In Vitro Assessment of the Cytocompatibility of Magnesium-Based Implant Materials with Osteoclasts

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Declaration on Oath

I hereby declare that except where reference to the literature as well as acknowledgement of collaborative research and discussions have been made, this PhD dissertation entitled "*In Vitro* Assessment of the Cytocompatibility of Magnesium-Based Implant Materials with Osteoclasts" represents my own original research work and effort and it has not been previously submitted, in whole or in part, to qualify for any other academic award.

Signed:

16.09.2015, Geesthacht

Abstract

Peripheral blood mononuclear cells (PBMC) freshly isolated from human whole blood were driven towards an osteoclastogenesis pathway. The effects of external addition of Mg salt (coming from magnesium chloride; $MgCl_2$) on osteoclastic differentiation as well as resorption behaviour were evaluated. It was demonstrated that $MgCl_2$ first accelerated osteoclastic proliferation and differentiation until a concentration of 10 or 15 mM $MgCl_2$ and then it was reduced.

In an attempt to distinguish the influence of magnesium ion (Mg^{2+}) from chloride ion (Cl^-) when the role of $MgCl_2$ on osteoclastogenesis was investigated, osteoclastic response to different concentrations of supplemented sodium chloride (NaCl) was assessed. While 50 mM Cl^- coming from the addition of 25 mM $MgCl_2$ was shown to decrease osteoclast resorption activity, the same amount of Cl^- from 50 mM NaCl was investigated to significantly increase osteoclastic resorption activity. Therefore, it might be concluded that the first elevated followed by reduced effects of $MgCl_2$ on osteoclastogenesis was due to Mg^{2+} rather than Cl^-.

A series of Mg extract dilutions was further prepared and their role on osteoclastic differentiation behaviour was investigated as well and compared with the results from the cultures supplemented with $MgCl_2$. While $MgCl_2$ first enhanced and then opposed osteoclastic differentiation, decreased cell metabolic activity whereas enhanced resorption activity per osteoclast were discovered at a lower concentration of Mg extract. It therefore could be concluded that: (i) Mg salt in the form of $MgCl_2$ and Mg extract exhibited different direct effects on osteoclast metabolism; (ii) while $MgCl_2$ could enhance osteoclastic proliferation and function activity up to a concentration of approximately 10 or 15 mM, Mg extract exerted its positive effect on the resorption activity per osteoclast at a lower Mg content (≈ 6 mM Mg^{2+}).

In the next step an osteoblast-osteoclast coculture was established. Their responses to a series of Mg extract dilutions were investigated. Phenotype characterization as well as late osteoclastogenesis analyses suggested that while 14.36 mM Mg^{2+} of Mg extract was extremely toxic to osteoclast monoculture, monocytes cocultured with osteoblasts exhibited a far more superior tolerance to higher concentrated Mg extract. Results from specific osteoblastic and osteoclastic markers (both gene and protein levels) revealed that 10.13, 14.36 and 26.67 mM Mg^{2+} of Mg extract were beneficial to osteoblastogenesis but exerted a detrimental effect on osteoclastogenesis.

In summary, coculture of osteoblasts and osteoclasts has been shown in the present work to be preferable for *in vitro* cytocompatibility assessment of Mg-based implants compared to their monocultures. While a contribution of high concentration of Mg extract to enhanced bone formation has been demonstrated, the impact of potentially decreased osteoclastic resorption should be taken into account as well. Coculture of osteoblasts and osteoclasts is a compromise way between *in vitro* monocultures and *in vivo* animal models for compatibility assessment of Mg-based alloys for orthopaedic applications.

Zusammenfassung

Die einkernigen Zellen des peripheren Blutes (Peripheral blood mononuclear cells, PBMC) wurden frisch aus humanem Vollblut isoliert und daraufhin die Osteoklastogenese induziert. Es wurde der Effekt von Mg Salz (in Form von Magnesiumchlorid; $MgCl_2$) auf die Differenzierung der Osteoklasten und deren Resorptionsverhalten studiert. Dadurch konnte gezeigt werden, dass $MgCl_2$ die Proliferation und Differenzierung der Osteoklasten bis zu einer Konzentration von 10 bzw. 15 mM $MgCl_2$ beschleunigt und danach abfällt.

Um zu sicher zu stellen, dass diese Reaktion an den Magnesiumionen (Mg^{2+}) und nicht an den Chlorionen (Cl^-) liegt, wurde der Einfluss von $MgCl_2$ auf die Osteoklastogenese untersucht und die Reaktion der Osteoklasten auf verschiedene Konzentrationen von Natriumchlorid (NaCl) bewertet. Die in den 25 mM $MgCl_2$ enthaltenen 50 mM Cl^- führen zu einer Reduzierung der Resorptionsaktivität der Osteoklasten. Außerdem konnte bei derselben Cl^- Konzentration, die in 50 mM NaCl enthalten ist, ein signifikanter Rückgang der Resorptionsaktivität der Osteoklasten beobachtet werden. Daher ist es wahrscheinlich, dass die beobachteten Effekte eher durch Mg^{2+} statt Cl^- ausgelöst werden.

Im nächsten Schritt wurden Mg-Extrakte hergestellt. Um die Rolle des darin enthaltenen Magnesiums auf die Osteoklasten zu untersuchen, wurde eine Verdünnungsreihe angefertigt und mit den Ergebnissen der mit $MgCl_2$ behandelten Kulturen verglichen. Zwar wird auch die metabolische Aktivität der Zellen verringert allerdings, konnte auch beobachtet werden, dass die Resorptionsaktivität der einzelnen Osteoklasten bei einer geringeren Mg Extrakt Konzentration erhöht wird. Daraus kann geschlossen werden: (i) Mg-Salz als $MgCl_2$ oder als Mg Extrakt zeigen einen unterschiedlichen Effekt auf den Metabolismus von Knochen resorbierenden Osteoklasten; (ii) $MgCl_2$ kann bis zu einer Konzentration von ca. 15 mM die Proliferation und die Osteoklastenaktivität steigern, der Mg Extrakt hingegen übt seinen positiven Effekt bei einer deutlich geringeren Mg Konzentration aus.

An diese Ergebnisse anschließend wurde die Osteoblasten-Osteoklasten Co-Kultur etabliert. Daraufhin wurde das Verhalten der Co-Kulturen auf verschiedene Verdünnungen des Mg Extraktes untersucht. Die folgende Charakterisierung des Phänotyps zeigte, dass Mg Extrakte mit einer Konzentration von 14.36 mM Mg^{2+} oder höher extrem toxisch für die Osteoklasten Monokultur ist. Hingegen weisen die Monozyten, die mit Osteoblasten gemeinsam kultiviert wurden, eine weitaus höhere Toleranz gegenüber höheren Mg Extrakt Konzentrationen auf. Ergebnisse von spezifischen Markern für Osteoblasten und Osteoklasten (auf Gen- und Proteinlevel) zeigen, dass Konzentrationen von 10.13; 14.36 und 26.67 mM Mg^{2+} im Mg Extraktes einen positiven Effekt auf die Osteoblastogenese aufweisen allerdings haben diese Konzentrationen einen schädlichen Effekt auf die Osteoklastogenese.

Zusammenfassend konnte in der vorliegenden Arbeit gezeigt werden, dass eine Co-Kultur, die aus Osteoblasten und Osteoklasten besteht, für die Untersuchung der *in vitro* Zytokompatibilität von Mg basierten Implantaten einer Monokultur vorzuziehen ist, da ein Zusammenhang zwischen einer hohen Konzentration des Mg Extraktes und einer

Steigerung der Knochenbildung gezeigt werden konnte. Allerdings sollte auch ein möglicher Rückgang der Resorptionsfähigkeit der Osteoklasten berücksichtig werden. Eine Co-Kultur von knochenaufbauenden Osteoblasten und knochenabbauenden Osteoklasten ist ein Kompromiss zwischen *in vitro* Monokulturen und dem *in vivo* Tiermodel für die Beurteilung der Zytokompatibilität von Mg basierten Legierungen für die orthopädische Anwendung.

Contents

1 Introduction

As a consequence of rising life expectancies, major traumatic injuries, resection of bone tumours, the demand for substitution or reconstruction of encountered bone loss with suitable materials remains to be a major concern in orthopaedic surgical applications [1-3]. Advances in material science and in medical technology allow the development of new implant material for loading bearing application. Nevertheless, since all of the elements will enter into the human physiological environment, it is ethically and financially deemed to be of excellent biocompatibility. There should be no toxic or side effects or enabling to be tolerated by the body without causing any potentially harmful perturbation [4]. This necessity limits the range of candidate materials selected for such investigations to a relatively small number. It includes mainly four types of materials: metals, ceramics, polymers and their composites of two or more of the preceding [5].

Metallic implants made of medical-grade metals such as stainless steels (316L) [6], commercially pure titanium (Ti) and Ti-6Al-4V alloys [7] as well as cobalt-chromium-based alloys (Co-Cr alloys) [8] are typically employed for load bearing applications. Due to their combination of more superior mechanical strength and fracture toughness, they are used to assist with the repair or replacement of bone tissue [9-12]. However, there are several major drawbacks associated with the utilisation of these conventional metallic implants. First, the mismatch of the mechanical properties between different metallic materials and the surrounding natural bone tissue may cause long-term adverse effects. For example, it leads to an increased risk of local inflammation or even stress shielding effects which can reduce stimulation of new bone growth and remodelling, thus decreased implant stability [13, 14]. Second, the possible release of toxic metallic ions, such as chromium, cobalt and nickel, into the body may result in inflammatory cascades from the body's immune system and should be considered potentially harmful to the surrounding tissues [15-25]. Third, a second surgical removal procedure after the tissue has healed sufficiently is needed due to its neutral properties *in vivo*, leading to an increase of health care costs and longer hospitalisation [26].

Alternatively, to reduce or avoid such complications, a considerable amount of researches has been focused currently on the investigation of new classes of so-called "biodegradable implants" in medical applications [27-30]. They compose of non-toxic materials that can be gradually dissolved or absorbed by the human body after a reasonable period of time. Therefore, it offers a less invasive repair and temporary support during tissue recovery [9, 31, 32]. Nowadays most of the biodegradable implants are polymer-based since they are lightweight, ductile in nature, biocompatible and biodegradable [33, 34]. However, even possessing such attractive performances, they exhibit relatively insufficient mechanical strength when compared to ceramics and metallic implants, making them unsuitable for high-load bearing applications. Litsky et al. [35] reported the need for a second surgical procedure because of the failure of the fixation by implants made of biodegradable polymers. Moreover, the reaction between some organic polymers and human host tissues has also been reported which results in severe osteolysis and eventually synovitis. Consequently, with respect to better mechanical performance compared to biodegradable polymers,

biodegradable metallic alloys are new promising materials for implant production whilst taking into consideration of both the biodegradation and the specific mechanical properties.

1.1 Magnesium (Mg) as a potential biodegradable metallic material in orthopaedic applications

Based on the requirements in terms of biocompatibility and their relatively fast biodegradability, currently, iron (Fe)-based and Mg-based alloys have been commonly investigated for this application [36, 37]. However, due to its higher elastic modulus (91 GPa) and density (7.8 g/cm^3) [38] as compared to human natural bone [38, 39], Fe is not recommended for using as orthopaedic implants. From the point of view of the mechanical performance, Mg alloys are therefore preferred as potential candidates for biodegradable implants owing to their rather low stiffness (i.e., low Young´s modulus) which is closer to that of human bone [9, 12, 40]. The major advantages are their excellent capacities for biodegradability, biocompatibility combined with appropriate mechanical properties over traditional metallic materials, ceramics and biodegradable polymers [9, 40, 41].

Biodegradability

One of the most desirable characteristics of Mg-based alloys is their ability to be degraded in physiological environment after the bone tissues have healed, avoiding the need for a secondary implant removal surgery [9, 31, 32]. The corrosion behaviour of unprotected Mg exposed to a typical aqueous environment proceeds by an electrochemical reaction in which magnesium hydroxide ($Mg(OH)_2$) and hydrogen gas (H_2) will be yielded [42].

It has been demonstrated that the grey oxide film of $Mg(OH)_2$ onto the Mg surface can further protect the metal from ongoing corrosion by slowing down their corrosion rate [43]. However, since these films of $Mg(OH)_2$ are slightly soluble in water, in an aqueous physiological environment enriched with Cl^-, a highly soluble $MgCl_2$ will be formed due to the dissolving of $Mg(OH)_2$ layer by Cl^-. It thus yields the soluble $MgCl_2$ salt, leading to severe corrosion [9, 43, 44]. Corrosion tests have been performed *in vitro* and particularly *in vivo* in a range of Mg alloy systems, such as aluminium/zinc (AZ), aluminium/manganese (AM) and yttrium/rare earth elements, indicating a high potential for medical applications [45-47]. Witte et al. have successfully inserted open porous metallic scaffolds made of Mg alloy AZ91D (containing (mass fraction, %) of 9.0 aluminium (Al), 1.0 zinc (Zn) and the balance of pure Mg) into the femur of New Zealand White Rabbits. It has been shown that Mg implant had substantially degraded after 3 months and then replaced with new bone [48, 49]. Nevertheless, Mg implants for bone applications are still not in clinical use, due mainly to their inappropriately high and in some cases unpredictable corrosion rates. Alloying, protective coatings as well as surface treatments can be applied to improve the mechanical properties and corrosion resistance of Mg implants [43, 50, 51].

Biocompatibility

Mg^{2+} is undoubtedly one of the most important elements present in the human body that is largely and mainly stored in muscle and bone tissues [52]. It has been estimated that the human body usually contains approximately 35 g Mg per 70 kg body weight and the daily Mg intake is recommended to be 375 mg/day [53]. Insufficient dietary Mg uptake has been

proved to be associated with low bone mass, thus increased risk of the development of osteoporosis, both in human epidemiologic studies [54, 55] and experimental animal researches [56]. Mg depletion is also estimated to be closely associated with cardiac arrhythmias, the development of atherosclerosis, vasoconstriction of coronary arteries and increased blood pressure in the cardiovascular system [57]. Moreover, Mg is highly involved in many metabolism processes and required for more than 325 biochemical reactions [9, 58-60].

In an attempt to secure fracture involving the bones of the lower leg, as early as 1907 [9], Mg materials were first utilised as orthopaedic implants by Lambotte, a French surgeon. However, it failed due to the rapid corrosion of the Mg metal, causing a large amount of gas beneath the skin. Around 1938, McBride used screws, pegs, plates and bands prepared from Mg alloys to secure 20 fractures and stated the non-toxicity of Mg metal as well as its stimulatory effect on the early proliferation of connective tissue [61]. Consistent with this research, in 1944, Troitskii and Tsitrin reported the success in securing various fractures with plates and screws made from Mg, alloyed with small levels of cadmium [62]. In all the 34 cases of patients, no increase in serum levels of Mg and inflammatory reactions to the implant was observed [62]. Similar results were also reported by Znamenskii in 1945 in which gunshot wounds in two young men were successfully healed by the implantation of Mg materials alloying with 10wt% Al [63]. While their biocompatibility was suggested by these previously-mentioned early clinical investigations, a very large amount of evidence coming from recent *in vivo* and *in vitro* researches also indicated that Mg-based implants are non-toxic [30-32, 40, 48, 64-70].

Excellent mechanical properties

Except for its excellent biodegradability as well as its good biocompatibility, another significant advantage of Mg-based alloys over currently used surgical metals is their unique mechanical properties which are closely matched to those of bone. Being considered as an exceptionally lightweight metal, Mg and its alloys possess densities ranging from 1.74-2.0 g/cm^3 which is 1.6 and 4.5 times less dense than Al and steel, respectively and very similar to that of bone (1.8–2.1 g/cm^3) [9]. Pure Mg possesses a modulus of elasticity of approximately 45 GPa, which is much more closely matched to that of natural bone than in the case for Ti alloys (110–117 GPa), stainless steels (189–205 GPa) and Co-Cr alloys (230 GPa). It thus minimizes the likelihood of stress shielding effect occurring and the associated loss of bone density and strength, rendering them desirable load-bearing hard-tissue implants [9].

Osteoconductivity and osteoinductivity

Apart from its biocompatibility, several studies have further reported its stimulatory effects on bone healing processes, indicating the good osteoconductivity and osteoinductivity [30, 40, 45, 62, 65, 66, 71-73]. Fig. 1 showed the enhanced new bone formation surrounding the Mg rods compared to the polymer control group. Both Precival [74] and Boanini et al. [75] have demonstrated the stimulatory role of the presence of Mg on bone growth as well as bone healing by enhancing the osteoblastic and osteoclastic activities. Similar results were also found in studies conducted by Zhang [76] and Duygulu [77] which implied that Mg alloy AZ31

(containing (mass fraction, %) of 3.0 Al, 1.0 Zn and the balance of pure Mg) implantation is likely to be physiologically beneficial than harmful to the growth of new bone tissues and biocompatible in animal experiments.

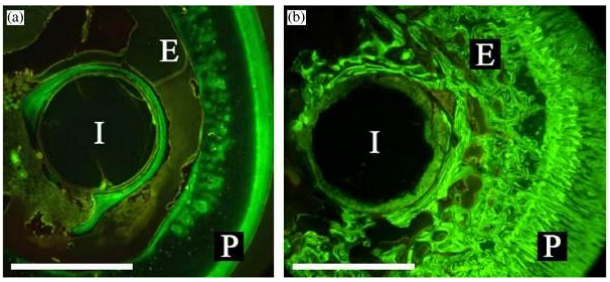


Fig. 1 Fluoroscopic images of cross-sections of a degradable polymer (a) and a magnesium rod (b) performed 10 mm below the trochanter major in a guinea pig femur. Both specimens were harvested 18 weeks postoperatively. *In vivo* staining of newly formed bone by calcein green. Bar = 1.5 mm; I = implant residual; P = periosteal bone formation; E = endosteal bone formation. Reprinted from ref. [40], copyright 2005, with permission from Elsevier.

1.2 Bone cells and bone remodelling

From a biological perspective, bone is a remarkable living, highly vascular, dynamic and mineralised tissue that is continuously maintained and renewed. Of the many cells associated with bone, there are three primary types that are of special interest: osteoblasts, osteocytes and osteoclasts. They are responsible for the elaboration, maintenance and resorption of bone tissue, respectively [78]. All their names start with the root ‘osteo’ meaning ‘bone’ in Greek. Their roles and the one of osteoprogenitor cells are shown schematically in Fig. 2 [79].

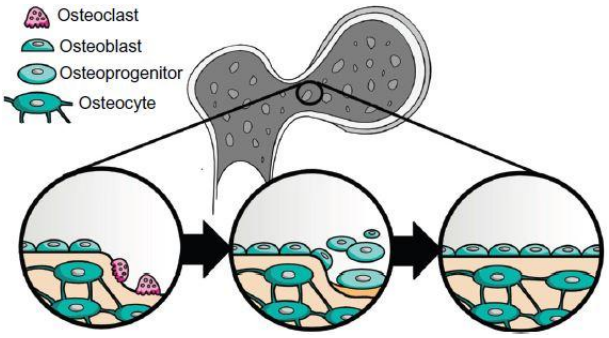


Fig. 2 Left: The differentiated osteoclasts break down bone tissue and generate resorption lacunae (‘pit’) by bone resorption. Centre: osteoprogenitor cells are recruited to the area and then differentiate into osteoblasts, while the osteoblasts are already present, line the surface of the bone tissue and proliferate. Right: the osteoblasts deposit new bone in the cavity through a process called ossification, while some of them differentiate into mature bone cells – osteocytes. Reprinted from ref. [80]. Non-commercial uses of the work are permitted without any further permission from Dove Medical Press Limited, provided the work is properly attributed.

Osteoblasts

Osteoblasts are the cells working in groups to generate new bone matrix as our body grows, i.e., bone-forming cells. The second part of the word, ‘blast’, comes from a Greek word that means ‘germ’ or ‘embryonic’. They are found on the surface of new bone and form uncalcified collagen-rich protein mixture known as "osteoid". Osteoid comprises bony matrix proteins such as type I collagen and other non-collagenous proteins including osteocalcin, bone sialoprotein, osteopontin as well as osteonectin [81]. It subsequently mineralises to become bone in which the stiffness and the compressive strength of the bone at the expense of its energy-storing capacity are increased. After finishing making bone, osteoblasts become either lining cells or osteocytes.

Osteocytes

Osteocytes are star shaped bone cells which are most commonly found in compact bone and considered to be the derivatives of the terminal differentiation stage of osteoblasts. They are embedded in the space known as osteocytic lacunae within the mineralised matrix. Osteocytes have many cytoplasmic processes (around 80) that are 15 µm in length for connecting them to neighbouring osteocytes probably for the purposes of exchanging minerals and communicating with other cells in the area [82].

Osteoclasts

Osteoclasts are multinucleated, terminally differentiated cells. They are derived from hematopoietic cells of the monocyte-macrophage lineage and responsible for bone resorption and remodelling during normal and pathological bone turnover [83]. The second part of the word, ‘clast’, comes from the Greek word which means ‘break’. They can be generally distinguished from other bone cells by their multinuclearity and positive tartrate-resistant acid phosphatase (TRAP) staining (Fig. 3). These cells work as a team with osteoblasts for reshaping bone. It has been well established that the survival, differentiation and activation of osteoclasts are regulated by numerous hormones and cytokines produced by macrophages and osteoblast/stromal cells [84]. Macrophage colony-stimulating factor (M-CSF) was first shown to play an essential role on osteoclast generation and differentiation. It acts through binding to its receptor, colony-stimulating factor 1 receptor (c-Fms), on early osteoclast precursors, thereby signals required for their survival and proliferation are provided. Although it has been known that M-CSF is important for osteoclast formation and survival, it alone failed to stimulate osteoclastic bone resorption. Receptor activator of nuclear factor kappa-B ligand (RANKL) on the other hand, is recognized as the other cytokine which plays a crucial role on osteoclast differentiation and activation. Moreover, the complete osteoclastogenesis has been proved to be achieved *in vitro* with pure populations of macrophages in the exposure of only to M-CSF and RANKL [85].

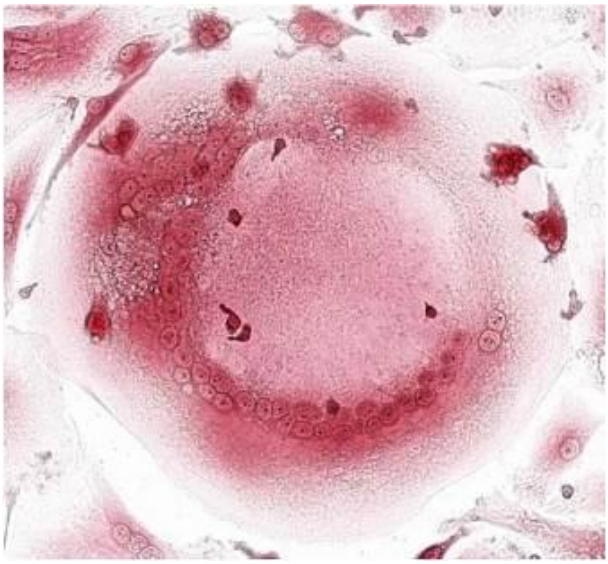


Fig. 3 Fully differentiated, multinucleated, TRAP-positive human osteoclast *in vitro* (magnification 400x). Reprinted from ref. [86], copyright 2006, with permission from Springer.

Bone remodelling by osteoclasts and osteoblasts

During adult life, bone continually undergoes a renewed process called bone remodelling. It is performed by clusters of bone-breaking osteoclasts and bone-generating osteoblasts to attain and preserve skeletal size, shape, and structural integrity and to regulate mineral homeostasis. It begins with the process conducted by multinucleated osteoclasts which are formed through the fusion of mononuclear precursors of hematopoietic origin [87]. After osteoclasts migrate to a resorption site, the sealing zone, a specific membrane domain, is formed. Through the sealing zone osteoclasts attach tightly to the bone surface. Then the production of another specific membrane domain, the ruffled border at the bone/osteoclast interface is performed. Together with sealing zone, an isolated microenvironment is created by osteoclasts. The subsequent resorption behaviour is completed by acidification of the extracellular compartments lying beneath the osteoclasts and dissolution of both organic and inorganic matrices [83]. On the contrary, the other process of bone remodelling, bone formation is carried out by osteoblasts which originated from mesenchymal stem cells and starts briefly after osteoclastic resorption stops. In physiological conditions, this sequence of events is tightly coordinated both temporally and spatially. It is necessary for preserving skeletal structural integrity, repairing damaged bone and maintaining mineral homeostasis [88]. In order to finally achieve replacement of the substitution material by healthy bone, interaction between the two cell types must be considered.

1.3 *In vitro* cytocompatibility assessment for Mg-based alloys

For any biodegradable Mg material to be developed for use in the biomedical applications, they become resorbed by the body after a certain period of time. All the products from the progressive corrosion will enter into the human physiological environment. Therefore, in the pre-clinical steps, the potential cytotoxicity of the constituent elements of an implant material should be seriously taken into consideration by submitting to a range of pre-toxicity tests, *in vitro* or *in vivo* or both.

In vivo biocompatibility assessments have been predominantly carried out in small animals, like rats, guinea pigs and rabbits. It has been proved that Mg or Mg alloys in general are suitable for the orthopaedic applications and even revealed that they are osteoinductive and they can promote bone remodelling [30-32, 40, 48, 49, 89]. However, *in vitro* toxicity examinations are preferable regarding ethical considerations (i.e., the attempt to restrict animal experimentation to a minimum) as well as the need for a fast investigation of the vast newly developed Mg-based materials. Furthermore, it has been pointed out that the results of tests examined outside the body are more reproducible [90, 91]. Thus, preliminary *in vitro* cytocompatibility assays are continually being preceded as an appropriate “pre-screen” means in order to characterize the potentially harmful effects of Mg-based materials before it is ultimately used for human application [92-94].

Osteoblast model

Currently, most *in vitro* biocompatible investigations of Mg and its alloys involve cell culture experiments performed predominantly using osteoblasts of murine or human origin to analyse the capability of the respective material to support new bone formation. For example, the human osteosarcoma cell line U2OS cells were cocultured with Mg specimen for one

week. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as well as visual observation through cell staining have revealed that there was no significant difference in cell cytotoxicity between control and cultures in the presence of the corroding Mg sample. It suggested that Mg could be suitable as a biodegradable implant material [38]. MC3T3-E1 cells were used for the cytotoxicity tests of nine binary Mg alloys (alloying elements Al, silver (Ag), indium (In), manganese (Mn), silicon (Si), tin (Sn), yttrium (Y), Zn and zirconium (Zr)). The results showed that there was no significant reduce in osteoblasts cell viability when cultured with Mg-1Al, Mg-1Sn and Mg-1Zn alloy extract [95]. Apart from Mg alloys, when Mg was incorporated into bone substitutes such as hydroxyapatite (HA), tricalcium phosphate (TCP) or Ti alloy implants, their beneficial effects on osteoblast adhesion, proliferation as well as differentiation behaviour have been demonstrated in a large amount of studies [65-68, 96]. For example, compared to the Mg-free CO_3Ap-collagen composite, osteoblast adhesion to the FG$MgCO_3$Ap-collagen composite was proved to be accelerated due to the free Mg^{2+} [65]. Similarly, a significantly enhanced level of osteoblastic adhesion activity for human bone-derived cells (HBDC) grown on Mg divalent cations enriched substrates was estimated compared to those grown on the native unmodified aluminium oxide (Al_2O_3). It accompanied with a subsequent increase in the expression of type I collagen extracellular matrix protein [66]. Mg incorporation in TCP was found significantly stimulated the adhesion and proliferation of human osteoblast-like SaOs2 cells [67]. More recently, similar beneficial effects of Mg-enriched materials (Mg incorporated fluoridated HA and Ti-based alloys) on bone cell attachment, proliferation and late differentiation have been reported [68, 96]. The proliferation of human osteoblast-like MG63 cells was significantly increased in cultures with the external addition of Mg ions [69].

Osteoclast model

While the extensive utilisation of osteoblasts or their precursors for the *in vitro* cytocompatibility assessment of Mg-based alloys, cellular and molecular characterization of osteoclastic response to Mg-based alloys has been extremely rare. This is mainly due to two properties of these cells. First, osteoclasts are very scarce in number relative to other cell types in bone, particularly in physiological states in adult primates, covering only 1% of the bony surface [97]. Second, osteoclasts are situated on calcified bone surfaces within the medullary cavity. It is extremely difficult to harvest them or obtain pure populations of cells in large number for biochemical and molecular analyses. Due to the above reasons, the generation of osteoclasts from their precursors via *in vitro* models has been of major interest. The discovery of RANKL and M-CSF has made the *in vitro* generation of osteoclasts from mononuclear precursors possible, without the support of osteoblastic stromal cells [98].

In this present work, the initial effect of variable concentrations of Mg that comes from $MgCl_2$ solution on osteoclasts differentiation and function was investigated first. PBMC blood mononuclear cells were driven towards an osteoclastogenesis pathway via stimulation with RANKL and M-CSF for 28 days in the exposure of variable concentrations of $MgCl_2$. Their influence on osteoclast proliferation and function was evaluated based on cell metabolic activity, total protein content, TRAP activity, cathepsin K (CK) and calcitonin receptor (CTR) immunocytochemistry. The cellular ability to form resorption pits was assessed as well.

Moreover, in order to distinguish the influence of Mg^{2+} from Cl^- when the effect of $MgCl_2$ on osteoclastic differentiation was investigated, the effect of various concentrations of NaCl on the differentiation and function of human osteoclasts was tested. While 50 mM Cl^- from 25 mM $MgCl_2$ was investigated to decrease osteoclastic function, the same concentration of Cl^- coming from the addition of 50 mM NaCl was found to significantly increase osteoclast resorption activity. It indicated that it might be Mg^{2+} rather than Cl^- that played a role on osteoclastic differentiation behaviour. Surprisingly, a direct, parathyroid hormone (PTH)-independent, concentration-dependent effect of hypernatremia on osteoclastic resorption was investigated as well. The study suggested that the reported enhanced bone resorption after diets rich in NaCl and in diseases with high serum sodium (Na) may not only be secondary to the urinary loss of calcium (Ca) but also due to a direct, cell-mediated effect of increased Na concentration on osteoclastogenesis.

In the comparison with the cultures exposed to different concentrations of $MgCl_2$, the effects of various dilutions of Mg extract on osteoclastic differentiation and function behaviour were also estimated. Compared to $MgCl_2$, Mg extract exhibited a different mode influencing osteoclastogenesis. It suggested that (i) variations in the *in vitro* Mg^{2+} affects osteoclast metabolism and (ii) Mg extract should be used preferentially for indirect *in vitro* biomedical tests as they mimic the *in vivo* environment more closely compared to Mg salt.

Osteoblast-osteoclast coculture model

Bone is a dynamic, living organ that is constantly being reshaped through a precisely coordinated process called bone remodelling. *In vivo* osteoblasts and osteoclasts are usually coexisting on the surface of the bone tissue. It has been well estimated that they interact with each other through a precisely coordinated process in which the crosstalk between them plays a key role [88]. However, the interaction between them or even the role of osteoclasts on the success or longevity of biomedical implants is often underestimated [86]. Therefore, new *in vitro* models involving investigation of both osteoblast and osteoclast differentiation and activity on bone substitute are of great interest for biocompatible investigation of new biomaterials. They are one step closer to natural conditions and allow investigation of the complex communication between bone-building osteoblasts and bone-breaking osteoclasts.

Different cell combinations of osteoblasts and osteoclasts have been reported recently. For example, the coculture systems were established based on the combination of primary osteoblasts or osteoblast cell lines with PBMC or other isolated monocytes [99-104]. Human mesenchymal stem cells (hMSCs) have been widely considered maintaining the potential to differentiate into a variety of mesenchymal tissues [25]. However, due to their senescence-associated growth arrest during the culturing period, only a limited amount of cells can be generated [26]. For example, it has been reported that bone marrow stromal cells (BMSC) had a markedly diminished proliferation rate and they gradually lost their multiple differentiation potential after the first passage [27]. Human telomerase reverse transcriptase (hTERT) transduced mesenchymal stem cells (SCP-1) is a well-established hMSCs model cell line. It was immortalised through constitutive overexpression of *hTERT*

and has been further demonstrated to be comparable to primary hMSCs because of their potential to undergo osteogenic and adipogenic differentiation [28].

Therefore, in the present study, a human osteoblast-osteoclast coculture model derived by hMSCs line SCP-1 and human monocytes PBMC was established for Mg-based biomaterial testing. The cells' responses to various dilutions (concentrations) of Mg extract were investigated.

2 Motivation and Objective

In the present work, osteoclast monoculture as well as osteoblast-osteoclast coculture were conducted in the supplement with enriched Mg^{2+} (coming from either Mg salt or Mg extract). I attempt to pursue the following major goals:

1. Evaluate the influence of increased extracellular $MgCl_2$ content on the osteoclastogenesis process via various parameters concerning osteoclast differentiation and function behaviour;
2. Establish the osteoclast monoculture system in the exposure of different concentrations of NaCl to assess their cellular responses in order to distinguish the influence of Mg^{2+} from Cl^- when the effect of $MgCl_2$ on osteoclastic differentiation behaviour is investigated;
3. Investigate the effect of Mg extract on osteoclastogenesis in terms of cell differentiation and function. The results will be in comparison with that from the cultures supplemented with $MgCl_2$;
4. Develop an attractive coculture procedure consisting of osteoblasts and osteoclasts to investigate both qualitatively and quantitatively the *in vitro* cellular response to Mg extract. The outcomes will be compared with that from osteoclast monoculture.

3 Materials and Methods

3.1 Preparation of cell culture media

Preparation of $MgCl_2$-containing media

A $MgCl_2$ stock solution at a concentration of 1 M (Sigma-Aldrich Chemie GmbH, Munich, Germany) was prepared in double-distilled water (ddH_2O) and subsequently sterilised through a 0.2 µm membrane filter (Merck KGaA, Darmstadt, Germany). Appropriate dilutions (0, 2, 5, 10, 15 and 25 mM) were prepared by diluting the 1 M $MgCl_2$ stock solution directly in the cell culture medium, i.e., minimum essential α-minimum (α-MEM) (Biochrom AG, Berlin, Germany) supplemented with 10% (v/v) foetal bovine serum (FBS; PAA Laboratories GmbH, Linz, Austria).

Preparation of NaCl-containing media

A 1 M NaCl stock solution was prepared from NaCl powder (purchased from Sigma-Aldrich Chemie GmbH, Munich, Germany) in ddH_2O followed by sterile filtration through a membrane filter (0.2 µm; Merck KGaA, Darmstadt, Germany). The NaCl stock solution was appropriately and directly diluted in the cell culture medium α-MEM (Biochrom AG, Berlin, Germany) supplemented with 10% (v/v) FBS (PAA Laboratories GmbH, Linz, Austria) for the production of external addition of 0, 2, 5, 10, 15, 25 and 50 mM NaCl. The basal concentration of Na^+ in the medium was calculated at 142 mM according to the manufacturer's formulation (see Na composition in Table 1). Four groups were resulted in based on the clinical standards. They were normal (135-145 mM; two NaCl treatment groups obtained by the external addition of 0 and 2 mM NaCl plus the basal 142 mM NaCl in α-MEM), mild (146-149 mM; one NaCl treatment group obtained by the external addition of 5 mM NaCl plus the basal 142 mM NaCl in α-MEM), moderate (150-169 mM; three NaCl treatment groups obtained by the external addition of 10, 15 and 25 mM NaCl plus the basal 142 mM NaCl in α-MEM) as well as severe hypernatremia (≥ 170 mM; one NaCl treatment group obtained by the external addition of 50 mM NaCl plus the basal 142 mM NaCl in α-MEM) conditions, respectively. An overview about the modifications differing in media composition is present in Table 2.

Table 1 Na composition in α-MEM

Substance	Concentration (mg/l)	Concentration (mM)
NaCl	6800.0	116.4
$NaH_2PO_4 \cdot 2H_2O$	158.3	0.8
$NaHCO_3$	2000.0	23.8
Na-pyruvate	110.0	1
Total Na^+		**142**

Table 2 Modifications differing in media composition

Modifications (mM)	Na^+ in α-MEM (mM)	External addition of Na^+ (mM)	Total Na^+ in the media (mM)
Normal (135-145)	142	0, 2	142, 144
Mild (146-149)	142	5	147
Moderate (150-169)	142	10, 15, 25	152, 157, 167
Severe (≥ 170)	142	50	192

Preparation of Mg extract-containing media

Cuboid Mg specimens with dimensions of 1 cm x 1 cm x 0.5 cm were cut from a cast ingot of pure Mg (99.98 wt%; Hydro Magnesium, Norway) via electrical discharge machining. The samples were sterilised via sonication for 20 min in 100% isopropanol (2-propanol, Merck, Darmstadt, Germany). Subsequently, they were incubated in the extraction medium α-MEM (Biochrom AG, Berlin, Germany) supplemented with 10% FBS (PAA Laboratories GmbH, Linz, Austria) for 72 h under physiological conditions (5% CO_2, 20% O_2, 95% humidity, 37°C). The extraction medium to specimen weight ratio is 0.2 g/mL for the 1x extract preparation according to EN ISO standards 10993:5 [105] and 10993:12 [106]. The Mg extract stock solution was serially diluted (2, 3, 5, 10 and 30 times) in cell culture medium (referred to as 2x, 3x, 5x, 10x and 30x dilution of Mg extract, respectively). While five different dilutions (1x, 2x, 5x, 10x and 30x dilution of Mg extract) was used for studying the effect of Mg extract on osteoclast monoculture, six dilutions (1x, 2x, 3x, 5x, 10x and 30x dilution of Mg extract) was performed for osteoblast-osteoclast coculture system. Normal cell culture medium without Mg extract was considered as control. As all the PBMC in the exposure of 2x and 1x dilution of Mg extract (14.36 and 26.67 mM Mg^{2+}, respectively) were observed dead on day 3, only the results from osteoclast monocultures in the exposure of 5x, 10x and 30x dilution of Mg extract (6.08, 3.50 and 1.46 mM Mg^{2+}, respectively) were exhibited in the text below.

The osmolality, pH values as well as Mg and Ca contents in the series of Mg extract solutions were further measured. Gonotec 030-D cryoscopic osmometer (Gonotec, Berlin, Germany) and an ArgusX pH Meter (Sentron Europe BV, Roden, the Netherlands) were used for osmolality and pH quantification, respectively. Mg concentrations were determined via the metallochromic dye xylidyl blue followed by a colorimetric method. 10 µL Mg extract, extraction medium, or different concentrations of $MgCl_2$ (standard curve) was mixed with 1.5 mL of a xylidyl blue solution (200 mmol/L trishydroxymethylaminomethane buffer, pH 12, containing 0.12 mmol/L xylidyl blue II, 0.05 mmol/L ethylene glycol tetraacetic acid (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). The mixture was incubated at room temperature (RT) for 10 min. The absorbance of the samples was measured at 520 nm via an enzyme linked immunosorbent assay (Elisa) reader (Tecan Sunrise; TECAN Deutschland GmbH, Crailsheim, Germany). Unknown concentrations of Mg content were determined by the standard curve. Moreover, Calcium O-cresolphthalein kit (Futura system Srl, Rome, Italy) and a calcium chloride ($CaCl_2$; Sigma-Aldrich Chemie GmbH, Munich,

Germany) standard curve were used for the determination of Ca content. An aliquot of 50 µL of the samples were mixed with 2 mL of reagent. After incubation at RT for 5 min, the absorbance of the mixtures was measured at 580 nm with a Tecan Sunrise microplate reader (Tecan Sunrise; TECAN Deutschland GmbH, Crailsheim, Germany). Unknown concentrations were determined from the standard curve.

In order for better comparison with the results from the supplementation of different concentrations of $MgCl_2$, the concentrations of Mg^{2+} in the different dilutions of Mg extract (rather than Mg extract dilutions) are used in the following text.

3.2 Isolation of human PBMC

PBMC were freshly isolated from the whole blood obtained from healthy and consenting donors (purchased from the Institute for Clinical Transfusion Medicine and Immunogenetics Ulm, Ulm, Germany). The procedure was based on a density gradient centrifugation technique in which Ficoll Paque Plus (Amersham Biosciences, Uppsala, Sweden) was used. To prevent clotting, human peripheral blood (approximately 25 mL) was treated with 1% sodium-heparin and stored on ice before processing. It was then diluted 1:8 in phosphate-buffered saline (PBS; 37°C pre-warmed, without calcium ion (Ca^{2+}) and Mg^{2+}; Biochrom AG, Berlin, Germany). Then it was carefully layered over Ficoll Paque Plus (37°C pre-warmed, 1:1, v/v, Amersham Biosciences, Uppsala, Sweden) to avoid mixing of the two solutions followed by a centrifugation step without brake with 350 g at 20°C for 30 min. After this centrifugation step, the monocyte-enriched hematopoietic fraction accumulates at the interface between PBS (37°C pre-warmed, without calcium ion (Ca^{2+}) and Mg^{2+}; Biochrom AG, Berlin, Germany) and Ficoll Paque Plus (37°C pre-warmed, Amersham Biosciences, Uppsala, Sweden). These cells were carefully pooled and transferred in fresh tubes. These tubes were filled to 50 mL with PBS (37°C pre-warmed, without calcium ion (Ca^{2+}) and Mg^{2+}; Biochrom AG, Berlin, Germany) and centrifuged with 300 g at 20°C for 10 min with brake. The wash step was repeated with PBS twice. After the wash step, the pellets were collected.

The cell pellet was then resuspended in 1 mL α-MEM (Biochrom AG, Berlin, Germany) containing 10% (v/v) FBS (PAA Laboratories GmbH, Linz, Austria). 10 µL of cell suspension was stained with 990 µL Trypan blue solution for the assessment of cell viability. The number of isolated PBMC monocytes was determined by counting via a Neubauer counting chamber (Brand GMBH + CO KG, Wertheim, Germany). Cell suspension at a density of 2×10^6 cells/mL was prepared in α-MEM (Biochrom AG, Berlin, Germany) supplemented with 10% (v/v) FBS (Life Technologies GmbH, Karlsruhe, Germany), 2 mM L-glutamine (Life Technologies GmbH, Karlsruhe, Germany) and 1% antibiotic/antimitotic solution (PAA Laboratories GmbH, Pasching, Austria). The purification of the culture was performed by cell adherence in which the contaminating lymphocytes were disposed. Slices of sterilised dentin chips were kindly provided by German customs in accordance with the international laws for the protection of species. They were cut by a low speed saw with water cooling to dimensions of 6 mm x 6 mm x 0.9 mm and were previously placed into the culture wells in order to evaluate bone resorption activity (at least three slices per treatment per donor).

3.3 Cell culture

Osteoclast monoculture

Monocytes freshly isolated from the whole blood (obtained from the Institute for Clinical Transfusion Medicine and Immunogenetics Ulm, Ulm, Germany) were seeded in 48-well plate (Nunc, Roskilde, Denmark) at a cell density of 2 x 10^6 cells/mL. For osteoclast differentiation, the cells were cultured in the presence of factors promoting osteoclastic differentiation, i.e. RANKL (Peprotech Germany, Hamburg, Germany; 40 ng/mL) and M-CSF (Peprotech Germany, Hamburg, Germany; 20 ng/mL). Non-adherent cells were discarded after 24 h to eliminate the culture of contaminating lymphocytes. To evaluate how $MgCl_2$, NaCl and Mg extract influence the differentiation and function behaviour of osteoclast monoculture, different $MgCl_2$ concentrations (0 (control), 2, 5, 10, 15 and 25 mM), NaCl concentrations (0 (control), 2, 5, 10, 15, 25 and 50 mM) and Mg extract dilutions (control, 5x, 10x and 30x in culture medium) were added to the media, respectively. All the cell cultures were cultured at 37°C, 5% CO_2 and 95% H_2O saturation. Phase contrast inverted microscopy was conducted every day for monitoring of the cultures and half of the medium was replenished every other days.

Human osteoclasts were differentiated for a period of 28 days during which the cell culture medium was half refreshed every two days. In order to assess the impact of $MgCl_2$, NaCl and Mg extract on osteoclastogenesis, biochemical parameters including viability and TRAP activity were tested on days 3, 7, 14, 21 and 28. At the end time point of the culture period (day 28), stainings including TRAP staining, immunocytochemistry detection of CK and CTR were performed. Osteoclastic resorption activity on day 28 was also estimated by testing their ability to form resorption pits. Additionally, mRNA expressions for osteoclastic specific genes *CK*, osteoclast-associated immunoglobulin-like receptor (*OSCAR*) and receptor activator of nuclear factor κ B (*RANK*) were further performed to assess the molecular mechanism involved in the effect of NaCl on osteoclast resorption activity. For $MgCl_2$, the experiments were performed three times with three different donors, while for NaCl as well as for Mg extract the experiments were conducted twice with two different donors. The overview of osteoclast monocultures in $MgCl_2$-containing media, NaCl-containing media as well as Mg extract-containing media is present in Table 3.

Table 3 Overview of osteoclast monocultures

Osteoclast Monoculture	Donors	Concentrations	Culture time (days)	Time points (days)
$MgCl_2$	3	0, 2, 5, 10, 15, 25 mM	28	3, 7, 14, 21, 28
NaCl	2	0, 2, 5, 10, 15, 25, 50 mM	28	3, 7, 14, 21, 28
Mg extract	2	0, 5x, 10x, 30x dilution	28	3, 7, 14, 21, 28

The isolation of human PBMC and subsequent osteoclastogenesis for experiments concerning osteoclast monoculture is briefly described as in Fig. 4.

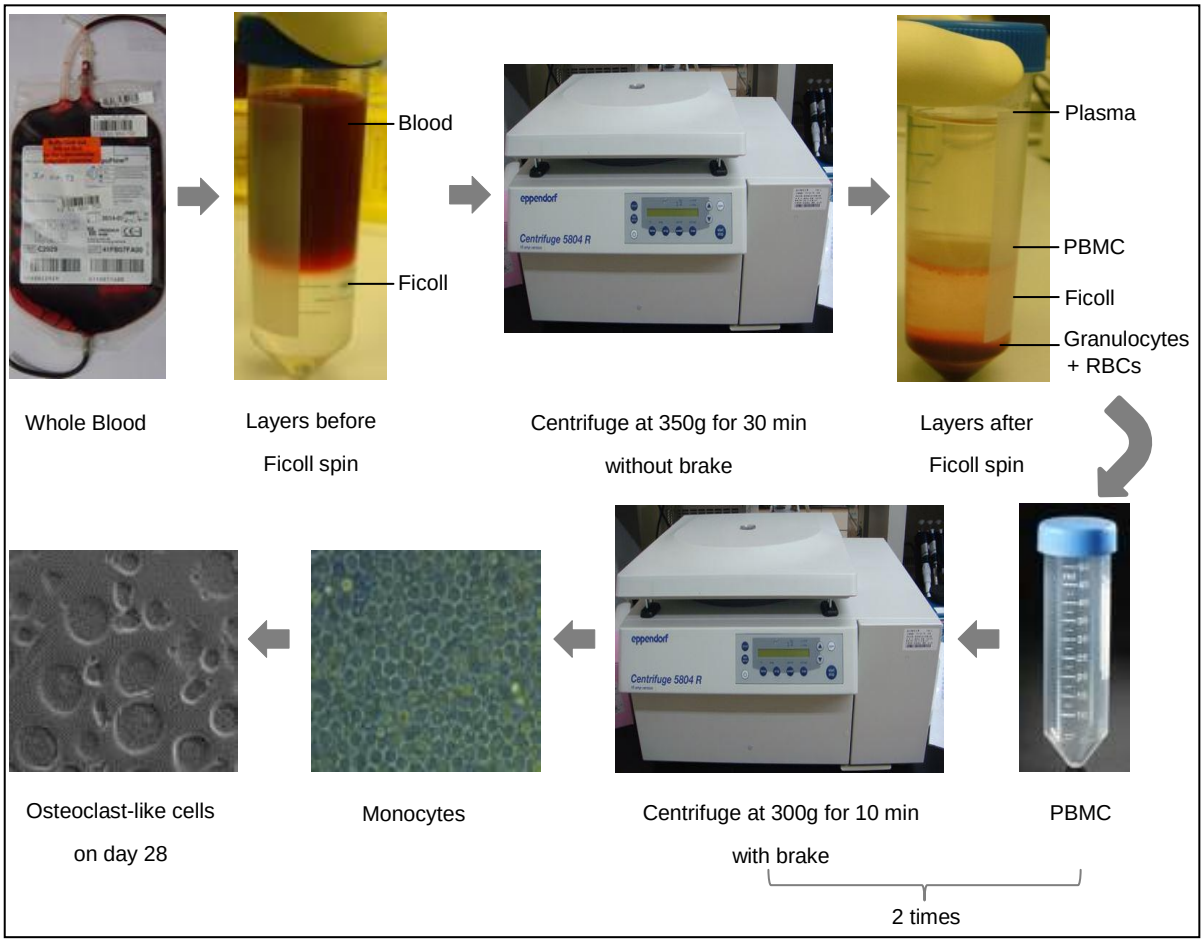


Fig. 4 Experimental procedure for the isolation of human PBMC from the whole blood. Pictures represent the different steps for the establishment of osteoclast monoculture system.

Osteoblast-Osteoclast coculture

For the osteoblast-osteoclast coculture experiment, hMSCs cell line SCP-1 was kindly provided by Prof. Schieker (Experimental Surgery and Regenerative Medicine, Department of Surgery, Ludwig-Maximilians-University). Böcker et al. have shown that they retained their ability to undergo osteogenic differentiation [107]. They were cultured at 37°C in a 5% CO_2 humidified atmosphere in the first osteogenic medium for 7 days. The first osteogenic medium contains α-MEM (Biochrom AG, Berlin, Germany) supplemented with 10% (v/v) FBS (PAA Laboratories GmbH, Linz, Austria), 2 mM L-glutamine (Life Technologies GmbH, Karlsruhe, Germany), 1% antibiotic/antimitotic solution (PAA Laboratories GmbH, Pasching, Austria) and osteoblastic differentiation factors (10^{-8} M Dexamethasone, 5 mM L-Ascorbic acid, 10^{-8} M 1α,25 Dihydroxyvitamin D3). The cells were then further cultured in the second osteoblast induction medium for another 21 days until the osteoblastic phenotype was detected. The second osteogenic medium consists of α-MEM (Biochrom AG, Berlin, Germany) supplemented with 10% (v/v) FBS (PAA Laboratories GmbH, Linz, Austria), 2 mM L-glutamine (Life Technologies GmbH, Karlsruhe, Germany), 1% antibiotic/antimitotic solution (PAA Laboratories GmbH, Pasching, Austria) and osteoblastic differentiation factors (10^{-8} M Dexamethasone, 5 mM L-Ascorbic acid, 10^{-8} M 1α,25 Dihydroxyvitamin D3 as well as 10 mM ß-Glycerolphosphate).

PBMC were isolated according to the procedure mentioned before and immediately cocultured with differentiated osteoblasts in 48-well plates (Nunc, Roskilde, Denmark, at a ratio of 1:100) to a final volume of 500 µL medium for 28 days. The coculture medium remained the same as the one used for SCP-1 cell culture but neither with osteogenic promoting factors nor osteoclastogenesis promoting factors (RANKL and M-CSF). Seven different dilutions of pure Mg extract (control, 30x, 10x, 5x, 3x, 2x and 1x in culture medium) were used and cultures were incubated in a humidified atmosphere at 37°C and 5% CO_2 for up to 28 days. The culture medium was carefully renewed every two days. The entire procedure was briefly described as in Fig. 5. The role of Mg extract on osteoblast and osteoclast differentiation was characterized either throughout the culture time (days 7, 14, 21 and 28) or at the end of culture period (day 28).

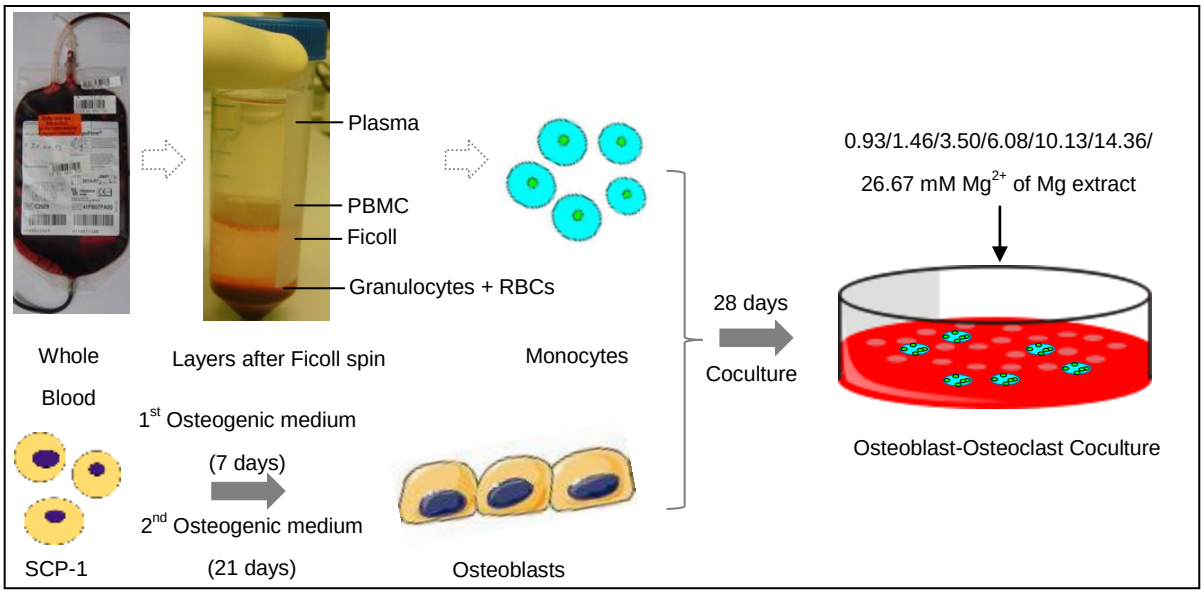


Fig. 5 Experimental procedures for osteoblastogenesis, isolation of human PBMC from the whole blood as well as the coculture of osteoblast and PBMC in the exposure of different concentrations of Mg extract. Pictures represent the different steps for the establishment of osteoblast-osteoclast coculture system.

3.4 Cell metabolism assay

Water soluble tetrazolium (WST-1) assay

For the experiments concerning the effect of $MgCl_2$, NaCl as well as Mg extract on the differentiation and function of osteoclasts monoculture, to assess cellular viability, a standard WST-1 (Roche Diagnostic GmbH, Mannheim, Germany) assay was used. It was performed according to the manufacturer's instructions. The colorimetric assay is based on the cleavage of the tetrazolium salt WST-1 by cellular enzymes to a highly coloured formazan. The formazan could absorb at 450 nm and the amount of the dye directly correlates to the number of metabolically active cells.

After 3, 7, 14, 21 and 28 days of culture, the cells were subsequently incubated with a 10 vol.% WST-1 dye solution (50 µL/well; n = 3 wells) under cell culture conditions for 2 h. Cell culture media (with corresponding Mg-containing, NaCl-containing as well as Mg extract-containing solutions, respectively) containing 10% (v/v) of WST-1 without any cells

were performed as blanks and treated similarly. After incubation, 200 µL of the culture supernatant (in triplicate) was collected and pipetted into 96-well plates (Nunc, Roskilde, Denmark). Their absorbance was subsequently measured using a spectrophotometer (Berthold Technologies GmbH, Bad Herrenalb, Germany) at 450 nm with a 620 nm reference wavelength.

Lactate dehydrogenase (LDH) assay

For osteoblast-osteoclast coculture experiment, LDH assay instead of WST-1 assay was performed by assessing LDH released into the media as a marker of dead cells. LDH is an oxidative enzyme extensively existing in cell membranes and cytoplasm. As it is released during tissue damage, determination of serum LDH activity is frequently considered as a good indicator of cellular damage for cytotoxicity studies [108].

In the present study, the cell culture supernatants (n = 3 wells) were harvested at 7, 14, 21 and 28 days of culture. The LDH assay via a LDH cytotoxicity detection kit (Roche Diagnostic GmbH, Mannheim, Germany) was conducted according to the manufacturer's instructions. Briefly, 100 µL aliquot of LDH substrate buffer was added into 100 µL cell supernatant (in triplicates) followed by incubation in dark at RT for 20 min. The absorbance was measured with a spectrophotometric microplate reader at 490 nm with 600 nm as a reference (Berthold Technologies GmbH, Bad Herrenalb, Germany).

3.5 Cell lysate preparation and Determination of total protein content

Preparation of cell lysate

The total protein synthesized by osteoblast-osteoclast coculture in the exposure of different concentrations of Mg extract was extracted on days 7, 14, 21 and 28. For osteoclast monocultures supplemented with various concentrations of $MgCl_2$, NaCl as well as a series of Mg extract concentrations, the total protein content was also extracted on day 3.

The cell lysate was obtained by using radioimmunoprecipitation assay (RIPA) lysis buffer. It contains 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). In order to avoid any sample degradation, all solutions and materials used for this procedure were previously cooled. Cell layers were rinsed twice with PBS and then covered with RIPA lysis buffer which has been freshly supplemented with a protease inhibitor cocktail (Sigma-Aldrich Chemie GmbH, Munich, Germany). The adherent cells were harvested with a plastic cell scraper (TPP, Trasadingen, Switzerland) and subsequently the cell suspensions were transferred into pre-labelled 1.5 mL micro-centrifuge tubes. After maintaining on ice for 30 min for lysis with pipetting up and down every 10 min, lysates were cleared of cell debris by centrifugation at 10, 000 g for 10 min at 4°C.

Determination of total protein content

Total protein content in the cell lysates (biological replicates = 3) was subsequently quantified with the BCA Protein Assay kit (Thermo Fisher Scientific, Bonn, Germany) in which the procedure recommended by the manufacturer for microplate was followed and bovine serum albumin (BSA) was used as protein standard. 25 µL BSA standard, blank (lysis buffer) and the unknown samples were added into 200 µL working reagent (in replicates; 50:1, Reagent A: B) and then incubated at 37°C for 30 min. A spectrophotometric microplate reader (Berthold Technologies GmbH, Bad Herrenalb, Germany) at 560 nm was used for the measurement of their absorbance and protein concentrations were determined correlating to a standard protein curve of absorbance.

3.6 TRAP & alkaline phosphatase (ALP) activity assay

Extracellular TRAP activity determination

For the experiments concerning the effect of $MgCl_2$, NaCl as well as Mg extract on the differentiation of osteoclasts, after culturing for 3, 7, 14, 21 and 28 days, osteoclast differentiation was evaluated based on the measurement of extracellular TRAP activity (three biological replicates per treatment).

P-nitrophenylphosphate (pNPP disodium hexahydrate; Sigma-Aldrich Chemie GmbH, Munich, Germany) was used as a substrate (pH 5.5-6.1). A 150 µL aliquot of the TRAP reaction solution which consists of 100 mM sodium acetate (Carl Roth GmbH, Karlsruhe, Germany), 50 mM disodium-tartrate dihydrate (Carl Roth GmbH, Karlsruhe, Germany) and 7.6 mM pNPP disodium hexahydrate (Sigma-Aldrich Chemie GmbH, Munich, Germany) was added to 50 µL of the samples in 96-well plates (in triplicates). The mixtures were incubated for 1 h at 37°C. Subsequently the enzymatic reaction was stopped by adding 50 µL of 3 M sodium hydroxide (NaOH; Carl Roth GmbH, Karlsruhe, Germany). The absorbance was measured at 405 nm (reference wavelength, 620 nm) using a microplate spectrophotometer (Berthold Technologies GmbH, Bad Herrenalb, Germany).

Intracellular TRAP activity determination

Osteoclastogenesis in the coculture system with different concentrations of Mg extract was quantified by the spectrophotometric measurement of intracellular TRAP activity (coming from the cell lysate) after 7, 14, 21 and 28 days of culture. pNPP disodium hexahydrate (Sigma-Aldrich Chemie GmbH, Munich, Germany) was used as a chromogenic substrate (3 biological replicates per Mg concentration). The measurement of intracellular TRAP content was performed according to the same procedure as that for the extracellular TRAP determination (three technical replicates; see ***Extracellular TRAP activity determination***).

Intracellular ALP activity determination

To evaluate osteogenic differentiation in the coculture experiment, the measurement of intracellular ALP activity was conducted on days 7, 14, 21 and 28. An aliquot of 25 µL cell lysate (biological/technical replicates = 3) was mixed with 75 µL ALP substrate buffer (pH adjusted to around 9.8). The ALP substrate buffer contains 9 mM pNPP disodium hexahydrate (Sigma-Aldrich Chemie GmbH, Munich, Germany), 1 M diethanolamine (Sigma-Aldrich Chemie GmbH, Munich, Germany), 1 mM $MgCl_2$ (Sigma-Aldrich Chemie

GmbH, Munich, Germany) and 1% Triton-100 (Carl Roth GmbH, Karlsruhe, Germany). After incubation at 37°C for 30 min, the enzymatic reaction was terminated by adding 25 µL NaOH solution (3 M; Carl Roth GmbH, Karlsruhe, Germany). ALP activity was then determined spectrophotometrically at 405 nm in a microplate reader (Berthold Technologies GmbH, Bad Herrenalb, Germany).

3.7 TRAP staining

Because of its abundant expression of TRAP by osteoclasts [109, 110], except for the determination of its extracellular/intracellular activity, the measurement of TRAP expression of the adherent cells by TRAP staining is also a common approach for the quantification of osteoclasts differentiation.

For the experiments concerning the effects of different concentrations of $MgCl_2$, NaCl and Mg extract on osteoclastogenesis, after 28 days of culture, TRAP staining was performed. The adherent cells (three replicates for each condition) were rinsed with PBS and then fixed with a TRAP fixation solution at RT for 5 min. The TRAP fixation solution contains 3.7% formaldehyde (Carl Roth GmbH, Karlsruhe, Germany) and 0.2% Triton X-100 (Carl Roth GmbH, Karlsruhe, Germany). Subsequently, after removal of the fixation solution, the cells were dyed with a TRAP staining solution. It contains 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 sodium tartrate (Carl Roth GmbH, Karlsruhe, Germany) and 40 mM sodium acetate (pH 5.0; Carl Roth GmbH, Karlsruhe, Germany). Followed by the removal of staining solution and replacement by PBS, red-stained TRAP-positive multinucleated cells that possessed three or more nuclei were scored as osteoclast-like cells (OCLs) and photographed. The means of at least five representative fields of view per condition were determined for quantification and formulated as the number of positive cells per mm^2.

3.8 CK and CTR staining

The cellular expression of CK and CTR in osteoclasts cultured with different concentrations of $MgCl_2$ and Mg extract was detected via immunocytochemistry (two replicates for each condition) after 28 days of culture. CTR staining was performed as well to investigate the effect of different NaCl concentrations on osteoclastogenesis.

After washing with PBS, the cells were fixed with 3.7% formaldehyde (Carl Roth GmbH, Karlsruhe, Germany) for 10 min at RT and then PBS was used for thorough rinse. For CK staining, the fixed cell layers were subsequently permeabilised 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 and then washed two times by PBS. The cells were immersed in BSA (10% in PBS (w/v); Carl Roth GmbH, Karlsruhe, Germany) for 1 h at 37°C in order to block the nonspecific binding sites. The cells were subsequently 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. Followed by two PBS washing steps (5 min/time), the samples were incubated with the appropriate secondary antibodies (Alexa Fluor 568 Goat Anti-Rabbit IgG (red) or Alexa

Fluor 488 Goat Anti-Rabbit IgG (Life Technologies GmbH, Karlsruhe, Germany (green)) 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). The nonspecific staining was identified by conducting a negative control in which staining omitting the primary antibodies was carried out. To visualise cell nuclei, 4-6-diamidino-2 phenylindole solution (DAPI; 1.5 µg/mL diluted in PBS; Sigma-Aldrich Chemie GmbH, Munich, Germany) was subsequently used and the cells were counterstained for 5 min at RT. The means of at least five representative images per condition were determined for quantification and formulated as the percentage of CK- or CTR-positive osteoclasts compared to control.

3.9 Resorption assay

Two-dimensional (2D) resorption assay

Functional assessment of osteoclast exposed to different concentrations of $MgCl_2$, NaCl and a series of concentrations of Mg extract was determined by a lacunar resorption assay. The resorption assay has been considered as the hallmark of the osteoclastic bone-breaking capacity. Osteoclast activity was determined in a lacunar resorption assay using cells cultured on slices of ivory which is a commonly used resorbable substrate for *in vitro* osteoclastic resorption assays (at least 3 chips per condition).

At the end of the culture period (day 28), cells were removed with 30% H_2O_2 (Carl Roth GmbH, Karlsruhe, Germany) and by wiping the surface of bone slices with tissue towels. To visualise resorption lacunae, dentine chips were stained with a 1% (w/v) toluidine blue (Carl Roth GmbH, Karlsruhe, Germany) solution (in ddH_2O) for at least 5 s at RT. After the chips being rinsed twice in tap water, the resorbed areas (pits) were microscopically visualised. At least five representative images were randomly captured from each dentine piece. ImageJ analysis software [111] was used for the quantification of pit resorption area.

Three-dimensional (3D) resorption assay

After 2D assessment of bone resorption area, the spatial resorption activity of osteoclasts in the conditions supplemented with different concentrations of NaCl was further determined by 3D resorption assay (at least 3 chips per condition). The volume of bone resorbed on the dentin slices was analysed with a Color 3D laser microscope (VK-9700; Keyence Corp, Japan) equipped with an optical laser unit and a scanning unit using dedicated VK Analyzer image analysing software.

Briefly, the sample surface was horizontally scanned in a point-by-point, line-by-line raster followed by vertical scanning through the movement of the lens in the direction of Z-axis. Thus, the resultant images were generated in which the surface of the dentine chips was characterized three-dimensionally. Five fields from each dentin slice were randomly selected and resorption volume for at least three dentin chips per NaCl concentration was assessed.

3.10 Real time quantitative polymerase chain reaction (RT-qPCR)

To analyse the differentiation of monocultured osteoclasts in the exposure of different concentrations of NaCl as well as cocultured osteoblasts and osteoclasts in the culture conditions with different concentrations of Mg extract, RT-qPCR was further conducted.

Cells were washed twice with PBS and immediately used for RNA preparation (biological replicates per condition). Total RNA isolation was performed by using the High Pure RNA Isolation Kit (Roche, Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's instruction. The concentrations of RNA samples were determined by a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). 150 ng of total RNA was reversibly transcribed into complementary DNA (cDNA) using a final volume of 20 µL reaction mixture with Oligo-dT primers (QuantiTect Reverse Transcription Kit, Qiagen). For quantitative RT-qPCR experiments, 1 µL of cDNA was used as a template and the analysis was performed using SsoFast EvaGreen Supermix and CFX96 Real-time PCR Detection System (both from Bio-Rad Laboratories; technical replicates = 3). The details of primer sequences (*RANKL*, osteoprotegerin (*OPG*), *M-CSF*, runt-related transcription factor 2 (*RUNX2*), distal-less homeobox 5 (*DLX5*), *CK*, *OSCAR*, *RANK*, spleen focus forming virus (SFFV) proviral integration oncogene (*SPI1*), nuclear factor-activated T cells c1 (*NFATc1*), nuclear factor-κB 1 (*NFκB1*) and the housekeeping genes *Actinβ*, β2 microglobulin (*B2M*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ribosomal protein L10 (*RPL10*)) are summarized in Table 4. The reference genes were carefully selected according to the geNorm algorithm method [112] automatically calculated with the CFX Manager Software (Bio Rad, Munich, Germany; version 3.0). The resulting PCR products were analysed with CFX Manager Software (Bio Rad, Munich, Germany; version 3.0) and the mRNA levels were normalised using the expression of the housekeeping genes.

Table 4 Primer sequences applied for RT-qPCR

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
RANKL	ATACCCTGATGAAAGGAGGA	GGGGCTCAATCTATATCTCG
OPG	CGCTCGTGTTTCTGGACAT	GGACATTTGTCACACAACAGC
M-CSF	TGCAGCGGCTGATTGACA	GATCTTTCAACTGTTCCTGGTCTACA
RUNX2	CAGTAGATGGACCTCGGGAA	ATACTGGGATGAGGAATGCG
DLX5	GAGTGTTTGACAGAAGGGTCC	GAATCGGTAGCTGAAGACTCG
CK	TCTCTCGGCGTTTAATTTGGG	AACCACCTCTTCACTGGTCAT
OSCAR	CCCCAGCTTCATACCACCCTA	CCGGCATCTCAAGGTCACG
RANK	GCTCAACAAGGACACAGTGTGC	CGCATCGGATTTCTCTGTCCCA
SPI1	ATGGAAGGGTTTCCCCTCGT	CCAGTAATGGTCGCTATGGCT
NFATc1	CACCAAAGTCCTGGAGATCCCA	TTCTTCCTCCCGATGTCCGTCT
NFκB1	CTGGAAGCACGAATGACAGA	CCTTCTGCTTGCAAATAGGC

(to be continued)

Reference Genes		
Actinß	CTTCCTGGGCATGGAGTC	TGATCTTCATTGTGCTGGGT
B2M	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT
GAPDH	GTCGGAGTCAACGGATTTG	TGGGTGGAATCATATTGGAA
RPL10	AGTGGATGAGTTTCCGCTTT	ATATGGAAGCCATCTTTGCC

Except for the above approaches described in common, several extra assays were performed for the osteoblast-osteoclast coculture experiment as well:

3.11 Cell morphology investigation

To evaluate the adhesion/proliferation and differentiation behaviour of osteoblast-osteoclast cocultures in the media supplemented with different concentrations of Mg extract, cells were co-seeded on the plastic surface of 48-well plates (Nunc, Roskilde, Denmark; at densities of 2×10^4 and 2×10^6 cells/mL for osteoblasts and PBMC, respectively) for 6 and 28 days. For each time point, three wells of each condition were monitored and light microscopy was performed for their morphological investigation.

3.12 Elisa

The quantitative determination of RANKL, M-CSF, and OSCAR in cell supernatant from the coculture experiment (in replicates) was performed with the Human TNFSF11/RANKL ELISA Kit (Biorbyt, catalogue No orb10264), Human M-CSF ELISA Kit (Biorbyt, catalogue No orb50070), and the OSCAR ELISA Kit (Uscn Life Science Inc., catalogue No SEC695Hu), respectively. It was conducted according to the protocol provided by the manufacturer (two technical replicates). After detection, the absorbance of the samples was measured at 450 nm in an Elisa plate reader (Berthold Technologies GmbH, Bad Herrenalb, Germany).

3.13 Alizarin Red S (ARS) staining

ARS is an anthraquinone derivative that can be used to identify Ca in tissue sections and forms an ARS-Ca complex in a chelation process, which is birefringent. Therefore, it has been used for decades as one of the important means to assess osteoblastic mineralisation.

In the osteoblast-osteoclast coculture experiment, after 28 days of culture, the late differentiation of cocultured osteoblasts was evaluated using ARS staining. Briefly, the medium was removed, and the cell layers from three wells of the culture plates were rinsed three times with PBS and fixed in 2% (v/v) formaldehyde solution (Sigma-Aldrich) at RT for 15 min. The cell layers were washed with ddH_2O and then stained for Ca deposit with 0.5% ARS (pH 4.0) (Sigma-Aldrich Chemie GmbH, Munich, Germany). The plates were incubated at RT for 20 min with gentle shaking until the production of nice red-orange staining of Ca was observed by light microscope. At least five representative fields of view were randomly captured per well.

3.14 Statistical analysis

All data was presented as mean ± SD. Statistical analyses were carried out by SigmaStat package (Systat software GmbH, Erkrath, Germany; version 11.0). One-way repeated

measures analysis of variance (ANOVA) was employed to compare all conditions for cellular proliferation and differentiation parameters.

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 when $P < 0.05$. Linear regression analysis was conducted with change in TRAP-positive staining counts as well as 2D and 3D resorption activity as the dependent variables and supplemented NaCl as independent variable via SPSS Statistics version 20. To detect different gene expression in RT-qPCR, Volcano Plot was employed in which the regulation threshold and *P* value were set to 2.00 and 0.05, respectively (directly calculated by CFX Manager Software; Bio Rad, Munich, Germany; version 3.0).

4 Results

4.1 Effects of extracellular $MgCl_2$ on the differentiation and function of human osteoclasts

The following observations were obtained from three independent experimental repeats using the PBMC isolated from three donors.

4.1.1 WST-1 assay

To assess the cellular viability and proliferation of human PBMC/osteoclasts cultured with different concentrations of $MgCl_2$, WST-1 assay was used at different time points (on days 3, 7, 14, 21 and 28). It measures the intracellular mitochondrial dehydrogenase activity of viable cells by forming water-soluble formazan dye with WST-1. Therefore, a change in the number of living cells results in an increase or decrease in the amount of formazan dye formed which could be quantified by measuring the absorbance at ë = 450 nm. From the figure (Fig. 6) we can see that although there is an increase in WST-1 activity with increased $MgCl_2$ concentrations on days 3 and 7, no significant difference is detected. For days 14, 21 and 28, a trend of first increase until 5 or 10 mM of $MgCl_2$ and then decrease was found without statistically significant difference.

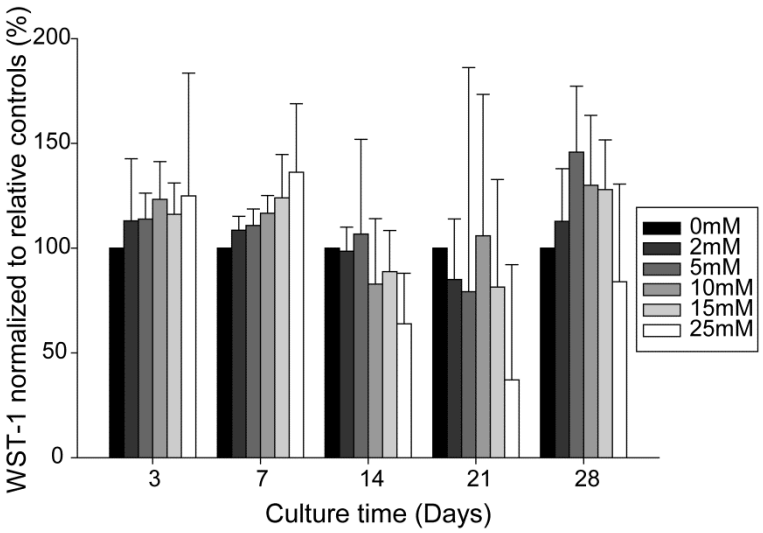


Fig. 6 Cellular metabolic activity of human PBMC/osteoclasts cultured in the media supplemented with different concentrations of $MgCl_2$ was conducted by WST-1 assay. For each time point, 0 mM (without external addition of $MgCl_2$) was set as the control (100%), to which the WST-1 for other conditions was normalised.

4.1.2 Total protein content

Normally, it is thought that the total protein content does not directly correlate with the cell number. However, it does show the protein-synthesising activity within the cells, which can reflect, to some extent, the proliferation and differentiation ability of the cultures. Thus, the total protein content of human PBMC/osteoclasts cultures exposed to different concentrations of $MgCl_2$ was determined at different time points (on days 3, 7, 14, 21 and 28) and the results are shown in Fig. 7. From the figure we can see, on days 3, 7, 21 and 28, the total protein contents of human PBMC/osteoclasts in the cultures supplemented with different concentrations of $MgCl_2$ increase first and then decrease. However, on day 14, a different trend is shown in which the decrease in total protein content is associated with the increase in the supplementation of $MgCl_2$.

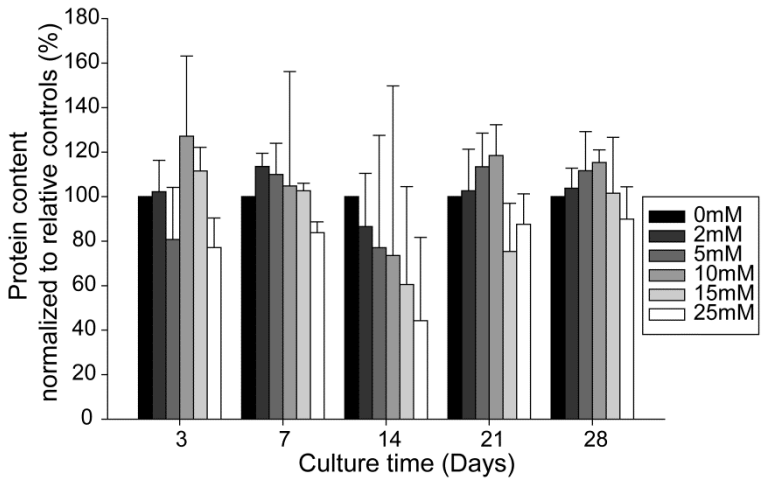


Fig. 7 Total protein was extracted from human PBMC/osteoclasts exposed to different concentrations of $MgCl_2$ on days 3, 7, 14, 21 and 28. For each time point, total protein content isolated from cells cultured in the control condition was set as 100% while that for other conditions was expressed as the percentage of the control (%).

4.1.3 TRAP activity assay

Since TRAP is an important marker for osteoclasts, determination of its serum level is widely used as an approach to monitor osteoclast differentiation. In the experiment concerning the effect of $MgCl_2$-containing media on human osteoclast differentiation, extracellular TRAP activity was measured over time (days 3, 7, 14, 21 and 28) to investigate the effect of $MgCl_2$ on osteoclast differentiation. As shown in Fig. 8, $MgCl_2$ has almost no effect on extracellular TRAP activity, i.e., no statistically significant difference is detected at all the time points.

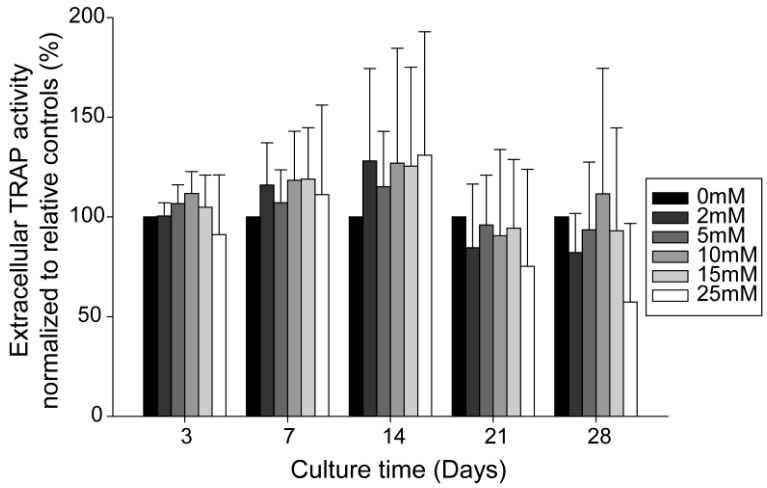


Fig. 8 Extracellular TRAP activities of human PBMC/osteoclasts cultured with different concentrations of $MgCl_2$ on days 3, 7, 14, 21 and 28 were evaluated by extracellular TRAP activity assay. For each time point, 0 mM (without external addition of $MgCl_2$) was set as the control (100%), to which the extracellular TRAP activities for other conditions were normalised.

4.1.4 TRAP staining

Except for the measurement of TRAP activity, TRAP staining is also widely used for the specific identification of osteoclasts. Therefore, it was carried out on day 28 to access the formation of multi-nucleated osteoclasts. Fig. 9 shows us the results of TRAP staining for human osteoclasts cultured with different concentrations of $MgCl_2$. A clear bell-shaped distribution pattern is observed with the maximum value at the culture condition supplemented with 15 mM $MgCl_2$. It is followed by a decrease at 25 mM (even lower than control; statistically significant difference is detected between 5/10/15 mM and the control group; see Fig. 9b).

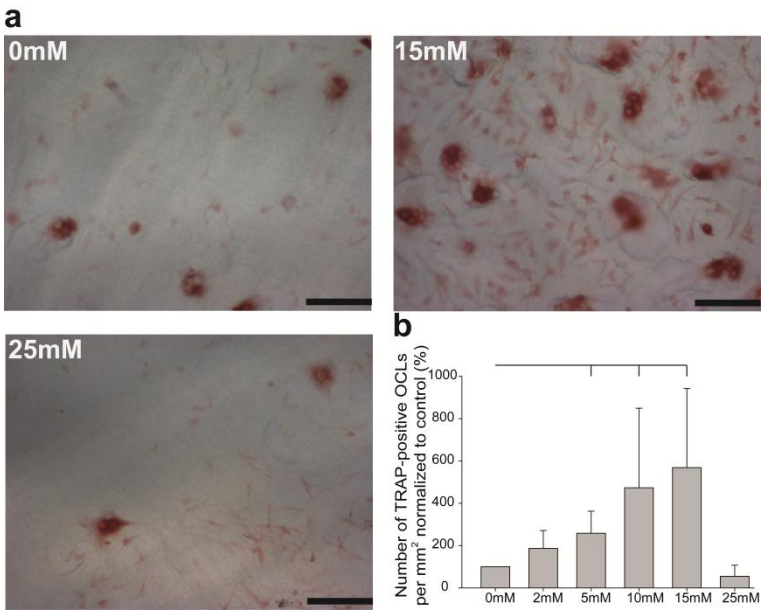


Fig. 9 TRAP staining of human osteoclasts cultured in different concentrations of $MgCl_2$ was conducted on day 28. (a) Micrographs of TRAP staining for OCLs cultured on dentine slice (scale bar = 100 µm). (b) The number of TRAP-positive cells recorded in 0 mM $MgCl_2$ was set as the control (100%), to which the numbers of TRAP-positive cells under other conditions were normalised. Symbols indicate significant differences between cultures supplemented with different concentrations of $MgCl_2$ and the control group ($P < 0.05$).

4.1.5 CK staining

Since CK is the enzyme that is responsible for the degradation of type I collagen in osteoclast-mediated bone resorption, its expression in the human PBMC/osteoclasts cultures with various concentrations of $MgCl_2$ was determined by fluorescence staining on day 28. The result is displayed in Fig. 10. An initial decrease in the number of CK-positive cells in the cultures with 2 mM $MgCl_2$ is detected. The number of CK-positive osteoclasts remains stable until 25 mM of $MgCl_2$. Then even fewer positive cells are observed compared to the control group (statistically significant difference is detected between the control condition and cultures with 2/25 mM $MgCl_2$; see Fig. 10b).

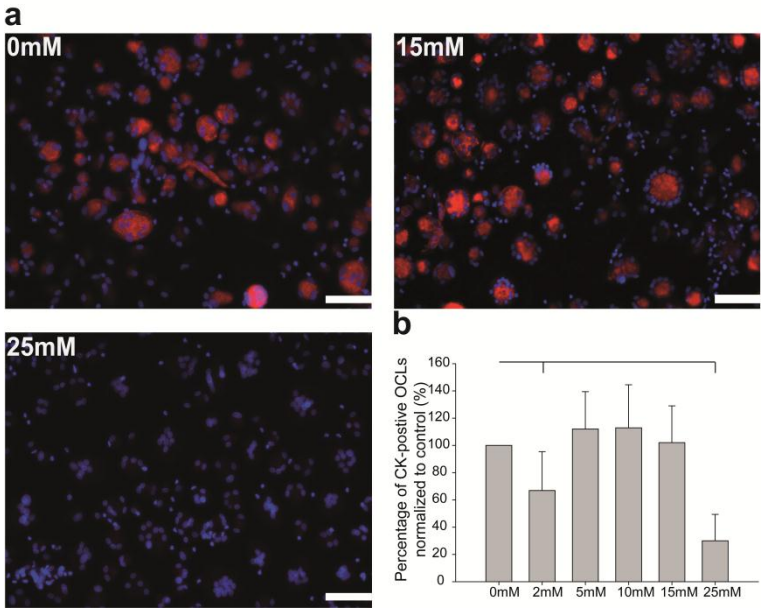


Fig. 10 The detection of osteoclast specific marker CK was performed on day 28. (a) Images of cultures with different concentrations of $MgCl_2$ (scale bar = 100 µm). The staining of nuclei was performed by DAPI staining and appears in blue colour. (b) The number of CK-positive cells under the control condition was considered as 100%, to which the count under other concentrations was normalised. Symbols indicate significant differences between cultures with different concentrations of $MgCl_2$ and the control group ($P < 0.05$).

4.1.6 CTR staining

CTR has been shown to have a strong correlation with the generation of active osteoclasts. Therefore, its expression in the human osteoclasts cultures exposed to different concentrations of $MgCl_2$ was accessed by CTR fluorescence staining at the last time point

(day 28). As for CK staining, the similar pattern is observed for CTR staining of the cells exposed to different concentrations of $MgCl_2$. The result is shown in Fig. 11. From Fig. 11b, we can see that an increased $MgCl_2$ content first increases and then decreases the number of CTR-positive cells (significant difference is observed between the cultures with 2/10/15/25 mM $MgCl_2$ and the control group; see Fig. 11b).

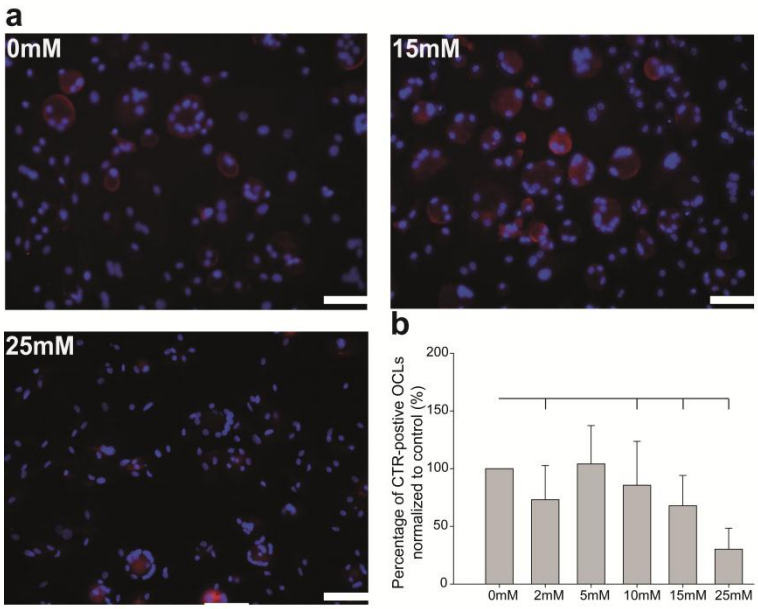


Fig. 11 CTR immunocytochemistry was conducted on day 28. (a) Images of CTR (red) staining (scale bar = 100 µm). Nuclei were stained with DAPI and appear blue. (b) The number of CTR-positive human osteoclasts counted for the control condition was set as 100%, to which the numbers of CTR-positive cells for other conditions were normalised. Symbols indicate significant differences between cultures supplemented with different concentrations of $MgCl_2$ and the control group ($P < 0.05$).

4.1.7 2D resorption assay

In order to estimate the effect of $MgCl_2$ on the osteoclast resorption activity, osteoclast-generated resorption was investigated via toluidine blue staining, followed by quantifying the surface area resorbed on dentine chips after culturing for 28 days. Fig. 12 shows us that osteoclastic resorption activity increases up to a certain $MgCl_2$ concentration and then decreases.

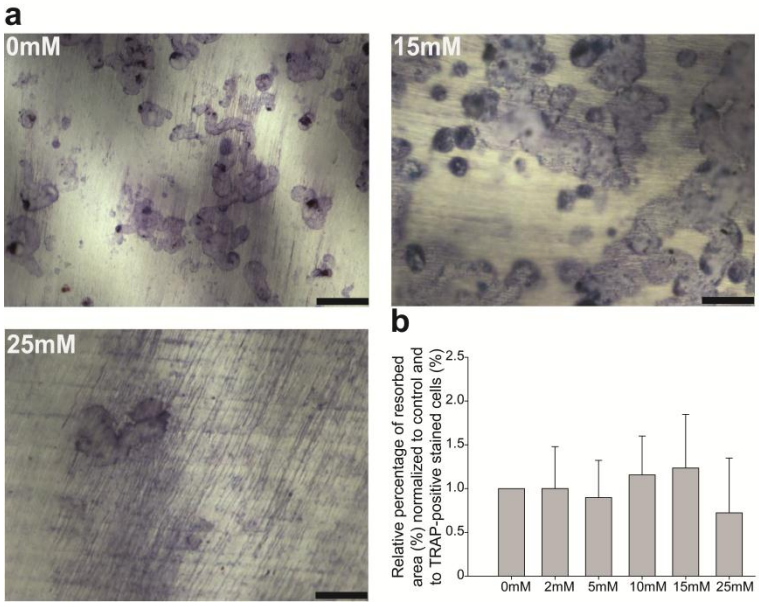


Fig. 12 Osteoclast resorption activity was assessed by pits absorption assay on day 28. (a) Images of dentine resorption (scale bar = 200 µm). (b) The resorbed area measured under the condition without the supplementation of $MgCl_2$ was set as 100%, to which the resorbed areas measured for other concentrations were normalised.

4.2 NaCl directly and dose-dependently increases osteoclastic differentiation and resorption

The following observations were obtained from two independent experimental repeats using the PBMC isolated from two different donors.

4.2.1 WST-1 assay

Cytotoxicity of different concentrations of NaCl against the human monocytes as well as osteoclasts was measured by WST-1 assay on days 3, 7, 14, 21 and 28. The result is presented in (Fig. 13). From the figure we can see, after 3 days of culturing, NaCl significantly stimulated cell proliferation with a maximum effect at mild cell culture condition. On days 7, 14, 21 and day 28, an increased NaCl content first increases (with the maximum values reached at moderate condition) and then decreases the activity of WST-1 (significant difference between moderate and the normal conditions found on day 14 while no significant difference detected on days 7, 21 and 28). Moreover, it should be noted that although not significant, the proliferating activity of cultures exposed to severe hypernatremia condition is found to be kept continuously reduced during the whole culture course compared to the control treatment.

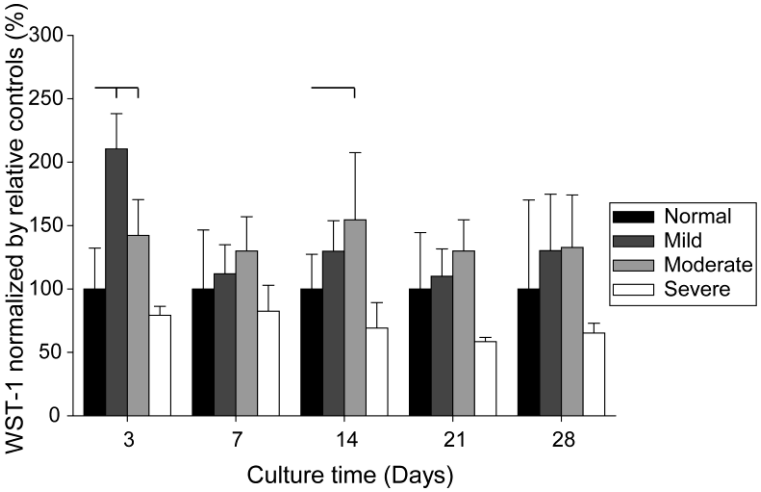


Fig. 13 Effect of NaCl on human PBMC/osteoclasts proliferation was evaluated by WST-1 assay on days 3, 7, 14, 21 and 28. For each time point, the value under normal culture condition was set as the control (100%), while results under other conditions were normalised as percentage of the control. Symbols indicate significant differences between the control group and other hypernatremia conditions ($P < 0.05$).

4.2.2 Total protein content

In order to access the protein-synthesizing activity of the human monocytes and osteoclasts cultures exposed to different concentrations of NaCl, total protein content was measured on days 3, 7, 14, 21 and 28. The results are shown in Fig. 14. From the figure we can see that on days 3, 14 and 28, an increase in the concentration of supplemented NaCl first increases and then decreases the total protein content of cultures in the exposure of different concentrations of NaCl (significant difference detected between normal and severe conditions on day 3). However, a different curve trend is observed on days 7 and 21 in which the increase in NaCl concentration is found to be associated with diminished protein content. It should be noted that the total protein content under severe hypernatremia condition is always lower as compared to less severe hypernatremia conditions at all time points although no significant differences were observed.

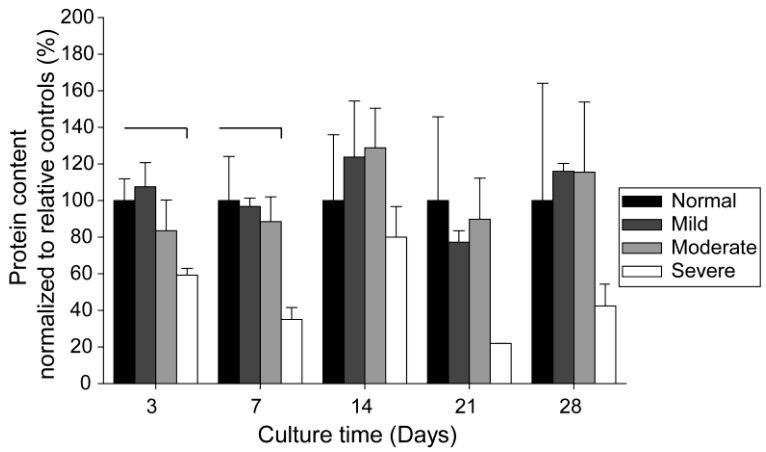


Fig. 14 Effect of different concentrations of NaCl on the total protein content was evaluated. For each time point, the protein content from cells under normal culture condition was set as the control (100%), to which that under other hypernatremia conditions were normalised. Symbols indicate significant differences between normal group and hypernatremia conditions ($P < 0.05$).

4.2.3 TRAP activity assay

As TRAP activity is widely regarded as an important cytochemical marker of osteoclasts, its expression was measured in the human PBMC/osteoclasts cultures with different concentrations of NaCl. A general bell-shaped distribution is observed on days 3, 14 and 21 (Fig. 15). The maximum levels are obtained at moderate condition on day 3 (statistically significant differences observed between normal and mild/moderate/severe condition), mild condition on days 14 and 21 (statistically significant differences detected between normal and mild/moderate/severe condition on day 14 - normal and mild/severe condition on day 21). On day 7, there is almost no difference in the extracellular TRAP activity in the cultures with different concentrations of NaCl. It is noteworthy that the extracellular TRAP amount under the severe hypernatremia condition is significantly lower relative to normal/mild/moderate condition after culturing for 28 days.

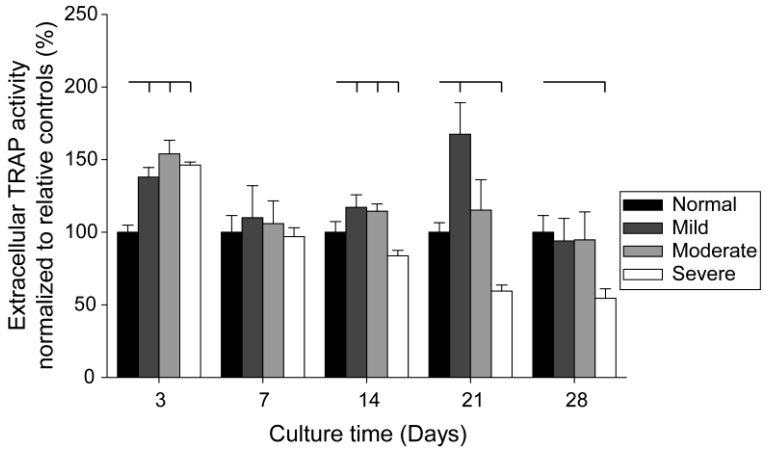


Fig. 15 Release of extracellular TRAP activity in response to the treatment of PBMC with NaCl was measured. Normal condition was set as the control (100%). Results for human PBMC/osteoclasts cultures with NaCl are expressed as the percentage of TRAP release in the cultures without NaCl (control). Symbols indicate significant differences between normal group and hypernatremia conditions ($P < 0.05$).

4.2.4 TRAP staining

In order to identify the number of TRAP-positive osteoclasts in the exposure of different concentrations of NaCl, TRAP staining was used on day 28. An increase in the number of TRAP-positive OCLs is found to be associated with the elevation in NaCl concentrations (Fig. 16). The formation of TRAP-positive OCLs is significantly enhanced in the severe hypernatremia condition (≈ 4-fold compared to the control; Fig. 16b). A significant linear correlation between NaCl concentration and the number of OCLs is investigated with $R^2 = 0.9008$, $P < 0.001$ (Fig. 16c).

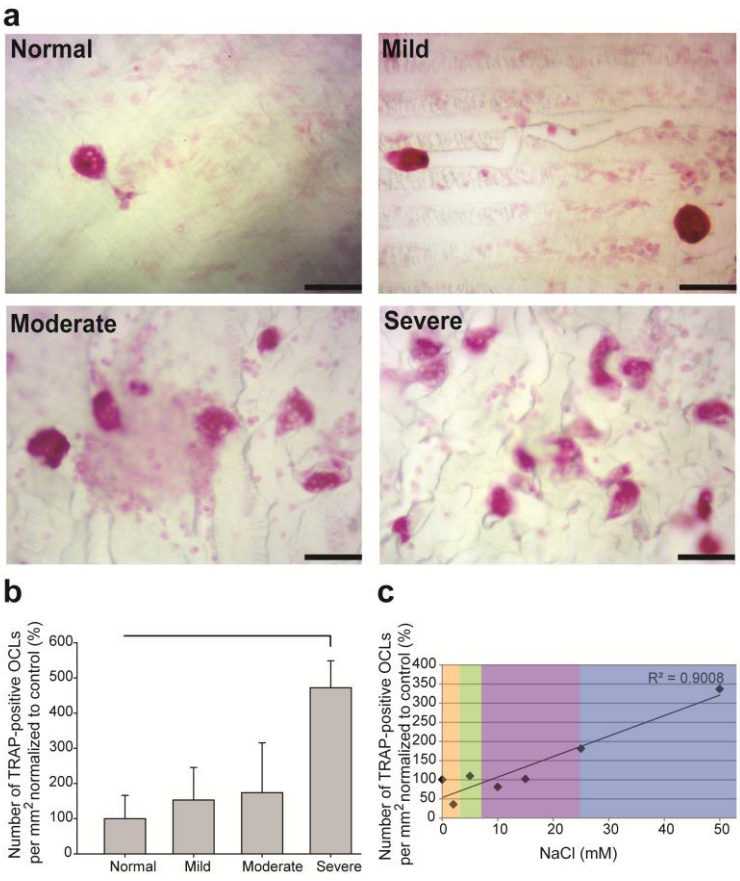


Fig. 16 The formation of TRAP expressing multinucleated human osteoclasts on dentin slices after 28 days of culture. (a) Micrographs of TRAP-positive staining of multinucleated OCLs. Scale bar = 100 µm. (b) The number of TRAP-positive cells with the normal treatment was set as control (100%) while the numbers of TRAP-positive cells under other hypernatremia conditions were expressed as the percentage of control. The statistical analyses are presented with symbols representing the statistical significances between the normal group and hypernatremia conditions ($P < 0.05$). (c) A linear relationship is suggested by regression analysis ($R^2 = 0.901$, $P < 0.001$).

4.2.5 CTR staining

As a specific marker of the differentiated osteoclasts, the number of CTR-positive osteoclasts exposed in different concentrations of NaCl was determined by CTR staining. The result is shown in Fig. 17. From Fig. 17b we can see that the number of CTR-positive cells increased with the increase in NaCl concentration. Significant difference is observed between cultures under severe hypernatremia and the normal condition.

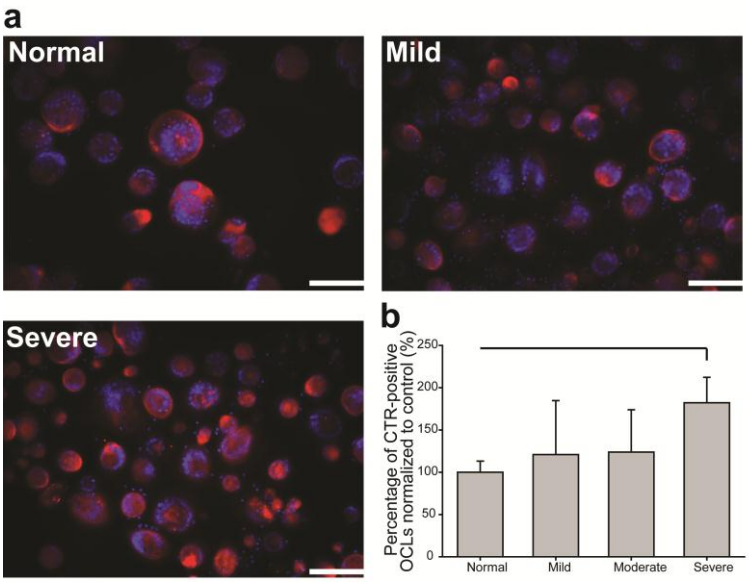


Fig. 17 CTR staining of human osteoclasts cultured in different concentrations of NaCl was performed on day 28. (a) Micrographs of CTR staining (scale bar = 100 µm). (b) The number of CTR-positive cells under the normal treatment was set as 100%, to which that under other hypernatremia conditions were normalised. Symbols indicate significant differences between the normal group and hypernatremia conditions ($P < 0.05$).

4.2.6 2D resorption assay

After 28 days of culture, the effect of NaCl supplementation on human osteoclasts resorption function was tested via 2D resorption assay. As described before, dentin slices were placed at the bottom of the culture well plates before seeding the monocytes in the presence of various conditions of NaCl supplementation as well as two osteoclastogenesis promoting factors (RANKL and M-CSF) for 28 days. As indicated in Fig. 18, similar osteoclast resorption patterns are observed with TRAP staining. Osteoclastic resorption activity (in terms of resorption area) is significantly and dosage-dependently increased with the increase in the additional NaCl concentrations. More than three times of bone resorption is obtained in the cultures under severe hypernatremia condition compared to the normal condition (Fig. 18b). Moreover, a significant, linear correlation between NaCl concentration and resorption area is detected by regression analysis with R^2 = 0.8917 and P < 0.001 (Fig. 18c).

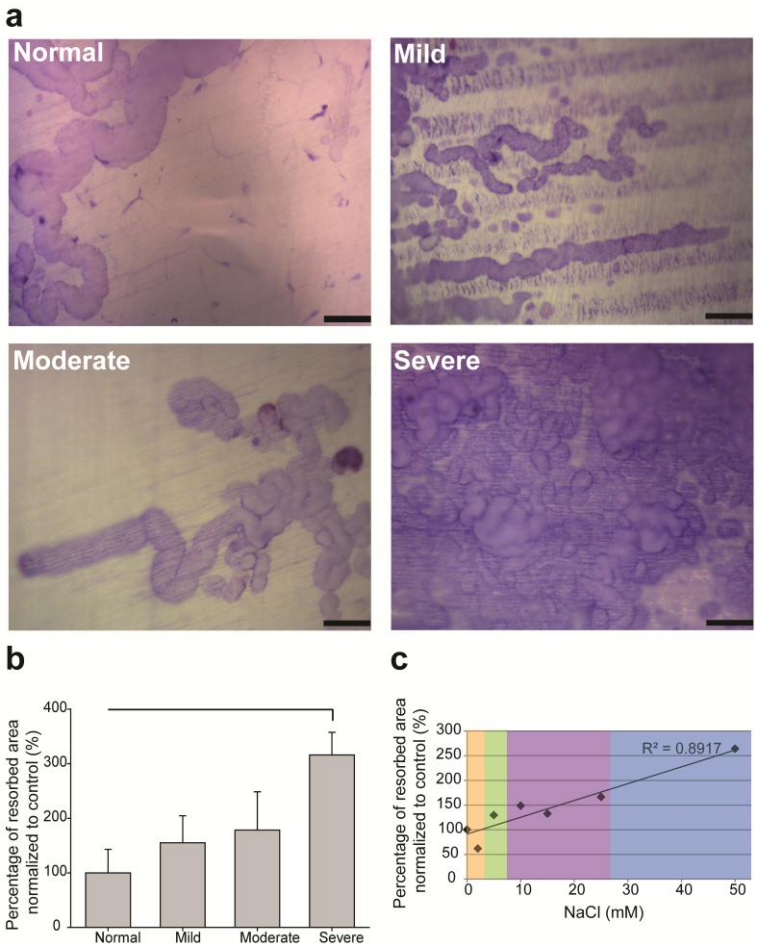


Fig. 18 Effect of NaCl on human osteoclasts resorption was evaluated by 2D resorption area assay on day 28. (a) Determination of osteoclastic resorption area by toluidine blue staining. scale bar = 200 µm; (b) The resorbed area measured for the normal treatment was set as the control (100%), to which the resorbed areas under other hypernatremia conditions were expressed as the percentage of the control condition. The respective statistical analyses are presented and symbols denote significant differences between the normal group and hypernatremia conditions (P < 0.05). (c) A positive linear-correlation was determined by regression analysis with R^2 = 0.892; (P < 0.001).

4.2.7 3D resorption assay

Except for the measurement of resorption area via 2D resorption assay, 3D resorption assay conducted by confocal laser scanning microscopy was further performed at the end time point (day 28) in order to evaluate osteoclast resorption activity in terms of resorption volume. Fig. 19a showed us the spatial resorption behaviour of human osteoclasts in various hypernatremia conditions (i.e., normal/mild/moderate/severe hypernatremia condition). It can

be clearly seen that both the shape and size of the individual resorption lacunae are highly variable among different hypernatremia conditions. The pits produced by cells treated with normal/mild/moderate hypernatremia condition are smaller and shallower when compared to those created by cultures in the exposure of the severe hypernatremia condition (Fig. 19a). When the resorption volume was quantified and normalised to the control (the normal condition; 100%), it could be seen from Fig. 19b that there is a significant increase between the normal condition and the mild/moderate/severe conditions (Fig. 19b). The excavated volume by osteoclasts under severe hypernatremia condition is significantly increased by 3.5-fold higher compared to osteoclastic resorption volume under the control condition. A linear correlation with R^2 = 0.9664 and P < 0.001 was further detected by regression analysis between the resorption volume and the concentration of supplemented NaCl (Fig. 19c).

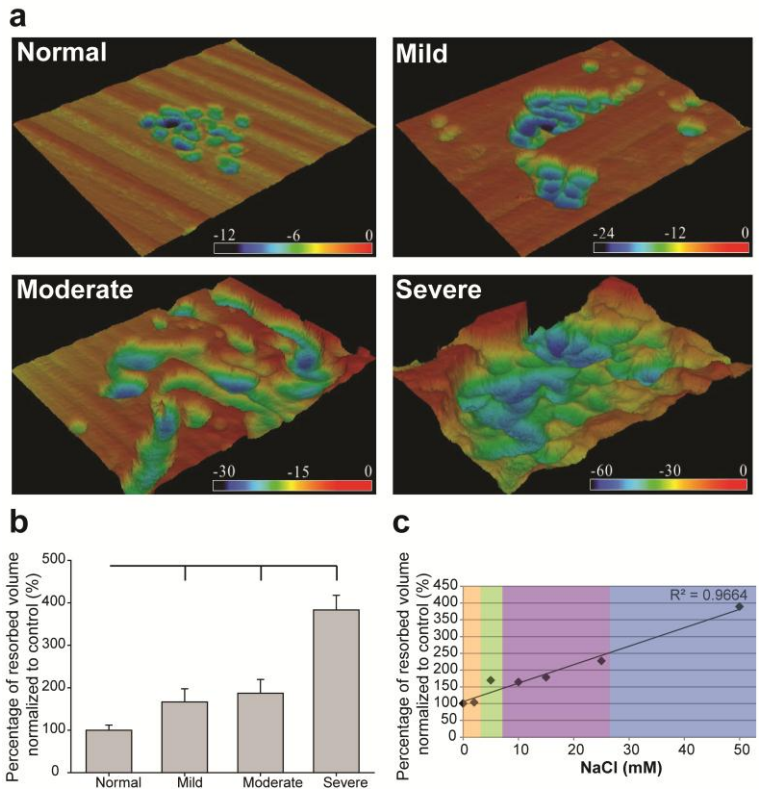


Fig. 19 Effect of NaCl on human osteoclast resorption activity was investigated by 3D resorption analysis via confocal laser scanning microscopy after 28 days' culture. (a) Images of resorption pits. The difference in the depths of the resorption pits in the vertical direction is represented by different colour contours (black, deep; red, shallow; µm); (b) VK Analyzer image analysing software was used to determine the resorbed volume. The resorbed volume of pits measured under the normal condition was recorded as the control (100%), to which the resorbed volumes evaluated under other hypernatremia treatments were normalised. The respective statistical analyses are presented. Symbols indicate significant differences between normal group and hypernatremia conditions (P < 0.05); (c) A linear-correlation relationship between the resorption volume and the concentration of supplemented NaCl is detected with R^2 = 0.9664 and P < 0.001 by regression analysis.

4.2.8 RT-qPCR

To access the effects of different concentrations of NaCl on the expression of human osteoclast specific genes, RT-qPCR was performed on day 28. As shown in Fig. 20, when cells were treated with varying concentrations of NaCl, mRNA expression for *CK*, *OSCAR* and *RANK* is not significantly modified in the samples under different hypernatremia conditions. It indicates that osteoclasts expressed *CK*, *OSCAR* and *RANK* mRNA constantly and that their expression was not affected by treatment with different concentrations of NaCl.

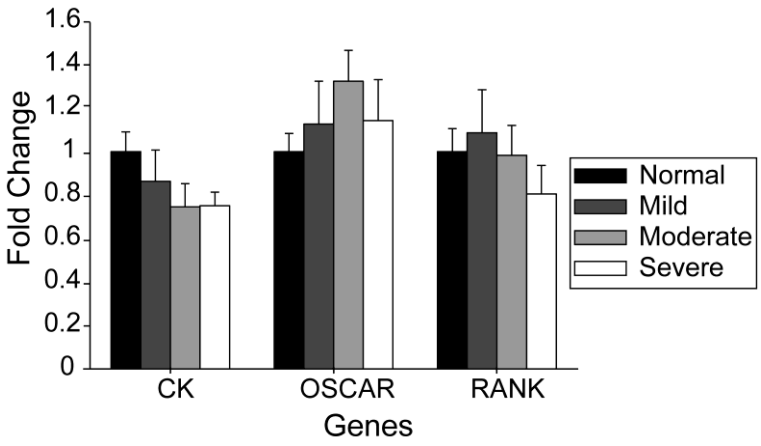


Fig. 20 Effect of various hypernatremia conditions on *CK*, *OSCAR* and *RANK* mRNA expression in human OCLs. Isolated PBMC cells were exposed to the factors RANKL and M-CSF as well as the supplementation of different concentrations of NaCl for 28 days. After removal of the media, RNA was extracted and reverse transcribed. cDNA was subsequently subjected to PCR using specific primers. Expression of the markers was normalised to the expression of the reference genes. For comparison, gene expression in normal condition was set to 1.

4.3 Effects of extracellular Mg extract on the differentiation and function of human osteoclasts

The following observations were obtained from two independent experimental repeats using the PBMC isolated from two donors.

4.3.1 Mg-containing media

The measurement of Mg and Ca contents as well as their pH values and osmolalities was performed in order to characterize the different concentrations of Mg extract. The results are presented in Table 5.

Table 5 Characterization of Mg extract solutions

Extract	pH	Osmolality (osmol kg^{-1})	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

4.3.2 WST-1 assay

Cell viability of the PBMC/osteoclasts cultures supplemented with different concentrations of Mg extract was determined using WST-1 assay on days 3, 7, 14, 21 and 28. As shown in Fig. 21, WST-1 activity is enhanced with the increase in the concentration of Mg extract on day 3 (statistically significant difference detected between 0.93 and 6.08 mM Mg^{2+} of Mg extract). However, the Mg extract concentrations decreases WST-1 activity at the following time points with the statistically significant differences detected on days 14 and 21 between the control and 3.50/6.08 mM Mg^{2+} of Mg extract as well as on day 28 between the control and 6.08 mM Mg^{2+} of Mg extract.

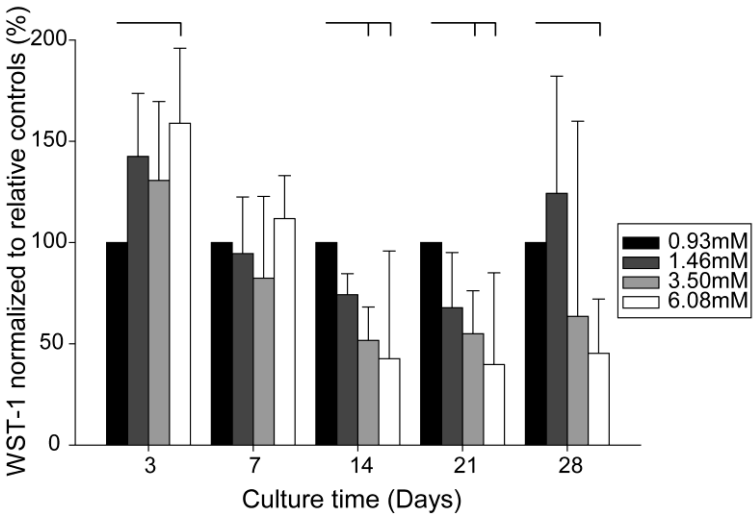


Fig. 21 Cellular metabolic activity of the human PBMC/osteoclasts cultured in the media supplemented with different concentrations of Mg extract was conducted by WST-1 assay. The absorbance was detected using a spectrophotometer at a wavelength of 450 nm with a reference wavelength of 620 nm to determine the cell viability in comparison with the control (with no additional Mg extract). For each time point, the control condition was set as 100%, to which the WST-1 for other concentrations was normalised. Respective statistical analyses are presented and symbols indicate significant differences between the control group and cultures supplemented with different concentrations of Mg extract ($P < 0.05$).

4.3.3 Total protein content

Except for WST-1, the direct approach, the measurement of total protein content was also performed which indirectly indicated the proliferation and differentiation ability of the human PBMC/osteoclasts cultures with different concentrations of Mg extract. As shown in Fig. 22, total protein content from cultures exposed to various concentrations of Mg extract exhibits different patterns in different time points. While a general time-dependent increase/decrease in total protein content occurred on day 3 and day 28, respectively, on days 7, 14 and 21, there is a decrease first until 3.50 mM Mg^{2+} of Mg extract and then followed by an increase. However, no significant difference is detected at all the time points.

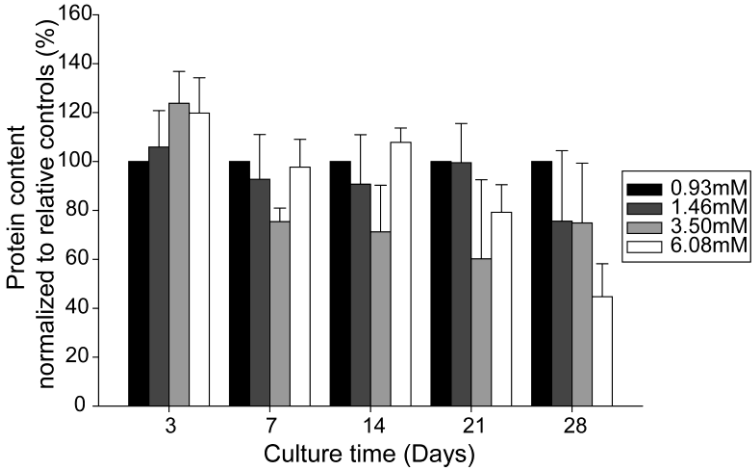


Fig. 22 Total protein content was extracted from PBMC/osteoclasts exposed to a series of Mg extract concentrations. For each time point, total protein content from cells cultured in the control condition was set as 100% while that for other conditions was expressed as the percentage of control. No significant differences between control and cultures supplemented with different concentrations of Mg extract are detected.

4.3.4 TRAP activity assay

The effects of various concentrations of Mg extract on human PBMC/osteoclasts were determined at different time points by measuring the extracellular TRAP activity as it is a biochemical marker of osteoclast function and degree of bone resorption. Fig. 23 shows the results of extracellular TRAP activity of cultures in the presence of a series of Mg extract concentrations. A clearer trend is observable, i.e., a constant decrease in the extracellular

TRAP activity is found associated with increased concentrations of Mg extract over days (statistically significant differences are detected between control and 6.08 mM Mg^{2+} of Mg extract on days 7, 14, 21 and 28 - control and 3.50 mM Mg^{2+} of Mg extract on days 3, 7, 14 and 21 - control and 1.46 mM Mg^{2+} of Mg extract on day 7).

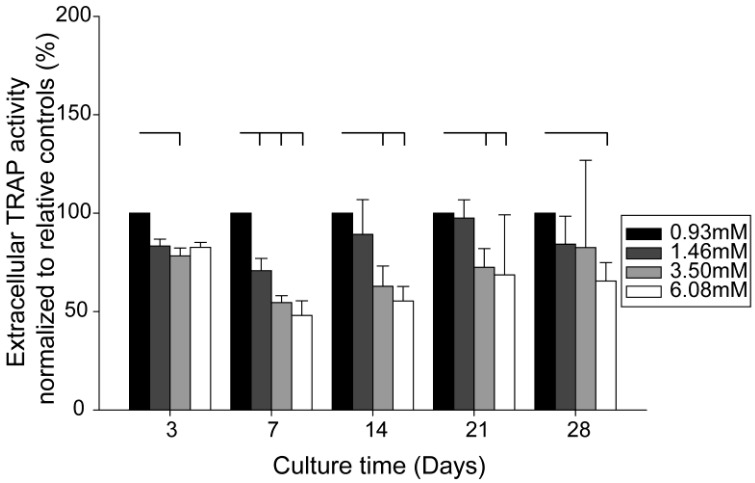


Fig. 23 Extracellular TRAP activities of human PBMC/ osteoclasts cultured with different concentrations of Mg extract were evaluated by TRAP activity assay. The absorbance was measured at 405 nm with a reference wavelength at 620 nm. For each time point, the culture without external addition of Mg extract was set as the control (100%), to which the extracellular TRAP activities for other conditions were normalised. The respective statistical analyses are presented. Symbols indicate significant differences between the control group and cultures supplemented with different concentrations of Mg extract ($P < 0.05$).

4.3.5 TRAP staining

The quantification of TRAP-positive human osteoclasts in the cultures with different concentrations of Mg extract was determined using TRAP staining. As shown in Fig. 24, a decrease in the number of TRAP-positive osteoclasts is observable associated with increased concentrations of Mg extract (see Fig. 24b; statistically significant difference observed between control and 3.50/6.08 mM Mg^{2+} of Mg extract).

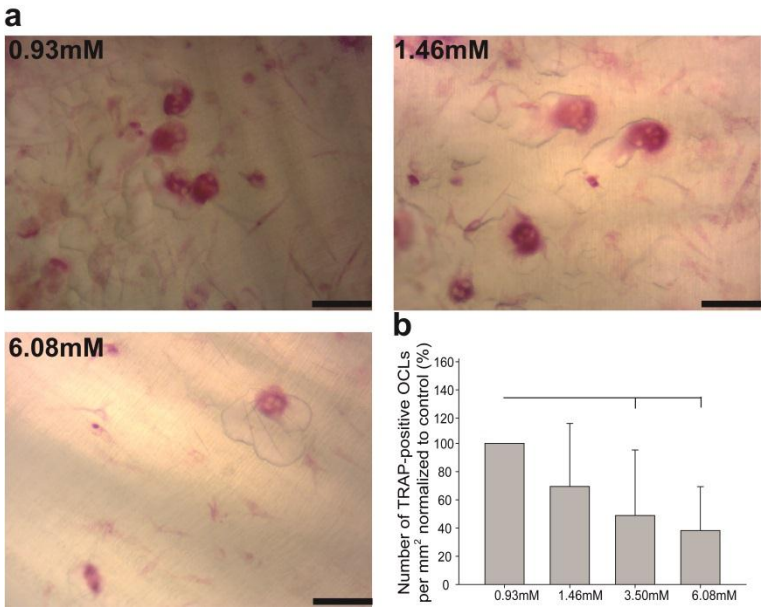


Fig. 24 TRAP staining of human osteoclasts cultured in different concentrations of Mg extract was performed on day 28. (a) Micrographs of TRAP staining (scale bar = 100 µm) (0.93/1.46/ 6.08 mM Mg^{2+} of Mg extract, respectively). (b) The number of TRAP-positive cells recorded in 0.93 mM Mg^{2+} of Mg extract was set as the control (100%), to which the numbers of TRAP-positive cells under other conditions were normalised. The statistical analyses are presented. Symbols indicate significant differences between control and cultures supplemented with different concentrations of Mg extract ($P < 0.05$).

4.3.6 CK staining

To quantify the effects of Mg extract on the number of CK-positive human osteoclasts, CK staining was done on day 28. The results of CK expression in osteoclasts exposed to different concentrations of Mg extract are displayed in Fig. 25. A continuous decrease in the number of CK-positive cells is found to be associated with increased concentrations of Mg extract (statistically significant decrease observed between the control and 1.46/3.50/6.08 mM Mg^{2+} of Mg extract treatments; see Fig. 25b).

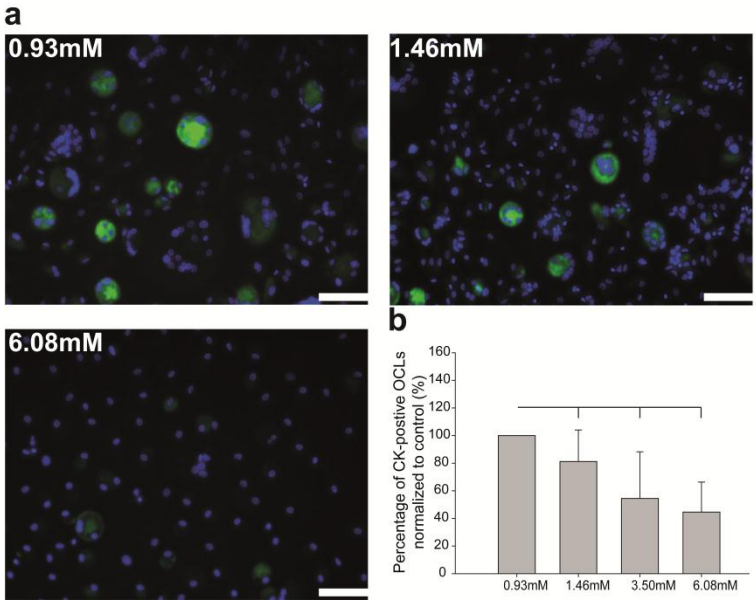


Fig. 25 The detection of osteoclast specific marker CK was performed on day 28. (a) Images of cultures supplemented with different concentrations of Mg extract (0.93/ 1.46/6.08 mM Mg^{2+} of Mg extract, respectively; scale bar = 100 µm). The staining of nuclei was performed by DAPI staining and appears in blue colour. (b) The number of CK-positive cells counted under the control condition was considered as 100%, to which the numbers of CK-positive cells under other conditions were normalised. Respective statistical analyses are presented and symbols indicate significant differences between the control group and cultures supplemented with different concentrations of Mg extract ($P < 0.05$).

4.3.7 CTR staining

The detection of osteoclastic specific marker CTR was performed by CTR staining on day 28 (Fig. 26). For the number of CTR-positive osteoclasts under the conditions exposed to various concentrations of Mg extract, no significant difference is detected (see Fig. 26b).

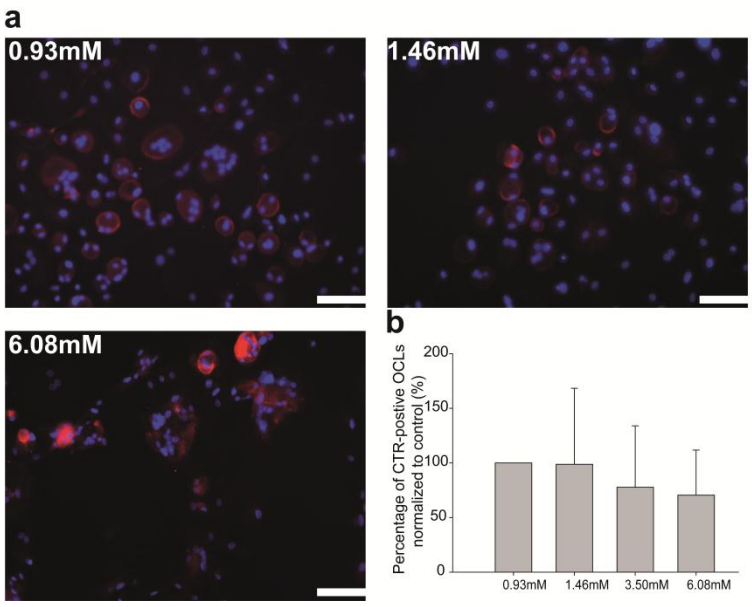


Fig. 26 CTR immunocytochemistry was conducted on day 28. (a) Images of CTR (red) staining (0.93/1.46/6.08 mM Mg^{2+} of Mg extract, respectively; scale bar = 100 µm). Nuclei were stained with DAPI and appear blue. (b) The number of CTR-positive human osteoclasts counted for the control treatment was set as 100%, to which the numbers of CTR-positive cells for other conditions were normalised.

4.3.8 2D resorption assay

Dentine resorption activity assay was performed on day 28 to estimate the effect of Mg extract on human osteoclast resorption activity (Fig. 27). The resorption activity per osteoclast for the cells cultured with different Mg extract concentrations was surprisingly increased up to 6.08 mM Mg^{2+} of Mg extract (Fig. 27b; significant difference was detected between the control group and the cultures supplemented with 6.08 mM Mg^{2+} of Mg extract).

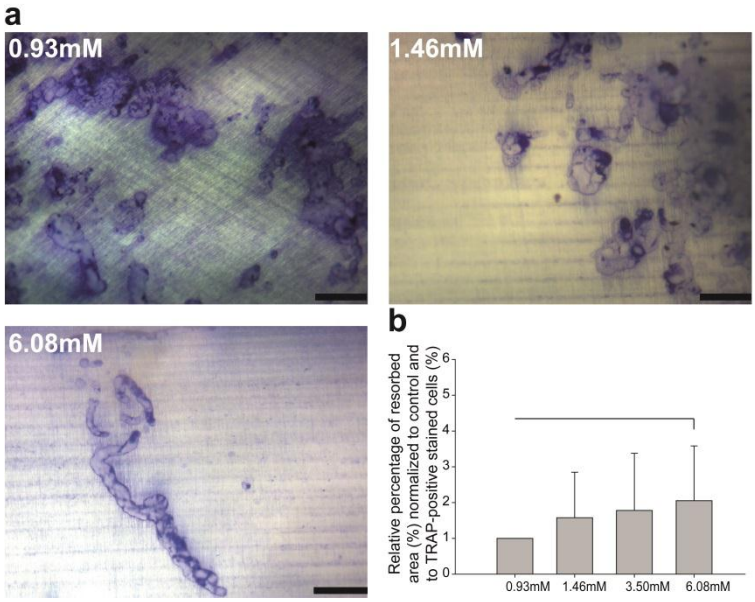


Fig. 27 Human osteoclast resorption activity was assessed by pits absorption assay on day 28. (a) Images of dentine resorption (0.93/1.46/6.08 mM Mg^{2+} of Mg extract, respectively; scale bar = 200 µm). (b) The resorbed area measured under control conditions was set as 100%, to which that measured for other conditions were normalised. The statistical analyses are presented. Symbols indicate significant differences between the control condition and cultures supplemented with different concentrations of Mg extract ($P < 0.05$).

4.4 Effects of extracellular Mg extract on the proliferation and differentiation of human osteoblast-osteoclast cocultures

The following observations were obtained from two independent experimental repeats using the PBMC isolated from two donors.

4.4.1 Mg extract-containing media

To characterize the different concentrations of Mg extract, their pH and osmolalities as well as the Mg and Ca contents were determined and the results are present in Table 6.

Table 6 Characterization of Mg extract and its dilutions

Extract	pH	Osmolality (osmol kg^{-1})	Mg content (mM)	Ca content (mM)
0	7.80	0.33	0.93	1.76
30x	7.80	0.34	1.46	1.70
10x	7.90	0.38	3.50	1.59
5x	8.04	0.46	6.08	1.42
3x	8.09	0.48	10.13	0.97
2x	8.17	0.49	14.36	0.43
1x	8.35	0.51	26.67	0.08

4.4.2 Cell morphology investigation

The initial cellular adhesion, proliferation and differentiation behaviour of human osteoblasts and monocytes/osteoclasts in the cocultures exposed to a series of Mg extract concentrations after 6 and 28 days of culture was estimated via light microscopy. As indicated in Fig. 28a (only results of cells cultured in 0.93, 3.50, 6.08 and 26.67 mM Mg^{2+} of Mg extract are presented), after 6 days of incubation osteoblasts were alive in all the cell conditions (as shown in black arrow). However, in addition to that layer, round-shaped monocytes could only be identified adherent in the cultures with lower concentrations of Mg^{2+} in Mg extract (as shown in white arrow). For those exposed to higher concentrations of Mg extract (for example, 14.36/26.67 mM Mg^{2+} of Mg extract), most of the monocytes were found detached from the surface of the plates with small cell debris visible (as indicated by the solid circles; Fig. 28a). Some degree of osteoclastogenesis or even activation was proved by the formation of multinucleated OCLs after culturing for 28 days with the initial direct physical contact of osteoblasts (Fig. 28b). Both osteoblasts and osteoclasts in the coculture conditions with different Mg extract concentrations spread well. While osteoblasts attached in a long, flattened manner, osteoclasts presented its large and multinucleated phenotype. It should be highly emphasised that osteoclasts were formed in the absence of external addition of RANKL and M-CSF. Obviously in the cultures with 26.67 mM Mg^{2+} of Mg extract osteoblasts were found existed in multiple layers compared with the cells in other Mg extract concentrations or the control condition (Fig. 28b).

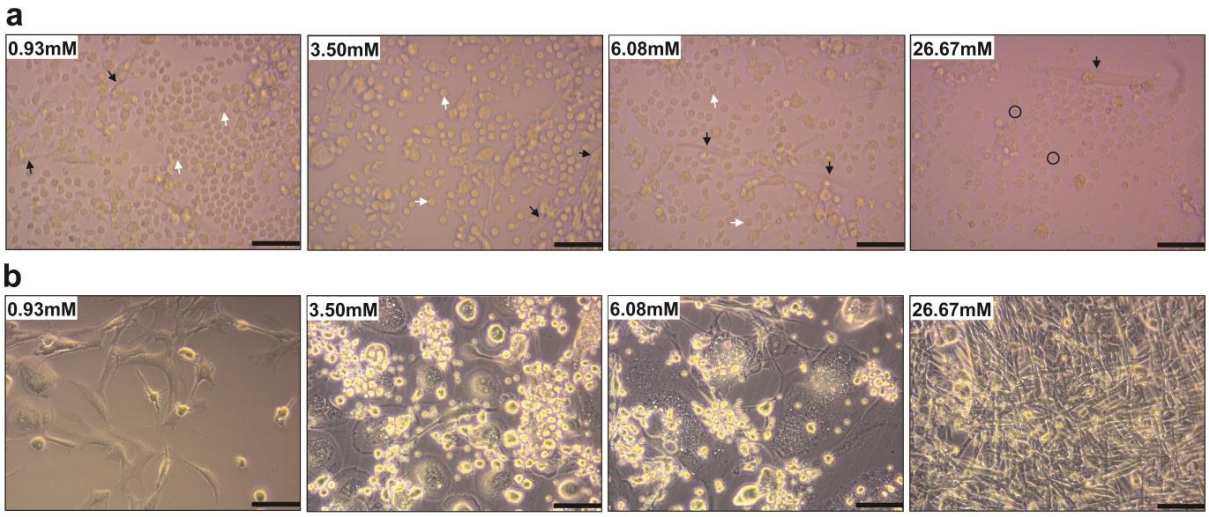


Fig. 28 Light microscopy of human osteoblast-osteoclast cocultures in the exposure of various concentrations of Mg extract was performed on day 6 (a) and day 28 (b). Images of the cocultures under the control condition as well as the culture conditions exposed to 3.50, 6.08 and 26.67 mM Mg^{2+} of Mg extract are shown. Black, white arrow as well as solid circle represents osteoblast, monocyte and cell debris, respectively. Scale bar = 100 µm.

4.4.3 Total protein content

To evaluate the activity of synthesising protein of the human osteoblast-osteoclast cocultures, the total protein content of the cocultures exposed to various concentrations of Mg extract was calculated on days 7, 14, 21 and 28 (Fig. 29). A general bell-shaped trend was observed with the maximum values being reached at cultures with 3.50 mM Mg^{2+} of Mg

extract on days 7 and 14. On days 21 and 28, total protein content first increases and then decreases with a higher concentrated Mg extract (no significant difference detected). Moreover, it was discovered that the total protein content in cultures with 14.36 mM Mg^{2+} of Mg extract was lower on day 7 but higher on day 14 compared to the control condition. For cultures in the condition with 26.67 mM Mg^{2+} of Mg extract, the total protein content was lower on day 7 and day 14 but higher on day 21 compared to that for control condition.

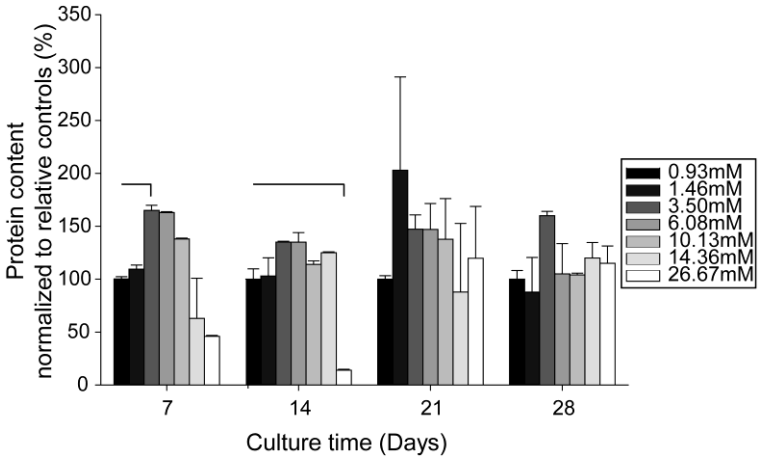


Fig. 29 Total protein content of the human cocultures with various Mg extract concentrations was measured. For each time point, total protein content isolated from cells cultured in the control condition was set as 100% while that in other conditions was normalised. Symbols indicate significant differences between the control group and other conditions ($P < 0.05$).

4.4.4 LDH assay

Since LDH in an indicator of cell death, its release into the supernatant of human osteoblast-osteoclast cocultures supplemented with various concentrations of Mg extract was measured to access the damage of Mg extract to the cells. As presented in Fig. 30, the absorbance of LDH leakage on day 7 was elevated gradually with the increase in Mg extract concentrations. The significant difference of LDH release between control and higher concentrations of Mg extract (especially 14.36 and 26.67 mM Mg^{2+} of Mg extract) was detected on days 7, 21 and 28.

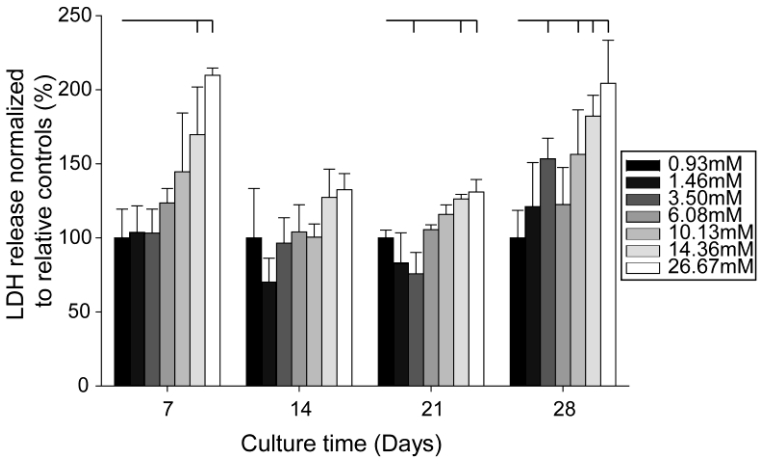


Fig. 30 Effect of Mg extract on LDH release was estimated by LDH assay on days 7, 14, 21 and 28. For each time point, the LDH in cultures without Mg extract was set as 100%, to which that under other conditions was normalised. Symbols indicate significant differences between the control group and conditions supplemented with different concentrations of Mg extract ($P < 0.05$).

4.4.5 TRAP activity assay

As a highly important osteoclast marker, the differentiation extent of human PBMC monocytes in the coculture systems supplemented with various concentrations of Mg extract was further estimated by the determination of intracellular TRAP activity on days 7, 14, 21 and 28.

A general, noticeable, bell-shaped distribution of TRAP activity on days 7 and 14 could be detected with significant difference between the cultures with 26.67 mM Mg^{2+} of Mg extract and the control group (Fig. 31). However, on the late culture time points (days 21 and 28), no significant difference is observed. Remarkably, in the cultures with 26.67 mM Mg^{2+} of Mg extract the intracellular TRAP release was continuously lower over the course of 28 days' culture.

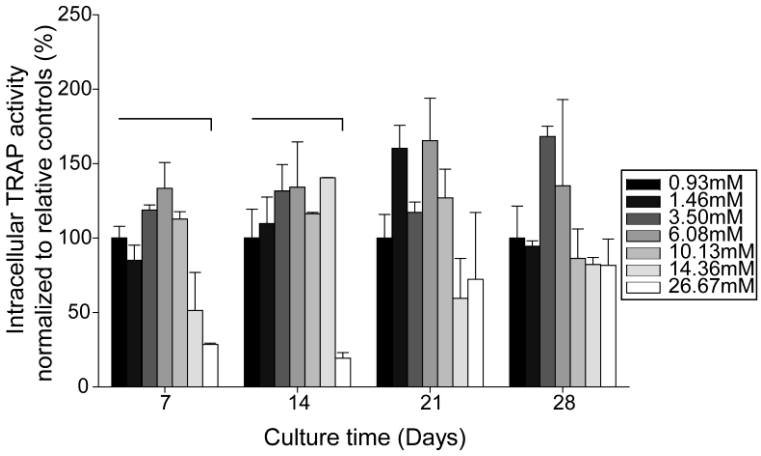


Fig. 31 Intracellular TRAP release was evaluated on days 7, 14, 21 and 28. TRAP release under the control condition was considered as 100% while that under other conditions was normalised. Symbols indicate significant differences between the control group and conditions supplemented with different concentrations of Mg extract ($P < 0.05$).

4.4.6 ALP activity assay

Since ALP is a byproduct of osteoblast activity, the osteoblastogenesis in the human osteoblast-osteoclast cocultures in the exposure of different concentrations of Mg extract was often assessed by the measurement of intracellular ALP activity. The results are shown in Fig. 32. As the PBMC/osteoclasts culture have no ALP production, the values of intracellular ALP activity measured for all the conditions are attributed to the SCP-1/osteoblasts cells. From the figure we can see, similarly, at almost all the assayed time points (except for day 28), a bell-shaped distribution of intracellular ALP activity could be observed. Notably, on day 28, ALP increased nearly linearly with increasing concentrations of Mg extract.

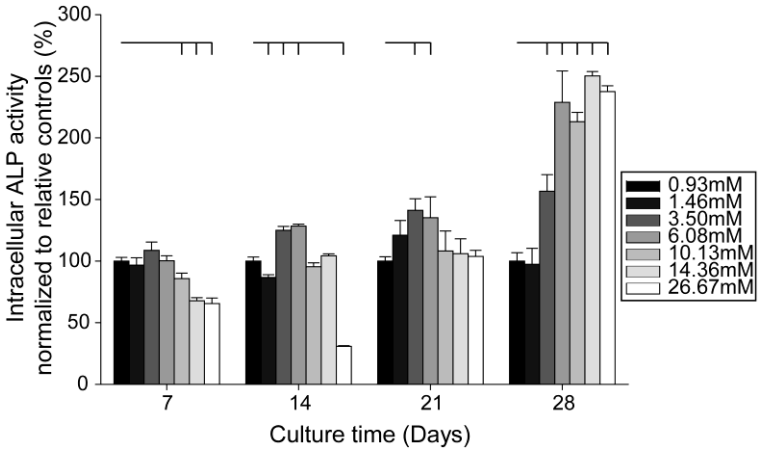


Fig. 32 Effect of Mg extract on enzymatic activity of intracellular ALP in osteoblast-osteoclast cocultures with different concentrations of Mg extract was quantified on days 7, 14, 21 and 28. ALP activity under the control culture was set as 100% while that under other conditions was normalised. Symbols indicate significant differences between the control group and other conditions, ($P < 0.05$).

4.4.7 Elisa

To estimate the effects of various concentrations of Mg extract supplements on the protein production of human osteoblasts and osteoclasts specific markers (RANKL, M-CSF for

osteoblasts and OSCAR for osteoclasts, respectively), Elisa was carried out for the various coculture conditions on the different culture time points (days 7, 14, 21 and 28). The results are shown in Fig. 33.

From Fig. 33a we can see that there is a significant increase in the production of RANKL in cultures treated with higher concentrations of Mg extract compared to the control culture on day 28 (Fig. 33a). Similarly, a general induction in M-CSF was detected in the cultures with 14.36/26.67 mM Mg^{2+} of Mg extract compared to the control group on day 14 (Fig. 33b). Whereas, the result shows a significant decrease in the production of OSCAR after being cultured with 26.67 mM Mg^{2+} of Mg extract for 28 days compared to the control culture (Fig. 33c).

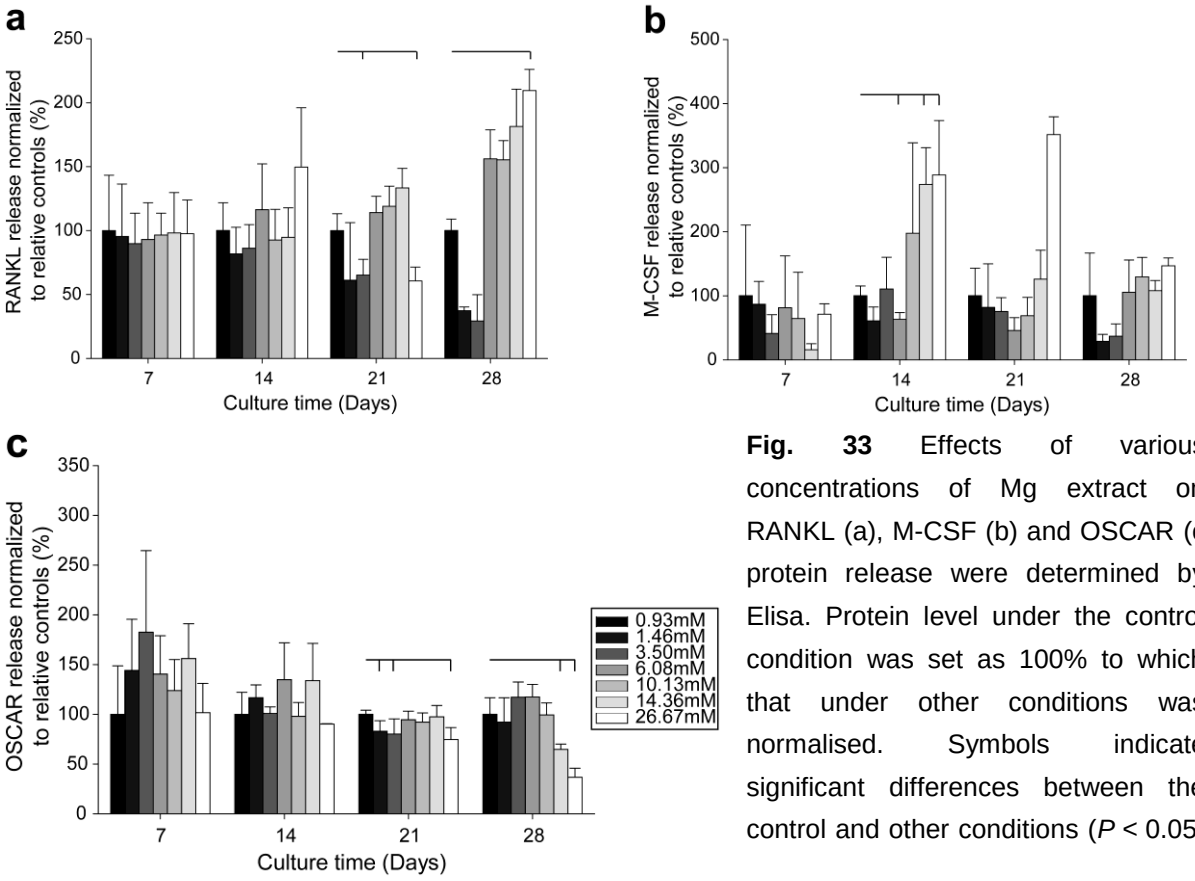


Fig. 33 Effects of various concentrations of Mg extract on RANKL (a), M-CSF (b) and OSCAR (c) protein release were determined by Elisa. Protein level under the control condition was set as 100% to which that under other conditions was normalised. Symbols indicate significant differences between the control and other conditions ($P < 0.05$).

4.4.8 ARS staining

Since ARS could be used to identify Ca, it has been commonly used as one of the specific osteoblast markers. At the end time point (day 28), ARS staining was conducted for the determination of the effect of Mg extract on human osteoblast mineralisation function in its coculture with osteoclasts. The extent of osteoblast mineralisation was visualised and the results are shown in Fig. 34. From the figure we can see that there is a significantly increased mineralisation in the cultures supplemented with 26.67 mM Mg^{2+} of Mg extract compared to the control condition as well as the cultures exposed with lower concentrations of Mg extract.

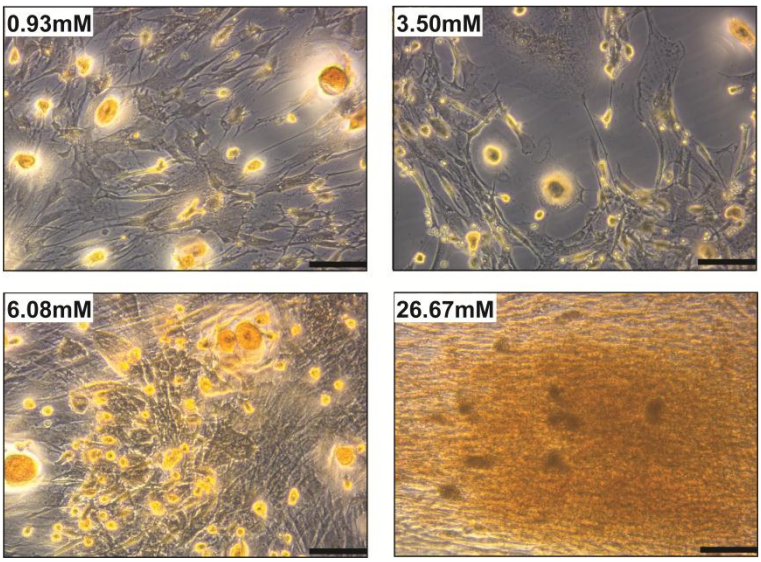
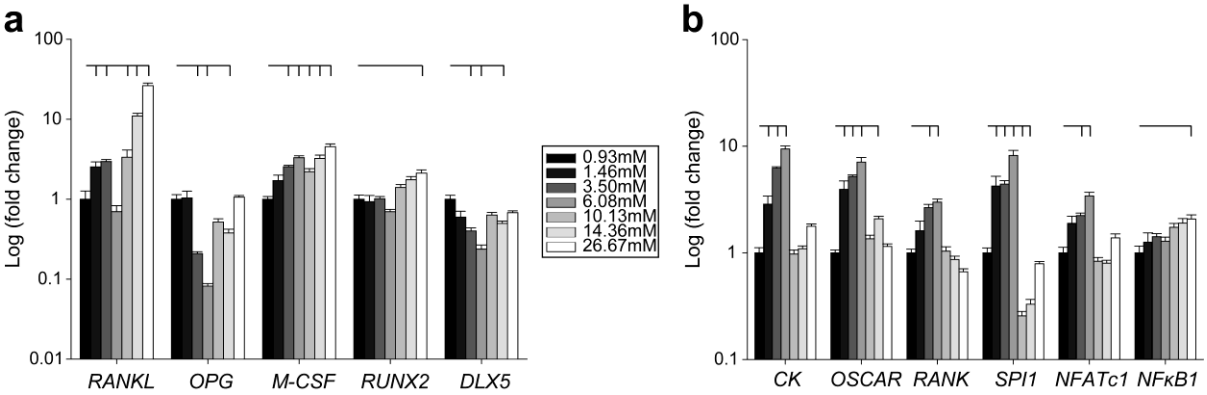


Fig. 34 The late differentiation behaviour of human osteoblasts was investigated in the cocultures by ARS staining on day 28. Images of cultures under the control condition as well as conditions exposed to 3.50/6.08/26.67 mM Mg^{2+} of Mg extract are shown. Scale bar = 100 µm.

4.4.9 RT-qPCR

RT-qPCR was conducted to characterize the influence of different concentrations of Mg extract on human osteoblasts and osteoclasts specific genes expression on day 28.

From the results exhibited in Fig. 35a, we could see a decrease in the mRNA expression of osteoblastic specific markers *RANKL*, *OPG*, *RUNX2* and *DLX5* until 6.08 mM Mg^{2+} of Mg extract and then followed by an increase with increasing concentrations of Mg extract. An opposite pattern was shown for the mRNA expression analyses of osteoclastic specific genes *CK*, *OSCAR*, *RANK*, *SPI1* and *NFATc1* (Fig. 35b). A bell-shaped distribution of the mRNA expression of osteoclast specific markers was detected, i.e., an increase until the concentration of 6.08 mM Mg^{2+} of Mg extract and then a significant decrease observable in the cultures with higher concentrations of Mg extract (10.13, 14.36 and 26.67 mM Mg^{2+} of Mg extract) (Fig. 35b). Additionally, the *M-CSF* and *NFκB1* gene expression was found to be gradually increased with the increase in the concentration of Mg extract (Fig. 35a and Fig. 35b, respectively). Furthermore, the ratio between *RANKL* and *OPG* was also calculated (Fig. 35c). The ratio > 1 was found in the cultures supplemented with 14.36 and 26.67 mM Mg^{2+} of Mg extract (1.5 and 1.3, respectively; Fig. 35c).



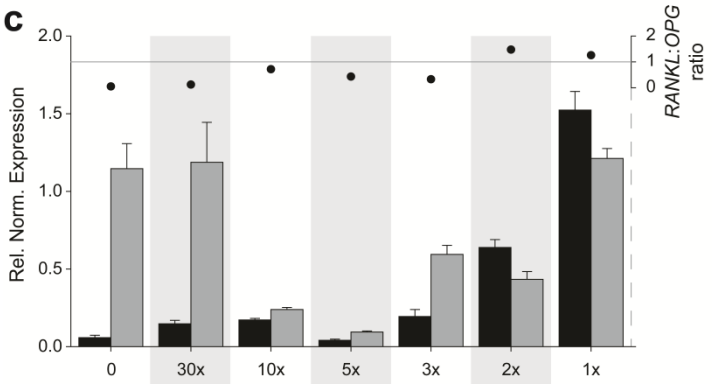


Fig. 35 The mRNA expression of osteoblastic and osteoclastic specific genes in the cocultures exposed to various concentrations of Mg extract was determined by RT-qPCR on day 28. (a) *RANKL*, *OPG*, *M-CSF*, *RUNX2* and *DLX5*, (b) *CK*, *OSCAR*, *RANK*, *SPI1*, *NFATc1* and *NFκB1* and (c) left axis: gene expression of *RANKL* (black) and *OPG* (grey) (column chart); right axis: *RANKL*:*OPG* ratio (scatter chart). Data are normalised to reference genes Actinβ, B2M, GAPDH and RPL10. The relative amount of gene expression under culture conditions with Mg extract was normalised to that of the control culture (without Mg extract). Symbols indicate significant differences between the control condition and conditions supplemented with different concentrations of Mg extract ($P < 0.05$).

5 Discussion and Conclusions

Currently, Mg and Mg-based alloys have attracted great attention as a biodegradable material for orthopaedic applications due to their excellent biodegradability [40], biocompatibility [41] and appropriate strength and elastic moduli compared to a natural bone [9]. When the materials are surgically introduced into the patient's bone and start to degrade, all the corrosion products will interact with the local bone cells, i.e., osteoblasts and osteoclasts. Since osteoblasts are the cells that are not only responsible for the bone generation but also regulate osteoclastogenesis, they have been widely considered as the most dominating cells for osseointegration of implants [80, 113]. Therefore, most researches concerning the *in vitro* biocompatible assessments for the newly developed Mg-based alloys were performed by the use of osteoblast or their precursors monoculture by analysing their cytocompatibility or osteoinductivity [32, 38, 89, 95, 114]. Although frequently been underestimated, it has been strongly suggested that the successful use or longevity of an implant is mainly determined by the action of bone-breaking osteoclasts. However, little is known about the influence of Mg degrading products to osteoclasts. Therefore, in the present work, procedures for osteoclast monoculture derived by PBMC in the supplement with RANKL and M-CSF as well as osteoblast-osteoclast coculture were successfully established. Their response to high/non-physiological extracellular Mg contents (coming from either $MgCl_2$ or Mg extract) in terms of cellular differentiation and function was investigated.

Mg salt in the form of $MgCl_2$ was first used and their influence on osteoclastic differentiation and activation was investigated based on osteoclastic monoculture. As indicated in Fig. 6, no significant difference in WST-1/formazan production was observable at all the other time points. It therefore suggests that $MgCl_2$ does not hinder cell viability. The results of TRAP staining (Fig. 9), CK (Fig. 10) and CTR immunocytochemistry (Fig. 11) showed that $MgCl_2$ increases the formation of osteoclasts until a certain concentration and then it decreases. Similarly, as shown in Fig. 12, there was an increase in the relative resorption activity per TRAP-positive osteoclast up to a certain $MgCl_2$ concentration followed by a decrease. As the specialised cell that is responsible for bone resorption, both the initial adhesion of osteoclasts to the bone surface and further migration along it while resorbing are two mechanisms involved in its functional resorption [115]. It is well known that osteoclasts express at least two á-integrins (á2 and áv) and three ß-integrins (ß1, ß3 and ß5) as cell surface adhesion receptors that mediate osteoclast attachment to bone matrix [116]. Another research performed by Shankar demonstrated that the binding between integrins and substrate requires divalent cations [117] and Mg^{2+} plays a more effective role than Ca^{2+} in facilitating cell adhesion to the substrates [118]. Therefore, initial adhesion may be facilitated. However, osteoclast migration and function were inhibited. Based on the above arguments, it can be suggested that $MgCl_2$ enhances osteoclast differentiation and function up to a certain concentration. This is exactly what we observed for $MgCl_2$.

In an attempt to distinguish the effect of Mg^{2+} from Cl^- when osteoclastic response to the supplementation of $MgCl_2$ was investigated, osteoclasts were cultured in the presence of a series of NaCl concentrations. As indicated in Fig. 18 as well as Fig. 19, a significant,

linear, concentration-dependent enhancement in the resorbed area (R^2 = 0.8917, P < 0.001) as well as in the resorbed volume (R^2 = 0.9664, P < 0.001) was investigated. Although it was difficult to completely rule out the role of Cl^- on osteoclast function, while 50 mM Cl^- (coming from the supplementation of 25 mM of $MgCl_2$) was shown to significantly decrease osteoclast resorption activity, the same amount of Cl^- (coming from the addition of 50 mM NaCl) was investigated to significantly increase osteoclastic resorption activity. Therefore, it might be concluded that the first elevated followed by reduced effects of $MgCl_2$ on osteoclastic differentiation and function was due to Mg^{2+} rather than Cl^-.

A contribution of high NaCl intake to the development of osteoporosis has been illustrated by previously published evidence coming from animal researches as well as clinical and epidemiological studies [119-122]. The oral NaCl supplements in growing rats have been estimated to increase the urinary excretion of Ca and phosphorus, however, the intestinal absorption of Ca didn't increase, thus leading to a depression in the accumulation of Ca and phosphorus in bone [123]. Need et al. [124] examined the effect of salt restriction on urine hydroxyproline excretion and revealed that salt restriction may be one way of reducing bone resorption in postmenopausal women as a restriction in Na^+ diet could lead to a decrease in urinary Na^+, Ca^{2+} and hydroxyproline/creatinine. On the other hand, evidence coming from both human subjects and experimental rats indicates that higher Na^+ intake is associated with increased hydroxyproline excretion via urine [125-128]. In a two-year longitudinal study of bone density in 124 postmenopausal women [119], Devine et al. suggested that there was a negative correlation between urinary Na excretion and changes in bone density at the total hip and the intertrochanteric sites. Based on an extensive review of the literature, MacGregor concluded that a high salt intake is suggested as a major aggravating factor in osteoporosis and a reduction in salt intake could have major benefits in bone density [129]. The beneficial effect of the reduction in Na^+ intake on bone health was documented in an interventional study by Lin et al. [130] as well. In an observational study conducted for four-week period, Harrington [122] also concluded that higher salt intake might lead to greater rates of bone resorption in postmenopausal women.

However, the mode of action, how NaCl affects bone density is still controversial. While a correlation between increased urinary Ca excretion and the production of serum PTH was indicated in some studies [131], other studies failed to show the elevation in PTH secretion due to NaCl loading [119, 132, 133], suggesting possible direct cellular effects of NaCl on bone-resorbing osteoclasts.

In the present study, a direct, progressive and PTH-independent influence of NaCl on human osteoclasts differentiation and function behaviour was revealed through an *in vitro* cell culture system in which human PBMC monocytes was induced into mature osteoclasts with the external addition of osteoclastogenesis promoting factors RANKL and M-CSF. WST-1 assay was first performed to evaluate the influence of high NaCl addition on the cell metabolism. As indicated in Fig. 13, a bell-shaped distribution of WST-1 was observed on days 3 and 14. An increase in the cells metabolic activity was obtained until the cultures supplemented with mild and moderate hypernatremia NaCl followed by a significant decrease in the cultures with severely high NaCl concentration. This result was consistent

with the previous study that was conducted by Arimochi [134]. In the study, the effect of high salt culture conditions on the growth of rat C6 glioma cells was examined and it was found that there was a reduction in the number of viable cells in a concentration-dependent manner when the cells were exposed to the culture medium containing high concentrations of NaCl. While most of the studies involved acute application of NaCl for only a few hours, the response of human cervical epithelial carcinoma (HeLa) cells as well as primary mouse embryonic fibroblasts to prolonged exposure to high NaCl culture condition was investigated for 18 and 10 days, respectively. The proliferation rate of HeLa cells treated with NaCl was reported to decrease gradually and display signs of senescence while similarly for the primary mouse embryonic fibroblasts, the appearance of cellular senescence was accelerated as well [135]. Except for the inhibitory effect of high NaCl on cell metabolic activity, total protein synthesis in cells exposed to hypertonic osmolarity of the extracellular medium was also found to be profoundly inhibited. Protein synthesis was found to be strongly inhibited when the chick embryo fibroblasts were exposed to the medium with 0.4 osM osmolarity within 30 minutes, although the cells appear to adapt to the altered environment with a gradual protein synthesis restoration reaching control values within 12-14 h of treatment [136]. Consistently, Petronini et al. also reported the existence of transient impairment of the rate of protein synthesis in cultured endothelial cells [137]. However, as shown in Fig. 14, a general bell-shaped distribution pattern of total protein content was observed on day 3. On days 14 and 28, although the difference is not significant, an increased concentration of NaCl first increases and then decreases total protein content. All of these indicate the stimulatory effect of low concentrations of NaCl on the proliferation/differentiation capacity of osteoclastic precursors. It should be noted that the total protein content of cultures under severe hypernatremia condition was always lower than that of relative controls at all the tested time points which suggested the inhibition of cell metabolism and protein production when exposed the cells to severe hypernatremia culture environment. Interestingly, however, in spite of the lower overall cell activity in the severe hypernatremia culture group, more osteoclasts that were more active than in any other group were observed which suggested that there were diverging effects of NaCl on osteoclasts at different stages of differentiation. A possible explanation would be that the increase in supplemented NaCl concentrations favoured its differentiation over proliferation, thus leading to a higher relative number of multinucleated osteoclasts. Indeed, this was confirmed by TRAP staining in which a positive linear relationship between the number of osteoclast-like TRAP-positive multinuclear cells per mm^2 and the concentration of supplemented NaCl in the extracellular culture medium was investigated with $R^2 = 0.9008$, $P < 0.001$ (Fig. 16). An inductive effect of high NaCl on the formation of bone-resorbing osteoclasts was thus indicated. Similar results were further obtained for CTR expression (Fig. 17).

On the contrary, the extracellular TRAP release (Fig. 15) was found to be decreased in the cultures exposed to severe hypernatremia condition while more TRAP-positive multinuclear cells were detected. The most probably explanations for the above discrepancy lie in the different isoforms of TRAP. It has been widely suggested that there are two distinctly glycosylated forms of TRAP in human blood circulation which are known as TRAP5a and TRAP5b [138]. While TRAP5a is mainly derived from macrophages as well as

dendritic cells, TRAP5b was reported to exclusively originate from both bone-resorbing and non-resorbing osteoclasts [139, 140]. The TRAP activity determined in the present work, however, is not isoform selective. Therefore, it might be that high NaCl supplementation may have led to a situation where the increased formation of TRAP5b in the multinucleated TRAP-positive OCLs is offset by the decreased number of activated monocytes or macrophages, thus leading to a diminished release of TRAP5a and consequently to a decrease of combined TRAP activity.

Moreover, in order to enable a more profound analysis of the influence of high NaCl on osteoclastic function, 2D resorption area measurement as well as 3D resorption volume evaluation was further performed. As indicated in Fig. 18c and Fig. 19c, a well-fit linear correlation between the resorption capacity and NaCl was found with R^2 = 0.8917 and 0.9664, in terms of resorption area and volume, respectively, suggesting a direct, dose-dependent positive effect of high NaCl concentration also on the function of osteoclasts.

A limitation of this study is that the effect of the monovalent anion Cl^- on osteoclast function couldn't be completely ruled out. However, in another setup, 50 mM Cl^- has been estimated to decrease osteoclastic resorption capacity. Furthermore, the osmotic or ionic effect in the exposure of high extracellular NaCl have not been attempted to dissociate. In the future it will be interesting to study, which molecular pathway results in the observable effect of NaCl on the bone-resorbing osteoclasts. Therefore, it could be concluded that NaCl directly, positively regulated osteoclastic differentiation and function. The previously reported enhancement in bone resorption after diets rich in NaCl, and in diseases with high serum Na^+ may not only due to the urinary Ca loss but also because of a direct, cell-mediated effect of increased Na^+ concentration on osteoclast resorption activity. These findings may help to explain the clinical findings in bone metabolism in patients with increased serum NaCl levels and may therefore be important for the prevention and treatment of osteoporosis.

In order to more closely mimic the *in vivo* environment, the osteoclast monoculture was exposed to different concentrations of Mg extract and their response was investigated in comparison of the experimental setup of $MgCl_2$. For the cultures in the exposure of different concentrations of Mg extract, however, the biphasic effect of $MgCl_2$ on osteoclast differentiation and function was not found. The results from WST-1 (Fig. 21), extracellular TRAP activity (Fig. 23), TRAP staining (Fig. 24) as well as CK staining (Fig. 25) consistently indicated a decrease associated with the increase in Mg extract content. However, as shown in Fig. 27, the resorption activity per osteoclast increased gradually with the increase in Mg extract content. The different influence patterns for $MgCl_2$ and Mg extract on osteoclast proliferation and differentiation behaviour are not surprising. In a study concerning the effect of Mg on the primary human osteoblasts, such differences have previously been highlighted in which Mg extract was found to increase the expression of genes involved in bone metabolism (e.g. osteocalcin) in a more effective manner than $MgCl_2$ (data not shown). The different effects can be explained by one of the main differences between $MgCl_2$ and Mg extract, i.e. Ca depletion in the pure Mg extract (see Table 5). It has been pointed out that the balance between Mg and Ca is very important for bone homeostasis and circulating Ca^{2+} has an important effect on bone turnover. When there is too much Ca^{2+}, osteoclast

resorption activity in bones will be inhibited by calcitonin which is primarily released via thyroid. On the contrary, when the Ca^{2+} is too low, osteoclast bone resorption will be promoted by calcitriol and PTH. The effects of Mg^{2+} and Ca^{2+} omission, depletion and excess have been extensively studied by Rubin and co-workers (see Reference [141]). It has been estimated that omission of Ca^{2+} could lead to a significant increase in cell permeability and Mg^{2+} can apparently substitute for Ca^{2+} in the maintenance of normal permeability, but in a less-efficient manner since 5-10 mM Mg^{2+} is required to maintain Na^{2+} and K^{+} at normal levels in the absence of Ca^{2+}. Furthermore, other evidence indicated that the depletion of Ca^{2+} will result in a shift of Mg^{2+} away from ATP toward other binding sites in the cell [142]. This is maybe one reason that Mg extract doesn't exert the effect observed under the supplementation of $MgCl_2$ salt.

Moreover, the different effect patterns of $MgCl_2$ and Mg extract on osteoclast differentiation and function behaviour could also be explained by the osmolality and pH which are considered to be two negative parameters for osteoclast formation and activity. It can be seen from Table 5 that both the osmolality and pH in the culture medium supplemented with Mg extract are high. It has been tested that when PBMC (coming from two separate donors) were cultured in the presence of 14.36 and 26.67 mM Mg^{2+} of Mg extract, all of them died on day 3. The role of pH on osteoclast activity has been also investigated by several studies. For instance, acidic conditions were proved to stimulate osteoclast activity [143, 144]. pH between 7.0-7.5 was estimated to be the optimal condition for the proliferation and differentiation of osteoclast from neonatal rabbits while the resorption activity was enhanced when the pH at 6.5-7.0 [145]. As indicated in Table 5, the pH ranged from 8.35 to 7.8 in the culture conditions with a series of Mg extract concentrations. While a negative effect of Mg extract on osteoclast metabolic activity was detected (as shown in Fig. 21), cell differentiation was less affected. As an inward transporter of Mg, transient receptor potential melastin 7 (TRPM7) has been reported to sense osmotic gradients rather than ionic strength and be inhibited by hypertonic conditions [146]. The positive effects observed in the cultures treated with $MgCl_2$ would then be abolished, resulting in no or a reduced increase in the intracellular Mg content until the extracellular osmolality was restored. Indeed, in the present experiments, it was observed that when osmolality was returned to normal level osteoclast activity was enhanced. The specific knockout or inhibition of TRPM7 (e.g. with non-specific lipoxygenase inhibitors like nordihydroguaiaretic acid or NDGA [147]) may help to uncover the role of this channel.

The differences observed for the two Mg-containing solutions could also be explained by Voltage-dependent Ca channels (VDCC). The VDCC are a group of voltage-gated ion channels that are activated by changes in electrical potential near the channel and they are generally found in excitable cells. For cells of the nervous system, it has been investigated that the external Mg ions can block VDCC [148]. In osteoclasts, long-lasting Ca channels that may promote movement can be also found [149]. The increased resorption observed at lower Mg concentrations in the case of the Mg extract (compared to $MgCl_2$) would then be explained by Mg blockage. This hypothesis could be validated through performing the measurements of osteoclast migration pattern.

Furthermore, the correlation between *in vitro* and *in vivo* condition would be discussed. It can be assumed that there is a decreasing Mg concentration gradient between the implant surface and the farther tissues during *in vivo* Mg-based implant degradation. This gradient could thus explain the bell-shaped distribution observed in the cultures supplemented with $MgCl_2$ and the constant decrease in the case of cultures in the presence of Mg extract measured for the *in vitro* osteoclastic activity. Therefore a depletion of osteoclasts may appear close to the implant, while osteoclasts may form further away. In this respect, it might be interesting for the analysis in the *in vivo* experiments. In fact, this may partly explain the different osteoclast behaviour in the peri-implantation site of Mg-based implants detected in previous studies. For example, while reduced osteoclast surface and fewer osteoclasts were described by Janning et al. [150], Huehnerschulte et al. [151] showed a large number of osteoclasts formation. Another essential issue in this respect is the coupling of bone-building osteoblasts to bone-resorbing osteoclasts. It may be possible that the deleterious effect of increased osteoclastic activity may be matched or even overcome by activation of osteoblasts via an osteoclastic relay. The establishment of a coculture procedure consisting of osteoblast and osteoclast will be necessary to test this hypothesis. Indeed, a high mineral apposition rates and increased bone mass/volume around degrading Mg implants in bone were estimated by Witte et al. [40]. Furthermore, the formation of implant particles in the degradation process could be one of the contributing factors. It has been pointed out that the production of osteoclastogenic cytokine could be influenced by the particles coming from different materials [152] and the cells reaction is shown to be dependent on the particle size [153].

Finally, in the present work, á-MEM supplemented with 10% FBS was used for the solvent to prepare extract, as it is complex and some chemical reactions may also take place during Mg degradation process, more detailed analyses of Mg extract and its differences with $MgCl_2$ salt by inductively coupled plasma mass spectrometry or liquid chromatography–tandem mass spectrometry, may be helpful in understanding the observed differences in the cell behaviour.

$MgCl_2$ salt and Mg extract exhibited different direct effects on the differentiation and function of human bone-resorbing osteoclast. While $MgCl_2$ was able to increase osteoclast proliferation and function up to a concentration of approximately 15 mM, Mg extract appeared to reduce cell metabolism, although the parameters concerning cell differentiation were less affected. Osteoclastic resorption activity was activated by both $MgCl_2$ and Mg extract, but the Mg extract exerted its positive effect at a lower Mg content. With respect to the *in vivo* degrading properties, Mg extract is believed to be the closest *in vitro* model for the cytocompatibility assessment of Mg-based biomaterials [105, 106].

The establishment of an osteoblast-osteoclast coculture system in which the crosstalk between bone-forming and bone-resorbing cells could be investigated for the *in vitro* cytocompatibility assessments for biomedical Mg-based alloy is of great interest. In the present work, a coculture system containing human osteoblasts (derived from SCP-1 cell line) and osteoclasts (obtained from the differentiation of human PBMC monocytes) was successfully established. The effect of a series of concentrations of Mg extract (7

concentrations: 0.93/1.46/3.50/6.08/10.13/14.36/26.67 mM Mg^{2+} of Mg extract) on the proliferation and differentiation behaviour of osteoblast-osteoclast coculture was evaluated at various time points (days 7, 14, 21 and 28). Opposite modes of Mg extract acting on osteoblastic and osteoclastic behaviour could be detected. On the one hand, osteoclast differentiation was elevated with the increase in the concentration of Mg extract (up to 6 mM Mg^{2+} of Mg extract) and then decreased in the cultures with high concentrations of Mg extract. On the other hand, osteoblast differentiation was first inhibited until 6.08 mM Mg^{2+} of Mg extract and then enhanced in higher concentrations (especially 14.36 and 26.67 mM Mg^{2+} of Mg extract).

The initial adhesion behaviour of the cocultures cultured with different concentrations of Mg extract was estimated by light microscopy after culturing for 6 days (Fig. 28). As shown in Fig. 28a, a bad attachment of the coculture especially the PBMC cells in the culture condition supplemented with 26.67 mM Mg^{2+} of Mg extract was observed. As indicated by the solid circles (see Fig. 28a), small cell debris were visible, suggesting the detachment for most of the monocytes from the surface of the plates. This is consistent with further evidences. For example, after culturing for 7 days, the total protein content of the cocultures under the condition supplemented with 26.67 mM Mg^{2+} of Mg extract was significantly decreased compared to the cultures with lower concentrations of Mg extract (3.50, 6.08 and 10.13 mM Mg^{2+} of Mg extract for instance, Fig. 29). Moreover, the LDH release from the cell destruction was found continuously increased on day 7 because of the exposure of higher concentration of Mg extract. Especially for the cocultures with 26.67 mM Mg^{2+} of Mg extract, significant increase in LDH release was detected compared to all the other conditions (Fig. 30). The bad attachment of osteoblast-osteoclast cocultures in the cultures with higher concentrations of Mg extract (especially for 14.36 and 26.67 mM Mg^{2+} of Mg extract) compared to control or cultures with lower concentrations of Mg extract on day 7 was also proved by the decreased intracellular TRAP and ALP activity (as indicated in Fig. 31 and Fig. 32, respectively). In a study conducted by Lorenz et al. [154], a similar conclusion was achieved, i.e., the high reactivity of polished commercially pure Mg samples in the cell culture medium might lead to a pH shift in the alkaline direction as well as an increase in osmolality and thus the reduced cellular adhesion and viability. A very recent paper compared the biocompatible properties of Mg-Ca alloy samples with and without microarc oxidation coatings [155]. While the coated ones showed great cytocompatibility, because of the low adhesion activity caused by the suffering from severe corrosion, no MG63 cells were observed on the uncoated samples. Furthermore, Ca^{2+} has long been reported playing a highly important role in the maintenance of bone mass which potentially antagonizes with Mg^{2+} in many other physiological activities [156, 157]. The balance between Mg and Ca is considered to be highly important for bones and for homeostasis in general [158]. Therefore, the concentration of Ca^{2+} in the culture media with various concentrations of Mg extract was also measured. As indicated in Table 6, a gradual decrease in Ca^{2+} concentration was observed with the increase in the concentration of Mg extract. The explanation has been well described in a recent study conducted by Willumeit et al. [159] which revealed the deposit of Ca^{2+} on the corrosion layer of the material. Such deposit of Ca^{2+} leads to its depletion in the Mg extract solution, thus the decrease of Ca^{2+} in the measured culture medium (Table 6).

Despite suffering from a relatively inhospitable environment with increased pH as well as osmolality, some cellular adhesion did happen. Compared to our very recent study [70], while osteoclast monoculture in the exposure of 14.36 and 26.67 mM Mg^{2+} of Mg extract was detected all dead after culturing for 3 days, being cocultured with osteoblasts, they were found to be still alive although only in a few amount (Fig. 28a). As indicated in Fig. 28b, with the increasing culture time up to 28 days, their cell division and even late cell differentiation were further confirmed by the formation of multinucleated osteoclasts. The superior capability of osteoclastic survival and differentiation in the coculture system with high concentration of Mg extract (especially for 14.36 and 26.67 mM Mg^{2+} of Mg extract) compared to osteoclast monoculture could be explained by the increased amount of osteoblasts. The stimulatory effects of high concentration Mg extract on osteoblasts lead to the increased amount of osteoblasts in the culture condition which causes cell culture media acidification, resulting in neutralisation of the Mg extract alkalinity.

Similarly to osteoclasts, osteoblasts were also found to be alive in the culture conditions supplemented with high concentration of Mg extract (14.36 and 26.67 mM Mg^{2+} of Mg extract). They exhibited their morphological appearance and produced late differentiation parameters. As shown in Fig. 29, when exposed to 14.36 mM Mg^{2+} of Mg extract, the total protein content of the cocultures was lower on day 7 but higher on day 14 compared to control, however, when exposed to 26.67 mM Mg^{2+} of Mg extract, it was lower on day 7 and day 14 but higher on day 21 (Fig. 29). It thus indicated that the more alkaline the culture medium is the longer it needed for the acid-alkaline neutralisation process. Furthermore, with having a closer look, on day 14 intracellular ALP activity in the cultures with 14.36 mM Mg^{2+} of Mg extract was found to be higher compared to control however, in the cultures with 26.67 mM Mg^{2+} of Mg extract, 21 days was needed for osteoblasts to produce higher level of ALP activity compared to control (Fig. 32). As depicted in Fig. 31, late osteoclastic differentiation behaviour was further assessed by the measurement of intracellular TRAP activity. Although a general increase was detected of intracellular TRAP activity during the entire culture period of 28 days, the enzymatic level in cultures with 26.67 mM Mg^{2+} of Mg extract was continuously lower compared to relative controls at all the time points. It thus suggested that osteoblasts were recovering faster compared to osteoclasts. This could be explained by the different modes of osteoblastic and osteoclastic differentiation. While osteoblasts could further proliferate in the high alkaline environment, as osteoclasts are generated by the fusing of monocytes and they are terminally differentiated cells, there might be not enough cells left for them to start the selection process if the fusing cells are depleted.

The polypeptide growth factor M-CSF and RANKL released by osteoblasts are the key factors that are essential and sufficient for osteoclastogenesis [160]. Binding of M-CSF to its receptor (colony-stimulating factor 1 receptor, or c-Fms) will activate cell proliferation and survival and the expression of RANK. The RANKL/RANK interaction will in turn triggers a sequence of events that leads to the downstream activation of transcription factors like NFκB and the expression of master transcription factors for osteoclastogenesis, i.e., NFATc1 and SPI1. NFATc1 and SPI1 will then induce the expression of osteoclastogenesis-related genes like *TRAP*, *CK* and *OSCAR* [161]. While RANKL induces osteoclastic destruction,

OPG produced by osteoblasts is reported as a decoy receptor that downregulates the bone destruction through preventing the binding of RANKL with its receptor RANK [162, 163]. Therefore, the bone resorption is regulated by the relative expression of RANKL and OPG and the ratio between them (*RANKL*:*OPG* ratio) has been estimated as an important marker for bone loss or more specifically, for osteoclast activation (i.e. ratio > 1 pro-osteoclastogenesis and < 1 pro-osteoblastogenesis; Fig. 35c) [164, 165].

Taken together, it can be concluded that higher concentrations of Mg extract could increase osteoblastic activity. The evidences came from the enhanced ALP activity (Fig. 32) and mineralisation capacity (revealed by ARS staining; Fig. 34). Osteoblast released pro-osteoclast factors were also found to be increased in the cocultures exposed to high concentration of Mg extract (i.e., RANKL and M-CSF, both protein and gene levels; Fig. 33a/35a, respectively). However, Fig. 30 showed an increase in LDH release with the increase in the concentration of Mg extract on day 28, indicating the high cytotoxicity. It seems incompatible with the enhanced osteoblastic proliferation and differentiation in that culturing condition. The high cell competition (as shown in Fig. 28b, multiple layers of osteoblast cells) resulted from the significantly stimulatory proliferation rate of osteoblasts in the culture environment with high concentration of Mg extract might be one of the main reasons.

Moreover, an opposite pattern of Mg extract acting on osteoclasts was detected, i.e., osteoclastogenesis markers (both on protein and gene levels; Fig. 33c/35b, respectively) are increased up to ≈ 6.08 mM Mg^{2+} of Mg extract and then decreased with increasing Mg extract. For example, a general bell-shaped distribution was observed for intracellular TRAP activity on days 7 and 14 with significant difference between the cultures with 26.67 mM Mg^{2+} of Mg extract and the control group, suggesting the dual effect of Mg extract on osteoclastic differentiation, i.e., lower concentrations of Mg extract induced osteoclast intracellular TRAP production until a concentration of 3.50 mM Mg^{2+} or 6.08 mM Mg^{2+} of Mg extract and then decreased (Fig. 31). Similarly, the pattern of dual effect of Mg extract on osteoclastic differentiation was also observable in terms of OSCAR protein expression and *CK*, *OSCAR*, *RANK*, *SPI1* as well as *NFATc1* mRNA expression as indicated in Fig. 33c and Fig. 35b, respectively. The inhibitory effects of high concentration of Mg extract (especially 14.36 and 26.67 mM Mg^{2+} of Mg extract) acting on osteoclastogenesis could be explained by the fact that neither osteoblast-released osteoclastogenesis promoting factors nor osteoblast-induced pH media neutralisation process are sufficient to counteract the negative effects caused by high pH and osmolality. This hypothesis can be proved by previously published work in which alkalosis has been considered to decrease osteoclastic resorption activity while increase osteoblastic formation *in vivo* [166]. Furthermore, although RANKL has been estimated to activate mature osteoclastogenesis in a dose-dependent manner *in vitro* [48, 49] and Fig. 35a/35c have shown the significant increases in *RANKL* and *M-CSF* mRNA expression as well as the *RANKL*:*OPG* ratio (respectively) in the cultures supplemented with 14.36 and 26.67 mM Mg^{2+} of Mg extract, we failed to get more osteoclast formation at the high Mg concentrations. This could be explained by the lower expression of *RANK* mRNA (Fig. 35b) which is essential for RANKL/RANK signalling in the same coculture

condition [50]. Furthermore, increased extracellular Mg concentration has been estimated to decrease osteoclast migration (i.e., function) through increasing its binding to integrins [167, 168].

Consistent with the *in vivo* study in which enhanced bone growth was observed in the contributions to both the enhancement in osteoblast activity and a decrease in osteoclast numbers [150], in the present study, the *in vitro* data showed a promotion in the formation of osteoblasts but an inhibitory effect on the differentiation of osteoclasts caused by exposure to higher concentrated Mg extract (especially 14.36 and 26.67 mM Mg^{2+} of Mg extract). The enhanced bone formation after implanting Mg-based alloys could be contributed to the dual modes of Mg on the bone cell level. Compared to monoculture, when cocultured with osteoblasts, PBMC exhibited higher tolerance to high concentration of Mg extract which can be attributed to the cell-cell communication. Therefore cocultures consisting of both bone cells (osteoblasts and osteoclasts) should be preferentially performed for *in vitro* cytocompatibility assessment of Mg-based implants.

6 References

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7 Abbreviations

Ag	Silver
Al	Aluminum
Al_2O_3	Aluminium oxide
ALP	Alkaline phosphatase
AM	Aluminium/manganese
α-MEM	Minimum essential α-minimum
ANOVA	Analysis of variance
ARS	Alizarin Red S
AZ	Aluminium/zinc
BMSC	Bone marrow stromal cells
BSA	Bovine serum albumin
B2M	β2 microglobulin
Ca	Calcium
Ca^{2+}	Ca ion
$CaCl_2$	Calcium chloride
cDNA	Complementary DNA
c-Fms	Colony-stimulating factor 1 receptor
CK	Cathepsin K
Cl^-	Chloride ion
Co-Cr alloys	Cobalt-chromium-based alloys
CTR	Calcitonin receptor
DAPI	4-6-diamidino-2 phenylindole solution
ddH_2O	Double-distilled water
DLX5	Distal-less homeobox 5
EDTA	Ethylenediaminetetraacetic acid
Elisa	Enzyme linked immunosorbent assay
FBS	Foetal bovine serum
Fe	Iron
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HA	Hydroxyapatite
HBDC	Human bone-derived cells
hMSCs	Human mesenchymal stem cells
hTERT	Human telomerase reverse transcriptase
H_2	Hydrogen gas
In	Indium
LDH	Lactate dehydrogenase

M-CSF	Macrophage colony-stimulating factor
Mg	Magnesium
Mg^{2+}	Magnesium ion
$MgCl_2$	Magnesium chloride
$Mg(OH)_2$	Magnesium hydroxide
Mn	Manganese
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na	Sodium
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NDGA	nordihydroguaiaretic acid
NFATc1	Nuclear factor-activated T cells c1
NFêB1	Nuclear factor-êB 1
OCLs	Osteoclast-like cells
OPG	Osteoprotegerin
OSCAR	Osteoclast associated immunoglobulin-like receptor
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
pNPP	P-nitrophenylphosphate
PTH	Parathyroid hormone
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
RIPA	Radioimmunoprecipitation assay
RPL10	60S ribosomal protein L10
RT	Room temperature
RT-qPCR	Real time quantitative polymerase chain reaction
RUNX2	Runt-related transcription factor 2
SCP-1	Single cell-picked clone 1
Si	Silicon
Sn	Tin
SPI1	Spleen focus forming virus (SFFV) proviral integration oncogene
TCP	Tricalcium phosphate
Ti	Titanium
TRAP	Tartrate-resistant acid phosphatase
TRPM7	Transient receptor potential melastin 7
VDCC	Voltage-dependent Ca channels
WST-1	Water-soluble tetrazolium salt

Y	Yttrium
Zn	Zinc
Zr	Zirconium
2D	Two-dimensional
3D	Three-dimensional

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9 Appendix

I. RISK AND SAFETY STATEMENTS

Following is the list of potentially hazardous materials as well as the respective hazard and precautionary statements as introduced by the Globally Harmonized System of Classification and Labelling of Chemicals (GHS).

Compound	**Chemical Abstracts Service No.**	**Hazard statements**	**GHS hazard**	**Precautionary statements**
ARS	130-22-3	H315-H319-H335	GHS07	P261-P305 + P351 + P338
$CaCl_2$	10043-52-4	H319	GHS07	P305 + P351 + P338
Deoxycholic acid	83-44-3	H302-H315-H319-H335	GHS07	P261-P305 + P351 + P338
Dexamethasone	50-02-2	H315-H317-H319-H334-H335	GHS07, GHS08	P261-P280-P305 + P351 + P338-P342 + P311
Diethanolamine	111-42-2	H302-H315-H318-H373-H412	GHS05, GHS07, GHS08	P273-P280-P301 + P312 + P330-P305 + P351 + P338 + P310
EDTA	60-00-4	H319	GHS07	P305 + P351 + P338
Ethanol	64-17-5	H225-H319	GHS02, GHS07	P210-P280-P305 + P351 + P338-P337 + P313-P403 + P235
Fast red violet LB salt	32348-81-5	H302-H312-H332-H351	GHS07, GHS08	P280
Formaldehyde solution	50-00-0	H301 + H311 + H331-H314-H317-H335-H341-H350-H370	GHS05, GHS06, GHS08	P201-P260-P280-P301 + P310 + P330-P303 + P361 + P353-P304 + P340 + P310-P305 + P351 + P338-P308 + P311-P403 + P233
H_2O_2	7722-84-1	H302-H318	GHS05, GHS07	P280-P301 + P312 + P330-P305 + P351 + P338 + P310
Isopropanol	67-63-0	H225-H319-H336	GHS02, GHS07	P210-P280-P305 + P351 + P338-P337 + P313-P403 + P235

Naphthol AS-MX phosphate	1596-56-1	H315-H319-H335	GHS07	P261-P305 + P351 + P338
Nonidet™ P 40 Substitute	9016-45-9	H302-H318-H400	GHS05, GHS07, GHS09	P273-P280-P305 + P351 + P338
Potassium carbonate	584-08-7	H302-H315-H319-H335	GHS07	P280-P301 + P312 + P330-P305 + P351 + P338-P337 + P313
Sodium azide	26628-22-8	H300 + H310-H373-H410	GHS06, GHS08, GHS09	P273-P280-P301 + P310 + P330-P302 + P352 + P310-P391-P501
Sodium hydroxide	1310-73-2	H290-H314-H318	GHS05	P280-P301 + P361 + P353-P304 + P340 + P310-P305 + P351 + P338
Triton X-100	9002-93-1	H302-H319-H411	GHS07, GHS09	P273-P280-P301 + P312 + P330-P337 + P313-P391-P501
1α,25 Dihydroxyvitamin D3	32222-06-3	H300 + H310 + H330-H361	GHS06, GHS08	P260-P264-P280-P284-P301 + P310-P302 + P350

GHS hazard statements

H225 Highly flammable liquid and vapour

H290 May be corrosive to metals

H300 Fatal if swallowed

H301 Toxic if swallowed

H302 Harmful if swallowed

H310 Fatal in contact with skin

H311 Toxic in contact with skin

H312 Harmful in contact with skin

H314 Causes severe skin burns and eye damage

H315 Causes skin irritation

H317 May cause an allergic skin reaction

H318 Causes serious eye damage

H319 Causes serious eye irritation

H330 Fatal if inhaled

H331	Toxic if inhaled
H332	Harmful if inhaled
H334	May cause allergy or asthma symptoms or breathing difficulties if inhaled
H335	May cause respiratory irritation
H336	May cause drowsiness or dizziness
H341	Suspected of causing genetic defects
H350	May cause cancer
H351	Suspected of causing cancer
H361	Suspected of damaging fertility or the unborn child
H370	Causes damage to organs
H373	Causes damage to organs through prolonged or repeated exposure
H400	Very toxic to aquatic life
H410	Very toxic to aquatic life with long lasting effects
H411	Toxic to aquatic life with long lasting effects
H412	Harmful to aquatic life with long lasting effects

GHS precautionary statements

P201	Obtain special instructions before use
P210	Keep away from heat/sparks/open flames/hot surfaces. — No smoking
P233	Keep container tightly closed
P235	Keep cool
P260	Do not breathe dust/fume/gas/mist/vapours/spray
P261	Avoid breathing dust/fume/gas/mist/vapours/spray
P264	Wash hands thoroughly after handling
P273	Avoid release to the environment
P280	Wear protective gloves/protective clothing/eye protection/face protection
P284	Wear respiratory protection
P301	If swallowed
P302	If on skin
P303	If on skin (or hair)
P304	If inhaled

P305	If in eyes
P308	If exposed or concerned
P310	Immediately call a poison centre or doctor/physician
P311	Call a poison centre or doctor/physician
P312	Call a poison centre or doctor/physician if you feel unwell
P313	Get medical advice/attention
P330	Rinse mouth
P337	If eye irritation persists
P338	Remove contact lenses, if present and easy to do. Continue rinsing
P340	Remove victim to fresh air and keep at rest in a position comfortable for breathing
P342	If experiencing respiratory symptoms
P350	Gently wash with plenty of soap and water
P351	Rinse cautiously with water for several minutes
P352	Wash with plenty of soap and water
P353	Rinse skin with water/shower
P361	Remove/Take off immediately all contaminated clothing
P391	Collect spillage. Hazardous to the aquatic environment
P403	Store in a well-ventilated place
P501	Dispose of contents/container to… [… in accordance with local/regional/ national/international regulations (to be specified)]

II. CURRICULUM VITAE

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09/2007-06/2010	Master, School of Life Sciences, Lanzhou University, China
09/2003-06/2007	Bachelor, School of Life Sciences, Lanzhou University, China

RESEARCH EXPERIENCE

Division of Metallic Biomaterials, HZG cooperated with **Department of Plastic Surgery and Hand Surgery**, Klinikum Rechts der Isar, Technical University Munich (TUM), 2010-Present

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Dissertation: "*In Vitro* Assessment of the Cytocompatibility of Magnesium-Based Implant Materials with Osteoclasts."
The cellular mechanism involved in the remodelling of magnesium-based implants has been revealed via osteoclast monoculture as well as osteoblast-osteoclast coculture models. The importance of the cocultivation system of bone cells has been highlighted as it could be considered as a compromise way between *in vitro* monoculture and *in vivo* animal model for biocompatible assessment of biomedical magnesium-based alloys.

School of Life Sciences, Lanzhou University, 2007-2010
Master thesis research supervised by Prof. Jian Quan Liu
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The complex response of *Allium przewalskianum* diploid-tetraploid complex on the Qinghai-Tibetan Plateau (QTP) to the Quaternary climatic oscillations was addressed through the examination of five chloroplast DNA fragments to determine the extent of sequence variation and its distribution within diploid and tetraploid populations.

III. PUBLICATION LIST

1. **L. L. Wu**, B. J.C. Luthringer, F. Feyerabend, A. F. Schilling and R. Willumeit-Römer (2015). Effects of extracellular magnesium extract on the proliferation and differentiation of human osteoblasts and osteoclasts in coculture. *Acta Biomaterialia* 27: 294-304. http://www.ncbi.nlm.nih.gov/pubmed/26318802. (Journal Article)
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3. **L. L. Wu**, B. J. C. Luthringer, F. Feyerabend, A. F. Schilling and R. Willumeit (2014). Effects of extracellular magnesium on the differentiation and function of human osteoclasts. *Acta Biomaterialia* 10: 2843–2854. http://www.sciencedirect.com/science/article/pii/S1742706114000622. (Journal Article)
4. D. Tie, R. G. Guan, T. Cui, **L. L. Wu**, L. L. Song and H. M. Qin (2013). Influence of strontium concentration on *in vitro* corrosion property and cytocompatibility of ternary Mg-Zn-Sr alloys. *European Cells and Materials* 26. Suppl. 5, 36. http://www.ecmjournal.org/journal/supplements/vol026supp05/pdf/vol026supp05a036.pdf. (Conference Proceeding)
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7. **L. L. Wu**, X. K. Cui, R. I. Milne, Y. SH. Sun and J. Q. Liu (2010). Multiple autopolyploidizations and range expansion of *Allium przewalskianum* Regel. (Alliaceae) in the Qinghai-Tibetan Plateau. *Molecular Ecology* 19: 1691–1704. http://onlinelibrary.wiley.com/doi/10.1111/j.1365-294X.2010.04613.x/abstract. (Journal Article)
8. **L. L. Wu**, B. J.C. Luthringer, F. Feyerabend, Z. Y. Zhang, H. G. Machens, R. Willumeit-Römer and A. F. Schilling (2015). Increased levels of sodium chloride directly and dose-dependently increase osteoclastic differentiation and resorption. Submitted to *Osteoporosis International*, under revision. (Journal Article)

IV. CONFERENCE CONTRIBUTIONS

1. **L. L. Wu**, B. J. C. Luthringer, F. Feyerabend, A. F. Schilling and R. Willumeit-Römer (2014). Effects of extracellular magnesium extracts on the proliferation and differentiation of human osteoblast-osteoclast co-cultures. Maratea, Italy: 6th Symposium on Biodegradable Metals, 24-29 Aug. 2014. (Oral & Poster presentation)

2. **L. L. Wu**, B. J. C. Luthringer, F. Feyerabend, A. F. Schilling and R. Willumeit (2014). Effects of extracellular magnesium on the differentiation and function of human osteoclasts. Smolenice Castle, Slovak Republic: Magnesium in Translational Medicine, 11-15 May 2014. (Poster presentation)

3. **L. L. Wu**, B. J. C. Luthringer, L. Grünherz, N. Wojtas, U. Hopfner, F. Feyerabend, R. Willumeit and A. F. Schilling (2013) $MgCl_2$ affects the proliferation, differentiation and function of human osteoclasts. Weimar, Germany: Osteologie, 6-9 Mar. 2013. (Poster presentation)