Preclinical evaluation of acute systemic toxicity of magnesium incorporated poly(lactic-co-glycolic acid) porous scaffolds by three-dimensional printing

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Key Words:
acute systemic toxicity; clinical translation; magnesium; median lethal dose; porous composite scaffolds

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ABSTRACT

Biodegradable polymer scaffolds combined with bioactive components which accelerate osteogenesis and angiogenesis have promise for use in clinical bone defect repair. The preclinical acute toxicity evaluation is an essential assay of implantable biomaterials to assess the biosafety for accelerating clinical translation. We have successfully developed magnesium (Mg) particles and beta-tricalcium phosphate (β-TCP) for incorporation into poly(lactic-co-glycolic acid) (PLGA) porous composite scaffolds (PTM) using low-temperature rapid prototyping three-dimensional-printing technology. The PTM scaffolds have been fully evaluated and found to exhibit excellent osteogenic capacity for bone defect repair. The preclinical evaluation of acute systemic toxicities is essential and important for development of porous scaffolds to facilitate their clinical translation. In this study, acute systemic toxicity of the PTM scaffolds was evaluated in mice by intraperitoneal injection of the extract solutions of the scaffolds. PTM composite scaffolds with different Mg and β-TCP content (denoted as PT5M, PT10M, and PT15M) were extracted with different tissue culture media, including normal saline, phosphate-buffered saline , and serum-free minimum essential medium , to create the extract solutions. The evaluation was carried out following the National Standard. The acute toxicity was fully evaluated through the collection of extensive data, including serum/organs ion concentration, fluorescence staining, and in vivo median lethal dose measurement. Mg in major organs (heart, liver, and lung), and Mg ion concentrations in serum of mice, after intraperitoneal injection of the extract solutions, were measured and showed that the extract solutions of PT15M caused significant elevation of serum Mg ion concentrations, which exceeded the safety threshold and led to the death of the mice. In contrast, the extract solutions of PT5M and PT10M scaffolds did not cause the death of the injected mice. The median lethal dose of Mg ions in vivo for mice was determined for the first time in this study to be 110.66 mg/kg, and the safety level of serum magnesium toxicity in mice is 5.4 mM, while the calcium serum safety level is determined as 3.4 mM. The study was approved by the Animal Care and Use Committee of Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences (approval No. SIAT-IRB-170401-YGS-LYX-A0346) on April 5, 2017. All these results showed that the Mg ion concentration of intraperitoneally-injected extract solutions was a determinant of mouse survival, and a high Mg ion concentration (more than 240 mM) was the pivotal factor contributing to the death of the mice, while changes in pH value showed a negligible effect. The comprehensive acute systemic toxicity evaluation for PTM porous composite scaffolds in this study provided a reference to guide the design and optimization of this composite scaffold and the results demonstrated the preclinical safety of the as-fabricated PTM scaffold with appropriate Mg content, strongly supporting the official registration process of the PTM scaffold as a medical device for clinical translation.

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Introduction

Critical-sized bone defects caused by trauma, tumours, inflammation, or sports injuries have always been considered a problem in orthopaedics, and have caused extensive medical and socioeconomic burdens in recent years.[1, 2] For bone repair, external intervention is essential to guide the bone reconstruction, since a bone with a large defect, also known as a critical-sized defect, cannot be naturally repaired. Autologous bone grafting and allografting are the usual strategies, but these methods remain challenging due to limited availability of graft material, donor site morbidity, and immune rejection.[3] Consequently the demand for alternative materials for bone defect repair is increasing.

Recently, incorporating magnesium (Mg)-based materials into biodegradable polymeric scaffolds has been found to provide a good option for efficient and effective clinical approach of critical-sized bone defects, simultaneously improving osteogenic ability and enhancing mechanical properties.[4] This combined materials system has several advantages as a bone scaffold: (1) Mg alloy materials have specific strength and it's Young's modulus is similar to natural cortical bone, meaning that they can improve the mechanical properties of polymeric scaffolds;[5] (2) Mg-based materials are degraded under physiological condition, and indeed the released Mg ions can promote osteogenic differentiation mediated by neuronal calcitonin gene-related polypeptide-α,[2] which enhances the osteogenesis induced by the composite polymeric scaffolds; and (3) the acidic degradation products of a polymer can be neutralized by the alkaline products of Mg, which provides benefits by relieving the inflammatory response in the microenvironment.[6, 7] Numerous studies have been devoted to the development of these combined materials, and promising results concerning enhancement in mechanics and osteogenesis have been reported.[2, 8-11] Therefore, incorporation of Mg-based materials into polymer systems is a potential clinical therapy, and preclinical evaluation, such as investigation of acute systemic toxicity, is essential. However, very limited studies focusing on preclinical evaluation of this combined system have been reported. In our previous study,[12] we demonstrated that the degradable three-dimensional (3D)-printed poly(lactic-co-glycolic acid) (PLGA) porous composite scaffolds (PTM) composite scaffolds can significantly improve bone regeneration and angiogenesis in the mice bone defect model by using micro-computed tomography, tissue section, and other methods. The magnesium in the scaffolds could enhance the mechanical strength of the PLGA/beta-tricalcium phosphate (β-TCP) scaffold to match the natural bone tissue and balance the pH value during the degradation process.[12]

In considering the potential clinical use of a Mg-based composite polymer system, the objective of this study was to conduct preclinical evaluation of the acute systemic toxicity of PTM. A common method of intraperitoneal (I.P.) injection of the scaffold extract solution into mice was used in this study. The evaluation was conducted following the standard.[13, 14] Composite scaffolds with different Mg and β-TCP content (1:2, 1:1, and 2:1, labelled as PT5M, PT10M, and PT15M, respectively) were fabricated using the low-temperature rapid prototyping technique. In this study, the *in vitro* degradation behaviour of scaffolds was investigated. The PTM scaffolds with different Mg content were extracted using three different tissue culture media (normal saline, phosphate-buffered saline (PBS), and minimum essential medium (MEM)) with different pH values (7.0 and 10.0) to create the extraction solutions. Then, the extraction solutions were intraperitoneally injected into mice to assess the acute systemic toxicity of the scaffolds. At 1 hour after injection, serum concentrations of Mg and calcium (Ca) ions were measured. Histological evaluations, including haematoxylin and eosin (H&E) staining and Mg indicator fluorescent staining of mice organs were performed. The mass ratio of Mg and Ca in organs was measured, and the *in vivo* median lethal dose after injection was calculated. Finally, we suggested that safety level of serum Mg toxicity and calcium serum safety level according to the results of acute systemic toxicity and median lethal dose study *in vivo*.

Methods

Materials and sample preparation

PLGA (lactic acid:glycolic acid ratio = 75:25, molecular weight: 250 kDa, analytical grade) was purchased from Shandong Institute of Medical Instruments (Jinan, China). β-TCP with particle size ranging from 48 μm to 74 μm was supplied by Shanghai Bio-lu Biomaterials Co., Ltd. (Shanghai, China). Pure Mg powder (purity of 99.81%) with a particle diameter of 48 μm to 74 μm was provided by Weihao (Tianjin, China), and 1,4-dioxane was purchased from Nantong Senxuan Pharmaceutical Co., Ltd. (Nantong, China).

Porous composite scaffolds of PLGA with β-TCP (PT) and Mg powder mixture (PTM) were fabricated using a low-temperature rapid-prototyping 3D printing technology as described in our previous study.[12, 15] In brief, Mg powder and β-TCP were mixed with different weight ratios (1:2, 1:1 and 2:1), and then the Mg powder and β-TCP mixture was added and homogeneously suspended into 15 w/v% PLGA 1,4-dioxane solution and fabricated in three dimensions. As described above, different content of Mg powder were added to the PLGA/TCP scaffolds to produce PT scaffold (0 wt%Mg), PT5M scaffold (5 wt% Mg), PT10M scaffold (10 wt% Mg), and PT15M scaffold (15 wt% Mg). The porous scaffolds were fabricated using a low-temperature rapid-prototyping machine at -30°C. The macroscopic and internal structures of the porous scaffolds were designed according to the stereolithography model for 3D porous scaffold blocks.[12]

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The dimensional parameters for each type of scaffold, such as porosity and porous interconnectivity, were similar. Due to rapid freezing and concreting of the materials mixture liquid, phase separation occurs in the solution formed the micropores. After fabrication, the scaffolds were lyophilized in a freeze dryer (Alpha 2-4 LDplus, Marin Christ, Osterode, Germany) for 48 hours to remove the solvent.[16] After lyophilization to evaporating the solvent, the scaffold with a gradient pore structure was generated and cut into 10 × 10 × 10 mm^3. Macroscopic images of each scaffold were captured with a digital camera, and the surface morphology of the scaffolds was imaged using a scanning electron microscope (JSM-6390, Jeol Ltd., Tokyo, Japan) after gold sputter-coating. Mechanical tests were detected by using dynamic material testing machine (Instron-E3000, Norwood, MA, USA) with load cell of maximum 1000 N and speed of 1 mm/min (n =3 in each group). Those methods were based on the GB/T 8813-2008.[17]

In vitro static degradation study

The *in vitro* static degradation assessments of scaffolds were performed by extracting the scaffolds in different solutions at 37°C. The solutions used were saline (0.9 wt% NaCl), PBS (Hyclone, Logan, UT, USA), and serum-free MEM (Gibco, Carlsbad, CA, USA). The ratio of mass of the scaffold to volume of the solution was 0.1 mg/L according to the GB/T 16886.12-2017 standard.[14] Each time-point for each type of sample was analysed independently, and three parallel samples were investigated for statistical analysis. After 24, 48, and 72 hours immersion, the pH value of the solutions was measured with a pH meter (S220 SevenCompact, Mettler Toledo, Shanghai, China). The Mg and Ca ion concentrations of the solutions were measured with an inductively-coupled plasma-optical emission spectrometry technique (ICP-OES; 700 Series, Agilent Technologies, Santa Clara, CA, USA).

In vivo acute systemic toxicity study

The *in vivo* acute systemic toxicity analyses of scaffolds were performed by injecting the scaffold extraction solutions into mice. This assessment was performed according to the GB/T 16886.11-2011 standard.[13] The specific-pathogen-free level Kunming mice (purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China), were 6 weeks old and 30 g in weight. All the animal treatments and all surgical procedures related to this study were performed according to the guidelines for the Animal Care and Use Committee, and all these were approved by the Animal Care and Use Committee of Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences, with the approval No. of SIAT-IRB-170401-YGS-LYX-A0346 on April 5, 2017. Every effort was made to minimize the pain and suffering of the animals. The extract solutions were prepared according to the GB/T 16886.12-2017 standard.[14]

Different extract solutions (normal saline, serum-free MEM and PBS), injected the various Mg content scaffold extract (PT, PT5M, PT10M, and PT15M), different Mg content composite scaffolds soaked in MEM and different pH values of MEM used to extract the PT15M were all incorporated in this experiment as shown in **Additional Tables 1–3**. The scaffolds were soaked in the extract solutions at 37°C for 72 hours. After preparation, the extract solutions were removed and used for I.P. injection with an injection rate of 0.5 mL/min. The injection volume of the extract solution for each animal was 50 mL/kg according to Appendix B of the GB/T 16886.11-2011 standard.[13]

After the I.P. injection, different groups of animals were observed to determine the acute systemic toxicity effects. The animal body weight was recorded and the tissue samples were collected for H&E staining at 72 hours after exposure. For those dead mice, tissue samples were immediately collected for further test and staining. Blood samples were collected after 1 hour and allowed to stand for 3 hours. Then, the samples were centrifuged at 3 × 10^3 r/min for 20 minutes. After harvesting serum, the concentrations of Mg and Ca ions in serum were analysed by ICP-OES. All mice were dissected to measure the Mg ion and Ca ion concentration in organs at 1 hour after I.P. injection, including the heart, liver, lung, spleen, and kidney. The harvested organs were cut into pieces of the volume about 14–16 mm^3 and then dissolved in 65% nitric acid. This dissolved tissue was further digested with a microwave. Finally, the concentrations of Mg and Ca ions in these tissue solutions were measured by ICP-OES. For histological evaluation, the harvested organs were fixed with 10% formalin at room temperature for 24 hours. After fixation, the organs were dehydrated with a series of increasing ethanol and xylene mixtures (70%, 80%, 90%, 100%) for 1 hour each, followed by embedding in paraffin. Cross-sections of the embedded tissues were cut into slices with a thickness of 4 μm and stained with H&E. The stained sections were imaged with an optical microscope (Olympus IX71, Olympus, Tokyo, Japan). For the Mg indicator fluorescent staining, after fixation with 10% formalin at room temperature for 3 hours, the organs were embedded in paraffin and then cross-sections of the organs were cut with a slice thickness of 8 μm. After the water in slices was evaporated at room temperature, the slices were fixed with acetone. The working solution used to stain Mg indicator fluorescence of slices was prepared in advance. Mg Green™ AM indicator fluorescent solution (5 μM; Thermo Fisher Scientific, Waltham, MA, USA) was added to cover the sections and incubated at room temperature for 1 hour. After that, the working solution was added into the cover section and incubated in the dark for 30 minutes. The purpose of the second step is to allow the complete de-esterification of intracellular AM esters before commencing fluorescence measurements. After staining, every sample was observed by fluorescence microscopy (DMi8, Leica, Wetzlar, Germany) to visualise the Mg-positive areas.

In vivo median lethal dose study

PT15M scaffolds extracted with MEM at 37°C for 72 hours were used as the extract solution for I.P. injection. More than 10 Kunming mice (half male and half female) were used to evaluate the lethal dose for each concentration. The critical dose of Mg content in diluted extract solution leading to LD100 (100% lethal dose) and LD0 were determined with a dichotomy method in mice. The class interval for adjacent concentrations was calculated following the modified Spearman-Karber statistical method:[18]

$$i = (\lg LD_{100} - \lg LD_0) / (n-1) \quad (1)$$

Five fixed doses (0, 79, 99, 119, 139, and 165 mg/kg) were selected for the test, and LD_{100} and LD_0 represented the critical dose of Mg content in intraperitoneally-injected extract solutions leading to 100% and 0% death of animals respectively. Accordingly, the initial extract solutions were diluted to give five dose groups with a calculated class interval. The median lethal dose (LD_{50}) was obtained according to the equation:

$$\lg LD_{50} = X_k - i(Ep - 0.5) \quad (2)$$

Where Xk is the log value of the maximum dose, and Ep stands for the summation of the death rate. The extract solutions of different concentrations were injected into the enterocoelia of the mice with an injection rate of 0.5 mL/min and an injection volume of 50 mL/kg. After I.P. injection of the extract solution, all mice were observed for 72 hours. If the animals died within 72 hours, blood was drawn immediately for further evaluation. The surviving animals were sacrificed at 72 hours according to the protocol and blood was drawn for further evaluation. The blood was allowed to stand for about 3 hours and then centrifuged under 4°C at 3 × 10^3 r/min for 20 minutes to prepare serum. The concentration of Mg and Ca ions in blood serum were measured using the ICP-OES method. Meanwhile, all the dead and sacrificed animals were anatomized, and the heart, liver, lung, spleen, and kidney were removed to measure the mass ratio of Mg and Ca in each organ according to the above-mentioned method.

Statistical analysis

All quantitative data are expressed as the mean ± standard deviation (SD). Statistical comparisons were conducted by using the GraphPad Prism v5.0 software (GraphPad Software Inc., La Jolla, CA, USA). The results were tested using one-way analysis of variance followed by Tukey's *post hoc* test to assess statistical significance at $P < 0.05$.

Results

Characterization of three-dimensional printed PTM composite scaffolds

Macroscopic image and scanning electron microscopy (SEM) images of 3D-printed PTM composite scaffolds are shown in **Figure 1**. The uniform porous structure of the scaffold is shown in a macroscopic digital photograph (**Figure 1A**). SEM images showed that the pore size of well-interconnected macropores in PT10M scaffolds were around 378.85 ± 36.89 μm, and micropores ranging from 5 to 50 μm were also distributed on the surface of the scaffold. Scanning electron microscopy observation of the composite scaffolds revealed more detail of the porous structure (**Figure 1B** and **C**). Mechanical tests revealed that the compression modulus of the PT10M composite scaffolds was 73.50 ± 27.52 MPa and the compressive strength of PT10M composite scaffolds was 3.23 ± 0.31 MPa. The experimental results proved that the PTM composite porous scaffold prepared by low-temperature rapid prototyping 3D-printing technology has good connectivity of porous structure, mechanical strength suitable for bone reconstruction, and mechanical properties similar to those of natural cancellous bone.[12, 19, 20]

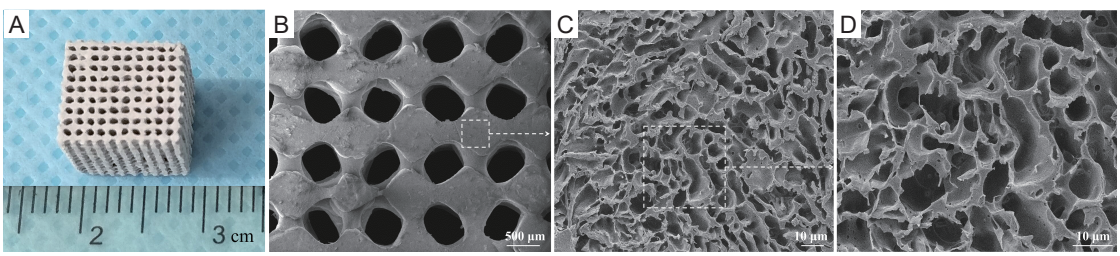


Figure 1. Macrograph image and scanning electron microscopy micrographs of 3D-printed PLGA/β-TCP/Mg composite porous scaffolds. (A) Macroscopic image of a fabricated scaffold. (B–D) Transverse section at a magnification of PT10M scaffold by SEM (original magnifications 30×, 500×, and 1000×). Scale bars: 500 μm in B, 10 μm in C, and D. 3D: three-dimensional; Mg: magnesium; PT10M: PLGA/β-TCP/10 wt% Mg porous composite scaffolds; PLGA: poly(lactic-co-glycolic acid); β-TCP: beta-tricalcium phosphate.

In vitro degradation studies of the scaffolds

To test their *in vitro* degradation, the PTM scaffolds were immersed in different solutions at 37°C for up to 72 hours. The pH value of the three solutions used to soak the PT15M scaffolds gradually increased with immersion time (**Figure 2A**). Meanwhile, the Mg ion concentrations of the different solutions also increased significantly with increased immersion time (**Figure 2B**), and a very low concentration of Ca ions was released over the 72 hours of extraction (the baseline concentration of Ca ions in MEM was deducted). Moreover, after 72 hours immersion, the Ca ions released by different PTM scaffolds in MEM remained at similar concentrations (**Figure 2C**). From the result in **Figure 2D**, we found that after 72 hours immersed in the MEM, the average concentration of Mg^{2+} in the MEM was increased from 50.20 ± 9.35 mM (PT5M scaffold) to 261.26 ± 5.40 mM (PT15M scaffold). When magnesium ions are released into solution, they could react with water molecules to form magnesium hydroxide and hydrogen gas, which would increase the pH value. Therefore, pH value in MEM was approximately linear increased from 7.07 ± 0.09 to 10.73 ± 0.21 with the increase of Mg content in scaffolds. In general, the Mg ions accumulations and pH value of the extraction were increased with the elevation of the Mg content of the scaffolds.

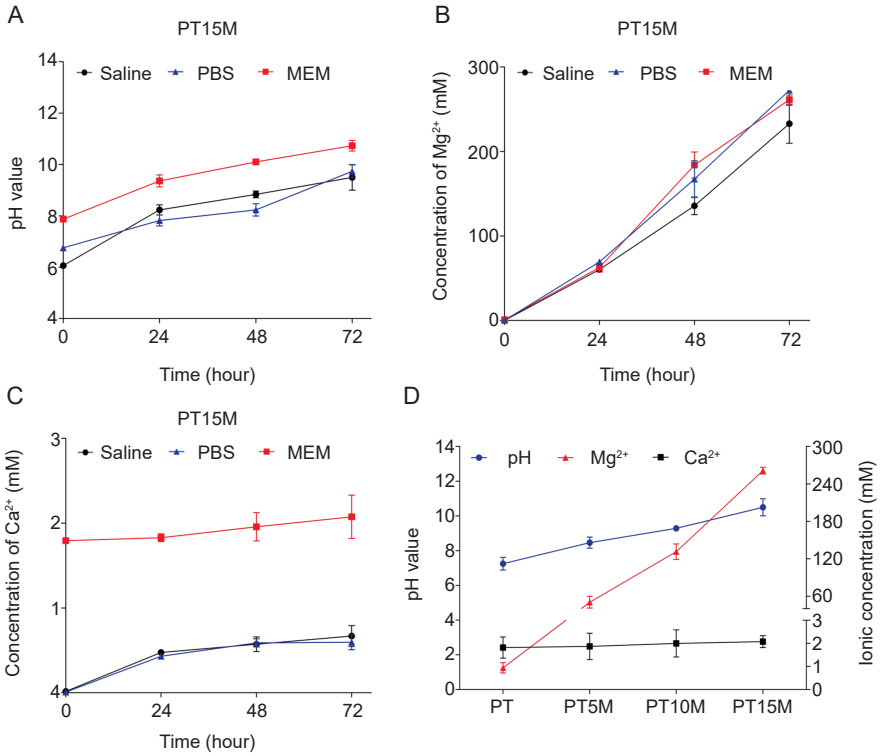


Figure 2. *In vitro* immersion degradation behaviour of scaffolds. (A) Changes in the pH value of different solutions used to soak the PT15M scaffold at 37°C for 24, 48, and 72 hours. (B , C) The concentrations of Mg ions (B) and Ca ions (C) released into serum-free MEM after incubation with PT15M at 37°C for 24, 48, and 72 hours. In the test results, MEM has a higher baseline as it contains calcium chloride. (D) The pH value, Mg, and Ca ion concentrations of MEM incubated with PT, PT5M, PT10M, and PT15M scaffolds at 37°C for 72 hours. Data are expressed as the mean ± SD. (n = 3 and experiments were repeated by twice). Ca: calcium; MEM: serum-free minimum essential medium; Mg: magnesium; PT: poly(lactic-co-glycolic acid)/beta-tricalcium phosphate porous composite scaffolds; PT5M: poly(lactic-co-glycolic acid)/beta-tricalcium phosphate/5 wt% Mg porous composite scaffolds; PT10M: poly(lactic-co-glycolic acid)/beta-tricalcium phosphate/10 wt% Mg porous composite scaffolds; PT15M: poly(lactic-co-glycolic acid)/beta-tricalcium phosphate/15 wt% Mg porous composite scaffolds.

In vivo acute systemic toxicity of Mg composite scaffolds

Different extraction solutions affect in vivo acute systemic toxicity

The above degradation study showed that the PT15M scaffold generated the highest Mg ion concentration after extraction for 72 hours. To further address the actual correlation of the *in vitro* observations to an *in vivo* situation, we used the extract solutions of the PT15M scaffold to challenge mice and evaluate whether the mice could tolerate the extract solutions with the highest Mg concentration. To verify whether different extraction media vary in their *in vivo* acute systemic toxicity, three tissue culture media (normal saline, PBS, and serum-free MEM) were selected to extract the PT15M scaffold at 37°C for 72 hours. All three extraction solutions of PT15M resulted in the death of all the mice after I.P. injection and all mice were survived in the control group (serum-free MEM, **Figure 3A**). For those mice in three PT15M extracts treated groups, the serum concentration of Mg ions was dramatically raised and far above the safety level of toxicity of serum Mg (5.4 mM,[21, 22] $P < 0.001$, *vs.* control group), while the serum calcium levels were near its safety level (3.4 mM,[23] $P < 0.05$, **Figure 3B**). H&E staining revealed that previously dense cardiac tissue was loosened by Mg ions (black arrows), which may suggest that the death of the mice might have been caused by myocardial weakness and paralysis due to hypermagnesemia. The Mg indicator staining results of the heart, liver, and lung of mice showed that Mg ions were enriched in these tissues after injection of all three of the extract liquids (white arrows, **Figure 3C**). In addition, the mass ratio of Mg to the organs was larger than that of the control group (**Figure 3D**). From those results, it showed that different extraction media had no significant effect on the *in vivo* acute systemic toxicity. Since the medium is similar to human body fluids, using serum-free MEM extraction can better simulate the actual Mg release behaviour in human body.

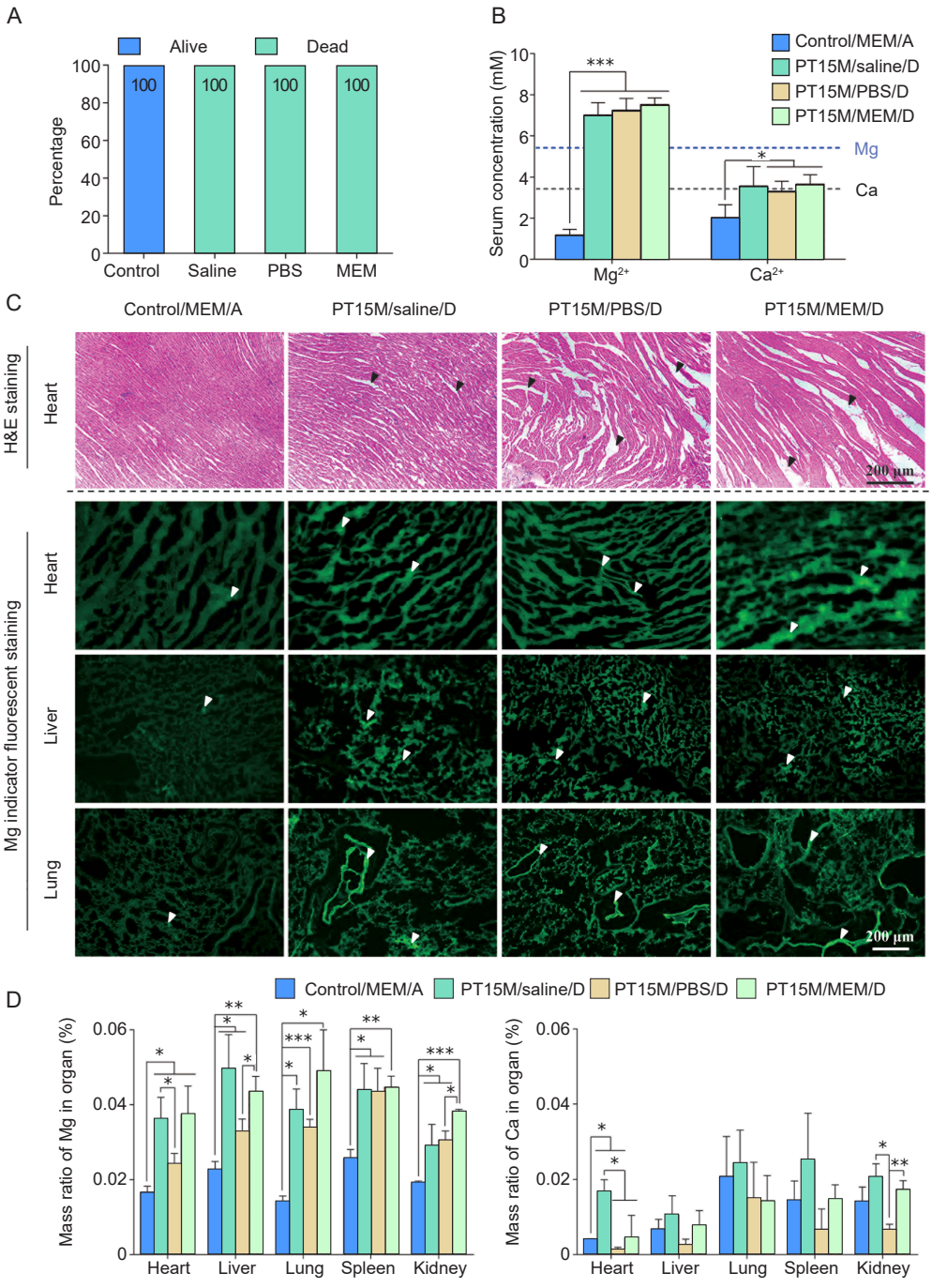


Figure 3. *In vivo* acute toxicity results in mice after intraperitoneal injection of different extract solutions. Each extract solution was prepared by soaking the PT15M scaffold in the solution at 37°C for 72 hours. (A) The percentage of live and dead animals. For those mice that survived or dead, corresponding to the extract medium they were injected, we grouped them into four groups which were control/MEM/alive (Control), PT15M/saline/dead, PT15M/PBS/dead, and PT15M/MEM/dead groups. (B) Mg and Ca ion concentrations of serum in mice, 1 hour after intraperitoneal injection of extract solution. (C) H&E staining of heart, and Mg indicator fluorescent staining (green) of heart, liver, and lung. Black arrows indicate loosened cardiac tissues, and white arrows indicate enrichment sites of Mg ions in tissues. Scale bars: 200 μm. (D) The mass ratios of Mg and Ca in different organs. The control group was injected with MEM that was not incubated with any scaffolds. Data are expressed as the mean ± SD (n = 10, 5 male and 5 female). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (one-way analysis of variance followed by Tukey's *post hoc* test). "A" stands for alive mice, and "D" means dead mice. Ca: calcium; H&E: haematoxylin and eosin; MEM: serum-free minimum essential medium; Mg: magnesium; PBS: phosphate-buffered saline; PT15M: poly(lactic-co-glycolic acid)/beta-tricalcium phosphate/15 wt% Mg porous composite scaffolds; Saline: normal saline.

Different Mg contents of the scaffolds affect in vivo acute systemic toxicity

According to GB/T 16886.11-2011,[13] MEM has a better buffer effect compared with saline or PBS. MEM was therefore selected as the extraction solution to conduct further experiments. We then injected the extraction liquid from scaffolds with different Mg content into mice, using the same extract solution (MEM) to further evaluate the *in vivo* acute toxicity. PT, PT5M, PT10M, and PT15M were soaked in MEM at 37°C for 72 hours. As in the previous experiment, the mice treated with PT15M extract were all dead after I.P. injection. However, the mice injected with extract from PT, PT5M, and PT10M scaffolds all survived (**Figure 4A**). The weight change of all the surviving animals at 72 hours after I.P. injection was less than 10%, which was regarded as normal weight variation (**Figure 4B**). Unlike the PT15M group, serum Mg ion concentrations in mice after I.P. injection of extract MEM incubated with PT, PT5M, or PT10M were within safe limits (**Figure 4C**). H&E staining (**Figure 4D**) showed that the myocardium of the PT15M group had a wavy appearance (black arrows), while the corresponding phenomenon was not found in any other groups. In addition, the space between the myocardium was dilated with increasing Mg concentration in the scaffold. The enlarged green areas shown by Mg indicator fluorescent staining corresponded with the gradient of Mg content in the scaffolds (**Figure 4D**, white arrows). With the increase of injection Mg concentration, Mg gradually accumulated in different organs, especially in the heart, liver, and lung. The mass ratio of Mg in the liver, lung, spleen, and kidney for the PT15M group was higher than the PT group. The PT10M group presented a higher mass ratio of Mg in the spleen and kidney compared to the PT group, while the ratio in the heart, liver, and lung showed no difference compared with the PT group. The Mg mass ratio in PT and PT5M groups remained at the basal level. The mass ratio of Ca in the organs evaluated showed that the Ca content in the heart was lower in the PT15M group compared with the PT group. The above data implied that the high concentration of Mg in the scaffold is responsible for the death of the mice. In addition, all mice survived after I.P. injection with extract solution incubated with 5% or 10% Mg content composite scaffolds, which indicated that PT5M and PT10M are non-acute-toxic and safe in Kunming mice.

In vivo pH value affects acute systemic toxicity

Since the pH value of the extract solution was alkaline after 72 hours of incubating at 37°C with the PT15M scaffold, it was necessary to consider the pH value as another possible factor responsible for the deaths of the animals. The animal death rate, weight loss of surviving animals, and serum Mg concentrations of the mice were investigated by injecting the extraction medium and scaffold extract into mice after adjusting the pH value to assess the effect of pH on acute systemic toxicity. After the PT15M was soaked in MEM for 72 hours at 37°C, the extract solutions were adjusted to two different pH values (7.0 and 10.0). All mice survived after injection with control MEM of different pH values. The death rate for I.P. injection of MEM incubated with PT15M with pH values of 7.0 and 10.0 was 67% and 100%, respectively. Among all the surviving mice, only the mice injected with MEM with a pH value of 10.0 showed a weight change of 11.9% (**Figure 5A** and **B**). As shown in **Figure 5C**, the Mg ion concentration in serum after injection with PT15M extract with a pH value of 10.0 was higher than the MEM with pH of 7.0 ($P < 0.001$), and the Ca ion concentration in serum of MEM/pH7/alive, MEM/pH10/alive, PT15M/pH7/alive groups were below the safety level of toxicity of 3.4 mM. H&E staining showed that the wavy appearance of the myocardium was only apparent in PT15M extract-treated dead mice, while the myocardium in the MEM, PT5M/alive, PT10M/alive, and PT15M/pH7/alive groups remained normal, indicating that the pH value of the solution had a certain effect on the animals, but was not the main cause of death. There were no abnormalities in the liver, lung, spleen, or kidney in any groups (**Additional Figure 1**). The heart, liver, and lung in PT15M extract-treated dead mice exhibited an obvious increase in Mg indicator-stained areas compared with other surviving mice (white arrows, **Figure 5D**). Analysis of the mass ratio of Mg in organs revealed that dead mice of the PT15M group had a higher Mg content in all investigated organs in comparison to the MEM group, with the exception of surviving mice of the PT15M group and the MEM/pH10 group in which the Mg content of the heart showed no difference to the MEM group. All other surviving groups showed no difference in Mg content compared with the MEM group. Analysis of Ca content showed that the PT15M/pH7/D group had a higher mass ratio in the heart and liver ($P < 0.05$, **Figure 5E**). When the pH value of the extract MEM incubated with PT15M for 72 hours was reduced to 7.0, the survival of mice increased to 33%. Those results implied that the pH value of the solution had a certain effect on animals, while it should not be the main cause of mice death.

In vivo median lethal dose study

To verify the median lethal dose of Mg ions, different ratios of diluted PT15M scaffold-extracted MEM were intraperitoneally-injected into mice after soaking the PT15M scaffolds at 37°C for 72 hours. We first identified the LD_0 of extract solution as 79.08 mg/kg, while the minimum dose of LD_{100} was 164.75 mg/kg (Mg content of extract liquid versus mice body weight). Therefore, five concentrations (mg/kg) of extract solution were set based on the Spearman-Karber statistical method, described in materials and methods,[18] which gave doses of 0, 79, 99, 119, 139, and 165 mg/kg. All mice survived 72 hours after injection with low doses of extract MEM solutions (0 and 79 mg/kg). As the Mg content in the extract solution increased from 99 mg/kg to 165 mg/kg, the death rate also increased from 30% to 100% (**Figure 6A**). Meanwhile, the weight fluctuation of the surviving mice remained less than 10% at 72 hours after I.P. injection (**Figure 6B**). The concentration of Mg ions in the serum of all dead mice was above the safety level of toxicity of serum Mg (5.4 mM), while blood Ca ion levels in all alive mice were within safe limits (**Figure 6C**). Clinical observations are shown in **Table 1**. After I.P. injection, the extract solution entered the circulatory system, and then Mg ions accumulated in the organs. Measurement of Mg ion concentration in tissues

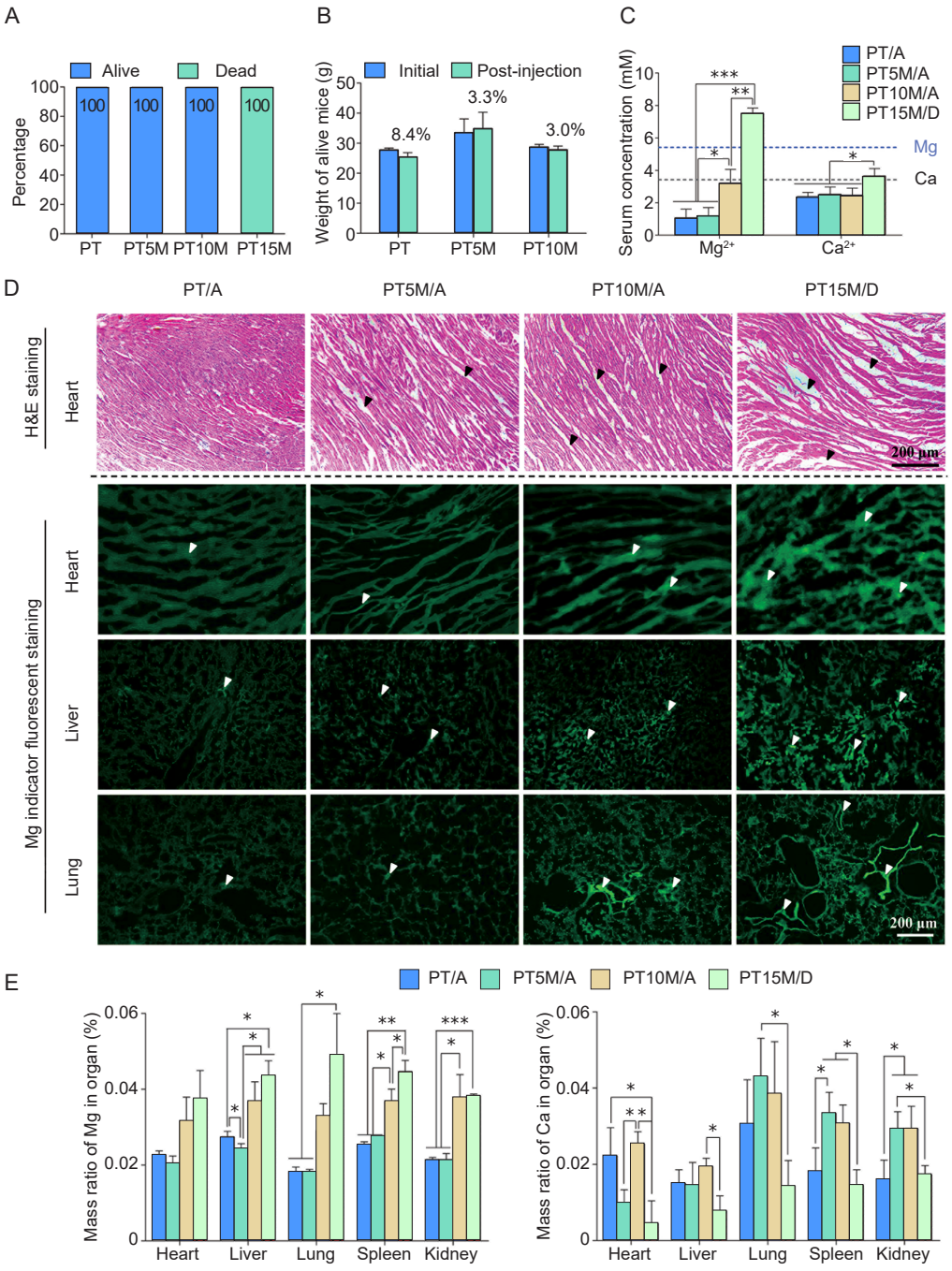


Figure 4. *In vivo* acute toxicity results of mice after intraperitoneal injection of different Mg content scaffold extract. Extract MEM solutions were prepared by soaking PT, PT5M, PT10M, and PT15M samples in MEM at 37°C for 72 hours. (A) The percentage of live and dead mice 1 hour after intraperitoneal injection of extract MEM. For those mice that survived or dead, corresponding to the scaffold extract they were injected, we grouped them into four groups which were PT/alive (PT/A), PT5M/alive (PT5M/A), PT10M/alive (PT5M/A), and PT15M/dead (PT5M/D) groups. (B) The weight change of live mice 72 hours after intraperitoneal injection of extract MEM. (C) Mg and Ca ion concentrations in serum 1 hour after intraperitoneal injection of extract MEM. Scale bars: 200 μm. (D) H&E staining of heart, and Mg indicator fluorescent staining of heart, liver, and lung. Black arrows indicate loosened cardiac tissues, and white arrows indicate enrichment sites of Mg ions in tissues. (E) The mass ratios of Mg and Ca in different organs at 1 hour after intraperitoneal injection of extract MEM. Data are expressed as the mean ± SD (n = 10–12, 5–6 male and 5–6 female). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (one-way analysis of variance followed by Tukey's *post hoc* test). "A" stands for alive mice, and "D" means dead mice. Ca: calcium; H&E: haematoxylin and eosin; MEM: serum-free minimum essential medium; Mg: magnesium; PT: poly(lactic-co-glycolic acid)/beta-tricalcium phosphate porous composite scaffolds; PT5M: poly(lactic-co-glycolic acid)/beta-tricalcium phosphate/5 wt% Mg porous composite scaffolds; PT10M: poly(lactic-co-glycolic acid)/beta-tricalcium phosphate/10 wt% Mg porous composite scaffolds; PT15M: poly(lactic-co-glycolic acid)/beta-tricalcium phosphate/15 wt% Mg porous composite scaffolds.

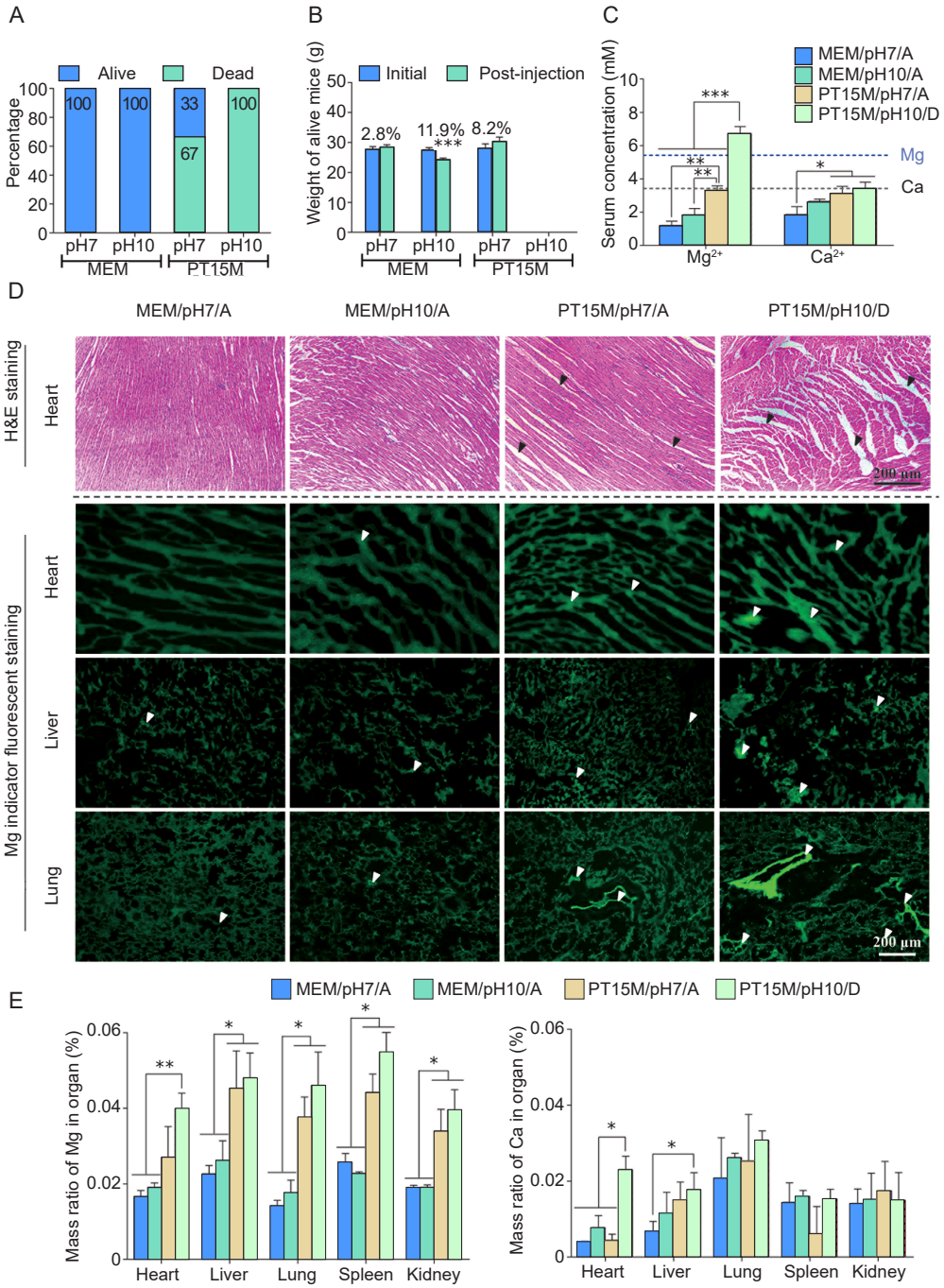


Figure 5. *In vivo* acute toxicity results in mice after intraperitoneal injection of extract MEM with different pH values (7.0 and 10.0). Extract MEM was prepared by soaking PT15M at 37°C for 72 hours. For those mice that survived or dead, corresponding to the different pH values of scaffold extract they were injected, we grouped them into four groups which were MEM/pH7/alive (MEM/pH7/A), MEM/pH10/alive (MEM/pH10/A), PT15M/pH7/alive (PT15M/pH7/A), and PT15M/pH7/dead (PT15M/pH7/D) groups. (A) The percentage of live and dead animals at 1 hour after intraperitoneal injection of extract MEM. (B) The weight change of live mice at 72 hours after intraperitoneal injection of extract MEM. (C) Mg and Ca ion concentrations of serum in mice 1 hour after intraperitoneal injection of extract MEM. (D) H&E staining of heart, and Mg indicator fluorescent staining of heart, liver, and lung. Black arrows indicate loosened cardiac tissues, and white arrows indicate enrichment sites of Mg ions in tissues. Scale bars: 200 μm. (E) The mass ratios of Mg and Ca in different organs at 1 hour after intraperitoneal injection with extract MEM. Data are expressed as the mean ± SD (n = 10, 5 male and 5 female). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (one-way analysis of variance followed by Tukey's *post hoc* test). "A" stands for alive mice, and "D" means dead mice. Ca: calcium; H&E: haematoxylin and eosin; MEM: serum-free minimum essential medium; Mg: magnesium; PT15M: poly(lactic-co-glycolic acid)/beta-tricalcium phosphate/15 wt% Mg porous composite scaffolds.

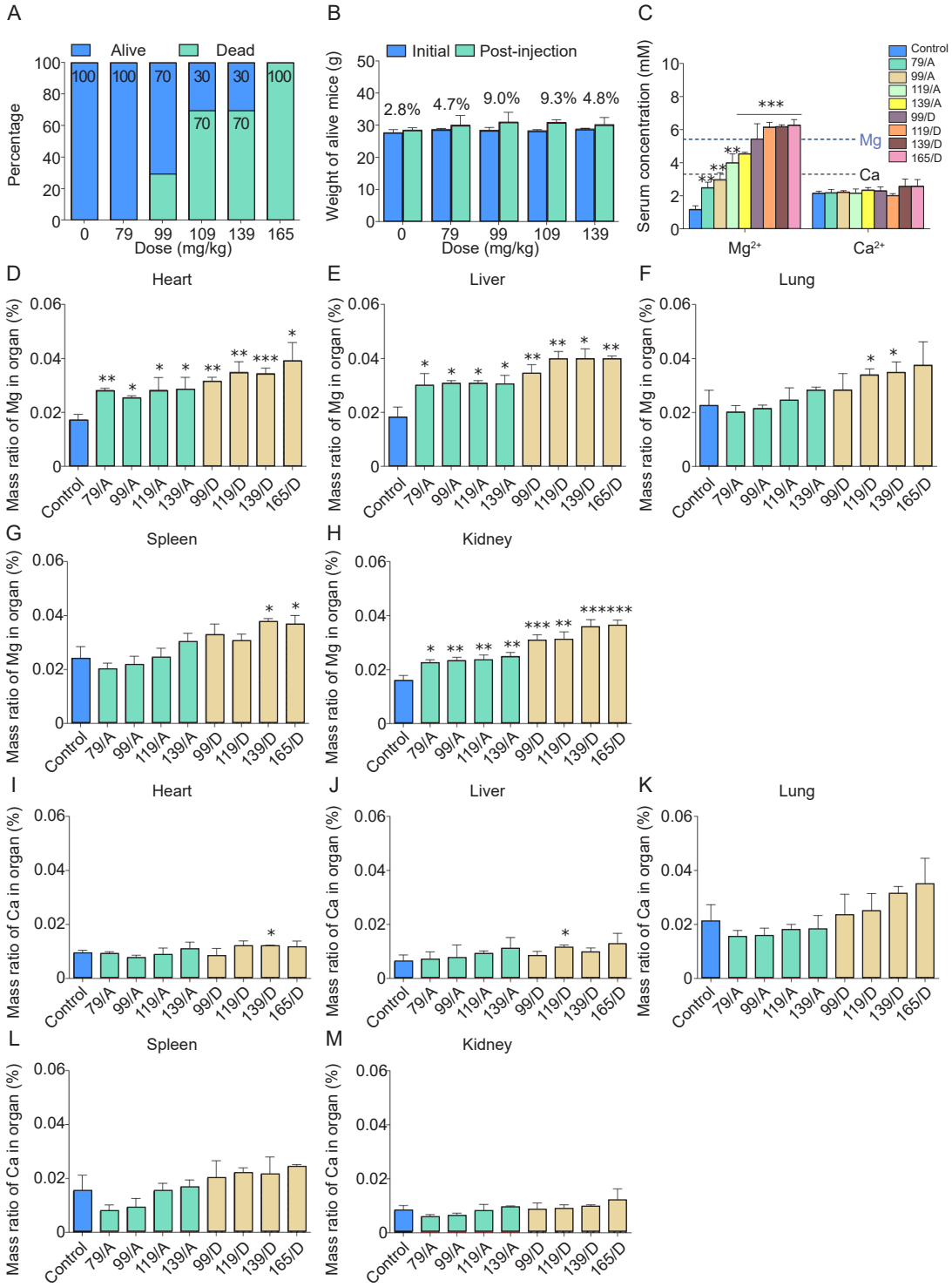


Figure 6. *In vivo* median lethal dosing results of mice after intraperitoneal injection of different concentrations of diluted extract MEM. The extract MEM was prepared by soaking the PT15M scaffold in MEM at 37 °C for 72 hours. (A) The percentage of live and dead animals. For those mice that survived or dead, corresponding to the different magnesium concentrations of MEM solutions they were injected, we grouped them into nine groups which were control group (serum-free MEM), 79 mg/kg Mg/A, 99 mg/kg Mg/A, 119 mg/kg Mg/A, 119 mg/kg Mg/D, 139 mg/kg Mg/D, and 165 mg/kg Mg/D groups. (B) The weight of the live animals. The *x*-axis indicates Mg content in extract solution *versus* the weight of the mice. (C) Mg and Ca ion concentrations in the serum of mice at 1 hour after intraperitoneal injection. (D–H) The mass ratios of Mg in different organs. (I–M) The mass ratios of Ca in different organs. The control group was injected with MEM with a pH value of 7.0. Data are expressed as the mean ± SD (n = 10, 5 male and 5 female). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (one-way analysis of variance followed by Tukey's *post hoc* test). "A" stands for alive mice, and "D" means dead mice. Ca: calcium; MEM: serum-free minimum essential medium; Mg: magnesium.

and organs from different experimental groups showed that the accumulated Mg ion content in dead mice was higher, and thus had a greater impact on the heart, liver, and kidney (**Figure 6D–H**). The accumulated Ca ion concentration in tissues and organs of the different experimental groups didn't significantly change compared with the control group (**Figure 6I–M**).

All mice were observed for clinical signs and symptoms at 24 and 72 hours after I.P. injection of different concentrations of diluted extract MEM. As shown in **Table 1**, the Kunming mice in the 99 mg/kg and 119 mg/kg groups had prostration symptoms after 24 hours, while the mice exhibited only diuresis after 72 hours in those two groups. At concentrations of extract solution above 139 mg/kg, mice presented dyspnoea, prostration, and convulsion at 24 hours after I.P. injection. The mice in the 165 mg/kg group did not survive up to 24 hours, while the mice in the 139 mg/kg group appeared normal but had diuresis symptoms after 72 hours. Accordingly, the *in vivo* LD50 of extract solution from Mg-containing composite scaffolds tested by I.P. injection of mice was calculated as 110.66 mg/kg based on the Spearman-Karber statistical method.

Table 1. The clinical signs and observed symptoms for mice after intraperitoneal injection of different ratio diluted extract MEM.

Dose (mg/kg)	Time	Dyspnea	Prostration	Convulsion	Ptosis	Salivation	Piloerection	Diuresis	Edema	Erythema
0	1 day	–	–	–	–	–	–	–	–	–
	3 day	–	–	–	–	–	–	–	–	–
79	1 day	–	–	–	–	–	–	–	–	–
	3 day	–	–	–	–	–	–	–	–	–
99	1 day	–	+	–	–	–	–	–	–	–
	3 day	–	–	–	–	–	–	+	–	–
119	1 day	–	+	–	–	–	–	–	–	–
	3 day	–	–	–	–	–	–	+	–	–
139	1 day	+	+	+	–	–	–	–	–	–
	3 day	–	–	–	–	–	–	+	–	–
165	1 day	+	+	+	–	–	–	–	–	–
	3 day	NA	NA	NA	NA	NA	NA	NA	NA	NA

Note: Data were based on GB/T 16886.11-2011.13 The original extract MEM was soaked with PT15M for 72 hours. "+" stands for the symptom, "–" means no abnormality detected. The dose (mg/kg) means Mg ions concentrations in diluted extract solution versus the weight of mice. MEM: serum-free minimum essential medium; Mg: magnesium; NA: not available; PT15M: poly(lactic-co-glycolic acid)/beta-tricalcium phosphate/15 wt% Mg porous composite scaffolds.

Discussion

The biomaterials community has shown great interest in the research and development of biodegradable polymers incorporating biodegradable Mg composite scaffolds for bone regeneration. As summarized in the introduction, numerous studies on Mg composite materials systems have been carried out, and many promising results showing improvements in mechanics and osteogenesis have been reported.[9, 12, 19] Based on these promising reports, it is essential and urgent to push forward this materials system to clinical use, a process which is meaningful for both the scientific community and patients with musculoskeletal disorders.[24] However, there is still a gap between scientific research and clinical use for Mg composite scaffolds, and very limited studies have been devoted to this aspect.

This study was intended to provide a comprehensive demonstration of ISO-10993 or GB/T 16886[13, 14] based acute systemic toxicity evaluation for PTM composite scaffolds, which aimed to bridge the gap between scientific research and clinical use and to push for this type of scaffold to be granted approval by the National Medical Products Administration of China. Therefore, all the assessments in this study were implemented in accordance with the GB/T 16886.11-2011 standard.[13] In these composite scaffolds, PLGA as the biodegradable material has already been approved by the U.S. Food and Drug Administration, which means that it can be safely used as artificial implants. Meanwhile, β-TCP, one of the calcium phosphates, is already widely used in orthopaedic surgery helping to facilitate bone reconstruction due to its bio-resorbability and osteoconductive properties.[25, 26] The degradation behaviour of calcium phosphates is dependent on their textural parameters,[27] such as composition, specific surface area, porosity, extract surroundings,[28] and implantation site. During the degradation, Ca ions would be released from the β-TCP. In PTM composite scaffolds, Ca ions were released at a slow rate regardless of the β-TCP content of the scaffold system during the *in vitro* degradation process. For that, we guessed that it was the insolubility of apatite in aqueous solutions that the release rate of Ca ions integrated in β-TCP was much lower than that of Mg compounds with better water solubility.[12, 27] Also, when implanted *in vivo*, the level of Ca ions in serum remained within safe limits throughout (as shown in **Figures 3–5**). Hence, the component in this composite scaffold that may be responsible for acute systemic toxicity is Mg metal. Mg is degraded easily and quickly in the physiological environment, when Mg ions are released into solution, they could react with water molecules to form magnesium hydroxide and hydrogen gas, the degradation of Mg follows the aquation: $Mg + 2H_2O \rightarrow Mg(OH)_2 + H_2$. It would lead to the fast accumulation of Mg ions and rapidly increased pH at the implant site.[29-31] This may cause side effects on the surrounding tissues or even lead to the death of animals.[21] Therefore, the primary purpose of this research was to illustrate the acute systemic toxicity in mice of Mg in composite scaffolds, and the *in vivo* median lethal dosing study was incorporated to investigate the acute systemic

toxicity of Mg composite scaffolds.

From the *in vitro* degradation result shown in **Figure 2B**, the similar Mg ion release profile for PT15M scaffolds immersed in different solutions evidenced that these solutions have a similar corrosive effect to Mg particles in scaffolds, and this might be attributable to the similarity of the chloride ions concentrations in these solutions.[32] The different pH variation profiles for PT15M scaffolds in the three extract solutions may be attributed to the buffering effect of the MEM and PBS, and the lack of this effect in normal saline.[6]

After I.P. injection, the extract solution entered the circulatory system and then accumulated in the organs. Therefore the instantaneous ionic concentration in serum, and mass ratio of Mg and Ca in organs are the important indicators to determine acute systemic toxicity of extract solution in live or dead animals.[31] After I.P. injection with different solutions incubated with PT15M, all the mice were dead, which suggested that the factors responsible for the deaths of the mice may be the high concentration of Mg ions (more than 240 mM) and increased pH value of the extract solution (**Figures 2** and **3**). When the pH of the MEM extract solution (incubated with PT15M) was reduced to 7.0, 33% of the mice survived (**Figure 5A**), which indicates that pH value is also a factor leading to the death of the mice. Further, all mice survived (**Figure 4**) after injection with MEM extract solution incubated with scaffolds with lower Mg contents (PT5M, PT10M). All these results confirmed that the death of the mice was due to the high concentration of Mg ions and increased pH, with the high concentration of Mg ions being the major reason, and pH value is a minor factor. Based on the results of acute systemic toxicity and median lethal dose study *in vivo*, we suggested that the safety level of serum Mg toxicity in mice is 5.4 mM, while the calcium serum safety level is determined as 3.4 mM. In addition, the mass ratio of Mg in all the investigated organs of dead mice was significantly higher than in the control group. For all the surviving mice, some organs showed no significant difference in the mass ratio of Mg in comparison with the control group (**Figures 4E** and **5E**). More important, compared with human physiological level,[33, 34] the mass ratios of Mg and Ca in different mouse organs were relatively low (basically less than 0.06%), which indicates the clinical application of scaffold in human may not have the acute toxicity effect.

As reported above, though the pH value of extract solutions was less critical in leading to the death of the mice, it should also be taken into consideration when determining the safe concentration of Mg ions in mouse serum. In addition, since the primary purpose of this research was to evaluate the *in vivo* acute systemic toxicity of our previously-developed scaffolds, we selected the limit interval points for Mg ion concentration and pH value of the extract solutions. Our further work in this area will certainly concentrate on and address these two aspects. Taken together, the results of this research will be valuable in that we have demonstrated preclinical evaluation of *in vivo* acute systemic toxicity of our bone scaffold, which indicated that PT5M and PT10M are non-acute-toxic and safe in Kunming mice.

In conclusion, preclinical evaluation of the acute systemic toxicity of our 3D-printed PLGA/TCP/Mg composite scaffolds was first comprehensively evaluated by I.P. injection of extract solution into mice. As expected, the pH value and released Mg ion concentration of extract solutions incubated with scaffolds both increased with the increase in the Mg content of the scaffolds or with immersion time. The Mg ion concentration of serum in all these dead mice was higher than in the control group. The *in vivo* LD50 of extract solution incubated with Mg-containing composite scaffolds by I.P. injection into mice was 110.66 mg/kg. Mg ion concentration and pH value of the extract solutions were responsible for the deaths of the mice, with the former being the major factor. This research presents a comprehensive example of acute systemic toxicity for this type of composite scaffold in translational medicine.

Author contributions

Conceptualization: YL, LQ; methodology: JL, WZ, XW; investigation: JL, BT, WZ, LL, YC, ZY; experimental studies: JL, XM; resources: MZ, XW, LQ; data acquisition and analysis and manuscript draft: JL; manuscript review: BT, YC; manuscript editing: BT; supervision, validation, funding acquisition, project administration: YL. All authors approved the final version of the manuscript.

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Conflicts of interest statement

None.

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Additional files

Additional Table 1: Verified the difference between different extract solutions effect on acute systemic toxicity *in vivo*.

Additional Table 2: Determined Mg effect of *in vivo* acute toxicity based on various Mg content scaffolds.

Additional Table 3: Effect of pH value on *in vivo* acute systemic toxicity.

Additional Figure 1: Haematoxylin and eosin staining of heart, liver, lung, spleen, and kidney 1 hour after intraperitoneal injection of extract MEM.

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