The effect of Mg-2Zn-0.5Nd alloy on the mTOR signalling pathway in L6 cells

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Abstract. Magnesium alloys have shown potential as biodegradable metallic materials for orthopaedic applications due to their degradability, and their resemblance to cortical bone and biocompatible degradation/corrosion products. However, the fast corrosion rate and the potential toxicity of their alloying element has limited the clinical application of Mg alloys. In the present study, a novel Mg-2Zn-0.5Nd alloy was prepared, and then the effects on the cell biological behaviour of the Mg-2Zn-0.5Nd alloy was compared with 317L stainless steel and titanium (Ti-6Al-4V) alloys as controls. The L6 cells were cultured in various leaching solutions. The proliferative effect of the Mg-2Zn-0.5Nd alloy was determined using the Cell Counting Kit-8 assay method on the L6 cells. Also, the regulation of key intracellular signalling proteins was investigated in the L6 cells by the western blot analysis. The Mg-2Zn-0.5Nd alloy showed no cytotoxicity and induced higher levels of proliferation in the myoblast cell line L6 than the other alloys. Molecular analysis demonstrated that Mg-2Zn-0.5Nd had stimulatory effects on bone morphogenetic protein-2 phosphorylation and on the activity of phosphorylated-mammalian target of the rapamycin (mTOR), protein kinase B and forkhead box protein O1. Mg-2Zn-0.5Nd also had no effect on P38 activity. These results suggested that Mg-2Zn-0.5Nd is likely to promote myoblast cell proliferation by activating the mTOR signalling pathway.

Introduction

Recently, degradable materials developed as orthopaedic implants have attracted much attention since their use could avoid the necessity for a secondary operation to remove the implants. Among the possible materials, magnesium and its alloys are the most promising due to their degradability, suitable mechanical properties and good biocompatibility (1,2). Several magnesium alloys, such as WE43 (3), AZ91 (4), Mg-Zn (5), Mg-Ca (2) and Mg-Mn-Zn (3) show great potential in clinical application. More specifically, previous *in vivo* experiments have identified the gradual degradability of Mg alloys in bone tissue. In addition, the degradation products induced an appropriate level of inflammatory response (6). However, the rapid corrosion rate of Mg alloys is still a significant obstacle in the process of clinical applications (5).

Researchers have tried various ways to deal with this challenge. Among these, it has been demonstrated that alloying is the most effective approach to manipulate the corrosion resistance and mechanical properties of Mg alloys. For the sake of safety and human body tolerance, only a small number of alloying elements are suitable for inclusion in biodegradable Mg alloys, such as Zn, Nd, Ca, Sr, Mn and several rare earth elements (7,8).

Zn is one of the essential elements in the human body (9). Mg-Zn-based alloys are very promising because not only are they the second strongest ductile alloy system, but their corrosion rates can also be greatly reduced by utilizing certain strategies. More importantly, Mg-Zn-based alloys may be RE (rare earth) free. It has been shown that Mg-Zn-based alloys are the second strongest alloying system with varying corrosion rates. They could be RE-free systems which compete with the Mg-RE-based alloys and which are used in RE-sensitive implants (10). Meanwhile, it is reported that Mg-2Nd alloys have characteristically high elongation ratios, and they improve the yield strength and degradation rate (11). The addition of light RE elements to a magnesium alloy can not only improve its corrosion resistance and mechanical properties, but also help to improve the anti-coagulation behaviour of biological implants. The element Nd is a rare earth element with minimal toxicity. A small amount of Nd can be added to a magnesium alloy, without causing any significant cytotoxicity in experiments (12). The skeletal muscle as the dynamical device of the motor system, is attached to the skeleton, which is of great significance to the movement of the joints. We may implant materials to repair injuries of the motor system. Whether it is suture or other internal fixation materials, it is inevitable that the muscle is contacted. But the biocompatibility of magnesium alloys to skeletal muscle is not clear, and it is not sure

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whether magnesium alloys has any effect on the adhesion and proliferation of skeletal muscle cells. So the skeletal muscle biocompatibility of magnesium alloys is of great significance in the research process of implant materials.

Bone morphogenetic protein 2 (BMP-2), which is a member of the transforming growth factor (TGF)-β superfamily, has profound effects on the osteoblast activity (13,14). Many studies have found that BMP-2 not only exists in the bone matrix, but it is also present in other tissues (15). In recent years, other researchers have found that BMP-2 is involved in the regulation of the proliferation, differentiation and apoptosis of many types of cells, thus affecting their biological behaviour (16,17).

Intracellular kinase signalling plays an important role in many biological functions including cell differentiation (18,19). Adenosine monophosphate-activated protein kinase (AMPK) is a principal intracellular energy sensor which activates energy-producing pathways (20). Moreover, AMPK activation can mediate the downstream signalling response of the phosphoinositide 3-kinase (PI3K)/Akt, mitogen-activated protein kinases (MAPK) and the mammalian target of the rapamycin (mTOR) pathway (21). mTOR as a serine/threonine protein kinase can regulate cell proliferation (22,23), and also plays an important role in cell apoptosis and survival (24,25).

These beneficial effects of Zn and Nd prompted us to investigate the feasibility of alloying Zn-Nd with Mg and the corresponding effects on the corrosion properties and biocompatibility of the resulting alloy. In this study, a Mg-2Zn-0.5Nd alloy was designed and prepared. To date there have been no systematic researches on Mg-2Zn-0.5Nd alloy systems for biomedical applications. The purpose of the present study was to investigate the effect of Mg-2Zn-0.5Nd on the expression of BMP-2- and mTOR-related signalling proteins. The purpose of this study is to clarify the effect of Mg-2Zn-0.5Nd alloy on the proliferation of skeletal muscle cells, and to explore the effect of Mg-2Zn-0.5Nd on the expression of BMP-2 in skeletal muscle cells and mTOR related signal proteins.

Materials and methods

Material preparation. Alloys of 317L, Ti-6Al-4V and Mg-2Zn-0.5Nd were prepared in the Institute of Metal Research (Chinese Academy of Science, Shenyang, China). Plate samples with a diameter of 10 mm and a thickness of 1 mm were prepared. Cylindrical rods with a diameter of 1 mm were machined for implantation into mice. All samples went through ultrasonic cleaning in acetone, absolute ethanol and distilled water for 10 min each and then sterilization with ethylene oxide.

The leaching solution was prepared in accordance with the ISO 10993-5: 2009 standard (26). Specifically, plate samples were immersed in complete DMEM with 10% foetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin and incubated at 37˚C for the indicated duration. The extracts were analysed using inductively coupled plasma optical emission spectroscopy (ICP-OES; VISTAPRO; Agilent Technologies, Inc., Santa Clara, CA USA) to determine the elemental concentrations of Mg, Zn and Nd.

Culture of L6 cells. The L6 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in complete Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were grown in a humidified atmosphere containing 5% CO_2 at 37˚C. Before the experiment, cells ($5x10^5$ cells/well in 6-well plates) were grown for 24 h. The next day, cells were treated with different concentrations of extraction medium. The biological morphology of skeletal muscle cells in each group was observed by an inverted microscope after 72 h.

Cell proliferation assay. The proliferative effect of the leaching solution on L6 cells was determined using the CCK-8 kit (Dojindo Molecular Technology, Kumamoto, Japan). Cells were plated in 96-well plates at $5x10^3$ cells/well in triplicate. After 1, 3 and 5 days of culture, 90 μl of culture medium and 10 μl of CCK-8 solution were added to each well at each time-point and incubated at 37˚C for another 4 h. The optical density (OD) was measured using an ELX800 absorbance microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA) at 450 nm (650 nm reference).

Western blot analysis. Aliquots containing $2x10^6$ cells per well were plated into 6-well plates and cultured in various leaching solutions for the periods indicated. Then the L6 cells were harvested and washed with cold PBS, lysed for 30 min on ice and then centrifuged for 10 min at 12,000 x g at 4˚C. The supernatants were collected, mixed with loading buffer, and boiled for 10 min. Electrophoresis was performed on 12% SDS-PAGE for 3 h and then proteins were transferred onto PVDF membranes in transfer buffer (containing 20 mM Tris, 20% methanol, and 150 mM glycine) at 200 mA for 70 min. The membrane was incubated in non-fat dried milk for 2 h. After washing with TBST three times the membrane was incubated with primary antibodies against BMP-2, p-mTOR, p-AKT, FoxO1 and p38 overnight at 4˚C. Membranes were incubated with the appropriate secondary antibodies conjugated with IRDye 800CW (molecular weight, 1,166 kDa), and antibody reactivity was detected by exposure in an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA). Each group was repeated 10 times and the gray value was calculated by Image J method. The gray value of BMP-2, t-mTOR, t-AKT, t-FoxO1 and t-P38 to GAPDH protein was used as the protein expression in BMP-2, t-mTOR, t-AKT, t-FoxO1 and t-P38 groups. The gray value of p-mTOR, p-AKT, p-FoxO1, and p-P38 to t-mTOR, t-AKT, t-FoxO1, and t-P38 is the relative expression of p-mTOR, p-AKT, p-FoxO1, and p-P38.

Statistical analysis. The data are presented as the mean ± standard error mean of three independent experiments. One-way analysis of variance was performed with a Bonferroni post hoc test to analyse the results using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

The biological morphology of skeletal muscle cells. As shown in Fig. 1, we found that after being cultured 72 h with different extracts, the cells of each group adherent growth, appears

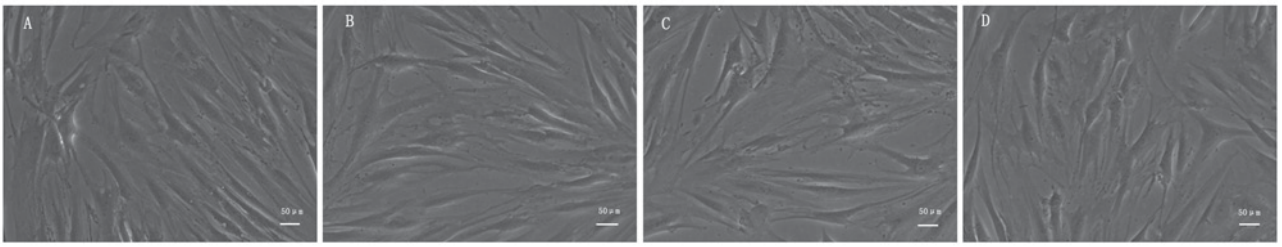

Figure 1. Rat skeletal muscle cells following culture with different extracts for 72 h. (A) Control group, (B) 317L alloys group, (C) Ti-6A1-4V alloys group and (D) Mg-2Zn-0.5Nd alloys group. The cells were cultured for 72 h with different extracts; the cells were observed to be spindle shaped and had grown well, without a visible difference between the different groups. Scale bars=50 μm.

cell clusters, the number of long spindle skeletal muscle cells increased significantly, gradually becoming slender and interconnected to a network. The growth state of the cells in each group was good, but no difference was observed between the different groups.

The effect of Mg-2Zn-0.5Nd on cell proliferation. Cell proliferation was determined by CCK-8 assay after incubating Mg-2Zn-0.5Nd with L6 cells for 24 h (Fig. 2). Cell proliferation is expressed as relative growth rates (RGR) as determined by RGR (%)=(OD sample/OD negative control) x100%. The CCK-8 values were calculated based on means ± standard deviations from 5 wells (SD, n=5). The differences between the groups were considered statistically significant at P<0.05. With Mg-2Zn-0.5Nd, we observed an increase in cell proliferation, indicating that Mg-2Zn-0.5Nd promoted cell growth and proliferation. In contrast to Mg-2Zn-0.5Nd, incubation with 317L alloys and the Ti-6Al-4V groups resulted is no significant increase in cell proliferation, indicating that Mg-2Zn-0.5Nd has significantly better bioactivity than the other two alloys.

Mg-2Zn-0.5Nd stimulates the phosphorylation of BMP-2 in L6 cells. In this study, the BMP-2 protein content of L6 cells cultured for 24 h with leaching solution from the indicated alloys was determined by the western blot analysis. As shown in Fig. 3, cells cultured with Mg-2Zn-0.5Nd exhibited the highest phosphorylation level of BMP-2. No increase in BMP-2 phosphorylation was observed when the L6 cells were cultured with 317L alloys and Ti-6Al-4V alloys.

Mg-2Zn-0.5Nd stimulates the activity of p-mTOR in L6 cells. In order to identify whether Mg-2Zn-0.5Nd promoted the proliferation of skeletal muscle cells via mTOR-related signalling pathway, the western blot analysis was performed to examine mTOR protein expression *in vitro*. As shown in Fig. 4, in comparison with the control group, Mg-2Zn-0.5Nd treatment significantly upregulated p-mTOR expression, while 317L alloys and Ti-6Al-4V alloys caused no significant change in p-mTOR expression.

Mg-2Zn-0.5Nd stimulates the activity of AKT in L6 cells. In this present study, our results show that the activation of p-AKT proteins is significantly increased in the Mg-2Zn-0.5Nd group, while co-culture with 317L alloys and Ti-6Al-4V alloys does not affect the expression of p-AKT proteins (Fig. 5). These results suggest that Mg-2Zn-0.5Nd affects mTOR activity of L6 cells partly through the AKT-mTOR axis.

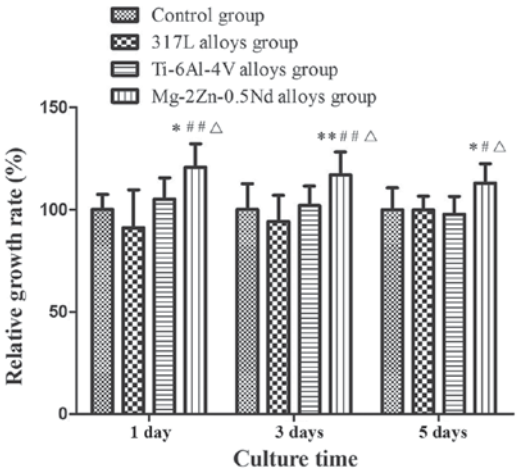

Figure 2. Proliferation rate of rat skeletal muscle cells cultured with different extracts (n=5). To test the effect of the degradation products of various alloys on cell biological behaviour, the present study compared the effects of the Mg-2Zn-0.5Nd alloys group with the other three groups. The L6 cells were cultured in various leaching solutions for the indicated times. The relative growth rates of the different groups of L6 cells were assessed using the Cell Counting Kit-8 assay at different time points following incubation. Compared with the control, 317L alloys and Ti-6Al-4V alloys groups, the cell proliferation rate of Mg-2Zn-0.5Nd following 1, 3 and 5 days markedly increased. *P<0.05 and **P<0.01 vs. control group; $^{\#}$P<0.05 and $^{\#\#}$P <0.01 vs. 317L alloys group; $^{\Delta}$P<0.05 vs. Ti-6Al-4V alloys group.

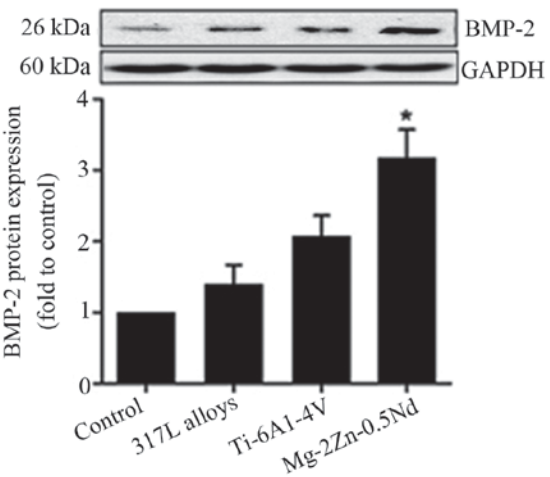

Figure 3. Expression of BMP-2 protein in the different groups. Western blot analysis was performed to investigate the phosphorylation of BMP-2 in the different groups of L6 cells. Compared with the control, 317L alloys and Ti-6Al-4V alloys groups, the expression of BMP-2 in the Mg-2Zn-0.5Nd group markedly increased. *P<0.05 vs. control. BMP-2, bone morphogenetic protein 2.

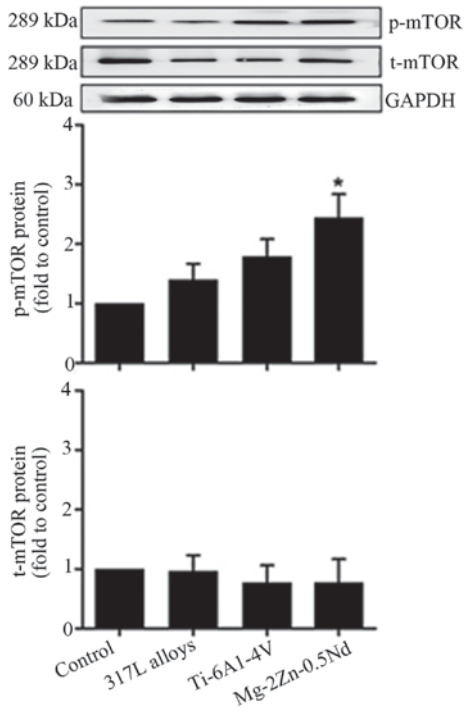

Figure 4. Expression of mTOR protein in the different groups. Western blot analysis was performed to investigate p-mTOR expression in the different L6 cell groups. Compared with the control, 317L alloys and Ti-6Al-4V alloys groups, the expression of p-mTOR in the Mg-2Zn-0.5Nd group markedly increased. *P<0.05 vs. control. mTOR, mammalian target of the rapamycin; p-, phosphorylated; t-, total.

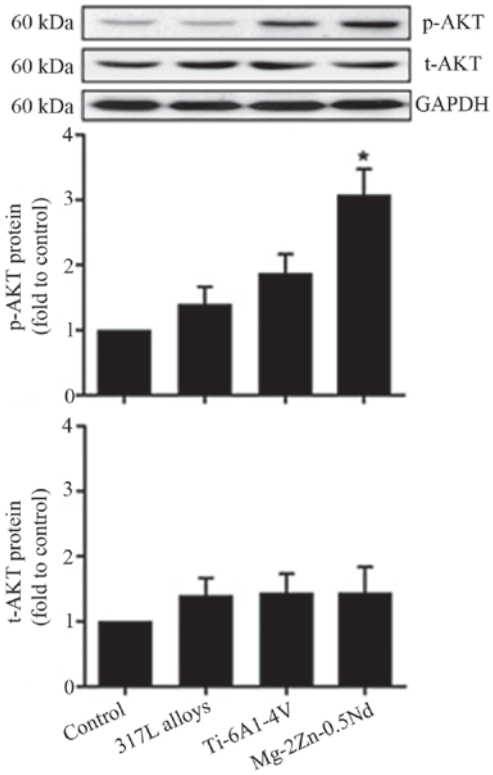

Figure 5. Expression of AKT protein in the different groups. Western blot analysis was performed to investigate AKT expression in the different L6 cell groups. Compared with the control, 317L alloys and Ti-6Al-4V alloys groups, the expression of p-AKT in the Mg-2Zn-0.5Nd group markedly increased. *P<0.05 vs. control. AKT, protein kinase B; p-, phosphorylated; t-, total.

Mg-2Zn-0.5Nd stimulates the activity of FoxO1 in L6 cells. In order to identify whether FoxO1 is involved in the Mg-2Zn-0.5Nd-induced proliferation of skeletal muscle cells *in vitro*. We undertook western blot experiments to study the effects of Mg-2Zn-0.5Nd on the activation of FoxO1 proteins. As shown in Fig. 6, our results show that the expression of p-FoxO1 proteins is significantly increased in both the Mg-2Zn-0.5Nd and Ti-6Al-4V alloy groups, while co-culture with 317L alloy does not affect the expression of p-FoxO1 proteins.

Mg-2Zn-0.5Nd has no effect on P38 activity in L6 cells. To gain further insight into the molecular mechanisms by which Mg-2Zn-0.5Nd participates in cell proliferation, the phosphorylation of p38/MAPK in L6 cells was examined. As shown in Fig. 7, the p38 protein expression could be identified in all groups, however there was no significant difference between the groups. These data suggest that p38/MAPK might not participate in Mg-2Zn-0.5Nd-induced changes in cell proliferation.

Discussion

Extensive studies have been carried out on Mg alloys as biodegradable materials. Mg alloy has been regarded as a promising candidate for bone implants because of its biodegradability and special mechanical properties (27). However the fast corrosion rate, release of hydrogen gas and the lack of long-term mechanical integrity of the implants are the most critical obstacles for the clinical applications of Mg alloys. Previous research provided various solutions such as polymer coatings, composition optimization, corrosion potential optimization and so on, in an attempt to retard the rapid corrosion reaction of Mg alloys (10).

Current research has mainly focused on reducing the degradation rate, and promoting the mechanical and biological properties (8,28,29). Specifically, researchers hope to develop a new magnesium alloy of the indicated ratios, one proved to be an ideal alloy system with appropriate mechanical performance, stable degradation speed and favourable biocompatibility. Since Mg-based alloys are biodegradable, Mg alloy degradation induces dynamic micro-environmental changes. Therefore, much effort is needed to fully understand how the alloy degradation process evokes physiological reactions, especially for the sake of safety in considering potential usage in humans (30,31).

Zn is one of the essential elements in the human body, acting in a pivotal role in mediating the activity of hundreds of enzymes (9). In its ionic form, Zn is also involved in the cell metabolism (32,33). As implant-related material, Mg-Zn-based alloys exhibit the lowest strength and ductility with varying corrosion rates. More importantly, Mg-Zn-based alloys could be RE-free systems which could compete with the Mg-RE-based alloys and could be used in RE-sensitive implants (10).

Meanwhile, the addition of light rare earth elements to a magnesium alloy can not only improve the corrosion resistance and mechanical properties of the alloy, but also help to improve the anti-coagulation behaviour of biological implants.

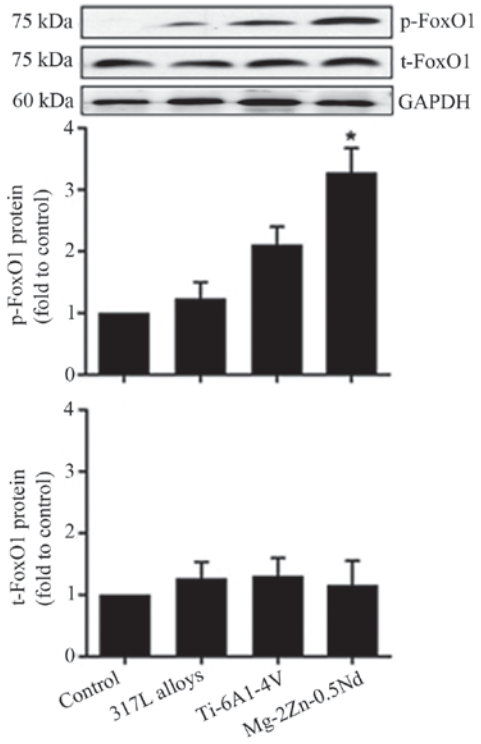

Figure 6. Expression of FoxO1 protein in the different groups. Western blot analysis was performed to investigate FoxO1 expression in the different L6 cell groups. Compared with the control group, 317L alloys and Ti-6Al-4V alloys groups, the expression of p-FoxO1 in the Mg-2Zn-0.5Nd group markedly increased. *P<0.05 vs. control. FoxO1, forkhead box protein O1; p-, phosphorylated; t-, total.

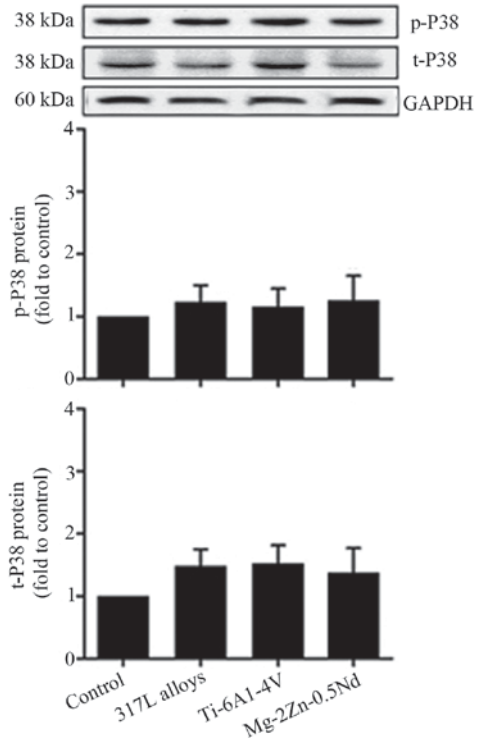

Figure 7. Expression of P38 protein in the different groups. Western blot analysis was performed to investigate P38 expression in the different L6 cell groups. p38 protein expression was observed in all of the groups; however, there no significant differences were identified between the groups. p-, phosphorylated; t-, total.

The element Nd is a rare earth element with minimal toxicity. When a small amount of Nd is added to a magnesium alloy in an experimental situation, no significant cytotoxicity is found (12). More importantly, it has been reported that Mg-2Nd alloys have the characteristic of a high elongation ratio, which improves the yield strength and degradation rate (11).

As a new kind of magnesium alloy, the microstructure, mechanical properties and degradation properties of Mg-2Zn-0.5Nd alloy have been previously studied. It showed excellent plastic deformation properties and moderate strength, and the corrosion resistance was significantly higher (34), so it has a good prospect of clinical application. But before the clinical application, it is necessary to evaluate its biocompatibility. At present; the research of magnesium alloy materials is mainly based on the study of bone tissue and osteoblasts. There are few studies related to the skeletal muscle. But without the study of the effect of magnesium alloy on skeletal muscle, the biocompatibility of magnesium alloy materials is not comprehensive. The skeletal muscle as the dynamical device of the motor system is attached to the skeleton, which is of great significance to the maintenance of the posture of the human body and the movement of the joints. We may implant materials to repair injuries of the motor system. Whether it is suture or other internal fixation materials, it is inevitable that the muscle is contacted. The implant material is good, not only need to meet the excellent biocompatibility of osteoblasts, but also to ensure the good biocompatibility of skeletal muscle cells. The skeletal muscle biocompatibility of implanted materials is of great significance in the research process of implant materials.

We attempted to study the effect of Mg-2Zn-0.5Nd alloy on the proliferation of L6 cells, by culturing different alloy extracts with rat skeletal muscle cells. The results showed that in the Mg-2Zn-0.5Nd alloy group, the relative cell proliferation rate was higher than in the 317L group or the Ti-6Al-4V alloy group, and the difference was significant. Our study showed that Mg-2Zn-0.5Nd alloy has the ability to improve the proliferation of L6 cells. In order to clarify the mechanism of Mg-2Zn-0.5Nd alloy involved in improving the adhesion and proliferation of L6 cells, we analysed the expression of intracellular related proteins in the experimental groups.

BMP is a member of the TGF family. Early studies have shown that BMP-2 can induce bone and cartilage formation *in vivo* and play an important role in bone regeneration and repair. Later it was found that BMP-2 not only exists in the bone matrix, but it is also found in other tissues. Musgrave *et al* (35) found that skeletal muscle satellite cells can also express BMP-2. In recent years, other researchers have found that BMP-2 is involved in the regulation of proliferation, differentiation and apoptosis of many types of cells, thus affecting their biological behaviour (36-38). *In vitro* experiments, Wei *et al* (39) found that BMP-2 could promote the adhesion and proliferation of skeletal muscle satellite cells.

Our experiment found that the expression of BMP-2 in the Mg-2Zn-0.5Nd alloy group was higher than that in the 317L alloy group or the Ti-6Al-4V alloy group, suggesting that Mg-2Zn-0.5Nd alloy can effectively promote the expression of BMP-2 and may play an important role in promoting proliferation via the action of BMP-2.

How does Mg-2Zn-0.5Nd promote the expression of BMP-2? Studies have shown that the BMP-2 receptor (serine/threonine kinase) is regulated by PI3K/AKT and the MAPK pathway (40). AKT kinase, which is activated by growth factors, hormones and drugs, regulates cell proliferation and survival (41). It has been proved that magnesium ions can activate the PI3K/Akt signaling pathway (42). In the process of degradation, magnesium alloys can release magnesium ions to regulate the expression of Akt and activate the downstream target proteins, and then affect cell adhesion, proliferation and differentiation. mTOR is a downstream factor of AKT which induces cell differentiation (22,43). mTOR as a serine/threonine protein kinase plays an important role in BMP2-induced changes in cell metabolism (44). A previous study demonstrated that the AKT-mTOR signalling axis plays a vital role in mediating proliferation and apoptosis (45). To be more specific, mTOR negatively regulates autophagy, which is manipulated by several upstream activators such as PI3K-AKT and MAPK (46). Phosphorylation refers to the addition of a phosphate group to a protein or other type of molecule, thereby changing its activity. p-mTOR is the active form of mTOR, and the activation of AKT is dependent on the phosphorylation of AKT. Therefore, we measured the levels of both p-AKT and p-mTOR, and found that both p-AKT and p-mTOR were increased in the Mg-2Zn-0.5Nd alloy group. Furthermore, we speculated that Mg-2Zn-0.5Nd can activate mTOR via the PI3K/AKT pathway, and thus increase the expression of BMP-2.

FoxO1 is also one of the essential transcription factors in the regulation of cell proliferation and differentiation (47). Specifically, FoxO1, which is expressed in skeletal muscle cells, Inhibit the proliferation of skeletal muscle cells (48). Yamashita *et al* (49) found that an increased expression of FoxO1 reduced the proliferation of muscle cells, and suggested that FoxO1 could inhibit the proliferation of skeletal muscle cells *in vitro*. In this study, p-FoxO1 levels were increased, suggesting that Mg-2Zn-0.5Nd alloy can activate p-FoxO1, thereby inhibiting the excessive growth of cells. In our experiments, we found that the expression of p-P38 was not increased. p-P38 is the active form of P38 and the upstream factor of the MAPK pathway. We therefore speculate that Mg-2Zn-0.5Nd alloy does not affect the differentiation of L6 cells through the p38/MAPK pathway.

Above all, in the present study we demonstrate that the novel alloy Mg-2Zn-0.5Nd shows no cytotoxicity *in vitro* and even exhibits a stimulatory effect on cell proliferation. Meanwhile, further studies into the molecular mechanisms suggests that Mg-2Zn-0.5Nd may affect BMP-2 protein expression through the PI3K/AKT/mTOR pathway and thus promote the proliferation of L6 cells.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WL performed the experiments and wrote the article. LG contributed to the design of the study and revised the manuscript. TJ and SN performed the data analysis and revised the manuscript. YZ performed the western blot analysis and revised the manuscript. All the authors read and approved the final version to be published.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Gu X, Zheng Y, Zhong S, Xi T, Wang J and Wang W: Corrosion of, and cellular responses to Mg-Zn-Ca bulk metallic glasses. Biomaterials 31: 1093-1103, 2010.
2. Li Z, Gu X, Lou S and Zheng Y: The development of binary Mg-Ca alloys for use as biodegradable materials within bone. Biomaterials 29: 1329-1344, 2008.
3. Xu L, Yu G, Zhang E, Pan F and Yang K: In vivo corrosion behavior of Mg-Mn-Zn alloy for bone implant application. J Biomed Mater Res A 83: 703-711, 2007.
4. Witte F, Fischer J, Nellesen J, Crostack HA, Kaese V, Pisch A, Beckmann F and Windhagen H: In vitro and in vivo corrosion measurements of magnesium alloys. Biomaterials 27: 1013-1018, 2006.
5. Zhang S, Zhang X, Zhao C, Li J, Song Y, Xie C, Tao H, Zhang Y, He Y, Jiang Y and Bian Y: Research on an Mg-Zn alloy as a degradable biomaterial. Acta Biomater 6: 626-640, 2010.
6. Duygulu O, Kaya RA, Oktay G and Kaya AA: Investigation on the potential of magnesium alloy AZ31 as a bone implant. Materials Science Forum 546-549: 421-424, 2007.
7. Rosalbino F, De Negri S, Saccone A, Angelini E and Delfino S: Bio-corrosion characterization of Mg-Zn-X (X=Ca, Mn, Si) alloys for biomedical applications. J Mater Sci Mater Med 21: 1091-1098, 2010.
8. Song G: Control of biodegradation of biocompatable magnesium alloys. Corrosion Science 49: 1696-1701, 2007.
9. Watt NT, Taylor DR, Kerrigan TL, Griffiths HH, Rushworth JV, Whitehouse IJ and Hooper NM: Prion protein facilitates uptake of zinc into neuronal cells. Nat Commun 3: 1134, 2012.
10. Chen Y, Xu Z, Smith C and Sankar J: Recent advances on the development of magnesium alloys for biodegradable implants. Acta Biomater 10: 4561-4573, 2014.
11. Seitz JM, Eifler R, Stahl J, Kietzmann M and Bach FW: Characterization of MgNd2 alloy for potential applications in bioresorbable implantable devices. Acta Biomater 8: 3852-3864, 2012.
12. Feyerabend F, Fischer J, Holtz J, Witte F, Willumeit R, Drücker H, Vogt C and Hort N: Evaluation of short-term effects of rare earth and other elements used in magnesium alloys on primary cells and cell lines. Acta Biomater 6: 1834-1842, 2010.

13. Liu H, Zhang R, Chen D, Oyajobi BO and Zhao M: Functional redundancy of type II BMP receptor and type IIB activin receptor in BMP2-induced osteoblast differentiation. J Cell Physiol 227: 952-963, 2012.
14. Tachi K, Takami M, Sato H, Mochizuki A, Zhao B, Miyamoto Y, Tsukasaki H, Inoue T, Shintani S, Koike T, *et al*: Enhancement of bone morphogenetic protein-2-induced ectopic bone formation by transforming growth factor-β1. Tissue Eng Part A 17: 597-606, 2011.
15. Bragdon B, Moseychuk O, Saldanha S, King D, Julian J and Nohe A: Bone morphogenetic proteins: A critical review. Cell Signal 23: 609-620, 2011.
16. Yang B, Lin X, Yang C, Tan J, Li W and Kuang H: Sambucus williamsii hance promotes mc3t3-e1 cells proliferation and differentiation via bmp-2/smad/p38/jnk/runx2 signaling pathway. Phytother Res 29: 1692-1699, 2015.
17. Wei L, Lei GH, Yi HW and Sheng PY: Bone formation in rabbit's leg muscle after autologous transplantation of bone marrow-derived mesenchymal stem cells expressing human bone morphogenic protein-2. Indian J Orthop 48: 347-353, 2014.
18. Lee SY, Auh QS, Kang SK, Kim HJ, Lee JW, Noh K, Jang JH and Kim EC: Combined effects of dentin sialoprotein and bone morphogenetic protein-2 on differentiation in human cementoblasts. Cell Tissue Res 357: 119-132, 2014.
19. Lee SK, Chung JH, Choi SC, Auh QS, Lee YM, Lee SI and Kim EC: Sodium hydrogen sulfide inhibits nicotine and lipopolysaccharide-induced osteoclastic differentiation and reversed osteoblastic differentiation in human periodontal ligament cells. J Cell Biochem 114: 1183-1193, 2013.
20. Hardie DG: AMP-activated/SNF1 protein kinases: Conserved guardians of cellular energy. Nat Rev Mol Cell Biol 8: 774-785, 2007.
21. Han D, Li SJ, Zhu YT, Liu L and Li MX: LKB1/AMPK/mTOR signaling pathway in non-small-cell lung cancer. Asian Pac J Cancer Prev 14: 4033-4039, 2013.
22. Pantovic A, Krstic A, Janjetovic K, Kocic J, Harhaji-Trajkovic L, Bugarski D and Trajkovic V: Coordinated time-dependent modulation of AMPK/Akt/mTOR signaling and autophagy controls osteogenic differentiation of human mesenchymal stem cells. Bone 52: 524-531, 2013.
23. Yeh LC, Ma X, Ford JJ, Adamo ML and Lee JC: Rapamycin inhibits BMP-7-induced osteogenic and lipogenic marker expressions in fetal rat calvarial cells. J Cell Biochem 114: 1760-1771, 2013.
24. Grozinsky-Glasberg S, Rubinfeld H, Nordenberg Y, Gorshtein A, Praiss M, Kendler E, Feinmesser R, Grossman AB and Shimon I: The rapamycin-derivative RAD001 (everolimus) inhibits cell viability and interacts with the Akt-mTOR-p70S6K pathway in human medullary thyroid carcinoma cells. Mol Cell Endocrinol 315: 87-94, 2010.
25. Grozinsky-Glasberg S, Franchi G, Teng M, Leontiou CA, Ribeiro de Oliveira A Jr, Dalino P, Salahuddin N, Korbonits M and Grossman AB: Octreotide and the mTOR inhibitor RAD001 (everolimus) block proliferation and interact with the Akt-mTOR-p70S6K pathway in a neuro-endocrine tumour cell Line. Neuroendocrinology 87: 168-181, 2008.
26. Fischer J, Pröfrock D, Hort N, Willumeit R and Feyerabend F: Reprint of: Improved cytotoxicity testing of magnesium materials. Materials Science and Engineering: B 176: 1773-1777, 2011.
27. Ding W: Opportunities and challenges for the biodegradable magnesium alloys as next-generation biomaterials. Regen Biomater 3: 79-86, 2016.
28. Hornberger H, Virtanen S and Boccaccini A: Biomedical coatings on magnesium alloys-a review. Acta Biomater 8: 2442-2455, 2012.
29. Seal CK, Vince K and Hodgson MA: Biodegradable surgical implants based on magnesium alloys-A Review of Current Research. IOP Conference Series: Materials Science and Engineering. IOP Publishing, Bristol, p012011, 2009.
30. Virtanen S: Biodegradable Mg and Mg alloys: Corrosion and biocompatibility. Materials Science and Engineering: B 176: 1600-1608, 2011.
31. Witte F, Hort N, Vogt C, Cohen S, Kainer KU, Willumeit R and Feyerabend F: Degradable biomaterials based on magnesium corrosion. Current Opinion in Solid State and Materials Science 12: 63-72, 2008.
32. Seo HJ, Cho YE, Kim T, Shin HI and Kwun IS: Zinc may increase bone formation through stimulating cell proliferation, alkaline phosphatase activity and collagen synthesis in osteoblastic MC3T3-E1 cells. Nutr Res Pract 4: 356-361, 2010.
33. Wang T, Zhang JC, Chen Y, Xiao PG and Yang MS: Effect of zinc ion on the osteogenic and adipogenic differentiation of mouse primary bone marrow stromal cells and the adipocytic trans-differentiation of mouse primary osteoblasts. J Trace Elem Med Biol 21: 84-91, 2007.
34. Li J, Tan L, Peng W, Yu X and Ke Y: Study on microstructure and properties of extruded Mg-2Nd-0.2Zn alloy as potential biodegradable implant material. Mater Sci Eng C Mater Biol Appl 49: 422-429, 2015.
35. Musgrave DS, Pruchnic R, Wright V, Bosch P, Ghivizzani SC, Robbins PD and Huard J: The effect of bone morphogenetic protein-2 expression on the early fate of skeletal muscle-derived cells. Bone 28: 499-506, 2001.
36. Feng J, Yang G, Yuan G, Gluhak-Heinrich J, Yang W, Wang L, Chen Z, Schulze McDaniel J, Donly KJ, Harris SE, *et al*: Abnormalities in the Enamel in Bmp2-Deficient Mice. Cells Tissues Organs 194: 216-221, 2011.
37. Kang MH, Oh SC, Lee HJ, Kang HN, Kim JL, Kim JS and Yoo YA: Metastatic function of BMP-2 in gastric cancer cells: The role of PI3K/AKT, MAPK, the NF-κB pathway, and MMP-9 expression. Exp Cell Res 317: 1746-1762, 2011.
38. Nakase T and Yoshikawa H: Potential roles of bone morphogenetic proteins (BMPs) in skeletal repair and regeneration. J Bone Miner Metab 24: 425-433, 2006.
39. Wei K, Pei G and Dan JI: Effects of recombinant human bone morphogenetic protein-2 on the proliferation and adhension of skeletal muscle satellite cells. Chin J Rehab Med 18: 416-417, 2003.
40. Ghosh-choudhury N, Abboud SL, Nishimura R, Celeste A, Mahimainathan L and Choudhury GG: Requirement of BMP-2-induced phosphatidylinositol 3-kinase and Akt serine/threonine kinase in osteoblast differentiation and Smad-dependent BMP-2 gene transcription. J Biol Chem 277: 33361, 2002.
41. Zheng W, Wang H, Zeng Z, Lin J, Little PJ, Srivastava LK and Quirion R: The possible role of the Akt signaling pathway in schizophrenia. Brain Res 1470: 145-158, 2012.
42. Akca E and Gursel A: The Effect of Diffusion Welding Parameters on the Mechanical Properties of Titanium Alloy and Aluminum Couples. Metals 7: 22, 2017.
43. Foster KG and Fingar DC: Mammalian target of rapamycin (mTOR): Conducting the cellular signaling symphony. J Biol Chem 285: 14071-14077, 2010.
44. Wang MH, Zhou XM, Zhang MY, Shi L, Xiao RW, Zeng LS, Yang XZ, Zheng XFS, Wang HY and Mai SJ: BMP2 promotes proliferation and invasion of nasopharyngeal carcinoma cells via mTORC1 pathway. Aging (Albany NY) 9: 1326-1340, 2017.
45. Park KR, Nam D, Yun HM, Lee SG, Jang HJ, Sethi G, Cho SK and Ahn KS: β-Caryophyllene oxide inhibits growth and induces apoptosis through the suppression of PI3K/AKT/mTOR/S6K1 pathways and ROS-mediated MAPKs activation. Cancer Lett 312: 178-188, 2011.
46. McGonnell IM, Grigoriadis AE, Lam EW, Price JS and Sunters A: A specific role for phosphoinositide 3-kinase and AKT in osteoblasts? Front Endocrinol (Lausanne) 3: 88, 2012.
47. Van der Vos KE and Coffer PJ: FOXO-binding partners: It takes two to tango. Oncogene 27: 2289-2299, 2008.
48. Behl Y, Siqueira M, Ortiz J, Li J, Desta T, Faibish D and Graves DT: Activation of the acquired immune response reduces coupled bone formation in response to a periodontal pathogen. J Immunol 181: 8711-8718, 2008.
49. Yamashita A, Hatazawa Y, Hirose Y, Ono Y and Kamei Y: FOXO1 delays skeletal muscle regeneration and suppresses myoblast proliferation. Biosci Biotechnol Biochem 80: 1531-1535, 2016.