RESEARCH ARTICLE

Effects of Antibacterial Co-Cr-Mo-Cu Alloys on Osteoblast Proliferation, Differentiation, and the Inhibition of Apoptosis

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Objectives: To investigate the effects of antibacterial Co-Cr-Mo-Cu alloys with different Cu contents on osteoblast proliferation, differentiation, and the inhibition of apoptosis to optimize the selection of surgical implantation.

Methods: Microstructure, phase structure, and ion release were evaluated using X-ray diffraction, scanning electron microscopy (SEM), and inductively coupled plasma (ICP) spectrometry. The effects on osteoblast proliferation, differentiation, and apoptosis were characterized by cell proliferation assay, alkaline phosphatase (ALP) activity assay, and western blotting, respectively.

Results: Compared to the original Co-Cr-Mo alloys, the released Cu ions from Co-Cu alloys promoted osteoblast proliferation and differentiation and inhibited apoptosis. It can be noted that the optical density (OD490) and the ALP activity have increased to 1.237 and 1.053, respectively, in Co-2Cu alloy (0.604 and 0.171 for original Co-Cr-Mo alloy). Meanwhile, these effects were evaluated through the upregulation of ROS levels and 4E-binding protein 1 (4E-BP1) expression and the downregulation of adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) and p-AMPK. Moreover, the antibacterial properties of the Co-Cu alloys were also enhanced, as demonstrated by the strong antibacterial activity of Cu phases in Co-Cu alloys incubated with Staphylococcus aureus, in which more than 99.8% of the bacteria has been killed.

Conclusions: The addition of Cu element in the Co-Cr-Mo alloys could induce OB proliferation and differentiation and inhibited OB apoptosis. Meanwhile, it can be recognized that the Co-Cu alloys with 2wt% Cu exhibit the highest performance among all the samples, indicating that the effects of osteoblast differentiation and the inhibition of apoptosis are highly dependent on the adding of Cu elements. Co-Cr-Mo-Cu alloys with an excellent antibacterial property could be used as a tool to improve osteogenic ability and antibacterial properties in orthopaedic implant operations.

Key words: Antibacterial property; Co-Cr-Mo alloy; Osteoblast differentiation; Osteoblast proliferation; Released Cu ion

Introduction

Artificial biomaterials with excellent osseointegration, strong antibacterial properties, and good mechanical properties have a broad range of clinical applications1–4. The ultimate implant material should have excellent biocompatibility and bioactivity and provide the environment necessary for the proliferation and differentiation of cells into desired tissues5–7. Combined with currently available clinical biomaterials, Co-Cr-Mo alloys have emerged as promising materials for hip or knee implants due to their excellent corrosion resistance and mechanical properties8,9. Unfortunately, aseptic loosening and microbial infections triggered by relatively low osseointegration and poor antibacterial properties are the main limitations10. Therefore, the development of the new
biomaterials based on Co-Cr-Mo alloys to improve osseointegration and antibacterial properties has become the main direction in bone implant materials research.

Recently, surface modification and the generation of novel composite alloys using functional elements significantly improved osseointegration and antibacterial properties of the implant materials.[11,12] For example, Ren et al.[13] reported that the Co-Cr alloy coating using Cu and Zn to substitute hydroxyapatite demonstrated good antibacterial properties. Zhang et al.[14] investigated the association between the addition of Cu and antibacterial properties, since the antimicrobial activity of Cu is well-established. Moreover, Pulissaar et al.[15] showed that the osteogenic differentiation was induced by coating TiO2 with alginate hydrogel containing simvastatin. Du et al.[16] reported that the carbon-based materials improved the osteogenic differentiation of human adipose-derived mesenchymal stem cells compared to nano-hydroxyapatite materials, suggesting that multi-walled carbon nanotubes (MWCNTs) could be used as a bone repair material. These studies suggest that antibacterial properties could be enhanced by adding antibacterial metal elements to the Co-Cr-Mo alloys; at the same time, to improve osteogenesis, a combination of active materials is necessary, which is difficult to achieve with Co-Cr-Mo alloys.

Here, a modified Co-Cr-Mo alloy with combined excellent osteogenic performance and strong antibacterial properties were developed. Recently, there was an increased interest in adding Cu to alloys to improve osteogenic performance[17,18], since it has been reported that Co-Cu alloys with 2 wt% Cu showed enhanced osteogenesis in a rabbit implant model[19]; however, the molecular mechanism responsible for these effects is not known. In this study, we prepared Co-Cr-Mo-Cu (Co-Cu) alloys with different Cu content (0, 1, 2, and 4 wt%, marked as Co-0Cu, Co-1Cu, Co-2Cu, and Co-4Cu, respectively) and investigated the effect of released Cu ions on osteoblasts (Ob). Our results showed that, in addition to excellent antibacterial properties, OB proliferation and differentiation were increased, while apoptosis was suppressed, particularly in the Co-2Cu alloy group. The aims of this study are: (i) to introduce a novel Co-Cu alloy as the future surgical implantation; (ii) to evaluate the cell toxicity and antibacterial performance of Co-Cu alloy; (iii) to understand the effects on osteoblast proliferation, differentiation, and the inhibition of apoptosis of different Cu contents.

**Materials and Methods**

**Preparation of Co-Cu Alloy and Cu Ion Concentration Analysis**

Co-Cu alloys with different Cu content (1–4 wt%) were prepared based on the GB17100-1997 (National Standard of China) using an investment casting method[14]. An X-ray fluorescence spectrometer (XFS, XRF-1800) was used to analyze chemical composition (Table 1). To measure ion release, samples were ground and then cleaned using an ultrasonic bath, rinsed, and immersed in 0.9% NaCl solution.

**Microstructure Characterization**

Phase structures of different alloys were obtained by X-ray diffraction (XRD, Rigaku Smaertlab) in the range of 30° to 100° (2θ) using Cu Kr (λ = 0.154 nm) incident radiation with 4° min⁻¹ scan step, 35 kV voltage, and 200 mA current. Microstructures of the alloy surface and the corresponding elemental distribution maps were determined using a scanning electronic microscope (SEM, Hitachi S3400, Hitachi, Japan) with an energy scattering spectrum (EDS, Inca, Oxford). The tested dimension of samples was fixed at 15 mm x 15 mm x 1 mm. All samples were pre-prepared using SiC emery paper (1500 grits) and diamond grinding paste (1.5 μm), followed by a 5 wt% HCl solution to etch surface oxidized layers for −15 s at 5 V voltage.

**Ion Release**

Four alloy samples were ground using the SiC emery paper and then cleaned in an ultrasonic bath in ethanol for −10 min. To evaluate the ion release from different samples, samples were incubated in a 0.9 wt% NaCl solution at a surface area-to-volume ratio of 1.5 cm²/mL for −24 h. Ion concentrations of Co²⁺, Cr³⁺, and Cu²⁺ were measured by the inductively coupled plasma (ICP-MS) spectrometer (Perkin Elmer, Optima 5300 DV, Perkin-Elmer Instruments, USA) with a detection limit of 5 μg/L (5 ppb).

**Antibacterial Properties**

The *Staphylococcus aureus* suspension was prepared in the nutrient broth (NB) at 1 x 10⁶ cfu/mL concentration. Next, the bacterial suspension was diluted to 1 x 10⁵ cfu/mL in the 0.9% NaCl solution using the 10-fold dilution method. Samples were cleaned using the SiC emery paper, ethanol, pure water, and then air dried. A clean coverslip was set as the blank control sample. The suspension of bacteria (0.2 mL) was added to different samples, and samples were incubated at 37°C and 90% humidity for 1 day. All samples were then washed with 4.8 mL 0.9% NaCl solution, 0.1 mL of the washing solution was added to the nutrient agar medium, and samples were incubated for 1 day to evaluate the antibacterial properties. Colony numbers were counted on a colony counting instrument (ShinesoV3). The antibacterial rate (R) was calculated using the following equation[15]:

\[
R = \left( \frac{N_{\text{blank}} - N_{\text{sample}}}{N_{\text{blank}}} \right) \times 100\%
\]

**Preparation of Extract Solution**

The Co-Cu alloy samples (25 mm x 25 mm x 2 mm) containing 0 wt%, 1 wt%, 2 wt%, and 4 wt% Cu were ground using the SiC emery paper. All samples were washed in an ultrasonic bath for 10 min, first, with acetone, then with dehydrated alcohol, and, finally, with deionized water. Next, samples were dried with a sterile filter paper and disinfected using ethylene oxide. Samples were incubated in a DMEM
culture medium at the surface area to volume ratio of $3 \text{ cm}^2/\text{mL}$ in a $37^\circ\text{C}$, 95% relative humidity (RH), 5% CO$_2$ incubator for 72 h. The extract solutions were collected, sealed, and then stored in a 4°C refrigerator until further use.

**Cell Proliferation Assay**
MC3T3-E1 mouse preosteoblast cell line was obtained from American Type Culture Collection (Manassas, VA, USA). Cells ($6 \times 10^4$ cells/mL) were plated into 96-well plates at 100 μL/well, and then cultured to confluence in a $37^\circ\text{C}$, 5% CO$_2$, 95% RH incubator. Next, 100% extract solution, the positive control (0.64% phenol culture medium), and the negative control (DMEM hyperglycemic culture medium) were added into the wells. Cells were cultured for 1, 2, and 3 days, and cell morphology was observed under an inverted phase-contrast microscope. Cell viability was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and the optical density (OD) was measured at 490 nm by a universal microplate spectrophotometer.

**Alkaline Phosphatase (ALP) Staining**
MC3T3-E1 cells were plated in 48-well culture plates at $2 \times 10^4$ cells/well and then cultured overnight in a $37^\circ\text{C}$, 5% CO$_2$ incubator. Next, medium was removed, 100% alloy extract solution was added, the cells were incubated for additional 48 h, and then lysed for 30 min. The activity of ALP and the protein content in the cell lysate were detected by the cell ALP kit (A059-2; Nanjing Jiancheng Biological Engineering Institute, China) and BCA Protein Concentration Assay Kit (Nanjing Kaiji Biological Products Co., Ltd., China), respectively, following the manufacturers’ instructions. The OD at 520 nm was measured using a universal microplate spectrophotometer.

**Detection of Reactive Oxygen Species (ROS) Concentration in Cells**
MC3T3-E1 cells were plated in 96-well plates at $1 \times 10^4$ cells/well (200 μL), with three parallel wells set up, and cultured for 24 h in a $37^\circ\text{C}$, 95% RH, 5% CO$_2$ incubator. Next, the growth medium was removed and serum-free DMEM culture medium containing 10 μmol/L DCFH-DA and 500 nmol/L mitochondrial probe MitoTracker Red CMXRos was added into each well, and cells were incubated in a $37^\circ\text{C}$, 5% CO$_2$, 95% RH incubator, protected from light, for additional 60 min. Cells were washed several times with PBS, and 100% Co-xCu alloy extract solution with or without 5 mmol/L NAC was added to the cells for 60 min, and ROS green fluorescence intensity (FI) 30 and 60 min after the addition of extract solution, with or without NAC, was measured at 488 and 525 nm using a universal microplate spectrophotometer.

**Detection of ROS and Mitochondria in Cells Using Confocal Microscopy**
MC3T3-E1 cells were plated in a 15-mm confocal microscopy-dedicated cell culture dish at $1 \times 10^4$ cells/well (200 μL) and cultured in a $37^\circ\text{C}$, 95% RH, 5% CO$_2$ incubator for 24 h. Next, a 500 μL serum-free DMEM culture medium containing 10 μmol/L DCFH-DA and 500 nmol/L mitochondrial probe MitoTracker Red CMXRos was added into each well, and cells were incubated in a $37^\circ\text{C}$, 5% CO$_2$, 95% RH incubator, protected from light, for additional 60 min. Cells were washed several times with PBS, and 100% Co-xCu alloy extract solution with or without 5 mmol/L NAC was added to the cells for 60 min, and ROS green fluorescence (excitation wavelength: 488 nm) images and mitochondria red fluorescence (excitation wavelength: 579 nm) images were acquired using laser scanning confocal microscope (LSCM); 50 μg/mL ROS$_{up}$-treated group was used as the positive control.

**Detection of Caspase-3, AMPK, p-AMPK, and 4E-BP1 Protein Expression**
MC3T3-E1 cells were treated with the Co-xCu alloy extract solution for 48 h and then collected. The total protein (TP) was extracted according to the kit (KGP2100, Key-GENBioTECH, China) instructions, and then subjected to electrophoresis, transferred to a membrane, and blotted with the corresponding primary antibodies, followed by the secondary antibodies. Antibodies were obtained from the following commercial sources: Caspase-3 (#9662 CST, 1:1000), AMPK (#2532 CST, 1:1000), p-AMPK (#2535 CST, 1:1000), 4E-BP1 (#9452 CST, 1:1000), β-actin (A1978 Sigma, 1:1000). The images were acquired using a BIO-RAD gel electrophoresis image analyzer, and the quantitative analysis was performed with the ImageJ software.

**Statistical Analysis**
The GraphPad Prism 6.0 software was used for the statistical analysis of data. The multiple comparisons of data were

### Table 1: Chemical composition of Co-Cu alloys with different Cu contents (wt.%)

| Alloys   | Cr   | Mo | Fe | Ni | Si | C  | Mn | Cu | Co  |
|----------|------|----|----|----|----|----|----|----|-----|
| Co-0Cu   |      |    |    |    |    |    |    |    | Balance |
| Co-2Cu   | 29   |    | 0.8| 0.6| 0.5| 0.2| 0.5| 1.8| Balance |
| Co-4Cu   |      |    |    |    |    |    |    |    | 3.8 Balance |

with added DCFH-DA were used as the positive control, the negative control, and the blank control, respectively. For the ROS scavenger experiment, 5 mmol/L N-acetyl cysteine (NAC) was added to the cells together with extract solutions, and the fluorescence intensity (FI) 30 and 60 min after the addition of extract solution, with or without NAC, was measured at 488 and 525 nm using a universal microplate spectrophotometer.
performed with one-factor analysis of variance (ANOVA) and multi-factor ANOVA, respectively. $P < 0.05$ indicated that the difference was statistically significant.

**Results**

**Microstructure**

Co-Cu alloys with different Cu content were obtained using the investment casting method, and the elemental content was characterized by the X-ray fluorescence spectrometry (Table 1). The phase structures of different alloys were analyzed by XRD, as shown in Figure 1. Two crystalline structures including face-centered-cubic (FCC) structural $\gamma$-Co phase and hexagonal-close-packed (HCP) structural $\varepsilon$-Co phase could be observed. However, none of the Cu phases could be detected in any of the alloys. The surface element distribution of the Co-2Cu and Co-4Cu alloys is shown in Figure 2. Different elements, including Co, Cr, Mo, and Cu, have been marked in four colors (pink, yellow, green, red) to demonstrate the corresponding distribution. The element distribution was tested at three different points, in which a uniform elemental distribution has been exhibited. The element concentrations of all the samples are listed in Table 2. An outlier could be observed in Co-2Cu alloy data, which could be related to the element segregation during the synthesis.
**Ion Release**

The ion release by different Co-Cu alloys is presented in Figure 3. At higher composite Cu content, the concentration of released Cu ions in the NaCl solution had also increased. It can be observed that the concentrations of Cu ions released from Co-1Cu and Co-2Cu alloys were 12.2 ± 8.0 and 18.5 ± 12.0 μg/L ($F = 0.49$, $P = 0.52$), respectively, while Cu ion concentration from the Co-4Cu alloy increased to 430 ± 150 μg/L ($F = 23.15$, $P = 0.0086$). Meanwhile, the concentrations of Cr and Co ions showed an opposite trend: the concentrations decreased with the increasing Cu content, with the concentrations reduced from 17.8 to 8.1 μg/L for Cr, and from 788 to 510 μg/L for Co.

**Antibacterial Properties of Co-Cu Alloys**

The number of bacterial colonies on the surfaces of different Co-Cu alloys after 24 h incubation was illustrated in Figure 4. A large number of bacterial colonies was observed on the blank control sample and Co-0Cu alloy, indicating that the Co-Cr-Mo alloy does not possess any antibacterial properties. However, the number of bacterial colonies on Co-1Cu alloy samples was reduced, while that number for Co-2Cu alloy samples was dramatically decreased. Furthermore, almost no bacterial colonies could be observed on Co-4Cu alloy samples. These findings suggested that the bacteria were killed by the Co-Cu alloys, with the rate of 50.89%, 2.35%, and 0.20% corresponding to 1wt%, 2wt%, and 4wt% Cu content, respectively. These data indicate that the Co-2Cu alloy is an antibacterial material ($R > 90\%$) and Co-4Cu is a strong antibacterial material ($R > 99\%$).

**Proliferation of Mouse OBs**

The OD values of OBs cultured with extracts from different Co-Cu alloys are summarized in Figure 5. Compared to the control group, the proliferation of OBs was induced by both Co-1Cu (0.433 ± 0.008) and Co-2Cu (0.452 ± 0.013) at 24 h, while the difference between these two alloys was not statistically significant ($F = 4.95$, $P = 0.091$). At the same time, there was no significant difference between Co-0Cu (0.252 ± 0.008) and Co-4Cu (0.239 ± 0.009) ($F = 3.98$,

**TABLE 2 EDS analysis of different alloys at different random points(at%)**

| Elements | Point A | Point B | Point C |
|----------|---------|---------|---------|
|          | Co-2Cu  | Co-4Cu  | Co-2Cu  | Co-4Cu  | Co-2Cu  | Co-4Cu  |
| Co       | 57.44   | 59.49   | 55.37   | 52.84   | 55.98   | 56.66   |
| Cr       | 31.12   | 29.11   | 37.28   | 35.77   | 34.43   | 34.81   |
| Mo       | 6.32    | 5.32    | 5.04    | 5.22    | 4.37    | 3.92    |
| Cu       | 1.85    | 3.53    | 1.48    | 4.10    | 4.22    | 3.47    |
Fig. 4 Typical colonies of Staphylococcus aureus after 24 h incubation on different Co-Cu alloys. (A) Blank control; (B) Co-0Cu alloy; (C) Co-1Cu alloy; (D) Co-2Cu alloy; (E) Co-4Cu alloy.

Fig. 5 (A) The effect of Co-Cu alloys on OB proliferation at different time points (the line represents the comparison with the control group, and the square brackets represent a comparison between two groups); *p < 0.05 and **p < 0.01. (B) Effect of different Co-Cu alloys on the ALP activity in OBs (the line represents the comparison with the control group); **p < 0.01. (C) Caspase-3 protein expression in OBs at 48 h after treatment by different Co-Cu alloys.
Groups with different Co-Cu alloy extracts showed a significant increase in cell proliferation at 48 h. The Co-0Cu group showed a proliferation rate of 0.344 ± 0.024, while the control group had a rate of 0.223 ± 0.021. At 72 h, the proliferation rate increased significantly in the Co-1Cu (0.856 ± 0.026) and Co-2Cu (1.237 ± 0.051) groups, particularly in the Co-2Cu group (F = 133.13, P = 0.00032), while there was no significant difference between Co-0Cu (0.604 ± 0.064), Co-4Cu (0.570 ± 0.022), and control groups (0.624 ± 0.015) (F = 0.74, P = 0.44), suggesting an uncorrelated behavior.  

### Differentiation of Mouse OBs

ALP expression is the early marker of OBs extracellular matrix (ECM) maturation. Figure 5B indicates that ALP expression is increased in the Co-1Cu and Co-2Cu groups, and this increase is more pronounced in the Co-2Cu group.
activity in Co-1Cu (1.275 ± 0.001) and Co-2Cu (1.053 ± 0.002) groups was significantly higher compared to other groups (F = 48.40, P = 0.0022), demonstrating the effect on cell differentiation; however, ALP activity was not affected in Co-0Cu (0.171 ± 0.00002) and Co-4Cu (0.196 ± 0.0002) alloy groups and control group (0.264 ± 0.002), indicating that the differentiation of OBs was not affected (F = 7.75, P = 0.050). These results suggest that ALP activity increases with the Cu content, while high Cu ion concentrations show the opposite effect, confirming that proper concentration of Cu ions could accelerate mineralization.

Apoptosis of Mouse OBs
It can be recognized that after 48 h incubation with extract solutions from Co-Cu alloys, Caspase-3 protein levels in OBs were decreased compared to control and Co-0Cu groups, indicating that OB apoptosis was inhibited by the treatment (Figure 5C).

ROS Production in Mouse OBs
The FI of DCFH-DA is an indirect measurement of ROS levels produced by cells. As shown in Figure 6, after adding 50 μg/ml ROSup, the FI of cells was continuously increased at 30 and 60 min, indicating that OBs responded to ROS inducers. In Co-1Cu and Co-2Cu groups, the ROS concentration in cells was increased at 30 min (13,125 ± 240 and 14,101 ± 184 for Co-1Cu and Co-2Cu) (F = 22.33, P = 0.001), and ever further increased at 60 min (14,843 ± 294 and 16,431 ± 424 for Co-1Cu and Co-2Cu) (F = 6.50, P = 0.0035); however, there was no significant difference between Co-0Cu (10,798 ± 255 and 10,836 ± 281 for 30 and 60 min) and Co-4Cu (9856 ± 142 and 10,055 ± 226 for 30 and 60 min) groups and the control group (9588 ± 289 and 9503 ± 376 for 30 and 60 min) (F = 0.55 and 2.23, P = 0.48 and 0.17). In response to 5 mmol/L NAC, the effects of the ROS level within Co-1Cu (9877 ± 299 and 9582 ± 314 for 30 and 60 min) and Co-2Cu (9547 ± 222 and 9152 ± 345 for 30 and 60 min) are offset, compared with non-treated CoCu alloys groups (F = 9.21 and 22.13, P = 0.016 and 0.0015). These findings suggest that Co-Cr-Mo-Cu alloys with different Cu content could stimulate ROS production in OBs at a certain Cu ion concentration.

The confocal microscopic observation of all the samples was exhibited in Figure 7. At a consistent level of red MitoTracker fluorescence, the strongest and the weakest green DCFH-DA fluorescence was observed in Co-2Cu and Co-4Cu groups, respectively, compared to the Co-0Cu group, corresponding to RFI data presented in Figure 6. The localization of ROS fluorescence overlapped with mitochondrial localization in all groups, confirming that ROS was mainly produced by mitochondria.

AMPK, p-AMPK, and 4E-BP1 Protein Expression in Mouse OBs
The protein expressions of different Co-Cu alloys were demonstrated in Figure 8. After 48 h incubation with alloy extracts, the protein level of p-AMPK was decreased compared to the Co-0Cu group, and no significant differences were observed between Co-0Cu and control groups. Furthermore, compared to the Co-0Cu group, 4E-BP1 protein expression in OBs was increased after the treatment with Co-1Cu, Co-2Cu, and Co-4Cu alloy extracts.

Discussion
Microstructures and Phase Structural Characterizations
Here, the effects of antibacterial Co-Cr-Mo-Cu alloys on osteoblast proliferation, differentiation, and the inhibition of apoptosis have been demonstrated. Co-Cr-Mo-Cu alloys
usually crystallize into two structures: (i) FCC γ-Co phase, appearing at high temperatures, and (ii) HCP ε-Co phase, appearing at low temperatures. Good ductility and deformability could be achieved by the γ-Co phase; however, these parameters in the ε-Co phase are relatively poor. Therefore, as alloys are designed, to maintain excellent deformability and ductility, the FCC γ-Co phase is always preferred\(^{20,21}\). It has been reported that the HCP phase could be reduced by adding nitrogen, carbon, and nickel to the alloy\(^ {22-24}\). In this study, the HCP phase was decreased by adding the copper, as verified by the XRD peak patterns shown in Figure 1.

Since the corresponding Cu phase has not been found in the XRD pattern, the SEM coupled with an EDX analysis was utilized. According to EDS mapping images for Co-2Cu and Co-4Cu alloys, different elements (Co, Cr, and Mo), as well as the Cu, showed a uniform distribution in the alloys. Therefore, we concluded that Cu was successfully added to the Co-Cr-Mo alloy using the investment casting method\(^ {14}\). An aberrant distribution was detected in the Co-2Cu alloy, and we believe that this was triggered by element segregation during the cooling process.

**Cell Toxicity and Antibacterial Performances**

Ion release by different alloys plays an important role in cell toxicity. It has been well-established that the excess ion release would result in cell damage. For example, it was reported that in the MO3.13 cells, TC50 for three ions, such as Co\(^{2+}\), Cr\(^{3+}\), and Cu\(^{2+}\), was 215.5, 2083.7, and 178.8 μM, respectively\(^ {25}\). At the same time, in human gingival fibroblasts, the TMC50 concentrations for these three ions (Co\(^{2+}\), Cr\(^{3+}\), and Cu\(^{2+}\)) were 705.8, 1971, and 344 μM, respectively\(^ {25}\). Here, in the corresponding study, released ion concentrations for Co, Cr, and Cu were 787.6 μg/L (13.1 μM), 17.8 μg/L (0.3 μM), and 430.2 μg/L (7.2 μM), respectively, the concentrations far lower than that of the safety concentrations in recent reports and, therefore, would not cause cell toxicity after implant placement.

As previously reported, Cu has always been preferred as an antibacterial material\(^ {14,26-29}\). Meanwhile, such antibacterial properties could only be launched as the concentration can satisfy the critical value\(^ {30,31}\). For example, Popover et al.\(^ {32}\) suggested that 20–640 mg/L of Cu element in MIC90 could be activated in *Neisseria gonorrhoeae*. However, Hu et al.\(^ {33}\) reported that Cu-MMT with 2.5wt% Cu content demonstrated antibacterial properties; furthermore, the extracts also displayed a low level of antibacterial activity. Moreover, the antibacterial activity of the Ti-Cu alloys could only be activated on the surface of the samples, and the enhancement of the superficial area in the fine ε-Cu phase could obtain an excellent antibacterial activity\(^ {34}\). Therefore, we believe that Co-Cu alloys described in this study, show strong antibacterial properties due to Cu phases associated with the increased superficial area, while released Cu ions could further contribute to the antibacterial activity in alloy extracts. At the same time, another important antibacterial element, silver (Ag) has also been considered as an addition to alloys to improve the toxicity of Cu ion; however, its use has been restricted by alloying rules between Ag and Co, Cr, Mo, and will require further investigation in the future.

**Effects on OBs Differentiation and Inhibited Apoptosis**

In addition to strong antibacterial properties, biological activity (e.g. effect on cell proliferation, differentiation) is another key requirement for Co-Cu alloys. Burghardt et al.\(^ {35}\) reported that the Cu-coated titanium alloy suppressed the growth of bacteria, while 0.3–0.05 mM released from galvanically deposited copper induced the differentiation of bone marrow stem cells into Ob. Duan et al.\(^ {36}\) found that there was a dose-dependent Cu concentration effect: \(1 \times 10^{-8} \text{ mol/L}\) Cu inhibited human keratinocyte proliferation by more than 87.8%, while \(1 \times 10^{-6} \text{ mol/L–}1 \times 10^{-5} \text{ mol/L}\) Cu showed a proliferative effect, and the \(1 \times 10^{-8} \text{ mol/L–}1 \times 10^{-7} \text{ mol/L}\) Cu had no effect. Furthermore, it has been previously reported\(^ {37}\) that titanium alloy containing 5% Cu induces the proliferation, adhesion, and migration of osteoblasts.

Our results also showed that both Co-1Cu and Co-2Cu with a Cu ion concentration in the range of \(1 \times 10^{-6} \text{ mol/L}\) and \(1 \times 10^{-7} \text{ mol/L}\) induced the proliferation of OBs at 24 h time point. At 48 h, this effect was observed in Co-1Cu, Co-2Cu, and Co-4Cu groups, and the effect was weakened at 72 h in the Co-4Cu group, while it was still significant in Co-1Cu and Co-2Cu groups. Therefore, our findings indicate that the released Cu ion concentration in the \(1 \times 10^{-7} \text{ mol/L–}1 \times 10^{-6} \text{ mol/L}\) range contributes to the proliferation of OBs.

The early marker of OBs ECM maturation is the activation of ALP\(^ {38}\). From the rapid proliferation phase to the ECM maturation phase of OBs, ALP would increase from a low level to the peak level, and its activity would decrease during the mineralization phase\(^ {39}\). Zhang et al.\(^ {40}\) reported that after 48 and 72 h incubation, \(1 \times 10^{-8} \text{ mol/L–}1 \times 10^{-7} \text{ mol/L}\) concentration of released Cu ions increased the ALP activity of OBs. Our study demonstrated that ALP activity markedly increased in Co-1Cu and Co-2Cu groups after 48 h, suggesting that Cu ion release concentration from these alloys could induce the ECM maturation of OBs.

Caspase-3 plays a key role in cell apoptosis\(^ {41}\). Here we showed that Caspase-3 protein levels in OBs were decreased in Co-1Cu, Co-2Cu, and Co-4Cu groups compared with the Co-0Cu group. These results suggest that Co-x Cu alloy could inhibit OBs apoptosis.

Several studies have reported that Cu ions can change the redox state of cells and increase the ROS content, the process associated with cytotoxicity\(^ {42,43}\). Currently, ROS are perceived as “double-edged swords” due to their role in the regulation of cellular physiological processes. For example, high ROS levels could induce several pathological processes, such as ischemia–reperfusion injury, atherosclerosis, and aging. However, low ROS levels could play an important role in intracellular signal transduction, as well as cell
proliferation and differentiation. In this study, we showed that in OBs ROS levels were significantly increased after 30 and 60 min incubation with Co-1Cu and Co-2Cu extracts; however, in the presence of NAC, OB ROS levels were restored in Co-1Cu and Co-2Cu groups, suggesting that cell proliferation could be induced by the upregulation of ROS.

AMPK activity maintains a balance between ATP production and consumption and plays an important role in the regulation of energy metabolism in cells. AMPK is also responsible for the regulation of cell growth and proliferation, the physiological rhythms, as well as the establishment and stabilization of the cell polarity, making it one of the essential signal molecules involved in the normal physiological functions of organisms44. Studies of AMPK in B lymphoblastic cells showed that high-concentration Cu ion treatment (250–1000 μM) caused excessive ROS production and apoptosis. Here, we observed that AMPK was involved in the regulation of OBs even at the low concentrations of Cu ions released from Co-xCu alloys.

Eukaryotic translation initiation factor 4E-BP1 is a highly conserved low-molecular-weight protein45. 4E-BP1 is regulated by several signaling pathways, such as PI3K/AKT/mTOR and MEK/ERK/AMPK. Mitochondria-dependent apoptosis is regulated via the mTOR/4E-BP1 pathway46. Inhibition of AMPK phosphorylation leads to the activation of mTOR, resulting in increased cell proliferation and protein synthesis via a signaling cascade that involves 4E-BP147. Here we showed that at 48 h time point, 4E-BP1 protein levels in Co-1Cu, Co-2Cu, and Co-4Cu groups were increased, while AMPK and p-AMPK levels were decreased. These data suggest that Cu ions could activate mTOR signaling by downregulating AMPK phosphorylation, leading to the upregulation of 4E-BP1 and the induction of OB proliferation.

Conclusion

In summary, here we characterized antibacterial Co-Cr-Mo-Cu alloys, which induced OB proliferation and differentiation and inhibited OB apoptosis. These results further verify that Cu could be used as a tool to improve osteogenic ability and antibacterial properties. Our data indicate that this effect on proliferation, differentiation, and apoptosis inhibition was due to the upregulation of ROS levels and 4E-BP1 expression and the downregulation of AMPK and p-AMPK levels. Moreover, the antibacterial properties of Co-Cu alloys have also been enhanced, as demonstrated in experiments with Staphylococcus aureus, confirming the strong antibacterial activity of Cu phases in Co-Cu alloys. This study presents an effective way to optimize the properties of traditional Co-Cr-Mo alloy and could provide a potential selection method for future clinical bone-implant materials. Furthermore, the correlation between the additional Cu content and the effects on proliferation, differentiation, and apoptosis inhibition in OBs has been clarified.

Ethical Statement

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Authorship declaration

All authors listed meet the authorship criteria according to the latest guidelines of the International Committee of Medical Journal Editors, and all authors are in agreement with the manuscript.

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