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

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ABSTRACT

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 *in vitro* and *in vivo* Mg ions and the capability of *in vivo* circulation system to dilute local degradation products 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* cytotoxicity test specified in ISO 10993 part 5 for pure Mg developed as potential orthopedic implants based on literature review and our specifically designed *in vitro* and *in vivo* tests presented in the study. Our work may contribute to the progress of biodegradable metals involved translational work.

1. Introduction

Biodegradable magnesium (Mg) based medical implants have attracted increasing attention from researchers and clinicians over the past century [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 [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 [5]. The improvement of corrosion resistance in Mg-based biometals remotivated medical researchers and metallurgists to continue their endeavors to develop new generations of orthopedic implants with

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appreciated 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 vivo* 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 biometals 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 as the concentrations of protein and other biomolecules in blood are much higher than those in the culture medium [21]. However, the removal of all the inorganic constituents in contacting solution could not reflect the *in vivo* environment. 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, Califorlia, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Thermo Scientific, Massachusetts, USA) for 72 h under cell culture conditions (5% CO_2, 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 CrO_3 (200 g/l) and $AgNO_3$ (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 m_0 and treated weight m_t according to the formula: $mass\ loss\ ratio = (m_0 - m_t) \cdot 100\%/m_0$. 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 (5520, 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,