Salt impregnation of implant materials

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Implant infection and peri-implantitis are recurrent and serious complications in implantology. Antibiotic treatment of infected implants in bone is complicated because of limited vascularization of the surrounding tissue on the one hand, and biofilm formation on the implant surface on the other. Therefore, contamination of the implant surface has to be prevented. For this purpose we developed a method of coating Ti6Al4V samples with NaCl in order to reduce the water activity on the sample surface. The resulting NaCl-coated surfaces showed good biocompatibility with osteoblastlike cells and resulted in a significant reduction of bacterial strains. The presented surface modification is hence a promising method to reduce bacterially induced implant failure. **(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2009;xx:xxx)**

Implant failure due to deep endoprosthesis infection and peri-implantitis is a serious problem in orthopedics as well as in dental implantology. In many cases the systemic therapy with antibiotics is ineffective because of poor accessibility of the infection site.[1,2] Additional complications emerge from increasing amounts of multiresistant bacterial strains, which are less treatable by conventional antibiotic therapies.[3,4] The increased occurrence of antibiotic-resistant microorganisms is to be partly blamed on antibiotic misuse. It is common practice to provide patients with antibiotic prophylaxis, although there are studies indicating that there may be no need for this procedure in the treatment of healthy individuals, at least in dental implant surgery.[5,6] A total of 10% of all common antibiotics in the United States are prescribed by dentists.[1] Critical conditions are in effect in intraoral surgery, since the mouth provides a contaminated environment that cannot be sterilized, which enables germs to penetrate the operation field. Insertion of implants is therefore classified as clean-contaminated surgery. Various precautions have to be taken to avoid contamination of the surgical field, like rinsing with chlorhexidine, aspirating saliva, and reducing saliva production.[7,8] Implant contamination with microorganisms originating from the hospital environment, the surgeon, or the patient may be reduced by providing antimicrobial implant surfaces. For orthopedic implants this is achieved by impregnating the surfaces with antibiotics directly[9-11] or coating with antibiotic-loaded polymers and ceramics respectively.[12-14] The critical situation concerning antibiotic resistances and the semisterile conditions inside of the mouth cavity demands the development of alternative antimicrobial agents. One of these alternatives was found in coating implant surfaces with silver-based antimicrobials due to the known biocompatibility of these agents for human cells[15] and their simultaneous antimicrobial activity.[16,17]

Another possibility avoiding surface contamination would be an impregnation with sodium chloride. It is a commonly used food preservation agent and its action is based on the water inactivation capacity of the salt. Most of the microorganisms cannot live in an environment with low water activity. Therefore, we planned to decrease the water activity locally on the implant surface by coating sandblasted Ti6Al4V samples with sodium chloride. This may be a simple and cost-reducing possibility to keep the surfaces free of microbial contaminants during surgery. Especially during the first days after implant placement, microorganisms have to be kept off the surface to provide space for the body cells colonizing the surface. In these early hours, a process called "race to the surface" takes place where microorganisms and body cells compete for the colonization of the substrate.[18]

MATERIALS AND METHODS

Sample preparation and characterization

As test specimen, round platelets made from Ti6Al4V (ASTM F 136-84) were used. The height of the specimen was 1.5 mm and the diameter was 15 mm. These dimensions were chosen according to the size of

The surfaces analyzed in this work have been developed by S. Ihde and are currently under commercial use of the Dr. Ihde Dental AG, Switzerland.

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the incubation wells for cell culture. The platelets had been sandblasted with corundum powder (grain size 50 μm). Subsequently, the samples were washed in 10% NaCl (Sodium chloride 10%, Streuli Pharma AG, Uznach, Switzerland) solution and air dried.

As the test specimen for antimicrobial tests, original compression screw implants of the STO-type (4.1 × 15 mm, made from Ti6Al4V, ASTM F 136-84, sandblasted with Al-Oxide particles of 180 μm in size) were used. They were also washed in 10% NaCl solution and air dried.

Cell culture

Human osteoblast cell line MG63 (ATCC no. CRL-1427, Rockville, MD) was cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen Life Technologies, Karlsruhe, Germany). The culture medium was supplemented with 10% fetal calf serum, 1% penicillin and streptomycin, respectively (all from Invitrogen Life Technologies), and the cells incubated in a humidified 5% CO_2 incubator at 37°C. For biocompatibility tests the samples were placed in triplicate into the wells of a 24-well plate (Nunc, Wiesbaden, Germany). The cells were seeded onto salt-coated surfaces, reference surfaces, and on polystyrene, with an initial density of 50,000 cells per well.

Biocompatibility testing

The biocompatibility of the salt-modified surfaces was determined by cell counting and a cell activity test after 3, 5, 7, and 10 days of culture on the surfaces as described earlier.[19] Cell proliferation was analyzed by electronic cell counting using a CASY 1 TTC cell analyzer (Schärfe System, Reutlingen, Germany). Cell viability was analyzed by using the cell proliferation reagent WST 1 (Roche Diagnostics, Mannheim, Germany). After incubating the cells for 30 minutes with the WST reagent 1:10 in DMEM at 37°C, the adsorption of the supernatant was quantified in a Tecan spectra fluor plus photometer (Tecan, Crailsheim, Germany). For each method and sample, 3 measurement readings were recorded, from which the averages and standard deviations were calculated.

Immunofluorescence microscopy

Vinculin distribution in cells grown on the coated surfaces in comparison to reference surfaces was analyzed by indirect immunofluorescence microscopy. Cultured cells grown on the samples were rinsed in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 7.0 mM $Na_2HPO_4 \times 2\ H_2O$, 1.5 mM KH_2PO_4) and fixed for 10 minutes in –20°C methanol followed by dehydration for 4 minutes in –20°C acetone. After air drying for 30 minutes the cells were incubated with the primary antibody recognizing vinculin (diluted 1:100 in PBS, Sigma, Taufkirchen, Germany) for another 30 minutes at room temperature. Specimens were then washed in PBS and incubated for 20 minutes at room temperature with the appropriate secondary antibody conjugated to Cy2 (diluted 1:75 in PBS, Dianova, Hamburg, Germany). The samples were counterstained with the DNA-specific dye Hoechst 33258 (5 μg/mL), washed in PBS, air dried from ethanol, and mounted in Mowiol (Hoechst, Frankfurt, Germany).

Scanning electron microscopy

Cells grown on modified and reference surfaces were rinsed in PBS and fixed for 15 minutes in ice-cold glutaraldehyde (6% in PBS, Merck, Darmstadt, Germany). After 4 rinses in PBS, cells were dehydrated in ascending acetone concentrations (30%, 50%, 75%, 90% and 4 times 100%). After critical point drying, the samples were coated with gold and analyzed in a Zeiss scanning electron microscope DSM 940 (Zeiss, Oberkochen, Germany).

Antimicrobial properties

For testing the antimicrobial activity of the modified samples, 4 test organisms, namely *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 9027), *Bacillus atrophaeus* spores (ATCC 9372), and *Fusarium solani* (ATCC 36031) were used. These organisms were resuspended in 0.9% NaCl solution and 10 μL of each challenge suspension containing 10^3 to 10^7 colony-forming units (CFU) was separately transferred to the test samples. The specimens were incubated at 30°C to 35°C in a moist chamber. As control, each of the challenge suspensions was also transferred to 4 uncoated test items and incubated under the same conditions. After an exposure time of 1, 3, and 7 days the total number of recoverable organisms was determined by counting the CFU.

RESULTS

Surface coating with a saline layer and sample characterization

The Ti6Al4V surfaces coated with a thin layer of pure NaCl showed a crystalline structure (Fig. 1). The NaCl layer did not appear as a uniform layer; some areas were covered with huge crystals, about 200 μm to 300 μm in width, while other areas were uncoated. This phenomenon occurred because of the manual coating process of the tested sample.

Cells in contact with the surface

The morphology of cells growing on surfaces covered with a saline layer was analyzed. There was no

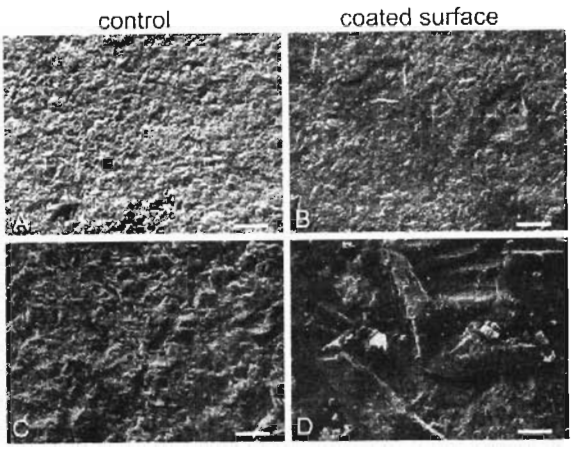

Fig. 1. SEM microphotograph of control Ti6Al4V surfaces and salt coated Ti6Al4V. The control samples show the typical appearance of sandblasted surfaces (**A, C**). Coating with NaCl leads to large crystals covering the surface (**B, D**). The coating is not continuous. Bar A, B 200 μm; C, D 50 μm.

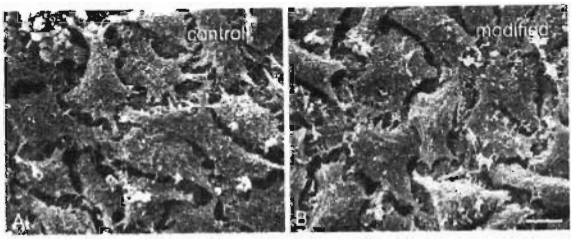

Fig. 2. SEM microphotograph of osteoblast cells grown on uncoated Ti6Al4V control surfaces (**A**) and NaCl-coated Ti6Al4V surfaces (**B**). There is no obvious difference in cell morphology. Bar 10 μm.

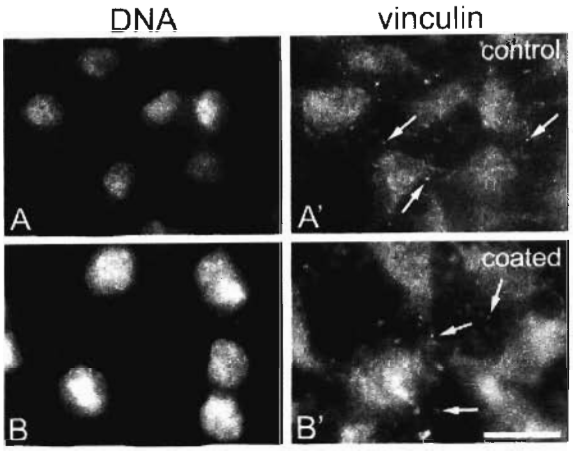

Fig. 3. Osteoblasts cultivated on Ti6Al4V (**A**) surfaces and cells grown on NaCl-coated Ti6Al4V surfaces (**B**) show a comparable distribution of the focal contact protein vinculin (A', B'). Some vinculin spots are depicted by arrows. The corresponding Hoechst staining for chromatin is shown (**A, B**). Bar: 25 μm.

obvious difference in morphology between cells cultivated on the reference sample and the test item. In both cases the cells were well spread, attached to the substrate with filopodia, and the samples were confluently covered (Fig. 2). To examine the correct formation of focal adhesions, the distribution of vinculin was determined by indirect immunofluorescence microscopy (Fig. 3). Cells grown on the control as well as the cells on the test samples showed a considerable amount of focal adhesions in a comparable distribution (depicted with arrows in Fig. 3, *A'* and *B'*).

Evaluation of the biocompatibility of the saline-coated specimen in comparison with the tissue culture polystyrene revealed good results. The parameters cell number, resembling cell growth, and cell activity, considering mitochondrial activity, have been determined. Cells grown on the metallic surfaces showed a 20% reduced growth on day 7 in comparison to polystyrene. But there was no significant difference in cell number between the cells cultivated on the test item and cells grown on reference surfaces (Fig. 4, *A*). Also on day 3, when the salt concentration in the culture medium was considered highest, the cell growth was not significantly reduced. At this time point, just before the first change against fresh medium, the initial medium contained the highest salt concentration due to the complete dissolution of the NaCl coating. An analogous result was obtained with the WST-test specific for mitochondrial activity. On day 7 the activity on the metallic surfaces was reduced by about 20%, whereas the activity on the metallic samples remained the same (Fig. 4, *B*). After 3 days of culture the activity on the reference and the coated surface were comparable. Taken together, the saline-coated surfaces showed the same biocompatibility as the reference samples representing a surface modification that is already being used for the fabrication of dental implants.

Antimicrobial properties

For testing the antimicrobial properties of salt-coated Ti6Al4V surfaces, we used *Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus atrophaeus* spores, and *Fusarium solani* as test organisms. The reaction of these organisms to the salt coating is depicted in Fig. 5; the total CFU counts are given in Fig. 5, *A*. The saline coating showed no antifungal activity. There were even more *F. solani* colonies on the coated surfaces than on the reference (Fig. 5, *B*). By contrast, there was a

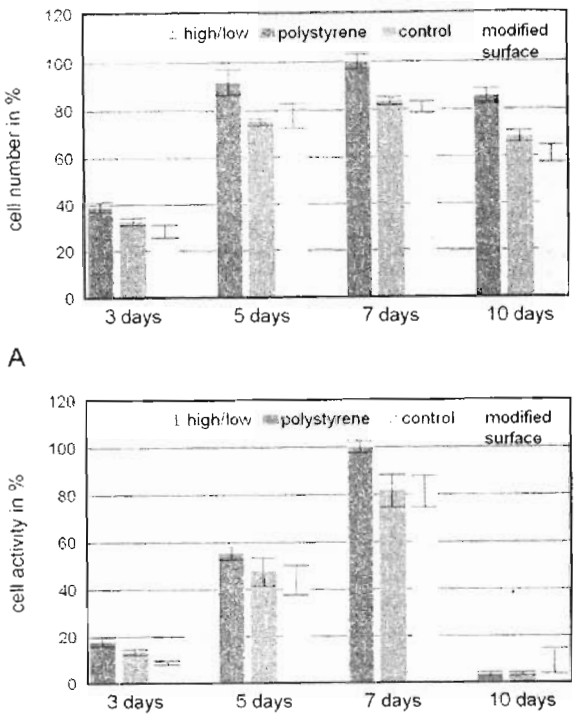

Fig. 4. NaCl-coated Ti6Al4V surfaces are biocompatible. **A,** Osteoblast growth is comparable on control and on test samples. **B,** Osteoblast cell activity is not reduced on the coated surfaces.

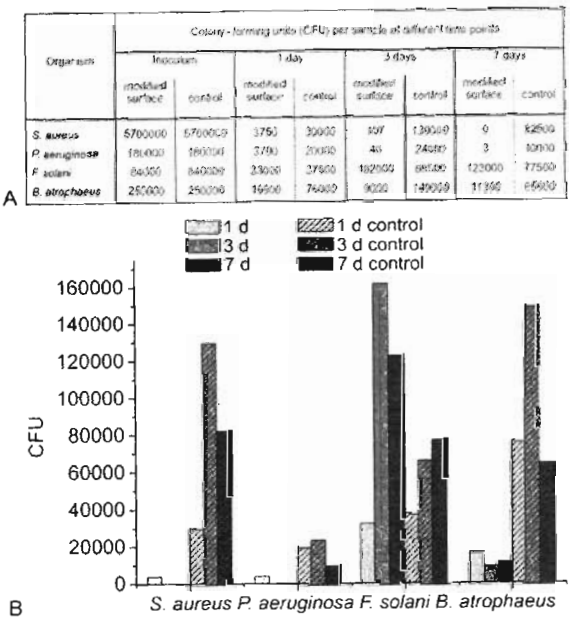

Fig. 5. Adhesion of different test organisms on NaCl-coated Ti6Al4V surfaces. **A,** Colony-forming units of the different test organisms per sample measured at different time points. **B,** Diagram of the results presented in **A**. Bacterial contamination is effectively reduced on coated samples when compared to control. Surface coating has no effect on fungi and a moderate inhibitory effect on *Bacillus* spores.

considerable effect on bacterial growth. After 1 day of incubation there were still some *S. aureus* and *P. aeruginosa* colonies detectable, but these were only 12.5% and 18.5% respectively of the control surface (compare Fig. 5, *A*). On day 7 there were no *S. aureus* and only 3 *P. aeruginosa* colonies detectable. The surfaces showed a moderate effect on spores. *B. atrophaeus* contamination was reduced by 80% compared to the control surface on day 1. The reduction was 6% on day 3 and 17% on day 7. Altogether, contamination with the tested bacteria was effectively inhibited, the impact to *B. atrophaeus* spores was moderate, and there was no affect on the tested fungi.

DISCUSSION

A severe complication after implantation is biofilm formation on the implant surface. Biofilms can be found on short-term devices like catheters as well as on long-term implants, like artificial hip prostheses. These structures consist of a population of microorganisms surrounded by a dense extracellular matrix composed of proteins, glycoproteins, and carbohydrates. The biofilm is formed successively by few organisms, which first bind transiently to the surface, followed by a strong adhesion. After a process of aggregation and microcolony formation the biofilm matures and becomes impermeable for substances like antibiotics.[20,21] For this reason, implant surfaces have to be designed in a way avoiding the initial adhesion of microorganisms, especially during handling of the device, and so preventing biofilm formation. Often, implant contamination is a result of patient-derived skin organisms or airborne organisms during surgery.[22] At the same time, the elimination of the microorganisms from implant surfaces allows the unrestricted adhesion of host cells to the device. Usually bacteria and body cells compete for the substrate in a process called "race to the surface"[17] and therefore microorganisms have to be reduced to ensure a proper ingrowth of the device.

A lot of work has been done to design surfaces with antimicrobial properties.[12-14,19] They can be impregnated directly by antibiotics,[23] or they can be modified by the addition of antimicrobial metals like silver, which are either partially or fully released over a certain period of time.[24,25]

In the food industry, a common preservative agent is NaCl. NaCl coatings on implant devices may present a simple, cost-efficient and effective method to prevent

microorganisms from adhering to the surface during the transfer of the implant from the dry aseptic packing into the implantation site. This inhibition of microorganism attachment is important in handling the implant device during the surgical procedure, especially under semi-sterile conditions like in the mouth cavity. Because of osmotic conditions, the microorganisms will lose water in contact to the salted surface and therefore be killed. Once inside the tissue, the NaCl coating will be dissolved in the body fluid and thus washed off the implant, enabling tissue cells to interact with the implant surface. In the present study we could show the significant reduction of bacterial strains *S. aureus* and *P. aeruginosa*. The coating showed no antifungal effect and was moderately active against spores. It may be possible that *F. solani* are not susceptible because these fungi can also be found in costal regions with higher salt concentrations.[26]

Growth of MG 63 osteoblastlike cells on the NaCl-modified surfaces was comparable to the growth of the cells on Ti6Al4V reference surfaces, which are already used as dental implants. Buser et al.[27] described enhanced "bone apposition" to sandblasted, acid-etched titanium surfaces stored in 0.9% NaCl. The NaCl concentration on these surfaces is lower than on the samples examined in this study; however, it is an indication for the NaCl tolerance of osteoblasts. The findings of Buser et al.[27] have to be regarded with caution, since it does not seem justifiable to describe nonmineralized tissues around dental implants as bone. Furthermore, it has to be taken into account that their experiment created void spaces: those spaces will not allow conventional screw implants to be inserted with primary stability. The salt concentration in the bone is more than double of that in the blood; hence, the description "physiologic" corresponds only to blood and not to bone and NaCl 0.9% is a hypo-physiologic concentration in bone.

When dental implants with the coating developed in this study are inserted into bone, the NaCl crystals on the implant will dissolve quickly in the body fluid and adjacent bone. This agent will be momentarily effective as an antibacterial agent eliminating germs that attached during the surgical procedure, whereas no such effect can be expected from NaCl 0.9%.

We expect that the NaCl-coated Ti6Al4V surface properties will also help stabilize the implant, because the high osmotic pressure will lead to an inflow of liquids toward the implant surface. This may help to reestablish the intra-bony liquid and salt pressure. Compared with antibiotic coatings, the saline-coated Ti6Al4V surface will not show typical side effects of antibacterial coatings.

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