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Bisphosphonates offer protection against prosthetic joint infections caused by *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms

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ABSTRACT

Medical device-associated osteomyelitis is a complication in orthopedic surgery, and often caused by *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. There is an urgent need for the discovery of biodegradable therapies with bone restoring properties, which can additionally hamper biofilms development. Bisphosphonates inhibit osteoclastic bone resorption and are used e.g. in the treatment of osteoporosis. In turn, bioactive glass is a biodegradable material, which is used to resolve infection-induced bone defects due to its anti-biofilm and osteostimulative properties. Combining bioactive glass with bisphosphonates has attracted interest due to their synergistic effects on improved bone formation in orthopedic and dental applications. Our previous study showed that the addition of bisphosphonates (alendronate, etidronate, risedronate and zoledronate) improves the anti-biofilm effect of bioactive glass (S53P4; BAG) against periodontal biofilms (*Aggregatibacter actinomycetemcomitans*). In the present study, we studied the anti-biofilm effects of these bisphosphonate-BAG combinations on *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms as a plausible therapeutic alternative for the treatment of medical device-associated osteomyelitis. It was demonstrated that alendronate, etidronate and risedronate displayed anti-biofilm activity either when used alone or in combination with BAG. Overall, these results support a promising role of bisphosphonates in managing medical device-associated osteomyelitis, which it is worth exploring further.

1. Introduction

Biofilms are defined as *"aggregated, often sessile bacteria, which differ from free floating cells by slow growth and tolerance to antibiotics and immune cells"* [1]. Biofilms caused by staphylococci are frequent causes of medical device-associated osteomyelitis. *Staphylococcus aureus* is usually responsible for early post-interventional prosthetic joint infections, while chronic infection forms are frequently caused by *Staphylococcus epidermidis* [2–4]. These device-associated infections are in many cases impossible to eradicate with regular systemically administered antibiotics. Currently applied treatment protocol requires debridement of the infected tissue and revision of the infected device either in one stage or two stages. Between the two stages, surgical implantation of antibiotics embedded in polymethyl methacrylate (PMMA) spacers can be used. Still, these spacers are not biodegradable and need to be subsequently removed with revision surgery. They can also promote biofilm formation on them causing secondary infections [5,6]. The re-surgeries and prolonged hospitalization periods are responsible for a huge economic burden in addition to the decreased quality of life of the patients [7]. Moreover, inadequate antibiotic release kinetics from PMMA induce emergence of multi-resistant bacterial strains, hindering the usability of this kind of local antibiotic therapy [8]. Thus, there is a major need for the discovery of biodegradable and bone-restoring therapies with biofilm formation-disallowing features.

Combining bisphosphonates with bioactive glass has gained interest due to their synergistic improved bone formation in orthopedic [9,10] as well as dental applications [11]. Bioactive glasses (synthetic silica-based materials) are often used topically to resolve infection-induced bone defects due to their anti-biofilm [7,12] and

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osteoproductive properties [13]. Bioactive glass S53P4 (BAG), used in this publication, is composed by weight SiO_2 53%, Na_2O 23%, CaO 20%, P_2O_5 4% and is indicated in the treatment of bone defects caused by bone tumors, trauma and chronic infections, e.g. osteomyelitis and chronic sinusitis [14]. The completely divergent bactericidal mechanism (elevated pH and osmotic pressure, which generate hostile environment for bacteria) compared to regular antibiotics makes bioactive glasses a novel choice for managing antibiotic resistance [15]. In turn, bisphosphonates inhibit osteoclastic bone resorption and are used e.g. in the treatment of osteoporosis [16]. Previously, we found that the addition of bisphosphonates (alendronate, etidronate, risedronate and zoledronate) ameliorates the anti-biofilm effect of bioactive glass (S53P4) against periodontal biofilms (*Aggregatibacter actinomycetemcomitans*) [17]. In this study, we aim to further study the anti-biofilm effects of these bisphosphonate-bioactive glass combinations on *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms as a potential therapeutic approach for the treatment of prosthetic joint infections.

2. Materials and methods

2.1. Materials

Tryptic soy agar (TSA), tryptic soy broth (TSB), Tween® 20, etidronic acid monohydrate and vancomycin hydrochloride hydrate were purchased from Sigma-Aldrich (Steinheim, Germany). Zoledronic acid monohydrate was acquired from Kemprotec Limited (Carnforth, UK). Risedronic acid monohydrate was from AK Scientific (Union City, CA, USA), while alendronate sodium trihydrate was obtained from Cayman Chemical Company (Ann Arbor, MI, USA). Clodronate disodium tetrahydrate was produced by PharmaZell GmbH (Raubling, Germany). Bioactive glass S53P4 (BAG; granule size: 500–800 μm) was provided by BonAlive Biomaterials Ltd. (Turku, Finland). Inert glass beads (IG; particle size: 230–320 μm) were acquired from Jencons Ltd. (Bedfordshire, UK).

2.2. Methods

2.2.1. Implant-based biofilm model using the Static Biofilm assay

The bisphosphonate-BAG combinations were investigated in an implant-based biofilm model (Fig. 1), which was optimized here. This model is based on the Static Biofilm method, (originally presented in Ref. [18] that has been further developed by our research group in Refs. [19] and [17]. The general protocol of the Static Biofilm method was performed as follows: first a sterile Whatman filter paper (70 mm-diameter, qualitative grade 2, GE Healthcare, Little Chalfont, UK) was placed on a tryptic soy agar (TSA) plate. For biofilm formation, the pre-culture of methicillin-sensitive *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* Newman or *Staphylococcus epidermidis* RP62A ATCC 35984 (1 μL of colonies suspended into 100 mL of TSB); grown under aerobic conditions at 37 °C with 220 rpm for 20 h) was diluted 1:10 in TSB in order to contain approximately 10^8 CFU mL^{-1} bacteria. The filter paper (facilitates bacterial suspension to distribute evenly) was inoculated with 1.5 mL of the bacterial dilution. This was followed by addition of sterile coupons, and this assembly was incubated under humidified aerobic conditions at 37 °C for 24 or 48 h. After the incubation the biofilms (usually formed underside of the coupons) were disaggregated for quantification. First, the coupons were transferred into Falcon tubes of 50 mL containing 1 mL of 0.5% (w/v) Tween® 20-TSB solution. After that, the tubes were sonicated in a water bath in Ultrasonic Cleaner 3800 (Branson Ultrasonics, Danbury, CT, USA) at 25 °C, for 5 min at 35 kHz. The tubes were mixed vigorously for 20 s with Vortex mixer SA8 (Stuart, Stone, UK) prior to and after the sonication step. Serial dilutions were performed from the resulting bacterial suspensions, and plated on TSAs. The bacterial attachment on coupons is expressed on a $\log_{10}$ scale and the anti-bacterial effect of the samples are expressed as logarithmic reduction (logR) of the bacterial burden [20].

$$logR = log_{10}\langle (CFU/ml)_{control} \rangle - log_{10}\langle (CFU/ml)_{compound} \rangle$$

where $\langle \cdot \rangle$ denotes averaging over samples.

2.2.2. Selecting suitable biofilm formation substrate and time

Before performing the susceptibility trials, an appropriate biofilm formation time and a suitable substrate were determined. Substrates tested included coupons made of borosilicate glass, plexi glass, titanium (dimensions: 0.4 cm height, 1.27 cm diameter) and hydroxyapatite (dimensions: 0.25 cm height, 1.27 cm diameter) (BioSurface Technologies Corp., Bozeman, MT, USA). The appropriate formation time was determined by comparing two incubation times: 24 and 48 h (48-h-old biofilms were remoistened with 1.5 mL of 10-fold diluted TSB halfway of the incubation period).

2.2.3. Exploring anti-biofilm effects of bisphosphonate-BAG and bisphosphonate-IG combinations

The combination samples were prepared with a mixture ratio (1:10) similar to the one used for clodronate-BAG in Ref. [11]. In a similar manner to [11], excessive saline was let to evaporate from the samples on a filter paper to obtain a semisolid paste. First, in order to investigate the anti-biofilm effects of bisphosphonate-BAG combinations 25 mg of bisphosphonate (alendronate, clodronate, etidronate, risedronate and zoledronate) or vancomycin (as a positive control) or inert glass beads (IG; as a negative control) and 250 mg of BAG in 225 μL of 0.9% (w/v) saline were mixed. Second, three most active selected bisphosphonates were assayed in terms of their intrinsic anti-biofilm activity. Herein, 25 mg of bisphosphonate (alendronate, etidronate or risedronate) or vancomycin (as a positive control) was combined with 250 mg of IG in 225 μL of 0.9% saline. A negative control composed of 275 mg of IG in 225 μL of 0.9% saline was included. The samples were then applied on coupon undersides. A second layer of filter papers (25 mm-diameter; one per coupon) was placed on the larger papers to assist in scraping of the compound samples into Falcon tubes in the disaggregation phase, like described in Ref. [17].

2.2.4. Data processing and statistical analysis

Data processing and statistical analysis was performed with Microsoft Excel 2013 software and GraphPad Software (Prism, version 5.0 for Mac). For paired comparisons of the data, an unpaired *t*-test with Welch's correlation was utilized. $p < 0.05$ was considered statistically significant. Experiments were performed at least in triplicates.

3. Results and discussion

To resemble the conditions of an infected implant (small amount of liquid, low shear stress) in a realistic manner, the Static Biofilm method was applied. This method enables testing of versatile substrates and can be used in any basic-equipped laboratory [19]. Moreover, it is a better alternative to the ASTM-standardized biofilm reactors, as those require vast amounts of compounds to be used (not typically available at the investigational stage) or to microtiter well plates, which are not suitable for testing combination samples (such as paste formulations).

Suitable biofilm-forming conditions were first studied on four substrate materials and two incubation times. The material tests included three clinically relevant materials (plexi glass, titanium