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In vitro release of insulin and biocompatibility of in situ forming gel systems

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Abstract

The purpose of this study was to develop single-dose insulin delivery system based on in situ forming gel to provide basal insulin level for a prolonged period. The in situ forming gel formulation was prepared by dissolving poly(D,L-lactic acid) (PLA) in hydrophobic (benzyl benzoate) and hydrophilic (benzyl alcohol) solvent mixtures. In vitro release was carried out in phosphate buffered saline (PBS) (pH 7.4) and the amount of released insulin was quantified by MicroBCA assay. In vivo biocompatibility study of in situ forming gel system was based on the histological evaluation of the tissue samples retrieved from injection sites at different time points. The tissue reaction was evaluated over 12 weeks. Throughout this period, all formulations showed normal inflammatory and foreign body reactions characterized by the presence of macrophages, fibroblasts and foreign body giant cells. Neither necrosis nor tissue damage could be identified. At the end of 12 weeks, no distinct histological differences were observed in comparison to the control tissue samples. The comparable results between blank and insulin-loaded in situ forming gel system indicated that the insulin itself did not induce additional inflammatory reactions. The results suggested that in situ forming gel system was biocompatible.

Keywords: Biocompatibility; Poly(lactic acid); In situ forming gel; Insulin; Benzyl benzoate; Benzyl alcohol

1. Introduction

Diabetes mellitus leads to complications such as blindness, end-stage renal disease, neuropathy, foot or leg amputation, coronary artery disease, stroke, etc. (Steil, 1999). Strict glycemic control is an effective way to reduce the incidence and progression of the above complications. Insulin replacement therapy provides better chance of glycemic control. However, it has been a challenge to supply exogenous insulin that mimics the physiological secretion found in non-diabetic populations (Riddle, 1983).

Current insulin replacement therapy requires multiple insulin injections daily to mealtime and basal insulin for type 2 and many type 1 diabetes patients. Much effort has been made to develop fast-acting insulin to meet the mealtime insulin requirement

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(Brange, 1997). To date, less progress has been made on basal insulin supply. Although the basal insulin level is low, it plays an important role. The continuous low-level basal insulin secretion into portal circulation modulates the rate of overnight hepatic glucose secretion and glucose output during prolonged periods between meals. It reduces hepatic glucose production but allows for circulating glucose levels sufficient for cerebral energy production at bedtime. In this way, basal glucose levels are maintained constant and within a narrow concentration range (Galloway and Chance, 1994). More recently, type 2 diabetes mellitus treatment has been reassessed due to recognition of the basal and postprandial components of hyperglycemia in tandem with other clinical research. In the future, basal insulin supplement will be used earlier. Basal insulin therapy with combination of oral agents can provide a smoother transition to insulin and reduce the risk of loss of glycemic control (Riddle, 2004).

The current insulin formulations for basal insulin supply still require one to two injections daily. To decrease injection frequency of basal insulin supply, several research groups have investigated the potential use of biodegradable polymers as a sustained–release carrier. Poly(lactic acid) (PLA) and its copolymers with poly (glycolic acid), poly(D,L-lactic/glycolic) acids (PLGA), have received wide attention in recent years as excipients for sustained release of parenteral drugs. The choice of PLA and PLGA as polymers for sustained–release formulations originated from their use in the medical device industry to make bioabsorbable sutures. PLA is more hydrophobic than PLGA, which should provide drug release for longer duration due to its slower degradation. Triblock copolymer PLGA–PEG–PLGA has been studied as a delivery system for continuous release of insulin (Kim et al., 2001; Choi and Kim, 2003). Insulin-loaded PLGA microspheres could deliver insulin at 36.2–140.7 μU/ml level within 9 days after single dose subcutaneous administration (Takenaga et al., 2002). Pluronic F-127 gels and its combination with PLGA nanoparticles have been evaluated for controlled insulin delivery (Barichello et al., 1999).

In situ forming gel system provides various advantages such as depot formation without surgery, easy drug loading and dose adjustment. Biocompatibility of polymeric delivery systems is of utmost importance for clinical application. A desirable response of an implanted biomedical polymer would show a short-lived inflammatory response with a minimum of fibrosis resulting from the normal healing response of wounds (Galloway and Chance, 1994). In this study, we investigated in situ forming gel for controlled delivery of insulin to meet basal insulin level requirement. The biocompatibility of delivery system was also investigated.

2. Materials and methods

Human recombinant insulin was obtained by Serologicals Corporation (Norcross, GA). PLA (intrinsic viscosity 0.19 dl/g) was purchased from Birmingham Polymer Inc. (Birmingham, AL). Benzyl benzoate and benzyl alcohol were obtained from Sigma Chemical Company (St. Louis, MO). MicroBCA Kit was purchased from Pierce (Rockford, IL). All the other reagents were of analytical grade.

2.1. Preparation of insulin–PLA solution

Benzyl benzoate or the mixture of benzyl benzoate and benzyl alcohol was used as solvent(s) to dissolve PLA. The sealed vial containing the known amount of PLA and solvent(s) was placed in a shaker water bath overnight at 37 °C to dissolve the polymer. Insulin powder was added in the PLA solution and mixed by homogenization at 8000 rpm for 2 min. All formulations were prepared with insulin loading at a level of 4% (w/v). Compositions of formulations are given in Table 1. The formulation was readily injectable through 21-gauge needle.

2.2. In vitro release of insulin from in situ forming gel

The insulin–polymer–solvent(s) solution (0.5 ml) was injected through a 1-ml syringe with a 21-

Table 1
Insulin-loaded in situ forming gel formulations

Insulin (%, w/v)	PLA (%, w/v)	BB (%, v/v)	BA (%, v/v)
4	15	85	15
4	15	95	5
4	15	100	0
4	30	95	5
4	30	100	0

BB, benzyl benzoate; BA, benzyl alcohol.

gauge needle directly into a vial containing 10 ml of phosphate-buffered saline (PBS, pH 7.4, 0.05%, w/v, NaN_3). The system turned into a gel in the buffer. In vitro release study was carried out by placing these vials in a water bath (Model 50 Reciprocal Shaking Water Bath, Precision Scientific, Winchester, VA) maintained at 37 °C and shaken at 40 rpm. At predetermined time intervals, 4 ml of the release buffer was sampled after gentle mixing. Thereafter, 4 ml of the fresh buffer was added to the vials in order to maintain constant volume of release medium. Insulin content in release buffer was quantified by MicroBCA assay (Smith et al., 1985). Three replicates were carried out for each formulation.

2.3. In vivo biocompatibility of in situ forming gel

New Zealand white rabbits were chosen to evaluate the biocompatibility of the in situ forming gel formulations. Five rabbits were included in this study. One rabbit without receiving injection was used as the control. Four rabbits were injected blank and insulin-loaded in situ forming gel formulations at different area of the back region. At each time point of 1, 4, 8 and 12 weeks after injection, one rabbit was euthanized by injecting an overdose of Nembutal (100 mg/kg body weight). Tissue samples from the injection sites were removed using a scalpel. Samples were evaluated by light microscopy.

2.3.1. Light microscopy

Tissue samples were fixed by immersion into 10% buffered formalin solution and then embedded in paraffin. Transverse sections (5 μm) were cut using a microtome. Slides of the skin sections were prepared and stained with hematoxylin and eosin. The slides were observed under light microscope (Meiji Microscope, Osaka, Japan).

3. Results and discussion

3.1. In vitro release of insulin from in situ forming gel

Fig. 1 shows the in vitro release profile of insulin from in situ forming gel formulations. Three formulations containing 15% (w/v) PLA showed large burst (35–50%) followed by a prolonged period of little

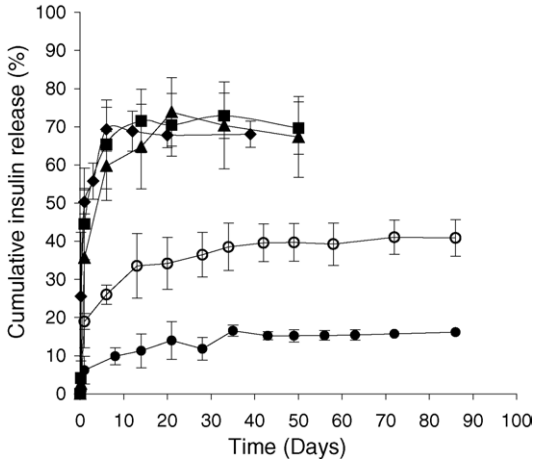

Fig. 1. In vitro release of insulin from in situ forming gel with different solvent compositions and polymer D,L-PLA concentrations. Key: (♦) BB:BA = 85:15%, D,L-PLA 15%; (■) BB:BA = 95:5%, D,L-PLA 15%; (▲) BB = 100%, D,L-PLA 15%; (◯) BB:BA = 95:5%, D,L-PLA 30%; and (●) BB = 100%, D,L-PLA 30%.

insulin release up to 50 days. We did not observe significant difference in release profiles by changing the ratio of benzyl benzoate and benzyl alcohol in the solvent systems. Once the PLA concentration was increased to 30%, a significant decrease in burst was observed. Increasing the benzyl benzoate from 95 to 100% in solvent system led to further decrease in the burst release.

Many factors have been reported to affect the drug release from in situ gel forming systems. These factors include polymer properties (e.g., molecular weight, hydrophobicity, co-monomer ratio) (Ravivarapu et al., 2000; Eliaz and Kost, 2000), polymer concentration (Duenas et al., 2001), drug loading (Eliaz and Kost, 2000), and solvents used (Brodbeck et al., 1999). It was found that solvent/non-solvent affinity played a key role that governed the protein release from this system (Brodbeck et al., 1999). For a water-miscible solvent like *N*-methyl-2-pyrrolidone (NMP), a relatively faster phase inversion was found, resulting in a porous, rubbery gel structure. Its release profile was characterized by an initial burst followed by much slower release rate for a long period. On the other hand, solvents that have low solvent/non-solvent affinity such as triacetin and ethyl benzoate gave a relatively slower phase inversion, resulting a less porous, more fluid structure matrix, which released protein more uniformly. A solvent system composed of both hydrophilic (e.g., NMP,

ethanol, and benzyl alcohol) and hydrophobic (e.g., benzyl benzoate, miglyol, and tiracetin) components was proposed (Cleland, 2001). The hydrophilic solvent leaves the gel upon injection, resulting in a shell around the exterior of the depot, whereas the hydrophobic solvent slows the water penetration and therefore decreases the polymer hydrolysis. Insulin is expected to release from the gel formulations by a combined mechanism of diffusion and polymer erosion (degradation). At the early stage, insulin released from gel formulations in a way mainly depending on the diffusion process. A consistent and complete release in vivo due to the progressive structural erosion of the PLA is expected. However, due to the complexity of polymer degradation, in vitro studies may not predict the in vivo performance of formulations. We found higher polymer concentration, in this study, led to lesser burst and extended release in vitro due to higher viscosity of gel depot.

We demonstrated in our previous studies that the phase sensitive smart polymer-based delivery systems completely released a model protein, lysozyme (Singh and Singh, 2004). However, it is not true for insulin. Almost all the formulations showed incomplete release of insulin. Formation of aggregates for another protein, erythropoietin, caused an incomplete release of the protein from the delivery systems (Lu and Park, 1995a,b; Bittner et al., 1998; Morlock et al., 1998). Therefore, further studies such as gel electrophoresis (SDS-PAGE) and size-exclusion chromatography (SEC) need to be performed in order to determine the formation of insulin aggregate (Van de Weert et al., 2000).

3.2. Biocompatibility

Since the formulation with 30% PLA in solvent mixture of benzyl benzoate/benzyl alcohol (95/5%, v/v) showed better control on the insulin release. The above formulation was selected for the biocompatibility studies. The inflammatory responses in rabbits were evaluated up to 12 weeks after subcutaneous injection of the formulation. The histological evaluation was performed using light microscopy.

Figs. 2 and 3 show light micrographs of the tissue responses after 1 week of subcutaneous injection of in situ forming gel formulation. A large number of macrophages and a few polymorphonuclear (PMN) cells were observed infiltrating into the connective tissue. The presence of large number of macrophages indicates a chronic inflammatory reaction in response to the foreign body invasion and injury (Anderson and Shive, 1997). Similar inflammatory reactions were observed with blank and insulin-loaded formulations.

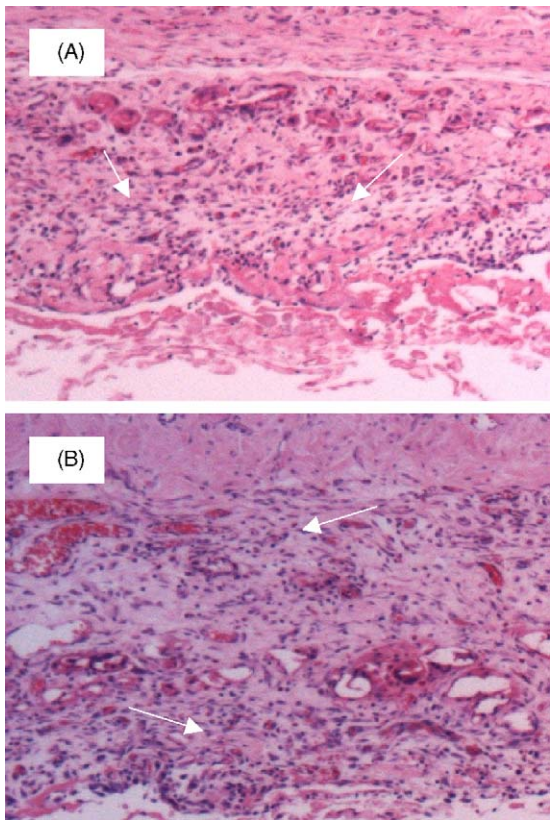

Fig. 2. Light photomicrograph of subcutaneous tissue 1 week after subcutaneous injection of blank (A) and insulin-loaded (B) in situ forming gel formulation, showing inflammatory responses to the implants, represented by the inflammatory cells (indicated by arrows) surrounding the tissues. Original magnification 5×.

Figs. 4 and 5 show the tissue responses after 4 weeks of subcutaneous injection of the in situ forming gel formulation. Compared to those tissue samples after 1 week, less inflammatory cells were identified. The in situ forming gel was broken into small pieces and surrounded by macrophages and foreign body giant cells.

Fig. 6 shows the tissue responses after 8 weeks of subcutaneous injection of the formulation. A small number of macrophages were identified in the tissue. No signs of cell damage such as rough endoplasmic

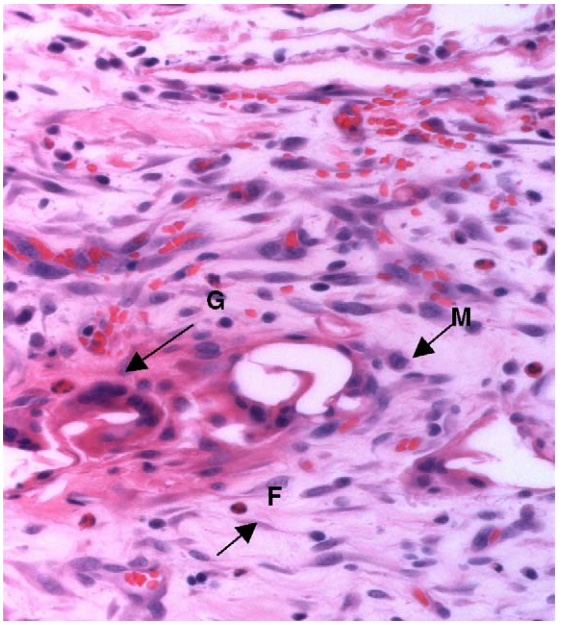

Fig. 3. Light photomicrograph of subcutaneous tissue 1 week after subcutaneous injection of insulin-loaded in situ forming gel formulation. The inflammatory cells are mainly macrophages (M) and foreign body giant cells (G). Original magnification 40×.

reticulum or swollen mitochondria were observed in any of the tissue samples after 12 weeks of injection (Fig. 7). Normal connective and muscular tissue were seen in all samples, which were highly comparable to the control tissue sample (Fig. 8).

Both the polymer and solvent used in drug delivery systems play an important role in the toxicity to the body (Royals et al., 1999). The preferred solvents used for administration of drugs are isotonic sodium chloride solution, glucose solution (5% glucose) and distilled water. However, some organic solvents are also used due to the poor water solubility of some polymers or active agents. For in situ forming gel system, organic solvents such as NMP and dimethyl sulfoxide (DMSO) have been used to dissolve polymer PLGA. The tissue response results suggested that these delivery system using NMP and DMSO are considered acceptable for use as injectable implant systems (Royals et al., 1999).

Organic solvents were used to dissolve polymer PLA and were injected subcutaneously to form semisolid gel depot. The organic solvent used were benzyl benzoate and benzyl alcohol in a ratio of 95:5% (v/v) and the total injection volume was 0.5 ml. Ben-

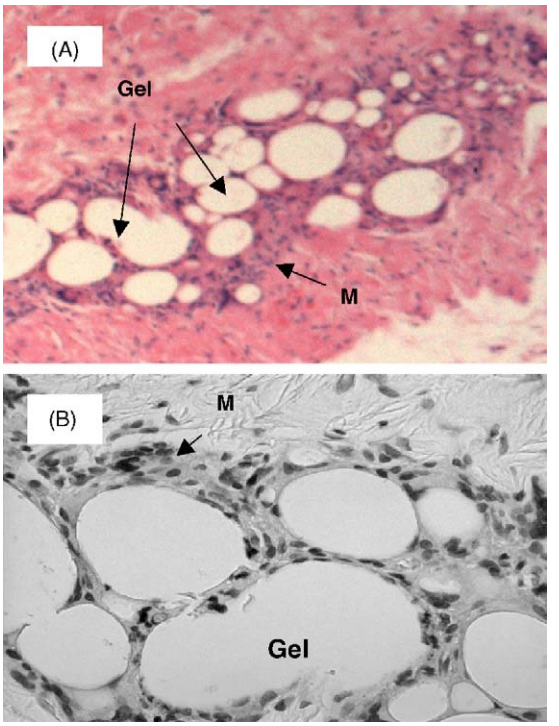

Fig. 4. Light photomicrograph of subcutaneous tissue 4 weeks after subcutaneous injection of blank in situ forming gel formulation. (A) Original magnification 5×; (B) original magnification 40×. Gel beads were surrounded by macrophages (M).

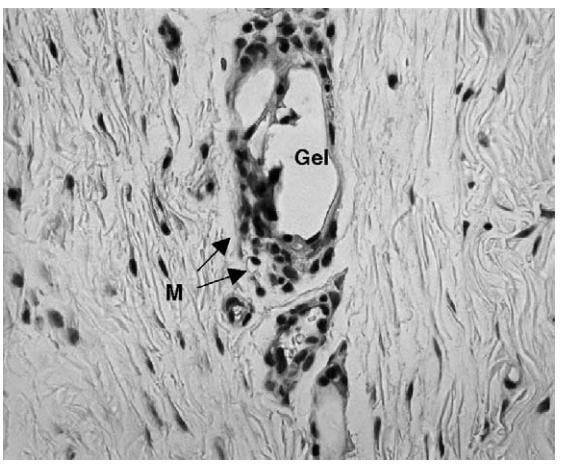

Fig. 5. Light photomicrograph of subcutaneous tissue 4 weeks after subcutaneous injection of insulin-loaded in situ forming gel formulation. Gel beads were surrounded by macrophages (M). Original magnification 40×.

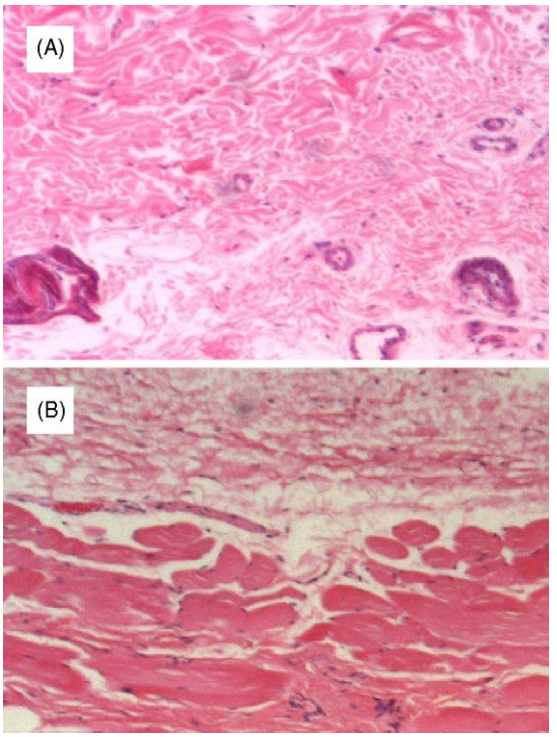

Fig. 6. Light photomicrograph of subcutaneous tissue 8 weeks after subcutaneous injection of blank (A) and insulin-loaded (B) in situ forming gel formulation. A few macrophages (M) were identified. Original magnification 5×.

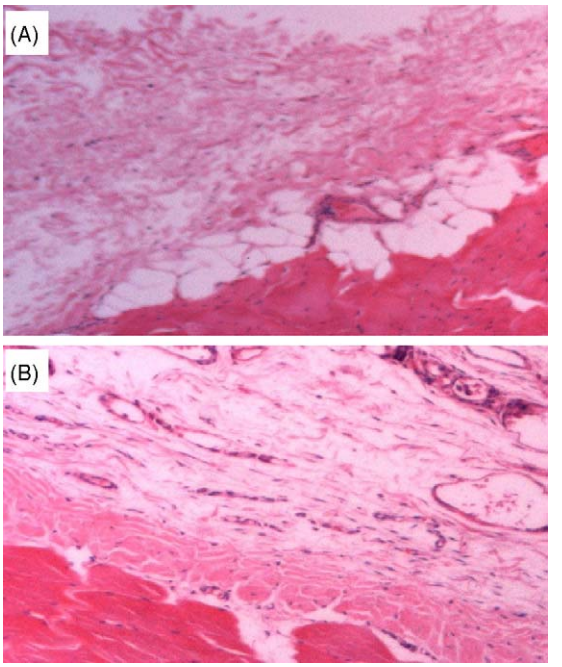

Fig. 7. Light photomicrograph of subcutaneous tissue 12 weeks after subcutaneous injection of blank (A) and insulin-loaded (B) in situ forming gel formulation. No significant inflammatory cells were identified. Original magnification 5×.

zyl alcohol is used in a wide variety of pharmaceutical formulations, including oral and parenteral preparations, as a antimicrobial preservative. The acute oral and skin LD_{50} values in rabbits for benzyl alcohol are 1.58 and 2.0 g/kg, respectively. The LD_{50} values in rats for oral, IP and IV routes are 1.23, 0.4 and 0.06 g/kg, respectively (Wade and Weller, 1997). In this study, the amount of BA injected subcutaneously in rabbits was 0.025 g per rabbit (∼3 kg body weight), equivalent to 0.008 g/kg. This value is well below the reported acute LD_{50} toxicity values. As for benzyl benzoate, it has been used as a solubilizing agent and nonaqueous solvent in intramuscular injections at concentrations between 0.01 and 46% (v/v) (Spiegel and Noseworthy, 1963). It is also widely used as a 25% (v/v) topical therapeutic agent in the treatment of scabies and as an excipient in intramuscular injections and oral products. The oral LD_{50} in rabbits is 1.68 g/kg (Wade and Weller, 1997). In this study, the amount benzyl

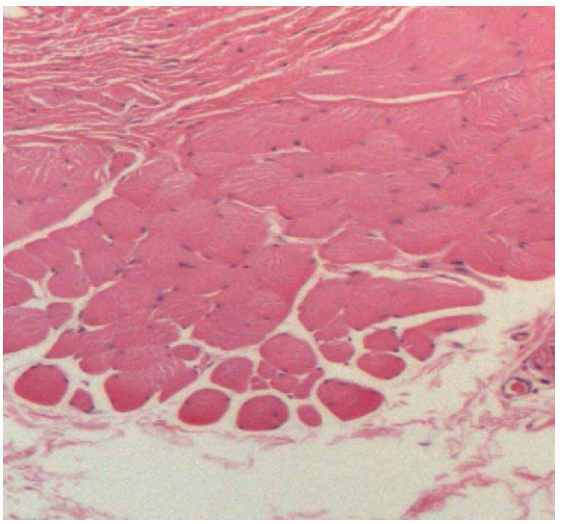

Fig. 8. Light photomicrograph of subcutaneous tissue from control tissue sample. No inflammatory cells were identified. Original magnification 5×.

benzoate injected subcutaneously in rabbits was about 0.18 g/kg. We would not anticipate any systemic toxicity due to the small amount of solvent(s) used in gel formulations.

The primary concern in this study is the local tissue responses instead of systemic toxicity to the polymer formulations. Histological evaluation of tissue samples retrieved at different time points within 3 months showed a typical foreign body and wound healing responses. There is always trauma at the implant site due to injection. An inflammatory response to tissue injury, even in the absence of the implant, would start and proceed through the steps of wound healing. This response is considered as components of the tissue or cellular host reaction to injury (Anderson and Shive, 1997). The first phase of inflammation is acute inflammation characterized by the presence of polymorphonuclear lymphocytes. They usually disappear in 24–48 h. Chronic inflammatory response is the next phase of reaction. Monocytes migrate into the site of injury and differentiate into macrophages. The acute and chronic inflammatory reactions are generally similar regardless of the degradation rate of polymer and may contain components of injury caused by the injection itself (Anderson and Shive, 1997). Macrophage is the predominant cell type in the tissue reaction to the polymer devices. Since macrophages initiate a cascade of responses, infiltration of macrophages towards the implantation site is important. Macrophages can secrete polypeptide hormones and enzymes, which are of importance for the sequence of events during wound healing process (Ziats et al., 1988). Macrophages also play an essential role in the phagocytosis of the polymer and its degradation in vivo.

4. Conclusions

We investigated in situ forming gel systems for their potential use to deliver basal insulin. Insulin release, especially the burst, from in situ forming gel can be readily modulated by controlling the composition of solvent system and polymer concentration. Histological studies of tissue samples from the injection sites showed an initial mild tissue irritation response and the inflammatory reaction diminished with time of implantation. Insulin itself did not induce additional inflammatory reactions from the in situ forming gel systems based on the similar histological observations between control (i.e., delivery system without insulin) and insulin-loaded systems.

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