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Antimicrobial activity of gentamicin palmitate against high concentrations of *Staphylococcus aureus*

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Abstract The reduction of implant related infections plays a pivotal role in orthopaedic surgery as an increasing number of people require implants (up to 200,000 per year in the United States (source: Joint Implant Surgery & Research Foundation 2010)). The aim of the current study is to prevent and thus decrease the number of bacterial infections. Both pre and post operative systemic antibiotic treatment and gentamicin containing bone cements (polymethylmethacrylate, PMMA) are commonly used strategies to overcome infections. In this study, the antimicrobial efficacy of gentamicin sulfate loaded bone cement was compared with titan discs coated with a new form of gentamicin, gentamicin palmitate. Adherence prevention, killing rates and killing kinetics were compared in an in vitro model, using *Staphylococcus aureus (S. aureus)*, which together with *Staphylococcus epidermidis* (*S. epidermidis*) represents 60% of bacteria found responsible for hip implant infections (An and Friedman, 1996, J Hosp Infect 33(2):93–108). In our experiments gentamicin, which was applied as gentamicin palmitate on the surface of the implants, showed a high efficacy in eliminating bacteria. In contrast to gentamicin sulfate containing bone cements, gentamicin palmitate is released over a shorter period of time thus not inducing antibiotic resistance. Another benefit for clinical application is that it achieves high local levels of active ingredient which fight early infections and minimize toxic side effects. Furthermore, the short term hydrophobic effect of gentamicin palmitate can successfully impede biofilm formation. Thus, the use of self-adhesive antibiotic fatty acid complexes like gentamicin palmitate represents a new option for the anti-infective coating of cementless titan implants.

Abbreviations

Ti1a/Ti1b	Titan discs with high/low concentration of gentamicin palmitate
Ti2a/Ti2b	Titan discs with high/low concentration of gentamicin palmitate
Ti3a/Ti3b	Titan discs with high/low concentration of gentamicin palmitate
PMMA1	Commercially available bone cement without gentamicin
PMMA2	Commercially available bone cement with gentamicin sulfate

1 Introduction

Aminoglycosides were first introduced in the 1950s, and in 1963 gentamicin was isolated from *Micromonospora pupurea*. The antimicrobial efficacy of aminoglycosides is based on the mechanism of inhibiting protein synthesis by binding to the 30 s subunit of the ribosome. Gentamicin has a broad spectrum against gram-positive (including *mycobacteria*) and gram-negative bacteria. Besides binding to the ribosome, gentamicin is supposed to disrupt the packing order of lipopolysaccherides of the outer membrane and to induce holes [1]. As gentamicin has to be

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applied parenterally and leads to nephrotoxicity and ototoxicity if given over a longer period of time, its use in clinical therapy has decreased. Chemically, gentamicin sulfate consists of a mixture of gentamicin C1, C1a and C2a + b, the protonic gentamicin base being the antimicrobial active substance [2].

Alongside with pre- and post-operative systemic antibiotic therapy [3], the addition of antibiotics to bone cement is an important factor in fighting infections in orthopaedic surgery [4]. This new field of application for gentamicin was established when Buchholz and Engelbrecht [5] described the addition of gentamicin sulfate to bone cement. Studies from Wahlig and Dingeldein [6] showed interesting details about bone cements with antibiotics, especially gentamicin. The observed patients showed low concentrations of gentamicin (as base) in serum and urine, but high concentrations in wound exudate. In addition, high concentrations of gentamicin could be measured in samples taken from the vicinity of the implant for a long period of time. The release rate of gentamicin was extended and 2–3 fold higher compared to the release rate of other antibiotics.

Biofilm formation on an implant or bone cement, which is one of the characteristics of bacteria, is triggered by the adherence of proteins on the implant surface. The exposure to biological material such as blood or compounds of the wound secretion system facilitates this step [7]. Additionally, the surface conditions, e.g. roughness or porosity, affect biofilm formation [8]. Palmitic acid with its hydrophobic quality hinders the adherence of proteins on a surface [9]. Therefore, an antimicrobial coating of the implant itself, the rapid release of the active ingredient, and the hydrophobic quality of gentamicine palmitate could facilitate fighting biofilms at a very early stage.

The use of self-adhesive antibiotic fatty acid complexes represents a new option for the anti-infective coating of implants. Gentamicin sulfate can be converted easily into a gentamicin palmitate by ion exchange [10]. It does not contain biofilm-forming polymers and no critical degradation products are produced. The antibiotic itself functions as the anti-infective layer and the fatty acid can be metabolized in the human organism by β-oxidation, which generates carbon dioxide and water. In both gentamicin palmitate and gentamicin sulfate, the protonic gentamicin base functions as the antimicrobial active substance.

The application form of gentamicin palmitate (as a coating) leads to high local levels and not to the induction of a burden to organs or blood [6, 11]. In contrast to bone cement (PMMA) or poly-L-lactide (PLLA), its dissolution from the surface is completed within 2 weeks and is not likely to induce resistance to endogenous bacteria [12–14].

2 Aim of the study

In the present study we compared the antimicrobial efficacy of titan discs coated with gentamicin palmitate (representing titan cementless implants) to bone cements loaded with gentamicin sulfate because these bone cements have been in successful clinical use for a long time. The experiments were designed in order to resolve questions on the antimicrobial efficiency, on the inhibition of adherence and on the kinetics of microbial elimination of gentamicin palmitate in an in vitro model using *S. aureus*.

3 Materials and methods

3.1 Microbiology

All incubation experiments and dilutions were performed in CSL Media (Casein soya bean digest broth, 30 g/l, Oxoid LTD., England) and, in order to detect bacterial growth, the colony forming units (cfu) were counted on casein soja bean agar plates (CSA, Casein soya bean digest broth, 40 g/l, Oxoid LTD., England). We used *S. aureus* ATCC 6538 strain for all experiments. All experiments and controls were done in triplicates. Inhibiting aerola testings were done on Müller Hinton Agar plates (inhouse production).

3.2 Titan discs

Flat titan discs (Tilastan®, diameter 15.6 mm) with three different surfaces were used: corundum blasted titan discs (Ti-1), corundum blasted titan discs with an additional HX-layer of calcium-phosphate (Ti-2), and titan discs with an additional porous plasma coating (CPT, porosity 35 ± 5%) and an HX-layer (Ti-3). The titan discs Ti-1, Ti-2 and Ti-3 are routinely used in the manufacture of commercially available uncemented endoprosthesis (C.F.P®, MP® hip, Gemini® knees, all Waldemar Link GmbH & Co. KG).

3.3 Coating of the discs

All titan discs were coated with gentamicin palmitate (GP; Heraeus Medical GmbH) [15]. Gentamicin palmitate was converted into a gentamicin–fatty acid complex by metathesis of gentamicin sulfate with endogenous palmitic acid. Gentamicin palmitate was then applied in form of alcoholic solution on the titan discs [10]. The titan discs were thus coated with two amounts of gentamicin, 100 µg (low) and 220 µg (high) gentamicin base (GB) per cm^2, respectively. The employment of a special spraying process [16] finally resulted in the absolute amount of 400 µg, and 840 µg of gentamicin base for the discs. The coating

process was monitored by weighing. Uncoated titan discs and commercially available gentamicin loaded bone cements were used as reference.

3.4 Bone cement discs

The cements were mixed according to manufacturer instructions and flat discs of the same size as the titan discs were produced: bone cement 1 (plain without gentamicin sulfate Palacos® R), and bone cement 2 (bone cement with 0.5 g gentamicin sulfate in 40 g polymer powder) resulting in a total amount of 8 mg gentamicin base per disc.

All discs were sterilised by gamma irradiation (25–40 kGy).

For kinetic experiments gentamicin sulfate (Sigma-Aldrich, Austria) was used as "gold standard" in concentrations ranging from 8 to 84 μg/ml.

Discs were washed with phosphate buffered saline (PBS, Biochrom AG, Germany).

3.5 Ability of adherence prevention of gentamicin palmitate coating

The test discs were incubated in 12-well plates, filled with 1.13 ml *S. aureus* suspension containing 1×10^3, 5×10^3 and 1×10^4 (cfu)/ml. The discs could move freely in their wells and were shaken carefully during incubation period (at 37°C). After 24 h (h) the discs were removed and washed in 5 ml of PBS over 30 s at 200 rpm. Afterwards they were transferred into fresh CSL media and incubated for another 24 h at 37°C. After this period, aliquots of the media were taken out and either plated directly on CSA or diluted and plated, to determine cfu numbers. The test setup was based on ISO DIN EN 17025. In contrast to this standard, however, not only the growth of the test bacteria by change of turbidity was measured, but also every single colony could be found by plating aliquots after the 2nd incubation period.

3.6 Elimination of bacteria in 24 h suspension

Discs were incubated in 12 well plates for 24 h at 37°C with either bacteria (*S. aureus*, 10^2, 10^4, 10^6 cfu/ml) or media alone. After the incubation period, cfu were detected.

3.7 Diffusion of the active ingredient in inhibiting areola testing

100 μl of a bacterial suspension (1.5×10^6 cfu/ml) were swabbed all-over Müller Hinton Agar plates. Discs were put on the Agar. On the contact side, the discs were moistened with 10 μl of PBS and kept for 1 h at room temperature to facilitate adherence. The diffusion of the active ingredient into the agar plate leads to circular inhibition of the plated bacteria (inhibiting areola). Diameters of the clear area were measured on the bottom side of the petri dishes after 24 h incubation at 37°C.

3.8 Kinetics of bacterial reduction

An overnight culture of *S. aureus* was diluted in pre-warmed media (37°C) to a bacterial number of $\times 10^6$ cfu/ml resulting in a final volume of 10 ml. This bacterial suspension was then incubated at 37°C and shaken at 96 rpm for 2 h. Subsequently, antibiotics and discs were added. Samples were drawn after 15, 30, 45 and 60 min, and then by the hour. The last sample was taken after 24 h. Aliquots of the samples were plated on agar plates to determine germ number.

3.9 Data analysis

For all data, mean and standard deviation of the mean were calculated, graphs were calculated with GraphPadPrism™ 5.01 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

4 Results

4.1 Ability of adherence prevention of gentamicin palmitate coating

These experiments were designed to provide evidence if gentamicin palmitate could prevent bacterial settlement on the surface.

All coated titan and PMMA discs with antibiotics did not show bacterial growth after the second incubation step (after 48 h). PMMA or titan discs without gentamicin did not inhibit bacterial growth over the incubation period (Table 1).

All test objects remained sterile after transfer for another 24 h into fresh media. Uncoated titan discs and PMMA moulds without gentamicin did not inhibit bacterial growth (Table 1).

Discs with half of the amount of antibiotics (1b, 2b, 3b) did not show a weaker effect in their antimicrobial activity (within 48 h).

4.2 Elimination of bacteria in 24 h suspension test

The gentamicin palmitate surface coatings (high and low) showed the same bactericidal effect on different *S. aureus* concentrations (Table 2) as gentamicin sulfate containing

Table 1 Adherence testings

Material	cfu	cfu	cfu	Material	cfu	cfu	cfu
	1×10^3	5×10^3	1×10^4		1×10^3	5×10^3	1×10^4
Ti1a	0	0	0	Ti1b	0	0	0
Ti2a	0	0	0	Ti2b	0	0	0
Ti3a	0	0	0	Ti3b	0	0	0
PMMA1	4.7×10^9	2.8×10^9	2.5×10^9	PMMA1	4.7×10^9	2.8×10^9	2.5×10^9
PMMA2	0	0	0	PMMA2	0	0	0
K_{PLU}	2.3×10^9	2.5×10^9	1.7×10^9	K_{PLU}	2.3×10^9	2.5×10^9	1.7×10^9
K_W	1.1×10^9	2.3×10^9	2.3×10^9	K_W	1.1×10^9	2.3×10^9	2.3×10^9
K_Z	1.0×10^3	5.1×10^3	1.1×10^4	K_Z	1.0×10^3	5.1×10^3	1.1×10^4
K_0	0	0	0	K_0	0	0	0

cfu colony forming units, K_{PLU} control discs without gentamicin, K_W growth control, K_Z initial cfu, K_0 sterile control. Gentamicin palmitate coated titan discs show the same antimicrobial effect as antibiotic bone cement moulds (1a, 2a, 3a, 5 as well as 1b, 2b, 3b, 5). Growth of *S. aureus* was not inhibited by the discs alone (K_{PLU})

Table 2 Suspension testing

Material	cfu	cfu	cfu	Material	cfu	cfu	cfu
	1×10^2	1×10^4	1×10^6		1×10^4	1×10^6	1×10^2
Ti1a	0	0	0	Ti1b	0	0	0
Ti2a	0	0	0	Ti2b	0	0	0
Ti3a	0	0	0	Ti3b	0	0	0
PMMA1	2.0×10^9	1.8×10^9	1.6×10^9	PMMA1	2.0×10^9	1.8×10^9	1.6×10^9
PMMA2	0	0	0	PMMA2	0	0	0
K_{PLU}	1.1×10^9	4.4×10^8	1.4×10^9	K_{PLU}	1.1×10^9	4.8×10^8	2.5×10^9
K_W	1.3×10^9	1.2×10^9	1.4×10^9	K_W	1.3×10^9	1.5×10^9	1.4×10^9
K_Z	1.3×10^2	1.4×10^4	1.0×10^6	K_Z	1.3×10^2	1.4×10^4	1.0×10^6
K_0	0	0	0	K_0	0	0	0

cfu colony forming units, K_{PLU} control discs without gentamicin, K_W growth control, K_Z initial cfu, K_0 sterile control. Gentamicin palmitate coated titan discs show same antimicrobial effect as antibiotic bone cement moulds (1a, 2a, 3a, 5 as well as 1b, 2b, 3b, 5). Growth of *S. aureus* was not inhibited by the discs alone (K_{PLU})

PMMA moulds. Growth was not inhibited by discs or PMMA moulds without gentamicin.

All discs and PMMA moulds with antibiotics showed antibacterial activity. No viable bacteria could be recultivated after 24 h.

4.3 Diffusion of the active ingredient in inhibiting areola testing

Inhibiting areolas were comparable on all different kinds of discs. The PMMA moulds showed slightly reduced diameters reaching 88% of the diameter of 1a, 2a or 3a (Table 3). The reference discs (uncoated) did not show any inhibiting areolas.

All titan discs containing gentamicin palmitate showed comparable inhibition diameters irrespective of the additive on the surface (CaP) or the surface structure (sand blasted). Even discs with half the amount of the antibiotics showed similar diameters. PMMA discs containing gentamicin sulfate showed slightly reduced but comparable diameters (Table 3).

Table 3 Diameters of inhibiting areola testing

Material	Mean diameter (mm)
Ti1a	40 mm ± 0
Ti2a	40 mm ± 1
Ti3a	40 mm ± 0
Ti1b	39 mm ± 2
Ti2b	39 mm ± 1
Ti3b	39 mm ± 0
PMMA2	35 mm ± 0

Discs without antiobiotics did not show any inhibition

4.4 Kinetics of bacterial reduction

In our kinetic studies all concentrations of the active ingredients eliminated the starting bacterial number of *S. aureus* within the first hour, with the exception of 8.4 µg/ml gentamicin base in the media. We could detect slight differences between the different materials and gentamicin concentrations. 1a, 2a, 3a and 84 µg/ml of gentamicin base in suspension reduced the starting cfu from 2 to 4 10^6 cfu/ml to around zero within the first half hour. Within the next half hour, 42 µg/ml of gentamicin base and PMMA completely eliminated the bacteria (Figs. 1, 2). Only the lowest concentration of gentamicin base (8.4 µg/ml) allowed a small number of bacteria to survive up to 4 h and to grow again to a final number of x-fold of 10^9 after 24 h (data not shown). All the other gentamicin concentrations did not show bacterial growth after 24 h.

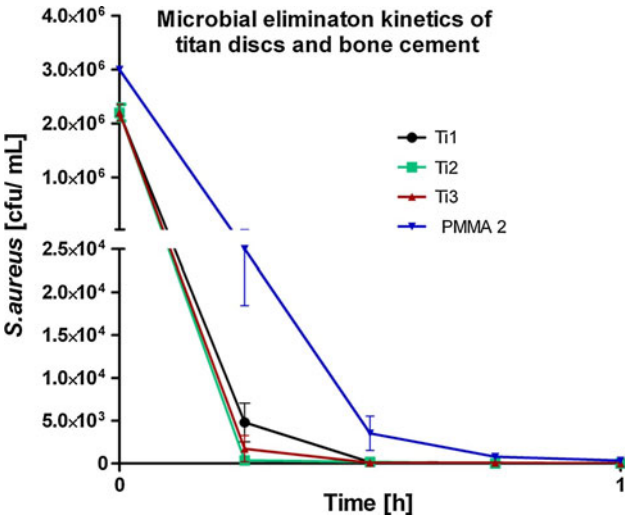

Fig. 2 Kinetics of bacterial elimination over 1 h with the three titan discs with GP (Ti1, Ti2, Ti3) and bone cement with gentamicin sulfate (PMMA2). Mean was calculated out of three values and bars show the standard error of the mean (SEM)

5 Discussion

Orthopaedic implant surgery wants to achieve three main goals: the best possible reconstitution of the patient, a short residence time in hospital, and low costs for the public health system. In a study about revisions of total hip arthroplasty by Bozic et al., data of 51,345 patients were analyzed and evaluated. They found out that infections were responsible for revision in 14.8% and the most common reason for arthrotomy or removal of the prothesis in 74.3% of the cases [17]. The period of hospitalisation was lengthened by 6.2 days for all types of revision together, and the average of total charges was $54,553 per case.

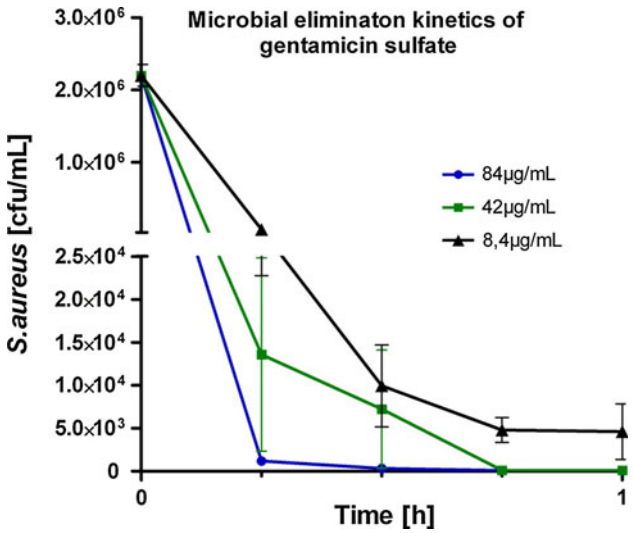

Fig. 1 Kinetics of bacterial elimination over 1 h with 3 different concentrations of gentamicin sulfate (84 µg/ml, 42 µg/ml and 8.4 µg/ml). Mean was calculated out of three values and bars show the standard error of the mean (SEM)

These figures are not only important for insurances and the public health system; what is more, they mean pain and life threatening complications for the patient.

Surface modifications of implants are a current issue in orthopaedic surgery. In principle two types of modification are of general interest: enhancing osseointegration of the implant and adding anti-infective functionality [18, 19]. Modifications on osseointegration are currently achieved with hydroxyapatite [20] or by enhancing the biophysical properties of the implant [19]. These modifications do not contribute to an anti-infectious prophylaxis.

Anti-infective aid is mostly given by either adding bone cement or antibiotic-eluting medical devices [21]. Newer tries focus on poly-L-lactide (PLL) as a carrier also for gentamicin [22] or on dipping the titan implant with chitosan-vancomycin [18]. The realization of a biodegradable antibiotic delivery system is therefore the most wanted feature [23].

There are two ways of administering the antimicrobial agent which follow similar principles: bone cements containing antibiotics or the new surface coating with gentamicin palmitate. Meta-studies with a large sample size in the United States of America showed a sustainable reduction of infection risk when using bone cements containing antibiotics [24]. On cementless implants, however, antibiotic protection from bacterial colonisation is not possible so far. The application of gentamicin palmitate could offer a solution to this problem, because gentamicin palmitate delivers its load in the implant surrounding area and is very effective in fighting local infections [15, 16].

The kinetic experiments revealed very interesting details about how fast gentamicin base is released. As the

calculated "pool" of gentamicin base on a "high" titan disc is 840 μg, we used 84 μg/ml (10 ml of culture volume yield in a total amount of 840 μg) gentamicin base (calculated from gentamicin sulfate anhydrous) in our cultivation media so that the calculative concentration of both cultures was equal. The difference was that the antibiotic was added in solution all at once whereas the gentamicin palmitate had to dissolve. Release rates of gentamicin base out of gentamicin palmitate are known to be high enough for antimicrobial effects within a time span of the first 24 h [25]. In our experiments, moreover, we found out that they were already high enough within the first 30 min to compete efficiently with the directly transmitted antibiotic. Our graphs clearly demonstrate the potential of the gentamicin palmitate coating. Within the first half hour of the experiment the cfu/ml were already reduced to amounts below 10^2 cfu (Fig. 1). In addition, we could not detect bacterial growth after 24 h on any of the used titan discs or on the PMMA moulds.

All concentrations of gentamicin sulfate eliminated our test bacteria except the (8.4 μg/ml). At this concentration the bacterial number decreased within the first 4 h, and increased after this point of time to reach a final cfu number after 24 h equal to the growth control, findings which are consistent to the work of Schafer et al. [26] (Fig. 2).

In general, bone cement moulds with gentamicin sulfate and titan discs coated with gentamicin palmitate are very similar in their effectiveness against *S. aureus*. Both can eliminate cfu/ml numbers ranging from 10^2 to 10^6 within 24 h and still have enough reservoir to prevent further growth of bacteria. The release of gentamicin base per cm^2 on day four to five is still high enough to inhibit growth of both gram negative and gram positive bacteria [27]. In addition to the quick availability of gentamicin base out of gentamicin palmitate, palmitic acid changes the implant surface characteristics and interrupts first steps in biofilm formation [28].

To sum up, the high release rates of the active ingredient and the repellent surface of the implant both impede biofilm formation [29, 30]. Gentamicin palmitate surface coatings could therefore represent a significant improvement in orthopaedic surgery by eliminating bacteria in a rapid and efficient way over a short period of time.

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