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Research paper

Preparation of bupivacaine-loaded poly(ε-caprolactone) microspheres by spray drying: drug release studies and biocompatibility

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Abstract

Poly(ε-caprolactone) microspheres containining bupivacaine were prepared by the spray-drying process. The average size of drug loaded microspheres was less than 3 μm in diameter, and the percentage of entrapment efficiency was 91 ± 3%. In vitro drug release kinetic in phosphate buffer at 37°C showed a hyperbolic profile, with a burst-effect during the first hour. Subcutaneous injection of bupivacaine-loaded microspheres in the back of rats caused an increase in drug concentration in plasma. Maximum bupivacaine concentration in plasma was 237 ± 58 ng/ml at 105 h, and drug was detected in plasma for 16 days. The half-life time of the drug was increased by more than 125 times with regard to that of the drug administered in a solution by intraperitoneal injection. After 30 days of injection, a mass formed by microspheres surrounded by a thin fibrous capsule was observed. Small blood vessels and multinucleate foreign body giant cells with macrophagic function around microspheres were detected. After 60 days of injection a subcutaneous mass was also observed, which was formed of more degraded dispersed microspheres in conjunctive tissue, which had a normal structure. Thus, bupivacaine-loaded poly(ε-caprolactone) microspheres could be considered as a device to be used in the treatment of severe pain that is not responsive to opioids for example in cancer-related syndromes or in intractable herpetic neuralgia.

Keywords: Poly(ε-caprolactone) microspheres; Spray-dryer; Bupivacaine; In vitro and in vivo drug release; Histological studies

1. Introduction

One of the microencapsulation techniques used to obtain microspheres is the spray-drying process, widely used in the pharmaceutical industry [1]. This process has been used to obtain drug-loaded microspheres of poly(lactide) and poly(lactide-co-glycolide) [2,3] due to its advantages with regard to water-in-oil-in-water emulsion processes. Thus, microspheres are obtained in only one step from solutions, dispersions or emulsions. On the other hand, this process allows to obtain microspheres of very small diameter that can be suitable for drug administration by injection, probably in a more convenient way than those of larger diameter obtained from solvent evaporation processes [4].

Poly(ε-caprolactone) is a biodegradable polymer, which is capable of entrapping very different chemical compounds. This polymer has been studied as a carrier for oral vaccines [5,6], and also to entrap non-steroideal anti-inflammatory drugs [7] and estrogens [8]. This polymer is a water-insoluble but water-permeable polymer, whose degradation is slow in aqueous medium and does not cause an acid environment. In vitro biodegradation assays of poly(ε-caprolactone), crosslinked by gamma rays as well as uncrosslinked, have been carried out in the presence of lipase AK, and it was confirmed that enzymatic degradation occurs even for high crosslink density of the polymer [9]; the degradation of microparticles prepared by emulsification-solvent evaporation was also enhanced in the presence of lipase enzyme [10]. Poly(ε-caprolactone) has also been blended with alphatic polyesters [11,12] to obtain microspheres of different biodegradability for the release of different drugs. Implantation of polycaprolactone-b-poly(ethylene glycol) copolymer in the back or small intestine of rats has shown that the degradation is faster than that in in vitro assays in the presence of lipase due to the physiological conditions of the sample being implanted in the body of animals [13]. Furthemore, cellular internalization of polycaprolactone-b-poly(ethylene oxide) copoly-

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mer micelles by endocytotic uptake has been observed in cell cultures [14]. Thus, devices prepared with polycaprolactone as well as its copolymers could be degraded or internalized in cells when implanted in the organism, which allow them to have different uses in vivo. This way, assays carried out with rabbits have shown that polycaprolactone might be a promising local antibiotic delivery vehicle for the treatment of osteomyelitis [15].

On the other hand, local anesthetic drugs can be used for regional control of major pain. One of these drugs is bupivacaine, which is characterized by its long action and high therapeutic power [16]. There is a substantial population with intractable pain that is not responsive to opioids. In these cases non-opioid agents including local anesthetics, such as bupivacaine, are used [17–19]. The efficacy of this sort of drugs could be improved by an administration using sustained drug delivery systems [20].

The aim of this work is the preparation of small size poly(ε-caprolactone) microspheres loaded with bupivacaine by spray-drying process, the study of drug release in vitro, and the evaluation of these devices for drug administration in vivo by injection, determining drug plasma levels, device biodegradation and histological findings of tissue surrounding the implant.

2. Materials and methods

2.1. Materials

Poly(ε-caprolactone) ($\bar{M}_w = 6500$ g/mol-Aldrich) dipotassium monohydrogen phosphate (K_2HPO_4) (Panreac, Barcelona, Spain), potassium di-hydrogen phosphate (KH_2PO_4) (Panreac), di-hydrogen sodium phosphate (NaH_2PO_4) (Panreac), acetonitrile (Panreac), dichloromethane (Panreac), heparin (Leo Laboratories, Madrid, Spain), heptane (Panreac), ethyl acetate (Panreac), sulphuric acid (Panreac), sodium acetate (Panreac), Tween 80 (Panreac) were used as received.

Bupivacaine ($C_{18}H_{28}N_2O$) was kindly supplied by Inibsa Laboratories (Spain). Milli-Q® (Millipore, Madrid, Spain) water was used.

2.2. Preparation of bupivacaine-loaded microspheres

Preparation was carried out by the spray-drying process. Poly(ε-caprolactone) (910 mg: 91% w/v) and bupivacaine (90 mg: 9% w/v) were dissolved in dichloromethane (100 ml). The obtained solution was sprayed through the nozzle (0.5 mm diameter) of a spray-dryer (Mini Spray-dryer B-190, Büchi, Switzerland). Assay conditions were: inlet air temperature 39°C, outlet air temperature 26°C, spray flow 4 ml/min, and compressed spray air flow (represented as the volume of the air input) 700 l/h. Microspheres collected from the spray-dryer cyclone were used for drug release experiments.

2.3. Bupivacaine stability in solution

Bupivacaine stability in solution at 37°C was studied. Bupivacaine (1 mg/ml) in phosphate buffer (1 mM, pH 7.5) was maintained at a constant stirring rate for 21 days. At intervals, 100 μl was withdrawn from the solution in order to follow the change in bupivacaine concentration. A similar study was carried out in acid solution (HCl 7% v/v: pH 1.5).

The concentration of bupivacaine was determined by high performance liquid chromatography (HPLC) (Spectra-Physics SP8800 HPLC pump, SP 100 UV absorbance detector and SP 4400 computing integrator). The stationary phase was Lichrosorb RP8 5 μ (15 × 0.46 cm; Teknokroma). The eluent was 0.01 M dihydrogen sodium phosphate with acetonitrile (70:30 v/v) pH 2.1 [21]. The flow rate was set at 1.5 ml/min and the detector wavelength was 205 nm. Bupivacaine standards of 1–1000 μg/ml were run for external standardization and linear curves with a correlation coefficient of 0.999 were generated from the area under the peak measurements. The bupivacaine retention time was 5.5 ± 0.2 min.

2.4. Determination of bupivacaine included in poly(ε-caprolactone) microspheres

The stability of bupivacaine in solution was very high in acidic solution for a long period of time (Fig. 1). Thus, the amount of the drug included in microspheres was determined by placing these in acidic solution for 48 h at 37°C with vigorous stirring. The concentration of bupivacaine was determined by HPLC. The amount of drug entrapped per weight of microspheres was calculated. The percentage of entrapment efficiency was expressed by relating the actual drug entrapment to the theoretical drug entrapment [5].

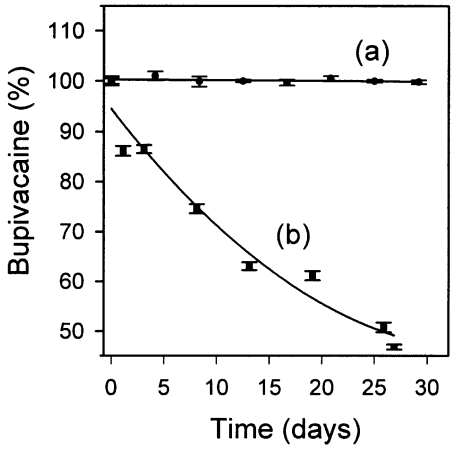

Fig. 1. Percentage of bupivacaine as a function of time of incubation at 37°C. The drug is dissolved in (a) acid solution, HCl 7% v/v, pH 1.5, and (b) phosphate buffer 1 mM, pH 7.5.

2.5. Bupivacaine release from microspheres in vitro

Bupivacaine-loaded microspheres (40 mg) were added to 7 ml phosphate buffer 1 mM pH 7.5 inside a dialysis bag (Spectra/Por membrane MWCO: 6-8000) which was placed in a vessel containing 50 ml phosphate buffer at constant temperature (37°C) and stirring rate. At intervals, 100 μl samples were withdrawn from the solution in order to follow the change in bupivacaine concentration by HPLC. The volume removed from the vessel was replaced with phosphate buffer.

2.6. Animals: bupivacaine administration

Male Wistar rats, weighing 250 ± 10 g, were obtained from the Animalario of the Universidad Complutense de Madrid (Spain) which operates according to the requirements relating to animal experimentation regulations (DC 86/609/CEE; RD 223/1988; OM 13/X/1989). The animals were kept on a 12:12 h light:dark cycle and were fed standard rat food and water ad libitum. The surgical material used in the experiments was previously autoclaved. Just before injection, solutions were put under ultraviolet light (Ecogen Lamp, Vilber Lourmat, Intensity 7 mW/cm^2) at 254 nm for 4 s, because of the germicidal action of this wavelength.

The animals were divided into three groups. (A) The control group was subcutaneously injected in the back with a saline solution (NaCl 0.9%) containing 0.06% v of Tween-80. (B) The implanted group consisted of rats implanted with bupivacaine-loaded microspheres: 30 mg of microspheres, whose bupivacaine content was 2.46 mg, were dispersed in 1.5 ml of saline solution (0.9% NaCl) containing 0.06% v of Tween-80. The animals were anaesthetized with halotane an then the microsphere dispersion was subcutaneously injected in the back of the rat using a sterile syringe with a 1.2×40 mm^2 nozzle. (C) The third group of animals was intraperitoneally administered with a single injection of bupivacaine containing the same amount of drug than that administered by microspheres, 2.46 mg of bupivacaine solved in 1 ml of saline solution (0.9% NaCl). Intraperitoneal injection is a parenteral via of drug administration, usually used in animal experimentation due to its very simple use and fast absorption of the drug into the blood, essentially by the porta vein [22]. This form of administration allows establishing a comparison between the drug plasma levels and its pharmacokinetic parameters with regard to drug administration using a sustained drug release device.

2.7. Plasma bupivacaine determination

At predetermined times after the injection of bupivacaine-loaded microspheres, the animals were anaesthetized with halotane. Blood (1 ml) was collected by puncturing the jugular vein in heparinized (75 units = 15 μl) polypropylene tubes. The heparinized blood was centrifuged at 8000 *g* for 10 min in a Sigma 202 M centrifuge, immediately after collection so as to obtain plasma, which was then stored at −20°C. When bupivacaine was administered by injection of a drug solution, blood was collected 15 min, 30 min, 1, 2 and 4 h after the injection.

Bupivacaine was extracted from plasma samples by heptano-ethyl acetate (90:10 v/v), and in a second step by 0.05 M sulphuric acid [21]. The acid solutions were later buffered with sodium acetate and were injected into the chromatograph. The HPLC method used was the one previously described. For calibration, known amounts of bupivacaine were added to pooled drug-free plasma to obtain a concentration between 10 and 1000 ng/ml. These plasma aliquots were extracted as described above. The retention time of bupivacaine was 7.5 ± 0.2 min.

Animals were sacrificed with diethyl ether 30 and 60 days after implantation of drug-loaded microspheres. An incision was made on the rat back to remove the tissue around the place of injection.

2.8. Pharmacokinetic parameters

Plasma bupivacaine data were analyzed using a non-compartment model. The elimination constant (K_e) and the corresponding half-life ($t_{1/2} = \ln 2/K_e$) were derived from the terminal slope of the semilogarithmic plots of plasma concentration vs. time. The areas under curve (AUC) were determined by the trapezoidal method. The total body clearance (TBC) was derived from dose/AUC.

2.9. Scanning electron microscopy

The morphology, average diameter as well as the size distribution of bupivacaine-loaded microspheres prepared by the spray-drying process, was studied by scanning electron microscopy (SEM) (Jeol JSM-6400 Electron Microscope). Microspheres were fixed with an adhesive sheet on a rigid support and coated with gold. For the in vivo studies, the morphology of the microspheres after 30 and 60 days of injection was studied. The removed tissue was divided in two pieces, and one of them was cut into slices, which were then dried. These slices were fixed with an adhesive sheet on a rigid support and shadowed with gold.

2.10. Histological studies

A piece of the removed tissue, fixed with formol (10% v/v), was immersed in paraffin. Cuts (10 μm) were carried out with a paraffin microtome (Minot type). Samples were dyed using the alcian blue hemalum picro-indigo and the hematoxilin–eosin methods [23].

2.11. Statistical analysis

Results are expressed as mean ± SD of three experiments. Data analysis of the pharmacokinetic parameters

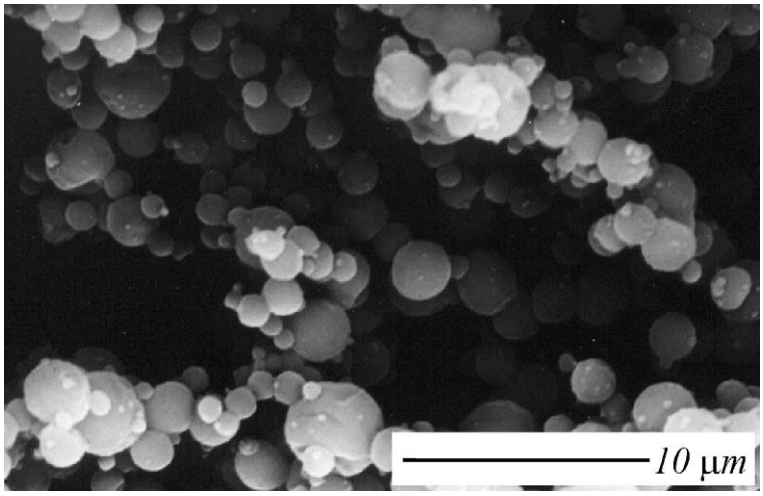

Fig. 2. Scanning electron micrography of bupivacaine-loaded poly(ε-caprolactone) microspheres prepared by the spray-drying process.

was performed by unpaired Student's *t*-test. A value of $P < 0.05$ was considered significant.

3. Results and discussion

3.1. In vitro studies

The average diameter as well as the size distribution of bupivacaine-loaded poly(ε-caprolactone) microspheres was calculated by direct measurement in SEM photographs. Thus, the diameter of 300 microspheres was measured. The diameter of bupivacaine-loaded poly(ε-caprolactone) microspheres was in the range of 0.61–4.24 μm. The size of 25% of them was between 0.61 and 1.21 μm; 54% of microsphere size was between 1.52 and 2.54 μm, where 33% of them had a diameter of 2.12 μm; and 21% of microspheres were between 2.73 and 4.24 μm. The average diameter of bupivacaine-loaded poly(ε-caprolactone) microspheres was 2.2 ± 0.8 μm. They were completely round and their surface was smooth (Fig. 2). They formed groups. Similar sizes and morphologies have been described for microspheres of poly(ε-caprolactone) loaded with albumin [5]. Photomicrographies of poly(ε-caprolactone) microspheres loaded with ketoprofen indicated that they were totally smooth and their size was lower than 10 μm [7].

The amount of bupivacaine included in poly(ε-caprolactone) microspheres was 82 ± 3 μg of the drug per milligram of microspheres, which corresponds to a percentage of entrapment efficiency of 91 ± 3%. This percentage of entrapment efficiency was very high due to chemical–physical characteristics of the drug and the preparation method used. The high solubility of both bupivacaine and poly(ε-caprolactone) in dichloromethane allows obtaining a solution that can be sprayed through the nozzle of a spray-dryer. The use of polymer and drug solutions improves the entrapment efficiency of drugs with regard to the results obtained when the drug is not soluble in the same solvent as the polymer. Thus, the entrapment efficiency of BSA in poly(ε-caprolactone) microspheres was about 43% in the absence of emulsion stabiliser [6]. On the contrary, the entrapment efficiency of ketoprofen-loaded poly(ε-caprolactone) microspheres was about 97% since both compounds were soluble in the organic solvent [7].

The stability of bupivacaine in phosphate buffer at 37°C was studied before drug release experiments were carried out. Degradation of the drug as a function of time was observed (Fig. 1), the amount of bupivacaine being 50% at 27 days (650 ± 10 h). The in vitro release kinetic of bupivacaine from poly(ε-caprolactone) microspheres showed a hyperbolic profile (Fig. 3). Maximum amount of released bupivacaine from 40 mg of drug-loaded microspheres was 3.1 ± 0.2 mg (54 ± 4 μg/ml) and it was detected at 26 ± 2 h. This maximum concentration of the

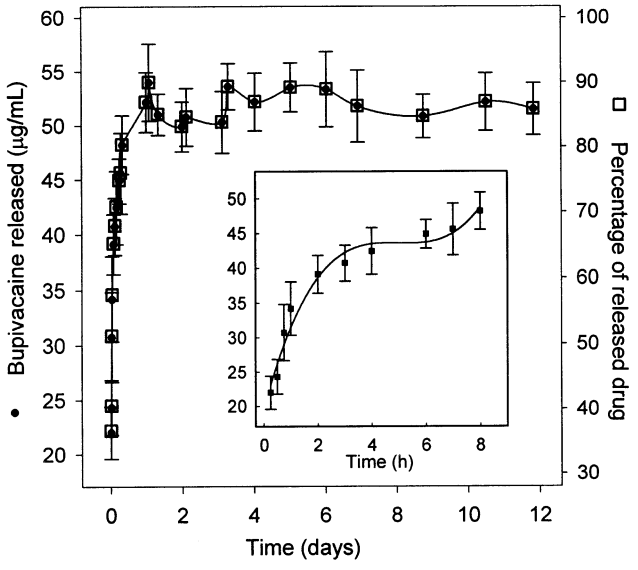

Fig. 3. Bupivacaine released from poly(ε-caprolactone) microspheres as a function of time: (•) μg/mL, (□) percentage. Phosphate buffer 1 mM, pH 7.5, 37°C.

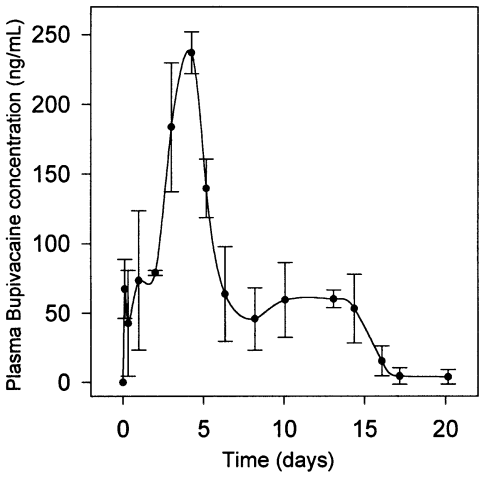

Fig. 4. Plot of bupivacaine plasma concentration as a function of time for drug released from subcutaneously injected bupivacaine-loaded poly(ε-caprolactone) microspheres.

drug was maintained almost constant for 12 days. The only way to maintain this drug concentration in the release medium during this period of time was with a constant and slow release of bupivacaine from microspheres, since pH of the buffer did not change during the experiment, and the drug in solution was degraded at pH 7.5. A burst effect was observed, thus a 59 ± 7% of the included drug was released in the first hour and then a slower release took place. The release of bupivacaine from poly(ε-caprolactone) microspheres was controlled during the first stages by diffusion due to the small molecular weight of this drug; thus the drug placed on the most superficial part of the microspheres was released quickly, where the contribution of the smaller microspheres was bigger. In addition, the semicrystallinity of the polymer, which had amorphous and crystalline regions contributed to the drug being released at different rates since the distribution of bupivacaine in microspheres would not have been homogeneous. This burst phase has been also described for the release of bupivacaine from albumin microspheres [24], the release of BSA from poly(ε-caprolactone) microspheres [5], and for the release of ketoprofen from poly(ε-caprolactone) microspheres [7].

3.2. In vivo studies

One of the objectives pursued by sustained release devices is that an active susbtance is administered in an efficient and convenient way. Controlled drug release devices can be designed for drug administration close to its target or by subcutaneous via, and in these cases the injection of the device is the most appropriate. Different solutions were assayed to obtain a dispersion of bupivacaine-loaded microspheres that could be injected, the best results were obtained with a saline solution (NaCl 0.9%) containing 0.06% v/v Tween-80. In order to establish the innocuous effect of this solution, animals were injected with 1.5 ml of it, and after 30 days they were sacrificed and an histological study of the tissue of the injection zone was carried out. The results indicated that the solution was absorbed and the structure of the surrounding tissue was normal. Thus, 30 mg of bupivacaine-loaded poly(ε-caprolactone) microspheres that included 2.46 mg of the drug, which means a dose of 9.8 mg/kg, were dispersed in 1.5 ml of the mentioned solution to be subcutaneously injected in the back of rats. The Le Corre's group has administered bupivacaine intraperitoneally to rabbits by poly(lactide) and poly(lactide-co-glycolide) microspheres, using doses between 6.9 and 15.6 mg/kg [25]. Also, pellets of polymer matrices of 1,3-bis(*p*-carboxyphenoxy)propane-sebacic acid anhydride (1:4) including 60 mg of bupivacaine have been used for drug administration to rats [26].

The plasma concentration of bupivacaine released from microspheres is shown in Fig. 4. The drug was slowly released from poly(ε-caprolactone) microspheres and its plasma concentration increased as a function of injection time of the device. The maximum plasmatic concentration of bupivacaine was 237 ± 58 ng/ml and it was reached 105 h after implantation. From this time, plasma drug concentration gradually decreased and the drug was not detected after 16 days. When the drug was administered in solution at the same dose by intraperitoneal injection, the maximum drug concentration was obtained at 1 h and the drug was detected in plasma only for 2 h. Pharmacokinetic data are shown in Table 1. The elimination half-life of the drug administered by microspheres injection increased by more than 125 times with regard to that administered by solution, and the maximum drug concentration was far from toxic levels, since the first symptoms of toxicity generally occur when bupivacaine plasma concentration exceed 2 μg/ml [27].

In order to determine the biological degradation and the biocompatibility of the bupivacaine-loaded poly(ε-caprolactone) microspheres some of the implanted animals were sacrified after 30 days. At the injection site, a mass was observed under the back skin of the rat, which was removed together with the surrounding tissue. The capsule was cut in

Table 1
Pharmacokinetic parameters of bupivacaine (BP) administered by drug solution and by drug-loaded poly(ε-caprolactone) microspheres

	BP-microspheres[a]	BP solution[b]
$k_e \cdot 10^3 (h^{-1})$	7 ± 2*	700 ± 100
$t_{1/2}$(h)	115 ± 41*	0.9 ± 0.2
AUC (μg h ml^{-1})	48.8 ± 0.7*	0.9 ± 0.2
TBC $\cdot 10^3$ (l h^{-1})	48.4 ± 0.7*	2700 ± 500
C_{max}(ng/ml)	237 ± 58*	617 ± 90

[a] Subcutaneous injection of bupivacaine-loaded microspheres (2.46 mg BP).
[b] Intraperitoneal injection (1 ml) of bupivacaine solution (2.46 mg BP).
K_e, elimination constant; $t_{1/2}$, elimination half-life; AUC, total area under the blood level-time curve; TBC, total body clearance; C_{max}, maximum plasma concentration. *$P < 0.05$.

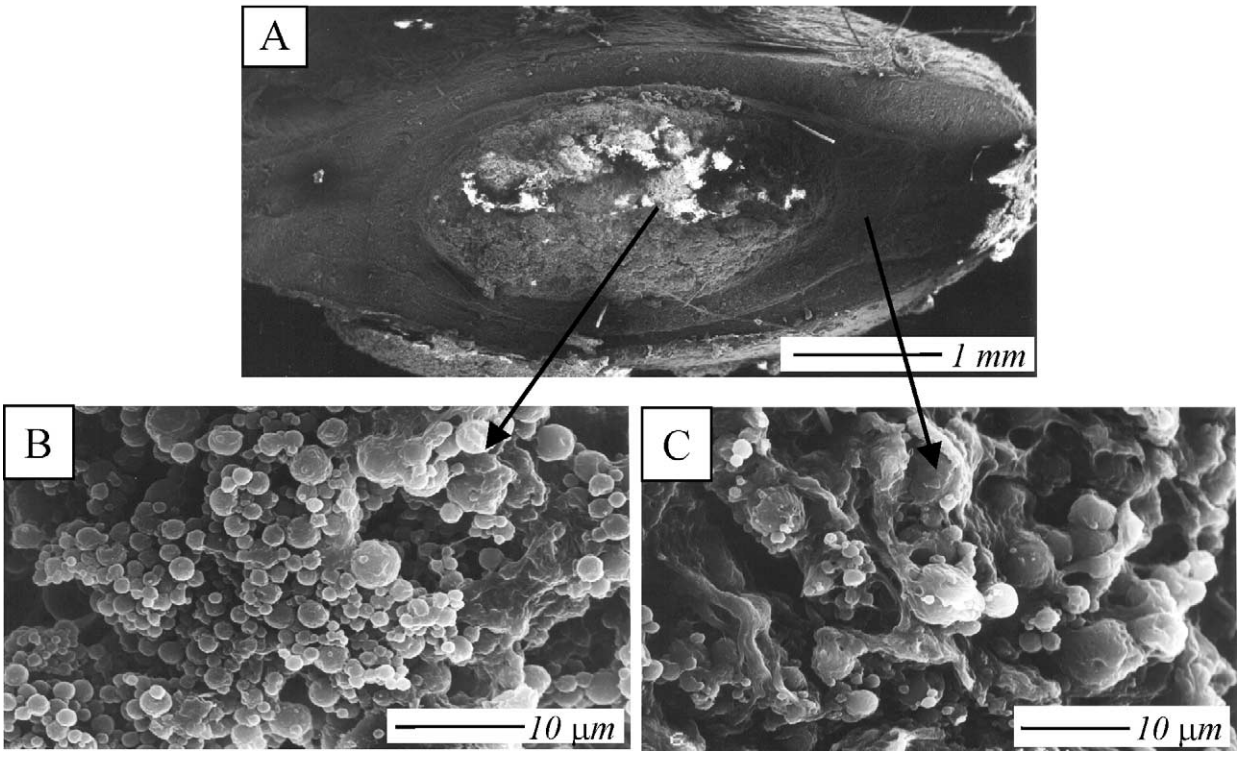

Fig. 5. Scanning electron micrograph of the capsule formed at the injection place after 1 month of subcutaneous implantation of bupivacaine-loaded poly(ε-caprolactone) microspheres: (A) view of a transversal cut of the capsule. (B) Magnification of the central zone of the capsule. (C) Magnification of the interface between central and external part.

two pieces, one of them was cut in thin slices and dehydrated for SEM studies, and the other one was placed in formaldehyde (10%) for histological determinations. With regard to SEM studies, in Fig. 5A a view of a cut of the implant after 1 month of injection is shown. Two defined zones are observed, in the central part there is a large amount of microspheres, and around them a thin fibrous capsule. The magnification of the central zone (Fig. 5B) shows a large number of microspheres, which are aggregated and do not show signs of degradation, and some fibers of conjunctive tissue. In the interface between central and external part (Fig. 5C), polymer microspheres surrounded by tissue are observed. Histological studies indicated that there was vascularization in the removed mass, and microspheres form groups surrounded by conjunctive tissue (Fig. 6A). A large amount of nucleuses were observed around polymer, which indicated a very active tissue. A significant number of multinucleate foreign body giant cells with macrophagic function, which is a normal reaction of the organism, were observed (Fig. 6B).

A second group of implanted animals were sacrificed 60 days after microsphere injection. A mass was also observed

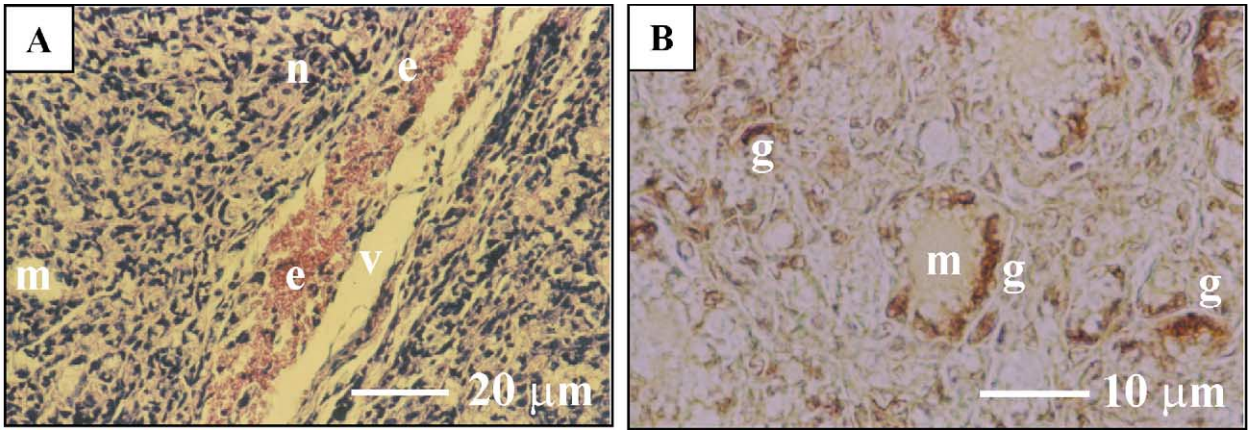

Fig. 6. Photomicrographs of microspheres and the surrounding tissue after 30 days of implantation of bupivacaine-loaded poly(ε-caprolactone) microspheres by subcutaneous injection in the back of rats: (A) microsphere groups of different sizes sorrounded by conjuntive tissue (m, microspheres; e, erythrocyte; v, blood vessel; n, nucleus). (B) Magnification of the implant zone. Groups of microspheres (m) and multinucleate foreing body giant cells (g) can be observed.

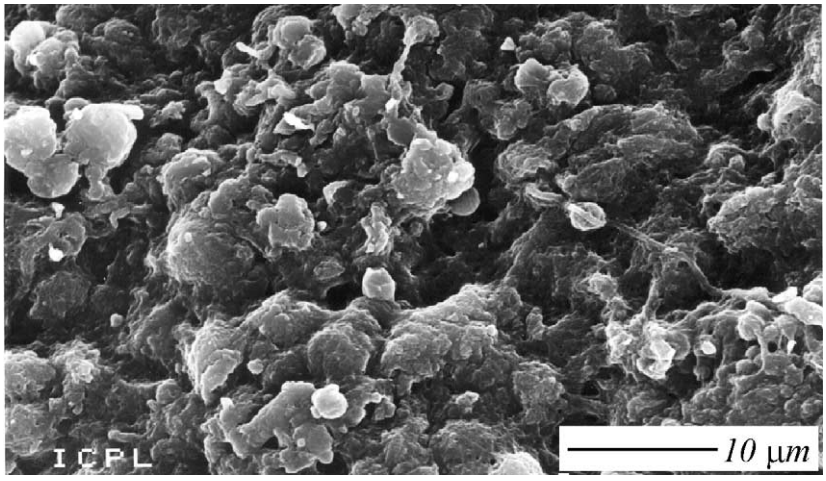

Fig. 7. Scannig electron micrograph of bupivacaine-loaded microspheres after 2 months of implantation by subcutaneous injection in the back of rats.

at the injection site under the back skin of rats. The SEM studies showed that there were microspheres, although a central zone formed by groups of microspheres was not detected. The degradation of the polymer was evident, the number of microspheres seemed to be lower, and microspheres were surrounded by tissue (Fig. 7). Histological studies did not indicate significant differences with the previous one (Fig. 8). However, the conjunctive tissue was more homogeneous, with a lower density of microspheres. Multinucleate foreign body giant cells around polymer aggregates were observed, which indicated the macrophagic activity that took place in this region. Thus, after 2 months of the subcutaneous injection of bupivacaine-loaded poly(ε-caprolactone) microspheres, microspheres were dispersed in the conjunctive tissue of the capsule, their number was lower and they were more degraded, the surrounded tissue showed a normal structure, and lymphocytes were not detected, which indicated that there was no infectious process. Furthermore, injury effects were not detected in the animals.

These bupivacaine-loaded poly(ε-caprolactome) microspheres could be considered safe for subcutaneous implantation in the organism because of (A) there were multinucleate foreign body giant cells, which are macrophagic cells present in normal processes of degradation; (B) there were not mast cells, and so histamine granules among microspheres were not observed. Thus, there were no inflammatory and immunitary processes; (C) groups of lymphocytes were not present in the implants, which indicated that the implanted microspheres were not rejected; (D) the implant was surrounded by conjunctive tissue, from which thin septa penetrate through the inside part of the implant surrounding microspheres. This characteristics was indicative of the integration of the implant in the body of the animal, and thus the absence of rejection; (E) accumulation

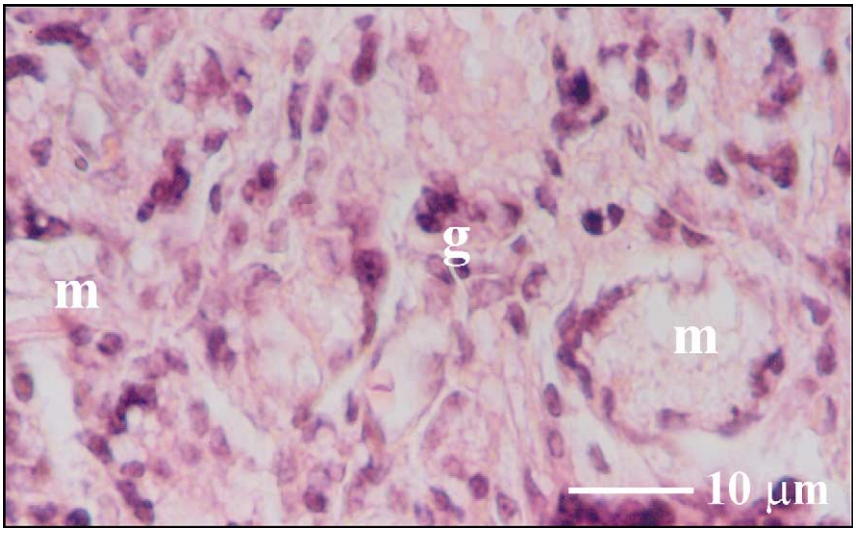

Fig. 8. Photomicrograph of a histological cut of the implant after 2 months of the subcutaneous injection of bupivacaine-loaded poly(ε-caprolactone) microspheres. Microspheres and groups of microspheres are surrounded by conjunctive tissue (m, group of microspheres; g, multinucleate foreing body giant cells).

of extravessel liquids in the implant area was not detected, which was indicative of that there was not an acute inflammatory response.

Microspheres of poly(ε-caprolactone) loaded with bupivacaine could be successfully prepared by the spray-drying process. Their diameter was low enough to allow the injection, and the percentage of entrapment efficiency was very high, which made possible the administration of a significant amount of the drug using a small amount of microspheres by subcutaneous injection. This resulted in a sustained release of the drug, which allowed the drug to be detected in plasma for a long period of time. This makes possible to use the devices in the control of intractable pain. Although these bupivacaine-loaded poly(ε-caprolactone) microspheres did not induce inflammatory effects or rejection, and they were very efficient as drug release devices, their biodegradation rate probably could be improved. In any case, the administration of bioerosionable drug-loaded microspheres by injection avoids surgery, which makes this kind of sustained drug release more convenient.

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