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Controlled release of growth hormone from thermosensitive triblock copolymer systems: *In vitro* and *in vivo* evaluation

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Abstract

The purpose of this study was to design injectable controlled release polymer formulations for growth hormone using triblock copolymer PLGA–PEG–PLGA (MW 1400–1000–1400). Porcine growth hormone (pGH) formulations were prepared by adding pGH into 30% (w/v) aqueous solution of triblock copolymer. pGH concentrations in the released samples were determined using a standard MicroBCA method. *In vitro* release studies demonstrated that there were no initial burst of pGH from both formulations containing a low dose (0.12%, w/v) and a high dose (0.42%, w/v) of pGH. *In vivo* absorption study of pGH in rabbits showed that constant serum levels of exogenous pGH (3–7 ng/mL from high dose and 2–4 ng/mL from low dose) were detected for nearly 4 weeks from delivery systems upon single subcutaneous injection. The absolute bioavailability of pGH enhanced from the thermosensitive polymer-based systems, which was ∼5–15-fold those of subcutaneous aqueous solution. MTT assay and light microscopy were used to investigate the *in vitro* and *in vivo* biocompatibility of thermosensitive polymer delivery systems, respectively. Both *in vitro* and *in vivo* results support the biocompatible nature of these polymer delivery systems. Thus, the triblock copolymer used in this study was able to control the release of incorporated pGH *in vitro* and *in vivo* for longer duration and the delivery system was biocompatible.

Keywords: Controlled release; Protein; Thermosensitive polymer; Biocompatible; Growth hormone; Subcutaneous

1. Introduction

Exogenous, pituitary-derived human growth hormone (hGH) was first used for the treatment of short stature in a child with growth hormone deficiency (GHD) in the early 1950s. Subsequently, the availability of recombinant hGH (rhGH) has contributed to an increasing use of GH therapy in a variety of indications, including pediatric GHD, adult GHD, chronic renal insufficiency, Turner's syndrome, and cachexia, secondary to AIDS (Capan et al., 2003; Mahan, 2006). The main disadvantage of current rhGH therapy is its short half-life, which necessitates for multiple injections; thus rhGH is an ideal therapeutic agent for controlled release formulation. A reduction in frequency of administration would greatly benefit these patients in terms of quality of life and compliance as well as potentially increased efficacy.

The development of a sustained release formulation of rhGH has focused on depot preparations such as microsphere (Johnson et al., 1996; Cleland et al., 1997a; Reiter et al., 2001; Capan et al., 2003; Kim et al., 2006; Wei et al., 2007), gel (Katakam et al., 1997; Brodbeck et al., 1999; Okumu et al., 2002), and implant (Garcia et al., 2002; Santovena et al., 2006). Entrapment of proteins in biodegradable poly(lactide-*co*-glycolide) (PLGA) microspheres has been widely investigated as a technique to produce sustained release formulations for GH administration. However, obtaining suitable GH release kinetics and preservation of GH integrity are still a problem in the PLGA system because most proteins do not maintain full activity when exposed to an organic solvent/water interface during preparation of the formulation (Cleland et al., 1997b; Kim and Park, 2001; Takada et al., 2003), meanwhile, there is considerable concern about the effects of PLGA on GH stability (Cleland and Jones, 1996; Johnson et al., 1997; Kim and Park, 1999; Yamagata et al., 2003). In 1999, the US FDA approved a PLGA microsphere formula-

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tion, Nutropin Depot®, as a once a month alternative to daily injections of hGH. However, the most frequent adverse reactions for Nutropin Depot® were injection-site reactions, which occurred in nearly all patients. Once injected, microsphere formulations may cause an acute tissue reaction (e.g. nodule), and possible transient irritation resulting from the presence of particles. Thus, development of solventless and painless methods to produce delivery systems is clearly of significant interest. An envisaged method would be to incorporate the protein directly into aqueous polymer solution by simple mixing, without the use of heat or solvents, followed by a conventional subcutaneous or intramuscular injection.

It is generally known that polymers bearing both hydrophilic and hydrophobic parts in their molecular structure exhibit thermosensitive properties and usually have a lower critical solution temperature (LCST) in aqueous solution. The LCST of such thermosensitive polymers in water results from a delicate balance between the hydrophilicity and hydrophobicity within the polymer molecules (Wang et al., 1999; Jeong and Gutowska, 2002). Thermoreversible block copolymers composed of poly(ethylene glycol) (PEG) (A) and biodegradable polyesters (B), such as polylactide (PLA), polyglycolide (PGA), PLGA, poly(ε-caprolactone) (PCL) and poly(ethylene terephthalate) (PET) were studied as controlled release drug carriers (Cohn and Younes, 1988; Cerrai et al., 1989; Li and Kissel, 1993). PEG/PLGA hydrogels are particularly attractive systems for pharmaceutical applications since they are biodegradable and generally have a good safety profile (Hatefi and Amsden, 2002; Huh et al., 2003; Packhaeuser et al., 2004). Compared to the widely utilized PLGA, protein delivery systems based on hydrophilic–hydrophobic block copolymers may have some important advantages. Incorporation of hydrophilic blocks in a hydrophobic polymer can be utilized to modify the degradation rate as well as the permeability of the matrix, leading to release kinetics which can be readily modulated by adjusting the copolymer composition. Their compositions can be tailored to provide drug delivery over weeks or months after single injection (Matsumoto et al., 1999; Chen and Singh, 2005a,b).

The objective of this study is to investigate the suitability of PLGA–PEG–PLGA (MW 1400–1000–1400) triblock copolymer as a matrix material for a controlled release system for proteins. Our previous study already showed this copolymer can control the release of a model protein, lysozyme, in biologically active form for about 1 month (Chen et al., 2005). In this study, we prepared the triblock copolymer-based delivery system for a therapeutic protein (pGH) and investigated its *in vitro* release and *in vivo* absorption in rabbits as well as *in vitro* and *in vivo* biocompatibility of the delivery system.

2. Materials and methods

2.1. Materials

PLGA–PEG–PLGA (MW 1400–1000–1400) was synthesized and characterized in our laboratory. MicroBCA protein assay reagent kit was purchased from Pierce Biotechnology, Inc. (Rockford, IL). pGH was obtained from Sigma (St. Louis, MO). pGH ELISA kit was purchased from Linco Research Inc. (St. Charles, MO). All other chemicals used were of analytical grade.

2.2. In vitro release of pGH

pGH (low dose 0.12% (w/v) and high dose 0.42% (w/v)) was added to 30% (w/v) triblock copolymer PLGA–PEG–PLGA (MW 1400–1000–1400) aqueous solution and homogenized at 8000 rpm for 30 s at room temperature to form a homogeneous clear solution. A 1-mL polymer solution formulation of pGH was injected into a 5-mL test tube and transferred to water bath maintained at 37 °C for 10 min to form a gel. A 2.5 mL of phosphate buffer saline (pH 7.4) containing NaN_3 (0.025%, w/v) was added to the tube as release medium. The tube containing gel was kept in a reciprocal shaking water bath maintained at 37 °C and 35 rpm for the entire period of study. The releasing medium was withdrawn and replaced with the same amount of fresh releasing media at intervals. The amount of pGH in the released samples was determined by MicroBCA protein assay method using the microplate described in previous study (Chen et al., 2005).

2.3. In vivo absorption of pGH in rabbits

New Zealand White (NZW) rabbits (Oryctolagus cuniculus), 10 weeks old and about 2.0–3.0 kg body weight, were used in the study. All the animal experiments were performed according to the protocols approved by Institutional Animal Care and Use Committee (IACUC) of North Dakota State University. The rabbits were anesthetized by 30 mg/kg pentobarbital sodium (Nembutal®) administered intraperitoneally. A 1-mL thermosensitive polymer formulation containing a high or low dose of pGH was injected subcutaneously at the back neck of the rabbits. At designated times, the blood samples were taken, and serum pGH levels of the rabbits were analyzed by a commercially available RIA kit (Porcine/Canine growth hormone RIA kit). The RIA kit utilizes ^{125}I-labeled pGH and a pGH antiserum to determine the level of pGH in serum. pGH aqueous solution without polymer was also administered intravenously and subcutaneously as controls. At the end of the experiment, the rabbits were euthanized by administering pentobarbital (100 mg/kg body weight) intravenously through the ear marginal vein and the tissue sample was taken for biocompatibility study.

2.4. In vitro biocompatibility

2.4.1. Preparation of extracts

The thermosensitive polymer-based delivery systems (without drug) were extracted by injecting 1 mL of the solution in PBS (pH 7.4) and maintaining for 10 days at both 37 °C and 70 °C because polymers degrade faster at elevated temperatures. The later extract should simulate the long-term effects of an *in situ*, gel-formed implant (artificial ageing extract). After an incubation period of 10 days, the pH of the extracts was measured and adjusted to 7.4 with NaOH. The extracts were filtered through a 0.2 μm filter and handled aseptically. The extracts were diluted

with double concentrated growth medium to a series of ratios (1:1 to 1:16).

2.4.2. MTT assays

The *in vitro* biocompatibility of the delivery systems was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The above extracts of the polymer materials were prepared and cultured with cell line to determine the mitochondrial succinate dehydrogenase activity. This assay is based on the ability of living cells to reduce a water-soluble yellow dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a water-insoluble purple formazan product by mitochondrial succinate dehydrogenase. Briefly, BALB/3T3 cells (Mouse embryonic fibroblast cell, 8000 cells/well) were plated into 96-well microtiter plates. After 24 h plating, 100 μL/well of a freshly prepared dilution series of the extracts was added. Medium produced in the same way without addition of polymer extract provided the negative control (PBS, pH 7.4), while 2% dimethyl sulphoxide (DMSO) in growth medium served as positive control. At least eight replicates were prepared for each dilution and for the positive and negative controls. The extracts were exposed to the cells for 24, 48, and 72 h at 37 °C in a humidified 5% CO_2 atmosphere. Ten microliter per well of an MTT solution (5 mg/mL in PBS, pH 7.4) was added and incubated for 4 h. Formazan crystals formed was dissolved by adding 100 μL per well of a freshly prepared acidified isopropanol. The colorimetric staining of the plates was evaluated on a multiwell plate reader at 570 nm. To ensure that the polymers themselves do not contribute to the reduction of the dye, polymer solutions alone were assayed in a microtiter plate by the MTT assays (Ignatius and Claes, 1996). Absorbance readings obtained were indicative of viability of cells.

2.5. In vivo biodegradability and biocompatibility of drug delivery systems

The biodegradability of the system was evaluated by injecting subcutaneously 1 mL of formulation (with or without pGH) into the upper portion of neck of rabbits. A visibly apparent gel lump was formed immediately which was monitored regularly. At the end of the study, the injection sites were surgically removed and examined visually. Briefly, representative tissue samples were excised and fixed in 10% neutrally buffered formalin solution (Accustain®). Test samples were washed with water to remove excess fixative. Then, the samples were dehydrated by transferring successively to increasing strengths of alcohol, cleared by passing through toluene. Finally, the samples were infiltrated and embedded in molten paraffin (Accumate™), cooled at room temperature and paraffin blocks were prepared. Sections of 10 μm thick were cut by rotatory microtome, mounted on a glass slide with the help of glycerogelatin, stained with chrysoidine, hematoxylin and counter stained with eosin. At last, they were dehydrated, cleared, and cover slipped (Humasons, 1972). The slides were examined under light microscope (Olympus) and photographed using an attached digital camera (SPOT RT, Diagnostic Instruments, Sterling Heights, MI) for the presence of any signs of chronic inflammation, granulation tissue, foreign body giant cells, and fibrous capsule formation (Voskercian et al., 2003). A stage micrometer was used to calibrate the object and the scale bar was inserted on to the micrograph.

2.6. Data analysis

The results were expressed as a mean ± S.D. ($n \geq 3$). Statistical comparisons were made using Student's *t*-test and one-way analysis of variance (ANOVA). The probability value of less than 0.05 was considered as significant. Pharmacokinetic analysis was based on the AUC data. The area under the serum pGH concentration versus time curve ($AUC_{0-\infty}$) was determined by means of trapezoidal rule. The following equations were used to calculate each AUC segment including the last segment

$$AUC_{0-t} = \sum_{i=0}^{n-1} \frac{t_{i+1} - t_i}{2}(C_i + C_{i+1}) \quad (1)$$

$$AUC_{0-\infty} = AUC_{0-t} + \frac{C_n}{k_{el}} \quad (2)$$

where C_n is the last concentration, and the overall elimination rate constant (k_{el}) was calculated from the slope of the terminal elimination phase of a semilogarithmic plot of concentration versus time after subjecting it to linear regression analysis. The absolute bioavailability of pGH from gel formulations and subcutaneous solutions in comparison to reference (IV solution) was calculated by dividing their AUC with that of IV solution.

3. Results and discussion

3.1. In vitro study

Fig. 1 displays the results of pGH release from hydrogel depot *in vitro*. There is no initial burst of pGH from both high dose and low dose polymer formulations. The releases of pGH from hydrogel formulations show constant release kinetics with the duration of 28 days. With higher dose of pGH, the release profile exhibited greater amount of the protein release. After 4 weeks, both lower and higher dose formulations reached plateau which means that higher dose formulation had an incomplete release of pGH.

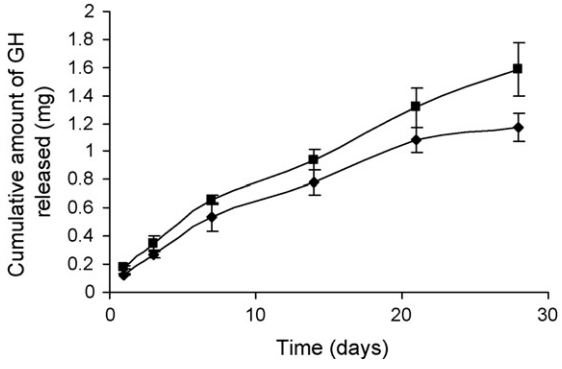

Fig. 1. *In vitro* release of growth hormone from thermosensitive polymer formulations (copolymer, 30%, w/v). Key: (♦) low dose (0.12% GH) and (■) high dose (0.42% GH).

In drug delivery systems, bioactive molecules can be physically entrapped in a hydrogel or chemically attached to the polymeric network (Molina et al., 2001). In the case of physical entrapment, the hydrogel is loaded with a drug to form supersaturated solutions or suspensions of particles. Depending on the hydrogel structure, the drug can be released through different mechanisms. A matrix-type system usually leads to a first order release profile, whereas a reservoir-type system leads to zero-order or pseudo-zero-order release kinetics, the most common mechanism for passive diffusion controlled by the concentration gradient between the device and the surrounding aqueous medium (Molina et al., 2001). If the hydrogel is degradable, drug release can be driven by both diffusion and degradation phenomena (Sawhney et al., 1993). Although there was no clear evidence for the mechanism of GH release from the gel, erosion of the gel and possibly some diffusion seem to be the predominant mechanisms (Katakam et al., 1997; Zentner et al., 2001; Qiao et al., 2005). The release profiles shown in Fig. 1 which is close to zero-order profile would be expected by the gel erosion. However, the less than perfect shape of the profile could be resulting from additional mechanisms, where diffusion of GH from the bottom layer may take longer than from the preceding layer (Katakam et al., 1997).

A concentrated aqueous solution of the GH forms within the network, permitted rapid aggregation of the GH (Pitt, 1993). All of the reactions, like primarily covalent cross-linking, deamidation, and peptide cleavage, could be involved in the decomposition of GH. Aggregation of pGH leading to insoluble precipitates has been reported *in vitro* experiments (Fan et al., 1996). The irreversible precipitation of GH was concentration-dependent. At high concentrations, the hormone was highly susceptible to aggregation (Hageman et al., 1992). This aggregated protein, which was not redissolved by dilution into PBS, may be responsible for the incomplete release of GH. Fan et al. (2000) reported that GH concentration in solutions affected the rate of hormone aggregation and degradation and GH was more likely to form aggregates when at high concentration, the formation of a covalent dimeric species with a MW of 44,000 Da was also confirmed by electrophoresis.

3.2. *In vivo* absorption of pGH in rabbits

Fig. 2 shows the standard curve for pGH ($Y = -19.871 \ln X + 96.169$, $R^2 = 0.9784$). Fig. 3a–c shows

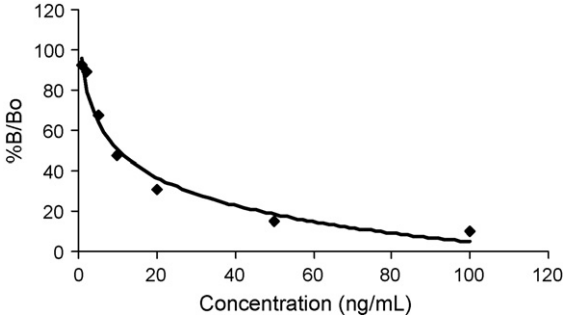

Fig. 2. Calibration curve for growth hormone *in vivo*.

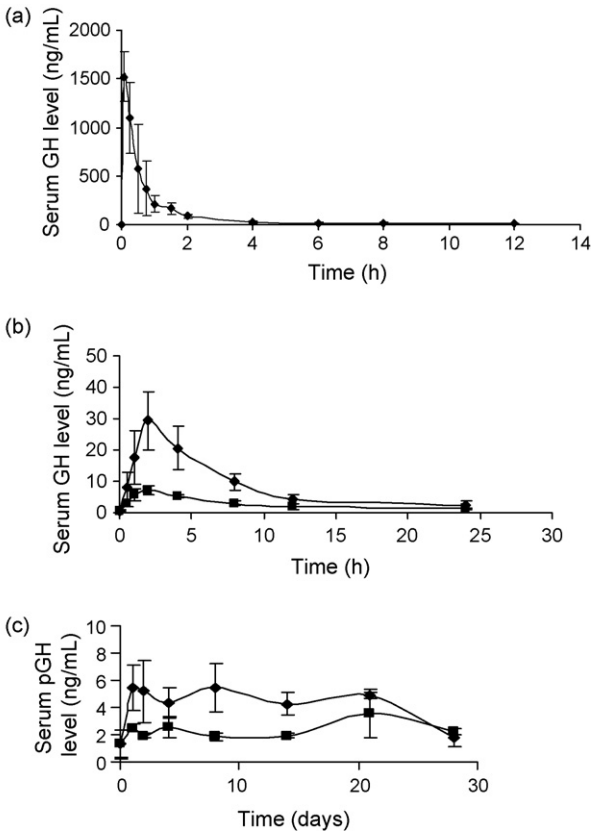

Fig. 3. *In vivo* absorption of growth hormone in rabbits after administration of different formulations. (a) i.v. administration (4.2 mg/mL); (b) s.c. administration; (c) thermosensitive polymer formulations (copolymer 4, 30%, w/v). Key: (■) low dose (0.12% GH) and (♦) high dose (0.42% GH).

the absorption profiles of pGH from different formulations in rabbits. An intravenous administration of pGH resulted in its high initial concentration followed by an immediate decrease concentration for 60 min and then the concentration was gradually decreased over the 12-h period (Fig. 3a). Fig. 3b shows the blood profile of subcutaneous formulation, which displayed an initial increase in pGH concentrations for 2 h and a gradual decrease over the 24-h period. In contrast, constant serum levels of exogenous pGH (3–7 ng/mL for high dose and 2–4 ng/mL for low dose) were detected for nearly 4 weeks from polymer formulations indicating constant rate of pGH release *in vivo* upon single subcutaneous injection (Fig. 3c). Absolute bioavailability was calculated based on AUC value. When compared to intravenously administered pGH, the absolute bioavailability of pGH enhanced by using thermosensitive polymer were in the range of 38.2–86.03%, which were about 4.5–14.5 times those of subcutaneous injection. Table 1 list the pharmacokinetic data for this study.

Kagatani et al. (1998) has shown that a GH nasal preparation based on absorption enhancers, didecanoylphosphatidylcholine (DDPC) and α-cyclodextrin (α-CD), can improve relative bioavailability up to 32% in rabbits. In this study, the bioavailability of pGH by s.c. injection in rabbits is only about 8%. A significant amount of administered pGH seems to be unabsorbed or unaccounted for. This may be due to degradation of pGH at

Table 1
Pharmacokinetic parameters after administration of GH in rabbits ($n = 3$)

Groups	GH (mg)	C_{max} (ng/mL)	T_{max} (h)	$AUC_{0-infinite}$ (ng h/mL)	F (%)
i.v.	4.2	1521.02 ± 258.44	0.083 ± 0.00	1865.81 ± 1283.91	100
s.c.	4.2	29.30 ± 9.38	2.00 ± 0.00	153.59 ± 58.73	8.23
s.c.	1.2	7.05 ± 1.31	2.00 ± 0.00	32.27 ± 6.51	6.06
t.p.	4.2	–	–	713.43 ± 28.64	38.20
t.p.	1.2	–	–	458.64 ± 242.97	86.03

GH: porcine growth hormone; C_{max}: maximum serum pGH concentration after dosing; T_{max}: time to reach C_{max} after dosing; $AUC_{0-infinite}$: area under the serum pGH concentration vs. time curve ($AUC_{0-\infty}$); F: absolute bioavailability. *Note*—i.v.: intravenous injection group (GH solution). s.c.: subcutaneous injection group (GH solution). t.p.: thermosensitive polymer group (controlled release formulation).

the site of injection. It has been shown that there is a significant amount of degradation of pGH at the site of s.c. injection (Jorgensen et al., 1988). A polymer system (poloxamer) was reported to protect GH against local proteolytic enzymes as long as GH is entrapped in the gel and not after passing from the gel to the tissue and fluids at the injection site (Katakam et al., 1997). PLGA–PEG–PLGA has been reported to extend the release and enhance the stability of a protein, insulin (Zentner et al., 2001).

3.3. *In vitro* compatibility study

Fig. 4a and b shows absorbances measured in MTT cell viability assay of BALB/3T3 cells containing growth media diluted with control extract or polymer extracts in the range of 1:1 to 1:16. We did not find significantly ($p > 0.05$) lower cell viabilities in growth media diluted with polymer extracts than diluted with control for all the dilutions during exposure periods.

Evaluation of the biocompatibility of a whole medical product is often not realistic, thus the use of representative portions, or extracts, of selected materials may be the only practical alternative for performing the assays. Many tests have been designed to determine the biological activity of mammalian cell cultures following contact with the polymeric materials or specific extracts prepared from the materials under test (USP 28, 2005). To study

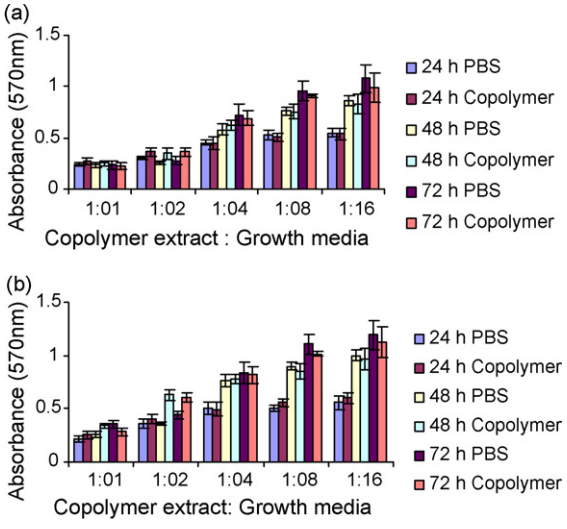

Fig. 4. *In vitro* biocompatibility of copolymer extract by MTT cell viability assay. (a) 70 °C 10 days; (b) 37 °C 10 days [PBS: phosphate buffer saline; copolymer: PLGA–PEG–PLGA (MW 1400–1000–1400)].

the final degradation and disintegration of biomaterials and avoid long follow-up periods, *in vitro* pre-degradation at elevated temperatures is a good method to simulate physiologically degraded material based on the fact that hydrolysis is probably the only degradation mechanism for PLA and PLGA (Rozema et al., 1992).

MTT assay has been proved to distinguish quantitatively toxic from non-toxic materials. The main advantage of the assay is the speed and possibility of testing large series of samples, generating large amounts of data for statistical analysis. The MTT test appears to be a useful method for measuring the cytotoxicity of biomaterials and to reveal the subtle toxicity of some materials, which do not kill the cells rapidly, i.e. within 24–72 h which is the usual time for a cytotoxicity test, but are able to affect cell metabolism and functions.

In the present study, the polymer was extracted at 37 °C and 70 °C in PBS. To exclude pH effects and to focus on the degradation products themselves, the pH of the extract was adjusted to 7.4. Neutralized extracts of thermosensitive polymer prepared at both 37 °C and 70 °C did not show cytotoxicity in BALB/3T3 cells.

A very sensitive method for testing the toxicity of soluble polymers and their degradation products is the assessment of *in vitro* toxicity. *In vitro* cell culture studies have the advantage of relatively well-controlled variables and are generally accepted as a very effective method for biocompatibility testing; the sensitivity is equal or greater than that of *in vivo* studies (Ignatius and Claes, 1996). The obtained results are good indications when thinking on the future clinical application of the polymers (Marques et al., 2002). However, *in vitro* results from different studies can be compared only in a restrictive manner because the test conditions, such as cell lines, exposure to polymers, and preparation of extracts, vary widely (Ignatius and Claes, 1996). Moreover, conditions could be very different depending on the amount of implanted polymer, the local ability of tissues to clear degradation products, and the degradation of the polymers which, in turn, depends on the initial molecular weight, the copolymer composition, the shape and size of the implant, and the mechanical conditions at the implantation site. Therefore, it is important to study biocompatibility *in vivo*.

3.4. *In vivo* biocompatibility

Fig. 5a shows a light micrograph of tissue samples taken from the control site where no formulation was injected. Fig. 5b–e

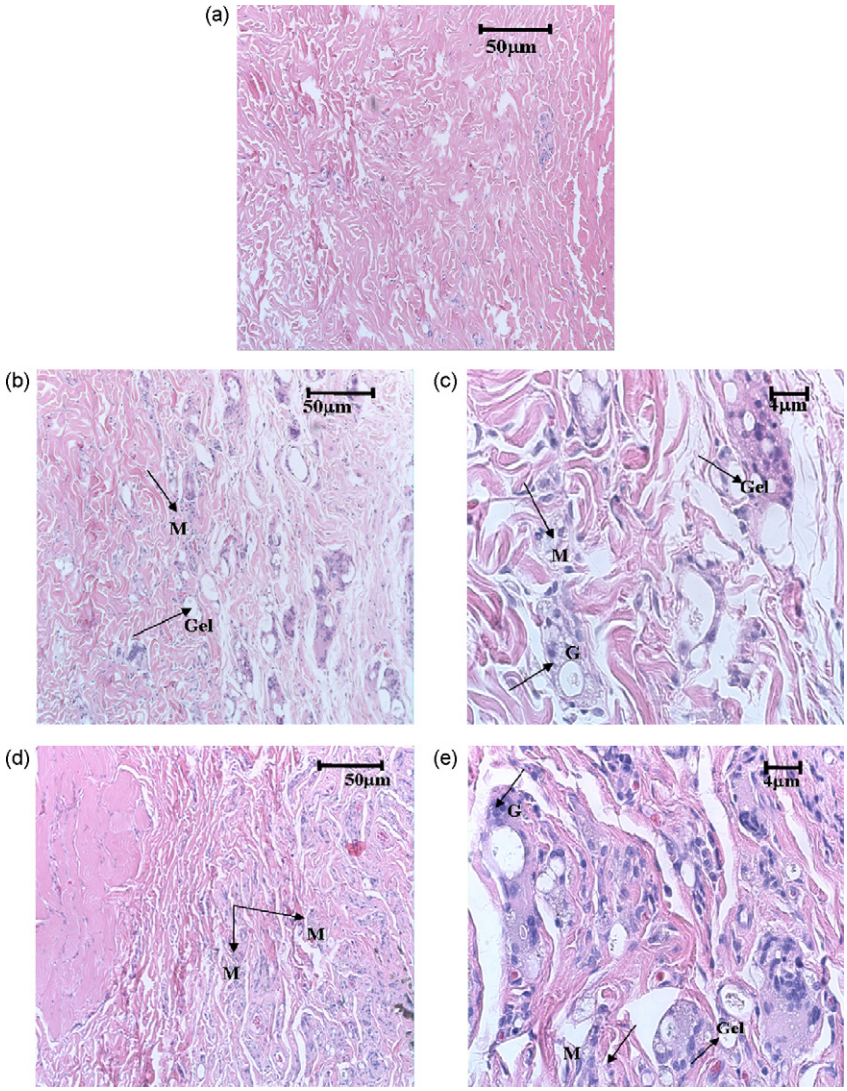

Fig. 5. Light micrographs of skin sample from different sites of injections. (a) Control, 10×; (b) blank thermosensitive polymer 1 month, 10×; (c) blank thermosensitive polymer 1 month, 40×; (d) GH formulation using thermosensitive polymer 1 month, 10×; (e) GH formulation using thermosensitive polymer 1 month, 40×. The inflammatory cells are mainly macrophages (M) and foreign body giant cells (G) (indicated by arrows).

shows light micrographs of tissue samples taken from sites of administration of delivery systems with/without pGH. We did not observe any significant histological difference between blank and formulation-administered tissue samples.

Fig. 5b–e shows the light micrographs of the tissue after 1 month of subcutaneous injection of thermosensitive polymer with/without pGH. After injection, the tissue adjacent to the gels consisted of an inner area close to the gels containing mostly inflammatory cells, and a peripheral area with fewer inflammatory cells. A large number of macrophages were seen infiltrating into the connective tissue (Fig. 5b and d). A cluster of gel particles was observed at the injection sites (Fig. 5c and e). Particles were surrounded by macrophages and foreign body giant cells. The presence of large number of macrophages indicates a chronic inflammatory reaction in response to the foreign body invasion and injury. This response is considered as a component of the normal tissue or cellular host reaction to injury (Anderson and Shive, 1997). A number of copolymers of lactide with glycolide have been approved by the FDA in parenteral drug delivery products (Royals et al., 1999). These biomaterials degrade *in vivo* by hydrolysis into lactic acid and glycolic acid, which are then incorporated into the tricarboxylic acid cycle and excreted (Athanasiou et al., 1996). Similar inflammatory reactions were observed for both blank and drug-loaded formulations. These *in vivo* results are in conformity with *in vitro* results, which support the biocompatible nature of these formulations.

4. Conclusions

This study provides evidence that thermosensitive triblock copolymer PLGA–PEG–PLGA (MW 1400–1000–1400) can control the delivery of pGH both *in vitro* and *in vivo* for a month after a single subcutaneous injection. The delivery system was biocompatible and biodegradable. Thus, PLGA–PEG–PLGA (MW 1400–1000–1400) is a good polymer for developing long-

acting controlled release injectable solution delivery system for growth hormone.

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