BIODEGRADABLE IMPLANTS OF GENTAMICIN SULFATE FOR EFFECTIVE MANAGEMENT OF OSTEOMYELITIS

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تهدف هذه الدراسة لتحضير وتقييم زرعات دواء كبريتات جنتاميسين العصوية الشكل والقابلة للتحليل الاحيائي باستخدام عديد كابرولاكتون. وقد تم تحضير الزرعات بطريقة مد الصهارة بنسب مختلفة من الدواء ومن عديد كابرولاكتون (1:1 ، 1:1.5 ، 1:2) وتقييم المحتوى الدوائي، وإطلاق الدواء معملياً، ودراسات الثباتية، والتقدير الأحيائي في الأرانب السليمة. وتبين أن الزرعات متناسقة في وزنها وقطرها وطولها ومحتواها الدوائي. أما في دراسات إطلاق الدواء معملياً، فقد أطلقت الزرعات الدواء بنمط ثنائي الطور مع إطلاق مبدئي مفاجئ متبوع بإطلاق بطيئ على مدار ثمانين يوماً. وفي دراسات الإطلاق الأحيائي، بقي تركيز دواء جنتاميسين في العظم فوق أدنى تركيز مثبط للمكورات العنقودية البرتقالية على مدار أربعة أسابيع. ولم يتغير محتوى الدواء في الزرعات في دراسات الثباتية بعد ستة أشهر من التثليج وحرارة خمس وعشرين درجة مئوية. وتوضح هذه الدراسة صلاحية زرعات جنتاميسين القابلة للتحلل الأحيائي في علاج اعتلالات العظام.

The purpose of the present study was to prepare and evaluate rod shaped biodegradable implants of gentamicin sulfate using poly(ε-caprolactone) (PCL). The implants were prepared by melt extrusion method with different ratios of drug and PCL (1:1, 1:1.5 and 1:2) and evaluated for drug content, *in vitro* drug release, stability studies and i*n vivo* evaluation in healthy rabbits. The implants were found to be uniform with respect to weight, diameter, length and drug content. In the *in vitro* drug release studies, implants released the drug in a biphasic pattern with an initial burst release followed by a slow release over a period of 80 days. In the *in vivo* experiment, the gentamicin concentration of bone was maintained above the minimum inhibitory concentration for *Staphylococcus aureus* over a period of 4 weeks. In the stability studies, the implants exhibited no change in the drug content after 6 months at refrigeration temperature and 25 °C. The present study reveals the applicability of biodegradable gentamicin implants to treat bone disorders.

Key words: Gentamicin, poly(caprolactone), biodegradable, bone implant

Introduction

Osteomyelitis is an inflammatory bone disease caused by microbial infection of the bone medullary cavity and / or cortex. Due to numerous reasons, present therapy for osteomyelitis still has deficits for successful treatment since systemic antibiotic delivery entails certain drawbacks such as systemic toxicity, poor penetration into ischemic tissues and hospitalization to monitor drug levels and effects. Surgical treatment such as bone grafting, debridement, irrigation, radial resection, etc. also lagged behind satisfactory results (1). Local antibiotic therapy has become an accepted and common adjunct to systemic antibiotics in osteomyelitis. Nonbiodegradable polymers such as polyethylene, poly(hydroxyethyl methacrylate) and ethylene-vinyl acetate co-polymers have been used for drug delivery and in the area of bone infections, poly(methylmethacrylate) is the forerunner (2). But

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these polymers are non-biodegradable necessitating second surgery for the removal of drug-depleted system (3). Recently biodegradable polymers have been used in drug delivery devices, which obviate the requirement of removing the drug depleted delivery devices. In the recent past, implants of gentamicin sulphate prepared by biodegradable polymers mainly polyanhydrides have been reported (4-12); but there are hardly any reports on the *in vivo* testing of the biodegradable implants of gentamicin sulphate. In our laboratory, biodegradable polymers like poly(ε-caprolactone), chitosan and poly(lactide-co-glycolide) have been investigated for the delivery of protein/peptidal drugs and to prolong the action of anticancer, contraceptives, antiinfective and antiinflammatory drugs (13-21). In the present work, implantable drug delivery system of gentamicin sulfate with poly(ε-caprolactone) for the treatment of osteomyelitis has been prepared and evaluated.

Experimental

Gentamicin sulfate was supplied as a gift from M/s Lyka Labs, India. Poly(ε-caprolactone) (Molecular weight 50000 to 60000) was purchased from Sigma Inc., USA. Ketamine hydrochloride Injection was purchased from Neon Laboratories Ltd., India. Bone wax was purchased from Ethicon Inc., USA. All other chemicals used were of analytical grade. Healthy male rabbits (Swiss Albino strain), weighing between 1 to 1.5 kg, were obtained from Central Animal House, Kasturba Medical College, Manipal.

Preparation of implants:

The melt extrusion method was employed to prepare rod shaped implants. Three different drug and polymer ratios (1:1, 1:1.5 and 1:2) were attempted. Poly(ε-caprolactone) was melted at 60 ºC over a water bath and gentamicin sulfate was thoroughly dispersed in the polymer melt. The melted mass was transferred into the barrel of a 10 ml disposable syringe and extruded out into a glass mold (10.25 cm length; 2.35 mm internal diameter) fixed on the tip of the syringe. The glass mold was then detached and allowed to cool at room temperature. The solidified rod was then pushed out from the glass mold with a glass plunger. The weight, length and diameter of the implants were determined. The implants obtained were cut into 1 cm pieces for further studies.

Sterilization of implants:

The implants were sterilized by ethylene dioxide using Steri-Vac gas sterilizer under cold cycle (37º C for 5.5 h). The sterility testing of the implants was carried out as per Pharmacopoeia of India at Department of Microbiology, Kasturba Medical College, Manipal by direct inoculation method using Alternative Fluid Thioglycolate as culture medium (22).

Drug content determination:

The content of gentamicin sulfate in the implants was determined by dissolving 1 cm length implant in 100 ml dichloromethane and then extracting the drug with 100 ml Phosphate Buffer Saline from dichloromethane. The drug content in PBS was determined by High Performance Liquid Chromatography after suitable dilution with PBS.

In vitro drug release studies:

The *in vitro* release studies were carried out by vial method as previously reported by us (14, 15). The implants (1 cm length) were placed in 20 ml vials containing 10 ml of phosphate buffered saline, pH7.4. The vials were kept in a water bath maintained at 37 °C with continuous shaking using thermostatic shaking water bath (Remi, Mumbai, India). The entire volume of the release media was replaced with fresh aliquots of PBS every hour for the first eight hours and every 24 hours for the next 10 days, followed by every 5 days for 30 days and for every 10 days upto 80 days. Drug concentration in PBS was determined at the different time intervals.

Analysis of gentamicin sulfate:

Gentamicin sulfate was assayed by paired-ion HPLC method (23). The procedure uses pre-column derivatization with o-phthaldehyde reagent. The eluent was monitored at 350 nm. A Shimadzu Class VP series HPLC with two LC-10AT pumps, a SPD-10A variable wavelength programmable UV/Vis detector, a SCL-10A system controller and a RP C-18 column (Luna, Phenomenex, USA; 250 mm x 4.6 mm; particle size 5 µm) were used. The system was equipped with Class VP series version 6.12 software.

In vivo studies:

The *in vivo* studies of the implants were conducted in twenty-one healthy male rabbits, weighing between 1 to 1.5 kg. They were housed in aluminum cages one in each cage and food and

water was allowed *ad libitum*. The rabbits were divided into 7 groups of 3 each. The *in vivo* experimental protocol was approved by Institutional Animal Ethical Committee, Kasturba Medical College, Manipal.

The over night fasted animals were anaesthetized by injecting ketamine hydrochloride (40 mg/kg) through marginal ear vein. The right thigh region of the rabbits was shaved and a medial incision was made to expose the femur. The surrounding muscular tissues were held apart using retractors to get an easy access to femur. A cavity (about 0.75 cm diameter and 1.10 cm length) was made in the femur using hand drill (Renda motor, India). The implant was placed inside the cavity and sealed using bone wax. The surrounding muscular tissues were sutured; the incision was closed and sutured (24). The animals were observed daily for inflammation at the surgical site, any changes in water and food intake and weight loss. Three animals were sacrificed periodically by injecting lethal dose of pentathal sodium (Abbott Ltd., India) through the ear vein at 1, 3, 5, 7, 14, 21 and 28 days after implantation and the implants were removed for estimation of the residual drug. The femur was also collected to determine the bone concentration of drug.

Bone concentration of gentamicin sulfate was determined in 1 g of bone tissue cut at the vicinity of implantation. The bone was powdered in a glass mortar with 10 ml distilled water. The dispersion was treated with 5 ml of trichloroacetic acid (5% w/v) to precipitate proteins. The whole content of the mortar was centrifuged at 4 ºC and 3500 rpm for 20 min. The supernatant was collected and analyzed for the drug content. Student's *t*-test (GPIS software) was employed to analyze the results and $p<0.05$ was considered to be significant.

Stability studies:

The stability studies for the implants were conducted by placing the implants in hermetically sealed amber coloured vials at refrigeration temperature (2-8 ºC) and at 25 ºC. Initially and at different time intervals, the implants were evaluated for physical appearance (colour, texture, diameter and brittleness) and drug content.

Results and Discussion

The implants passed the test for sterility according to Pharmacopoeia of India as there was no turbidity in implant contained culture medium upto 14 days (22). The prepared implants were found to possess satisfactory hardness (the implants were hard enough to handle properly without breakage) for easy placement into the bone cavity. The weight, length and diameter of implants from different batches were found to be uniform (Weight: 59.12±0.80 mg/cm; Length: 10.10±0.05 cm; Diameter: 2.34±0.03 mm). Good uniformity in the drug content was observed among the batches and varied from 96-99%.

Fig. 1 shows the results of *in vitro* release studies of gentamicin sulfate from the implants. The results show that drug and polymer ratio plays an important role in the release of drug from the implants. Drug release decreased as the ratio of polymer in the implant increased from 1 to 2 fold. Generally the release of the drug from biodegradable polymer depends mainly on its biodegradation; but at the same time, as the polymer content increases, the drug exposed to external environment will be less, which might have decreased the drug release with an increase in polymer content.

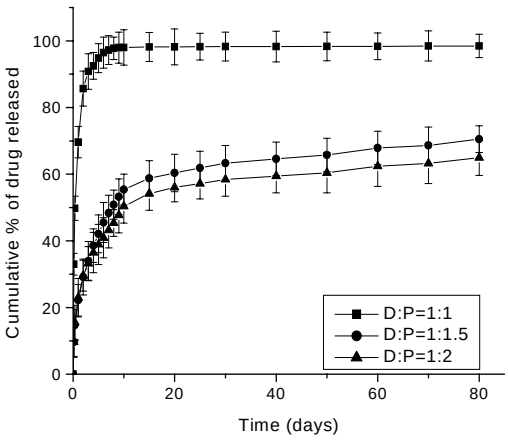

Fig. 1. In vitro release profile of gentamicin sulfate from 3 different implants having different drug and polymer ratios.
Each point is presented as Mean±SD; n=3.

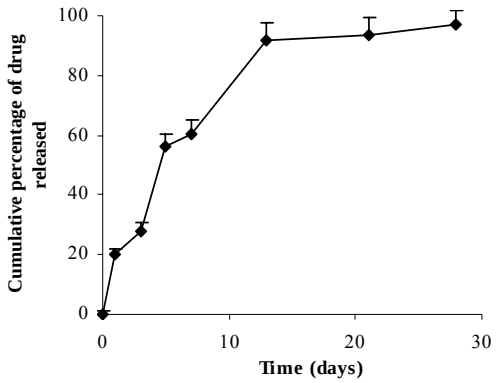

Fig. 2. In vivo release profile of gentamicin sulfate from implants in rabbit femur.
Each point is presented as Mean±SD; n=3.

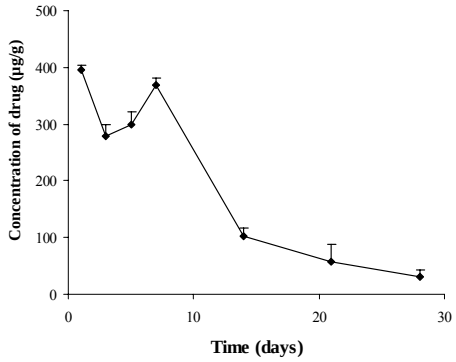

Fig. 3. Bone concentration of gentamicin sulfate at the vicinity of implantation.
Each point is presented as Mean±SD; n=3.

The implants released the drug in a biphasic manner with an initial burst effect followed by a slow release over a prolonged period. The burst effect in the initial stages could be due to the entrapped drug on the surface of the implant. Another reason put forth to explain the burst effect is the percolation limited diffusion, where the initial drug loading is theorized to be composed of two separate pools; a pool of mobile active agent which is free to diffuse upon hydration of the matrix, and a pool of immobilized active agent which can diffuse only after pore size increases due to hydrolytic degradation of the matrix (25). Secondary slow release of the drug can be explained by a permeability reduction due to changes in the morphology of the polymer and alternatively, a reduction in the concentration gradient across the polymer wall produced by a declined rate of drug dissolution in the polymer. The morphology is potentially relevant to the constancy of the release rate because poly(ε-caprolactone) is a semi-crystalline polymer subject to slow hydrolysis *in-vitro* and *in-vivo* (26). The resulting molecular weight decrease, coupled with annealing, is associated with a considerable increase in crystallinity, which in turn reduces the polymer permeability (27). Initial burst release of the drug could be favorable particularly in the treatment of osteomyelitis so as to provide high amount of drug to eradicate the pathogenic microbial flora followed by a slow release to prevent the recolonization and rehabilitation of pathogens. Based on *in vitro* drug release characteristics, the implants with drug and polymer ratio of 1:1.5 were selected for further *in vivo* studies in rabbits.

The results of *in-vivo* studies of gentamicin sulfate implants (Fig. 2) show that about 20% of the drug was released at the end of 24 h. A burst effect was observed at the end of 3 days, which could be due to the entrapped drug on the surface of the implant. The implants released more than 90% of the loaded drug over a period of 4 weeks. The drug release rate in *in-vivo* conditions was faster compared to *in vitro* release studies as components of the fluid could have influenced the drug release in *in-vivo*. Fig. 3 depicts the gentamicin sulfate concentrations in the bone tissue at the vicinity of the implantation at different time intervals. Results indicate that the concentrations were maintained above the minimum inhibitory concentration against *Staphylococcus aureus* (MIC_{90}=2 to 16 µg/ml and ≤ 4µg/ml as per National Committee for Clinical and Laboratory Standardization Institute) and *Pseudomonas auruginosa* (MIC_{90} =1 to 2 µg/ml Clinical and Laboratory Standardization Institute), the common pathogens for osteomyelitis, throughout the period of the study. The animals showed little inflammation at surgical site only for first two days of implantation and the same was not observed during the rest of the testing period. No significant changes were observed in water intake, food consumption and weight of the animals during *in vivo* testing period.

The results of stability studies are presented in Table 1. The implants exhibited no changes in the drug content at both refrigeration and 25 ºC during the whole testing period. All the implants maintained initial physical properties like colour, texture and diameter during stability testing period. But the implants placed at refrigeration temperature, showed brittleness after 4 months; whereas the implants stored at 25 ºC did not show brittleness during the testing period. These results are in accordance with the earlier report, where the biodegradable gentamicin implants, prepared using polyanhydride copolymer, were subjected to stability studies at -15 ºC and 25 ºC. The drug in polymer matrix was stable for at least 12 months when stored at both these temperature conditions. But cracking of the -15 ºC samples was evident, but the 25 ºC samples remained intact (28).

The present study shows that biodegradable gentamicin sulfate implants can be successfully used for the effective management of osteomyelitis. However, preclinical chronic toxicity studies have to be carried out prior to use the implants for human application.

Table 1: Stability studies of implants

Time (Months)	**Refrigeration temperature**		**25 ºC**	
	Drug content (%)	Physical appearance	Drug content (%)	Physical appearance
0	100±0.00	-	100±0.00	-
1	99.95±0.75	-	99.86±1.02	-
2	99.75±0.98	-	99.75±0.86	-
3	99.66±0.98	-	99.68±0.76	-
4	99.56±1.01	-	99.32±0.96	-
5	99.51±0.96	B	99.26±0.46	-
6	99.31±0.95	B	99.00±0.98	-

Physical appearance: --- = No change; + = Slight change; ++ = Moderate change; +++ = Extreme change; Brittleness = B

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