

In vivo and in vitro evaluation of effects of Mg-6Zn alloy on apoptosis of common bile duct epithelial cell

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Abstract Biodegradable magnesium alloy implants have attracted much attention because of their excellent biocompatibility and good mechanical properties. However, effects of Mg alloy on cell apoptosis remain unclear. The aim of this study was to investigate the effects of the Mg-6Zn alloy on the apoptosis and necrosis of common bile duct (CBD) epithelial cells. In the in vitro experiments, primary mouse extrahepatic bile epithelial cells (MEBECs) were exposed to Mg-6Zn alloy extracts with different concentrations (0, 40, 80, and 100 %). Flow cytometry analysis indicated that low concentration Mg-6Zn extract can

induce apoptosis of MEBECs, and high concentration Mg-6Zn extracts may relate to necrosis and/or 'apoptotic necrosis'. Real-time PCR results showed that when MEBECs were treated with 40 % extracts for 3 days, the relative apoptotic genes including Bax, Bax/Bcl-2 ratio, NF- κ B and caspase-3 were higher than those in the control group. In the in vivo experiments, Mg-6Zn alloy stents were implanted into rabbits' CBD for 1, 2, 3 weeks, respectively. Based on the hematoxylin and eosin (H&E) staining of peri-implant CBD tissue, no apoptotic bodies and necrotic cells were observed. Results of immunohistochemical staining also showed Mg-6Zn stents did not increase expression levels of apoptosis related gene such as Bax, Bcl-2, Bax/Bcl-2 ratio, TNF- α , NF- κ B and caspase-3 in CBD, which indicating Mg-6Zn

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did not induce significant apoptosis in the *in vivo* experiments. The different results of *in vitro* and *in vivo* experiment may result from the low corrosion rate of Mg-6Zn alloy stents *in vivo* and local Mg²⁺ ion concentration in CBD.

Keywords Biodegradable stent · Mg-6Zn alloy · Common bile duct · Apoptosis

Introduction

It is important to place supporting materials into common bile duct (CBD) to prevent post-operative CBD stenosis (Wu and Soper 2002; Isla et al. 2004). After biomaterials were implanted, the foreign matter can frequently trigger inflammation and immunological reaction (Tang et al. 1996). These events are commonly accompanied by apoptotic processes which are necessary to clean the injured and inflamed tissue and to support the healing and regeneration mechanisms (Bartsch et al. 2012). Kerr et al. first found that there are two types of the cell death, named necrosis and apoptosis. Apoptosis, in Greek languages means the fall of autumn leaves from trees, is the process of cell actively involved in itself death (Delhalle et al. 2003). One of important roles of apoptosis is acting as a defense mechanism such as in immune reactions or when cells are damaged by disease or noxious agents (Norbury and Hickson 2001). Necrosis, usually caused by cell poison effects, is essentially different to apoptosis on morphology and cell biological characteristics (Casiano et al. 1998). Unlike the apoptosis, the cytoplasmic contents (usually the proteolytic enzyme) of necrotic cells released, will cause the surrounding tissue inflammation (Stea et al. 2000). In summary, necrosis often occurs in pathological changes, whereas apoptosis is a normal physiological phenomenon.

Clinical widely used CBD supporting materials include silicone rubber, titanium and polymer. T-tube (mostly made of silicone rubber) in CBD may induce numerous complications such as dehydration, inability to remove the drain tube and biliary fistula (Daldoul et al. 2012; Wu et al. 2012). Titanium alloy and polymeric substance can cause sludge accumulation, bacteria-laden bio-films and epithelial hyperplasia, and needed be removed Xu et al. (2009). Currently,

various kinds of biodegradable CBD stents have been designed to replace the non-degradable stents (Ginsberg et al. 2003). The advantages of these biodegradable stents include not need to remove stent, large stent diameter and decrease of biofilm accumulation (Meng et al. 2006). Magnesium alloy is one of attractive biodegradable implanting materials due to their excellent biocompatibility and good mechanical properties (Zberg et al. 2009; Song 2007). Increasing studies have been focused on the application of Mg alloy on bone implant, vascular stent, surgical clips, staples and surgical sutures etc. (Xu et al. 2009; Hausmann et al. 2006). In the field of biliary surgery, few studies have been addressed. Our previous studies have shown that Mg-6Zn alloy can gradually degraded in CBD, and does not harm the function of the CBD, kidney, pancreases or liver (Chen et al. 2013a; 2014). Yet, it remains unknown whether Mg alloys induce beneficial apoptosis or harmful necrosis, or both after they were implanted.

In the present study, we aim to investigate the influence of Mg-6Zn alloy on apoptosis or necrosis through *in vitro* cell experiments and *in vivo* animal experiments. Primary mouse extrahepatic bile epithelial cells (MEBECs) were exposed to Mg-6Zn alloy extracts at different concentrations to evaluate expressions of apoptotic genes such as Bax, Bcl-2, TNF- α , caspase-3 and NF- κ B. Moreover, Mg-6Zn alloy stents were implanted into rabbits' CBD, hematoxylin and eosin (H&E) and immunohistochemical staining were taken to investigate the influence of Mg-6Zn alloy on apoptosis of peri-implant CBD tissues.

Materials and methods

Materials and extract preparation

The biodegradable Mg-6Zn alloy, donated by Suzhou Origin Material and Medical Technology Co. Ltd., Jiangsu, China, was prepared from high purity Mg (99.99 %) and zinc (Zn; 99.999 %) as previously described (Zhang et al. 2010). The composition of this alloy was given in Table 1. The as-extruded Mg-6Zn rods were processed into two shapes (discs samples and tube-shaped samples) to meet different experimental needs. Disc samples for the *in vitro* cell experiments, with a diameter of 11.3 mm and a height of 2.0 mm, were machined from extruded Mg-6Zn

Table 1 Chemical compositions of Mg-6Zn alloy

Material	Chemical compositions (wt%)							
	Fe	Si	Ni	Cu	Al	Mn	Zn	Mg
Mg-6Zn	0.0038	0.0016	0.0005	0.0005	0.0085	0.0004	5.6210	Balance

rods. Tube-shaped samples of Mg-6Zn alloys were designed to put into the CBD of rabbits, which had a luminal diameter of 1.0 mm, thickness of 0.1 mm and a length of 5 mm and were fabricated by machining and drilling. Before the samples were used in the *in vitro* and *in vivo* experiments, they were all cleaned ultrasonically by acetone and ethanol successively and finally sterilized with 29 kGy of ^{60}Co radiation.

Extracts were prepared according to ISO 10993-5. Disc samples were immersed in Dulbecco's Modified Eagle's medium-high glucose (DMEM, Gibco TM, Invitrogen), supplemented with 10 % fetal bovine serum (FBS, Hyclone). The surface area to extraction medium ratio was 1.25 cm²/ml, and the immersed samples were kept in a humidified atmosphere with 5 % CO₂ at 37 °C for 24 h. Then the extraction medium was collected and the supernatant was removed, centrifuged, and refrigerated at 4 °C. To observe a dose–response relationship, the extracts were serially diluted with DMEM to three concentrations (100, 80 and 40 % concentration extracts). Using the ICP-AES (inductively coupled plasma-atomic emission spectroscopy), the concentration of released Mg ions and Zn ions of 100 % Mg-6Zn alloy extracts were determined as 15.91 ± 4.42 mM and 0.014 ± 0.003 mM.

Animal

All animal experiments were authorized according to the Guidance Suggestions for the Care and Use of Laboratory Animals (issued by the Ministry of Science and Technology of the People's Republic of China), and was approved by the Ethics Committee of the 6th Hospital of Shanghai Jiao Tong University. Animals were supplied by the Sino-British Sippr/BK Lab Animal Ltd, Co, China (License No:SCXK (hu) 2008-0016). Two kinds of animal were used in our study. One is the New Zealand rabbit, which was taken for the *in vivo* Mg-6Zn alloy implanting experiment. Another is the BALB/C mice; they were used to isolate MEBECs.

For the *in vitro* test, ten BALB/C mice were used. MEBECs were isolated and cultured as described by previous studies (Chen et al. 2013a; Chai et al. 2010).

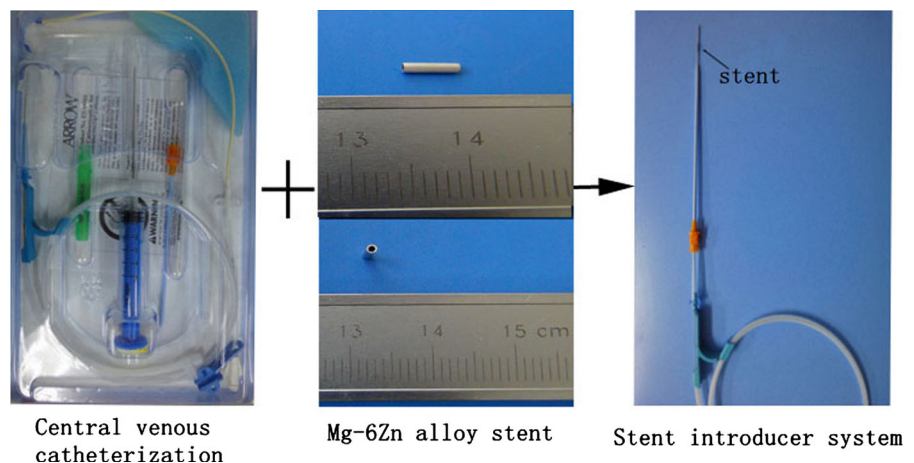
Briefly, each mice was injected 0.2 ml newborn bovine serum (GIBCO, Grand Island, NY) by intraperitoneal injection every 24 h to induce proliferation of MEBECs. After 7 days, the mice were sacrificed, and the CBD of each mouse was minced and then placed into a centrifuge tube. After centrifuged at 800 rpm for 5 min the supernatant was discarded, and 10 ml of collagenase IV with the concentration 200 U/ml (Invitrogen) was added. The mixture was incubated for 30 min at 37 °C in an incubator with 5 % CO₂ humidified air. Then, the mixture was centrifuged at 800 rpm for 5 min and re-suspended in 5 ml DMEM/HamF12 containing 10 % FBS. The detached cells were separated by sequential filtration. In order to remove fibroblast, the filtered cells were subsequently incubated on glass Petri dished three times for a total of 45 min. The non-adherent cells were counted, and cell density was adjusted to the range from 5 × 10⁵/ml to 1 × 10⁶/ml. Finally, the cells were seeded in a rat tail type I collagen-coated (Sigma) plastic culture flask and incubated at 37 °C in an incubator with 5 % CO₂ humidified air.

In the *in vivo* experiment, 42 adult New Zealand rabbits with a mean body weight of 1.2 ± 0.5 kg were used in our experiments, which were randomly and equally assigned to two groups. In research group, the tube-shaped Mg-6Zn alloy stent was implanted into the CBD of each rabbit. The control group used stainless steel stent instead of the Mg-6Zn stent. A central venous catheterization set (REF CS-24301-E; Arrow International, Inc., Reading, PA, USA) was used as a special stent introducer system (Fig. 1). The processes of surgery were described in our previous study Chen et al. (2013). In both Mg-6Zn alloy and control groups (stainless steel stent groups), seven rabbits were sacrificed postoperatively at 1, 2 and 3 weeks, respectively.

Flow cytometry analysis of apoptosis cell

Apoptotic kit (Molecular Probes, Invitrogen) containing Alexa Fluor 488 annexin V and propidium iodide (PI) was performed to evaluate the effect of Mg-6Zn

Fig. 1 The stent introducer system is made of a central venous catheterization and an Mg-6Zn alloy stent



alloy extracts on apoptosis of MEBDECs. Briefly, MEBDECs were cultured in 40, 80 and 100 % concentrations of Mg-6Zn alloy extracts for 1 and 3 days along with the control group (0 % extract). After the cultivation period, the cells were recovered by digestion with 0.25 % trypsin. The cells were then centrifuged and resuspended in Annexin-binding Buffer. After the cell density was determined and adjusted, annexin V conjugate and PI working reagent were added to each cell suspension. After incubation 15 min at room temperature, Annexin-Binding Buffer was added with gentle mixing, and the samples were kept on ice. Then the stained cells were analyzed by flow cytometry (Navios[®] Flow Cytometers, Beckman Coulter) through measuring the fluorescence emission at 530 nm and >575 nm. All data were acquired and analyzed using Navios analysis software.

mRNA isolation and real-time PCR

MEBECs were cultured on different concentrations (0, 40, 80 and 100 %) of Mg-6Zn alloy extracts for different time periods (1 and 3 days), respectively. MEBECs treated in 0 % extracts served as control group. Total cellular RNA was isolated from cell layers using a Trizol reagent (Life, Carlsbad, CA). The concentration and quality of RNA were determined using a ACTGene ASP Spectrophotometer (ACT-Gene, Piscataway, USA) and purity (A_{260}/A_{280}) of >1.8 was used. Complementary DNA (cDNA) was synthesized using the Prime-Script[™] RT reagent Kit (TaKaRa Biotechnology, Dalian, China) according to the manufacturer's instructions. In brief, RNA (500 ng) was transcribed in a volume containing of

5 × PrimeScript[™] buffer (2 μl), PrimeScript[™] RT Enzyme Mix I (0.5 μl), 50 μM Oligo dT Primer (0.5 μl) and 100 μM Random 6 mers (0.5 μl) in a PCR tube (Eppendorf, German) and added RNase-free water to total volume 10 μl. The reaction mixture was incubated at 37 °C for 15 min for reverse transcription, the reverse transcriptase then being inactivated at 85 °C for 5 s. Primers for the real-time PCR analysis Bax, Bcl-2, TNF-α, caspase-3 and NF-κB genes were designed (Table 2) and synthesized (Sangon Biotech Co., Ltd., Shanghai, China). Real-time PCR was performed using a quantitative real-time amplification system (TaKaRa Biotechnology, Dalian, China). The SYBR[®] Premix Ex Taq TM kit (TaKaRa Biotechnology, Dalian, China) was used in each reaction. Briefly, cDNA (2 μl) was reacted with 10 μl 2 × SYBR[®] Premix Ex Taq TM buffer, 0.5 μl of 10 μM each primer and 7.2 μl dH₂O in a 20 μl final reaction volume. PCR conditions were as follows, the initial denaturation at 95 °C for 30 s, followed by 40 PCR cycles: 95 °C for 5 s, 62 °C for 20 s.

H&E and immunohistochemical analysis

At predetermined time, the rabbits were sacrificed, and their CBD tissue was removed. Tissue samples of 5 cm² surrounding the implants were fixed in 10 % buffered formaldehyde. Sections were then cut to a thickness of 4 μm and mounted on a glass slides. Histological slices were processed, and stained with H&E to assess apoptosis or necrosis. Immunohistochemical analysis was conducted to investigate the expression of apoptosis related genes. Tissue sections were deparaffinized in xylene, and then rehydrated in

Table 2 Real-time PCR primer sequence of target genes

Gene	Refseq accession no	Primer sequence 5′–3′	Amplicon
β-actin	NM_007393.3	F:TTGCTGACAGGATGCAGAAGGAGA R:ACTCCTGCTTGCTGATCCACATCT	59 bp
Bax	NM_007527.3	F:GCGAGTGTCTCCGGCGAATT R:GCCCCAGTTGAAGTTGCCATCAG	146 bp
Bcl-2	NM_009741.4	F:TTGTGGCCTTCTTTGAGTTCGGTG R:AATCAAACAGAGGTCGCATGCTGG	195 bp
NF-κB	NM_008689	F:CCACGAGGCAGCACATAGAT R:CCGTGGGGCATTGTTTCAG	142 bp
Caspases-3	NM_001284409	F:GAGCTTGGAACGGTACGCTA R:CCGTACCAGAGCGAGATGAC	234 bp
TNF-α	NM_013693.3	F:ACCGTCAGCCGATTTGCTAT R:CCGACTCCGCAAAGTCTAA	209 bp

graded concentrations of ethyl alcohol (100, 95 and 75 %, then water). The sections were then microwave-treated twice in citrate buffer (pH 6.0) at 99 °C for 6 min. After the sections were placed in 3 % H₂O₂ for 10 min to inhibit the endogenous peroxidase activity, they were washed three times with phosphate-buffered saline (PBS) buffer for 5 min and placed in normal mouse serum as blocking antibody at room temperature for 10 min. The sections were evaluated by antibody for Bax (ab16837, 1:400, abcam, UK), Bcl-2 (bs-4563R, 1:200, bioss, China), caspase-3 (ab4051, abcam, UK), NF-κB (ab16502, abcam, UK) and TNF-α (ab5571, 1:100, abcam, UK). After incubation at 4 °C for 24 h, sections were washed three times with PBS buffer for 10 min. Biotinylated anti-mouse/rabbit immunoglobulin was used as the second antibody. 3, 3-Diaminobenzidine tetrahydrochloride (DAB) was used as a chromogen. Cytoplasmic staining was interpreted as positive for Bcl-2 and caspase-3, and membranous and cytoplasmic staining was considered positive for Bax, TNF-α and NF-κB. The sections were evaluated in the light microscope using the MICRO IMAGETM software (Olympus Optical Corp. Ltd., Tokyo, Japan). The expression of all apoptosis relative indicators was examined by the integrated optical density (IOD) using Motic Fluo 1.0 software (Motic China Group Co. Ltd., Shenzhen, China).

Statistical analysis

Statistical analysis was performed with the SPSS 18.0 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 calculated to determine differences between groups for each evaluated parameter that was evaluated at each time point. Non parametrical tests [χ independent samples tests (Kruskal–Wallis test)] were calculated when equal variances were not assumed in one-way ANOVA. The level of significance was defined as $P < 0.05$.

Results

Flow cytometric analysis of apoptosis

Figure 2a showed a representative experimental picture depicting fluorescence intensity of MEBECs cultured in Mg-Zn alloy extracts. There are three distinct cell distribution patterns. The lower left quadrant represents normal viable cells, and the lower right one is apoptotic cells, and the upper right one is necrotic and/or ‘apoptotic necrotic’ cells. Statistics showed that after 1 day, no significant difference ($P > 0.05$) on the apoptosis or necrosis/‘apoptotic necrosis’ of MEBECs was observed when they were cultured in different concentration Mg-6Zn alloy extracts (Data not shown). While after 3 days, as summarized in Fig. 2b, the apoptotic MEBECs were higher in the 40 and 80 % extracts than those in the control group. On the contrary, the necrotic/‘apoptotic necrotic’ MEBECs were much higher in 100 % extracts than those in the control group. In 80 %

extracts group, both apoptotic cells and necrotic/‘apoptotic necrotic’ cells were higher than control group.

In vitro Real-time PCR quantification of Bax, Bcl-2, NF- κ B, caspase-3 and TNF- α gene expression

Figure 3 presented the relative mRNA expression levels of Bax, Bcl-2 of MEBECs when cultured in the Mg-6Zn alloy extraction media for 1 and 3 days. After 1 day the mRNA expression of Bax in 100 % extracts was significantly less than other three groups ($P < 0.05$). After 3 days, the mRNA expression of Bax was evidently higher in 40 % extracts than control group and other two groups ($P < 0.05$). For the expression of Bcl-2, there were no significant differences between 40, 80 and 100 % extracts group and control group. The ratio of Bax to Bcl-2 was calculated due to it could more accurately reflect cell sensitivity to apoptotic stimuli than the individual expression of these two genes (Magistrelli et al. 2006). The results presented in Fig. 3c showed that when MEBECs were treated with 40 % extracts for 3 days, the Bax/Bcl-2 ratio was higher than those in the control group, which might mean the apoptotitis of MEBECs was improved when they were cultured in 40 % Mg-6Zn extracts.

Figure 4 presented the relative mRNA expression levels of NF- κ B, TNF- α and caspase-3 of MEBECs cultured in the extraction media for 1 and 3 days. There was no statistic difference between extract groups and control group at 1 day in the expression of NF- κ B. After 3 days, the expression of NF- κ B in 40 % extract group was significantly higher than that of control group. For TNF- α mRNA expression, no significant differences were observed between extract groups and control group at both 1 day and 3 days. The expression of caspase-3 at 1 day were higher in 80 and 100 % extracts than in control group, and after 3 days all the experiment groups increased in caspase-3 expression as compared to control group.

In vivo H&E staining

After implantation of the Mg-6Zn stents, no rabbits displayed unexpected deaths and inflammation. The rabbits were sacrificed 1, 2 and 3 weeks postoperative. In both the control and experimental groups, the CBD did not show obvious expansion or bile siltation. Based on the results from H&E stain (Fig. 5), most of the

Fig. 2 The results of the flow cytometric analysis: **a** a representative experiment depicting fluorescence intensity in MEBECs treated with extracts for 3 days; **b** percentage of apoptotic cells and necrotic cells when MEBECs cultured in the extraction media for a period of 1 and 3 days

cells present normal morphology. No significant cell swelling, vascular congestion, infiltration of neutrophils and monocytes were detected at 1, 2 and 3 weeks postoperative. There were also no significant apoptotic bodies and necrotic cells in the peri-implant CBD tissue both at each groups.

In vivo immunohistochemical evaluation

Figure 6a and b showed a representative immunohistochemical picture depicting the experiment of Bax and Bcl-2 genes in the peri-implant’s CBD tissues; Fig. 6c, d and e showed the statistical results of the immunohistochemical analysis. Based on our results, only the IOD value for Bax expression in Mg-6Zn alloy group was higher than those in the control group ($P < 0.05$) at 1 week post-operation. However, the more valuable expression of Bax/Bcl-2 ratio did not show significant difference at all the experimental time, which might mean Mg-6Zn alloy did not induce apoptosis in the in vivo experiment.

The results for immunohistochemical staining of NF- κ B, TNF- α and caspase-3 were presented in Fig. 7. There were no significant statistical differences for expression of NF- κ B between Mg-6Zn alloy groups and control group at all the experimental time. At 2 weeks postoperative, the IOD value for TNF- α expression in Mg-6Zn alloy group were significantly less than control group ($P < 0.05$). However, at 1 and 3 weeks, the expression of TNF- α were no significant statistical differences between Mg-6Zn alloy and control group. For caspase-3 expression, at 1 week postoperative, the IOD value of Mg-6Zn alloy group were significant higher than control group ($P < 0.05$), and after 2 or 3 weeks, the IOD value of Mg-6Zn alloy group were significant less than control group.

Discussion

Apoptosis is believed to have protective effects such as remove unwanted cells and maintain host stability. Necrosis is usually harm to bodies (Schultz and Harrington 2003). The in vitro study showed that Mg-

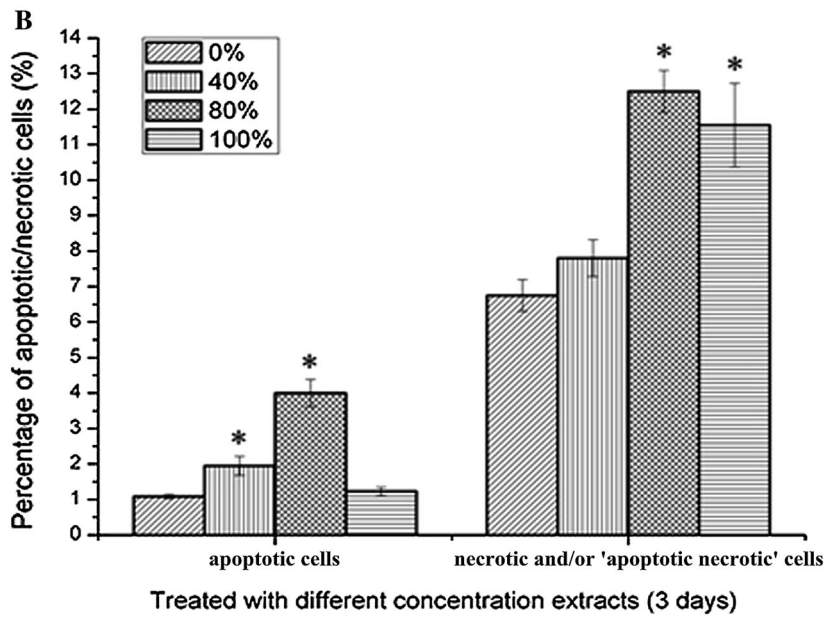
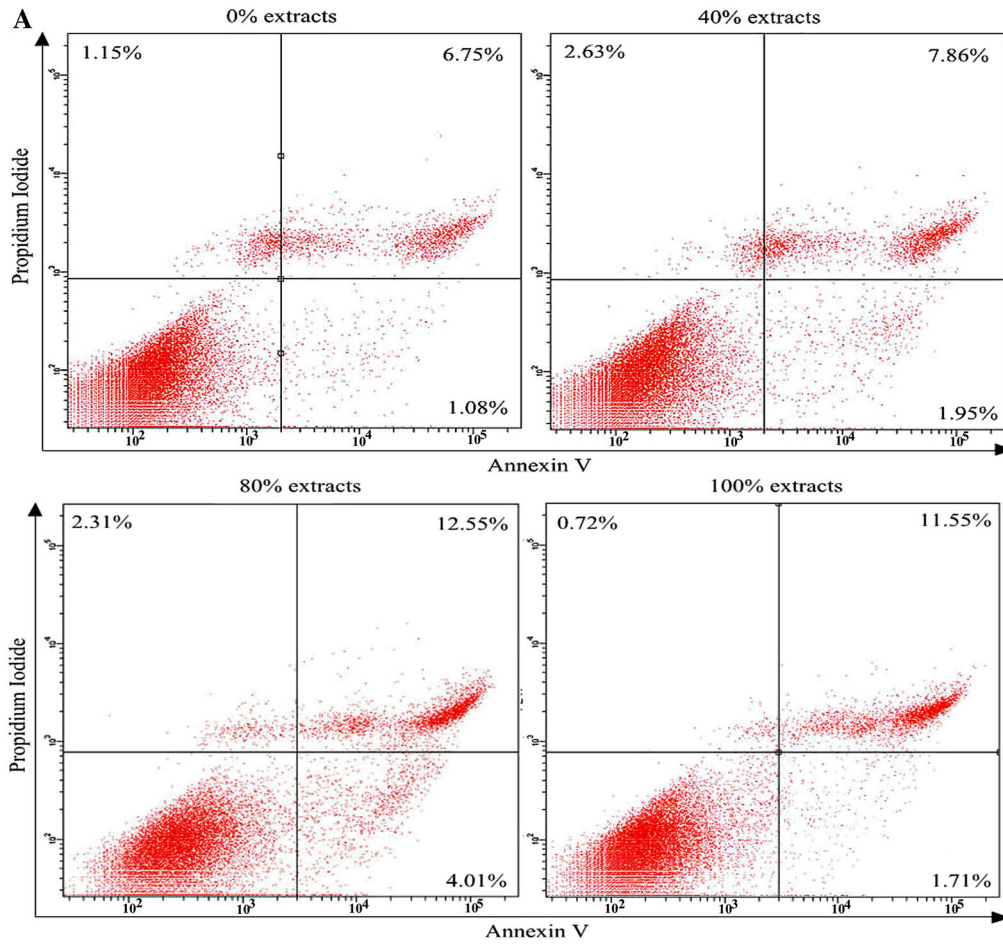
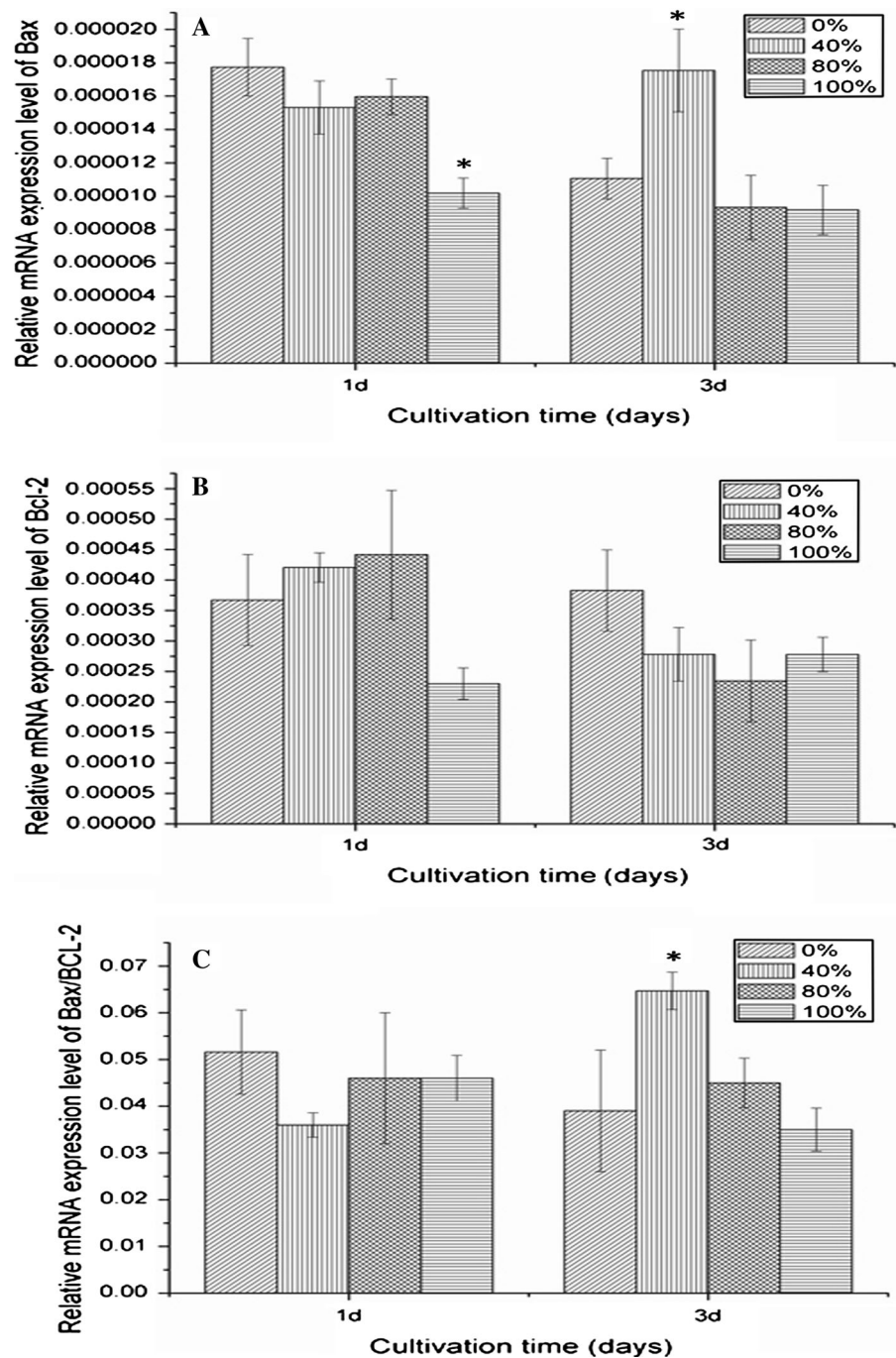


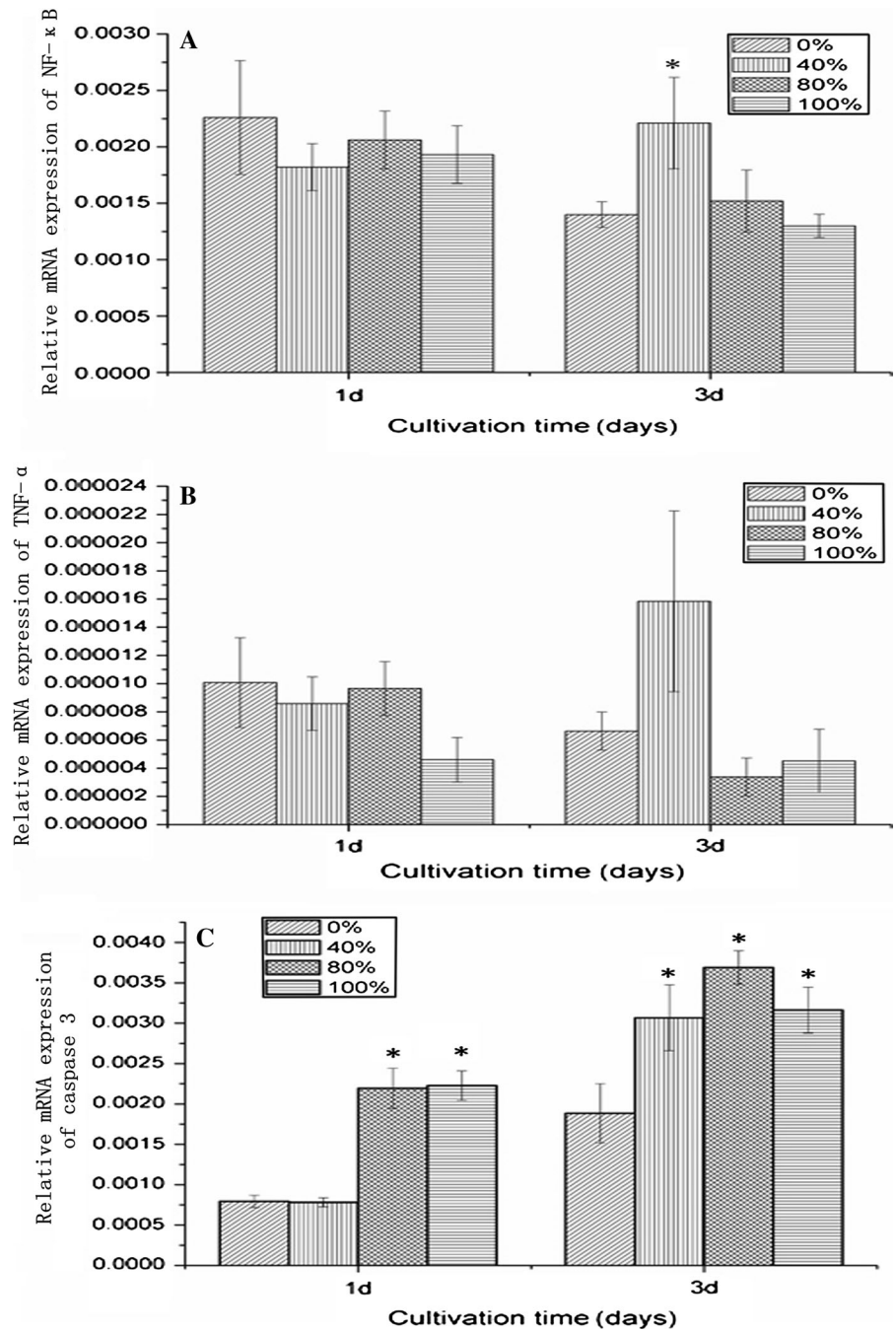
Fig. 3 The relative mRNA expression levels of **a** Bax, **b** Bcl-2, **c** Bax/Bcl-2, when MEBECs cultured in the extraction media for a period of 1 and 3 days



6Zn alloy extracts with a low concentration can promote MEBECs apoptosis, while along with the increasing Mg-6Zn concentration, the number of necrosis/'apoptotic necrosis' MEBECs gradually increased. The apoptosis of MEBECs cultured in Mg-6Zn alloy extracts with a 40 % concentration were significantly

higher compared with those in control group after 3 days. When the concentration reached 80 %, both apoptosis and necrosis/'apoptotic necrosis' increased. Finally, in the 100 % extracts the necrosis/'apoptotic necrosis' of MEBECs were significantly promoted instead of apoptosis. Bax is a pro-apoptotic protein, and

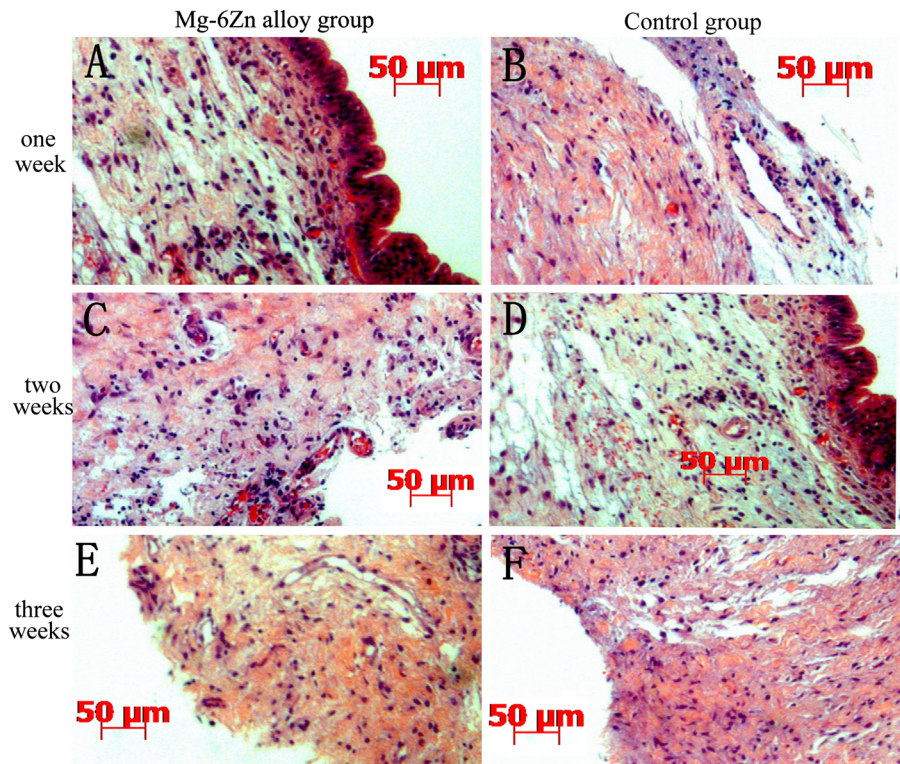
Fig. 4 The relative mRNA expression levels of **a** NF- κ B, **b** caspase-3 and **c** TNF- α when MEBECs cultured in the extraction media for a period of 1 and 3 days



Bcl-2 is anti-apoptotic protein of the Bcl-2 family (Falleni et al. 2005). Our in vitro results also showed that after 3 days cultured, the mRNA expression of Bax and Bax/Bcl-2 were evidently higher when MEBECs were treated with 40 % extracts than control group. These results may suggest that 40 % extracts of Mg-6Zn alloy can induce MEBECs apoptosis after 3 days in the

in vitro environment. However, it should be noted that the results did not consider the difference between necrosis and apoptotic necrosis. In longer cultures, apoptotic cells will undergo secondary necrosis, which has frequently been called post-apoptotic necrosis or late apoptosis (Silva et al. 2008). In this study, after 3 days cultures some of the cells classified as necrotic

Fig. 5 The results of *H&E* staining of the peri-implant CBD tissue at 1, 2 and 3 weeks postoperative. **a** is the representative picture of the Mg-6Zn alloy group at 1 week postoperative; **b** is the representative picture of the control group at 1 week postoperative; **c** is the representative picture of the Mg-6Zn alloy group at 2 weeks postoperative; **d** is the representative picture of the control group at 2 weeks postoperative; **e** is the representative picture of the Mg-6Zn alloy group at 3 weeks postoperative; **f** is the representative picture of the control group at 3 weeks postoperative



by the flow cytometric analysis may be due to apoptosis converting into secondary necrosis. Therefore, our results about necrosis may be influenced by the post-apoptotic necrosis or late apoptosis.

The mechanism of apoptosis is complex. NF- κ B, TNF- α and caspase-3 are important apoptosis mediators by transactivating the expression of anti-apoptotic genes (Kucharczak et al. 2003; D'amelio et al. 2009). NF- κ B signal pathway is a decisive factor in the cell's response to apoptotic stimuli (Kucharczak et al. 2003). TNF- α is an important NF- κ B downstream protein, which can lead to apoptosis via ligand binding and protein cross-link (Van Ophoven et al. 1999; Elmore 2007). Data showed that low concentration Mg-6Zn extracts can elevate the expression of NF- κ B. In 40 % Mg-6Zn alloy extracts, the expression of NF- κ B significantly increased. Although there is no statistically significant, the expression of TNF- α also presented an increasing trend in 40 % Mg-6Zn alloy extracts (Fig. 4b). Caspase-3 is considered to be a key executor and the final step of many apoptosis pathway (Rehm et al. 2002). In our study, the caspase-3 expression was more sensitive to the changes of the Mg-6Zn concentration. The expression of caspase 3 in all extracts was

higher than control group after 3 days. In summary, in the 40 % Mg-6Zn extracts group the expression level of both the important apoptosis relative genes (Bax and Bax/Bcl-2 ratio) and the critical apoptotic mediate protein (NF- κ B and caspase 3) increased. Jun Sugimoto et al. have reported that magnesium plays a critical regulatory role in NF- κ B activation (Sugimoto et al. 2012), which is agreement with this study. Our previous studies also reported that Mg-6Zn alloy could induce changing of apoptosis genes both in intestinal tract tissues and in intestinal epithelial cells (Yan et al. 2013; Wang et al. 2012). However, the mechanism of magnesium influence NF- κ B signal pathway and caspase-3 expression are unclear, and it deserves to further research.

The in vivo experiment results showed that when Mg-6Zn alloy contacted with bile, there are no significant difference in the H&E staining analysis and the expression levels of apoptosis relative genes. Although at 1 week post-operation, the Bax expression in Mg-6Zn alloy group is higher than control group, Bax/Bcl-2 ratio have no significant statistical differences between Mg-6Zn alloy and control group at all the experimental time. Moreover, expression

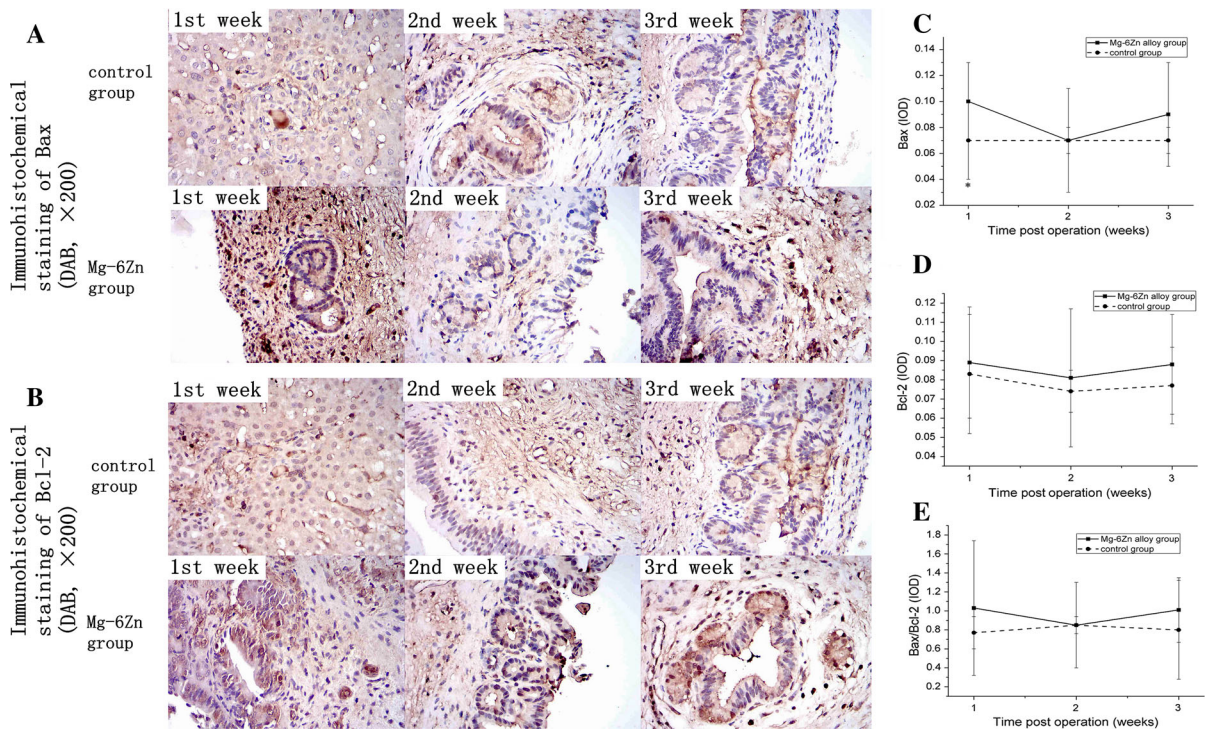


Fig. 6 The results of immunohistochemical staining of Bax and Bcl-2. Magnifications are at original 200×. **a** and **c** showed the positive expression of Bax in the Mg-6Zn alloy groups and control groups. Membranous and cytoplasmic yellow or brown staining was considered positive for Bax. The positive expression of Bax was higher than the control group after

1 week post-operative; **b** and **d** showed the positive expression of Bcl-2 in Mg-6Zn alloy group and control groups. Cytoplasmic yellow or brown staining was interpreted as positive for Bcl-2; **e** showed the result of Bax/Bcl-2. *Mg-6Zn alloy group vs control group ($P < 0.05$). (Color figure online)

levels of apoptotic mediators such as NF-κB, TNF-α and caspase-3 are inconsistent with the in vitro results.

Why apoptosis can be induced by Mg-6Zn in vitro experiments and cannot be induced in vivo? There are some possible explanations. First, the local Mg²⁺ ion concentration in CBD was lower than it in the culture dishes. The in vitro corrosion rate of Mg-6Zn alloy were much higher than that of the in vivo (~0.72 mm·year⁻¹ vs. ~0.107 mm·year⁻¹) (Chen et al. 2014). Therefore, the released Mg ion in CBD was less than it in the in vitro experiment. Moreover, in the CBD part of the Mg and Zn ion released by Mg-6Zn alloy stent are discharged into the intestine. For human, the CBD pressure is 12 cmH₂O, and approximately 1,500 ml of bile is secreted by the liver into the intestine daily. In these dynamic internal environment of CBD, the effect of Mg-6Zn alloy on apoptosis is differ with they in the cell culture dishes. Second, the of CBD stent is easy to be covered by sediments in vivo. Bile has more complex substance than cell culture media, and it contain many

inorganic and organic solutes such as cholesterol, bile acids, phospholipids, Na⁺, Cl⁻, K⁺, Ca²⁺ and HCO₃⁻. Recent studies reported that sludge accumulation and epithelial hyperplasia often occurred on the surface of CBD stent (Meng et al. 2006; van Boeckel et al. 2009). In a sense, there is a middle layer between Mg-6Zn alloy stent and CBD wall. Therefore, when Mg-6Zn alloy in CBD, the effect of Mg-6Zn alloy on apoptosis of CBD cells is not obvious as when Mg-6Zn alloy in cell culture media.

Mg and Zn are essential microelements in human body, and critical for many cellular functions (Sgambato et al. 2001; Wu and Veillette 2011). Among men and women aged ≥31 y, the estimated average requirement are 350 and 265 mg/d for Mg and 11 and 8 mg/d for Zn, respectively (Ford and Mokdad 2003; Maret and Sandstead 2006). Zn not only elevates the corrosion resistance of Mg alloys, but also plays a regulatory role in apoptosis. Zn ion can modulate cellular signal recognition, second-messenger metabolism, and protein

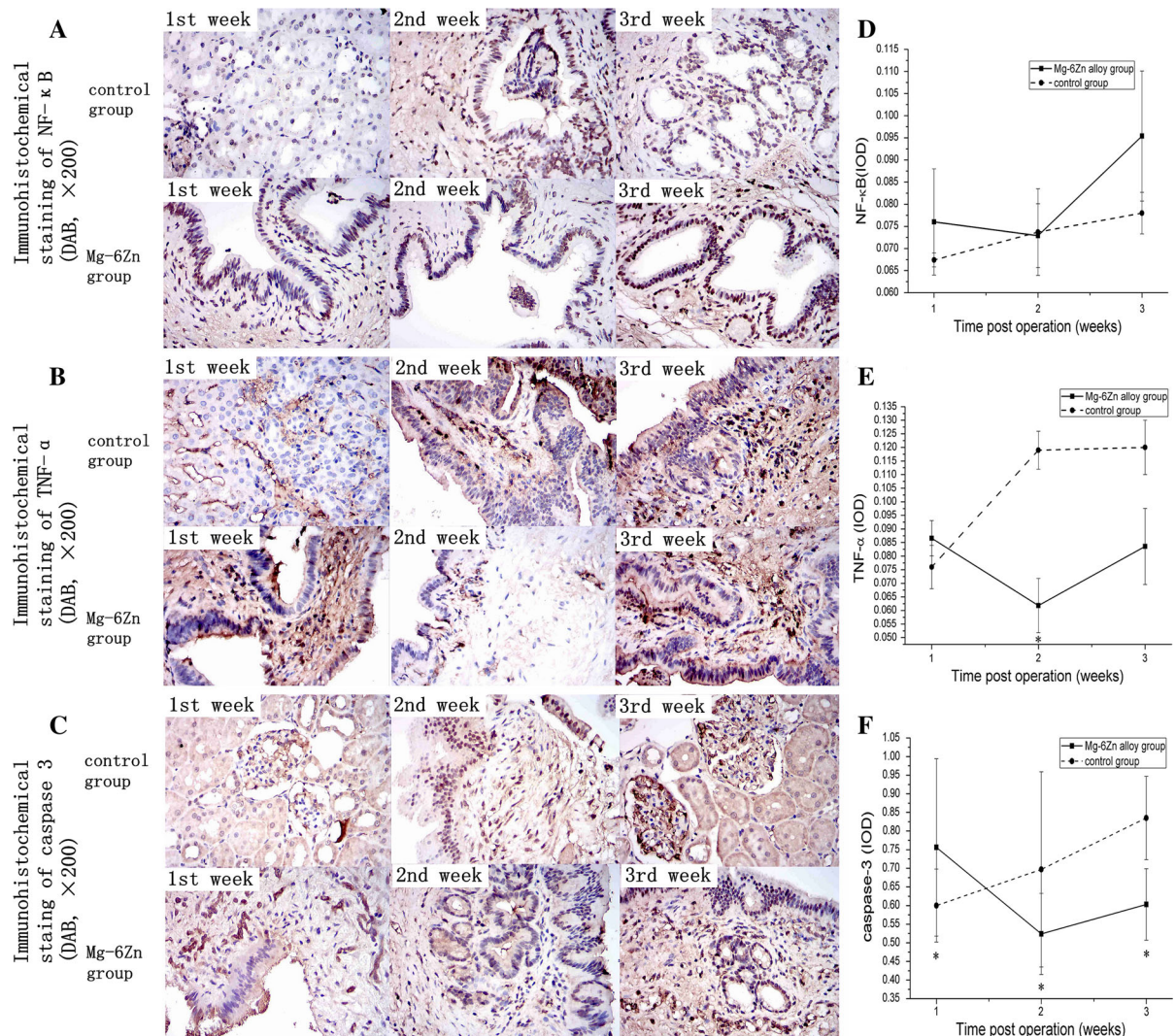


Fig. 7 The results of immunohistochemical staining of NF- κ B, TNF- α and caspase-3. Magnifications are at original $200\times$. **a** and **d** showed the positive expression of NF- κ B in the Mg-6Zn alloy groups and control groups. Membranous and cytoplasmic yellow or brown staining was considered positive for NF- κ B; **b** and **e** showed the positive expression of TNF- α in Mg-6Zn alloy group and control groups. Membranous and cytoplasmic yellow or brown staining was considered positive for TNF- α ; The positive expression of TNF- α was lower than the control

group after 2 week post-operative; **c** and **f** showed the positive expression of caspase-3 in the Mg-6Zn alloy groups and control groups. Membranous and cytoplasmic yellow or brown staining was considered positive for caspase-3. The positive expression of caspase-3 was higher than the control group after 1 week post-operative, and lower than the control group after 2 and 3 weeks post-operative. *Mg-6Zn alloy group vs control group ($P < 0.05$). (Color figure online)

kinase and protein phosphatase activities (Truong-Tran et al. 2001). In our research, the concentration of released Zn ions of 100 % Mg-6Zn alloy extracts was only 0.014 ± 0.003 mM. We speculated that the effect of Zn ions on apoptosis would be very weak at such a low concentration. However, the role of Mg on apoptosis of cells remains controversial. Patel et al.

reported that increases of intracellular Mg promote glycodeoxycholate-induced apoptosis in rat hepatocytes (Patel et al. 1994). On the contrary, Martin et al. suggested that extracellular Mg deficiency has a negative effect on the survival of cultured cells by inducing apoptosis, and supplementation of extracellular Mg did not reduce the spontaneous apoptosis (Martin

et al. 2003). The result of our study supports the former. When the concentration of Mg ion increased (from an Mg-6Zn extract with a 0 % concentration to 40 or 80 % concentration), the apoptosis of MEBECs was increased. However, the role and mechanism of Mg or Zn ion alone on cell apoptosis still need further studies.

Conclusion

In this study, both in vitro and in vivo experiments were performed to investigate the effect of Mg-6Zn alloy on expression of apoptosis of CBD epithelial cells. Mg-6Zn extracts with a low concentration can induce apoptosis of MEBECs and a high concentration perhaps relate to necrosis/‘apoptotic necrosis’ in the in vitro environment. When an Mg-6Zn stent was implanted into CBD, it did not induce evidently apoptosis or necrosis of MEBECs and increase expression of related apoptotic gene.

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