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제2형 당뇨병 치료제인 Pioglitazone을 봉입하기 위한 PLGA 나노입자 제조 및 분석

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Preparation and Characterization of Pioglitazone Loaded PLGA Nanospheres for the Treatment of Type 2 Diabetes

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초록: Pioglitazone을 봉입한 poly(lactide-*co*-glycolide) (PLGA) 나노입자를 emulsion-evaporation 방법을 이용하여 제조하여 최적의 나노입자와 봉입률을 조절하였다. 제조된 나노입자의 크기는 125~170 nm이었으며 30% pioglitazone이 봉입된 나노입자(3% PVA)의 봉입률은 85% 이상이었다. 이러한 나노입자들은 40일 동안 일정하게 용출이 되었다. 당뇨병 모델을 이용한 동물실험에서 글루코오스 농도를 저하시켰을 뿐만 아니라, 조직검사에서는 낮은 독성을 가지고 있는 것을 확인하였다. 이러한 결과는 pioglitazone 경구투여를 위한 약물전달을 위한 운반체로 사용될 수 있음을 확인하였다.

Abstract: The pioglitazone loaded poly(lactide-*co*-glycolide) (PLGA) nanospheres were prepared by emulsion-evaporation method and optimized for particle size and entrapment efficiency. The optimized particles were 125~170 nm in size with narrow size distribution and showed above 85% entrapment efficiency at 30% of pioglitazone loading when prepared with 3% w/v of poly(vinyl alcohol) (PVA) as a surfactant. These particulate carriers exhibited a controlled *in vitro* release of pioglitazone for 40 days at a nearly constant rate. The pioglitazone loaded PLGA nanospheres were not only effective to reduce the blood sugar level of diabetic rats but also non-toxic for the animal body, in particular for sensitive organs like kidney, liver, heart, lung and spleen. These results indicate that PLGA nanospheres have a great potential for oral delivery of pioglitazone.

Keywords: pioglitazone, PLGA nanospheres, type 2 diabetes, drug delivery, controlled release.

Introduction

Controlled drug delivery systems offer numerous advantages compared to conventional dosage forms including improved efficacy, reduced toxicity, and improved patient compliance and convenience.[1-3] Such systems often use synthetic polymers as carriers for the drugs. Polymers were employed to delay drug dissolution time to slow the rate at which drug molecules are exposed to water from the aqueous environment surrounding the drug delivery system.[4-6] This may be achieved by a polymer coating or a matrix that dissolves at a slower rate than the drug itself.

Pioglitazone is a drug that reduces the amount of glucose in the blood. It is in a class of anti-diabetic drugs called "thiazolidinediones" that are used in the treatment of type II diabetes.[7-9] Pioglitazone is often referred to as an "insulin sensitizer" because it attaches to insulin receptors on cells throughout the body and causes the cells to become more

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responsive to insulin. As a result, more glucose is removed from the blood.[10-12] At least some insulin must be produced by the pancreas for pioglitazone to work. Pioglitazone also lowers the level of glucose in the blood by reducing the production and secretion of glucose into the blood by the liver.[13-15] In addition, pioglitazone may alter the concentrations of lipids in the blood.[16] Specifically, it decreases triglycerides and increases the "good" cholesterol (HDL).

However, pioglitazone is not dissolved in aqueous condition but in organic solvents such as DMF and DMSO. This low solubility in aqueous condition limits the enhancement of pharmacokinetics and bioavailability of the drug, and induces side effects of the drug due to organic formulation method. For more extensive and effective drug delivery, hydrophobic drugs have been encapsulated or surface modified to boost absorption and water solubility.[17,18] The various strategies have been used to make them water-soluble, such as surface functionalization with water-soluble ligands,[19] PEG attachment,[20] and encapsulation within block-copolymer micelles.[21] Among polymeric carriers, poly(lactide-*co*-glycolide) (PLGA) and its various derivatives have been the center of focus for developing drug encapsulating therapeutic nano/micro particles for controlled release applications. It is because they have inherent advantages over the conventional devices that include extended release rate up to days, weeks or months, in addition to their biocompatibility/ biodegradability.[22-24]

In this study, we designed pioglitazone loaded PLGA nanospheres showing improved water solubility and sustained release of pioglitazone. They were prepared by a emulsion evaporation method and their physical chemistry characteristics were observed by SEM, DLS and HPLC analysis. A possibility as an effective therapeutic for diabetes and their toxicity were evaluated through *in vivo* animal study and *ex vivo* experiments including H&E staining.

Experimental

Materials. PLGA(50:50, MW: 40000~70000) and poly(vinyl alcohol) (PVA, 87~90%) were purchased from Sigma-Aldrich (St. Louis, MO). Ethyl acetate and methanol were obtained from Fisher Scientific Inc. (Waltham, MA). Pioglitazone was supplied by Dong Woo Syntech co., Ltd. (Seoul, Korea).

Preparation of Pioglitazone Loaded in PLGA Nanospheres. Pioglitazone-loaded PLGA nanospheres were prepared by a emulsion evaporation method. Four hundred mg of PLGA was dissolved in 20 mL of ethyl acetate and then 5 mL of methanol containing different amounts of pioglitazone was added. The solution was homogenized at 13500 rpm for 5 min after dispersed in 30 mL of water containing 3% PVA. Subsequent evaporation of ethyl acetate was carried out with a rotary evaporator. The formed nanospheres were collected by centrifugation and washed by dispersion in water and subsequent centrifugation. This final step was repeated three times to separate the pioglitazone loaded PLGA nanospheres from free pioglitazone. Finally, the particles were freeze-dried and obtained as a powder type. The feed amount of the drug was controlled from 5% to 60% (w/w) as shown in Table 1.

Table 1. Characterization of Pioglitazone Loaded PLGA Nanospheres

Samples	PVA (%)	Drug (%)	Size (nm)	Shape	Entrapment efficiency(%)
PLGA	0.1	0	625±56	Spherical	-
	1	0	315±22	Spherical	-
	3	0	165±13	Spherical	-
	5	0	85±9	Spherical	-
PLGA20	3	5	12.5±25.3	Spherical	89
PLGA40	3	10	168.9±44.1	Spherical	92
PLGA100	3	20	137.9±22.4	Spherical	92.4
PLGA200	3	30	144.0±49.8	Spherical	85
PLGA400	3	50	170.8±44.7	Spherical	73.7
PLGA600	3	60	-	Spherical	-

Size and Morphological Observation of Pioglitazone Loaded PLGA Nanospheres. For the morphological evaluation, the pioglitazone loaded PLGA nanospheres were sputter-coated with 5 nm platinum for SEM analysis. Photographs were obtained by FE-SEM (JEOL, Japan) to determine the morphology of nanospheres. The average size and size distribution of the pioglitazone loaded PLGA nanospheres were estimated by particle size analyzer (ELS-Z2, Otsuka Electronics Co., Ltd, Japan). The molecular weight of PLGA was measured by GPC (Agilent 1100 series, Santa Clara, California).

Determination of the Entrapment Efficiency. To measure the entrapment efficiency of pioglitazone in PLGA nanospheres, the nanospheres were mixed in mobile phase [(CH_3CN: $C_2H_7NO_2$ (0.1 mol/L): $C_2H_4O_2$=25:25:1)] using ultrasonication (3 min, 80 w). The precipitated PLGA nanospheres was removed by a syringe filter (0.45 μm, Millipore, Billerica, MA). The concentrations of pioglitazone in mobile phase were measured using isocratic reverse-phase HPLC (Futecs, Korea) equipped with a Prontosil 120-5-C18-ace-EPS column (5.0 μm, 250×4.6 mm). The flow rate of the mobile phase was 1.0 mL/min and the detection wavelength was 269 nm.

***In Vitro* Release Test.** For *in vitro* release experiments, the pioglitazone loaded PLGA nanospheres (10 mg) were put into a dialysis bag (spectra/por membranes, MWCO 8000, Spectrum Laboratories, Inc., Rancho Dominguez, CA) and the bag was sealed and immersed in 90 mL of PBS (pH 7.4)

buffers at 37 ℃. The tubes were incubated in a shaking incubator at 37 ℃ at a shaking frequency of 100 rpm. The release medium was exchanged totally with fresh PBS buffer solution of an equal volume when the concentration of the released drugs was determined. The collected solution (100 μL) was extracted to collect free pioglitazone released. The amount of pioglitazone in the sample were measured by HPLC under the following analysis conditions: Prontosil 120-5-C18-ace-EPS column (5.0 μm, 250×4.6 mm) and the mobile phase (CH_3CN:$C_2H_7NO_2$ (0.1 mol/L):$C_2H_4O_2$= 25:25:1) was used. The column effluent was monitored at 269 nm by using an UV detector. Quantitative value for pioglitazone was determined from a standard curve of pioglitazone. Each batch of samples was measured in triploid.

Degradation of PLGA Nanosphere. For degradation experiments, PLGA nanospheres (10 mg) were put into a 10 mL-vial and dispersed in PBS (pH 7.4) buffers at 37 ℃. The vials were incubated in a shaking incubator at 37 ℃ at a shaking frequency of 100 rpm. At time interval, the molecular weight of PLGA nanospheres was measured by GPC (Futecs, Korea).

***In Vitro* Cytotoxicity Study.** MTT assay was performed on KB cells (human epidermal cancer cells) by incubating them at 37 ℃ for 1 day with each different concentrations of each compound in quadruplicate. The control was incubated at 37 ℃ for 1 day without adding a drug. This assay is based on the reduction of the yellow tetrazolium component (MTT) to an insoluble purple-colored formazan produced by the mitochondria of viable cells. After 48 h incubation, 100 μL of medium containing 20 μL of MTT solution was added to each well and the plate was incubated for an additional 4 h, followed by the addition of 100 μL of MTT solubilization solution (10% Triton X-100 plus 0.1 N HCl in anhydrous isopropanol, Sigma-Aldrich) to each well. The solution was gently mixed to dissolve the MTT formazan crystals. The absorbance of each well was read with a microplate reader at a wavelength of 570 nm. The background absorbance of well plates at 690 nm was measured and subtracted from the 570 nm measurement. The results are expressed as % cell viability, obtained by dividing the optical density values (OD) of the treated groups (T) by the OD of the controls (C) ([T/C×100%]).

***In Vivo* Animal Study.** Fifty female Sprague-Dawley rats (6 weeks old and weighing average 140 g) were purchased from Orient-Bio Co. (Kyunggi-Do, Korea) and maintained in individual cages with a 12 h/12 h light-dark cycle. All animals were allowed free access to water. At the beginning of the study, before making the diabetes model, rats were checked if their blood sugar levels are within normal ranges with a glucose tolerance test (GTT). And then streptozotocin (STZ, Sigma, St. Louis, MO), dissolved in 0.1 M citrate buffer (pH 4.5), was injected into rats intraperitoneally with the concentration of 50 mg/kg after 18 h starvation. One week after STZ injection, a GTT was carried out and rats were fed with a high glucose-fat diet (containing 23.7% casein, 46% glucose, 23.4% fat) to make a type II, insulin-resistant diabetes model. After 4 weeks on a high glucose-fat diet, weight-matched, fasting-blood glucose-matched STZ rats were randomly divided into four groups (STZ, free pioglitazone, 33% PLGA-pioglitazone, 50% PLGA-pioglitazone) (n=6). STZ rats were given vehicle solution and each treatment group was orally administered (pioglitazone equivalent dose, 30 mg/kg) three times per a week for nine weeks. Meanwhile, a GTT was performed once per two weeks, totally four times and body weight was measured once a week. At the end of treatment period, the entire groups of rats were sacrificed and their main organs, such as the liver, spleen, kidney, heart, and lung were collected for H&E staining. All experiments were approved by Institutional guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Catholic University of Korea, College of Medicine in accordance with NIH guidelines.

Statistics. Statistical analysis was done using ANOVA. $p<0.01$ was accepted as statistically significant. Error bars represent standard deviation.

Results and Discussion

Pioglitazone Loaded in PLGA Nanospheres. Pioglitazone hydrochloride, a water insoluble drug, was encapsulated within PLGA nanospheres by a emulsion evaporation method. The drug was dissolved in the organic polymer solution followed by emulsification into an external aqueous phase to form an O/W emulsion (Figure 1). PLGA nanospheres were successfully manufactured by a emulsion-evaporation method. In all formulations, the method formed nanospheres were less than 700 nm in size. Figure 2 shows the SEM image of PLGA nanospheres prepared by changing the amount of PVA from 1 to 10%. In the presence of 1% PVA, the mean particle size of PLGA nanospheres were 315±22 nm by dynamic light scattering method.

At 3% and 5% of PVA concentrations, significant differences in size distributions were observed. In the presence of 3% PVA, the mean particle sizes of PLGA nanospheres were 165±13 nm and this was well suited to preserve the particle stability of PLGA during lyophilization. The optimum size range for efficient cellular uptake has been observed. The size of PLGA nanospheres were controlled to obtain the optimum size and shape by controlling PVA concentration.

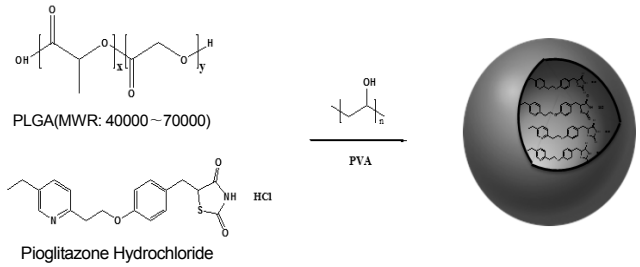

Figure 1. Schematic illustration of pioglitazone loaded PLGA nanosphere.

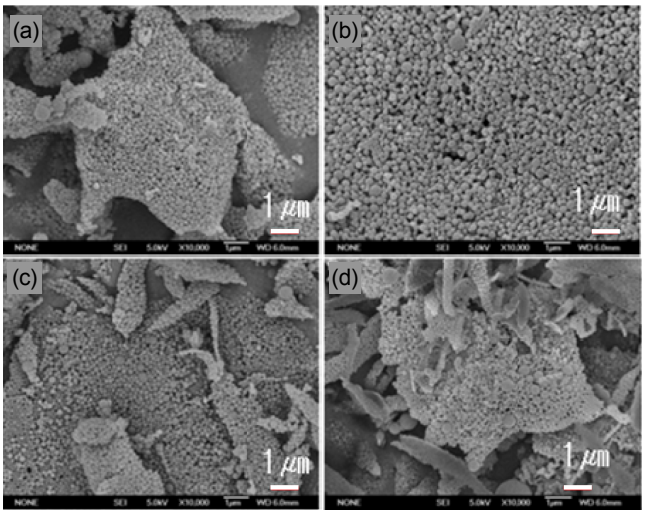

Figure 2. Scanning electron micrographs showing size distribution changes of PLGA nanospheres by different amounts of PVA: (a) 0.1%; (b) 1%; (c) 3%; (d) 5%.

In case 5% of PVA, the PLGA nanospheres found aggregated, which are not suitable for drug loading and delivery. So this formulation has been avoided.

Figure 3 showed the size distribution and morphology of pioglitazone loaded PLGA nanospheres (3% PVA) by scanning electron microscopy. The results have shown that the size distribution of the nanospheres was not dramatically altered according to loading amounts of pioglitazone within PLGA nanospheres. The size distributions of pioglitazone loaded in PLGA nanospheres were 125~170 nm in diameter, indicating pioglitazone in PLGA nanospheres are tightly protected from contacting the outside environment, their hydrodynamic behaviors is mainly controlled by the surface coating layer.

However, the spherical shape of PLGA nanospheres was changed to a rod type when 50% pioglitazone was loaded into the PLGA nanospheres. It indicates that all pioglitazone was not loaded into the inside of PLGA nanospheres.

In Vitro Controlled Release of Pioglitazone. Controlled release pattern is highly co-related with loading efficacy of the drug,

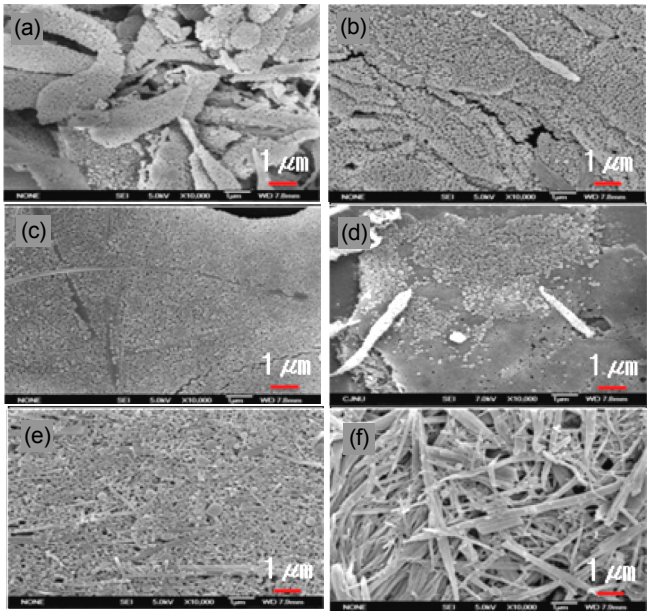

Figure 3. Scanning electron micrographs showing morphological changes of pioglitazone loaded PLGA nanospheres (400 mg, PVA: 3%) by drug loading: (a) 5%; (b) 10%; (c) 20%; (d) 30%; (e) 50%; (f) 60%(w/w).

polymer degradation rate, matrix swelling and erosion of carrier polymer (Jain, 2000). PLGA nanospheres showed a high loading efficiency up to a certain feed amount. For *in vitro* release test, we selected PLGA40 and PLGA200 to observe different drug release rates, cumulative release pattern regardless of drug amount (Figure 4). The initial burst effect was observed for PLGA200 for 10 days, and then maintained a sustained release for 30 days.

The release amount of the drug for 40 days was 50% of total amount of the drug. However, it lasted for only about a day in case of PLGA40 and the drug release rate then gradually tapered to slower rates. About 20% of the total drug has been released from PLGA40 sample for 40 days of observation. The release pattern has shown almost a steady state/constant release profile after 10 days of cumulative release. In our study it has been proved that the loaded amount of drug could control the release pattern.

Macromolecules are released from biodegradable matrixes by a mechanism involving polymer degradation followed by matrix erosion and diffusion of the released macromolecules through the aqueous channels was generated during the erosion process (Zolnik *et al.,* 2008). From the results, degradation profile of PLGA nanospheres has shown an initial phase which was a rapid degradation where major change in molecular weight and secondary phase was almost constant up to 10 days (Figure 5).

The degradation of PLGA nanospheres is related with

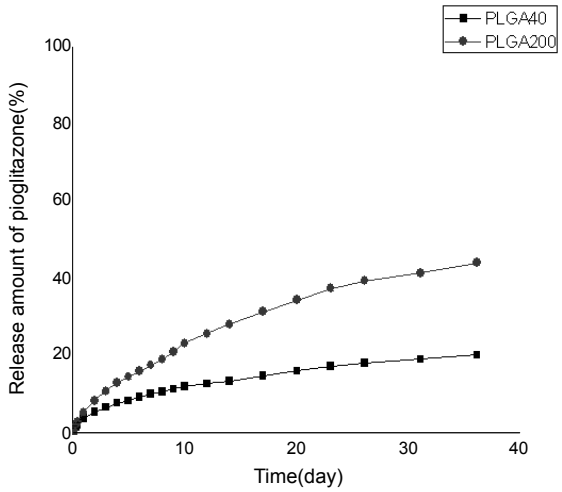

Figure 4. *In vitro* release rates of pioglitazone from PLGA nanospheres. Each symbol represents (■ PLGA40, ● PLGA200).

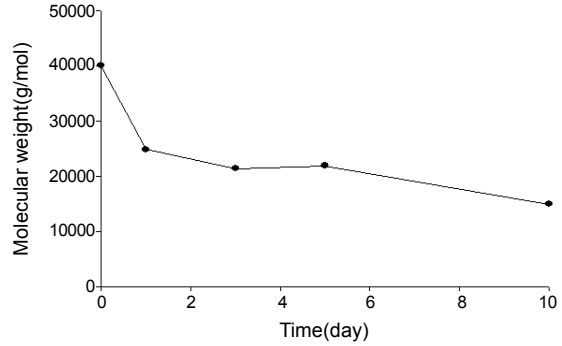

Figure 5. Degradation of PLGA nanospheres. Circle represents molecular weight of PLGA measured by GPC.

the release of pioglitazone from the core of nanospheres. The degradation study of PLGA also proved that; it losses the molecular weight according to time due to degradation of PLGA in aqueous solution.

In Vitro Cytotoxicity of Pioglitazone Loaded PLGA Nanospheres. KB cells were selected to evaluate the toxicity of pioglitazone loaded PLGA nanospheres. The cells were incubated for 48 h with blank PLGA nanospheres, pioglitazone loaded PLGA nanospheres and free drug. As shown in Figure 6, the cell viabilities of blank PLGA nanospheres were approximately 70% at the highest concentration (10 μg/mL) while the cell viabilities were approximately 35% when treated with free drug at the same concentration. The cell viabilities increased to 55% from 35% as the concentration (1 μg/mL) of added free pioglitazone decreased. It demonstrated the cell viability was dependant on the concentration of the free drug. Pioglitazone loaded PLGA nanospheres (PLGA40 and PLGA200) were also incubated with KB cells and it shown approximate same amount of cell viabilities (70%) for highest concentration (10 μg/mL).

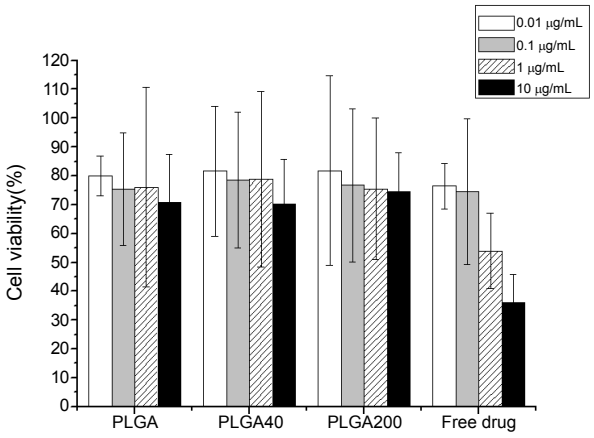

Figure 6. Cytotoxicity of pioglitazone, PLGA and pioglitazone loaded PLGA nanospheres against KB cells for 48 h incubation, respectively (p <0.001).

Though the loaded amounts of drug were different for each formulation but they have shown approximate same toxic effects. This data demonstrated, drug loading efficacy and release amount of drug did not affect on cell viabilities.

Effect of Pioglitazone Loaded PLGA Nanospheres on the Reduction of the Blood Glucose Level. The diabetes induced rats were divided into four groups for treatment policy and the therapeutic efficacy of free and loaded pioglitazone was observed. The control group was administered saline and the other groups were fed with free pioglitazone, PLGA40 and PLGA200. All rats were treated by the same protocol and same condition. Body weight and blood sugar level were measured every one week for all rats since oral administration of sample was started. The blood sugar level of sample rats steadily decreased in contrast that of control rats increased day by day (Figure 7(a)).

The reason that the pioglitazone loaded nanospheres showed better therapeutic efficacy than free drug, is probably because every drug needs a carrier molecule or polymer to reach the site of action. The free pioglitazone might not able to reach its site of action. The free pioglitazone was thought to reach its site of action less effectively compared to drug, which is conjugated with any carrier molecule or polymer.

Controlled release of drug from the PLGA nanospheres is another most important factor to have the better therapeutic effect. Our *in vivo* study has shown that pioglitazone loaded PLGA nanospheres started to release the drug and it continued up to 10 days. Consequently blood sugar level of the treated rats started to reduce from the first day of oral administration and it continued up to 6 days, and the blood sugar level reached the optimum condition. After that, it did not increase furthermore, and that means drug release was

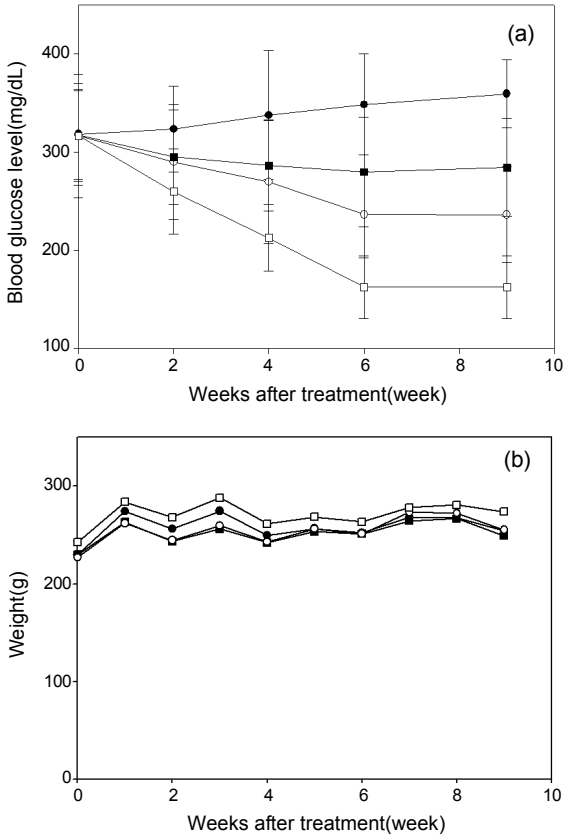

Figure 7. Effect of pioglitazone loaded PLGA on blood-sugar level (a); body weight (b) in animal model(●: control (saline), ■: Free pioglitazone, ○: PLGA40, and □: PLGA200).

not inhibited after 6 days of administration. The encapsulated drug was continuously released from the nanospheres and it maintained the blood sugar level for a long time. The average body weights of treated rats were similar to that of control rats and maintained constant during the course of experiments (Figure 7(b)). It was confirmed that pioglitazone loaded PLGA nanospheres were not only effective to reduce the blood sugar level of diabetes rats but also non toxic for the animal body.

Toxicity of Pioglitazone Loaded PLGA Nanospheres (Histopathologic Study). Histopathological analysis was performed on the tissues obtained from the harvested organs (heart, kidney, liver, spleen, and lung) to assess the signs of potential toxicity. Tissues were harvested after the completion of drug administration, fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

As seen in Figure 8, no apparent tissue or cellular changes were observed between mice receiving pioglitazone-loaded PLGA nanospheres and control mice. No pathological changes were observed from the spleen, kidney, heart, lung, and liver.

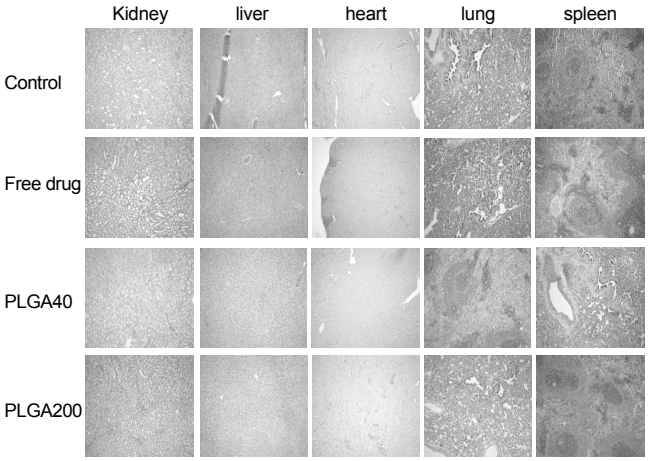

Figure 8. Histological evaluation of tissue treated with saline, pioglitazone, pioglitazone loaded PLGA nanospheres (H&E stain, magnification ×100).

Conclusions

In this study, PLGA nanospheres with a water-soluble characteristic were prepared for oral delivery of pioglitazone. The *in vitro* toxicity study demonstrated the safety of the prepared PLGA nanospheres after oral administration. Additionally, the pharmacodynamic evaluation of orally administered PLGA nanospheres in diabetic rats indicated that the intestinal absorption of pioglitazone was significantly enhanced. These results suggested that the PLGA nanospheres developed in the study might be used as a potential approach for the oral delivery of therapeutic drugs.

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