Embolization with Polyhydroxybutyrate (PHB) Microspheres: In-vivo Studies

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ABSTRACT: In this study polyhydroxybutyrate (PHB), produced by a methanol-utilizing bacteria, was used to prepare microspheres in the 120–200 μm size range for embolization. A solvent evaporation technique was utilized to obtain...

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INTRODUCTION

ChemoeMBOLIZATION combines two therapeutic techniques, i.e., chemotherapy and embolization, which involves sequential or concurrent injection of a drug and embolizing agents [19,21]. Alternatively microcapsules or microspheres carrying immobilized drugs can also be utilized [5,13,15,20]. Several materials, both natural or synthetic origin have been used for embolization including particles, sponges or oil emulsions [3,4,6,13,15,20], but no single agent has been completely satisfactory.

Recently, we attempted to prepare microspheres carrying drugs from a microbial biodegradable polyester, i.e., polyhydroxybutyrate (PHB) as a potential chemoembolization agent. PHB was produced by a methanol-utilizing bacteria. Details of the production, isolation and characterization of PHB were given elsewhere [22]. The PHB microspheres, either plain or carrying rifampicin (as a model drug) were produced by a solvent evaporation technique. Production of the PHB microspheres with different size, size distributions, different drug loadings, as well as in-vitro degradation and drug release from these microspheres were reported in our previous paper [12]. In this communication, we present their use in animals as an embolization agent.

MATERIALS AND METHODS

Preparation of PHB Microspheres

The biodegradable polymer used in this study, polyhydroxybutyrate (PHB), was produced by a methanol-utilizing bacteria, Hyphomicrobium zawitaense, in a two stage continuous fermentation system conducted at 35°C and pH 7.2, in a growth medium containing (NH₄)₂SO₄ (0.6 g/L), NaCl (0.5 g/L), EDTA (50 mg/L), phosphate (1.5 g/L) and methanol (4 ml/L) (see Reference [22] for details). PHB extraction and purification from the cells were performed by Law and Slookey’s method [9,22]. The weight-average (Mw) and number-average (Mn) molecular weights of the PHB, determined by GPC using polystyrene standards, used for the preparation of the microspheres were 1.8 x 10⁶ and 5.7 x 10⁵, respectively [22].

The PHB microspheres, which were prepared by the solvent evaporation technique similar to that reported in earlier papers, were in the size range of 120–200 μm [5,7,12]. In a typical procedure, 2 g of PHB was dissolved in methylene chloride (Merck, Germany) and then this solution was added drop-wise to 200 ml of dispersion medium (distilled water at 5°C) containing 1.2% (wt/v) of polyvinyl alcohol (PVA) emulsifier (average molecular weight of 10 kD, Sigma, USA). The mixture was stirred at 600 rpm with a mechanical stirrer (IKA-WERG-RW 20, Germany) for 5 min. The medium temperature was raised to 45°C and the solvent was evaporated (about 1 h). The microspheres, which were 120–200 μm in size, were collected by centrifugation at 4000 rpm for 5 min and washed twice with distilled water and vacuum dried at 25°C.

Embolization Studies

The PHB microspheres were sterilized in an ethylene oxide sterilizer (AMSCO, 3028 Model, Erie, PA, USA) deaerated and stored at 4°C until use. For embolization about 50 mg of the sterilized PHB microspheres were suspended within a 10 mL solution containing 240 mg/mL of the contrast material (i.e., Iohexol or Omnipaque, Nycomed, AS International Division, Oslo, Norway) to allow fluoroscopic observations during the injection and 10 mL of wetting agent (i.e., Plasmasteril, Fresenius AG, Bad-Homburg, Germany).

Two adult mongrel dogs weighing about 20 kg were used. The test animal was first anesthetized by intravenous injection of 8 mL of a mixture of 50 mg/mL ketanin (Eczacibaşı, A.Ş., Istanbul, Turkey) and 2% rompun (211) (Bayer AG, Leverkusen, Germany). The puncture site was prepared for embolization after shaving and cleaning with an antibacterial agent. The left femoral artery was catheterized by means of the Seldinger technique [18]. An 18G Seldinger needle was advanced slowly in the direction of the femoral artery until the return of a pulsating stream was observed. Immediately a 150 cm long and 0.038 inch diameter guide wire (Cordis, Boston, USA) was inserted through the needle into the artery, and the needle was removed. A Benson type 5F introducer sheath (Cordis, Boston, USA) was then placed through the femoral artery. After preparing the introducing site, a 5F selective catheter (Renal Double Curve, Cook, Denmark) was inserted toward the renal artery to deliver the contrast agent and the embolic mixture. A confirmatory preembolization renal angiogram was obtained immediately after injection of 7 ml of the contrast agent. Subsequently, 6 ml of the embolization suspension containing the PHB microspheres was injected through the catheter at a rate of 3 ml/sec, and another angiogram was obtained. The angiograms, before and after the embolization, were obtained using a digital subtraction unit (DSA) (Philips DVA, Optimus M200, Amsterdam, The Netherlands). After the embolization was complete, the
catheter and introducer sheath were removed and hand pressure was applied to the puncture site to prevent the blood leakage. One animal was killed after 24 h and the other 7 days after embolization, with a lethal injection (15 mL) of pentothal (Fako, Abbott, Istanbul, Turkey). The kidneys were removed and fixed in 10% formalin (Merck, Darmstadt, Germany) for histopathological examinations.

**Histopathological Evaluations**

The kidneys were removed from the animals and subjected to macro- and microanalysis to evaluate the renal histopathology. The kidneys were bisected longitudinally for microanalysis evaluation by light microscopy. Tissue specimens were taken from the cortex and a cortico-medullary of the fixed kidneys. Both embolized animal and untreated animal kidneys were embedded in paraffin for comparison. Sections (6 μm in thickness) were sliced and stained with hematoxylin and eosin (Merck, Germany) for examination by a pathologist using an optical light microscope (Olympus BX 50 F4, Japan).

**RESULTS AND DISCUSSIONS**

**Properties of PHB Microspheres**

Microbial biodegradable polyesters such as polyhydroxyalkanoates (PHA), are being evaluated for biomedical applications such as drug delivery systems, surgical sutures and implants, because of their high biocompatibilities [2,10,14].

We propose to use PHA particles as potential embolization agents. As discussed in a previous paper, we are able to produce PHB microspheres in appropriate sizes, both plain and drug loaded, which can be used for embolization or chemoembolization, respectively, in vascular channels [9].

In the present study healthy dogs were used as test animals, and renal arteries were subjected to embolization. For this purpose we produced PHB microspheres in the range of 120–200 μm according to information in the literature [1,11,16]. The formulation and preparation procedures were given elsewhere [9]. Two representative micrographs of the PHB microspheres, one taken by an optical microscope (A) and the other taken by a scanning electron microscope (B) are given in Figure 1, respectively. The PHB microspheres are spherical and have large size openings (pores). Based on our earlier studies [5,7,12] we used methylene chloride and polyvinyl alcohol, as the solvent and the emulsifier, respectively, to obtain well-shaped spherical microspheres with relatively narrow size distributions. The pore structure created during particle formation provided diffusion-controlled but very fast drug release rate when the drug-loaded PHB microspheres were analyzed [12]; further evaluation is ongoing in our chemoembolization studies.

**Embolization/Angiography**

The suspensions containing PHB particles were prepared by adding the contrast material and plasmaderm to the microsphere suspension just before the embolization. This homogenous suspension showed no cloudiness or settling which allowed for easy injection during catheterization. The narrow size distribution of the particles ensured an accurate occlusion in the target blood vessels. The commercial PVA suspensions (Ivalon, Laboratoire Ingenor, Paris, France), which are one of the most well-known and widely used embolization agents, were found to be cloudy after shaking. This may be true due to coalescence of the particles with time, which could pass through the capillary beds or especially through an arterio-venous malformation (AVM) and lead to mortality of the treated patients [17].

The pre-embolization angiograms in Figure 2 show the kidney and its vasculatures (A) and the renal parenchymal phase of the angiogram (B), respectively. The opacification seen in both figures are indications of normal renal anatomy and renal function.
Embolization was performed by injection of the embolization suspension containing the PHB microspheres through a 5-F catheter in two steps. Each step was followed with an angiograph to examine the effectiveness of the embolization. At the first step, a 4 mL suspension containing 10 mg of the PHB microspheres was found sufficient to slow the renal arterial blood flow with subsequent partial occlusion of the pre-capillaries. The angiograph following the injection showed a decrease in the vascularity of the lower pole of the kidney, accumulation of the contrast agent inside the kidney and that no contrast agent reached the renal venous which indicated embolization of lower branches of the intrarenal arteries (Figure 3).

In the second step of embolization, a small amount of the particle suspension (2 mL) was injected to complete the embolization. The angiogram obtained after injection showed neither passage of the contrast material through the renal artery toward the kidney nor distribution through the parenchyma, but reflux of the contrast material (Figure 4).
Histopathological Evaluations

Histopathological examination of the kidneys were carried out in the samples taken 24 hours and 7 days after embolization (Figure 5). All the embolized kidneys showed pathological changes associated with kidney infarction due to renal artery obstruction and blockage of the blood supply to the kidneys. Abnormal gross appearances such as wedge-shaped kidney enlargement with a pale appearance and a white area on the surface were observed. The cross sections of the embolized kidneys showed a renal cortical necrosis complicated by hemorrhage; the pale necrotic areas with hemorrhage margins (Figure 6).

The microscopic examinations of the sections taken from the embolized kidneys indicated necrosis of both the tubule and glomerular cells. Tubule cells were more sensitive to embolization than the glomerular cells. The sections obtained 24 hours after embolization exhibited early necrosis of the tubule cells and a chronic inflammatory infiltrate.
Figure 6. Representative cut sections of the embolized kidneys: (a) 24 h after embolization; (b) 7 days after embolization.

Figure 7. Photomicrographs of the sections taken from the embolized kidneys compared with the normal kidneys 24 h after embolization.

Figure 8. Photomicrographs of the sections taken from the embolized kidneys compared with the normal kidneys 7 days after embolization.

stimulated presumably by products of autolysis, while glomerular cells did not (Figure 7). The sections obtained from the kidney 7 days after embolization showed complete necrosis of both the tubule and glomerular cells in the embolized kidney (Figure 8).

Microscopic examination of the vasculated sections revealed fresh thrombus formation and distorted morphology of the PHB microspheres within the renal-blood vessels (Figure 9). There was no evidence of any inflammatory reaction tissue toxicity due to the PHB microspheres.

Based on the successful embolizations and the pathological finding re-
ported above, it was concluded that PHB microspheres have potential as embolization and/or chemoembolization agents. Further animal studies and subsequent clinical applications are being worked on now.

REFERENCES


