Recommendation for modifying current cytotoxicity testing standards for biodegradable magnesium-based materials

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\textbf{A B S T R A C T}

As one of the most promising medical metal implants, magnesium (Mg) or its alloys have shown significant advantages over other candidates attributed to not only their excellent biodegradability and suitable mechanical properties but also their osteopromotive effects for bone applications. Prior to approval mandated by the governmental regulatory body, the access to the medical market for Mg-based implants requires a series of testing for assurance of their safety and efficacy via preclinical evaluations and clinical tests including phase 1 and 2 evaluations, and phase 3 of multi-center randomized double blind and placebo-controlled clinical trials. However, as the most widely used protocols for biosafety evaluation of medical devices, current ISO 10993 standards should be carefully reevaluated when directly applying them to predict potential health risks of degradable Mg based biomaterials via cytotoxicity tests due to the huge gap between in vitro and in vivo conditions. Therefore, instead of a direct adoption, modification of current ISO standards for in vitro cytotoxicity test is desirable and justified. The differences in sensitivities of cells to Mg ions and the capability of Mg ions to induce apoptosis were fully considered to propose modification of current ISO standards. This paper recommended a minimal 6 times to a maximal 10 times dilution of extracts for in vitro study and in vivo tests presented in the study. Our work may contribute to the progress of biodegradable metals involved translational work.

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\section{1. Introduction}

Biodegradable magnesium (Mg) based medical implants have attracted increasing attention from researchers and clinicians over the past century\cite{1}. Mg or its alloys designed as pins, wires, screws, sheets, nails, and plates were tested clinically and also evaluated systemically using animal models for potential orthopedic applications with or without surface coating\cite{2–4}. Although the published records did not mention any health risks induced by the degradation of Mg-based implants, the fast degradation of less pure Mg accompanied by rapid deterioration of their mechanical properties impeded further research and development (R&D) of Mg-based medical implants for clinical applications. With advancement in metallurgy and alloying technology, Mg with high purity or its alloys have been achieved in recent years and also successfully fabricated and applied in many industrial fields\cite{5}. The improvement of corrosion resistance in Mg-based biometals re-motivated medical researchers and metallurgists to continue their endeavors to develop new generations of orthopedic implants with...
appraised clinical indications [6]. Apart from bioabsorbable properties, Mg-based implants could remarkably induce new bone formation and stimulate angiogenesis, the two coupling biological events relevant for accelerating bone fracture healing [7–10]. These biological advantages of Mg-based implants over other orthopedic materials, e.g. inert metals and polymers, may significantly contribute to the cost reduction of medical implants. More importantly, it could avoid implant removal surgery due to its biodegradability after completion of fracture repair. However, evaluation of biosafety and efficacy of such novel implants must be systematically performed prior to application for product registration at respective regulatory bodies [11]. Up to date, a significant number of in vitro experiments involving animal models as well as a few most recent clinical evaluations on biodegradable Mg-based fixators have further indicated that degradation products of Mg-based implants could be tolerated in the body across the entire treatment periods [12,13]. However, the currently available in vitro cytotoxicity tests documented by ISO 10993 series of standards were designed without considering clearance of degradable ions from the implanted biometals via body circulation in vivo, and this might cause to the discrepancy of in vitro and in vivo studies [8,10,14]. The critical step influencing outcomes of cytotoxicity tests is preparation of extracts from implants. Currently, the preparation of the extracts from medical devices for cytotoxicity evaluation should be strictly performed following Part 5 and 12 of ISO 10993 Standards [15,16]. For non-degradable metals, only a tiny amount of ions from the inert metals would be released and accumulated in the extracts within the given immersion time, e.g. commonly recommended for 72 h. Therefore, the outcomes of biosafety evaluation for the inert metals designed as medical devices are largely dependent on the selection of the incorporated elements as the highly toxic elements may cause safety concerns for humans even at trace level. Biodegradable polymers always show a very slow degradation rate in the initial stage as the induction period is always required prior to the cleavage of the polymer chains for release of oligomers and monomers [17]. Therefore, both inert metals and polymers show high stability in solutions especially in the early stage. However, Mg-based metals could react with water immediately and release Mg ions accompanied with higher pH value and osmolality in the surrounding medium [18]. Generally, in vitro degradation behavior of Mg-based implants largely depends on the constituents of the medium [19]. In order to mimic in vivo environment, cell culture medium supplemented with serum is commonly recommended [20]. However, the routine excretion of degradation products formed around the implants via the circulatory system in the body has never been taken into consideration for establishing in vitro testing model(s). These incomparable in vitro and in vivo environments may attribute to the different and even contradictory results. Therefore, how to re-design or modify the current protocols for cytotoxicity tests relevant to biodegradable Mg-based implants is critical for R&D and registration of innovative biomaterials for orthopedic applications. In order to reduce the accumulated Mg dose in the extracts to match in vivo findings, the inhibition of in vitro corrosion rates and the direct dilution of the extracts are the two feasible approaches. Use of full bovine serum instead of cell culture medium was proposed by Scheideler for the preparation of the extracts [15,16]. For in vivo experiments involving animal models [6], Fischer recommended diluting 10 times of the extracts to control extracellular osmolality (below 400 mOsmol/kg) for cytotoxicity results [20]. Although the rise of Mg ion concentration is accompanied with a linear increase of osmolality in the medium, it cannot be directly concluded that the rising osmolality in the extracts is the sole or critical parameter influencing cell viability. In addition, the recommended dilution factor of the extracts is lack of in vivo data support. The current work is therefore designed to address two fundamental issues prior to establishing a modified in vitro cell-toxicity test protocol relevant to R&D and registration of biodegradable Mg as potential medical devices. Firstly, we calculated the individual contributions of the relevant variables involved in the extracts of pure Mg implants to reduction of cell viability and identified the predominant factor(s) influencing the cytotoxicity results, including three distinguished variables, i.e. ion concentration, pH value and osmolality. Then a recommended diluted factor of the extracts would be proposed based on the most tolerated dose and the accumulated level of the predominant factor in vitro and in vivo. Similarly, the adoption of our proposed method might facilitate the production of a series of the proposed dilution factors responsible for corresponding Mg alloys with or without surface coating before their clinic tests.

2. Materials and methods

2.1. Material preparation and sterilization

Pure Mg (99.99%) was prepared by authors’ group through a double vacuum distillation process and then remelted for extrusion into rods by E-ande corporation in Dongguan, China [22]. Cylindrical specimens with 1.2 mm in diameter were processed and prepared from the original rods. Besides, screws with a diameter of 3.0 mm and a length of 8.0 mm were fabricated from these rods. Their surfaces were then polished with SiC abrasive paper to remove oxidative films. These samples were then ultrasonically cleaned with absolute acetone and ethanol to reduce organic substances on the surface. Ultraviolet (UV) was used for specimen sterilization.

2.2. Extract preparation

Mg specimens were immersed into Dulbecco’s modified eagle medium (DMEM, Invitrogen, California, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Thermo Scientific, Massachusetts, USA) for 72 h under cell culture conditions (5% CO2, 95% humidity, 37 °C) with a fixed mass ratio to medium volume (0.2 g/ml) for preparing extracts according to ISO 10993 Part 12 [16]. The extracts were then collected without any filtration for cytotoxicity tests [16].

2.3. In vitro corrosion tests to determine mass loss

After a 72 h incubation in the cell culture medium, Mg implants were cleaned with distilled water twice to remove surrounding particles before immersion into a mixture of CrO3 (200 g/l) and AgNO3 (10 g/l) for dissolution of corrosion layers (e.g. Mg(OH)2). The relative mass loss of Mg pins was calculated through the comparison of original weight m0 and treated weight mt according to the formula: mass loss ratio = (m0 – mt) / 100% m0. Sample size was six (n = 6) for statistical analysis.

2.4. Measurement of osmolality, Mg ion concentrations and pH values

The osmolality of the extract was measured using a vapor pressure osmometer (3520, Wescor, Utah, USA). Mg ion concentrations were quantified with a inductively coupled plasma mass spectrometer (ICP-MS, Agilent Technologies, Tokyo, Japan). The pH values of the extracts were measured with a pH meter (S209,
Mettler Toledo, Ohio, USA). All above measurements were repeated three times for data analysis.

2.5. Preparation of culture medium with varying osmolality, Mg ion concentrations and pH values

A sterilized solution of concentrated Mg chloride (1.5 M) was used to increase the Mg ion concentration (by 2, 3, 5, 10, 20, 50 and 100 mM) in the culture medium (0.8 mM). A sterilized solution of NaCl (5 M) was used to increase the osmolality of the culture medium to 350, 400, 450, 500 and 600 mOsm/kg, respectively. Finally, a sterilized NaOH solution was used to increase the pH value in medium to 8.0, 8.5, 9.0 and 9.5.

2.6. In vitro cell tests

2.6.1. Cytotoxicity tests

Primary osteoblasts were isolated from the calvaria of rabbits according to a published protocol [23]. In brief, after removal of the attached soft connective tissue, the calvaria was washed with phosphate buffer solution (PBS) for three times and cut into small pieces, then transferred to DMEM culture medium (Invitrogen, California, USA) supplemented with 10% FBS (Gibco, Thermo Scientific, Massachusetts, USA) and 1% penicillin & streptomycin (Gibco, Thermo Scientific, Massachusetts, USA) for cell proliferation according to our established protocol [24]. Besides, bone marrow-derived mesenchymal stromal cells (BMSCs) were isolated from the bone marrow of rabbits and cultured in alpha-MEM medium (Invitrogen, California, USA), supplemented with 10% FBS (Gibco, Thermo Scientific, Massachusetts, USA) and 1% penicillin & streptomycin (Gibco, Thermo Scientific, Massachusetts, USA). Cell culture medium was refreshed for removal of suspended cells (blood cells) after 7 days of incubation [25]. Apart from these two primary cell types, murine fibroblast cells (L-929) and murine calvarial pre-osteoblasts (MC3T3-E1) were also employed for cytotoxicity study. The primary osteoblasts in the 3–4 passage were seeded on 96-well plates with a density of 4000 cell per well and precultured in normal cell culture medium for 24 h for facilitating cell attachment. BMSCs, MC3T3-E1 and L929 were seeded with 3000 cells per well as they proliferated much faster than osteoblasts. Then the medium was refreshed with the extracts or the adjusted medium with various pH values, osmolality or Mg ion concentrations for cytotoxicity tests via MTT assay. 10% DMSO added medium was selected as the positive control for toxic tests. The primary osteoblasts were incubated for 1, 3 and 5 days under cell culture conditions to test cell viability, while the other three cell types were performed with the viability test on the 1st and 3rd day. Twelve millimolar MTT solution was prepared by adding 5 mg MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)) into 1 ml PBS prior to filtration and sterilization. The 5 mg/ml MTT solution was added into the wells with a volume ratio of 1:11 at each time point. The cells were incubated for 4 h. Then 150 μl dimethyl sulfoxide (DMSO) was added into each well to dissolve the formazan crystals for absorbance measurement at 570 nm within 30 min using a microplate reader (ELx800, Bio-Tek, Vermont, USA). For cytotoxicity tests, experiments were repeated 4 times and 6 replicates (n = 6) for each group at each time point to evaluate toxic responses of cells.

2.6.2. Fluorescence imaging of intracellular Ca and Mg ions

Intracellular Ca2+ and Mg2+ imaging were acquired using confocal microscopy as described previously [26]. Briefly, osteoblasts grown on 25 mm diameter glass coverslips were washed 3 times with Mg-free Margo solution after removal of the culture medium. The Margo solution was composed of 130 mM NaCl, 5 mM KCl, 2.5 mM CaCl2, 20 mM HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulphonic acid) and 10 mM glucose (pH = 7.4). The cells were then incubated in the Mg-free Margo solution supplemented with 2 μM Mg-Fura-2 (Molecular Probe, Invitrogen, California, USA) or Ca-Fura-2 (Molecular Probe, Invitrogen, California, USA) and 1 μM Fluoic F-127 (Sigma, USA) for 30 min in a 37 °C incubator. After loading of dyes, the cells were washed twice with Mg-free Margo solution and kept in the Mg-free Margo solution for another 30 min at room temperature in the dark. Then the coverslips were taken out and transferred to a mini-chamber and maintained in Mg-free Margo solution for imaging acquisition. The medium with a series of Mg ion concentrations (i.e. 0.8 mM, 5 mM, 10 mM and 20 mM) were then added on the coverslips to record changes of intracellular fluorescence intensity over time. For measuring ATP-induced intracellular Ca oscillations in cells incubated with multiple Mg concentrations mentioned above, Mg concentrations increased and 100 μM ATP was added onto coverslips to stimulate C a influx into cells. An epifluorescence microscope (Nikon Eclipse Ti, Japan) equipped with a CCD camera (Spot Xplorer, USA) and a Fluor 20X objective lens (0.75 NA) (Nikon, Japan) were used for quantification, with a dual excitation at 340 and 380 nm for emission collection at 510 nm. A series of ratios calculated by $F_{\text{max}}/F_{\text{base}}$ were used to represent changes of fluorescence intensity. $F_{\text{max}}$ here means the maximal values of fluorescence intensity and $F_{\text{base}}$ represents the minimal values of fluorescence intensity.

2.6.3. Cell apoptosis analysis

The primary osteoblasts after the 5th passage incubated with the medium containing a series of Mg ion concentrations (0.8, 5.0, 10.0 and 20.0 mM) were harvested for apoptosis analysis according to a published protocol [27]. Briefly, the trypsinized cells were centrifuged at 200 g for 5 min at room temperature prior to twice washing with the PBS. Then, the cell pellets resuspended in 500 μl of PBS were added in 4.5 ml of 70% (v/v) cold ethanol for overnight fixation. After fixation, the cells were then washed twice with 5 ml of PBS. One-half milliliter DNA extraction buffer composed of 0.2 M Na2HPO4 and 0.1% Triton X-100 (2:1 in volume ratio) was added for incubation of suspended cells in 0.5 ml PBS for 5 min. Then, cells were stained with 20 μg/ml propidium iodide (PI) (Sigma–Aldrich, Missouri, USA) in the presence of 200 μg/ml RNase A (Sigma, USA) for at least 30 min at room temperature in the dark. The treated cells were analyzed by a flow cytometer (FACSaria II, BD, USA) with 488 nm laser line for excitation.

2.7. Animal experiment

Eight month old male rabbits (n = 6) and 5 month old rats (n = 6) were used to investigate in vivo degradation of Mg specimens according to our protocol approved by the Animal Ethics Committee of the Chinese University of Hong Kong (Ref. No. 13/041/MIS-5). General anesthesia was performed using a combination of ketamine (35 mg/kg) and xylazine (5 mg/kg) intramuscularly for rabbits. In brief, an incision was made on the skin and the muscle of the lateral side of the left hindlimb to expose the femoral shaft. Then the transcortical implantation of Mg screws into the femoral shaft was performed along the direction perpendicular to the longitudinal axis of the femora. Six screws were uniformly distributed along the femoral shaft with a distance of approximately 0.8 mm from the centers of two neighboring holes to meet the requirement of 0.2 g/ml according to the calculated volume of intraosseous cavity [16]. Then, the rabbits were housed in environmentally controlled cages that allowed free movement. After implantation of 72 h, the screws were pulled out from rabbits prior to the insertion of a microprobe (Mettler toledo, Ohio, USA) to determine the pH of plasma around the implants. The plasma was aspirated and centrifuged to remove cells for the determination of Mg ions and osmolality. As the implantation of Mg screws may...
simultaneously contact multiple sites in vivo, e.g. bone marrow and bone, etc., Mg pins were inserted into cancellous bone and marrow cavity of femora in rats, respectively. After 72 h implantation of pins, the implanted pins were carefully separated for calculation of weight loss according to the protocol described above.

2.8. Identification of degradation products

After 72 h implantation or immersion as mentioned above, Mg-based implants were collected and rinsed with distilled water for three times prior to air drying. Energy dispersive X-ray spectroscopy (EDS) (Carl Zeiss SMT Ltd, UK) was performed to analyze the chemical composition of the degradation products deposited on Mg implants both in vitro and in vivo.

2.9. Statistical analysis

All continuous data are presented as mean ± standard deviation, including in vitro and in vivo measurements. Statistical analysis was performed with SPSS 10.0. Differences between groups were analyzed using a one-way ANOVA followed by post hoc Tukey’s test. 

p < 0.05 was considered statistically significant.

3. Results

3.1. Cell culture results

For all the four cell types, the extracts prepared from pure Mg implants in the cell culture medium showed severe toxic effects and the average viability of the four cell types at each time point was below 50%. As the degradation of Mg was accompanied by the release of Mg ions and the rise of pH and osmolality in surrounding environments, it is necessary to distinguish the predominant factors influencing cell viability. Therefore, a series of culture media with ascending Mg ion concentrations were used to test the Mg dose tolerance of osteoblasts, MC3T3-E1, BMSC and L929 (Fig. 1). According to the current ISO standards of Part 5, cell viability higher than 75% could be considered with no toxic risks for medical devices, so we defined the Mg ion concentration with 75% cell viability as the safety level. The cell viability of the four cell types incubated for 72 h (the most commonly used time point) in a range of Mg ion concentrations was plotted to get the most tolerated dose of Mg ions (cell viability above 75%). For L929 and osteoblasts, the safety level was 35 mM, while for BMSCs and MC3T3-E1, the safety level was only 15 mM (Fig. 2).

Interestingly, the maximal Mg ion dose without induction of any negative effects on cell viability was 10 mM for all the four cell types (Fig. 2) and 10 mM was therefore defined as the critical dose not adversely influencing cell growth. When the Mg dose in the medium reached the same level in the extracts (42 mM), it was estimated that less than 65% cell viability for L929 and osteoblasts and less than 50% cell viability for BMSCs and MC3T3-E1 would be
obtained. As shown in Fig. 3, osmolality in media raised with increasing Mg ion concentrations, so a series of media with ascending osmolality values adjusted with 5 M sodium chloride were applied to test cell responses to high extracellular osmolality. Although an increase in osmolality of the medium could induce inhibitory effects on cell proliferation, only 10% cell viability was reduced when osmolality values in medium increased from 300 mOsmol/kg to 500 mOsmol/kg matching with the osmolality values in the extracts (Figs. 4 and 8). Meanwhile, sensitivity of cells to alkaline environments might be greatly dependent on cell types selected. Higher pH (over 8.5) showed detrimental effect on cell viability (approximately 10% decrease) when L929 and osteoblasts were chosen for cytotoxicity tests, while the alkaline environment simulated cell growth of BMSCs and MC3T3-E1, especially at the early time point (Fig. 5).

3.2. In vitro and in vivo degradation performance

As shown in Fig. 6, 3% of mass loss was found in pins after 72 h of immersion in cell culture medium. In contrast, the overall mass of pins reduced approximately 7% and 4% within 72 h implantation in cancellous bone and medullary cavity of the rat femur, respectively. The chemical composition of degradation products also showed huge differences for Mg specimens located in different locations. Only trace amounts of Ca (0.555 wt.%) and P (1.0555 wt.%) could be detected in corrosion particles deposited on the in vitro immersed samples. Interestingly, very low intensity of Ca signals (assigned to 1.692 wt.%) while rich amount of P (12.012 wt.%) were observed in degradation products of samples implanted in bone marrow cavity. Meanwhile, much higher amounts of Ca (11.656 wt.%) and P (17.856 wt.%) were detected around Mg implants inserted in cancellous bone. As in vivo circulation system may effectively dilute degradation products, Mg ion concentrations, pH values and osmolality were measured in plasma around Mg screws for comparison with in vitro data (Figs. 7 and 8). The average pH values of in vitro medium increased from initial 7.40 to 8.75 after 72 h immersion, indicating alkalization of tissue fluid around the implants with the presence of degradation of Mg metals. The abundant accumulation of Mg ions (from 0.8 to 42 mM) was measured, which was also accompanied with an increase in osmolality (from 332 to 480 mOsmol/kg) of the extracts. In contrast, except a slight increase in Mg concentration (from 0.9 to 1.1 mM) after 72 h implantation in femoral medullary cavity, no significant differences in pH and osmolality values were found between pre- and post-implantation of the Mg specimens.
Fig. 5. Cell viability of osteoblasts (a), MC3T3-E1 (b), BMSC (c) and L929 (d) cultured in medium with different pH values. Asterisks stand for statistically significant differences in comparison to the control group ($P < 0.05$, $N = 4$).

Fig. 6. Comparisons of degradation behavior of Mg implants in different environments. (A) Weight loss ratio of Mg pins after a 72 h immersion in cell culture medium or implantation in cancellous bone and medullary cavity in rats. (B) EDS analysis of the region in degradation products marked by the cross symbol (left: in vitro; middle: bone marrow; right: cancellous bone). Asterisks stand for statistically significant differences ($P < 0.05$, $N = 6$).
3.3 Fluorescence imaging for dose analysis of intracellular Ca and Mg

Incubation of osteoblasts in Mg-free medium did not induce influx or efflux of intracellular Mg ions, indicating maintenance of balance for cellular Mg ions after 30 min pre-culture treatment of cells in Mg-free conditions (Fig. 9(a)). Mg ion channels were immediately activated to allow Mg ion influx when Mg incorporated medium was added to replace Mg-free medium. However, homeostasis of cellular Mg ions could not be re-established within a short time, i.e. approximately 5 min, as extracellular Mg ions in the medium were well controlled and regulated for gradual influx instead of abrupt flow into the cells by Mg-selective ion channels. Although the influx rates of Mg ions into cells remained constant in spite of more Mg dose being added to the medium, higher extracellular Mg level would trigger longer activation time for Mg ion relevant channels to facilitate more Mg entry. Interestingly, no dose changes in intracellular free Ca ions could be observed when extracellular Mg ion concentrations increased (Fig. 9(b)).

In order to test if lower cell viability was partially attributed to more severe cell apoptosis induced by higher Mg ion concentrations, identification of hypodiploid cells was necessary for precise evaluation of cellular DNA content using a flow cytometer. The fluorescent intensities in the range of 50–150 in the X axis was known as “PE-Texas Red-A” that represented DNA contents in normal nuclei of cells at different phases (G0, G1, S, G2 and M). In terms of apoptotic cells stained with PI (propidium iodide), they displayed a broad hypodiploid (sub-G1) peak, which could be easily discriminated from the narrow peak of cells with normal (diploid) DNA content in the red fluorescence channels. However, the percentage of apoptotic cells was too small to directly discriminate the apoptotic cells from normal cells. Thus, after the data of the debris and residuals of necrotic cells (lower diameter (FSC-A) and reduced fluorescent signals) were eliminated, we counted the number of normal cells in the labeled circle “P1” for calculating the percentage of the apoptotic cells in the whole gated events.

4. Discussion

The present study was designed to understand the applicability of in vitro testing results for in vivo situation for making a recommendation for potential modification of current ISO standards with regard to in vitro biosafety testing protocols relevant to biodegradable pure Mg or even Mg-based medical implants.

4.1 Predominant factors influencing cell viability

For cytotoxicity tests of biologically inert medical devices, the major parameter influencing cell responses to biomaterials is the released ions [28]. With regard to biodegradable Mg implants, more variables must be considered as the rise of Mg ion concentrations would induce higher pH values and osmolality in cell culture medium, which could be interpreted as Eq. (1).

\[
\text{Mg} + \text{H}_2\text{O} \rightarrow \text{Mg}^{2+} + \text{OH}^- + \text{H}_2
\]  

(1)

Generally, the range of the optimal pH values for cell growth is considered between 7.4 and 7.8 as alkalosis or acidosis may have an inhibitory effect on protein synthesis for some cells, ultimately
inducing cell apoptosis [29]. Therefore, alkaline stress caused by accumulation of OH⁻ ions during degradation of Mg implants has been regarded as negative stimuli for the cell population. Besides, an increase in medium osmolality as a result of increased ions dose may impose potential osmotic shock to cells, leading to DNA damage [30]. Therefore, apart from defining the critical dose or the safety level of Mg ion concentration for cell growth, the influences of pH value and osmolality, the other two variables to cell viability must be studied independently to distinguish the key parameter. After 72 h immersion of Mg implants in cell culture medium, the alkaline environment (pH value: 8.75) accompanied with high Mg ion concentrations (over 42 mM) and osmolality (above 480 mosmol/kg) was observed in the extracts, in which all these changes might contribute to the reduction of cell viability as the viabilities of the four cell types/lines were below 50%. The inhibitory effects of the Mg based extracts on cell growth were completely ascribed to the rise of extracellular osmolality (may induce osmotic shock on cells) in a previous study [20]. However, the biological toxicity of ion levels and the cell responses to increasing osmolality should be studied individually for evaluation of their own contributions to cell viability. Generally, osmolytes are divided into organic and inorganic substances. Sorbitol and NaCl were compared for their biocompatibility via cytotoxicity tests and the results showed that the medium with the same osmolality adjusted with NaCl induced significantly less adverse effects on cells, indicating higher biological toxicity for sorbitol (refer to Supplementary Fig. A.1). More importantly, the organic solute sorbitol could partially transport through the plasma

Fig. 9. Images of fluorescent intensity changes with regard to intracellular Mg ions (a), Ca ions (b) and ATP induced intracellular Ca ions over time under different extracellular Mg ion concentrations (c)–(g).
membrane to elevate intracellular osmolality and reduce osmotic pressure [31]. Therefore, NaCl was used here for osmolality regulation in culture medium to study the tolerance of different cell types under hyperosmotic conditions. As shown in Fig. 4, ascending osmolality in culture medium adjusted by NaCl gradually inhibited cell growth due to the differences of extracellular and intracellular osmolality. The adverse effects of increased osmolality in NaCl adjusted medium on cell viability was slightly enhanced with incubation over time, but the cell viability was still close to 90% even when the osmolality in the medium exceeded 500 mOsmol/kg (480 mOsmol/kg for extracts). As the cell membrane was partial permeable for Mg ions via relevant ion channels from extracellular solution [31], it could be estimated that the lower osmotic pressure would be induced to cells incubated in medium with supplementation of Mg ions with the same osmolality to that in NaCl adjusted medium [32,33]. The average cell viability for the four cell types/lines was less than 50% after a 72 h incubation in the extracts, indicating that the maximal contribution of the increased osmolality to reduction of cell viability was less than 20%. In terms of pH effects on cell viability, it was hard to mimic in vivo alkaline microenvironments just through medium replacement due to the removal of OH− by the existing acidic gas (CO2) in the incubator. It only took 1.5 h for CO2 to neutralize the alkaline substances in the medium, indicating the short time for triggering cell responses in alkaline conditions (refer to Supplementary Fig. A.2). Therefore, the stimulatory effect of pH values to cells might be only concentrated at the initial stage of cell incubation. More importantly, it seemed that the responses of cells to pH values were largely dependent on cell types. For MC3T3-E1 and BMSC, a slight increase in pH values could even stimulate cell growth. However, the alkalization (above 8.5) of the culture medium might induce adverse effects on cell viability of other two cell types (osteoblasts and L929), leading to about 10% loss in cell viability. This would account for approximately 20% contribution to inhibitory effects of the extracts on cell growth. The results regarding responses of L929 and osteoblasts to alkaline environments were consistent with a previous publication focusing on endothelial cells [29]. According to above analysis regarding individual contributions of potential factors influencing cell viability, the biological toxicity induced by high dose of Mg ions in the extracts was the predominant factor to detrimentally influence cell growth. Therefore, it is essential to measure Mg ion concentrations in all extracts from pure Mg or its alloys and local tissue around the implants made of either pure Mg or its alloys for health risk evaluation.

As one of the most important co-enzymes, Mg ions regulate more than 300 biochemical reactions in the human body [34]. However, over influx of Mg ions into cells might induce potential adverse effects on cells as high levels of Mg may block Ca ion channels and compete with Ca for binding sites on various Ca-binding proteins besides inducing disorders of Mg involved enzyme reactions [35]. Generally, intracellular Ca2+ levels were maintained and regulated by numerous voltage-dependent (e.g. T-type calcium channels, etc.) and ligand-gated (e.g. ryanodine receptor (RyR), etc.) calcium ion channels and pumps [36–38]. These Ca-selective ion channels might be blocked by high dose of Mg ions, leading to lower influx of Ca ions into cells [36,39]. However, the loss of fluorescent intensity of the intracellular Ca was not significant due to the storage of plenty of Ca in the endoplasmic reticulum (ER) required for maintaining biological functions [38,40]. However, the over-influx of Mg ions would compete with Ca2+ for common intracellular binding sites and even displace Ca from binding proteins to form a new complex with higher affinity [41]. Besides, as a new member of the subfamily of transient receptor potential (TRPC), the activity of LTRPC7 was largely dependent on cytosolic Mg-ATP levels [42]. As LTRPC7 was an important mediator of cellular energy metabolism, cell viability might be regulated by intracellular Mg-ATP concentrations [42]. More Mg ions into cells at high Mg level might facilitate the formation of Mg-ATP (accounted for over 90% ATP) while overexpression of Mg-ATP might inhibit LTRPC7 function [43]. In the presence of high Mg concentration (above 20 mM), the loss of Ca involved binding reactions and the overexpression of LTRPC7 might partially contribute to the inhibition of cell proliferation although more evidence was still required for illustration of their individual effects on cell responses.

4.2. Basis for proposing a dilution factor of extracts for cytotoxicity tests

The preparation of the extracts from inert meals and biodegradable polymers could be guided by current ISO Standards Part 12 without any limitations for cytotoxicity tests documented in the Part 5 protocol. However, the testing protocols should be modified for biosafety evaluation of resorbable metals, such as Mg or its alloys, as the in vitro rapid accumulation of degradation products in the local surrounding environments could not simulate or reflect the in vivo situation regulated by the circulating body fluid. The contradiction of the cytotoxicity evaluation results to our previously reported in vivo findings [8–9] and current data (Supplementary Fig. A.3) might provide misleading information that prevented R&D and registration of Mg-based medical implants. Therefore, to bridge the gap or discrepancy between in vitro and in vivo tests, this study provided relevant evidences for forming guidelines applicable for testing Mg-based implants before clinical application. As only pure Mg implants were studied in this work, the measurement of the most tolerated Mg dose in vitro and in vivo and the detection of the Mg level around the implants after 72 h of immersion (in vitro) or insertion (in vivo) should be the two key aspects for defining the dilution factor for the extracts. As cells in tissue (in vivo) and culture medium (in vitro) might have different tolerated doses of Mg ions, it was necessary to standardize in vitro and in vivo data to facilitate their comparison. As shown in Fig. 2, 10 mM of Mg ions could be considere...
(298 mM) could be observed in the surrounding plasma of the insertion sites after a 72 h implantation of Mg screws. Interestingly, higher mass loss ratio was observed among in vivo implants, causing contradictory results to our previously reported findings obtained following ASTM (American Society Testing Materials) protocols [46]. However, these guidelines from ASTM might not be applicable for prediction of in vivo degradation behavior of biodegradable metals as the degradation rates of implants were largely time/situation-dependent. Besides, the selection of methodologies for measuring corrosion rates might significantly affect the outcomes due to limitations of current technologies, e.g. the interference induced by degradation products to the volume estimation of Mg substrate by micro-CT imaging, etc. Therefore, we recommended quantifying the changes of implant mass as an index of Mg degradation in this study. Despite higher degradation rates for in vivo implants, no abrupt rise of Mg ions in the local tissue was observed, which could be ascribed to the excretion and dilution function of the circulatory system [47]. As the intramedullary Mg ion concentration was only 1.1 mM after 72 h implantation, the adjusted Mg level range via standardization should be 3 mM and 4.7 mM (1.1 mM × L). Then, the dilution factor could be calculated based on the conversion of in vivo adjusted Mg concentrations and in vitro Mg level in the extracts for cytotoxicity tests. As Mg ion concentration in the extracts was 42 mM, it suggested that a dilution factor between 9 and 14 times should be applied to the extracts. It is worth to mention that the surrounding tissue of the implanted material(s) would significantly affect their degradation characteristics, so it was necessary to consider the potential application of Mg-based implants in various locations of the human body and establish the corresponding dilution factor for the extracts corresponding to their intended use. However, due to limitations of current technology, there was no real-time method to measure ion concentrations in the solid tissue directly. Therefore, we had to propose herewith an alternative method based on the hypothesis that Mg ion concentrations at different locations in vivo could linearly be increased with degradation rates of implants. The average mass loss ratio of Mg pins in cancellous bone was approximately 50% higher than that located in bone marrow environments, which might be ascribed to physical effects induced by the surrounding bone tissue via load transfer [48]. According to the study hypothesis, the calculated Mg ion concentrations in the cancellous bone should be 1.5–1.6 mM. With regard to cytotoxicity evaluation for Mg implanted in bone-rich regions, the recommended range of the dilution factor of the extracts should be 6 and 10. Therefore, if we choose the biosafety level (15 mM) to match in vivo tolerated Mg dose, 6 times or 9 times should be diluted for extracts according to the selection of implantation regions, otherwise 10 times or 14 times should be diluted with the critical dose (10 mM) selected for consideration. Although the fixation of bone fracture would inevitably require simultaneous contact of the implants with bone marrow and bone, the dilution times of the extracts for in vitro test should be chosen in the lower margin for full assurance of biosafety. Therefore, the dilution range of Mg based extracts between 6 times and 10 times should be acceptable for in vitro biosafety evaluation. A draft guidance concerning use of ISO 10993 standards was distributed by U.S. Food and Drug Administration (FDA) for comments in 2013, which revealed that the dilution of the extracts was acceptable for special implants if justification could be provided [49]. Our testing model fully accommodated the characteristics of in vivo conditions to establish a reliable linkage between in vitro and in vivo evaluation systems.
4.3. Selection of cell types for cytotoxicity tests in vitro

Apart from adjustments of the extracts, selection of cell types (primary cells and cell lines) for cytotoxicity evaluation was also critical to influence results due to their sensitivity differences to environments [15,50]. In general, these murine cell lines, including CCL 1 (NCTC clone 929), CCL 163 (Balb/3T3 clone A31), CCL 171 (MRC-5) and CCL 75 (WI-38), CCL 81 (Vero) and CCL 10 [BHk-21 (C-13)] and V-79 379A, were recommended for cytotoxicity evaluation in ISO standards [15]. Generally, the selection of relevant cell types for cytotoxicity evaluation should depend on the in vivo situation of interest to simulate in vivo environment as much as possible [28]. With consideration of the potential use of Mg based implants in the orthopedic field, primary cells including bone marrow stem cells (BMSCs) and osteoblasts were used for comparison of their responses with the two most commonly used cell lines (i.e. MC3T3-E1 and L929). The sensitivity of the four cell types to Mg ions showed similar results to other reported cell types [44]. As our selected cell types/lines did not show significant differences in sensitivity to Mg ions, ISO recommended cell lines (L929 in our study) might be more preferable to primary cells for cytotoxicity evaluation due to their high purity. However, we would consider more cell types to test cell responses to extracellular Mg dose for validation of our proposed critical dose or biosafety level in the following study. Table 1 summarizes our justifications or rationales for modifying current Part 5 and 12 of ISO Standards for biodegradable Mg as potential implantable medical devices.

Table 1: Rationales for modifying current ISO standards on cytotoxicity tests for biodegradable Mg-based orthopedic implants.

<table>
<thead>
<tr>
<th>No. of items</th>
<th>Key conditions of regulations</th>
<th>Sources of standards</th>
<th>Our proposed recommendations</th>
<th>Rationales</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Extraction conditions:</td>
<td>ISO 10993.12 (37 ± 1 °C, (72 ± 2) h)</td>
<td>For long-term implantable medical devices (Class III), 72 h instead of 24 h for implant immersion for the preparation of extracts is necessary and body temperature is relevant due to vigorous reactions between Mg and solutions.</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Extraction media:</td>
<td>ISO 10993.12 Cell culture medium with serum</td>
<td>Serum added cell culture medium is more close to in vivo conditions and has been widely accepted.</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Ratio of implants to medium volume:</td>
<td>ISO 0.2 g/ml</td>
<td>Irregular shapes for Mg based implants (e.g. pins, screws, plates, etc.)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Extraction atmosphere:</td>
<td>ISO 10993.12 5% CO2 in cell culture incubator</td>
<td>The presence of CO2 in the human body will influence degradation rates of Mg implants</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Sterilization:</td>
<td>ISO 10993.12 UV may be an appropriate sterilization tool</td>
<td>No observation of infection of cell culture medium</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Dilution of extracts:</td>
<td>ISO 10993.12 At least 6 times but not more than 10 times dilution with the original medium</td>
<td>In vitro data should match in vivo data for cytotoxicity tests</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Preferred cell types:</td>
<td>ISO 10993.5 Current ISO recommended cell lines</td>
<td>No observation of significant differences regarding results of cytotoxicity tests between L929 &amp; MC3T3-E1 and selected primary cell types (osteoblasts and BMSCs)</td>
<td></td>
</tr>
</tbody>
</table>

4.5 Conclusion

As the current ISO regulations (Part 5 and 12) could not be directly applied to prescreening toxic evaluation of degradable biometals, here specifically referred to Mg or Mg-based alloys as potential medical devices in orthopedics, the current study aimed to provide rationales for modifying the current guidelines toward a more applicable prediction of their potential health risks. The rationale for establishing such protocol was to link in vivo degradation behavior or characteristics of Mg-based materials with in vitro testing models for reliable adjustments of the extracts. The correlation between in vitro and in vivo testing models was established based on Mg ion concentrations, which was the predominant variable to influence cell viability, where the 10 mM and 15 mM of Mg ion concentrations could be considered to be the critical dose without inhibiting cell viability and the biosafety level with cell viability above 75% for cell growth. The combined effects of the loss of LTRPC7 function and the decrease of Ca involved binding sites/reactions in high Mg level might be one of main reasons influencing cell viability. The recommended dilution range for the extracts to perform cytotoxicity evaluation was between 6 and 10. ISO recommended cell lines (L929) showed no significant difference in cytotoxicity evaluation compared to the primary cells, indicating that the use of these cell lines in priority was acceptable. Besides, other minor comments including preparation conditions of the extracts were also mentioned for specification of regulations concerning the evaluation of Mg-based implants in this study. More advanced technologies, e.g. fluorescent imaging, etc., might be explored for precise measurement of in vivo data to validate our conclusion. The significance of this work was to lay down a foundation for regulatory bodies to adopt our recommendation as the cell toxicity test protocol to be used for Mg-based medical devices and facilitation of clinical translation of Mg-based medic implants.

Disclosures

There are no potential conflicts of interest to disclose for authors of this work.
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Appendix A. Figures with essential color discrimination

Certain figures in this article, particularly Figs. 1–10, are difficult to interpret in black and white. The full color images can be found in the online version, at http://dx.doi.org/10.1016/j.actbio.2015.04.011.

Appendix B. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.actbio.2015.04.011.

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