Published in final edited form as:

J Biomim Biomater Tissue Eng. 2013 ; 17: 25–44. doi:10.4028/www.scientific.net/JBBTE.17.25.

Biocompatibility Assessment of Novel Bioresorbable Alloys Mg-Zn-Se and Mg-Zn-Cu for Endovascular Applications: *In-Vitro* Studies

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Abstract

Previous studies have shown that using biodegradable magnesium alloys such as Mg-Zn and Mg-Zn-Al possess the appropriate mechanical properties and biocompatibility to serve in a multitude of biological applications ranging from endovascular to orthopedic and fixation devices. The objective of this study was to evaluate the biocompatibility of novel as-cast magnesium alloys Mg-1Zn-1Cu wt.% and Mg-1Zn-1Se wt.% as potential implantable biomedical materials, and compare their biologically effective properties to a binary Mg-Zn alloy. The cytotoxicity of these experimental alloys was evaluated using a tetrazolium based- MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay and a lactate dehydrogenase membrane integrity assay (LDH). The MTS assay was performed on extract solutions obtained from a 30-day period of alloy immersion and agitation in simulated body fluid to evaluate the major degradation products eluted from the alloy materials. Human foreskin fibroblast cell growth on the experimental magnesium alloys was evaluated for a 72 hour period, and cell death was quantified by measuring lactate dehydrogenase concentrations. Both Mg-Zn-Se and Mg-Zn-Cu alloys exhibit low cytotoxicity levels which are suitable for biomaterial applications. The Mg-Zn-Cu alloy was found to completely degrade within 72 hours, resulting in lower human foreskin fibroblast cell viability. The Mg-Zn-Se alloy was shown to be less cytotoxic than both the Mg-Zn-Cu and Mg-Zn alloys.

Keywords

Biodegradable alloys; Magnesium; Biocompatibility; Cytotoxicity; Cell Adhesion

1. Introduction

One of the attractive features of biodegradable materials is their ability to serve as a temporary scaffold for biological tissue growth and degrade thereafter [1]. This degradable feature is highly desired because it minimizes the need for repeated corrective post-surgical procedures which increases the likelihood of patient complications. Currently, several metallic and polymeric materials possess a degradative quality suitable for a biodegradable device. For example magnesium alloys Mg-Zn, Mg-Zn-Al, Mg-Zn-Zr, Mg-Zn-Ca, iron

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alloys Fe-Mn, and Fe-Mn-Si, and polymeric materials like (co)polyesters from aliphatic hydroxyl acids (PHAs), polyglycolide (PGA), and poly(lactide-co-glycolide) (PLGA) have been shown to be suitable for these applications [2–5]. However, the widespread application of these materials is inhibited by inherent challenges for each material, such as controlling and profiling degradation kinetics for magnesium and iron alloys, and polymeric materials [6]. Polymeric materials were originally desirable; however, they have yet to overcome the challenges of producing repeatable and accurate degradation kinetic profiles, long-term performance integrity, minimizing host immune responses, and costly time-intensive research and development [5, 6].

Previous attempts at developing magnesium alloys for these applications include Mg-Zn, Mg-3Al-1Zn, and Mg-Zn-Zr. The inclusion of ternary elements ‘Zr’ and ‘Al’ to binary Mg-Zn was made in attempts to control the corrosion rate of the alloys, improve alloy strength and refine grain structure [7, 8]. However, a major downfall of these alloy compositions is that the alloying elements have no functional role within the body and are not entirely absorbable by endogenous tissues [9]. Furthermore, the degradation products of such alloys contain elements that have no functional role within the body and could impose significant complications to the human excretory system once implanted *in-vivo*.

The study presented in this paper explores novel Mg-Zn-Se and Mg-Zn-Cu degradable alloys with the inclusion of biologically essential trace elements, selenium (Se) and copper (Cu). It is expected that since the degradation products are composed of elements that have a functional role within the body, it would result in total material reabsorption and use by biological and enzymatic processes. Previous studies have extensively shown that selenium has an important role in fighting free oxidative radicals within the body, reducing viral expressions, preventing heart disease, and plays an important role in muscle formation [10, 11]. Additionally, copper has been utilized to treat diseases since the ancient society of Hippocrates 400 B.C. [12]. Copper also has several roles as a co-factor for essential enzymatic processes within the body, like cytochrome ‘c’ oxidase, superoxide dismutase, feroxidase, and peptidylglycine monooxygenase, amongst others [13]. It is believed that the inclusion of selenium and copper elements to form novel magnesium alloys will result in alloys with more favorable degradation kinetics required for endovascular use and wound healing properties that can promote neo-intimal growth. It has previously been shown by Persaud-Sharma et al., that these magnesium alloys possess improved mechanical properties when compared to other as-cast magnesium alloys such as Mg-3Al-1Zn, and martensitic Nitinol [14]. The trace element of copper has been shown to have harmful effects on living tissues; however, its role is vital for maintaining proper metabolic and neurological function [15]. Thus, the inclusion of 1 wt.% of copper and 1 wt.% selenium in the design of the alloy compositions, serves to evaluate the gross property differences of the alloys when compared to a binary Mg-Zn alloy without ternary elements copper and selenium.

In this study, the biocompatibility of experimental alloys Mg-1Zn-1Cu wt.% and Mg-1Zn-1Se wt.% is qualitatively and quantitatively assessed. Cytotoxicity effects of major degradation products are measured when used to culture human foreskin fibroblast cells *in-vitro*. Attempts at characterizing cell-surface interactions were made using scanning electron microscopy (SEM) techniques. Lactate dehydrogenase concentrations were measured to quantify cell death after the cells were in direct contact with the experimental magnesium alloys for a 72-hour period.

2. Materials and Methods

2.1. Alloy Manufacturing and Sample Preparation

The alloy compositions were synthesized using an arc-melting method in inert argon gas by ACI Alloys, Inc. (CA, USA). Both alloy compositions were composed of wt.% ratios (98/1/1) Mg-Zn-Cu/Se as seen in Table I. Square samples were then cut from a 1 × 1 × 6 cast mold bar of each alloy.

Square test specimens with an average dimension of 10 mm × 10 mm × 0.94 mm, were mechanically dry ground to a #1200 grit finish using silicon carbide abrasive paper. After mechanical polishing, samples were cleaned in acetone and rinsed with deionized water and blown dry with nitrogen gas. Materials were stored in a plastic container until further testing. Before any biological testing, all experimental alloy specimens were sterilized using an AMSCO Century Steris SV-120 pre-vacuum sterilizer at Gravity 20.

2.2. Alloy Sample Surface Characterization

The surface roughness of all alloy samples used in biological testing was characterized using a Veeco Instruments Wyko NT 9100 white-light profilometer system using the non-contact vertical scanning interferometry (VSI) measurement mode. 640 × 480 array size dimensions were used to quantify surface roughness using reported 'Ra' values. Optical profilometry equipment was used at the Nanotechnology Research Institute at the University of South Florida (Tampa, FL, USA).

Contact angle measurements using sessile drop methodology were made using a KSV contact angle goniometer with CAM 101 analysis software. The averages of the left and right contact angles were used to determine a mean contact angle value (°) used for surface energy calculations. Free surface energy or critical surface tensions of each alloy was calculated according to Zisman according to the Young Equation as shown in Equation 1. The γ_{SG} , γ_{SL} , γ_{LG} , and γ_c are the surface tensions of interfaces between solid/gas, solid/liquid, liquid/gas and critical surface energy, respectively. The contact angle θ is the angle between the liquid drop and a solid surface.

$$\gamma_{SG} = \gamma_{SL} + \gamma_{LG} \cos(\theta) \quad \text{Eq. (1)}$$

For this testing, deionized water and ethylene glycol were used as the two liquid probes for testing. Contact angle equipment was used at the Center for Drug Discovery and Innovation at the University of South Florida (Tampa, FL, USA).

2.3. MTS Assay

A cell proliferation MTS assay (G5421, Promega Corporation) was used to determine the percentage of viable human foreskin fibroblast cells grown in extract solutions of varying concentrations. To achieve this, Mg-Zn-Se, Mg-Zn-Cu, and Mg-Zn alloys were immersed in 300 mL of Hyclone's Dulbecco's Modified Eagles Medium (DMEM) without fetal bovine serum for 30-days at 37 °C with constant shaking. This method is conventionally used in the biomedical industry to obtain an extract solution in which the major toxic leachables from the material being tested is collected. Additionally, this process simulates the short-term effects of material degradation under dynamic conditions similar to what exists in the human cardiovascular system. The extract solution was filter sterilized using a 0.2 µm IC-Millex syringe filter with a hydrophilic PTFE millipore membrane to eliminate non-sterile contaminants. Initial cell media solutions were made using 100% extract, and a 50/50 solution composed of 50% extract with 50% fresh cell growth medium. Cells were grown in

the 100% and 50/50 extract solution for a period of 72 hours. After initial results showed that the fibroblast cell viability was low (33%) when cultured in the Mg-Zn-Cu 100% extract solution, but high (93%) when cultured in the 50/50 diluted Mg-Zn-Cu extract solution; subsequent testing to identify the toxic extract concentration between the 50% dilution and 100% extract was performed in 65% and 85% extract solution in which fibroblast cells were grown for a 7 day period with media being changed every 72 hours.

Human foreskin fibroblast cells (Global Stem®) were plated with a density of 3.3×10^3 cells/well into a 96-well micrometer plate. The plates were incubated for 24 hours at 37 °C in a humidified atmosphere of 5% CO₂ in air. Cells were counted before plating using a Bright-line hemacytometer. After the 24 hour incubation period, the 100% culture medium was replaced with the different concentrations of the extract dilutions and using pure culture medium (blank) without cells and pure culture medium with cells as control groups. After 72 hours of incubation in the extract solutions without changing media, the cell culture was treated with 20 µL/well of the combined MTS/PMS solution. The cells with the MTS assay treatment were further incubated for an additional 4 hours at 37 °C in a humidified atmosphere of 5% of CO₂. Immediately after 4 hours, absorbance measurements were made using a Tecan GeniOS instrument using Magellan software. Absorbance and excitation filters used were 470 nm and 690 nm, respectively. All material extracts were tested in 6 replicates for each extract concentration for 4 separate experiments with comparable results.

2.4. Fluorescent Imaging

To observe morphological changes to the cells and apoptosis or necrosis, the cells were fluorescently stained using Calcein-AM 5 mM in dimethyl sulfoxide (DMSO) (Molecular Probes) and propidium iodide 1mg/mL (Sigma-Aldrich). Imaging was recorded using a Nikon Eclipse TE-2000E microscope using a 10x objective and pseudo-colored using EZ-C1 software. Green cells were interpreted as being viable, dead cells were stained red, and apoptotic cells were imaged as yellow.

2.5. LDH Quantification

Human Foreskin Fibroblast cells were seeded at a density of 2.5×10^4 cells/well in a 24-well plate with culture medium (Hyclone DMEM, 5% Penicillin, 10% FBS). The cells were incubated for 24 hours at 37°C in a humidified atmosphere of 5% CO₂. Samples of each alloy possessed an average surface area of 1.1cm² and a thickness of 0.105 cm. The average growth area per well in a 24-well plate is 1.9 cm². Thus, each alloy specimen covered an average of 58% of the available growth area. After the 24-hour period of cell incubation, the alloy specimens were placed on the surface of the adherent fibroblast cells. The cells were then incubated for 72 hours without refreshing the culture medium. After each 24 hour period (day 1, day 2, day 3), the LDH concentration from each well containing cells was quantified using a Promega membrane integrity assay (G7890, Promega Corporation).

More specifically, after each 24 hour period, the plates were centrifuged for 4 minutes using an Eppendorf Vacuufuge vacuum concentrator with an orbital speed of 1400 rpm (250 × g). This was done to eliminate any contaminant particles resulting from cell lysis as a result of exposure to the experimental alloys. Subsequently, supernatant aliquots of 50 µL were transferred to a sterile black-bottom 96-well plate. 50 µL of the CytoTox reagent was then added to each 50 µL of experimental aliquot solution, bringing the total volume per well to 100 µL. Afterwards, the well plate was then left unexposed to light at room temperature for 10 minutes. Immediately after 10 minutes, the relative fluorescent units (RFU) was recorded using a Tecan GeniOS plate reader with Magellan software, with 555 nm and 590 nm excitation and emission filters, respectively. Wells containing pure culture media without cells and wells containing cells and pure culture media were used as controls. After each 24

hour period, lysis solution was added to control wells to obtain maximum LDH concentration values so that cytotoxicity percentages could be calculated.

2.6. Direct Contact Assay

Following the protocol stated above, human foreskin fibroblast cells were seeded at a density of 2.5×10^4 cells/well in 24-well plate with culture medium (Hyclone DMEM, 5% Penicillin, 10% FBS) containing the experimental alloys. This was done to characterize any cellular morphological changes from being in direct contact with the experimental alloys as well as to determine their cellular adhesion potential. Once the cells were seeded on the experimental alloys, cellular growth was monitored for 3 days (72-hours) at 37 °C in a humidified atmosphere of 5% CO₂. After the 72 hour period of growth, the samples were observed by scanning electron microscopy (SEM). The dimension of each alloy sample was tailored to cover 58% of the available growth area.

2.7. SEM Imaging Preparation

Cells were grown for 72-hours in contact with the experimental alloys as described in the direct contact assay section. After the 72-hour period of growth, the surface of the experimental alloys were washed with PBS and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 45 minutes at 4°C. After the fixation, the cells were washed for 10 minutes for each increasing concentration of ethanol (50%, 70%, 90% and 100%) to remove all water content from the specimens. The materials with the adherent cells were kept in 100% ethanol until being subjected to critical point drying (CPD) to avoid water contamination (Samdri-PVT-3D Tousimis). The samples were then coated with Au-Pd using a sputter coater (SPI Module). Lastly, secondary electron images (SEI) of the samples were obtained using a JEOL JSM 5900 LV SEM with an EDS-UTW Detector.

2.8. Statistical Analysis

A multivariate ANOVA test was conducted to assess whether there was any significant difference between the measurement parameters of surface roughness, contact angles values, liquid probe used for contact angle measurements (DI water, ethylene glycol), surface energy, cell viability percentages as determined by using the MTS Assay, and cytotoxicity results from the LDH assay for each alloy composition. Differences with p-values less than 0.05 were considered significant. This was followed by a post-hoc assessment using Fisher's least significant difference method. Additionally, Spearman's nonparametric correlation test was performed to evaluate the relationship between the tested variables and each alloy composition. Correlation was determined to be significant at both the 0.01 and 0.05 levels. Correlation analysis showed a significant positive correlation ($r_s = 0.830$) between liquid probe used (water and ethylene glycol) and contact angle formed. A negative correlation was found between the surface roughness parameter and the contact angle formed using ethylene glycol as a liquid probe ($r_s = -0.283$).

3. Results

3.1. Surface Properties of Ternary Magnesium Alloys

Table II summarizes the surface properties of the tested experimental ternary magnesium alloys. The average surface roughness values were not statistically different; however, the Mg-Zn alloy showed a higher mean surface roughness of 158 nm as compared to Mg-Zn-Se and Mg-Zn-Cu. Average contact angles values (left & right surface contact angles) for all samples were also very similar with mean values ranging from 70–77° for ethylene glycol measurements and 59–62° for DI water. Although not reaching differences of statistical significance, the Mg-Zn alloy with the highest mean surface roughness was also shown to have the highest mean contact angle values of 77 ° when using ethylene glycol as the liquid

probe, and the lowest mean contact angle of 59 ° when using DI water as the liquid probe. Surface energy calculations showed that Mg-Zn-Se had the highest mean critical surface tension of 70 mN/m, while Mg-Zn had the lowest mean critical surface tension of 57 mN/m. Differences in surface energy values also did not reach statistical significance.

3.2. Cell Viability: MTS Assay

The MTS assay results are represented in Figure 1. It was observed that cells maintained 100% viability when cultured in 50/50 extract solution from the Mg-Zn and Mg-Zn-Se alloys, while the cells cultured in the Mg-Zn-Cu alloy extract yielded the lowest cell viability of 93% after day 3. Additionally, the Mg-Zn-Cu alloy showed the lowest cell viability of 33% when cultured in the 100% extract solution. No statistical difference in cell viability percentages for cells cultured in the 100% and 50/50 extract solutions obtained from the Mg-Zn and Mg-Zn-Se alloys was observed.

Noting the low cell viability of fibroblast cells when cultured in the 100% extract obtained from the Mg-Zn-Cu alloy, further testing was performed to narrow the range of cytotoxicity between the 50/50 and 100% extract solution. Thus, cells were cultured in 65% and 85% extract concentrations for an extended period of 7 days.

Notably, there were a higher number of cells after the first day compared to the initial cell seeding density for both the 85% and 65% extract solutions of the Mg-Zn-Cu alloy with cell growth reaching 157% and 117% for each extract concentration, respectively. After the second day of growth, the percent cell growth remained above 100% for the 85% concentrated extract solution though decreasing from 157% to 107%, whereas the percent of viable cells in the 65% extract-solution decreased from 117% to 88%. Days 3, 4, and 5 showed the lowest percentage of viable cells (35–40%) when cultured in both extracts concentrations. Expectedly, cell growth remained slightly higher in the 65% concentrated extract solution than in the 85% concentrated solution for days 3, 4 and 5 because of the lower concentration of Cu in the 65% extract solution. Major cytotoxic effects were seen on day 3 of the cell growth period. Conclusively, extract concentrations ranging from 65%–100% were identified as inducing the most cytotoxic effect. Figure 3, shows a comparison of viable cells cultured in 100% extract media and diluted 50% extract media.

Figure 3 A, B, C all show a high percentage of viable cells using fluorescence microscopy. Viable cells appeared fluorescent green (Calcein-AM) for fibroblast cells grown in 50/50 culture medium for Mg-Zn, Mg-Zn-Cu, and Mg-Zn-Se alloys, respectively. Figure 3 D, E, F show lower percentages of viable cells (green) and more dead cells (red-PI) as these images correspond to cell growth in 100% pure extract solutions. Most notably with Figure 3E possessing the most dead cells (Mg-Zn-Cu).

3.3 Percent Cytotoxicity: LDH Quantification, Direct Contact Assay, and Cellular Adhesion

Figure 4, shows the cytotoxicity of human foreskin fibroblast cells cultured in direct contact with the Mg-Zn and the Mg-Zn-Se alloy for 3 days of growth without changing cell culture media. For all three days of growth, cytotoxicity was lower for the Mg-Zn-Se alloy with a mean cytotoxicity percentage of 22% on day 1, and a mean cytotoxicity percentage of 31% and 34% on days 2 and 3, respectively. Mg-Zn showed a slightly higher percentage of cytotoxicity of 26, 35 and 36 percent on days 1, 2, and 3, respectively. LDH concentrations could not be quantified for the Mg-Zn-Cu alloy because the alloy specimens for such a composition completely degraded within the 72-hour time period.

Surface images of the Mg-Zn and Mg-Zn-Se alloys were taken before and after the 72 hour period of growth to measure cell adhesion and characterize morphology changes. Figure 5A shows the Mg-Zn surface before cell exposure, while Figure 5D shows the same specimen

after experimental exposure and SEM sample preparation. Likewise, Figures 5B and 5E show the Mg-Zn-Se alloy surface both before and after experimentation. It can be seen for both surfaces after the experimental period of cell growth that no surface cellular adhesion is evident. Visually, the surfaces after experimentation and SEM sample preparation for imaging are coarser, have an increased surface roughness, and have more defined grain boundaries.

Within the 72 hour experimental period of cell growth, the Mg-Zn-Cu alloy samples with an average weight of 0.285g completely degraded forming a white precipitate; thus, negating any cell growth, cell adhesion, and LDH quantification. A true color optical image of the precipitate that resulted from the degradation of Mg-Zn-Cu in the DMEM culture medium with human foreskin fibroblast cells is seen in Figure 5F. Backscatter SEM images of the same precipitate is seen in Figure 5C. Characteristically, white deposits are visible on the surface of these alloys, with pronounced grain boundaries after the 72-hour period. Energy dispersive X-ray spectroscopy (EDS) was performed to chemically identify the composition of the features shown in Figure 6.

Figure 6A, shows the white deposits on the Mg-Zn alloy to consist of elevated levels of 35.49 wt.% oxygen, 23.92 wt.% carbon, 13.79 wt.% magnesium, 13.69 wt.% phosphorus, and 11.41 wt.% calcium. Comparatively, the Mg-Zn-Se alloy showed the deposits to be composed of 20.42 wt.% carbon, 38.47 wt.% oxygen, 24.11 wt.% magnesium, with 9.07 wt.% and 6.52 wt.% of phosphorus and calcium, respectively (Fig. 7B). EDS analysis of the precipitate resulting after the 72-hour period of cell growth on the Mg-Zn-Cu alloy revealed to consist of 51.04 wt.% oxygen, 30.62 wt.% magnesium, and 15.91 wt.% carbon. Generally, the deposits on the Mg-Zn, Mg-Zn-Cu, and the Mg-Zn-Se alloys, were shown to be composed of ionic salts commonly found in the cell culture medium that reacted with leachables from the alloys as evident from EDS analysis.

4. Discussion

Understanding the surface properties of an implantable device is vital to determining a fundamental relationship between fluid-cellular and material interactions. In this study, an essential parameter is the surface roughness of the test specimen. The corrosion current and pitting tendency of magnesium alloys has been widely reported to increase with increased surface area as a result of increased surface roughness, and to generally play a vital role in the passivation tendency of the alloys [16–18]. The influences of surface roughness on surface tension measurements are also critical as the deposition of a sessile drop on a rough surface could result in misleading contact angle values as the droplet volume would imbed within the crevices of the test surface [19]. From experimentation, it was observed that the Mg-Zn alloy composition revealed to possess the highest mean surface roughness, resulting in the highest mean contact angle value of 77° when using ethylene glycol as the liquid probe, and the lowest mean contact angle of 59° when using DI water as the liquid probe. This difference is attributed to the chemically heterogeneous surface on the magnesium alloys [14, 20]. As previously reported by Persaud-Sharma et al., the natural air formed oxide layer that develops on the surface of the alloys was shown to be comprised a mixture of elements that include carbon, oxygen, nitrogen, magnesium and aluminum as shown by X-ray photoelectric spectroscopy analysis (XPS) [14].

The contact angle hysteresis observed amongst the different alloy compositions is partially due to the varying surface roughness of the test specimens, but is also due to the different chemical composition of the air formed oxide layer which directly interacts with the liquid probe droplet used to measure the contact angles (Figures 7 and 8). Statistical correlation analysis showed a significant positive correlation ($r_s = 0.830$) between water and ethylene

glycol as liquid probes and the contact angle formed. A significant negative correlation was found between surface roughness and the contact angle formed when using ethylene glycol as a liquid probe ($r_s = -0.283$). This correlation is supported by contact angle and surface roughness parameters widely explored by Young, Wenzel, and Cassie & Baxter models [21–23]. Wenzel, Cassie & Baxter proposed that the liquid probe volumes imbed within the crevices of the rough surfaces or remain distended over trapped air within such crevices, thus resulting in contact angle hysteresis [21–23]. Correction factors developed by Wenzel, Cassie & Baxter are widely used to correct for such contact angle hysteresis imparted by rough surfaces. However, such corrections were not applied to contact angle data included in this paper. Backscatter images for the surfaces of the experimental alloys did not reveal any regions of contrast for distinguishable phases in the Mg-Zn-Se alloy, although notable phase contrast was seen for the Mg-Zn-Cu alloy along the grain boundary structure of the alloy as shown in Figures 7 and 8, respectively. Thus, despite the same grinding and polishing procedures employed for the three different alloy compositions, the variance in the surface roughness values are attributed to the higher mechanical property values for the air formed oxide layers on the alloys as compared to their bare surfaces as previously reported by Persaud-Sharma et al [14].

The cytotoxicological effect of leachables from the novel biodegradable Mg-Zn-Cu and Mg-Zn-Se alloys was quantified using different *in-vitro* biological assay tests. These findings were compared to a binary Mg-Zn alloy which has shown degradation rates appropriate for wound healing with desirable biocompatibility with host tissues and fluids [24]. Primarily, a Promega-MTS assay was utilized to perform this testing by exposing human foreskin fibroblast cells to the degradation products released from these experimental alloys after alloy samples were immersed and agitated in simulated body fluid for 30-days at 37°C (Fig. 1). Fibroblast cells were viable in the 50% diluted extract solutions obtained from the Mg-Zn-Se and Mg-Zn-Cu alloys, while only cells cultured in the Mg-Zn-Cu alloy extract showed to have poor viability (33%) when cultured in 100% concentrated extract media; whereas, the same composition showed to have a 93% viability in the 50/50 dilution. This difference in cell viability percentage can be explained by the increased copper concentration in the 100% extract, which can lead to copper toxicity in organisms when copper exceeds a critical threshold for survival [25]. This effect was aggrandized by the lack of nutrients in the 100% extract medium which was not supplemented with nutrients usually present in cell culture medium through the addition of fetal bovine serum (10% FBS) or antibiotic agents such as penicillin. While the toxicity of copper has been widely published, the necessity of copper as an essential trace mineral serving as an integral role in many enzymes involved in vital biological roles remains irrefutable [15]. Additionally, no alloy composition yielded complete cell cytotoxicity when cultured in the 100% extract media. This is a favorable quality for these experimental degradable magnesium alloys, because it has been reported that degradable polymer's such as polylactide materials have been shown to induce cytotoxic effects because of the dramatic changes in pH [26]. Alternatively, this is not observed when testing biodegradable alloys which fail to induce complete cytotoxicity [27, 28].

Further testing narrowed the cytotoxic concentration range of Mg-Zn-Cu extract from 65% to 100%, when cell growth was observed for a 7 day period. Days 1 and 2 of such experimentation, showed an increase in cell proliferation compared to the original seeding density with 157% ([85% extract]) and 117% ([65% extract]). A precipitous drop in cell growth was seen on Day 3 which remained low for the duration of the 7 day period (Fig. 3). The immediate increase in the percentage of viable cells above 100% observed on days 1 and 2 is attributed to the effect of copper promoting fibroblast proliferation. This notion is supported by Itoh et al., who previously showed that using copper can stimulate proliferation of mouse embryonic fibroblast cells (MEFs) using a copper chloride (CuCl_2) solution with a

copper mass of 47 percent [29]. Cell proliferation is seen at the 65% and 85% extract concentrations on day 1 and day 2, because the concentration of the solution has been diluted with pure culture media, thus reducing the concentration of the copper. Initially, the cells have a capacity to buffer and/or utilize the copper absorbed from the culture solution. Thus, the 85% extract concentration increased the number of fibroblast cells by 57% because it contained a higher concentration of copper, as compared to the 65% extract concentration which only increased the number of fibroblast cells by 17%. However, on day three of the experiment, the cells have most likely become saturated with any useable amount of copper and such saturation became toxic for fibroblast cell survival. The 47 percent copper solution concentration used by Itoh et al., similarly showed MEF proliferation and supports data from the study presented herein that culture media is toxic with extract concentrations of Mg-Zn-Cu ranging from 65 to 100 percent. Thus, the precipitous cell death observed on day 3 can be explained by the cell saturation of copper, which is ultimately the effect of copper toxicity. Therefore, the critical limit for human foreskin fibroblast cell survival to copper exposure from the Mg-Zn-Cu alloy is as high as an extract concentration of <65%.

LDH quantification showed that fibroblast cells cultured in direct exposure to the Mg-Zn-Se alloy had a lower percent cytotoxicity than those directly in contact with the Mg-Zn alloy, as both alloys showed a maximum cytotoxicity of approximately 35%. This means that more than 64% of the cells in direct exposure to these experimental alloys survived (Fig. 5). However, data from the direct contact assay evaluating cell adhesion on the surface of these alloys show that cells cannot adhere or grow on the surface of the materials (Fig. 6). According to studies performed by Huan et al., and Keim et al., the lack of cell growth on the substrate is common for many types of magnesium alloys and is attributed to the large amounts of hydrogen evolution from magnesium alloys which dramatically increases the pH levels within the immediate vicinity of the magnesium implants which prevents cellular adhesion [7, 30]. This is supported with physical observations of cell culture media with the Mg-Zn-Cu alloy specimens changing color from being red to faintly yellow/mostly clear. This color transition is only possible if the pH of the solution changed from neutral/basic conditions to more acidic conditions with a pH less than 6.8. This observation can be explained by the increased evolution of hydrogen gas from the Mg-Zn-Cu alloys which increase the presence of hydrogen ions within the cell culture media. Additionally, experimentation with the Mg-Zn-Cu alloy showed that the entire material corroded within the experimental time period, thus no cell adhesion or LDH amounts could be quantified. The lack of cellular adhesion seen on the alloys is further supported by the low Zisman surface energy values for each alloy, which is consistent with Baier et al. and van der Valk et al., who determined that materials with low surface energies show low cell attachment [31, 32]. It is important to note that all tests were conducted *in-vitro*, which is not a true presentation of an actual implant. In some instances when a device or material is implanted endovascularly, the fluid conditions will not be static and fresh nutrient-rich biological fluids will create an environment capable of sustaining cell growth and viability.

A particularly interesting finding was the complete degradation of the Mg-Zn-Cu alloy and the deposition of salts on the surface of the Mg-Zn and Mg-Zn-Se alloys as evidenced by SEM/EDS analysis (Fig. 6F, 6D, and 6E, respectively). As shown in Figure 6, the analysis of these products showed them to consist primarily of oxygen, magnesium, sodium, phosphorus, and calcium, which are all salts found in the simulated body fluid (DMEM) that reacted with the magnesium alloys and their leachables to form precipitates that deposited on the alloy surface. This is consistent with the findings of Keim et al., who also reported increased deposits of the same elements (Fig. 6) [30]. The most likely chemical products formed from the interaction of these materials are $\text{Mg}(\text{OH})_2$ and MgCl_2 according to equations (2), (3) and (4) [6, 33]. Generally, once the magnesium materials are immersed

within an electrolytic media, the alloys are attacked by the chloride salts or anion species within the media. In this case, the chloride, sulfate, phosphate, carbonate, sodium, and oxygenated species are attacking the surface of magnesium. The chlorides and fluorides are the most damaging species because of their high electronegativity values and affinity for metallic species. This would explain the increased detection of chlorine in the EDS analysis of the degradation product for the Mg-Zn-Cu alloy which underwent complete degradation. Xin et al., has proposed that the chloride ions can react with the Mg(OH)_2 layer that forms on the surface of magnesium alloys and transform it to a more soluble MgCl_2 intermediate as shown in Eq.(3) [33].

The presence of phosphorus, calcium, and magnesium deposited on the surface of the experimental alloys can be explained by the precipitation of insoluble tertiary magnesium phosphate. As the magnesium alloy undergoes corrosion, a substantial amount of Mg^{+2} ions are released into the solution. The increased concentration of Mg^{+2} ions prevents the precipitation of calcium phosphate which is found in the SBF, and rather forms magnesium phosphate ($\text{Mg}_3(\text{PO}_4)_2$) according to Eq. (5) [34]. The insoluble magnesium phosphate would then deposit on the surface of the implant. The formation of these insoluble products can potentially reduce the release of the Mg^{+2} ions resulting from the oxidation of the magnesium implant, as suggested by Keim et al. [30]. Keim suggests that the most efficient method of reducing Mg^{+2} ions from being released by the implant is the nearly spontaneous formation of a biomimetic coating composed of calcium, carbon, and phosphorus which are all elements identified in the degradation precipitate of Mg-Zn-Cu and the deposited layer on the Mg-Zn and Mg-Zn-Se alloys formed from their degradation products (Fig. 6). This nearly instantaneous protective layer has been shown to reduce the oxidation of the magnesium implant by as much as 60% as compared to polished and surface treated magnesium alloys [30].

5. Conclusions

In this study, *in-vitro* experimentation to assess the biocompatibility of novel ternary magnesium alloys Mg-Zn-Se and Mg-Zn-Cu was performed. This study presents the first evaluation of these biodegradable alloys, which have the potential to serve as effective endovascular medical devices. The cell viability of these alloys was examined by an MTS assay using human foreskin fibroblast cells. It is found that cells cultured in a diluted 50/50 (30-day extract solution/fresh DMEM) was shown to have cell viabilities above 90% for all alloy compositions (Mg-Zn-Cu, Mg-Zn, and Mg-Zn, Se). Cells cultured in the 100% and the 50/50 extract solution of the Mg-Zn-Se alloy showed higher cell viability than the binary Mg-Zn alloy without the element Se. Cell viability was 33% when cultured in a 100% extract solution obtained from the Mg-Zn-Cu alloy. It was further identified that extract concentrations from 65–100% of the Mg-Zn-Cu alloy is the most harmful for fibroblasts and lead to cell death by copper toxicity. Cytotoxicity of cells in direct contact with the magnesium alloys was quantified using a membrane integrity assay and showed that

fibroblast cells in direct contact with the Mg-Zn-Se alloy resulted in lower cytotoxicity than those in direct contact with the Mg-Zn alloy with cytotoxicity levels reaching 34 and 36 percent, respectively. Cells did not grow on the surface of any magnesium alloys, although cells surrounding the alloy test specimens remained viable. A significant positive correlation was revealed between the liquid probe used and the contact angle formed; while a negative correlation was determined between surface roughness and contact angle formed using ethylene glycol as the liquid probe.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This project was developed by Dharam Persaud (PI), who is also a recipient of a grant from the National Brain Aneurysm Foundation which financially supports this project. Dharam Persaud is also supported by NIH Fellowship number NIH/NIGMS R25 GM061347.

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Figure 1.

Percent of cell growth of human foreskin fibroblast cells when cultured in 100% concentrated 30- day extract and 50/50% diluted 30-day extract after 3-days of growth. Results collected using an MTS assay, and cell growth was measured relative to a control where the fibroblast cells were grown in 100% media. Data represented as mean with Std. Dev. Bars; N=4 (sample size), n=3 (repeated measurements per sample).

Figure 2.

Percent of cell growth of human foreskin fibroblast cells when cultured in 65% and 85% 30-day extract after 7 days. Results collected using an MTS assay, and cell growth was measured relative to a control where the fibroblast cells were grown in 100% media. Data represented as mean with Std. Dev. Bars; N=4 (sample size), n=3 (repeated measurement per sample). Cell growth/death was not statistically different between extract concentration solutions per day. Cell growth/death was significantly different relative to day 0.

Figure 3.

Human foreskin fibroblast cells cultured for 72 hours in diluted and pure 30-day extract solutions from the MTS Assay. Images after 72 hour growth period: A(Mg-Zn), B (Mg-Zn-Cu), C(Mg-Zn-Se) are cultured in 50% media: 50% extract solutions. D(Mg-Zn), E (Mg-Zn-Cu), F(Mg- Zn-Se) are cultured in 100% concentrated 30-day extract solution.

Figure 4.

Percent cytotoxicity of human foreskin fibroblast cells seeded on Mg-Zn and Mg-Zn-Se alloys measured by LDH quantification over a 3 day period of exposure. Data represented as sample mean with bars indicating one standard deviation; N=4 (sample size), n=3 (repeats per sample).

Figure 5.

Scanning electron microscope (secondary electron image) and optical microscope images of surface images of Mg-Zn and Mg-Zn-Se before and after exposure to cell growth duration of 72 hours and precipitate formation from the degradation of the Mg-Zn-Cu alloy. Surface images before/after experimentation: A/D) Mg-Zn showing no cell adhesion, B/E) Mg-Zn-Se showing no cell adhesion. C) SEM image of precipitate formation of Mg-Zn-Cu. F) Optical image with the true color of the precipitate formed from the Mg-Zn-Cu alloy.

Element	Wt.%	At.%
C	23.92	35.69
O	35.49	39.77
Na	1.71	1.33
Mg	13.79	10.17
P	13.69	7.93
Ca	11.41	5.11

Element	Wt.%	At.%
C	20.42	30.29
O	38.47	42.84
Na	1.41	1.09
Mg	24.11	17.67
P	9.07	5.22
Ca	6.52	2.90

Element	Wt.%	At.%
C	15.91	22.61
O	51.04	54.44
Na	01.09	00.81
Mg	30.62	21.49
Cl	01.35	00.65

Figure 6. Energy dispersive X-ray spectroscopy (EDS) analysis with backscatter detector SEM images of Mg-Zn (A), Mg-Zn-Se (B) surfaces, and precipitate formation of Mg-Zn-Cu (C) after cell growth for 72 hours.

Figure 7.

Scanning electron microscopy (SEM) images and X-ray photoelectron spectroscopy (XPS) analysis of oxide layer formation on the Mg-Zn-Se alloy [14]. A) Secondary Electron Image (SEI) of the alloy surface with an oxide layer, B) SEI image without a surface oxide layer, C) Backscatter image (BEI) of surface with an oxide layer, D) BEI image of surface without an oxide layer.

Figure 8.

Scanning electron microscopy (SEM) images and X-ray photoelectron spectroscopy (XPS) analysis of oxide layer formation on the Mg-Zn-Cu alloy [14]. A) Secondary Electron Image (SEI) of the alloy surface with an oxide layer, B) SEI image without a surface oxide layer, C) Backscatter image (BEI) of surface with an oxide layer, D) BEI image of surface without an oxide layer.

Table I

Weight Percentage of Alloy Compositions (wt.%)

Alloy Composition	Mg	Zn	Cu	Se
Mg-Zn (99/01)	99	1	-	-
Mg-Zn-Se (98/01/01)	98	1	-	1
Mg-Zn-Cu (98/01/01)	98	1	1	-

Table II

Surface Properties of Ternary Magnesium Alloys

Alloy Composition	3-Dimensional Optical Profilometry Image of the Alloy Surface	Average Surface Roughness (nm)	Average (Left & Right) Contact Angle Values (°) Ethylene Glycol DI Water	Zisman Surface Energy (γ_c) (mN/m)
Mg-Zn		158 ± 71	77 ± 5 59 ± 1.8	57 ± 35
Mg-Zn-Se		145 ± 107	76 ± 6 60 ± 1.9	70 ± 24

Alloy Composition	3-Dimensional Optical Profilometry Image of the Alloy Surface	Average Surface Roughness (nm)	Average (Left & Right) Contact Angle Values (°) Ethylene Glycol DI Water	Zisman Surface Energy (γ_c) (mN/m)
Mg-Zn-Cu		143 ± 91	70 ± 8 2 ± 1.7	64 ± 20

* Data represented as mean $\pm$ standard deviation; N=15(sample size), n=2 (repeats per sample).