Magnesium calcium alloys/mineralized collagen composites mediating macrophage polarization to promote bone repair

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Abstract

Magnesium-based composites are a focal point in biomaterials research. However, the rapid degradation rate of magnesium alloys does not align with the healing time of bone tissue. Additionally, the host reaction caused by magnesium implantation hampers its full osteogenic potential. To maintain an appropriate microenvironment, it is essential to enhance both the corrosion resistance and osteogenic activity of the magnesium matrix. In this study, a composite scaffold composed of mineralized collagen (nHAC) and magnesium alloy was utilized to investigate the regulatory effect of nHAC on RAW264.7 macrophages and evaluate its impact on mouse bone marrow mesenchymal stem cells (mBMSCs) in terms of osteogenesis, immune response, and macrophage-induced osteogenic differentiation. The findings revealed that Mg-Ca/nHAC biomaterials primarily induced osteogenic differentiation through M2 polarization of macrophages, while Mg$^{2+}$/Col I stimulated the integrin alpha2beta1-FAK-ERK1/2 pathway to promote bone formation. Cells treated with Mg-Ca/nHAC exhibited extensive spreading and flattening towards the surrounding area, facilitated by broad and abundant pseudopodia that firmly adhered them to the material surface and promoted growth as well as pseudopodia formation. These composite scaffolds based on prepared magnesium alloys hold significant potential for wide-ranging applications in bone tissue engineering.

1. Introduction

With the increasing number of elderly individuals, there has been a significant rise in the prevalence of bone-related diseases worldwide.$^{[1]}$ Annually, over six million fractures occur globally, with 56% requiring permanent or temporary internal fixation materials for surgical treatment.$^{[2-3]}$ Consequently, the development of biomaterials for internal fixation is an active area of research in orthopedics. Magnesium (Mg) and its alloys are biodegradable and absorbable materials that exhibit biocompatibility. Compared to other metals commonly used in clinical practice, Mg alloys have a similar modulus of elasticity to that of the bone cortex, thereby reducing the occurrence of the "stress shielding effect." However, while degradability is advantageous for Mg alloys, it also poses limitations as it can degrade rapidly without aligning with bone tissue healing time.$^{[4]}$ Moreover, host responses triggered by Mg alloy implantation hinder its full osteogenic potential. Therefore, one major limitation in traditional research lies in neglecting the significance of immune responses.$^{[5-6]}$ Timely and appropriate immune responses play a crucial role in promoting bone healing.$^{[7]}$ Henceforth, during synthesis techniques employed for Mg alloy composites fabrication, optimizing corrosion resistance within the Mg matrix and enhancing osteogenic activity become imperative to maintain an optimal "Bone immune microenvironment." This will enable matching between matrix degradation rate and osteogenesis during in vivo implantation while addressing key challenges associated with clinical applications involving Mg-based metals. Several researchers have implemented this strategy within their studies on artificial bone grafting materials. Zeng and colleagues$^{[8]}$ prepared Mg-Li-1Ca after plasma electrolysis (MAO) treatment and conducted experimental studies on its biocompatibility and corrosion resistance. They found that the coating of the MAO/PLLA composite materials greatly improved the corrosion resistance and compatibility of the cells.
Mineralized Collagen (nHAC) is a bionic composite material mainly composed of nano hydroxyapatite and type I collagen. Bone, cartilage, sinews, and dentine are made of nHAC.\textsuperscript{9} Therefore, nHAC can be used to repair bone defects, promote bone development, and accurately regulate bone immune responses. By regulating the polarization of macrophages during bone regeneration, nHAC can strongly induce enough M2 macrophages in vivo and in vitro to regulate the host immune response\textsuperscript{10-11}.

Regarding the regulatory role of bone biomaterials in osteogenesis, the immune response has both advantages and disadvantages. Adverse immune reactions can result in chronic inflammation and the formation of a fibrous capsule around the biomaterial, which hinders contact and integration between osteoblasts and the implant, thereby disrupting bone remodeling\textsuperscript{12}. Therefore, we applied a coating of nHAC material to the surface of the magnesium alloy. Mg\textsuperscript{2+}/Col I can stimulate the integrin α2β1-FAK-ERK1/2 pathway to promote osteogenesis\textsuperscript{13}. RANK (NF-kB receptor activating Ligand) receptor activating ligand is a cytokine belonging to the tumor necrosis factor (TNF) family. It induces differentiation of precursor osteoclasts into mature osteoclasts. By activating corresponding signal transduction pathways, RANK stimulates differentiated osteoclasts to express specific genes that enable mature osteoclasts to undergo bone resorption and maintain their survival\textsuperscript{14}. Hence, we investigated whether Mg-Ca/nHAC biomaterials promote osteogenic differentiation by regulating macrophage polarization and its underlying mechanism. The aim of this study was to investigate the osteogenic differentiation-promoting effect of Mg-Ca/nHAC materials by inducing macrophage polarization, as well as the influence of the scaffold on mouse bone marrow mesenchymal stem cells (mBMSCs), thereby elucidating the mechanism underlying Mg-Ca/nHAC composite scaffolds on bone repair.

2. Materials and methods

2.1. Preparation of materials

A magnesium-calcium (Mg-Ca) alloy sheet (with a calcium content of 1 wt.%; 10 mm in diameter and 1 mm in height) was provided by Beijing Union University. The nHAC bone powder (supplied by Beijing Aojing Medical Technology Co., Ltd.) is an artificial bone repair material. Its nano-based apatite accounts for 45% ± 5%. It has a porosity range of 80–90%, and pore sizes ranging from 50 to 500 nm. Preparation of Mg-Ca/nHAC: The nHAC solution (10 mg/mL in acetic acid) was evenly applied onto the surface of the Mg-Ca alloys.

2.2. Surface structure and characterization

We utilized the Oxford Quorum SC7620 Sputter Coating System to apply a gold coating (10 mA) for a duration of 45 seconds. Subsequently, the ZEISS GeminiSEM 300 scanning electron microscope was employed for sample observation, energy spectrum mapping, and other tests. The acceleration voltage was set at 3 kV during the recording of morphological characteristics. An SE2 secondary electron detector was utilized. Through this technique, we assessed the surface structure and element content distribution for each group of materials.
2.3. Osteogenic evaluation

2.3.1. Cell culture

According to the GB/T16886.5 standard, mouse bone marrow mesenchymal stem cells (mBMSCs, sourced from Shanghai Saibaikang) were introduced into DMEM supplemented with 10 mL/dL fetal bovine serum, 0.1 mol/L dexamethasone, 50 µg/mL Vitamin C, and 10 mmol/L β-Sodiumglycerophosphate, collectively known as the osteogenic induction medium. The cells were cultivated in a constant temperature incubator (5% CO₂, 37°C). When the cell density reached 80–90%, the culture medium was aspirated and the cells were subjected to digestion before use.

2.3.2. Cell proliferation

The alloy was extracted using a complete culture medium at a concentration of 0.1 g/mL for 24 hours. The Mg-Ca/nHAC sample was extracted at a ratio of 0.1 g/mL (Mg-Ca) and 60 mg/mL (nHAC). After filtration and sterilization, the experimental solution was divided into three groups: the control group, groups with an extraction solution to culture medium ratio of 1:2, and groups with an extraction solution to culture medium ratio of 1:1. To collect mBMSCs at the logarithmic growth phase, cell counting was performed, and the cell concentration was adjusted to maintain a cell density of 1 × 10⁴ cells/well in a 96-well plate. The cells were incubated at 37°C with 5% CO₂ for 24, 48, and 72 hours. Subsequently, the culture medium was removed, and each well was rinsed thrice with 100 µL of PBS. Following this, 100 µL of culture medium containing CCK-8 (Solarbio, China) was added to each well and incubated for 2 hours in a constant temperature incubator at 37°C. The absorbance was measured at 450 nm using an enzyme-linked immunosorbent assay.

2.3.3. Flow cytometry

The mBMSCs were seeded in Annexin V binding buffer at a concentration of 1×10⁶ cells/mL. The cell suspension was transferred to a test tube and stained with 5µL FITC/annexin V and 10µL propidium iodide solution (Soraibao, China). The cells were gently vortexed and then incubated for 15 minutes at room temperature. Finally, 400 µL of Annexin V Binding Buffer was added to each tube and mixed evenly. The mixed solution was analyzed by flow cytometry.

2.3.4. Cell morphology analysis

The mBMSCs were seeded onto cover slides of different groups and placed in a 24-well plate (2 per well, 2 × 10⁴ cells). Control cover glasses treated with polylysine were used for comparison. Subsequently, 50 ng/mL PMA was added to the culture medium for 24 hours to induce adherent cell formation. Following this, the cells were allowed to interact with the materials for one day. For SEM analysis, the sample was fixed in 3.7% glutaraldehyde and observed using a scanning electron microscope (SEM) at a voltage of 3 kV after undergoing a graded ethanol dehydration series.
2.3.5. Cell adhesion

The mBMSCs were seeded into confocal dishes at a density of $6 \times 10^4$ cells/mL, with 1 mL of cell suspension per group and 3 replicate wells per group. After 7 days, the culture medium was removed, and the cells were fixed with 10% paraformaldehyde for 10 minutes at room temperature. Subsequently, they were washed three times with PBS. A 0.5% Triton X-100 solution was applied to allow the cells to permeabilize for 5 minutes. The cells were then incubated with 200 µL of TRITC-labelled phalloidin working solution (Solarbio, China) for 30 minutes at room temperature in the dark. The nuclei were stained with 200 µL of DAPI solution (Sorabio, China) for 30 seconds. Confocal microscopy was used to observe the cells, and ImageJ software was employed to calculate cell areas.

2.4. Immunological evaluation

2.4.1. Cell culture

Following the guidelines outlined in GB/T16886.5, mouse macrophage RAW264.7 cells were provided by a company based in Shanghai, China. The macrophages were cultured using DMEM culture medium. The culture medium was aspirated and the cells were subjected to digestion before use, once the cell density reached 80–90%.

2.4.2. Cell proliferation

The alloy was extracted using a complete culture medium at a concentration of 0.1 g/mL for 24 hours, while the Mg-Ca/nHAC group was extracted at a ratio of 0.1 g/mL (Mg-Ca) and 60 mg/mL (nHAC). After filtering and sterilizing, the experimental groups were divided into three categories: the Control group, the groups with an extraction solution to culture medium ratio of 1:2, and the groups with an extraction solution to culture medium ratio of 1:1. The RAW264.7 cells were harvested during the logarithmic growth phase and then plated at a density of $1 \times 10^4$ cells/well in a 96-well plate. The cells were cultured at 37°C in 5% CO$_2$ for 24, 48, and 72 hours. Following this, the culture medium was removed, and each well was washed three times with 100 µL PBS. Subsequently, each well was incubated with 100 µL of CCK-8 solution (Solarbio, China) at 37°C for 2 hours. The absorbance was measured at 450 nm.

2.4.3. Analysis of the morphology of macrophages

Macrophages were seeded onto cover slides of different grouping materials and placed in a 24-well plate (2 coverslips per well, with $2 \times 10^4$ cells each). A cover glass treated with polylysine served as the control. Next, 50 ng/mL PMA was added to the culture medium for 24 hours to induce adherent cell formation. The cells were then allowed to interact with the material for one day. For SEM analysis, the sample was fixed in 3.7% glutaraldehyde and observed in a graded ethanol series at a voltage of 3 kV using a scanning electron microscope (SEM).

2.4.4. Immunofluorescence staining
RAW264.7 macrophages were fixed on coverslips cultured with various materials using 4% paraformaldehyde, permeabilized with 0.25% Triton X, and blocked with 1% BSA. Subsequently, IL-6 antibodies were diluted (1:100), applied to the coverslip, and allowed to incubate overnight at 4°C. Coverslips were then exposed to secondary antibodies conjugated with either fluorescein isothiocyanate or tetramethyl rhodamine isothiocyanate for 30 minutes. Finally, the cells were stained with DAPI for 5 minutes to visualize the nuclei. The samples were examined using a Zeiss laser scanning microscope (LSM510) equipped with LSM 5 release 4.2 software.

### 2.4.5. ELISA

The RAW264.7 macrophages were harvested for cell enumeration. A total of $2 \times 10^5$ cells per well were seeded in a six-well plate. Incubation was carried out at 37°C with 5% CO$_2$ for 72 hours. Subsequently, they were cooled in a refrigerator at 4°C for 30 minutes, followed by gentle agitation and collection. The cells were then washed with 1x PBS, and the supernatant was discarded. The samples underwent two freeze-thaw cycles in liquid nitrogen. The protocol provided with the ELISA test kit (Elabscience) was adhered to for assaying the supernatants of macrophages cultured on diverse materials. We assessed the expression of M1-related cytokines including iNOS, IL-6, and CD80, as well as M2-related cytokines such as IL-10, Arginase-1 (Arg-1), and CD206.

### 2.5. Induction of osteogenic differentiation by macrophages

#### 2.5.1. Cell culture

RAW264.7 cells were seeded in a 25 cm$^2$ cell culture flask with 4.0–5.0 mL of DMEM medium. They were cultured in a CO$_2$ incubator at 37°C with a 5% CO$_2$ concentration. The medium was refreshed every two to three days. For subculturing, cells were dissociated using 2.5% trypsin. The collected supernatant was then transferred to cultured mBMSCs. A 1:1 mixture of macrophage medium and complete DMEM medium was used to cultivate mBMSCs. The culture medium was replenished every 2–3 days.

#### 2.5.2. ALP (alkaline phosphatase) activity

The ALP activity of mBMSCs was assessed using the ALP kit (China Biyuntian Company). Cells were seeded into a 24-well plate at a concentration of $1 \times 10^4$ cells/mL, with 5 replicate wells per group. One milliliter of cell suspension was added to each well. After 7 days of incubation, the culture medium was aspirated and the cells were washed twice with PBS. Subsequently, 500 µL of 0.2% (v/v) Triton X-100 (Sigma, USA) was added to the plate to lyse the cells. The ALP activity in the lysate was determined using the ALP kit. Finally, the optical density(OD) at 520 nm was measured using a spectrophotometer.

#### 2.5.3. Real-Time QPCR assay
The expression levels of osteogenic-related genes, including alkaline phosphatase (ALP), runt-related transcription factor 2 (Runx2), bone morphogenetic protein 2 (BMP-2), osteocalcin (OCN), osteopontin (OPN), and vascular endothelial growth factor (VEGF), were assessed via real-time PCR. Cells were seeded at a concentration of $1 \times 10^5$ cells/well and cultured for 7 days. Total RNA was extracted using Trizol (Ambion, USA). Subsequently, 1 µg of RNA from each sample was reverse transcribed into complementary DNA (cDNA) using the Prime ScriptTM RT reagent kit (Vazyme, USA). Table 1 provides the forward and reverse primers for the selected genes. Beta-actin was used as an internal reference. Data analysis was conducted using iQTM5 optical system software version 2.0.

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>BMP-2</td>
<td>AAGCCAAACACAAACAGCGG</td>
<td>AAAGGCATGATAGCCGGAG</td>
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<td>ALP</td>
<td>AGCAGGTTTCTCTTGGGC</td>
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<td>Runx-2</td>
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<td>AGCCCTCTGCAGGTGATAGA</td>
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<td>OPN</td>
<td>TGCCAGTCAGAAGAAGAAG</td>
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<tr>
<td>VEGF</td>
<td>TCCGTAGTACGGTCTCCTC</td>
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<tr>
<td>β-actin</td>
<td>CGATATCGCTGGGAGTGC</td>
<td>AGGTGCTGGTCCAGATCTCTC</td>
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</tbody>
</table>

2.5.4. Western blotting

The mBMSCs were cultured for 7 days in DMEM supplemented with 10% fetal bovine serum. RIPA buffer (Beyotime, China) was employed for cell lysis. Protein concentration was determined using the BCA protein quantification kit (Beyotime, China). Subsequently, the protein sample was denatured by heating at 98°C for 5 minutes and then loaded onto an SDS-PAGE gel. Following electrophoresis, the protein was transferred to a PVDF membrane. The membrane was then incubated with 5% BSA for 2 hours at room temperature, followed by washing with TBST. Primary antibodies including anti-FAK, anti-integrin α2, anti-integrin β1, RANK, and anti-ERK1/2 were applied, followed by overnight incubation at 4°C. Subsequently, secondary antibodies were applied for 90 minutes. After washing with TBST, the membranes were treated with a chemiluminescence reagent and exposed. Immunoblotting images were semi-quantified using ImageJ.

3. Statistical Analysis

In all experiments, a minimum of three samples per group were analyzed. One-way analysis of variance (ANOVA) was employed to assess differences between the two groups. Statistical significance between
sample groups was determined. Quantitative data was represented as mean ± standard deviation for each group. Differences, both within and between groups, were considered statistically significant when p < 0.05.

4. Results

4.1. Preparation of materials

Preparation of Mg-Ca/nHAC: nHAC solution (10 mg/mL in acetic acid) was prepared and uniformly coated on the surface of Mg-Ca alloy (Fig. 1).

4.2. Surface structure and characterization

The SEM micrographs of electrodeposited Mg-Ca, nHAC, Mg-Ca/nHAC are shown in Fig. 2. Figure 2A shows the entire smooth surface and uniform microstructure of the Mg-Ca alloy, at the same time, a slight oxidation can be found on the surface. Figure 2B shows that the nHAC layer is a three-dimensional nanoscale structure that presents a sheet-like structure of disordered growth outward, forming a pore structure on the surface, paving the way for cells to be implanted from the outside. Figure 2C shows the three-dimensional nanoscale nHAC of the Mg-Ca/nHAC group growing on the surface of the Mg-Ca alloy. Most of the pores in nHAC nanostructures are interconnected, facilitating cell adhesion and growth. EDX spectra of MG-CA /nHAC composite coatings show the presence of Ca, Mg, P, and O (Fig. 2D-I). The high oxygen content may be the cause of sample oxidation. Because nHAC contains nano-hydroxyapatite, it is rich in calcium, phosphorus and other elements.

4.3. Osteogenic evaluation

4.3.1. Cell proliferation

Initially, we employed the CCK-8 kit (Fig. 3) to assess the growth of mBMSCs. The experimental groups consisted of a control, as well as groups with extract-to-medium ratios of 1:2 and 1:1. Each treatment was repeated three times. The results revealed that after 24 hours, there was no significant disparity in the proliferation of mBMSCs between the treatment groups and the control group. Only a slight reduction in cell viability was observed when the collagen extract-to-medium ratio was 1:1, although the difference was not substantial.

However, as the treatment duration extended to 48 and 72 hours, noticeable discrepancies in mBMSC viability emerged between the treatment groups and the control group. The highest level of cell activity was observed when the extract-to-medium ratio was 1:2. Consequently, for subsequent experiments involving mBMSCs, we opted for a 1:2 ratio of extract solution to medium.

4.3.2. Flow cytometry

Flow cytometry was employed to assess changes in cell apoptosis using GRN-B and RED-B channels. The results, as depicted in Fig. 4, indicated that the total number of apoptotic cells in all three treatment
groups was significantly lower compared to the control group. Additionally, both Mg-Ca and nHAC demonstrated a positive impact on cell growth. Particularly noteworthy was the substantial enhancement in cell growth observed after Mg-Ca/nHAC treatment, showcasing significant differences between the two groups.

Furthermore, the total number of apoptotic cells in the Mg-Ca/nHAC group was significantly reduced compared to both the nHAC and control groups. Among the three experimental groups, the Mg-Ca/nHAC group exhibited the lowest number of apoptotic cells, suggesting a significant positive impact on cell growth.

4.3.3. Cell morphology analysis

The impact of different materials on the morphology of mBMSCs was investigated by studying their morphological changes using scanning electron microscopy (SEM) after inoculation and culturing on various surfaces. The SEM analysis results are depicted in Fig. 5. The observations revealed distinct morphological characteristics for each group.

In the Mg-Ca group, cells show obvious stretching and expansion compared to the blank group, although their cell bodies remained spherical and not entirely elongated. In the nHAC group, cells also exhibited expansion, and pseudopodia were discernible, albeit in lower numbers. Conversely, in the Mg-Ca/nHAC group, all cells exhibited full expansion and flattened outward. The presence of wide and numerous pseudopodia facilitated cell adhesion to the material's surface, promoting the growth and development of additional pseudopodia. These findings corroborate earlier research, highlighting that Mg-Ca/nHAC treatment was the most effective in promoting osteogenic differentiation, followed by nHAC and Mg-Ca treatments.

4.3.4. Cell adhesion

F-Actin (Phalloidin) staining was utilized to investigate the organization of the cytoskeleton. The observed alterations in cell morphology corresponded with those depicted in the SEM images. In the control group (Fig. 6), cells exhibited a flattened and minimally extended appearance. Conversely, cells in the Mg-Ca group, nHAC group, and Mg-Ca/nHAC group displayed a widespread distribution, with a robust and pronounced morphology. Additionally, these cells appeared elongated and stretched, and their actin filaments were distinctly discernible. Notably, cells in the Mg-Ca/nHAC group exhibited a particularly dispersed appearance, further suggesting that Mg-Ca/nHAC treatment enhanced osteogenic differentiation of mBMSCs, promoting bone repair.

4.4. Immunological evaluation

4.4.1. Cell proliferation

The experimental group was divided into three subgroups (Fig. 7): the Control group, groups with an extraction solution to culture medium ratio of 1:2, and groups with an extraction solution to culture medium ratio of 1:1; each treatment subgroup was subjected to three replicate wells. The results revealed
that after 24 hours, when the ratio of extraction solution to culture medium was 1:1, the viability of cells in the Mg-Ca group and the Mg-Ca/nHAC group significantly decreased. However, compared to the cells in the control group, those in the nHAC group exhibited no significant difference in viability.

At 48 hours, with the extraction solution to culture medium ratio set at 1:1 across the three groups, cell viability was notably lower compared to the other groups. This trend persisted at 72 hours, with the same 1:1 ratio yielding lower cell viability in these groups. Treatment with an extraction solution to culture medium ratio of 1:2 significantly enhanced cell viability across all three treatment groups, aligning with the findings presented in Fig. 3.

Both mBMSCs and RAW264.7 cells exhibited the most substantial inhibition of proliferation when exposed to an extract ratio of 1:1. Conversely, cell activity reached its peak when the extract-to-culture medium ratio was set at 1:2. Consequently, we employed a 1:2 ratio of extraction solution to culture medium for treating RAW264.7 cells in subsequent experiments. These results suggest that varying concentrations of degradation product components exert differential effects on cell behavior over time.

### 4.4.2. Analysis of the morphology of macrophages

To assess the influence of different materials on macrophage morphology, macrophages were seeded and cultured on various surfaces. Morphological alterations in macrophages were examined using scanning electron microscopy, and the corresponding SEM images are presented in Fig. 8. The findings revealed distinct morphological features in each group.

In the Mg-Ca group, cells exhibited elongation, although their pseudopodial extensions were not fully pronounced compared to cells in the control group. Cells in the nHAC group also displayed elongation, with relatively fewer pseudopodia extending. These pseudopodia assumed a radiating sun rays pattern, predominantly indicative of the classic M1 phenotype. Conversely, cells in the Mg-Ca/nHAC group had spherical cell bodies extending towards the poles, resembling the typical M2 phenotype. The cell density in this group was akin to that of the M2 phenotype. The extensive pseudopodia firmly anchored the cells to the material's surface. This observation aligns with earlier results, underscoring that Mg-Ca/nHAC treatment was more conducive to cell adhesion and growth, mirroring previous findings.

### 4.4.3. Immunofluorescence staining

The experimental findings are presented in Fig. 9. The fluorescence intensity of IL-6 in the control group is notably high, whereas in the experimental treatment group, the fluorescence intensity of IL-6 is significantly reduced. This observation suggests that Mg-Ca/nHAC exerts a notable anti-inflammatory effect, favoring polarization towards the M2 phenotype, which is conducive to bone repair.

### 4.4.4. ELISA

We conducted ELISA assays in accordance with the manufacturer's instructions, using supernatants from macrophages cultured on different materials. The secretion of M1-related cytokines, namely iNOS, IL-6, and CD80, as well as M2-related cytokines, IL-10, arginase-1 (Arg–1), and CD206, were assessed. The
ELISA results demonstrated a significant upregulation of some markers associated with mouse M2 macrophages (IL-10, Arg–1, and CD206) in the mineralized collagen group compared to the control group. In the Mg-Ca/nHAC group, the expression of M2 markers (Arg–1 and CD206) was higher, while the M1 marker (IL-6) significantly decreased compared to the control group. These findings suggest that cells undergoing early-stage Mg-Ca/nHAC treatment likely experienced M2 polarization. The expression of iNOS and CD80 also decreased, though the extent of change was less prominent, indicating that Mg-Ca/nHAC treatment may modulate macrophage polarization (Fig. 10).

4.5. Induction of osteogenic differentiation by macrophages

4.5.1. Alkaline phosphatase activity

We investigated the impact of macrophage polarization on the osteogenic differentiation of bone marrow mesenchymal stem cells through an ALP activity assay, with results depicted in Fig. 11. Noticeable distinctions in blue nodules were observed across various treatment groups, particularly in the Mg-Ca/nHAC group. Positively stained cells appeared as dark bluish-purple.

Calculation was performed using the designated formula and the data was plotted using GraphPad Prism. The ALP activity of the Mg-Ca/nHAC group recorded the highest value. Moreover, both the Mg-Ca alloy group and Mg-Ca/nHAC group exhibited higher ALP activity compared to the blank control group. The ALP activity in the Mg-Ca/nHAC group significantly surpassed that of all other groups. The extent of new bone formation in the Mg-Ca/nHAC group was markedly greater than that in the nHAC group (p < 0.05).

The bone defect area in the Mg-Ca/nHAC group displayed evident bridging and a notable increase in new bone formation, while in the nHAC group, the bone defect area exhibited a cloudy appearance with comparatively less new bone formation. In the nHAC/CGF group, as most of the material had degraded, the defect area was effectively filled with new bone.

4.5.2. Expression of osteogenic genes

To assess whether macrophage polarization enhances osteogenesis, we collected macrophage supernatant for culturing mBMSCs and subsequently determined the expression levels of osteogenesis-related genes (OPN, OCN, ALP, RUNX2, BMP-2, and VEGF) using RT-qPCR. The results revealed a significant increase in the expression of osteogenic genes in the Mg-Ca group, nHAC group, and particularly the Mg-Ca/nHAC group, in comparison to the control group. These findings strongly suggest that macrophage polarization has a notable influence on the osteogenic differentiation of mBMSCs.

Moreover, treatment with Mg-Ca/nHAC led to a substantial upregulation in the expression of osteogenic genes, indicating the potential of Mg-Ca/nHAC to promote the osteogenic differentiation of mBMSCs by modulating macrophage polarization. The ELISA results further supported this observation,
demonstrating that macrophages polarized toward the M2 phenotype exerted a positive influence on osteogenic differentiation (Fig. 12).

4.5.3. Western blotting

Expanding on our previous discoveries, we aimed to explore its potential in upregulating the integrin α2β1-FAK-ERK1/2 pathway, while concurrently suppressing the RANK pathway, with the ultimate goal of promoting osteogenesis. Protein levels of FAK, α2, β1, ERK1/2, and RANK were evaluated through Western blot assays. The results revealed a significant enhancement in the expression of integrin α2 and ERK1/2 proteins within the Mg-Ca/nHAC group in comparison to the other groups. In contrast, the expression levels of FAK and RANK proteins demonstrated a substantial reduction (Fig. 13).

5. Discussion

In recent years, the role of Mg alloys as bone repair materials has garnered significant attention in research. However, the rapid degradation of magnesium alloys presents a challenge, as it may not align with the required healing time for bone tissue. Additionally, the accelerated degradation and formation of hydrogen gas may impact the host organism’s response. To address these limitations, researchers have successfully employed surface coatings or alloys to modify their physical and chemical properties.\[15–17\] Mg-Ca ternary or Zn alloyodegradable alloys composed of magnesium, zinc, and calcium have gained attention in recent years\[18–20\]. The corrosion resistance and degradation rate of Mg-Ca alloy were enhanced by coating it with nHAC. Furthermore, the host reaction triggered by magnesium alloy implants hinders its full potential in promoting osteogenesis\[21–23\]. Among various players, innate immune cells such as macrophages play a crucial role\[24–25\]. Cost discovered that magnesium alloys can stimulate inflammation; however, on the 5th and 10th day post implantation, magnesium alloy exhibited significant regulatory effects on macrophage polarization towards the M2 type. This process subsequently promotes tissue healing while reducing immune responses to degradation products from the alloy. These effects are attributed to ions (Mg, Zn, and Ca ions) released during early surface degradation interacting with intracellular ion channels\[26–27\]. Therefore, we conducted an in vitro simulated experiment using Mg-Ca/nHAC material to investigate the role and mechanism of Mg-Ca/nHAC in promoting the biological behavior of mBMSCs by regulating the bone immune microenvironment. The results of the CCK8 experiment (shown in Figs. 3 and 7) indicated that there was no significant difference observed in RAW264.7 cells when the extract vs media ratio was 1:1. Amongst the treatments applied to mBMSCs, only the Mg-Ca group and Mg-Ca/nHAC group exhibited noticeable cell damage. Notably, when the extract vs media ratio was 1:1, it resulted in the strongest inhibition of cell proliferation, which may be attributed to varying concentrations of Mg^{2+} affecting cellular behavior differently. Wang et al. also demonstrated that a concentration of 10 mM Mg^{2+} significantly enhanced adhesion and differentiation of osteoblasts, while a concentration of 18 mM Mg^{2+} inhibited osteoblast activity\[28\]. We treated the cells with a 1:2 ratio of extract to medium in order to determine the compatibility of different materials with mBMSCs through apoptosis and cell adhesion assays. The results from the apoptosis assay
demonstrated a significant reduction in apoptotic cells within the Mg-Ca/nHAC group, indicating better biocompatibility (Fig. 5). Compared to the control group, cells within the Mg-Ca/nHAC group exhibited full extension and flattening towards their surroundings, with broad and abundant pseudopodia firmly anchoring them to the material surface. This promoted cell growth and increased pseudopod count. The results indicated that the Mg-Ca/nHAC group had superior efficacy in promoting osteogenic differentiation, followed by the nHAC group and then the Mg-Ca group. Tritc labeling staining revealed dense distribution of cells within the Mg-Ca/nHAC group, suggesting that this treatment facilitated differentiation (Fig. 6). To assess whether materials from each treatment group could elicit an immune response, morphological analysis and ELISA were performed on RAW264.7 macrophages (Fig. 8). The Mg-Ca/nHAC group displayed higher cell density, enhanced growth state, and more extended synapses compared to other groups. Cells within this group exhibited elongation towards poles-characteristic features of M2 phenotype. Immunofluorescence staining further confirmed the anti-inflammatory and bone repair effects of this group (Fig. 9). ELISA results showed significant up-regulation of certain markers associated with M2 macrophages (IL-10, Arg-1, CD206) in comparison to the control group for Mg-Ca/nHAC treatment. Macrophages are innate immune cells that play an important role in substance-induced immune responses. Biomaterial implantation can induce the polarization of macrophages to M1 and M2 phenotypes. M1 is pro-inflammatory, while M2 is anti-inflammatory. Therefore, we investigated whether the combination of Mg-Ca could enhance the osteogenic differentiation of mBMSCs during macrophage polarization towards the M2 phenotype. Supernatants were collected and mBMSCs were cultured accordingly. The expression levels of osteogenic markers (OPN, OCN, ALP, RUNX2, BMP-2, and VEGF) were assessed by RT-qPCR and ALP activity assay. Notably, ALP is a crucial indicator for osteoblast differentiation. The results obtained from the ALP experiments (Fig. 11) demonstrated that the Mg-Ca/nHAC group exhibited significantly higher ALP activity with dark blue-purple positive cells compared to other groups. Furthermore, PCR analysis (Fig. 12) revealed significantly elevated levels of osteogenic genes in both Mg-Ca and nHAC groups. However, these levels were particularly pronounced in the Mg-Ca/nHAC group when compared to the control group. These findings suggest that M2-type polarization of macrophages promotes mBMSCs' osteogenic differentiation process effectively. Western blot experiments indicated that integrin α2 and ERK1/2 protein expressions were markedly upregulated in the Mg-Ca/nHAC group while FAK and RANK protein expressions showed significant downregulation when compared to other groups (Fig. 13). These differences may be due to the distance between integrins of adhesive related particles in the nano dimension. In stromal cells, activation of the integrin α2β1-FAK-ERK (MAPK) signaling pathway has been reported. The RANK protein plays a crucial role in bone and immune system regulation, making it a key component in the field of bone immunity. Osteoclasts release vesicles expressing RANKL, which interact with RANK on osteoblasts. This interaction triggers RANK reverse signaling, promoting bone formation. Our previous findings demonstrated that Mg²⁺/Collagen I can activate the integrin α2β1-FAK-ERK1/2 pathway, thereby enhancing osteogenesis. These results are consistent with those reported in other studies. However, the regulation of M2-type polarization of macrophages by Mg-Ca/nHAC biomaterial may involve a more intricate process.
The primary objective of this experiment was to investigate the impact of Mg-Ca/nHAC composites on bone formation. The findings revealed that Mg-Ca/nHAC exhibited superior osteogenic properties compared to both nHAC and Mg-Ca alloys. The fabrication process for these composites employed. In future studies, we can enhance the bonding method between these two materials to achieve a more tightly integrated composite structure. This material holds potential for application in load-bearing animal areas, allowing for its mechanical properties and subsequent improvement of its biological characteristics.

6. Conclusion

The objective of this study is to investigate the potential of Mg-Ca/nHAC biomaterials in promoting osteogenic differentiation of mBMSCs through modulation of the bone immune microenvironment, along with its underlying mechanism. The experiments were structured around three key aspects: osteogenic assessment, immune evaluation, and macrophage-induced osteogenic differentiation. Both macrophages and mBMSCs were cultured under various conditions, with assessments made for cell proliferation, adhesion, as well as osteogenic gene and protein expressions. In comparison to the other groups, Mg-Ca/nHAC biomaterials primarily may be induced osteogenic differentiation through M2 macrophage polarization. This effect was primarily mediated through the facilitation of the integrin α2β1-FAK-ERK1/2 signaling pathway, while concurrently inhibiting the RANK signaling pathway. The findings of this study establish a theoretical foundation for the prospective utilization of Mg-based composites in bone repair tissue engineering. Further exploration is needed to explore the specific role of macrophages in biomaterials research.

Declarations

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**Data and code availability:**

Datas can be found in the Mendeley Data, V3, http://dx.doi.org/10.17632/7zzpr4d8tz.3. Data will be submitted as required.

**References**


Figures
Figure 1

The process of nHAC coated on the surface of Mg-Ca alloy.
Figure 2

(A). The surface of Mg alloys materials viewed under an SEM (scale bar: 500nm). (B) The surface of nHAC materials viewed under an SEM (scale bar: 500 nm). (C) The surface of Mg-Ca/nHAC materials viewed under an SEM (scale bar: 500 nm). (D-I) The distribution of the content of the elements was observed (scale bar: 100 μm).
Figure 3

Changes in cell viability of mBMSCs with samples for 24, 48, and 72 h. (Compared to the Control group, *P < 0.05 and ** P < 0.01); Comparison between groups, # P < 0.05 and ## P < 0.01; (Es: extraction solution; Cm: Culture medium).
Figure 4

Flow cytometer analysis was performed using membrane-associated protein Annexin V and PI staining; *p < 0.05.
Figure 5

SEM images of the morphological characteristics of mBMSCs after treatment with different materials (scale bar: 50 µm and 10 µm).
Figure 6

The morphology of mBMSCs cultured with samples for seven days was analyzed by laser confocal microscopy (scale bar: 20 µm).
Figure 7

Changes in the viability of RAW264.7 cells with samples for 24, 48, and 72 h. (Compared to the Control group, *P < 0.05 and ** P < 0.01); (Comparison between groups, # P < 0.05 and ## P < 0.01); (Es: extraction solution; Cm: culture medium).
Figure 8

Scanning electron microscopy images of the morphology of macrophages in different material treatment groups (scale bar: 50 µm and 10 µm).
Figure 9

The luminescence of IL-6 in cells was observed under an inverted fluorescence microscope (scale bar: 20 µm).
Figure 10

Changes in the content of various indicators in cell lysate after they were co-cultured with RAW264.7 cells and different experimental treatment groups for 72 h; *p < 0.05 vs. control group and **p < 0.01 vs. control group.
Figure 11

The ALP activity of samples cultured with mBMSCs for 7 days (scale bar: 100µm).

Figure 12

The level of expression of the osteogenesis-related genes OPN, OCN, ALP, RUNX2, BMP-2, and VEGF after 7 days of treatment were quantified by PCR. *p < 0.05 vs. control group.
Figure 13

After seven days of incubation, the levels of expression of FAK, integrin α2, integrin β1, ERK1/2, and RANK were compared. *p < 0.05 vs. control group.