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A new concept of gentamicin loaded HAP/TCP bone substitute for prophylactic action: in vitro release validation

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Abstract Infections and their consequences are a considerable problem in orthopaedic surgery. Despite intravenous prophylactic antibiotic administration, infection rates can reach in some occasions more than 1%. Indeed, the concentration in bone tissues is very low with the majority of antibiotics. Because high local dose can be obtained, the local release of gentamicin from acrylic bone cements has been shown to be efficient in preventing infections. However, for surgical procedures other than cemented prostheses no other local antibiotic releasing device is clinically available. The purpose of this study was to validate the concept of a gentamicin loaded bone substitute. About 125 mg of gentamicin were introduced into a HAP/TCP bone substitute for prophylactic purpose, to enhance the efficiency of systemic antibiotic treatments. The release rate of gentamicin from the bone substitute was investigated in vitro, in 0.9% sodium chloride solution. The rate appeared to be related to the bone substitute volume. All the gentamicin was released in less than 48 h. This release rate corresponds to the recommendations for the prophylactic use of antibiotics: the duration of the treatment should be less than 48 h, not to select antibiotic-resistant bacterial strains.

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Introduction

Despite advances in prophylaxis against infection, post-operative osteomyelitis remains a considerable problem in orthopaedic surgery. For infections on prostheses, removal of the implant is often necessary and usually leads to severe functional disability. Such infections are very costly in terms of quality of life and public health expenditure.

In order to reduce the risk of infection, prophylaxis is carried out by intravenous injection of an antibiotic 1 h before surgery. The aim is that the patient tissues will be saturated with antibiotic from the first incision to the end of the surgery. However, bone is poorly vascularised and surgery disturbs the vascularisation of the operated site. As the antibiotic is driven by blood after intramuscular or intravenous injection, the local concentration in bone is low and bacteria can proliferate. Systemic antibiotics should be administrated in high concentrations for pro-

longed periods of time to yield adequate concentrations within bone tissues, but such high concentrations of drugs in blood for a long period may induce toxicity [1]. Furthermore, for long treatments, antibiotic-resistant microbial strains can emerge because of selective pressure induced by antibiotics. Such resistant-strains are a major problem in hospitals where antibiotics are widely used, because they may lead to catastrophic therapeutic dead ends.

Local delivery of antibiotics has the advantage of achieving high local levels of the drug with low risk of systemic toxicity. The only available commercial medical device able to achieve a local release of antibiotic is polymethylmethacrylate (PMMA) bone cement [2]. In 1970, Buchholz and Engelbrecht were the first authors to propose to add antibiotics to PMMA bone cement for prophylactic purposes. Today, the antibiotic most commonly incorporated is gentamicin. According to a review of 22,170 primary cemented total HIP replacements (reported to the Norwegian arthroplasty register from 1987 to 2001), Espehaug et al. [3] demonstrated the interest of this approach: infection rate was 0.4% when systemic antibiotic was combined to the use of gentamicin loaded cement whereas it was 0.7% with systemic antibiotic only. However the antibiotic loaded bone cement technology is perfectible. More than 90% of the antibiotic may be retained within the PMMA matrix [2, 4]. The remaining antibiotic generates a slow release at low levels during many months [1] and should select antibiotic-resistant strains [2].

Antibiotic loaded bone cements cannot be used when hydroxyapatite coated prostheses are used or when surgical procedures different from prosthesis setting are archived. For these procedures no local antibiotic delivery system is clinically available. For bone repair surgery, biphasic calcium phosphate bone substitutes processed from hydroxyapatite (HAP) and β tricalcium phosphate (TCP) are considered as the most promising alternative to autologous bone grafts [5]. They are now widely used in orthopaedic surgery and would be a useful scaffold for local delivery of antibiotics. In this study, gentamicin was introduced into the interconnected porosity of a commercial HAP/TCP bone substitute. In order to validate the concept of using a bone substitute to release an antibiotic in a prophylactic purpose, the release rate was investigated in vitro, in NaCl solution. The release duration should be less than 48 h, not to select antibiotic resistant bacterial strains.

Materials and methods

HAP/TCP bone substitute with gentamicin

A commercial bone substitute composed of 70% hydroxyapatite and 30% β-tricalcium phosphate (ATLANTIK, Medical Biomat, France) was used in this study. This biomaterial was manufactured by slip casting of slurry containing porogen particles, drying, debinding and sintering. The compromise between porosity (necessary for bone ingrowth) and mechanical strength (necessary for the workability of the implant) was investigated in a previous study [6]. The size and volume ratio of porogen particles was optimised to obtain an interconnected porosity with macro-interconnections of 15 µm, suitable for penetration of osteoblasts in the implant. The final bone substitute used in this study had a high ratio of porosity (70%) composed of macro-porosity (300–600 µm in size) and micro-porosity (1–2 µm in size). Its compressive strength was 10 MPa. After sintering, machining and cleaning of the bone substitutes, gentamicin was incorporated in the porosity by impregnation with a sterile-water solution of gentamicin sulphate (sterilised water for injection, Aguettant, France). The implants were then dried at low temperature in order to evaporate the sterile water. At last, the dry bone substitutes were packaged and sterilised (gamma sterilisation between 25 kGy and 40 kGy, Ionisos, France). Gentamicin sulphate and sterile water which conform to the European pharmacopoeia were used in this study [7, 8]. As different sizes of bone substitutes are used in orthopaedic surgery, the gentamicin release rate was studied on blocks of different shapes: parallelepipeds of minimum size $10 \times 5 \times 5$ mm or maximum size $50 \times 30 \times 15$ mm and cylinders of medium size Ø10H15 mm.

SEM observations

Observations of the porosity of the gentamicin loaded bone substitutes were performed with a Jeol 840 ALGS scanning electron microscope (SEM).

Gentamicin dose control

The amount of gentamicin sulphate loaded in bone substitutes was measured by weighing the bone substitutes before and after incorporation. The amount of active gentamicin was calculated as follows:

$$M_{\text{Gentamicin}} = 0.607 \times M_{\text{Gentamicin sulphate}}$$

with 0.607 being the ratio of active gentamicin versus gentamicin sulphate (i.e. the potency in I.U./µg), measured on the gentamicin sulphate powder by microbiological assay, according to the European Pharmacopoeia method [9].

The potency of the gentamicin in the bone substitutes at the end of the manufacturing process was investigated by microbiological assay after elution of gentamicin out of the biomaterial. The bone substitutes with gentamicin were immersed in 100 mL of 0.9% sodium chloride solution

(Aguettant, Lyon, France), in a flask thermostated at 37 ± 1 °C on a plate agitator regulated at 80 rpm. After 72 h, titration was performed by microbiological assay, according to the European pharmacopoeia method [9].

In vitro characterisation of the release rate

In vitro release of gentamicin from bone substitutes was carried out at 37 ± 1 °C. The bone substitutes were introduced in 100 mL of 0.9% sodium chloride solution (Aguettant) and placed on plate agitator at 80 rpm. As the solution was not renewed, and as the gentamicin concentration in the solution can influence the release rate, the volume of solution was chosen high enough for the final gentamicin concentration being negligible. About 500 µL of the release medium were collected at predetermined time intervals (0, 1, 4, 8, 12, 24, 48, 72 h). The gentamicin concentration of each sample was determined after dilution by using the COBAS INTEGRA fluorescence polarization system (Roche) [10]: the samples were incubated with a mouse monoclonal antibody, and then a tracer reagent was added. The light emission, which was proportional to the gentamicin concentration, was measured at 515 nm. The detection level for gentamicin was 0.14–10 µg/mL. The gentamicin concentrations obtained with the COBAS INTEGRA system were compared with an excellent correlation to those obtained using microbiological assay, according to the European Pharmacopoeia method [9].

Results

Physicochemical characteristics of HAP/TCP/Genta bone substitutes

Previous studies on the bone substitute without gentamicin [6] demonstrated that the biomaterial contained two types of porosities: macroporosities (300–600 µm size) and microporosities (1–2 µm size). In the gentamicin loaded bone substitutes, macroporosities are observed (Fig. 1) but microporosities are heterogeneously distributed: some areas are microporous whereas other areas seem to be dense (Fig. 1). It has been assumed that those later are filled with gentamicin. It has been confirmed by comparing the observations at the same magnification of the same implant before (Fig. 2a) and after releasing for 48 h in NaCl solution (Fig. 2b): the gentamicin present in the microporosities (Fig. 2a) has been eluted and the microporosities appear empty in Fig. 2b.

Control of gentamicin dose

The gentamicin amount in bone substitutes was controlled during the manufacturing process by weighing of the bone substitutes before and after incorporation of gentamicin in order to reach the specification: 125 ± 25 mg of gentamicin base per implant. The average weighting result on 20 samples (10 × 5 × 5 mm) was 109 mg of gentamicin base per implant with a standard deviation of 1.5 mg.

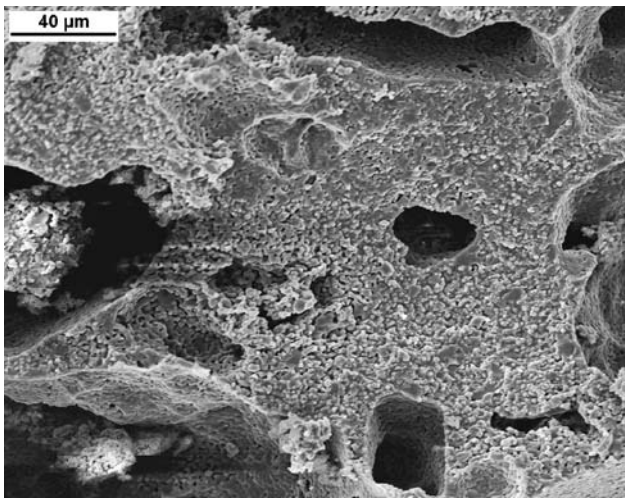

Fig. 1 SEM observation of the porosity of a gentamicin loaded bone substitute: macroporosity is free of gentamicin, whereas some microporosities are filled with gentamicin

Microbiological assay has been carried out on a sterilised bone substitute taken randomly in the same series of 20 samples. The microbiological assay result is 114 mg of gentamicin base. The precision of the microbiological assay being 5%, this result is coherent with the results obtained by the weighting method.

Release rate and microbiological assay of gentamicin

Figure3 shows that, for all shapes of bone substitute (50 × 30 × 15 mm, Ø10H15 mm and 10 × 5 × 5 mm), the concentration in the liquid increases until reaching a plate indicating the end of the release of gentamicin from the bone substitute.

The rates depend on the dimension of the bone substitute: the release durations for blocks of large size (50 × 30 × 15 mm), medium size (Ø10H15 mm) and small size (10 × 5 × 5 mm) are respectively 48, 24 and 6 h. It has been assumed that the release rate is governed by diffusion of the gentamicin through the porosities of the biomaterial and is described by the classical diffusion law:

$$X \propto \sqrt{D.t} \quad (1)$$

X being the distance of diffusion, D the diffusion coefficient and t the time.

For the samples tested, the release duration (t_{max}) corresponds to the diffusion distance of gentamicin from the

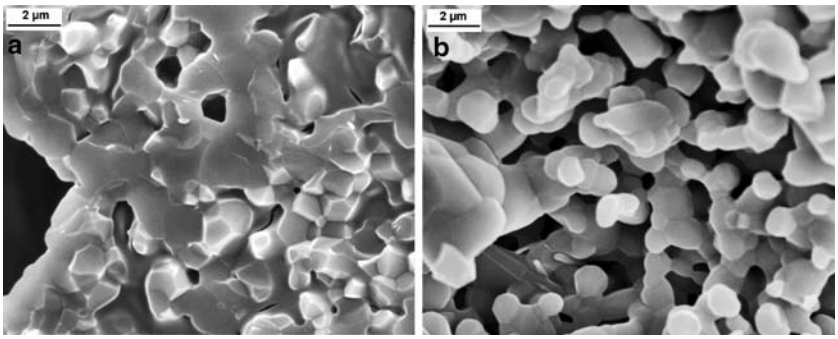

Fig. 2 SEM observation of the microporosity of a gentamicin loaded bone substitute: (**a**) microporosity is filled with gentamicin; (**b**) microporosity is empty after in vitro release during 48 h

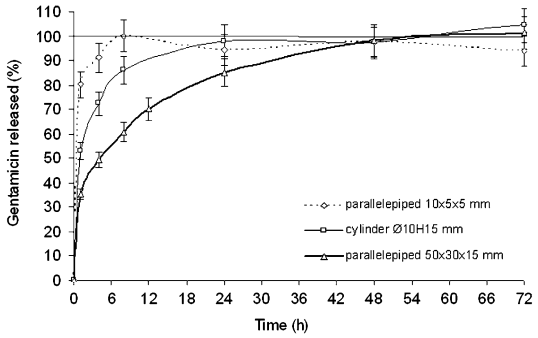

Fig. 3 In vitro release curves of gentamicin from bone substitute blocks of different shapes. The uncertainty was calculated from the uncertainties of dilution and titration

centre of the sample to the external surface (X_{max}). For $50 \times 30 \times 15$ mm, Ø10H15 mm and $10 \times 5 \times 5$ mm blocks, this distance X_{max} is respectively 7.5, 5 and 2.5 mm. Figure4, the relation between X_{max} and $\sqrt{t_{max}}$ is linear with a regression straight line passing close to the origin and a linear regression coefficient R^2 close to 1 :

$$X_{max}(m) = 0.00002\sqrt{t_{max}(s)} - 0.0003 \quad (2)$$

This result confirms that gentamicin release kinetic is governed by diffusion mechanisms through the porosity of the biomaterials.

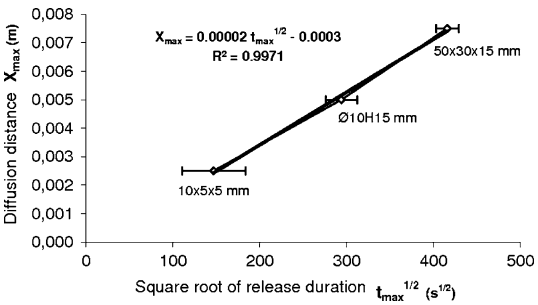

Fig. 4 Diffusion distance from the centre of the implants to the surface, versus the square root of the release time. The uncertainty on the evaluation of the release duration from Fig. 3 was 3 h

Discussion

The majority of the previous published data on antibiotic-loaded bone substitutes focuses on therapeutic applications, i.e. osteomyelitis. The treatment of such infections in a bone site requires large amounts of antibiotic for at least 10 days and sometimes several months. The antibiotic must specifically fit the bacteria responsible for the infection, identified beforehand. To delay the release of the antibiotic from the bone substitute, different solutions have been presented by authors: embedding the antibiotic in a polymer [11–13], encapsulating the antibiotic in the porosities of a poorly interconnected ceramic matrix [14–17], or increasing the specific surface area of the bone substitute [18]. But several problems should be taken into account: (i) the addition of polymer should not penalize bone ingrowth by closing porosities or by covering the HAP/TCP with a non-osteoconductive material; (ii) the main function of the bone substitute should remain bone healing and porosity should thus be interconnected; (iii) the volume of a bone substitute is not sufficient to entrap the dose of antibiotic necessary to reach efficient concentrations during a 10 days treatment in vivo. In the present study, the release duration for prophylactic application should be less than 48 h: the structure of the porosity of the bone substitute and its composition, optimized for bone ingrowth, were not modified to incorporate the antibiotic.

Gentamicin was chosen to be incorporated in bone substitutes because it is a broad spectrum antibiotic which is effective against most of the bacteria responsible for infections in orthopaedic surgery. Furthermore, gentamicin is a dose-dependant antibiotic: efficacy is related to the concentration in contact with bacteria, which should be very high in the case of local release.

The result of the microbiological assay with the gentamicin-loaded bone substitutes was 114 mg of gentamicin base per implant: dissolution, drying and gamma sterilisation did not influence the potency of the gentamicin used. The usual therapeutic dose of gentamicin is 3 mg/kg/day which corresponds to a dose of 150 mg for a patient weighing 50 kg. For antibiotic systemic prophylaxis, high

doses are recommended, usually equal to the therapeutic dose. The local release of the dose incorporated in the bone substitute should lead to high local gentamicin concentrations.

According to SEM observations of the bone substitute, gentamicin is stored in the microporosities of the biomaterial, while the macroporosities remain free. This can be explained by the incorporation technique used. During the impregnation of the bone substitute with the gentamicin solution, the solution penetrates by capillarity in macroporosities and microporosities. During the drying step, the liquid evaporates gradually. As the capillary pressure is higher in microporosities, evaporation begins in macroporosities and finishes in microporosities, leaving gentamicin in dry form in the latter.

The release duration was related to the diffusion distance of gentamicin from the centre of the implant to the external surface with a classical diffusion law. The lower the size of the bone substitute, the faster the release rate. From these results, it can be assumed that release proceeds in three steps:

(1) The liquid diffuses quickly in the bone substitute through the macroporosities which are interconnected and remain free of gentamicin.
(2) The most accessible gentamicin, close to the surface of the implant, diffuses out of the ceramic matrix immediately after immersion. As the majority of the gentamicin is close to the surface, the initial release rate is high.
(3) The diffusion of gentamicin which is in the centre of the bone substitute is driven by the diffusion rate through the bone substitute porosities. It can be assumed that the diffusion coefficient, determined in Eq. 2, depends on the characteristics of the porosity, especially the interconnectivity.

For all samples, the release duration was less than 48 h. This delay is the maximum duration recommended for prophylactic treatments in order to avoid selecting antibiotic-resistant bacteria.

Conclusion

The 125 ± 25 mg of gentamicin incorporated in the HAP/TCP bone substitute in this study were mainly stored in dry form in the micro-porosities of the biomaterial. This amount, close to the standard therapeutic dose (3 mg/kg/day), was released in less than 48 h. This rapid release combined with the high gentamicin dose should lead to high local concentrations in vivo. Such concentrations should be more effective against bacteria than the usual intravenous injections which generate weak concentrations in bone tissues. The release rate observed is compatible with the recommendations for antibiotic prophylaxis: high dose but limited in time, not to select antibiotic-resistant bacteria.

In a forthcoming study, the release rates observed in vitro will be compared to the results observed in vivo after implantation in bone tissue in sheep model.

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