Thin magnesium layer confirmed as an antibacterial and biocompatible implant coating in a co-culture model

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Abstract. Implant-associated infections commonly result from biofilm-forming bacteria and present severe complications in total joint arthroplasty. Therefore, there is a requirement for the development of biocompatible implant surfaces that prevent bacterial biofilm formation. The present study coated titanium samples with a thin, rapidly corroding layer of magnesium, which were subsequently investigated with respect to their antibacterial and cytotoxic surface properties using a *Staphylococcus epidermidis* (*S. epidermidis*) and human osteoblast (hOB) co-culture model. Primary hOBs and *S. epidermidis* were co-cultured on cylindrical titanium samples (Ti6Al4V) coated with pure magnesium via magnetron sputtering (5 μm thickness) for 7 days. Uncoated titanium test samples served as controls. Vital hOBs were identified by trypan blue staining at days 2 and 7. Planktonic *S. epidermidis* were quantified by counting the number of colony forming units (CFU). The quantification of biofilm-bound *S. epidermidis* on the surfaces of test samples was performed by ultrasonic treatment and CFU counting at days 2 and 7. The number of planktonic and biofilm-bound *S. epidermidis* on the magnesium-coated samples decreased by four orders of magnitude when compared with the titanium control following 7 days of co-culture. The number of vital hOBs on the magnesium-coated samples was observed to increase (40,000 cells/ml) when compared with the controls (20,000 cells/ml). The results of the present study indicate that rapidly corroding magnesium-coated titanium may be a viable coating material that possesses antibacterial and biocompatible

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properties. A co-culture test is more rigorous than a monoculture study, as it accounts for confounding effects and assesses additional interactions that are more representative of *in vivo* situations. These results provide a foundation for the future testing of this type of surface in animals.

Introduction

A total of 375,000 hip and knee endoprostheses implants are performed in Germany each year (1). In 1.5% of cases, implant-associated infections (IAI) occur following total joint arthroplasty (2). Despite the relatively low prevalence of IAI, it may have severe consequences for patients, physicians and the health care system (3). In the majority of cases, infections are initiated by *Staphylococcus aureus,* a biofilm-forming coagulase-negative *Staphylococcus epidermidis* (*S. epidermidis*), or gram-negative species (4). Early infections occur within weeks following surgery and are usually acquired intraoperatively. In addition, IAI may manifest as a late infection, occurring between 3 and 24 months following surgery, resulting from hematogenous spreading of the bacteria from other foci (5,6). IAI requires local and systemic antimicrobial therapy, which necessitates the removal of the contaminated endoprosthetic implant and insertion of a temporary cement implant (spacer) loaded with antibiotics (7-9).

There is currently a requirement for novel endoprosthetic materials with optimal surface and mechanical characteristics that prevent bacterial biofilm formation avoid bacterial resistance mechanisms and mediate biocompatibility and osseointegration (10). In recent years, magnesium has emerged as a promising implant material solution. Magnesium has various favourable properties, as it is a freely accessible metal and an essential cation serves an important role in the metabolism of human cells (11,12). The biocompatibility of magnesium as a coating is influenced by the corrosion rate, corrosion products and the varying pH-value of the solution (13). A previous *in vitro* study revealed that rapidly corroding magnesium in a thin layer on titanium (Ti6Al4V) was a suitable candidate for implant coatings with antimicrobial and biocompatible properties (14). Monocultures of *S. epidermidis* and human osteoblasts (hOB) revealed that magnesium coatings may prevent initial bacterial adhesion while remaining

biocompatible according to the cytotoxicity test standards of the International Organisation for Standardisation (15). In contrast to monocultures, co-cultures provide a more comprehensive model of the development of the infection, as demonstrated in a previous study (16).

The aim of the present study was to investigate the antibacterial potential and cytotoxic effects of a thin, rapidly corroding magnesium coating on titanium samples in a co-culture model of *S. epidermidis* and hOBs.

Materials and methods

Titanium samples coated with pure magnesium. The present study used Ti6Al4V discs (DOT GmbH, Rostock, Germany) coated with pure magnesium for the experiments. Uncoated Ti6Al4V discs (a common implant material) served as a control. The discs were 11 mm in diameter and 2.5 mm in thickness. Magnesium-sputtering targets (diameter, 20 cm; 3.5 mm thickness; purity, 3N5) were produced by Fhr Anlagenbau GmbH (Ottendorf-Okrilla, Germany). The magnetron sputtering process was conducted with a Von Ardenne CS730 cluster machine (Von Ardenne GmbH, Dresden, Germany) with a 200 W sputtering power and 2.3×10^3 mbar pressure. The samples were coated with layers of magnesium (5-μm thick) by breaking the vacuum and turning the samples. The temperature of the substrate holder did not surpass 76˚C, and all process parameters were performed in accordance with those described by Schlüter *et al* (17). The roughness (R_z) of the surfaces was measured via 3D laser scanning microscopy (VK-X260, Keyence Corporation, Osaka, Japan). The Ti6Al4V disc roughness was between 8 and 12.7 μm for the magnesium-coated discs as determined in our previous study (14). The samples were Y-sterilized at 25 kGy following the sputtering process.

Magnesium ion release and pH of the supernatants. The magnesium-coated Ti6Al4V samples were pre-incubated for 24 h in Modified Eagle's osteogenic cell culture medium (MEM; EMD Millipore, Billerica, MA, USA) without calcium or antibiotics, containing 10% foetal calf serum (EMD Millipore), 1% HEPES buffer (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and the following osteogenic additives: 100 nM dexamethasone, 50 μg/ml L-ascorbic acid and 10 mM β-glycerophosphate, which were all obtained from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany). Following 24 h pre-incubation and at 2, 4 and 7 days following infection, supernatants were transferred to 1.5-ml Eppendorf reaction tubes (Eppendorf, Hamburg, Germany). Digestion was achieved using 65% concentrated nitric acid (1.0% v/v; Sigma-Aldrich, Merck Millipore). The concentration of magnesium ions released from magnesium coatings was quantified using atomic absorption spectrometry ZEEnit 650P in combination with a graphite tube atomizer (all from Analytik Jena AG, Jena, Germany). The analysis process started with evaporation steps at 80, 90 and 110˚C. This was followed by a pyrolysis phase at 900˚C in the platform tube, heated to 2,000˚C to evaporate and transform compact fragments into atoms. This step involved elemental analysis using a resonating cathode lamp and a magnesium cathode at a wavelength of 202.6 nm. Following 0, 2 and 7 days, the pH value of the supernatants was determined using an InoLab® pH 720 pH meter (WTW GmbH, Weilheim, Germany).

Isolation and cultivation of hOBs. The isolation of hOBs was conducted according to the protocol previously described by Jonitz *et al* (18). The osteoblasts were obtained from the cancellous femoral head of 6 patients that underwent total hip replacement surgery (3 male donors, mean age, 72±8.6 years; 3 female donors, mean age, 78±3.8 years) at the Department of Orthopaedics University Medicine Rostock (Rostock, Germany). The present study was approved by The Local Ethics Committee of Rostock (Rostock, Germany; registration number, A2010-10) and written informed consent was obtained from each patient.

Co-culture of hOBs and S. epidermidis. The procedures used to co-culture hOBs and *S. epidermidis* were the same as those described by Zaatreh *et al* (16). hOBs (25,000 cells/ml) at passage three were transferred to a 24-well-plate (Sarstedt, Nümbrecht, Germany) and cultured on the Ti6Al4V sample discs in MEM with adjunctions for 24 h. Similarly, hOBs and *S. epidermidis* were cultured on Ti6Al4V sample discs coated with magnesium for 24 h. A prepared overnight culture of *S. epidermidis* (RP62A; ref. no. 35984; American Type Culture Collection, Manassas, VA, USA) was subsequently used for mono-species infections with a multiplicity of infection of 0.04 (25,000 osteoblasts/ml, 1,000 colony forming units/ml). Co-cultures were incubated for a period of 7 days under standard aerobic conditions (37˚C and 5% CO_2). At 2 and 4 days following infection the medium was replenished.

Determination of hOB viability. hOBs that adhered to the discs were washed with 1X phosphate-buffered saline (PBS; EMD Millipore), followed by the addition of 200 μl trypsin/EDTA (1X; Gibco; Thermo Fisher Scientific, Inc.) for 3 min under aerobic conditions (37˚C and 5% CO_2). The hOBs were then mechanically relocated from the discs with a pipette tip (Eppendorf). The solution with the osteoblasts was transferred to a 1.5 ml Eppendorf reaction tube (Eppendorf) centrifuged at 169 x g for 4 min at 4˚C, and washed with 1X PBS. Quantification of the number of viable primary osteoblasts on the test samples was determined by trypan blue staining (Sigma-Aldrich; Merck Millipore). Trypan blue enters the damaged membranes of dead cells, leaving viable cells unstained. The viable cells were counted using a Thoma cell counting chamber, obtained from Paul Marienfeld GmbH & Co. KG (Lauda-Königshofen, Germany) according to manufacturer's protocol (19) and using an Olympus CKX41SF optical light microscope, (Olympus Soft Imaging Solutions GmbH, Hamburg, Germany). Measurements were performed at 2 and 7 days of co-culture.

Quantification of biofilm-bound S. epidermidis. The test samples were transferred to glass test tubes (Greiner Bio-One International GmbH, Kremsmünster, Austria) containing 1 ml PBS (1X). *S. epidermidis* were removed by ultrasonic treatment with device settings at 100% for 4 min (BANDELIN BactoSonic, GmbH & Co. KG, Berlin, Germany) at days 2 and 7 of co-culture. The solution in the glass test tube was diluted in 1X PBS and plated onto tryptic soy broth (TSB)-agar

plates (Thermo Fisher Scientific, Inc.). Following 24 h incubation at 37°C and 5% CO_2, the number of colony forming units were quantified.

Quantification of planktonic S. epidermidis. The co-culture supernatants containing planktonic *S. epidermidis* were collected in 15 ml centrifuge tubes (Greiner Bio-One International AG, Kremsmünster, Austria) with 1 ml PBS (1X), and centrifuged at 3,345 x *g* for 10 min at 4°C on day 2 and 7 of co-culture. The number of planktonic bacteria were quantified by serial dilution in 1X PBS and then counting the number of colony forming units on TSB-agar plates (Thermo Fisher Scientific, Inc.) following 24 h incubation at 37°C and 5% CO_2.

Scanning electron microscopy (SEM). Test samples were fixed in a 2.5% glutaraldehyde solution at 4°C for 24 h. Specimens were subsequently washed with 0.1 M sodium acetate buffer, and dried using a graded ethanol series (5 min in 30% ethanol, 5 min in 50% ethanol, 10 min in 70% ethanol, 15 min in 90% ethanol and twice for 10 min in 100% ethanol). The samples were then dried by critical point drying with CO_2 (Critical Point Dryer, Emitech Ltd., Ashford, UK), sputter-coated with gold, and examined with a scanning electron microscope (Zeiss DSM 960A; Zeiss GmbH, Jena, Germany).

Statistical analysis. Data are expressed as the mean ± standard error for three independent experiments. Statistical significance was evaluated with an unpaired two-tailed t-test using SPSS software (version, 20.0; IBM SPSS, Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Magnesium ion release of the test samples and pH-values of the media. Ion release data were examined in order to characterize the strength of the corrosion process as it progresses over the 7 days. Fig. 1 shows the release of magnesium ions in the medium following one day of pre-incubation and at 2, 4 and 7 days following infection of the co-culture and the test sample alone. The magnesium-coated titanium alloy released 24 mM/day magnesium ions during pre-incubation and 11 mM/day over the first 2 days, decreasing to 3 mM/day during days 4-7. The magnesium-coated surface in the co-culture with osteoblasts and bacteria released 24 mM/day during pre-incubation and 8 mM/day over the first 2 days, which decreased to 2 mM/day during days 4 to 7. Media were replenished following 1 day of pre-incubation and after 2 and 4 days of co-culture. Magnesium ion release levels in the magnesium-coated samples maintained in medium without bacteria and cells were not significantly greater when compared with the hOB and *S. epidermis* co-culture samples. This indicates that hOB adhesion and *S. epidermidis* infection did not significantly alter the release of magnesium.

Bacterial growth is influenced by pH, therefore the pH-values of the media were monitored. Table I indicates the pH-values in the co-culture at 0, 2, 4 and 7 days. The magnesium-coated Ti6Al4V altered the pH-value to an alkaline environment whereas the uncoated Ti6Al4V control demonstrated a slightly acidic pH value.

Determination of hOB viability in co-culture with S. epidermidis. The number of viable hOBs was examined by trypan blue staining (Fig. 2). A total of 25,000 cells were seeded on the two samples. The number of osteoblasts on the magnesium-coated sample was 50,000 following 2 days ($P=0.01$) and 40,000 at 7 days ($P=0.01$). For the control, a value of 40,000 cells at day 2 and 20,000 at day 7 were observed. The number of viable osteoblasts was greater on the magnesium-coated sample when compared with the control by ~10,000 at 2 days and ~20,000 at 7 days.

Evaluation of the viability of S. epidermidis in co-culture with hOBs. Measurements of bacterial colonization in the test samples revealed that a greater number of *S. epidermidis* colonies survived on the control TiAI6V4 surface when compared with the magnesium-coated Ti6Al4V discs (Fig. 3). The magnesium-coated sample demonstrated a significant antibacterial effect, as indicated by the significant decrease in biofilm population by three orders of magnitude following 2 days ($P=0.02$), and 4 orders of magnitude following 7 days ($P=0.03$; Fig. 3). The effect was approximately 1 order of magnitude lower for the planktonic population. Complete results of the measurements for *S. epidermidis* are presented in Fig. 3.

SEM imaging. SEM was performed to qualitatively assess the layer structure and morphology of osteoblasts, as well as the growth patterns and distribution of bacteria on the test specimen. The SEM images shown in Fig. 4 confirm the results of the quantitative measurements of the biofilm-bound bacteria. *S. epidermidis* colonization was observed to be lower on the magnesium-coated surface when compared with the control on both days 2 and 7 (Fig. 4). The bacteria form biofilm-like structures on the human primary osteoblasts on the two surfaces.

Discussion

The aim of the present study was to investigate the antimicrobial properties of a rapidly corroding magnesium coating on titanium against *S. epidermidis,* as well as its effects on hOBs, in a co-culture model. Measurements of hOB and *S. epidermidis* populations revealed that hOB growth was improved and *S. epidermidis* growth was inhibited; these alterations differed by several orders of magnitude when compared with the uncoated titanium control.

In previously conducted monoculture experiments, the number of hOBs that colonized the magnesium-coated surface increased to ~80% when compared with the uncoated titanium control, due to the slightly less favourable growth conditions on the corroding surface (14). However, in the present co-culture experiments, hOB growth on the magnesium-coated samples increased to ~125% following 2 days and 200% following 7 days. The antibacterial surface significantly reduced the negative influence of *S. epidermidis* on the hOB. This result was achieved despite the presence of the magnesium coating, which demonstrates a negative effect on the growth potential of the hOBs. The negative influence of *S. epidermidis* on hOB may be due to competition for nutrients and pathogenicity (20). The uninhibited negative effects was observed on the control sample, where hOB numbers were markedly reduced following

Table I. pH-values for Ti6Al4V and Mg-coated Ti6A14V test samples in co-culture with hOB and *S. epidermidis* for 0, 2, 4 and 7 days.

Co-culture of hOB and *S. epidermidis*	Day 0	Day 2	Day 4	Day 7
MEM+10% FCS	7.94±0.07	8.03±0.15	8.01±0.07	8.05±0.03
Ti6Al4V	7.88±0.06	7.83±0.08	7.81±0.03	7.64±0.04
Mg-coating	8.82±0.08	8.56±0.08	8.19±0.01	8.12±0.04

Data are expressed as the mean ± standard deviation (n=3). MEM, Modified Eagle's osteogenic cell culture medium; FCS, foetal calf serum; hOBs, human osteoblasts; *S. epidermidis, Staphylococcus epidermidis*; Mg, magnesium.
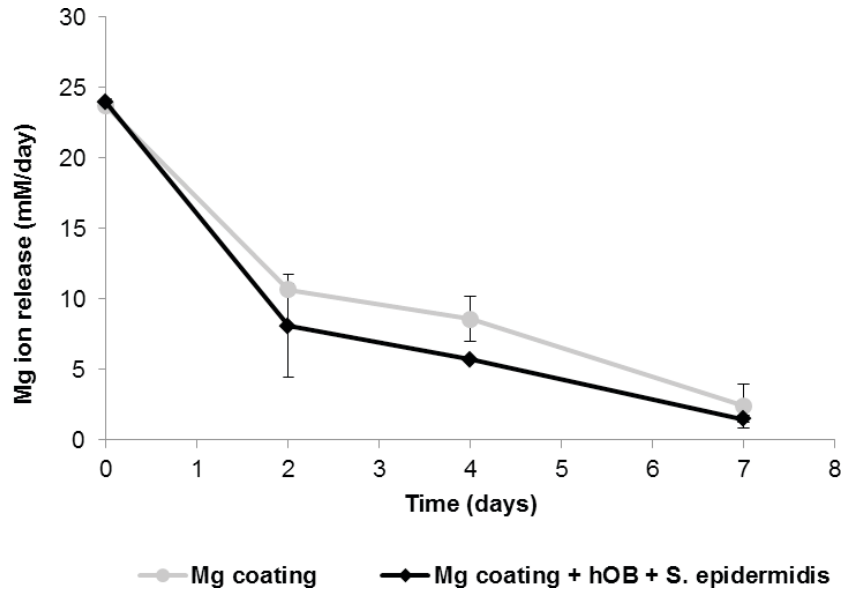
Figure 1. Magnesium ion release in the co-culture and test sample-only systems. Mg ion release was measured in the medium following 1 day of pre-incubation and at 2, 4 and 7 days for test sample in medium alone and test sample co-cultured with hOBs and *S. epidermidis*, as determined by atomic absorption spectrometry. The release declines from ~24 mM/day on day 1 of exposure to the medium, to 2-3 mM/day on day 7 in the co-culture and test sample-only systems. Data are expressed as the mean ± standard error (n=3). *S. epidermidis, Staphylococcus epidermidis*; hOBs, human osteoblasts; Mg, magnesium.
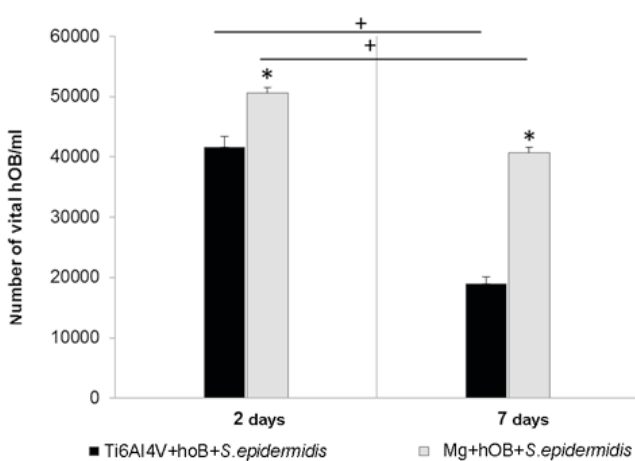

Figure 2. Viability of human hOBs in the co-culture on the test samples for 2 and 7 days. The number of osteoblasts on the titanium alloy sample was decreased by ~20,000 hOB/ml at day 7 compared to day 2, whereas a reduction of 10,000 hOB/ml was observed on the Mg-coated samples. Data are expressed as the mean ± standard error of the mean (n=3). *P<0.05 vs. Ti6Al4V + hOB + *S. epidermidis* (same day). +P<0.05 vs. day 2 (same material). *S. epidermidis, Staphylococcus epidermidis*; hOBs, human osteoblasts; Mg, magnesium.

7 days when compared with 2 days (a decrease of 20,000 from 40,000).

The increased growth of the hOBs on magnesium-coated samples, and the observed antibacterial effect, may be explained by the following factors: Prevention of bacterial adherence due to the corrosive dissolution process, osmotic stress in the strongest phase of initial corrosion, the microstructure of the surface, an unfavourable increase in pH-value for the bacteria or the inhibitory effects of magnesium ions on the bacteria (21-28). These effects are most prominent in the local environment close to the surface. The effects of dissolution and osmotic pressure decline over 2 days, however the pH shift may also have a negative effect on *S. epidermidis* growth over the 7-day period. The impact on the planktonic population may be less significant due to reduced exposure to these factors (13,29).

The antibacterial effects observed in the monoculture experiments were reproduced in the co-culture setup, however, they were observed to be one order of magnitude less prominent (14). These observations are in agreement with a prior comparison of a monoculture to co-culture experiment conducted by Zaatreh *et al* (16). Putative reasons for the
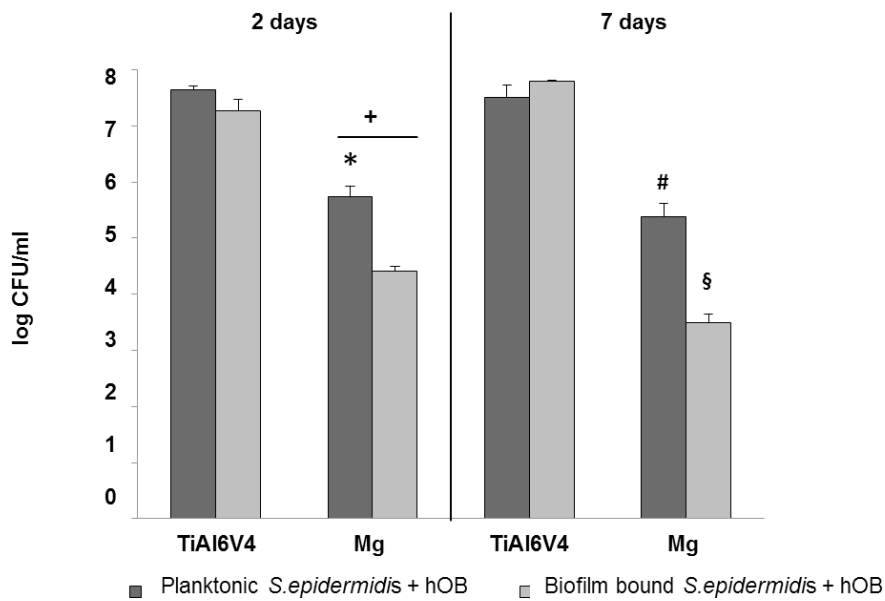
Figure 3. Quantification of viable biofilm-bound and planktonic *S. epidermidis* in co-culture with hOB cells on the test samples following 2 and 7 days. The number of viable biofilm-bound *S. epidermidis* on the Mg-coated specimen was significantly decreased when compared with the titanium control over a period of 7 days. Data are expressed as the mean ± standard error of the mean (n=3). [*]P<0.05 Mg vs. titanium alloy control, day 2, planktonic. [+]P<0.05 biofilm vs. planktonic, day 2, on Mg. [#]P<0.05 Mg vs. titanium alloy control, day 7, planktonic. [§]P<0.05 Mg vs. titanium alloy control, day 7, biofilm. *S. epidermidis*, *Staphylococcus epidermidis*; hOBs, human osteoblasts; Mg, magnesium; CFU, colony forming units.
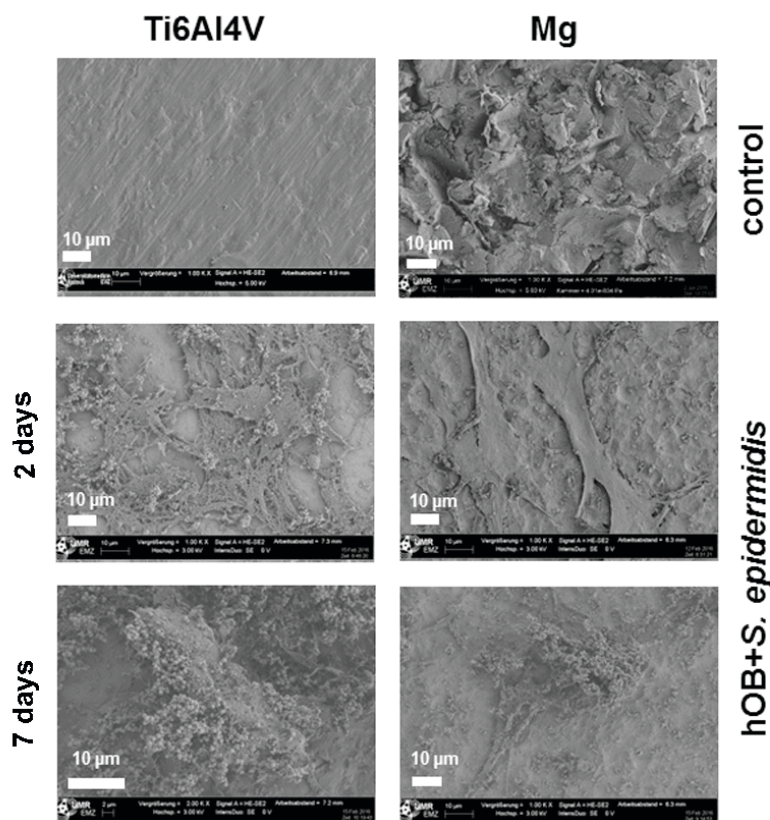

Figure 4. Scanning electron microscopy images of *S. epidermidis* in co-culture with hOB on the titanium-only test sample and the Mg-coated test sample for 2 and 7 days. A decreased density of *S. epidermidis* colonization was observed on the Mg-coated surface when compared with the titanium-only test sample (magnification, x1,000 or x2,000). Control images are test samples in medium only. *S. epidermidis*, *Staphylococcus epidermidis*; hOBs, human osteoblasts; Mg, magnesium.

diminished effect may include the favourable environment that the hOBs present for *S. epidermidis* and the protection that they may provide from antibacterial effects of the surface.

The planktonic population of *S. epidermidis* depends on the biofilm population, as this is where most of the bacterial reproduction occurs (30).

In previous studies, the authors demonstrated that mono-cultures may capture only a limited number of interactions among human cells, bacteria and the tested surface (14,16). Unpredicted behaviour may arise from the complex interaction among human cells, bacteria and implant surfaces. Therefore, the results of co-culture experiments are regarded as more robust (31,32). In the present study, the co-culture model captured the apparent protective effects of the hOB cells, whereby this layer of cells protects bacteria from the adverse effects of the surface. The antibacterial effects were diminished in the co-culture system, however a significant antibacterial effect persisted (three orders of magnitude following 7 days).

The investigation of different experimental parameters must be limited. Future work will aim to test different growth media, a greater number of bacterial strains and species, as well as different osteoblast cell lines. In addition, the length of time for infection to occur, and the ratio of initial hOB and bacterial numbers will be explored. The results of the present study provide significant observations with promising implications for implant material research.

In conclusion, novel implant materials are in high demand due to the severity of IAI, which may be life-threatening for patients with lengthy and demanding treatment procedures and may result in a marked decrease in quality of life. It has been previously demonstrated that titanium coated with a thin, rapidly corroding layer of magnesium may be a suitable candidate material, based on monoculture studies involving primary hOBs and *S. epidermidis*. The present study applied a more rigorous test system with hOBs and *S. epidermidis* in co-culture on the magnesium-coated titanium surface. The favourable qualities of this surface as an antibacterial and biocompatible material were verified. These results provide evidence for progression towards future animal model testing.

Acknowledgements

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