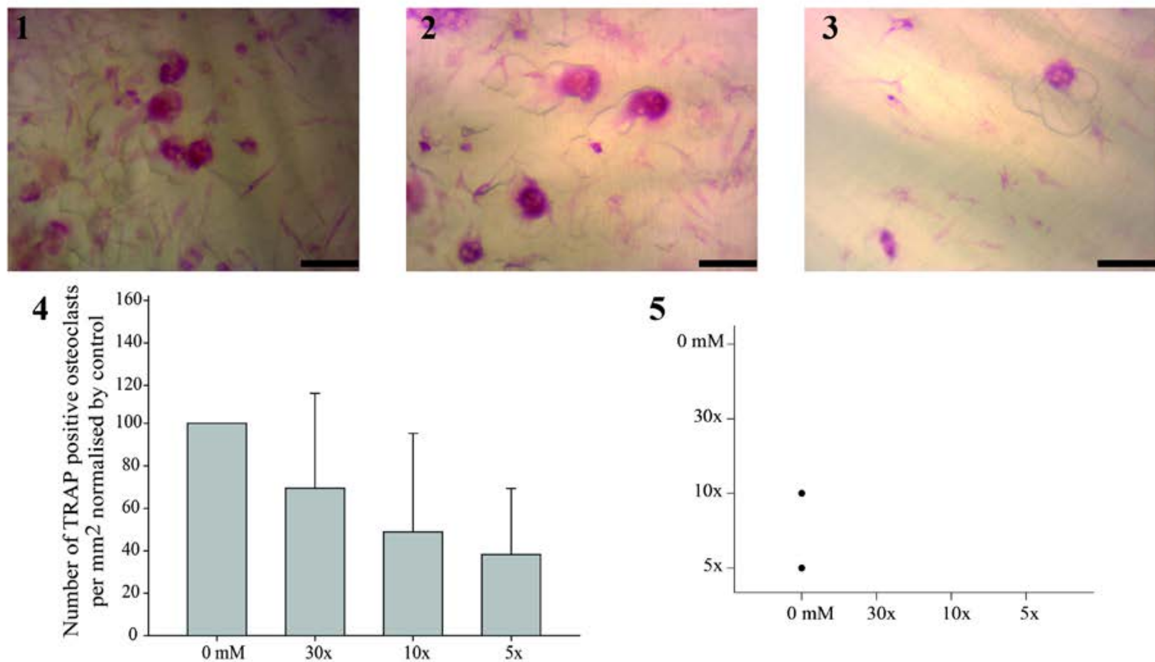


Fig. 4: TRAP staining at day 28: (A) $MgCl_2$ and (B) Mg-extract assays. (1-3) Micrographs of TRAP staining for dentine (A: 0, 15, and 25 mM, respectively, and B: 0 mM, 30x, and 5x, respectively; scale bar, 100 µm). (4) The number of TRAP-positive cells recorded for the 0 mM treatment was set as the control (100%), to which the numbers of TRAP-positive cells under the other concentrations (A) or dilutions (B) were normalised. (5) The respective statistical analyses are presented in multiple comparison graphs.

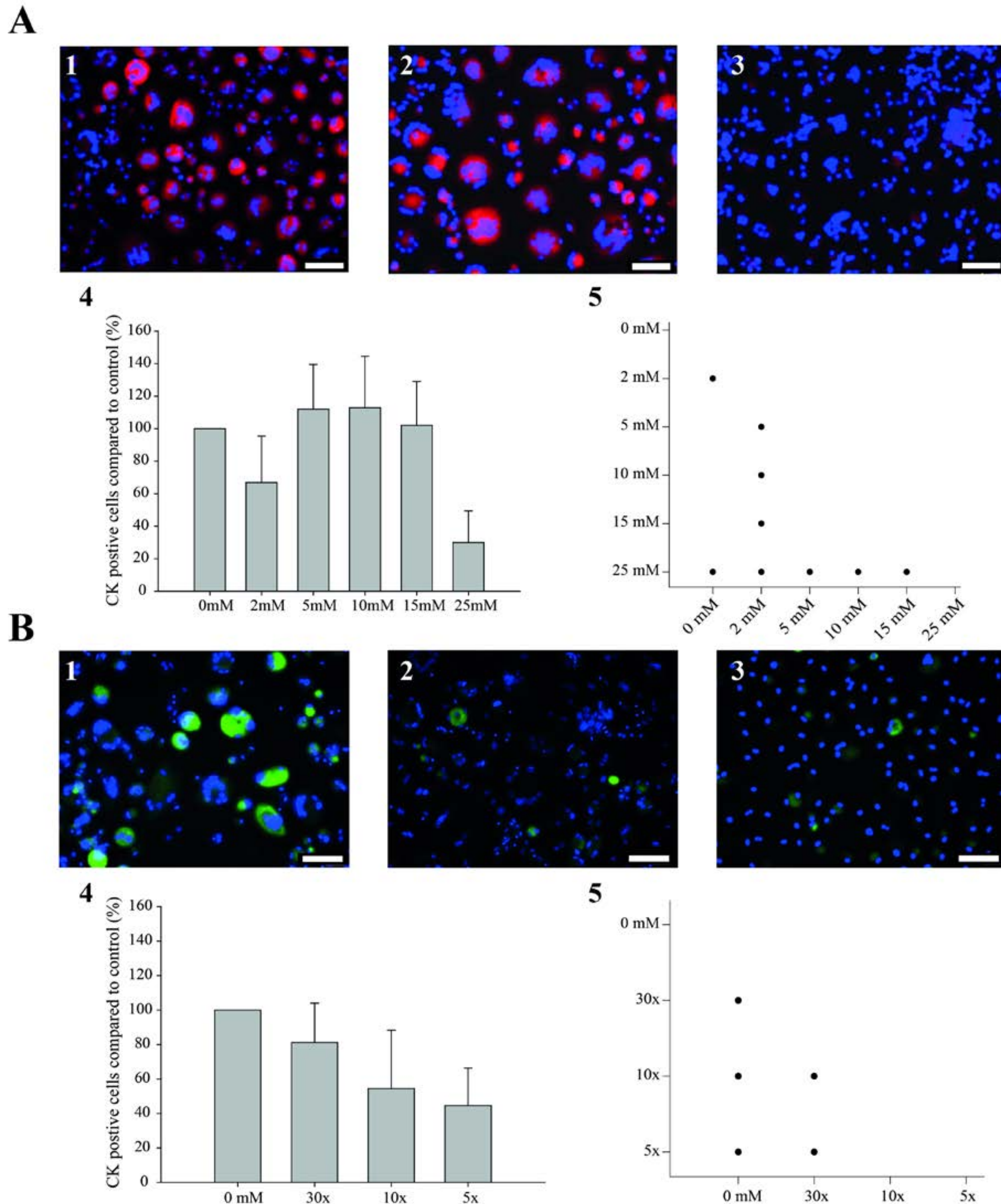


Fig. 5: CK immunocytochemistry at day 28: (A) $MgCl_2$ and (B) Mg-extract assays. (1-3) Micrographs of CK staining (A: red, 0, 15, and 25 mM, respectively, and B: green, 0 mM, 30x, and 5x, respectively; scale bar, 100 µm). Nuclei were DAPI stained and appear blue. (4) The number of CK-positive cells recorded for the 0 mM treatment was set as the control (100%), to which the numbers of CK-positive cells under the other concentrations (A) or dilutions (B) were normalised. (5) The respective statistical analyses are presented in multiple comparison graphs.

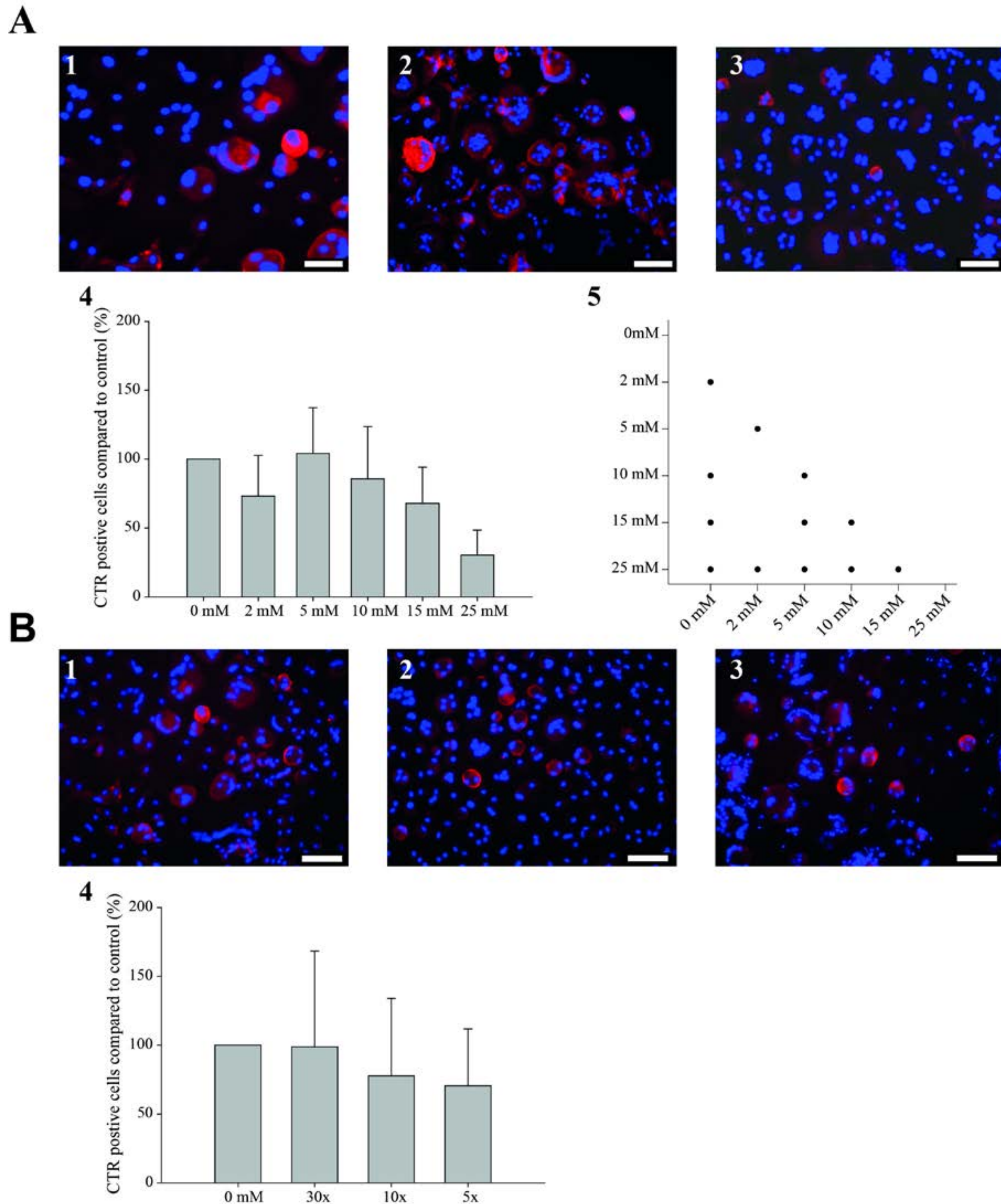


Fig. 6: CTR immunocytochemistry at day 28: (A) $MgCl_2$ and (B) Mg-extract assays. (1-3) Micrographs of CTR (red) staining (A: 0, 5, and 25 mM, respectively and B: 0 mM, 30x, and 5x, respectively; scale bar 100 µm). Nuclei were DAPI-stained and appear blue. (4) The number of CTR-positive cells recorded for the 0 mM treatment was set as the control (100%), to which the numbers of CTR-positive cells for other concentrations (A) or dilutions (B) were normalised. (5) The respective statistical analyses are presented in multiple comparison graphs.

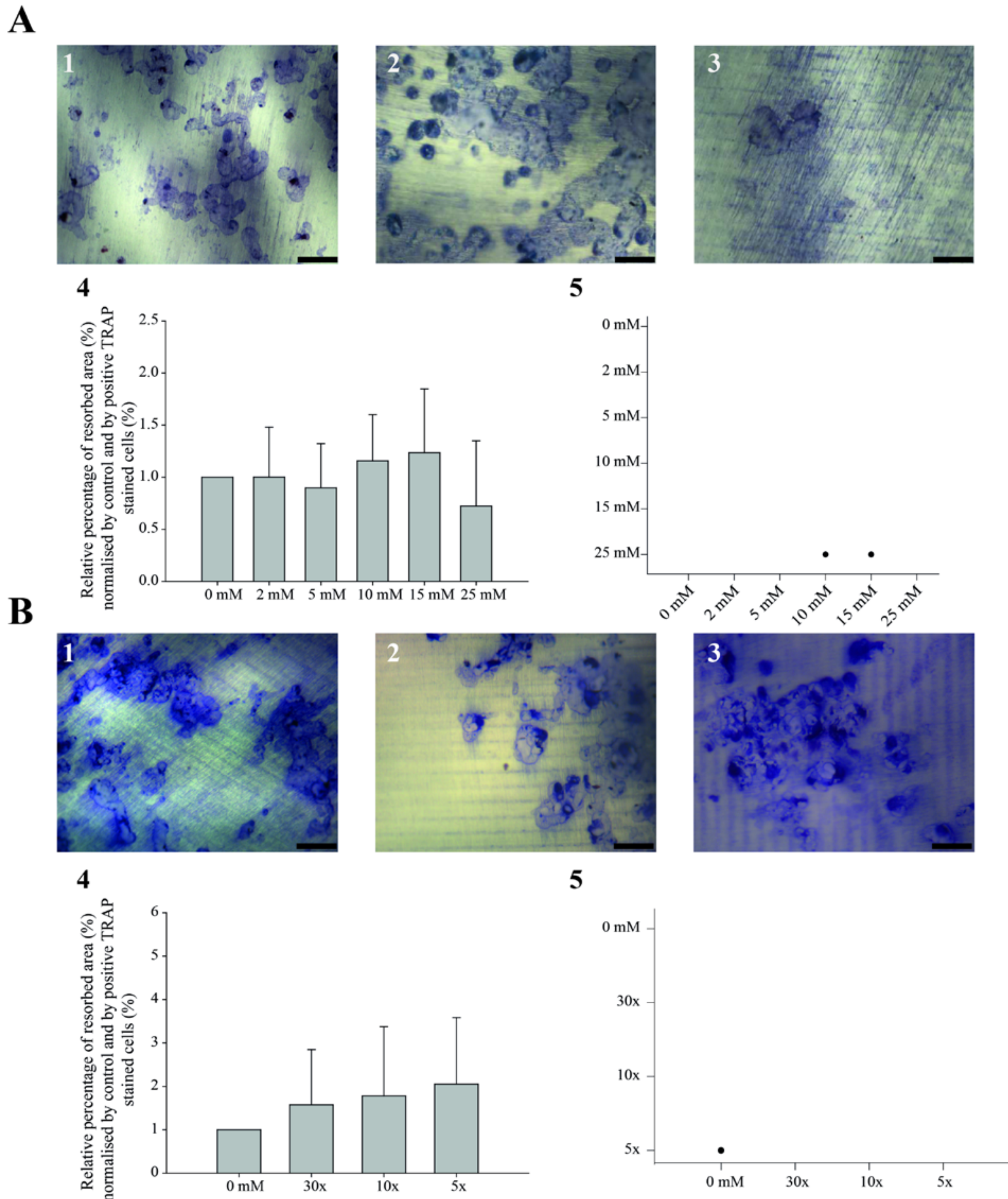


Fig. 7: Resorption assay at day 28: (A) $MgCl_2$ and (B) Mg-extract assays. (1-3) Micrographs of dentine resorption (A: 0, 15, and 25 mM, respectively and B: 0 mM, 30x, and 5x, respectively; scale bar 200 µm). (4) The resorbed area measured for the 0 mM treatment was set as the control (100%), to which the resorbed areas measured for the other concentrations (A) or dilutions (B) were normalised. (5) The respective statistical analyses are presented in multiple comparison graphs.

Tables

Extract	pH	Osmolality (osmol/kg)	Mg content (mM)	Ca content (mM)
α-MEM+10% FBS	7.80	0.33	0.93	1.76
1x	8.35	0.51	26.67	0.08
5x	8.04	0.46	6.08	1.42
10x	7.90	0.38	3.50	1.59
30x	7.80	0.34	1.46	1.70

Table 1: Characterisation of Mg-extract solutions.