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Biodegradable intraoperative system for bone infection treatment II. In vivo evaluation

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Abstract

A biodegradable implant containing gentamicin sulphate for the prevention or the treatment of bone infections was designed by using, as the polymer material, sodium alginate containing a high proportion of mannuronic sequences capable of forming a complex with the drug. By a crosslinking procedure with calcium ions, insoluble but biodegradable calcium alginate spheres were obtained and formed into a chain by means a surgical wire. To evaluate the implant effectiveness, the implant was inserted at the femur level of Wistar rats and gentamicin levels in plasma, bone and soft tissues were detected by microbiological assay. The gentamicin concentrations were found to be sufficiently high to control pathogens for at least 30 and 7 days in the bone and soft tissue, respectively, whereas plasma levels were low and detectable for only 1 day. The complete implant bioabsorption occurred within 8–10 days after implantation and no signs of rejection or inflammatory reactions were observed at the level of the surrounding tissues.

Keywords: Alginate; Biodegradability; Calcium; Gentamicin; Implant; Osteomyelitis

1. Introduction

Bone infection (osteomyelitis) is a serious bone disease caused by bacteria—usually *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Norden, 1971; Norden and Keleti, 1980)—directly contaminating the bone from an exogenous source such as open bone fractures or surgical proce dures. The treatment of the chronic osteomyelitis by irrigation suction dranage and systemic antibiotic therapy is often ineffective, though the dose of antibiotic was high, owing to the limited blood circulation to the bone tissue (Kahn and

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Pritzker, 1973; Norden, 1975; Glover and Geddes, 1981). Thus, a local antibiotic delivery system administered per-operatively represents an advantageous approach for the treatment as well as the prevention of bone infections. In fact, high local tissue antibiotic concentrations can be obtained without corresponding high toxic blood levels (Wahlig, 1982).

Recently, spherical, nonbiodegradable implants releasing antibiotic have been fabricated (Wahlig, 1982). However, nonbioabsorbable materials have the disadvantage of needing to be surgically removed after drug exhaustion. Besides, they determine incomplete and very slow drug release (Robinson and Sampath,. 1989). Therefore, more recently, a number of authors have attempted to incorporate antibiotics in bioabsorbable materials, e.g. poly-lactic acid (Wei et al., 1991; Sampath et al., 1992; Mauduit et al., 1993; Zhang et al., 1994) and ceramic composites usually used as a filling material for bone defects (Thoma et al., 1992; Gerhart et al., 1993). However, poly-lactic acid devices could contain residual organic solvents used in the preparation (Mauduit et al., 1993) and ceramic materials are generally very brittle to handle. Therefore, we propose calcium alginate as an alternative polymeric carrier for the development of an implant releasing an antibiotic to prevent or treat bone infections. Alginates are known to be biocompatible and biodegradable (Kronenthal, 1975). Moreover, they can be sterilized without decrease of molecular weight (Handbook of Pharmaceutical Excipients, 1986), have hemostatic properties (McDowell, 1986) which are responsible for sustention of the drug release from implants (Firsov et al., 1987), do not need organic solvents and have a low cost. The crosslinking reaction of alginates with calcium ions, which we referred to in Part I of the study by Iannuccelli et al. (1996) lead to water insoluble, but biodegradable devices which are mechanically strong and easy to handle. Besides, calcium alginate implants could act as a source of calcium, useful in repairing the bone defects generally combined with the infection.

The purpose of this paper is to report upon gentamicin levels in plasma, bone and soft tissues after implantation of calcium alginate spheres containing gentamicin sulphate as well as upon biodegradation and local tolerance of the implant.

An alginate with high mannuronic acid content was used for the preparation of the implant because the greater association capacities of the drug with the polymer, as reported in Part I of the study by Iannuccelli et al. (1996). Gentamicin was selected as the drug because it is a broad-spectrum antibiotic (Weinstein et al., 1963), active against osteomyelitis pathogens, widely used in the systemic therapy of the osteomyelitis and effective in low doses. Also gentamicin is stable in various pharmaceutical dosage forms and in biological systems (Rosenkrantz et al., 1980).

2. Materials and methods

2.1. Materials

The following chemicals were obtained from commercial suppliers and used without further purification. Sodium alginate manucol DM (MW about 147 000, extracted from Laminaria Digitata, containing 62% mannuronic acid and 38% guluronic acid) was donated by Kelco International (Bagnolet Cedex, France). Gentamicin sulphate (700 μg activity per mg) and calcium chloride dihydrate were purchased from Fluka Chemie (Buchs, Switzerland). Tween 20 (polyoxyethylensorbitan monolaurate) was purchased from Atlas Europol (Ternate, Italy). Sodium phosphate monobasic, potassium phosphate monobasic, sodium citrate, sodium hydrate, sodium chloride and all the solvents (analytical grade) were purchased from Carlo Erba (Milan, Italy). For the microbiological assay, tryptic-soy agar was purchased from Difco (Milan, Italy) and *Bacillus subtilis* ATCC 6633 from ISM S. Belfonti (Milan, Italy).

2.2. Methods

2.2.1. Preparation of the implant

The implant was formed by a chain of ten spheres of calcium alginate containing the drug.

The spheres were obtained using a technique described in Part I of the study by Iannuccelli et al. (1996). The spheres, in the hydrate state, i.e. before the drying process, were joined on a surgical nonabsorbable wire (butylester, 5-0 Novafil, Davis Geck, USA) by means of a needle and then dried under vacuum for at least 48 h.

2.2.2. Dimensional and morphological analysis

The morphology of the spheres was examined by scanning electron microscope (SEM) (XL-40, Philips, Eindhoven, The Netherlands). The mean size was determined by optical microscope (Carl Zeiss, Jena, Germany) on four spheres from four different batches.

2.2.3. Element analysis

The energy dispersive X-ray (EDS) analysis was used to evaluate the presence of sulphur atoms in the spheres. Sphere sections were carbonated (model CED 010, Balzers Uflnion, Liechtenstein) and analyzed by EDS analysis (EDAX 9900, Edax International, Prairie View, IL, USA) coupled with a SEM.

The emission map of S (2.31 keV) was obtained with the following experimental settings: 100×128 pixels; dwell time 200 ms; accelerating voltage 25 kV; detection limit about 0.3%.

2.2.4. Behaviour of the spheres in isotonic phosphate buffer

Dynamic swelling and loss of mass integrity of the spheres were examined by placing dry spheres in phosphate buffered saline solution of pH 7.4. The changes of the dry sphere diameter as a function of the time (d_t/d_0) were measured and the erosion phenomena were observed by optical microscope. The data are averaged on six determinations.

2.2.5. In vivo study

Wistar rats (200–250 g) were used for the animal studies. The implant, containing 3.8 ± 0.6 mg of gentamicin sulphate (about 85% associated to the polymer) and 7.1 ± 0.2 mg of calcium (about 55% associated to the polymer) as reported previously (Iannuccelli et al., 1996), was inserted through a small incision around the diaphiseal portion of each femur of twenty rats anaesthetized with ketamine (20 mg/kg). The animals were kept individually in cages and fed on a standard diet. Separate groups consisting of two rats per group were killed at post implantation time intervals of 12 h, 24 h, 3, 4, 7, 10, 14, 21 and 30 days.

Blood was collected just before death from the femural artery into EDTA tubes and immediately centrifugated. Each implant (or fragments of implant), femur and a specimen of soft tissue were removed from the implantation zone. The removed hydratated implant was observed by optical microscopy in order to determine the changes of the sphere initial diameter as a function of the time (d_t/d_0). The dried spheres (or their fragments) were observed by SEM to evaluate the biodegradation process.

The gentamicin concentrations were assayed in the implant, in the plasma, in the bone and in the soft tissue. The dried implant (or its fragments) was weighed and dissolved in 3% w/v sodium citrate solution. The bone, cleaned of all soft tissue and weighed exactly was frozen, crushed to a fine powder and suspended in 5 ml of NaOH (1 N). The soft tissue, exactly weighted, was homogenized in 5 ml of NaOH (1 N). It was demonstrated that NaOH treatment resulted in total recovery of aminoglycosides from animal tissues (Fox, 1989).

All the samples prepared as above along with the plasma were assayed for gentamicin concentration by the agar diffusion method using 10^5 cfu of *Bacillus subtilis* ATCC 6633 as the standard strain. Standard curves relating the size of the inhibition zones to known concentrations of gentamicin sulphate in NaOH (1 N) (plus tissues from untreated rats) for bone and soft tissues, in saline for plasma and in sodium citrate solutions for the implant were constructed.

The amount of antibiotic was expressed in μg/g of bone and soft tissues, in μg/mg of implant and in μg/ml of plasma. The minimal amount of gentamicin detectable was 0.2 μg/ml.

3. Results and discussion

3.1. Characterization of the spheres

The spheres forming the implant had a diameter of 2.2 ± 0.3 mm and a weight of 7.2 ± 0.9 mg. The scanning electron micrographs showed spheres with a nearly spherical shape, a rough surface (Fig. 1(a)) and a quite compact internal structure (Fig. 1(b)).

The drug contained in the spheres was homogeneously distributed, as indicated by the homogeneous distribution of the S atoms of the gentamicin sulphate molecules (Fig. 2(a)).

(a)

(b)

Fig. 1. Scanning electron photomicrographs of (a) a sphere and (b) a sphere cross-section.

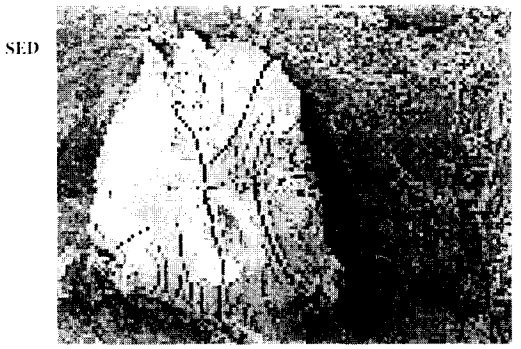

Fig. 2. Energy dispersive X-ray analysis (EDS) of a sphere cross-section: sulphur atom map (SK); scanning electron photomicrograph (SED).

To study the in vitro degradation of the spheres, an isotonic phosphate buffer of pH 7.4 was used in order to reproduce the calcium displacement process by non-crosslinking ions present in biological fluids. This process is thought to be the mean responsible of the biodegradation of alginates by biological environments, whereas the enzymic degradation process (β-elimination reaction), which leads to unsaturated uronic acid derivatives, is demonstrated to be slow at this pH value (Haug et al., 1963). The study revealed a first stage of hydration of the spheres with a maximum swelling value (d_t/d_0) of about 2.0 and, after about 1 h, a second stage of degradation with detachment of small fragments.

Fig. 3. Calcium alginate implant before drying (upper part) and after drying (lower part).

3.2. *In vivo investigations of the implant*

The implant was formed by a chain of ten spheres on a nonabsorbable surgical wire (Fig. 3) and inserted around the rat femur (Fig. 4). A nonabsorbable surgical wire was used to allow the removal of the implant from the implantation site during the animal experiments.

The biodegradation process of the implant, much slower than the in vitro degradation, underwent three stages. The first one (0–12 h) was the

Fig. 4. Calcium alginate implant at insertion site before wound closure.

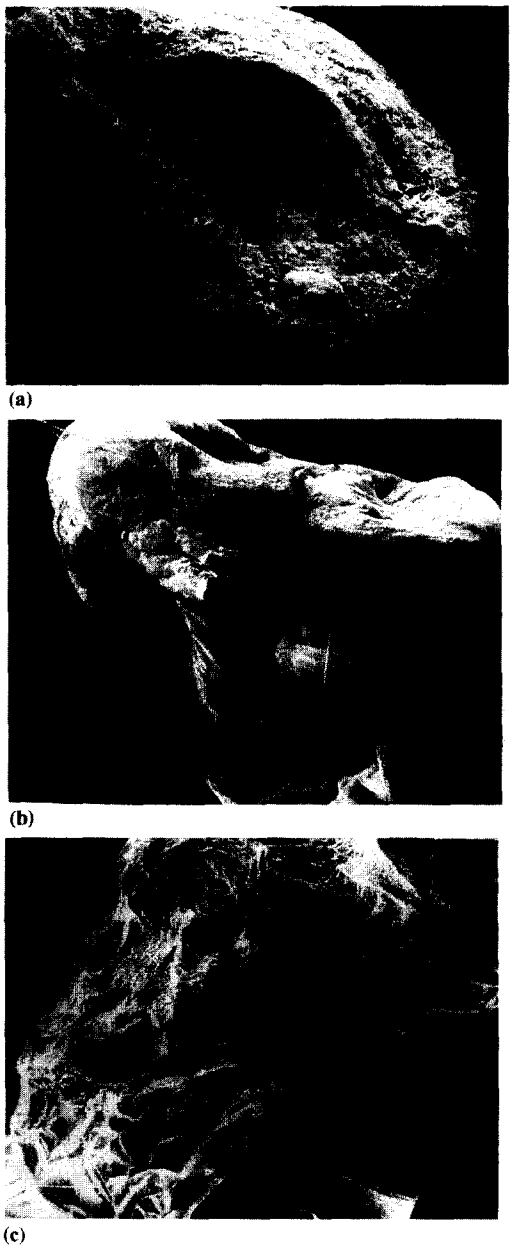

Fig. 5. Partially degraded implant removed from the insertion site at (a) 12 h, (b) 1 day and (c) 7 days post implantation.

hydration coupled with a partial crosslinking bonds cleavage. The hydration process led to a maximum swelling extent ($d_t/d_0 \approx 2.5$) at 12 h post implantation. Furthermore, the spheres removed at 12 h and dried showed a different shape and a

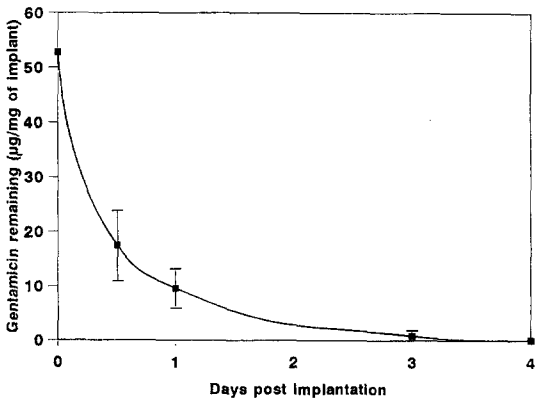

Fig. 6. Amounts of gentamicin sulphate remaining unreleased in the implant plotted against implantation time.

larger size in comparison with the initial ones (Fig. 5(a)). This fact could indicate a less restricted polymeric structure with a reduced strenght owing to partially broken crosslinking bonds. In this first stage of degradation the mass loss was slight (about 10%) and it could be attributed to both a surface erosion process (Fig. 5(a)) and the drug diffusion. In the second stage of degradation (12 h–3 days) the swollen spheres appeared still entire, but the mass loss process was more relevant (about 40% for all the period) (Fig. 5(b)). The mass loss could be reasonably attributed to the dissolution of soluble polymer fragments originated by the calcium displacement process. The third stage of degradation (3–7 days) involved the physical disruption of the spheres in small fragments which, after drying, appeared as thin slabs (Fig. 5(c)). The complete removal of the implant from the body tissues may be considered to happen within 8–10 days after implantation.

The release of the drug from the implant is given in Fig. 6 showing the antibiotic remaining unreleased plotted against the implantation time. Most of the drug was delivered within 1 day and the remaining fraction gradually decreased with time until the fourth day. However, the period of retention of gentamicin by the tissues at the implantation site was much greater. In fact, in bone and soft tissues, gentamicin could be detected over a period of 30 and 21 days, respectively (Table 1). Moreover, the gentamicin concentrations were sufficiently high to control pathogens, i.e. they exceeded the minimal inhibitory concentration (MIC) for the same inoculum of *S. aureus* and of *P. aeruginosa* (Weinstein et al., 1963; Casals, 1983), for at least 30 days in the bone tissue and for 7 days in the soft tissue (Fig. 7). These in vivo results showed that the antibiotic stored up in the tissues longer than the period of its complete release from the implant. It could be a consequence of the tendency of the drug to accumulate in body tissues, particularly in the bone tissue where the blood flow is limited and it may be further reduced by the hemostatic properties of alginates (McDowell, 1986; Firsov et al., 1987).

Gentamicin plasma levels were low—the toxic level is above 10 to 12 μg/ml (Reynolds, 1993 — and they were detectable only until 24 h after

Table 1
Gentamicin sulphate concentrations in plasma, bone and soft tissues

Days post implantation	Plasma (μg/ml)	Bone tissue (μg/g)	Soft tissue(μg/g)
0.5	2.6 (0.2)	29.6 (8.7)	34.3 (3.9)
1	1.1 (0.4)	27.4 (6.3)	51.3 (25.6)
3	—	14.0 (3.7)	12.2 (7.0)
4	—	10.2 (2.9)	3.9 (2.5)
7	—	9.7 (4.0)	6.4 (4.7)
10	—	12.6 (5.0)	1.3 (0.5)
14	—	6.5 (2.6)	1.1 (0.8)
21	—	4.1 (2.5)	0.2 (0.2)
30	—	3.2 (0.9)	—

Mean values (± S.D.)

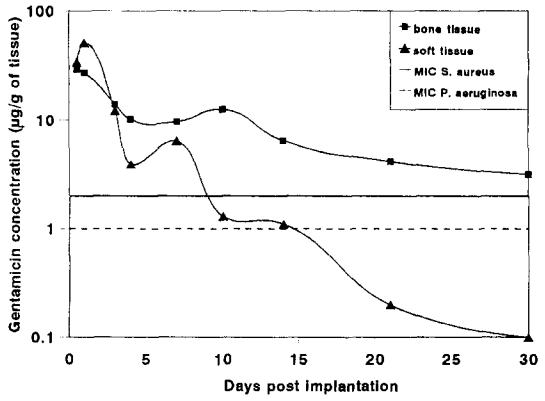

Fig. 7. Gentamicin sulphate concentrations in bone and soft tissue plotted against implantation time and minimal inhibitory concentration (MIC) for the same inoculum of *S. aureus* and of *P. aeruginosa*.

implantation (Table 1). Hence, it is expected that the risk of side effects is extremely small.

Visual observations did not reveal any sign of rejection or inflammatory reactions, indicating that the implant was well tolerated by the surrounding tissues.

4. Conclusions

Calcium alginate implants containing gentamicin provided effective antibiotic concentrations at the implantation site for a period longer than that considered sufficient to prevent or treat bone infections (10–14 days) (Wahlig, 1982; Mauduit et al., 1993).

The highest tissue levels of antibiotic were achieved within the first day, the most probable period for the infection development, and maintained for about 3 days without high systemic concentrations.

The nature of the polymeric material allowed a complete resorption of the implant and a good tolerance by the body tissues, besides, the technological feature of the implant could allow modification of the antibiotic dose by changing the number and the size of the spheres.

Therefore, such an implantable device could represent a useful and advantageous approach in the osteomyelitis management and it could be used by surgeons before wound closure to prevent or treat the infection.

Further investigations could be directed to establish the effectiveness of the implant in the presence of infection and the role of calcium in the osteogenesis process.

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