■ LOWER LIMB: RESEARCH

Osteoconductivity of thermal-sprayed silver-containing hydroxyapatite coating in the rat tibia

Y. Yonekura,
H. Miyamoto,
T. Shimazaki,
Y. Ando,
I. Noda,
M. Mawatari,
T. Hotokebuchi

From Saga
University, Saga,
Japan

A silver-containing hydroxyapatite (Ag-HA) coating has been developed using thermal spraying technology. We evaluated the osteoconductivity of this coating on titanium (Ti) implants in rat tibiae in relation to bacterial infection in joint replacement.

At 12 weeks, the mean affinity indices of bone formation of a Ti, an HA, a 3%Ag-HA and a 50%Ag-HA coating were 97.3%, 84.9%, 81.0% and 40.5%, respectively. The mean affinity indices of bone contact of these four coatings were 18.8%, 83.7%, 77.2% and 40.5%, respectively. The indices of bone formation and bone contact around the implant of the 3%Ag-HA coating were similar to those of the HA coating, and no significant differences were found between them (bone formation, $p = 0.99$; bone contact, $p = 0.957$). However, inhibition of bone formation was observed with the 50%Ag-HA coating.

These results indicate that the 3%Ag-HA coating has low toxicity and good osteoconductivity, and that the effect of silver toxicity on osteoconductivity depends on the dose.

The rate of primary infection for joint replacements is between 0.86% and 2.52% according to the National Nosocomial Infections Surveillance System.¹

Various antibacterial coatings such as vancomycin,² gentamicin,³ a combination of gentamicin and hydroxyapatite (HA),⁴ carbonated HA,⁵ nitric oxide-releasing xerogel⁶ and silver (Ag)-containing HA,⁷ have been developed for use on the surface of implants. Devices such as heart valves,⁸ central venous catheters,⁹ aortic grafts,¹⁰ urethral catheters,¹¹ burn wound dressings¹² and megaprotheses,¹³ have been coated with Ag or Ag compounds because of their broad antibacterial spectrum, strong bactericidal activity and low toxicity.^{14,15}

HA has good biocompatibility and osteoconductivity and is used on the surface of implants to improve the strength of the attachment to bone.¹⁶⁻²⁰ An antibacterial coating with HA containing silver oxide (Ag₂O), which has the properties of both Ag and HA, has been developed using thermal spraying.²¹ It has been reported that this coating has the properties of HA, induces the release of Ag ions, has high antibacterial activity with inhibition of bacterial adhesion and has low cytotoxicity *in vitro*.^{21,22} It also demonstrates antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) *in vivo*²³ as well as *in vitro*.²²

However, an *in vivo* study on the osteoconductivity of such an Ag-HA coating has not been carried out. We have evaluated the osteoconductivity of this coating on titanium (Ti) implants in rat tibiae.

Materials and Methods

Coating and implants. The implants used were Ti wires 20 mm in length and 1 mm in diameter (HOMS, Nagano, Japan). The Ag₂O powder (Kanto Chemical, Tokyo, Japan) was mixed with the HA powder (Japan Medical Materials, Osaka, Japan) in concentrations of 3% and 50% and shaken for five minutes in a plastic bag. The mix was then sprayed on to the Ti surface using the Flame Spraying System (Sulzer Metco Japan Ltd., Tokyo, Japan) which uses an acetylene torch. The temperature of the flame was about 2700°C. We prepared four types of implant as follows: Ti, HA coating on Ti, 3%Ag-HA coating on Ti and 50%Ag-HA coating on Ti. They were packaged singly and sterilised using a JS-8500 gamma steriliser (MDS Nordion, Ontario, Canada). The implants were obtained from Japan Medical Materials. Previous studies *in vivo* and *in vitro* had indicated that the concentration of Ag in the coating was responsible for the antibacterial activity.^{22,23} An amorphous structure of the Ag-HA coating was confirmed for concentrations $\leq 50\%$ Ag.

■ Y. Yonekura, MD,
Orthopaedic Surgeon
■ T. Shimazaki, MD, PhD,
Orthopaedic Surgeon
■ M. Mawatari, MD, PhD,
Professor
■ T. Hotokebuchi, MD, PhD
Department of Orthopaedic
Surgery
■ H. Miyamoto, PhD, Professor
Department of Pathology and
Microbiology
Faculty of Medicine, Saga
University, 5-1-1 Nabeshima,
Saga 849-8501, Japan.

■ Y. Ando, PhD, Research
Fellow
■ I. Noda, Research Fellow
Research Department
Japan Medical Materials
Corporation, 3-3-31 Miyahara,
Yodogawa-ku, Osaka, Japan.

Correspondence should be sent
to Dr Y. Yonekura; e-mail:
yoneyuta@acc.saga-u.ac.jp

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Fig. 1

Bar charts showing the mean increase in body-weight at two, four and 12 weeks in the Ti, hydroxyapatite (HA), 3%Ag-HA and 50%Ag-HA coating groups.

Operative technique. We used 38 10-week-old male Sprague-Dawley rats (Kyudo, Saga, Japan) with a mean weight of 364.6 g (335 to 430). The operation was carried out under general anaesthesia induced intra-peritoneally with pentobarbital (50 mg/kg body-weight). The right hind leg was shaved, cleaned with povidone-iodine and dried. A hole was drilled with an 18-gauge needle through the tibial tuberosity and the four different implants inserted into the medullary cavity of the tibiae. The rats were divided into four groups of three or four according to the implant inserted. They were housed individually with free access to food and water. After two, four and 12 weeks the body-weight was measured. The abdomen was then opened under general anaesthesia and 5 ml of blood was collected from the right common iliac vein. The tibia of the right hind leg was excised and the rats were killed. All the animal experiments were approved by the Animal Research Ethics Committee of Saga University and according to their recommendation that the number of experimental animals be reduced as much as possible, the sample was limited to three or four rats per group, which was the minimum number needed to calculate the mean and SD.

Radiological transmission imaging. At two, four and 12 weeks the radiographs were assessed for possible aseptic loosening, osteolysis and the formation of heterotopic bone, as described previously.¹³ The tibiae were analysed by radiological transmission imaging with microfocus x-ray apparatus (SMX-225CT-SV2; Shimadzu, Kyoto, Japan).

Histological examination. The tibial specimens were fixed in 10% phosphate-buffered formaldehyde, dehydrated in graded alcohol solutions and embedded in polyester resin. Sections 300 µm to 500 µm thick were cut by a diamond-bladed slicer (Maruto Co., Tokyo, Japan), ground to a thickness of 120 µm to 150 µm using a speed-track machine (Maruto Co.) and stained with toluidine blue. Osteoconductivity, bone formation and bone contact on

the surface of the implant were evaluated by light microscopy. Affinity indices, describing the percentage of bone formation and bone contact, were calculated by analysis software (Image-Pro Plus version 4.0; Media Cybernetics, Silver Spring, Maryland).^{16,18,24} The bone formation index was calculated as the length of bone formation on the surface of the implant divided by the total length of this surface, multiplied by 100. The bone contact index was calculated as the length of bone in direct contact with the surface of the implant divided by the total length of the implant, multiplied by 100.

Serum analysis. The concentration of Ag in the serum was determined by inductively-coupled plasma-mass spectrometric analysis (Perkin Elmer, Waltham, Massachusetts).

Statistical analysis. This was performed with Excel and SPSS version 12.0J software (SPSS Inc., Chicago, Illinois). One-way analysis of variance was used to evaluate the body-weight, affinity index and serum analysis. Where this was significant, Tukey's HSD (honestly significant difference) was applied as a *post-hoc* test. A *p*-value ≤ 0.05 was considered to be significant.

Results

Body-weight. The mean increase in body-weight at 12 weeks in the Ti, HA, 3%Ag-HA coating and 50%Ag-HA coating groups were 287.0 g, 309.0 g, 324.0 g and 337.0 g, respectively (Fig. 1). No significant differences in the increase in body-weight were found between the four groups at two, four and 12 weeks.

Radiological transmission imaging. The radiographs confirmed that the implants had been inserted correctly into the tibiae. There was no evidence of aseptic loosening, osteolysis or formation of heterotopic bone at two, four and 12 weeks.

Histological examination. The bone formation and contact around the implant in the 3%Ag-HA coating group were at the same level as those in the HA coating group at 12 weeks (Fig. 2). However, inhibition of bone formation was observed in the 50%Ag-HA coating group. The Ti group showed plentiful bone formation around the implant, although there were gaps between the Ti and the bone.

Affinity indices. The mean affinity indices of bone formation in the Ti, HA, 3%Ag-HA and 50%Ag-HA coating groups at two weeks were 61.0%, 70.4%, 65.7% and 29.1%, respectively. The value in the 50%Ag-HA group was significantly different from that in the HA and 3%Ag-HA groups (*p* = 0.031 and *p* = 0.033, respectively). At four weeks, the mean affinity indices of bone formation in the Ti, HA, 3%Ag-HA and 50%Ag-HA groups were 86.4%, 73.5%, 74.8% and 31.7%, respectively. There was a significant difference between the Ti and 50%Ag-HA groups (*p* = 0.048). At 12 weeks, the indices of bone formation in the Ti, HA, 3%Ag-HA and 50%Ag-HA groups were 97.3%, 84.9%, 81.0% and 40.5%, respectively (Fig. 3). No significant differences were found between the groups (*p* = 0.132). However, the 50%Ag-HA group showed less bone formation compared with the other groups.

Fig. 2a

Fig. 2b

Fig. 2c

Fig. 2d

Photomicrographs of rat tibiae at 12 weeks from the a) Ti group, showing plentiful bone formation around the implant, albeit with gaps between the two, b) hydroxyapatite (HA) and c) 3%Ag-HA coating groups, showing good bone formation and contact around the implant, and in d) the 50%Ag-HA coating group, showing less bone formation around the implant compared with the other groups (toluidine blue, $\times 40$ magnification).

The mean affinity indices of bone contact in the Ti, HA, 3%Ag-HA and 50%Ag-HA groups at two weeks were 16.2%, 70.4%, 65.7% and 29.1%, respectively. The values in the HA and 3%Ag-HA coating groups were significantly different from those in the Ti ($p = 0.007$ and 0.006 , respectively, Tukey's HSD test) and 50%Ag-HA ($p = 0.016$ and 0.016 , respectively, Tukey's HSD test) groups. At four weeks, the mean indices of bone contact in the Ti, HA, 3%Ag-HA and 50%Ag-HA groups were 31.6%, 63.3%, 71.8% and 29.6%, respectively. There were no significant differences between groups ($p = 0.105$). At 12 weeks, the mean indices of bone contact in the Ti, HA, 3%Ag-HA and 50%Ag-HA groups were 18.8%, 83.7%, 77.2% and 40.5%, respectively. The values in the HA and 3%Ag-HA coating groups showed significant differences compared with those in the Ti ($p = 0.005$ and 0.007 , respectively, Tukey's HSD test) and 50%Ag-HA ($p = 0.026$ and 0.041 , respectively, Tukey's HSD test) groups. The indices in the 3%Ag-HA group were almost the same as those in the HA group, and no significant differences were

found between the 3%Ag-HA and HA groups (ANOVA, $p = 0.943$) (Fig. 4).

Serum analysis. The mean serum concentration of Ag in the Ti and HA groups was undetectable at two, four and 12 weeks (Fig. 5). The 3%Ag-HA and the 50%Ag-HA groups showed a mean serum Ag concentration of 1.1 parts per billion (ppb) (0.5 to 1.3) and 5.3 ppb (3.1 to 8.3) at two weeks respectively. The concentration of Ag decreased gradually over the experimental period. The mean concentration of Ag in the 50%Ag-HA group was significantly different from that in the Ti and HA groups at two weeks ($p = 0.042$ and 0.045 , respectively, Tukey's HSD test). Furthermore, the mean concentration in the 50%Ag-HA group was statistically different from that in the other three groups at four ($p = 0.000$ for all comparisons, Tukey's HSD test) and at 12 weeks ($p = 0.046$, 0.025 and 0.020 for Ti, HA and 3%Ag-HA, respectively, Tukey's HSD test). The mean concentrations in the 3%Ag-HA group showed no significant difference compared with that in the Ti and HA coating groups at two, four and 12 weeks, (Ti: $p = 0.871$,

Fig. 3

Bar charts showing the mean affinity index of bone formation at two, four and 12 weeks (*, significant difference between the 50%Ag-hydroxyapatite (HA) coating group and both the HA and 3%Ag-HA coating groups at two weeks ($p < 0.05$). **, significant difference between the Ti and the 50%Ag-HA coating groups at four weeks ($p < 0.05$)).

Fig. 5

Bar charts showing the mean serum Ag concentration at two, four and 12 weeks (*, significant difference between the 50%Ag-hydroxyapatite (HA) coating group and the Ti and HA coating groups at two weeks ($p < 0.05$). ** and ***, significant difference between the 50%Ag-HA coating group and the other three groups at four weeks ($p < 0.01$) and 12 weeks ($p < 0.05$), respectively).

Fig. 4

Bar charts showing the mean affinity index of bone contact at two, four and 12 weeks (* and **, significant difference between the Ti group and both the hydroxyapatite (HA) and 3%Ag-HA coating groups at two and 12 weeks, respectively ($p < 0.01$). ■ and ■■, significant difference between the 50%Ag-HA coating group and both the HA and 3%Ag-HA coating groups at two and 12 weeks, respectively ($p < 0.05$)).

0.593 and 0.999, respectively; HA: $p = 0.898$, 0.593 and 0.999, respectively, ANOVA).

Discussion

There have been no previous studies *in vivo* of the osteoconductivity of an Ag-HA coating. In our study, the 3%Ag-HA coating was shown to have good osteoconductivity based on the affinity indices in the rat tibia.

The affinity indices of bone formation and bone contact in the 3%Ag-HA coating group were the same as those in the HA group, indicating that the 3%Ag-HA coating may have the same osteoconductivity and strength of attachment between the bone and implant as the HA coating. Because the affinity index of bone formation has been used to evaluate osteoconductivity, the affinity indices of bone formation and bone contact were calculated in order to reveal differences in osteoconductivity for each type of coating.¹⁶⁻¹⁸ However, measurement of the thickness of neonatal bone and bone maturation may be necessary for more precise evaluation of osteoconductivity.²⁵

The affinity index in the 50%Ag-HA coating group was lower than that in the 3%Ag-HA and HA groups, suggesting that the inhibition of osteoconductivity because of Ag toxicity depended on the dose. Alt et al²⁶ evaluated the cytotoxicity of bone cement *in vitro* with the addition of nanoparticulate Ag using human osteoblasts. They found that osteoblasts formed cellular processes on the nanoparticulate Ag bone cement as in the non-toxic control group, indicating good biocompatibility of the cement. Hardes et al²⁷ investigated the influence of elementary Ag on the behaviour of osteoblasts *in vitro* using human osteosarcoma cell lines, and observed a dose-dependent effect of Ag toxicity. Gosheger et al²⁸ analysed the toxicological side-effects of Ag-coated megaprotheses in ten rabbits, and found that the concentration of Ag in the blood and in the organs rose without either pathological changes in laboratory parameters or histological changes in tissues.

By contrast, it has been reported that a concentration of Ag in the blood of more than 300 ppb can cause argyria, argyrosis, leukopenia and liver and kidney damage.²⁹ Drake

and Hazelwood³⁰ found that the acute symptoms of over-exposure to Ag nitrate include a decrease in blood pressure, diarrhoea, irritation of the stomach and decreased respiration. Chronic symptoms resulting from the intake of a low dose of Ag salts are fatty degeneration of the liver and kidneys and changes in the blood cells.³⁰ Long-term inhalation or ingestion of soluble Ag compounds or colloidal Ag may cause argyria and/or argyrosis.³⁰

In our study, the highest concentrations of serum Ag resulting from the 3%Ag-HA and 50%Ag-HA coatings were 1.1 ppb and 5.3 ppb at two weeks, respectively. These levels were low enough to avoid harmful effects.

Several factors may influence the reduction in the Ag concentration in the Ag-HA coating group at two, four and 12 weeks. Because the surface of the Ag-HA coating was fully covered with a thick layer of HA after soaking in simulated body fluid for 24 hours, Ag may have been absent from the surface of the coating, or its release may have been suppressed.²¹ Schierholz et al¹⁵ observed that in an environment containing albumin and halide ions, serum Ag ions were decreased because of absorption processes with albumin and precipitation into insoluble Ag chloride.

Our study evaluated the antibacterial activity of a 3%Ag-HA coating *in vitro*.²² We have shown previously that the growth of *Escherichia coli*, *Staph. aureus* and MRSA was completely suppressed on Ti discs coated with 3%Ag-HA.²² Furthermore it was demonstrated that a 3%Ag-HA coating had *in vivo* antibacterial activity against MRSA, and that the concentration of Ag from Ti discs coated with 3%Ag-HA increased to more than 50 ppb at 48 to 72 hours.²³ Silver has been shown to have antibacterial activity at concentrations as low as 35 ppb without toxic effects to mammalian cells.²⁹ Although the concentrations of Ag were not measured during the first two weeks after implantation in our present study, the 3%Ag-HA coating may have had antibacterial activities. Further studies of the 3%Ag-HA coating are required for the evaluation of antibacterial activity *in vivo* in the rat tibia and for the evaluation of the concentration of Ag within two weeks of implantation.

One advantage of coating with Ag-HA is that the antibacterial activity and toxicity can be arbitrarily adjusted by increasing the amount of Ag₂O. The Ag-HA coating can be applied to add antibacterial activity to implants without toxicity to the recipient of the implant by adjustment of the level of Ag₂O. By adjusting the antibacterial activity and toxicity, the Ag-HA coating may have an important role in the prevention of microbial infection after implantation.

In the rat tibia a 3%Ag-HA coating showed good osteoconductivity, and was comparable with an HA coating and a 50%Ag-HA coating in the inhibition of bone formation. In addition, the serum concentrations of Ag with an Ag-HA coating were shown to be low enough to avoid harmful effects. The 3%Ag-HA coating had low toxicity with high osteoconductivity. However, no evidence of bactericidal activity was observed in the rat tibia. Further studies with the 3%Ag-HA coating are required for the evaluation of anti-

bacterial activity *in vivo* in the rat tibia. The 3%Ag-HA coating on implants may be a biologically safe antibacterial biomaterial and may be of value in the reduction of surgical-site infections related to implantation.

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