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Research Article

Potential Clinical Significance of Cytotoxicity Evaluation of Biodegradable Mg-6Zn Alloy on IEC-6, CT26 and A7r5 Cell Lines

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Abstract

The recent using of Mg-based Mg-6Zn alloys for biodegradable implants was not supported with sufficient toxicity evaluation data. This work presented a thorough cytotoxicity evaluation of Mg-6Zn alloy for biodegradable implants. Three cell lines (IEC-6, CT26 and A7r5 cells) were used to evaluate the cytotoxicity of Mg-6Zn alloy on intestinal wall cells *in vitro*. Cell viability and proliferation capability were assessed by MTT assay. Cell proliferation was investigated by flow cytometric analysis. Three types of cell lines were exposed to Mg-6Zn alloy extracts for 24h, 48h and 72 hours for subsequent research assay, the cytotoxicity assay of Mg-6Zn alloy extracts at different concentrations and the effect on cells factors expression levels were studied, and the biodegradation capability of Mg-6Zn alloy was also studied. The results indicated that, with the increasing of the concentrations of Mg-6Zn alloy extract, the impact of Mg-6Zn alloy on the viability and proliferation capability of three types of cell lines was not obvious, but when the culture time was prolonged, the Mg alloy extract as an obvious inhibitory effect on colon cancer cell CT26 growth ($P<0.05$). Although Mg-6Zn alloy extracts could have slight effect on the cell cycle of CT26 cell, there was no significant difference when compared with IEC-6. Mg alloys was subjected to slight corrosion after 3 weeks of immersion in the intestinal juice, and severe dramatic corrosion was observed at fourth weeks. In conclusion, Mg-6Zn alloy can be suggested as a suitable candidate of biodegradable implants to be used in biomedical applications.

Keywords: Magnesium alloy; Biodegradable implants; Cytotoxicity; Biomedical applications; Cell cycle

Introduction

Magnesium (Mg) alloys have attracted great interest for biodegradable implant applications since the early 2000s [1,2]. Mg alloys have mechanical properties close to natural bones. The mechanical and electrochemical properties of Mg alloys also make them suitable for broad biomedical applications, including orthopedic implants and vascular stents [1-3]. Mg alloys are more beneficial than non-degradable permanent metallic implants such as titanium, stainless steel and cobalt-chromium alloys. Application of Mg alloys reduces healthcare cost and patient morbidity by eliminating the second surgery to remove implants, this makes Mg-based alloys as potential bioresorbable implants [4,5].

Magnesium (Mg^{2+}) is the second most abundant cation within the cell. It is the cofactor for hundreds of enzymes and plays an essential role in a wide range of fundamental biochemical reactions and cellular functions, including cell cycle, channel regulation, membrane and nucleic acid stability [6]. Corrosion of the Mg-based implant in-vivo forms a soluble, non-toxic oxide that will be excreted to urine. Moreover, Mg may actually have stimulatory effects on the growth of new bone tissue [7]. The main drawback of Mg in clinical applications is low corrosion resistance, especially in aqueous environments, in the physiological pH (7.4-7.6) and high chloride environment, lack of mechanical integrity before sufficient healing of the bone tissue and production of hydrogen gas in the corrosion process [8]. The most practical techniques to improve the mechanical and corrosion properties of pure Mg is alloying with other elements such as aluminum (Al), zinc (Zn), calcium (Ca), tin (Sn), zirconium (Zr), yttrium (Y),silicon (Si) and manganese (Mn) [9,10]. Zn and Ca are the preferred alloying elements in medical application, no medical adverse effects are reported with Zn and Ca, they are beneficial elements for human health [11,12].

Zn is an essential element required for approximately 100 enzymes in numerous aspects of cellular function, including immune functions, protein and DNA syntheses. It also supports normal growth, wound healing and a proper sense of taste and smell [13,14]. There are numerous biodegradable Mg-based alloys containing zinc in amounts of several wt. %, like Mg-Zn, Mg-Zn-Mn-Ca, Mg-Zn-Y, and Mg-Zn-Si [13,14]. Additions of Zn improve the corrosion resistance and strength of the alloys. Mg-Zn eutectic contains about 51 wt.% Zn, that supports the high glass-forming ability of Mg–Zn-based alloys [15,16]. Mg-Zn-based alloys containing about 50 wt.% of Zn have excellent strength, high corrosion resistance and good biocompatibility in animals, therefore, they become promising candidates for biodegradable bone implants. But preparation and forming them to a final product are quite difficult [16]. We developed a novel binary Mg-6wt% Zn (Mg-6Zn) alloy and investigated its cytotoxicity and biocompatibility [17-20]. *In vitro* cytotoxicity to IEC-6 cells implies that the Mg-6Zn alloy is safe for cellular applications, with a cytotoxicity grade of 0-1 [21]. It is therefore imperative to comprehensively study the local cytotoxicity of Mg-6Zn alloys and to set a reference for further use as biodegradable implant materials in digestive tract.

Study on the effect of Mg-6Zn alloy extract to IEC-6, CT26 and A7r5cells was preliminarily performed in order to provide an evaluation of the effect of Mg alloy on surrounding cells when used as a degraded metal stent in the intestinal tract in interventional operations. We evaluated the effects of Mg-6Zn Alloy extracts on proliferation and apoptosis of three cell lines, rat IEC-6 cells were used to evaluate the cytotoxicity of intestinal implant materials on the intestinal epithelial cells *in vitro*; rat A7r5 cells were used to evaluate the cytotoxicity of

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intestinal implant materials on the intestinal smooth muscle cells *in vitro*; mouse CT26 cells were used to evaluate the cytotoxicity of intestinal implant materials on the colon and rectum cancer cells *in vitro*.

Materials and Methods

Mg-6Zn Extract Preparation

The magnesium alloy is provided by the materials science and engineering institute of Shanghai Jiao Tong University. It was prepared from high purity Mg (99.99 %) and zinc (Zn; 99.999 %). The composition of this alloy was given in Table 1. The as-extruded Mg alloy was processed into discs samples to meet experimental needs. All the Mg materials were cut into round sample discs with 10 mm in diameter and 1 mm in height, the area is 1.12 cm^2. Firstly, Mg alloy were ground with 1000 grit SiC paper and subsequently cleaned by two 10 min washes in pure ethanol under sonication. Mg alloy by 121℃ high pressure sterilization and ultraviolet light disinfection 2 h for both sides. According to ISO 10993-12 [22], the proportion of the alloy's surface area and volume of leaching solution, 1.25 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 1000 rpm to eliminate macro-particles. After obtaining a homogenous solution, the extracts were diluted at concentrations of 20%, 40%, 60%, 80%, 100% and un-diluted extract as well.

Cell Culture

IEC-6, CT26, A7r5 cells are all purchased from the Chinese Academy of Sciences Cell Bank. Three cell lines were chosen as the cytotoxicity test cells in this study. IEC-6 cell line (a rat intestinal epithelial cell line). CT26 cell line (a mouse colon cancer cell line) and A7r5 cell line (a rat vascular smooth muscle cell line) were cultured in DMEM (high glucose) with 10% FBS, 100 U/mL penicillin and100 μg/mL streptomycin.

Animals

Animal procedures were conducted according to the ethical guidelines of Shanghai Jiaotong University. Sprague-Dawley rats (250±50 g) were obtained from the Shanghai Laboratory Animal Center, Academia Sinica, and China. Rats were housed in stainless steel cages at a controlled humidity (60-65%) and temperature (22±2˚C) with a normal 12:12 h light/dark cycle for at least 7 days before the preparation of the intestinal juice.6 rats were sacrified by cervical dislocation. Segments of the colon and rectum were quickly removed, the lumens were washed by aseptic saline, the intestinal juice was prepared with 25ml constant volume when the intestinal juice was collected from each rat. The intestinal lavage solution was sealed and placed for 1 hour at room temperature and the liquid supernatant was taken for subsequent experiment.

Cytotoxicity Assessments by MTT Assay

Cells in logarithmic growth phase were seeded in 96-well plates (Sigma-Aldrich Co, Corning, USA) in a density of 5000 cells/100 μL in each well and incubated for 24 h to allow attachment. Thereafter, the culture medium was discarded and replaced with 100μL 0%, 20%, 40%, 60%, 80% and 100% alloys extracts 100 μL. Each experiment was performed four times. The cell morphology was checked after 48 h of incubation with inverted microscope after 1, 3, and 7 days of incubation, 20 μl MTT was added to each well and incubated for 4 h, and then the MTT solution was removed and 100 μL of Dimethyl Sulphoxide (DMSO) added in each well. The optical density of the eluted stains was evaluated with spectrophotometer at 570 nm wavelength.

Table 1: Chemical compositions of Mg-6Zn alloy.

	Mn	Si	Ni	Cu	Al	Fe	Zn	Mg
Mg alloy	0.0004	0.0016	0.0005	0.0005	0.0085	0.0038	5.6210	Bal.

Flow Cytometric Analysis of Cell Cycle

The concentration was 0%, 40%, 80%, and 100% metal alloy leaching solution was added to the medium of IEC-6,CT26,A7r5 cells respectively, All of three lines cell cultured for 48h, the cell cycle was measured by flow cytometry after Propidium Iodide (PI) staining. The evaluation of cell cycle was assessed using a flow cytometer (FACS Calibur, BD Bioscience, US). Data acquisition and analysis were performed using Cell Quest Pro 3.3 software (BD Bioscience, US).

Western Blot Analysis of Protein Level in NF-KB Signaling Pathway

All the cells were cultured as described by the previous studies. Firstly, IEC-6, CT26, cells were cultured on different concentrations (0, 20% and 40%) of Mg alloy extracts for different time periods (1, 3 and 5 d), respectively. Total protein extraction from cultured cells was used in electrophoresis and Western blot. Briefly, 20 micrograms of total protein were separated by standard SDS-PAGE and transferred onto polyvinylidene fluoride membranes. The membranes were washed, blocked and incubated with specific primary antihuman antibodies (1:1500), followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:5000). The reactions were detected by enhanced chemiluminescence assay. The anti-TGF-β1, anti-VEGF, anti-bFGF and anti-TNF-α antibody were purchased from Abcam (London, UK), and a concentration of 1:1500 was used.

Degradation of Mg alloys

The biodegradation behavior of Mg alloys was examined in long term exposure with intestinal juice. Disk shape Mg samples with dimension of 20 mm in diameter and 2 mm in thickness were immersed in 50 mL intestinal juice (50% dilution) and stored at 5% CO_2 and 37°C for 42 days to simulate the physiological conditions. Every 7 days, the intestinal juice Mg alloy simmersed was replaced by fresh frozen intestinal juice (50% dilution), and the surface of the alloy was observed by electron microscope.

Statistical Analysis

Statistical analysis was performed with the SPSS18.0 Software Package (SPSS Inc., Chicago, USA). The experimental values were analyzed using the paired-samples t test and were expressed by the mean values ±Standard Deviation (SD). Then, one-way ANOVA analysis was performed to determine differences between groups for each evaluated parameter that was evaluated at each time point. The level of significance was defined as $P<0.05$.

Results

Cell morphology

According to the morphological changes of IEC-6, CT26, A7r5 cells the toxicity of Mg alloy extracts solution to intestinal epithelial cells was evaluated. Figure 1 show IEC-6, CT26, A7r5 cells in 0% and 40% concentration of Mg alloy extracts on the third day. Further, it can be seen that all the cells contour are clear, with a long spindle or polygonal shape. The nucleoplasm ratio is normal and the cytoplasm is uniform, which is similar to that of the control cells.

MTT Assay

MTT assay was performed after 24, 48 and 72 h of culturing the IEC-6, CT26, A7r5 cells to check the cell viability and proliferation capability at the presence of 0%, 20%, 40%, 60%, 80% and 100% Mg alloys extracts. MTT results represent that all Mg alloys extracts have no significant toxic affects on IEC-6, A7r5 cells after culturing the cells for

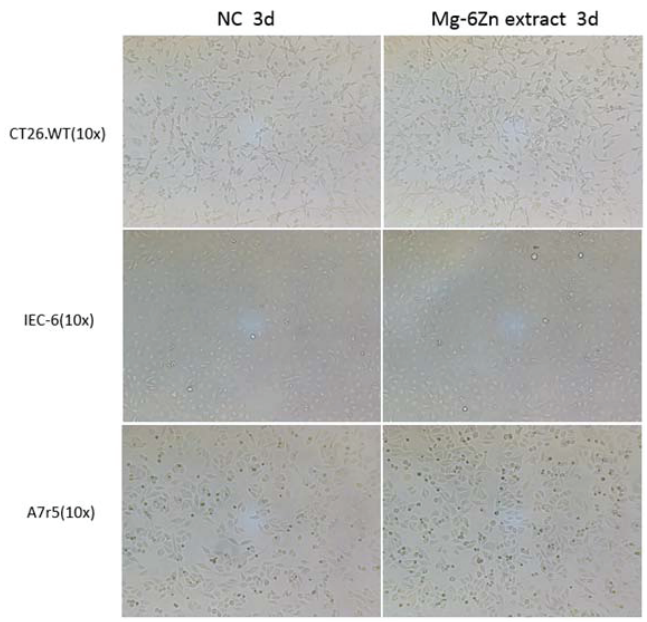

Figure 1: Cell morphology of IEC-6, CT26, A7r5 cells cultured in 40% concentration of Mg alloy extracts for 3d.

24, 48 and 72 h (Figure 2). In CT26 cells, MTT results were significantly difference under the stimulation of 40%, 60%, 80%, 100% concentration conditions when compared with the control group (0%) after 48 and 72h.While no significant difference was observed in CT26 cells after 24h (Figure 2). It was indicated that the Mg alloy had an obvious effect on colon cancer cell growth when the time of Mg alloys extracts culture was prolonged ($P<0.05$).

Cell Cycle

Cell cycle analysis demonstrated that 80% of IEC-6 cells accumulated in the G1 phase on third day after cultured with 0%, 40%, 80%, and 100% concentration of Mg alloys extracts. In CT26 and A7r5 cells, G1cells account for about 70%, along with decreased populations in the S and G2/M phases (Figure 3). There was no significant change in the cell cycle of three types of cell lines cultured by Mg alloys extracts for 3 days when compared each other among different concentrations ($P>0.05$). When compared the percentages of G1 phase of IEC-6 and CT26 cells, there were no significant difference ($P>0.05$).

Western Blot

Figure 4 presented the relative protein expression levels of TGF-β1, VEGF, bFGF and TNF-α of IEC-6 and CT26 when cultured in the 0%, 40%, 80%, and 100% concentration of Mg alloy extracts media for 3 day (Figure 5) indicated the statistical analysis of protein expression levels of TGF-β1, VEGF, bFGF and TNF-α of IEC-6 and CT26 in different concentrations. From the results,TGF-β1, VEGF, bFGF and TNF-α

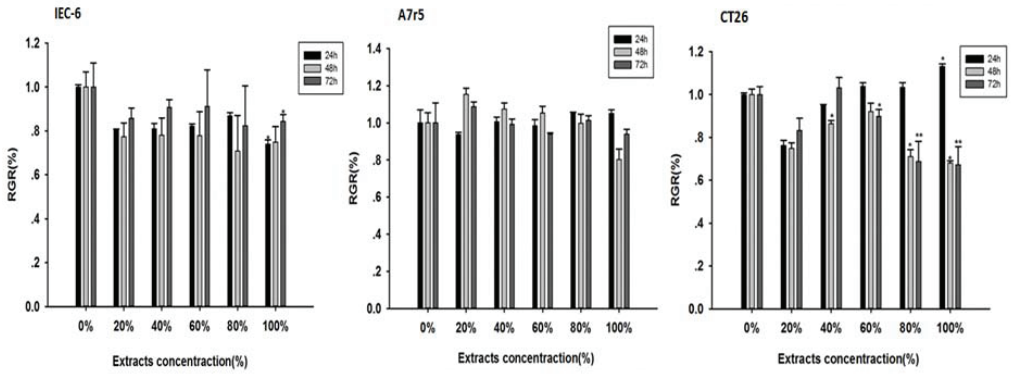

Figure 2: The cell Relative Growth Rate (RGR) of IEC-6, CT26, A7r5 in different extracts (100%, 80%, 60%, 40%, 20% and 0 %concentrations) after 24, 48 and 72 h by the MTT assay. The statistical significance was indicated by *($P<0.05$).

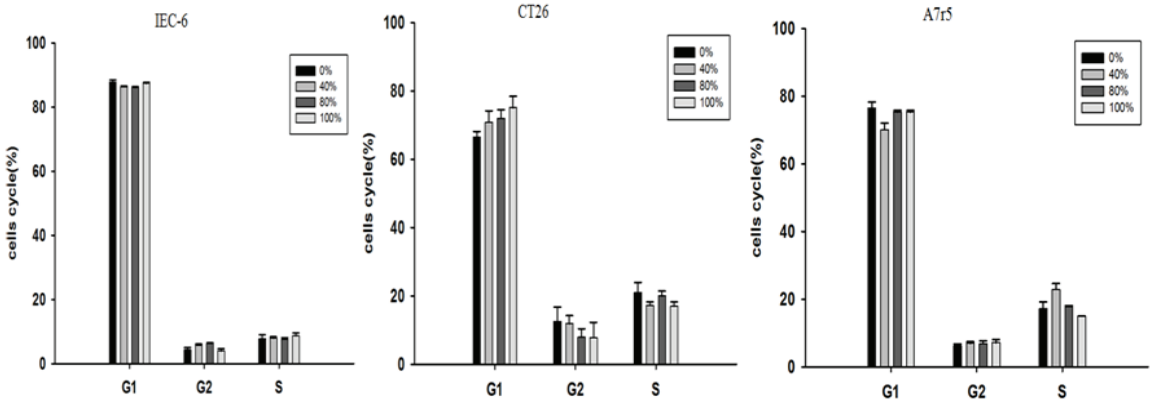

Figure 3: Comparison of Cell cycle of IEC-6, CT26, A7r5 in different concentrations of Mg alloys extracts for 3days.

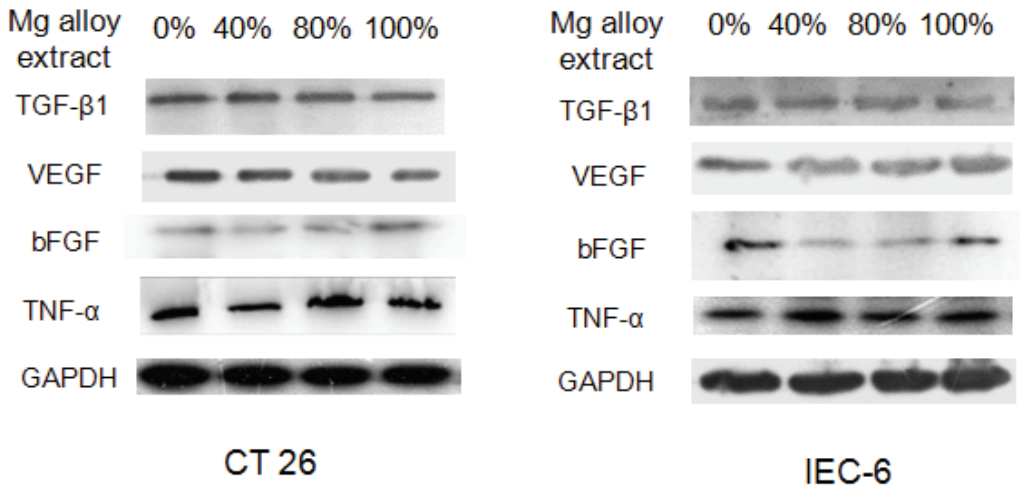

Figure 4: Western blot results of IEC-6, CT26 in0%, 40%, 80%, 100% concentrations of Mg alloy extracts media.

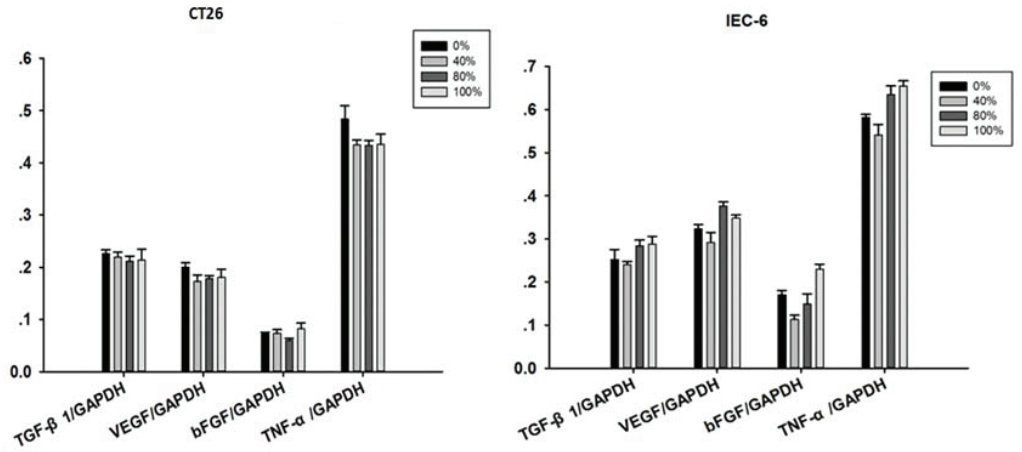

Figure 5: Statistical analysis of expression levels of TGF-β1, VEGF, bFGF and TNF-α at different Mg alloy extracts concentrations in IEC-6 and CT26 cells.

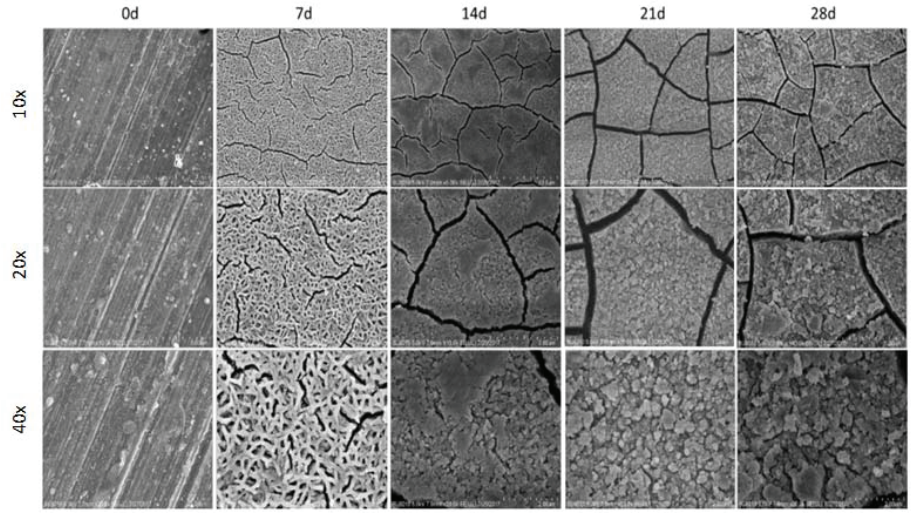

Figure 6: Surface morphology of Mg alloys in intestinal juice for 4 weeks under the electron microscope.

expression levels were slight up-regulated in IEC-6 cells, and slight down-regulated in CT26. No significant differences were observed both in CT26 cells and IEC-6 cells when compared with each other in different concentrations (P>0.05).

Degradation of Mg Alloys

In Figure 6, the long term immersion of Mg alloys samples is illustrated with different timelapse photography. The polished samples were immersed in intestinal juice and the degradation morphology was examined. The black corrosion product was formed on the surface of all Mg samples at 7d. After 3 weeks of immersion, Mg alloys was subjected to slight corrosion, losing the structural integrity. After 4 weeks of immersion, Mg alloys faced severe dramatic corrosion in the intestinal juice.

Discussion

Metallic biomaterials are widely used in tissue engineering applications and regenerative medicine. The mechanical and electrochemical properties of Mg alloys also make them suitable for broad biomedical applications, including orthopedic implants and vascular stents [1-3]. Application of Mg alloys reduces healthcare cost and patient morbidity by eliminating the second surgery to remove implants, this makes Mg-based alloys as potential bioresorbable implants [4,5].

There are several critical criteria's to evaluate metallic biomaterials: biocompatibility, bio-corrosion, and non-toxic corrosion products. Mg and Zn ions are some of the main ions in the body, without any toxic effect. Chemical compositions of Mg-6Zn alloy are beneficial elements for human health [6,11,12].

Clinically there are some patients with acute intestinal obstruction caused by advanced tumor, cannot tolerate surgery because the whole physical conditions are bad, then the minor trauma intervention operation is optional, by placing the intestinal stents, acute intestinal obstruction can be solved, thus win opportunities for further treatment. Biodegradable properties of magnesium alloys in combination with its good biological compatibility probably make Mg alloys stents be an ideal choice for the treatment of acute intestinal obstruction in patients with advanced intestinal cancer.

Mg-based alloys used as a degraded metal stent and the effect on intestinal tract after interventional operations were rarely reported, while there were many reports about biodegradable Mg-based alloys in orthopedic and cardiovascular areas [2,3]. After Mg alloys stents are implanted in the intestinal tract, the interaction between Mg alloys stents and tissue cells in the intestinal wall is a highly dynamic process and involves complex interactions, the effect of Mg-6Zn alloy on the proliferation, apoptosis and protein expression of the intestinal wall cells such as intestinal epithelial cells, smooth muscle cells and colon cancer cells should be considered.

Based on this novel consideration, an *in vitro* study on the effect of Mg-6Zn alloy extract to IEC-6, CT26 and A7r5 cells was preliminarily performed in order to provide an evaluation of the effect of Mg alloy on surrounding cells when used as a degraded metal stent in the intestinal tract in interventional operations. Cytotoxicity assays were among the first *in vitro* bioassay methods used to predict toxicity of substances to various tissues. MTT assay is well-characterized, simple to use and remains popular in several laboratories worldwide. The MTT assay is a colorimetric viability assay based on enzymatic reduction of the MTT molecule to formazan when it is exposed to viable cells. The outcome of the reduction is a color change of the MTT molecule. Absorbance measurements relative to a control determine the percentage of remaining viable cells following their treatment with varying concentrations of a tested compound [23,24].

In our study, MTT results represented that all Mg alloys extracts had no significant toxic effects on IEC-6, A7r5 cells after culturing the cells for 24, 48 and 72 h. But in CT26 cells, there were significant differences in cell Relative Growth Rate (RGR) under the stimulation of 40%, 60%, 80%, 100% concentration conditions when compared with the control group (0%) after 48,72h. Mg alloy has an obvious effect on colon cancer cell growth when the time of Mg alloys extracts culture was prolonged (P<0.05), this result suggested that the interactive time might be an important factor in the cytotoxicity evaluation of biodegradable Mg-6Zn alloy.

Magnesium (Mg) based alloys, have been proved to be easily corroded, it has been well recognized that degradation of Mg based metals in body fluid could obviously increase the local alkalinity around the Mg metals [25-27]. There were studies indicating that an acidic environment could be beneficial for survival of cancer cells, but they could not be easily survived in the alkaline environment, which implies that a great increase of alkalinity around cancer cells in human body may help to kill them or prevent the cancer recurrence or proliferation [28]. This good biological compatibility and effect on colon cancer cells probably make Mg alloys be an ideal choice for bioresorbable implants to resolve some clinic problems, such as stents to treat acute intestinal obstruction in patients with advanced intestinal cancer.

The toxicity of alloys may relate to the arrest of cells in specific cell cycle phases. Induction of cell cycle delay may provide more time for the repair of damaged DNA and prevent gene mutation. However, prolonged exposure to toxicants and cell cycle delay may lead to cell death and apoptosis [26,27]. We performed flow cytometric to analysis cell cycle distribution of cells, there was no significant change in the cell cycle of three types of cell lines cultured by Mg alloys extracts for 3 days when compared each other among different concentrations (P>0.05), and the percentages of G1 phase of IEC-6 and CT26cells were no significant difference (P>0.05). This result indicated that the Mg alloy has good biological compatibility with IEC-6 and A7r5 cells. Although Mg-6Zn alloy extracts had no effect on the cell cycle of CT26 cell, but biodegradable properties of magnesium alloys in combination with its good biological compatibility still made Mg-6Zn alloy as a suitable candidate to be used in biomedical applications.

Complex interactions might be involved between Mg-6Zn alloy stents and the intestinal wall cells including Extracellular Matrix (ECM) molecules, soluble mediators, various resident cells, and infiltrating leukocyte subtypes. The synthesis of numerous potent growth factors was observed in this study, such as transforming growth factor-β (TGF-β), TGF-α Basic fibroblast Growth Factor (bFGF), Platelet-Derived Growth Factor (PDGF), and Vascular Endothelial Growth Factor (VEGF), which promote angiogenesis, cell proliferation and the synthesis of ECM molecules by resident cells [29-31]. The function of transforming growth factor beta 1 (TGF-β1) is to adjust cell growth and proliferation. TGF-β1 activates repair cells and induces other growth factors. TGF-β1 plays an important role in wound repair by adding signals important for the initiation of the healing cascade and by attracting macrophages and stimulating them to secrete additional cytokines, including Fibroblast Growth Factor (FGF), Platelet-Derived Growth Factor (PDGF), tumor necrosis factor (TNF-α), and interleukin-1 (IL-1) [32,33]. Vascular Endothelial Growth Factor (VEGF) is released by a variety of cells and stimulates multiple components of the angiogenic cascade. It is up-regulated during the stage of tumor development. VEGF stimulates tissue growth via multiple mechanisms including collagen deposition, angiogenesis and epithelization [34,35]. Tumor necrosis factor (TNF, tumor necrosis factor alpha, TNFα) is a cell signaling protein (cytokine) involved in systemic inflammation and is one of the cytokines that make up the acute phase reaction [36,37]. TNF was thought to be produced primarily by macrophages, but it is produced also by a broad variety of cell types [38]. A local increase in concentration of TNF will cause the cardinal signs of inflammation to occur: heat, swelling, redness, pain

and loss of function [39]. Previous studies reported that Mg-6Zn alloy had good biocompatibility *in vivo* and performed better than medical Ti (Ti-3Al-2.5 V alloy) in promoting healing and reducing inflammation [19,40-42].

In this study, western blot results indicated that the protein expression levels of TGF-β1, VEGF, bFGF and TNF-α in IEC-6 and CT26 cells had no significant differences in the Mg alloy extract media for 3 day. Different concentrations of Mg alloy extracts had no effect on protein expression levels in three kinds of cells, the concentrations of Mg alloy extracts might not be an important factor to affect the expression levels of cell factors at one time point, as for time effect was an factor to affect the expression levels of cell factors or not, further studies are needed to confirm.

Finally, we had assessed the biodegradation capability ofMg-6Zn alloy with long term immersion in physiologic intestinal juice. The intestinal lavage solution prepared in this study kept the micro-environmental conditions of the intestinal tract as much as possible, such as the contents of the intestinal fluid, and the intestinal microorganism, so that the conditions *in vivo* could be simulated better. After 3 weeks of immersion, Mg alloys was subjected to slight corrosion, losing the structural integrity. After 4 weeks of immersion, Mg alloys faced severe dramatic corrosion in the intestinal juice, this result was consistent with previous study [19]. The results indicate that the Mg-6Zn alloys have good biodegradation capability under intestinal physiologic condition. There are numerous biodegradable Mg-based alloys containing zinc in amounts of several wt.%, like Mg-Zn, Mg-Zn-Mn-Ca, Mg-Zn-Y, and Mg-Zn-Si [13,14]. Mg-Zn-based alloys containing about 50 wt.% of Zn have excellent strength, high corrosion resistance [16]. Additions of Zn or other metal coating [43] could improve the corrosion resistance and strength of the alloys to meet the conditions of biomedical implants.

Conclusion

Different concentrations of Mg-6Zn alloy extracts had no effect on the proliferation and apoptosis of IEC-6, A7r5 and CT26 at 24h. But when the culture time was prolonged, Mg alloy extracts had an obvious inhibitory effect on colon cancer cell CT26 growth. Mg-6Zn alloy had good biodegradation capability under intestinal physiologic condition. Mg-6Zn alloy could be suggested as a suitable candidate of biodegradable implants to be used in biomedical applications.

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Compliance with Ethical Standards

Animal procedures were conducted according to the ethical guidelines of Shanghai Jiao Tong University.

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