Biodegradable Mg-6Zn Alloy Down-Regulation the NF-κB Signaling Pathway of Intestinal Epithelial Cells

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Nuclear factor kappa B (NF-κB) signaling pathway plays an important role in the processes of immunity, inflammation, and cancer. As studies of magnesium (Mg)-zinc (Zn) alloy have increased, it becomes necessary to investigate effects of Mg-6Zn alloy on NF-κB signaling pathway. The Mg-6Zn alloy was investigated in both in vitro and in vivo experiments. The mRNA expression of P65, P50 and IκBα, and the protein expressions of pIκBα were tested using real time-PCR and western blot analysis. In the in vitro experiments, pins of Mg-6Zn alloy were implanted into the intestinal wall of SD rats. After 1 week, H and E staining and immunohistochemical analysis of NF-κB, was performed. Results of this study showed the expression of IκBα, pIκBα, P50 and P65 did not significantly fluctuate in the 20% and 40% concentrations of Mg-6Zn alloy extracts. In the 80% and 100% extracts, the mRNA expression of IκBα was higher than that in the 0% extract. The mRNA expression of P50 and P65 were significantly lower in the 60%, 80% and 100% extracts than those in the 0% extract. The protein level of pIκBα was lower in the 60%, 80% and 100% extracts. In the in vivo experiments, there were fewer inflammation and lower levels of expression of NF-κB in the Mg-6Zn alloy group than those in the control group. The NF-κB signal pathway of IEC-6 cells is sensitive to the concentration of the Mg-6Zn extracts. High concentrations extracts (60%, 80% and 100%) partly inhibit the NF-κB signal pathway. The IκBα is the main acting site of Mg-6Zn extracts. The High concentrations Mg-6Zn extracts increases level of IκBα and decrease IκBα degradation.

Keywords: Magnesium Alloy, NF-κB Signaling Pathway, Intestinal Epithelial Cells.

1. INTRODUCTION
Magnesium and magnesium alloys are regarded as promising biodegradable implant materials and have been implanted into blood vessels and bone tissue, exhibiting good biocompatibility and mechanical properties.1–3 Currently, the manual suture using suture threads has basically been replaced by the anastomot suture using anastomotic staples for intestinal anastomosis.4 However, few literatures have reported the application of magnesium and magnesium alloy materials in intestinal anastomosis surgery. It is necessary to investigate effects of Mg-6Zn alloy on intestinal epithelial cells. The homemade Mg-6Zn alloys showed favor to the growth of intestinal epithelial cells in vitro, and it performed better than the titanium counterpart in promoting healing and reducing inflammation in vivo in our previous work.5,6 We also observed that the cell cycle of the intestinal epithelial cells and gene expression of caspase-1 and bcl-2 were strongly affected by the concentrations of Mg-6Zn alloy extracts.7 However, the mechanism remained unclear.

In recent years, the nuclear factor kappa B (NF-κB) signaling pathway has provided numerous insights into how signaling events influence gene expression, and it has served as a paradigm for inducible transcription factors.8 Based on a large body of epidemiological and experimental data, the role of NF-κB in the linking immunity, inflammation, and cancer became highly anticipated.9,10 It is well known that NF-κB complexes are comprised of homo or heterodimers that are formed from the multigene families of RelA (p65), c-Rel, RelB, NF-κB1 (p50/p105) and NF-κB2 (p52/p100). Typically, NF-κB activation is associated with exposure to inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin 1 (IL-1),...
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Fig. 1. The classical NF-κB signaling pathway. In the cytoplasm, IκBα retains P50 and P65, preventing their nuclear translocation and subsequent DNA binding. When IκB phosphorylation takes place, released NF-κB dimers translocate to the nucleus where they bind to specific DNA sequences and promote transcription of target genes. (B) The effects of high concentration Mg-6Zn alloy extracts on NF-κB signaling pathway (red parts). After add high concentration Mg-6Zn alloy extracts, levels of IκBα increased and IκB phosphorylation decreased. The NF-κB signaling pathway was partly inhibited.

or bacterial products such as lipopolysaccharide (LPS). Several reports have shown that metal ions of nickel, mercury, gold, or palladium can affect NF-κB activation, as they modulate NF-κB-DNA binding. These signals promote phosphorylation and subsequent degradation of IκB proteins, consequently, free NF-κB (p65 and p50) can enter the nucleus and induce downstream gene expression. Figure 1(A) shows a process diagram depicting the classical NF-κB signaling pathway.

Magnesium ions can function as a signals in the immune system. Recently, Sugimoto et al., reported that magnesium plays a critical regulatory role in NF-κB activation, cytokine production, and disease pathogenesis. Similarly, Zinc ions also plays important role in NF-κB signaling pathway. Through this signaling pathway, zinc ions can influence the expression of specific cellular genes that are involved in tumors and inflammation. Therefore, in this study, we hypothesize that Mg-6Zn alloys can affect the NF-κB signaling pathway. The intestinal epithelial cells (IEC-6) were cultured in different concentrations of Mg-6Zn extracts. Then, expression levels of IκBα (binding p65 and p50), phosphorylation of IκBα and free NF-κB (p65 and p50) were investigated in vitro. After Mg-6Zn pins implanted in the cecum wall, the expression levels of NF-κB (p65) was examined.

2. MATERIALS AND METHODS

2.1. Materials and Preparation of Extracts

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. The chemical composition of the Mg-6Zn alloy (Table I) was determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES, Iris Advantage 1000, TJA, USA). The disc samples with a diameter of 11.3 mm and a height of 2.0 mm were processed from the as-extruded Mg-6Zn rod. The pin with a size 5 × 1 × 1 mm used for the in vivo test were machined from the rod, and ground on metallographic emery paper up to 1,000#, followed by ultrasonic wash in acetone, ethanol, and distilled water. Prior to testing, both implants were γ-sterilized with 29 kGy of cobalt-60 radiation.

The Mg-6Zn extracts were prepared according to ISO 10993-5. Disc samples were immersed in Dulbecco’s Modified Eagle’s medium (DMEM, Gibco...
TM, Invitrogen). The ratio of the surface area of the disc samples to the volume of the extract medium was 1.25 cm²/mL, and the immersed samples were maintained in a humidified atmosphere with 5% CO₂ at 37 °C for 24 h. The extracts were then collected and refrigerated at 4 °C. To determine a dose–response relationship, the extracts were serially diluted with DMEM (20, 40, 60 and 80%). The pH values and ion concentration of Mg and Zn were determined by pH meter (PHS-3C, INESA, China) and ICP-AES (Iris Advantage 1000, TJA, USA).

### 2.2. Real Time PCR of IκBα, P50 and P65 In Vitro

The IEC-6 cells (1 × 10⁴) were cultured in 0%, 20%, 40%, 60%, 80% and 100% concentrations of Mg-6Zn alloy extracts for 1 day. Total cellular RNA was isolated from cell layers using a Trizol reagent (Life, Carlsbad, CA). The concentration and quality of RNA were determined using an ACTGene ASP Spectrophotometer (ACTGene, Piscataway, USA) and a purity (A260/A280) of >1.8 was used. Complementary DNA (cDNA) was synthesized using the Prime-ScriptTM RT reagent Kit (TaKaRa Biotechnology, Dalian, China) according to the manufacturer’s instructions. Briefly, RNA (500 ng) was transcribed in a solution containing 5× PrimeScriptTM buffer (2 μL), Prime-ScriptTM 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 RNase-free water was added to a total volume 10 μL. The reaction mixture was incubated at 37 °C for 15 min for reverse transcription, and the reverse transcribe was then being inactivated at 85 °C for 5 s. Primers for the real-time PCR analysis of IκBα, P50 and P65 genes were designed (Table II) and synthesized (Sangon Biotech Co., Ltd., Shanghai, China).

Real-time PCR was performed using a quantitative real-time amplification system (TaKaRa Biotechnology, Dalian, China) according to the manufacturer’s instruction. Typically, 20 μg of the protein was loaded per lane. Protein samples were resolved using SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were blocked with 5% non-fat dry milk for 1 h at room temperature and incubated at 4 °C overnight with pIκBα at a 1:200 dilution (ab12135, mouse monoclonal to IκB alpha (phospho S32 + S36), Abcam, UK) and GAPDH at a 1:2000 (ab9485, Rabbit polyclonal to GAPDH, Abcam, UK). After washing three times with TBST, the blots were incubated with horseradish peroxidase-labeled secondary antibody (926-32210, IRDye 800CW goat anti-mouse IgG (H + L), 0.5 mg, Licolir, USA) for 1 h at room temperature. The levels of pIκBα proteins were determined using Cooamassie Blue staining. Blots were visualized using Beyo ECL Plus (Beyotime, China) according to the manufacturer’s instructions and the protein bands were quantitatively analyzed using an image analysis system (QuantityOne software, USA).

### 2.3. Western Blot Analysis of pIκBα Proteins

The IEC-6 cells were cultured in 0%, 20%, 40%, 60%, 80% and 100% concentrations of Mg-6Zn alloy extracts for 1 day. Total protein was extracted using a Qproteome Mammalian Protein Prep Kit (Qiagen, Germany) in accordance with the manufacturer’s instruction. Typically, 20 μg of the protein was loaded per lane. Protein samples were resolved using SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were blocked with 5% non-fat dry milk for 1 h at room temperature and incubated at 4 °C overnight with pIκBα at a 1:200 dilution (ab12135, mouse monoclonal to IκB alpha (phospho S32 + S36), Abcam, UK) and GAPDH at a 1:2000 (ab9485, Rabbit polyclonal to GAPDH, Abcam, UK). After washing three times with TBST, the blots were incubated with horseradish peroxidase-labeled secondary antibody (926-32210, IRDye 800CW goat anti-mouse IgG (H + L), 0.5 mg, Licolir, USA) for 1 h at room temperature. The levels of pIκBα proteins were determined using Cooamassie Blue staining. Blots were visualized using Beyo ECL Plus (Beyotime, China) according to the manufacturer’s instructions and the protein bands were quantitatively analyzed using an image analysis system (QuantityOne software, USA).

### 2.4. H&E Staining and Immunohistochemical Analysis of NF-κB In Vivo

All animal experiments were performed 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), approved by the Ethics Committee of the Sixth People’s Hospital Affiliated to Shanghai Jiao Tong University. Animals were supplied by the Sino-British Sippr/BK Lab Animal Ltd, Co, China (License No: SCXK (hu) 2008-0016).

Fourteen adult, clean, male Sprague-Dawley rats with a mean body weight of 275 ± 25.6 g were randomly and equally assigned to two groups (7 rats in each group): the Mg-6Zn pins group and negative control group. Rats were acclimatized to laboratory conditions for three days before operation, and housed at 21 °C with a 12-hour light-dark cycle, and allowed free access to tap water and standard rodent chow. After the onset of anesthesia (an intraperitoneal injection of pentobarbital sodium 50 mg/kg), a median incision of 2 cm was made in the abdomen. In the Mg-6Zn alloy group, pins were implanted...
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into the cecum wall. In the negative control group, nothing was implanted and the 5 mm incisions were directly sutured.

At 1 week post-operation, the rats were sacrificed, and the embedded Mg-6Zn alloy pins were removed. The tissue samples surrounding pins were fixed immediately in 10% buffered formaldehyde. Sections were embedded in paraffin, then cut to a thickness of 4 μm and mounted on a glass slides. Histological slices were processed, and stained with H and E. Immunohistochemical analysis was conducted to determine the levels of expression of NF-κB. Tissue sections were deparaffinized in xylene and then rehydrated in graded concentrations of ethyl alcohol (100, 95, 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) for 5 min and then placed in normal mouse serum as blocking antibody at room temperature for 10 min. The sections were evaluated using antibody for NF-κB (at0552, 1:400, CMCTAG, UK). After incubating at 4 °C for 24 h, sections were washed three times with PBS 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 and membranous staining was considered positive for NF-κB. The sections were evaluated in a light microscope using the MICRO IMAGETM software (Olympus Optical Corp. Ltd., Tokyo, Japan). Expression of NF-κB was examined by integrated optical density (IOD) using Motic Fluo 1.0 software (Motic China Group Co. Ltd., Shenzhen, China).

2.5. Statistics
Statistical analysis was performed using the SPSS 18.0 software package (SPSS Inc., Chicago, USA). The experimental values were analyzed using the paired samples t-test and expressed as the mean ± standard deviation (SD). One-way ANOVA analysis was performed to determine differences between groups for each evaluated parameter at each time point. Non-parametrical tests [k 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 \( P < 0.05 \).

3. RESULTS
3.1. pH Values and Ion Concentration
The pH values of the 100, 80, 60, 40 and 20% extracts were 8.67 ± 0.06, 8.63 ± 0.05, 8.56 ± 0.03, 8.51 ± 0.07 and 8.42 ± 0.05, respectively. The ion concentration of Mg and Zn of 100% Mg-6Zn alloy extracts were 15.91 ± 4.42 mM and 0.014 ± 0.003 mM; 80% Mg-6Zn alloy extracts were 12.86 ± 2.51 mM and 0.013 ± 0.002 mM; 60% Mg-6Zn alloy extracts were 9.52 ± 2.62 mM and 0.010 ± 0.002 mM; 40% Mg-6Zn alloy extracts were 6.36 ± 1.57 mM and 0.006 ± 0.001 mM, and 20% Mg-6Zn alloy extracts were 3.31 ± 0.65 mM and 0.003 ± 0.000 mM.

Fig. 2. Relative mRNA expression levels of IkBa, p50 and p65 for IEC-6 cells cultured in 0, 20, 40, 60, 80 and 100% Mg-6Zn alloy extraction media for 1 day. (A) Relative mRNA expression levels of IkBa; (B) relative mRNA expression levels of p50; Figure 2(C) Relative mRNA expression levels of p65. * means \( P < 0.05 \).
3.2. mRNA Levels of IκBα, P50, P65
Figure 2 showed the relative mRNA expression levels of IκBα, p50 and p65. The levels of mRNA expression of IκBα were higher when IEC-6 cells were cultured in the 80% and 100% extract group than that in other groups for 1 day. No significant differences were found for the mRNA expression of IκBα in the 20%, 40% and 60% extracts compared with that in the 0% group ($P > 0.05$). Starting from the 60% concentration, there were significant differences in the P50 and P65 expression. Both the expressions of P50 and P65 were significantly lower in 60%, 80% and 100% extracts than those in the 0% extracts ($P > 0.05$). No significant differences were found between the 40%, 20% and 0% extracts groups. Decreasing IκBα phosphorylation.

3.3. Protein Expressions of pIκBα
Figure 3(A) showed a representative image of protein levels of pIκBα for IEC-6 using the western blot method. The calculated protein levels of pIκBα for IEC-6 cells exhibited in Figure 3(B). Starting from the 60% concentration, there were significant differences in the pIκBα expression. The levels of pIκBα in the 100%, 80% and 60% extracts were significantly lower than those in the 0% extracts ($P > 0.05$), while no differences were found between the 20%, 40% and 0% extract groups.

3.4. H and E and Immunohistochemical Analysis of NF-κB In Vivo
There were no unexpected deaths after the rats were implanted with Mg-6Zn alloy pins. All rats survived until the completion of the experiment and had stable body weights. The rats in the Mg-6Zn alloy and control group grew well and could do free activities with normal diet and drinking. Figure 4 showed a representative image of the morphology of the intestinal tissue at the implant region using H and E staining. There was less dynamic inflammatory cell infiltration in the Mg6Zn group than those in the control group.

Figure 5 shows a representative immunohistochemical image depicting the expression of NF-κB in the cecum tissues surrounding Mg-6Zn alloy pins at 1 week post-operation. Based on the immunohistochemical IOD results, the expression of NF-κB in the Mg-6Zn alloy group
were significantly lower than that in the control group ($P > 0.05$) at 1 week post-operation.

4. DISCUSSION

In a previous study, we reported that low concentrations of Mg-6Zn alloy extracts did not induce changes of apoptosis genes; extreme high concentrations of Mg-6Zn alloy extracts can cause abnormal expression of apoptosis genes of intestinal epithelial cells.\(^7\) Numerous studies have demonstrated that NF-κB signal pathway plays a pivotal role in maintaining a balance between apoptosis and anti-apoptosis, and inflammation and anti-inflammation.\(^20\)\(^{-}\)\(^23\) Cellular stimulation can activate NF-κB/Rel transcription family.\(^24\) Once the NF-κB signal pathway is abnormally induced, over-apoptosis or apoptosis deficiency and inflammation will take place.\(^25\)

The results of this study indicate that the NF-κB signal pathway of IEC-6 cells is sensitive to the concentration of the Mg-6Zn extracts. The NF-κB signal pathway of IEC-6 cells is tolerant to the low concentrations (20 and 40%) extracts, while high concentrations extracts (60%, 80% and 100%) partly inhibit the NF-κB signal pathway. Our \textit{in vivo} results are similar to the \textit{in vitro} results. At 1 week after surgery, the inflammation and expression levels of NF-κB in tissues surrounding the Mg-6Zn pins are lower than the negative control group. In this study, the change of pH value is little (from 8.67 ± 0.06 to 8.42 ± 0.05). Therefore, the influence of pH value on NF-κB signaling pathway is small.

The high concentrations Mg-6Zn extracts increase levels of IκBα and decrease IκBα degradation. In the cytoplasm, IκBα retains P50 and P65, preventing their nuclear translocation and subsequent DNA binding. This study shows that the IκBα is the main acting site of high concentrations of Mg-6Zn extracts (Fig. 1(B)). The expression levels of IκBα increase and IκBα phosphorylation decrease in the cell when there is a magnesium zinc alloy outside the cell. More IκBα and less IκB phosphorylation induce the binding sites of P65 and P50 increased, and the free P65 and P50 decreased. Then, the NF-κB signaling pathway was partly inhibited.

Mg and Zn ion are quite important for the NF-κB signaling pathway. Mg can increase basal IκBα levels and plays a critical regulatory role in NF-κB activation, cytokine production, and disease pathogenesis.\(^16\) Zn is required for structural and functional integrity of several transcription factors.\(^17\) A major target of Zn may be NF-kB. The effects of Zn on translocation of NF-κB have been attributed to suppression of phosphorylation and degradation of the inhibitory proteins that normally sequester NF-κB in the cytoplasm.\(^14\) The tumor necrosis factor (TNF-α) is a downstream targeted gene of NF-κB signaling pathway. In an \textit{in vivo} study, the Mg-6Zn alloy reduced the expression of the TNF-α at different stages and decreased inflammatory response.\(^6\) This decreasing of expression of TNF-α and inflammatory response might result from the inhibit effects of Mg-6Zn alloy on NF-κB signal pathway.

5. CONCLUSION

In this study, \textit{in vitro} and \textit{in vivo} experiments were performed to determine effects of concentrations of Mg-6Zn alloy extracts on NF-κB signaling pathway of intestinal epithelial cells. We found that NF-κB signaling pathway is inhibited in the high concentrations of Mg-6Zn alloy extracts. The high concentrations Mg-6Zn extracts increase levels of IκBα and decrease IκBα degradation. The IκBα is the main acting site of high concentrations of Mg-6Zn extracts.

Conflict of Interest

The authors declare that they have no competing interests.

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References and Notes

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