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Cytotoxicity assessment of adipose-derived mesenchymal stem cells on synthesized biodegradable Mg-Zn-Ca alloys

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ABSTRACT

Magnesium (Mg)-based alloys have been extensively considered as biodegradable implant materials for orthopedic surgery. Mg and its alloys are metallic biomaterials that can degrade in the body and promote new bone formation. In this study, the corrosion behavior and cytotoxicity of Mg-Zn-Ca alloys are evaluated with adipose-derived mesenchymal stem cells (ASCs). Mg-2Zn and Mg-2Zn-*x*Ca ($x = 1$, 2 and 3 wt.%) alloys were designated. Mg alloys were analyzed with scanning electron microscopy and potentiodynamic polarization. To understand the in-vitro biocompatibility and cytotoxicity of Mg-2Zn and Mg-2Zn-*x*Ca alloys, ASCs were cultured for 24 and 72 h in contact with 10%, 50% and 100% extraction of all alloys prepared in DMEM. Cell cytotoxicity and viability of ASCs were examined by MTT assay. Alloying elements including Zn and Ca improved the corrosion resistance of alloys were compared with pure Mg. The cytotoxicity results showed that all alloys had no significant adverse effects on cell viability in 24 h. After 72 h, cell viability and proliferation increased in the cells exposed to pure Mg and Mg-2Zn-1Ca extracts. The release of Mg, Zn and Ca ions in culture media had no toxic impacts on ASCs viability and proliferation. Mg-2Zn-1Ca alloy can be suggested as a good candidate to be used in biomedical applications.

1. Introduction

In the last decade, magnesium (Mg) alloys were fabricated as biocompatible and biodegradable metallic biomaterial to heal and repair bone tissue disorders [1–3]. This tangible vision makes Mg alloys more beneficial than non-degradable permanent metallic implants such as titanium, stainless steel and cobalt-chromium alloys due to the reduction of healthcare cost and patient morbidity by eliminating the second surgery to remove implants. An in-vivo study showed that common metallic biomaterials were essentially neutral, remaining as permanent fixtures, which in the case of plates, screws and pins, are used to secure serious fractures [4]. By fabrication of biodegradable Mg alloys, the biomaterialists aim to design an implant that degrades after implantation. This idea means that after curing the damaged bone, biodegradable Mg implants will degrade slowly in physiological conditions without toxicity. Also, in-vitro, in-vivo and early clinical studies suggested that Mg-based implants showed good biocompatibility [5].

Mg is an essential element for bone metabolism and may promote the formation of new bone tissue [6]. Also, Mg^{2+} is the fourth most abundant cation in the human body and is largely stored in the bone tissues. It is a co-factor of many enzymes, and a key component of ribosomal machinery that translates the genetic information encoded by messenger ribonucleic acid (mRNA) into polypeptide structures. Over 300 different enzymes and co-factors require Mg in osteogenesis, nerve functions and muscle relaxation to stabilize the structures of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) [7–9].

The main drawback of Mg in engineering applications is low corrosion resistance, especially in aqueous environments that makes an intriguing property for this material for biomedical applications, where in-vivo corrosion of the Mg-based implant involves the formation of a soluble, non-toxic oxide that is harmlessly excreted in the urine. Moreover, Mg may actually have stimulatory effects on the growth of new bone tissue [10,11]. The unfortunate complications of pure Mg are very quick corrosion in the physiological pH (7.4–7.6) and high chloride environment, lack of mechanical integrity before

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sufficient healing of the bone tissue and production of hydrogen gas in the corrosion process. Thus, Mg alloys can be considered as lightweight, degradable, load bearing orthopedic implants which maintain mechanical integrity during bone tissue repair over a time scale of 12–18 weeks, eventually being replaced by natural tissues [12,13].

One of the most practical techniques to improve the mechanical and corrosion properties of pure Mg is alloying with elements such as aluminum (Al), zinc (Zn), calcium (Ca), tin (Sn), zirconium (Zr), yttrium (Y), silicon (Si) and manganese (Mn) [14–17]. However, Zn and Ca are the preferred alloying elements in medical application [18,19]. Aluminum ions are released from the AZ (Mg-Al-Zn) alloys like AZ91, AZ61 and AZ31 which could easily combine with inorganic phosphates, leading to a lack of phosphate in the human body and an increased concentration of Al ions in the brain which enhances Alzheimer's disease [20–22]. In WE (Mg-Y-rare-earth-Zr) alloys, extensive toxicity has been detected after the administration of rare earth (RE) elements such as cerium, praseodymium and yttrium [23–26]. Meanwhile, no medical adverse effects are reported with Zn and Ca; these elements are beneficial for human health. Zn is an essential element [27] involved in numerous aspects of cellular metabolism. It is required for the catalytic activity of approximately 100 enzymes [28], protein synthesis, DNA synthesis and cell division [29]. Zn has a stimulatory effect on bone formation and mineralization in vivo and in vitro [30]. Also, many enzymes require Ca ions as a co-factor, those of the blood-clotting cascade being notable examples. Approximately, 99% of the body's Ca is stored in the bones and teeth [31]. Extracellular Ca is also important for maintaining the potential difference across excitable cell membranes, as well as proper bone formation [32,33].

In the current study, synthesized Mg-2 wt.% Zn and Mg-2 wt.% Zn-*x* wt.% Ca ($x = 1, 2$ and 3) alloys system is considered as the base for the development of a biomaterial alloy, in which Zn and Ca were used as an alloying elements for biocompatibility study with adipose-derived mesenchymal stem cells (ASCs). Adipose-derived mesenchymal stem cell (MSC) can be considered as a pluripotent cell with the ability to differentiate into osteoblast, the main cell in the bone tissue. During the bone repair, MSCs are involved in bone formation by differentiating into bone cells. Therefore, the biocompatibility, attachment properties of alloys and induction of cell proliferation are critical criteria to induce MSC differentiation, regeneration and remodeling of the bone tissue.

2. Materials and methods

2.1. Mg alloys preparation

The melting process to produce homogenous molten alloys was performed using an induction furnace in a graphite crucible. The protecting gas consisted of SF_6: $CO_2 = 1$: 10 which was blown into the crucible at a flow rate of 5 mL/s to prevent the ignition, combustion of the melt and prevent the oxidation during the melting. Commercially pure 99.95% Mg ingot (Gallium Source, LLC, USA), calcium grains (99.5%, HongRui, China) and zinc rod (99.9%, RotoMetals, USA) were used to prepare Mg-Zn and Mg-Zn-Ca alloys. The melting point of Mg is 650 °C; hence, the super-melting point temperature was fixed at 750 ± 5 °C for each alloy, and the Mg alloys melt was cast into a stainless steel 430 mold. In order to cast Mg-2Zn, Mg-2Zn-1Ca, Mg-2Zn-2Ca and Mg-2Zn-3Ca (all in wt.%), the molten metal was poured in preheated permanent molds. After performing the casting process, to obtain a homogenized structure for Mg-2Zn alloy, the homogenization temperature and time were chosen at 320 °C and 48 h, whereas for aging process, temperature and time were 200 °C and 10 h. In Mg-Zn-Ca alloys, for the homogenizing procedure, the samples were heated isothermally up to 300 °C for 48 h and subsequently increased to 450 °C for 96 h and then quenched immediately in cold water (4 °C). Finally, the samples were aged isothermally for 12 h at 250 °C [34].

2.2. Mg alloys characterization

Field-emission Scanning Electron Microscopy (FE-SEM) was performed using TESCAN MIRA3-XMU to investigate (a) microstructural features and (b) elemental distribution in homogenized alloys. Images were collected in the back scatter electron imaging mode with 15 kV working voltage and 10–12 mm working distance. The composition analysis is performed with an energy dispersive spectroscopy (EDS) with RÖNTEC GmbH, Germany X-ray setup. According to ASM handbook instruction, to reveal intermetallic phases in Mg alloys microstructure, acetic picral with the following composition was used as etchant of Mg-Zn-Ca alloy: 5 mL acetic acid, 6 g picric acid, 10 mL H2O and 100 mL ethanol (95%) [35].

The corrosion behavior of Mg alloys was studied by potentiodynamic polarization (PDP) examinations in Dulbecco's Modified Eagle Medium ((DMEM-Low Glucose), Biowest Inc., France). Electrolyte for electrochemical analysis was set up according to simulated human body conditions at 37 °C in water bath and pH 7.2–7.4. The electrochemical measurements were carried out by using PARSTAT® potentiostat (Princeton Applied Research, USA) system and conventional three-electrode cell with the examination sample as working electrode, saturated calomel electrode (SCE-Ag/AgCl) as the reference electrode and platinum auxiliary electrode as the counter. Polarization Tafel measurements were performed at a potential range of −1 V to −2 V at a scanning rate of 1 mV/s in DMEM medium.

2.3. Mg alloys extracts

Firstly, Mg alloys disks were sterilized in contact with ultraviolet irradiation and subsequently with 70% ethanol. According to ISO 10993-12 [36], the proportion of the alloy's surface area and volume of leaching solution, 1 cm^2/DMEM medium containing 10% FBS, was added to each well of a 6 well plate. After 72 h of incubation at 37 °C and 5% CO_2, the media containing Mg alloy extracts were collected and centrifuged at 4000 rpm to eliminate macro-particles. After obtaining a homogenous solution, the extracts were diluted at concentrations of 10%, 50% and un-diluted extract as well (100%).

The osmolarity of the Mg alloys extracts was also checked by Gonotec Osmomat 030 cryoscopic osmometer (Gonotec, Berlin, Germany). Distilled water and standard solution with 300 mOsmol/kg were used to calibrate the osmometer.

2.4. Adipose-derived stem cells (ASCs) isolation and characterization

2.4.1. Isolation of ASCs

Adipose tissues were isolated from the breast of normal individuals undergoing mammoplasty surgery with informed consents according to the University ethics committee guidelines. The tissues were washed with phosphate buffered saline (PBS), minced in small pieces and digested with 0.2% collagenase type I in Hank's balanced salt solution (HBSS) at 37 °C on a shaker for 2 h. The resulting soup was centrifuged at 2000 rpm for 10 min. The pellet was incubated for 10 min in red blood cells lysis buffer and centrifuged for 10 min at 1300 rpm. The pellet including the adherent stromal cells was carefully put on Ficoll and centrifuged at 2000 rpm for 30 min. The second layer, stromal vascular fraction, was re-suspended in DMEM culture medium (GIBCO, Germany) containing 10% fetal bovine serum (FBS, GIBCO, Germany) and 1% penicillin/streptomycin (Biosera, UK). Non-adherent cells were discarded after 24 h of culturing. The adherent cells were cultured by changing the medium every 4 days and harvested at the third passage.

2.4.2. Characterization of ASCs by flow cytometry

ASCs were harvested and 5×10^6 cell/mL washed twice with PBS and stained with phycoerythrin (PE) conjugated mouse anti-human, CD29, CD44, CD10 and CD166 (BD Biosciences, USA), fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD14, CD34, CD105 and