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Prostaglandin E_1 encapsulated into lipid nanoparticles improves its anti-inflammatory effect with low side-effect

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ABSTRACT

Prostaglandin E_1 (PGE1) shows various pharmacological activities including anti-inflammation. However, the rapid metabolization and inactivation of the intravenously administered PGE1 during the first passage through the lungs result in significant non-compliance in clinical trials which greatly limits its application. The aim of this work was to prepare the lipid nanoparticles loading PGE1 to improve its anti-inflammatory effect with low side-effect. The experimental results showed that PGE1 loaded lipid nanoparticles (PLNs) could be successfully prepared by high pressure homogenization with particle size 68.1 ± 4.7 nm, zeta potential -3.32 ± 0.37 mV and entrapment efficiency $92.1 \pm 1.3\%$. PLNs exhibited a sustained release with low burst drug release. PLNs could improve the inhibition effects of PGE1 on lipopolysaccharides (LPS)-induced TNF-α expression on macrophage RAW264.7 cells, and improve the inhibition of lymphocyte to endothelial cell adhesion and ICAM-1 adhesion molecule expression on HUVEC and MDA-MB-468 cell membrane. No allergenicity, vascular and muscle irritation were induced in animals by PLNs even at double of the highest drug concentration of clinical infusion. As a result, PLNs could be a more potential delivery system for PGE1 in the treatment of inflammation-related diseases.

1. Introduction

Prostaglandin E_1 (PGE1), also called Alprostadil, shows various pharmacological activities such as vasodilation, inhibition of leukocyte adhesion and platelet aggregation, amelioration of the rheological property of blood, and anti-inflammation (Kerins et al., 1991). Therefore, it has been widely used in the treatment of peripheral vascular disease, ulcers, hepatopathy, pulmonary hypertension, ischaemic heart disease and so on (Mizushima et al., 1983; Murota et al., 2008; Shen et al., 2005). However, the rapid metabolization and inactivation of the intravenously administered PGE1 during the first passage through the lungs lead to the requirement of long-term infusion to reach the valid treatment concentration which causes severe clinical side effects such as hypotension, peripheral oedema, severe allergic reactions, and bleeding, bruising, pain, swelling or redness at the injection site (Golub et al., 1975; Schramek and Waldhauser, 1989).

So far, great efforts have been made to develop reliable delivery system for PGE1 such as lipid microspheres (Mizushima and Hoshi, 1993), cyclodextrin complexes (Gu et al., 2005), liposomes (Kawakami et al., 2001) and polymeric conjugates (Takeda et al., 2009) in order to improve its stability or modulate its pharmacokinetics, and finally enhance therapeutic effects and improve clinical compliance. Complexation of PGE1 by α-CD (PGE1-CD) which is clinically used worldwide could improve dissolution characteristics and chemical stability of PGE1 (Davis and Brewster, 2004). The lipid emulsions of PGE1 (Lipo-PGE1) clinically used in Japan, South Korea, and China are known to accumulate in the sites of inflammation or vascular lesions, and thus show a significantly enhanced clinical efficacy for peripheral vascular disease (Mizushima et al., 1983, 1990; Mizushima and Hoshi, 1993). However, PGE1 encapsulated in emulsion containing soybean oil as the emulsifier showed some drawbacks including chemical instability and rapid leakage of drug from emulsions in blood (Igarashi and Mizushima, 1996) and the local side effects were still found in patients receiving PGE1-CD (Toyota et al., 1993) or Lipo-PGE1 (Shen et al., 2005). So it is still a challenge to develop new delivery system of PGE1 with chemical and biological stability, enhanced pharmacological activity with low side effect.

Recently, lipid nanoparticles have attracted great attentions because this drug delivery system could effectively protect drug from inactivation, prolong drug retention time in blood thus increase its efficacy (Müller, 2007; Joshi and Müller, 2009; Puglia et al., 2008). In addition, lipid nanoparticles also show some advantages including good safety of the component materials and easy preparation over other nano-based drug delivery systems. We are interested in designing and developing a new delivery system for PGE1. In this work, the PGE1 loaded lipid nanoparticles (PLNs) were prepared, and the physiochemical characteristics, the in vitro

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anti-inflammatory effects of PLNs, and the in vivo allergenicity, vascular and muscle irritation of PLNs were evaluated.

2. Materials and methods

2.1. Materials

Prostaglandin E_1, Freund's adjuvant complete, Freund's adjuvant incomplete, hematoxylin, eosin and *Escherichia coli* lipopolysaccharide (LPS) were obtained from Sigma (St. Louis, MO, USA). Soybean lecithin was obtained from Shanghai Taiwei Pharmaceutical Co. Ltd. (Shanghai, China). Poloxmer 188 was purchased from BASF (Ludwigshafen, Germany). Lipo-PGE1 (Kaishi) was purchased from Tide Pharmaceutical Co. Ltd. (Beijing, China). Trypsin-EDTA and phosphate buffered saline (PBS, pH 7.4) were obtained from Gibco-BRL (Burlington, ON, Canada). The Dulbecco's modified Eagle medium (DMEM), RPMI 1640 medium, M199 medium, antibiotics and fetal bovine serum (FBS) were purchased from Invitrogen GmbH (Karlsruhe, Germany). Bradford protein assay kit was purchased from Beyotime Institute of Biotechnology (Jiangsu, China). PE conjugated mouse IgG1 (K Isotype control), PE conjugated anti-human CD54 (ICAM-1), and PE conjugated anti-human CD106 (VCAM-1) were purchased from eBioscience (San Diego, CA, USA). Mouse TNF-α and IL-1β ELISA kits were purchased from Bender MedSystems (Burlingame, CA, USA). Lymphocytes separation medium was obtained from Huajing Biological Hi-tech Co. Ltd. (Shanghai, China). Bovine serum albumin (BSA) was purchased from Junchuang Bioscience Co. Ltd. (Shanghai, China). Urethane and Evans Blue dye were obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). All other chemicals and solvents were of analytical grade and used without further purification.

2.2. Cell culture

Human lymphocytes (HL) from healthy donors were separated by centrifugation with lymphocyte separation medium (Oostingh et al., 2006). The HUVEC (human umbilical vein endothelial cells) and MDA-MB-468 (human breast cancer cells) were purchased from the ATCC (American Type Culture Collection, Manassas, VA, USA). RAW264.7 cells (mouse macrophages) were obtained from CBCAS (Cell Bank of the Chinese Academic of Sciences, Shanghai, China). HUVEC were grown in M199 containing 20% fetal bovine serum (FBS), 100 Unit/ml penicillin G sodium and 100 μg/ml streptomycin sulfate. MDA-MB-468 cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% antibiotics (100 Unit/ml penicillin G sodium and 100 μg/ml streptomycin sulfate). RAW264.7 cells were cultured in DMEM supplemented with 10% FBS and 1% antibiotics (100 Unit/ml penicillin G sodium and 100 μg/ml streptomycin sulfate). Cells were maintained at 37 °C in a humidified and 5% CO_2 incubator.

2.3. Animals

Healthy female guinea pigs (180–220 g) were purchased from Shanghai Institute of Biological Products (Shanghai, China). Male Sprague–Dawley rats (180–200 g) were purchased from Sino-British Sippr/BK Lab Animal Ltd. (Shanghai, China). Male and female New Zealand white rabbits (1.8–2.2 kg) were obtained from Shanghai Shengwang Experimental Animal Ranch (Shanghai, China). All animal procedures were performed according to the protocol approved by the Institutional Animal Care and Use Committee of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

2.4. Preparation and characteristics of PLNs

The PLNs were prepared by high pressure homogenization as described elsewhere with minor modification (Puglia et al., 2008). Briefly, soybean lecithin and alprostadil (w/w, 300:1) were dissolved in ethanol at a concentration of 0.2 mg/ml. The solution was mixed with 0.5% Poloxamer 188 solution containing 20% sucrose to obtain a final drug concentration of 5 μg/ml, and the mixture was ultrasonicated for 2 min. The obtained premix was then passed through a high pressure homogenizer (EmulsiFlex-C3, Avestin, Canada) at 20,000 psi for three cycles. The PLN suspension was lyophilized and stored at 4 °C.

The mean particle size and zeta potential of PLNs after dispersed in water were determined by dynamic light scattering method using Nicomp 380/ZLS analyzer (Particle Sizing Systems, Inc., Santa Barbara CA, USA). The morphological examination of PLNs was performed using a transmission electron microscope (TEM, CM12, Philips, Netherlands) after negative staining with sodium phosphotungstate solution (0.2%, w/v). The amount of alprostadil in PLNs was determined by ultrafiltration/centrifugation. Briefly, 500 μl of PLNs dispersed in water was added to Ultrafree-MC (50KD, Millipore) and centrifuged for 10 min at 5000 rpm. The amount of alprostadil in the filtrate which was not entrapped in the nanoparticles, W_{EX}, was thus determined. Total alprostadil concentration W_{TO}, was measured by HPLC as described below after dissolution of the colloidal dispersions by methanol. The encapsulation efficiency (EE) of PLNs was calculated as:

$$\mathrm{EE}\,(\%) = \frac{W_{TO} - W_{EX}}{W_{TO}} \times 100\% \quad (1)$$

For measurement of alprostadil, a sensitive HPLC method with post-column derivatization was used. Aliquots of 20 μl were analyzed on HPLC system (Agilent 1100) with the sequence of separation, post-column reaction and detection. Separation was carried out by a column of SB-C_{18} (250 × 4.6 mm i.d., pore size 5 μm) at a flow rate of 1.0 ml/min with the mobile phase: 6.7 mM potassium dihydrogen phosphate solution (pH 6.3)–CH_3CN (3:1, v/v). The T_{98} post-column reaction system (Beijing, China) was placed between the detector and the column consisting of poly(tetrafluoroethylene) pipe (0.5 mm × 10 m) and 1 mol/l KOH as reaction solvent with reaction temperature 60 °C. The reaction product was detected at 278 nm.

2.5. In vitro release of PLNs

In vitro release of PLNs was performed using pure water as release medium. The PLNs dispersed in water (5 μg/ml) was diluted with water (1:5, v/v) and shaken horizontally (100 rpm) at 37 °C in dark place. At predetermined time intervals, 1 ml of solution was taken out and the amount of released drug was measured by HPLC after separated from nanoparticles by ultrafiltration/centrifugation. The same volume of fresh water was added to maintain the total volume after solution removal. As a control, Lipo-PGE1 was used, and in vitro release experiment was performed as PLNs.

2.6. In vitro anti-inflammation experiment

2.6.1. ELISA for detecting tumor necrosis factor alpha (TNF-α) and interleukin-1beta (IL-1β)

The concentration of TNF-α and IL-1β in the culture supernatants of RAW264.7 cells after LPS stimulation was analyzed by the commercially available ELISA kits according to the manufacturer's protocol. Briefly, RAW264.7 cells were seeded in a 24-well plate with 0.5 ml growth medium and allowed to attach for 24 h. Then, cells were incubated with free PGE1 (first dissolved in ethanol

then diluted with water) or PLNs with different concentrations for 30 min followed by adding 1 μg/ml LPS. After incubation for 8 h, supernatants from control or LPS-stimulated cells were collected, and chemokine levels were determined by ELISA kits. The samples were diluted at 1:10. The absorbance was read at 450 nm using a microplate reader (Bio-Rad Labs, Hercules, CA). Samples and standards were run in triplicate.

2.6.2. In vitro adhesion assay

The HL used in the adhesion assays were centrifuged and resuspended at 1×10^6 cells/ml in M199 medium containing 1% FBS. HUVEC were seeded in a 96-well plate with 200 μl growth medium until confluence. The HUVEC were further cultured for 24 to allow the formation of monolayers. The cells were incubated with or without LPS (2 μg/ml) in the absence or presence of various concentration of free PGE1 or PLNs for 8 h. Cells were washed with Hank's solution twice before adding 100 μl human lymphocytes for 1 h at 37 °C. Non-adherent lymphocytes were then washed away with PBS twice. The concentration of protein in each well was detected using bradford protein assay kit according to the manufacturer's protocol. W_{SUM} stands for the concentration of protein of HUVEC and adherent HL in the well. W_{HUVEC} stands for the concentration of protein of HUVEC. W_{HL} stands for the concentration of protein of added HL. The adherent efficiency (AE) was calculated as below:

$$\text{AE } (\%) = \frac{W_{SUM} - W_{HUVEC}}{W_{HL}} \times 100\% \quad (2)$$

2.6.3. Cell staining and flow cytometry analysis of adhesion protein expression

HUVEC and MDA-MB-468 cells were seeded in a 6-well plate with 2 ml growth medium and incubated until confluence. Then, cells were incubated with or without LPS (1 μg/ml) in the absence or presence of various concentrations of free PGE1 or PLNs for 8 h. Finally, cells were detached, centrifuged and washed twice with PBS. The cells resuspended in 100 μl PBS were stained with PE conjugated antibody anti-human CD54 (ICAM-1) or anti-human CD106 (VCAM-1) for 30 min at room temperature then subjected to flow cytometry detecting through fluorescence channel 2 (FL2) and analyzed with CellQuest software.

2.7. Allergenicity test

For active systemic anaphylaxis test, guinea pigs were divided into 4 groups ($n = 6$) including saline solution group, 5% bovine serum albumin group, and PLNs groups at doses of 0.86 μg/kg and 1.72 μg/kg, which were given intraperitoneally on day 1, 3 and 5, respectively. Two weeks later, a systemic anaphylaxis reaction was respectively induced by injecting the saline solution, 5% bovine serum albumin, PLNs groups at a dose of 1.72 μg/kg and 3.44 μg/kg intravenously into the animals. The reactions were monitored for 3 h and scored.

For passive cutaneous anaphylaxis (PCA) test, SD rats were divided into 4 groups ($n = 4$) and immunized intravenously with saline solution (negative control), 5% bovine serum albumin (positive control) and PLNs (0.8 μg/kg and 1.6 μg/kg) every other day for three times, respectively. Eleven days after the last immunization, animals were intraperitoneally anaesthetized with 25% urethane, and bleeding of the animal was done through abdominal aorta. Sera samples were collected for further analysis.

Untreated SD rats (4 groups, $n = 6$) were shaven on the abdomen and injected intradermally with 0.1 ml of the test sera in serial dilutions using saline solution. 48 h later, animals were intravenously administered with 1.6 ml/kg of a 1:1 mixture solution consisting of saline solution, 10% bovine serum albumin or PLNs (1 μg/ml and 2 μg/ml) and a solution of Evans Blue (1%), respectively. Evans Blue dye was used to detect the local increases in vascular permeability due to antigen-antibody interaction. After 30 min, the animals were anaesthetized with 25% urethane and the diameter of cutaneous reactions was measured. A positive (IgE) response was recorded if the challenge resulted in area >5 mm^2 blue lesion in the skin of the recipient animals. The response was also calculated quantificationally by immersion of the blue lesions with a 7:3 mixture of acetone and water, and the optical densities were detected at 610 nm.

2.8. Irritation test

For vascular irritation test, New Zealand rabbits were divided into 4 groups ($n = 4$). Lipo-PGE1 (0.47 μg/kg and 0.94 μg/kg) and PLNs (0.47 μg/kg and 0.94 μg/kg) were infused intravenously into the right auricular vein of rabbits and the saline solution into the left auricular vein as a control once a day for three days. Two days and 14 days after last infusion, rabbits randomly chosen were sacrificed respectively by exsanguination under 25% urethane anesthesia. The auricular veins 4.5 cm away from the injection site were removed, fixed in 10% (v/v) neutral buffered formalin, and embedded in paraffin wax. The histological sections were stained with hematoxylin and eosin, and examined by light microscopy (OLYMPUS BX51).

For intramuscular irritation test, New Zealand rabbits were divided into 4 groups ($n = 4$). Lipo-PGE1 (0.47 μg/kg and 0.94 μg/kg) and PLNs (0.47 μg/kg and 0.94 μg/kg) were injected into right quadriceps femoris muscle of rabbits once a day for three days. The left quadriceps femoris muscle injected with saline solution was served as a control. Two days after last injection, rabbits randomly chosen were sacrificed respectively by exsanguination under 25% urethane anesthesia. The quadriceps femoris muscle was dissected and the lesions were then observed grossly. The irritation scores were assigned based on the semiquantitative scoring system which was as follows: individual score, 0 = none; 1 = minimal hemorrhage; 2 = mild hemorrhage; 3 = intense hemorrhage; 4 = necrosis of muscle fibers. Some of the muscles served as samples for histological examinations were removed, fixed in 10% neutral formalin and embedded in paraffin wax. The muscle sections were stained with hematoxylin and eosin, and examined by light microscopy.

2.9. Statistical analysis

The mean ± SD was determined for each treatment group. Statistical analysis was performed using a Student's *t*-test. The differences were considered significant for $p < 0.05$, and very significant for $p < 0.01$.

3. Results and discussion

3.1. Preparation and characteristics of PLNs

The virtual of simplicity of the lipid nanoparticles makes the delivery system so promising (Müller, 2007). In this work, a conventional high pressure homogenization was used to successfully prepare PLNs. The soybean lecithin, a well-tolerated, regulatory accepted lipids which was utilized in a wide variety of food products, and the emulsifier poloxamer 188, both of which had been approved by FDA, were selected to prepare PLNs. Sucrose was selected as lyoprotectant during lyophilization. After lyophilization, PLNs showed little change in particle size and zeta potential. The particle sizes of PLNs before and after lyophilization were 75.8 ± 5.7 nm and 68.1 ± 4.7 nm, respectively, and the zeta potentials of PLNs were −3.46 ± 0.42 mV and −3.32 ± 0.37 mV before and after lyophilization, respectively. The morphology of PLNs after lyophilization observed through TEM showed round and pretty uniform (Fig. 1A). These results demonstrated that the lyophilization process would not destroy the nanostructure of PLNs. As a

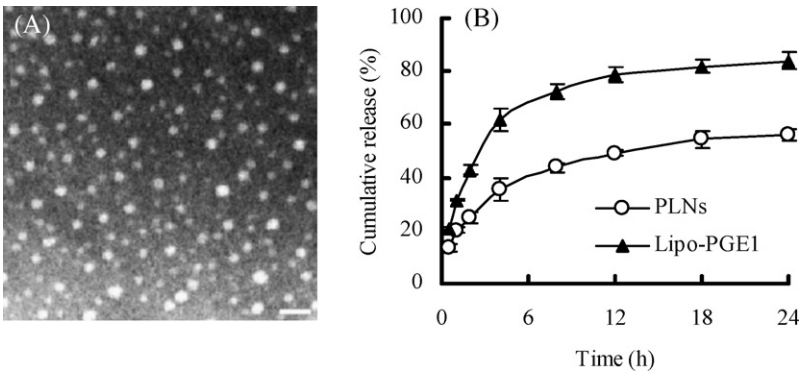

Fig. 1. The physicochemical characteristics of PLNs. (A) TEM image of PLNs (bar = 200 nm). (B) In vitro release profiles of PLNs and Lipo-PGE1 in water. Data represent mean ± SD of triplicate.

chemical-instable drug, PGE1 loaded in lipid nanoparticles could be protected from degradation, and lyophilization could improve their storage stability than the liquid formulation.

To quantitate the drug content and calculate the EE of PLNs, a sensitive HPLC method with post-column derivatization was developed. Post-column involves the modification of the chromatographic system to allow the reaction to take place prior to entering the detector by inserting a post-column reactor between the column and the detector. Because some of the PGE1 could be degraded into PGA1 due to its chemical instability, and both PGE1 and PGA1 could be converted to PGB1 in the presence of hydroxide ion, the sensitive HPLC method with post-column derivatization could firstly separate PGE1 from PGA1 with column, then convert both of them into PGB1 in the post-column reactor, and finally detect them separately under 278 nm. The EE of PLNs before and after lyophilization was 94.3 ± 1.9% and 92.1 ± 1.3%, respectively.

3.2. In vitro release experiment

Due to the light, pH and ionic strength of buffered solution all bring about effects on stability of PGE1 (Paul et al., 2005), the in vitro release was performed in distilled water away from light to minimally eliminate the PGE1 degradation thus to improve the test accuracy. The in vitro release profiles of PLNs and Lipo-PGE1 were shown in Fig. 1B. The low burst release was observed from PLNs. Compared with Lipo-PGE1, PGE1 entrapped in nanoparticles could be released more sustainedly. More than 80% PGE1 released to the outer of emulsion from Lipo-PGE1, while only 56% PGE1 released from the lipid nanoparticles after shaking for 24 h. The remaining drug within the nanoparticles could release sustainedly after 24 h. The results demonstrated that the lipid nanoparticles without soybean oil as emulsifier could significantly improve physical stability of PLNs. The sustained-release manner of PLNs could not only improve stability of PGE1 but also prolong the drug retention time in blood. It was often reported that the drug encapsulated into nano-formulation which exhibited sustained release manner could change drug pharmacokinetics and improve drug efficacy (Zhang et al., 2008; Manjunath and Venkateswarlu, 2005). After intravenous injection, PGE1 could release from PLNs slowly, and the occurrence of metabolization and inactivation of drug would be reduced. This could increase the valid drug concentration to the sites of inflammation or vascular lesions, and correspondingly reduce the requirement amount of drug for infusion, and finally reduce the side effect of PGE1.

3.3. In vitro anti-inflammation test

As a vasodilator, PGE1 has been proved to have anti-inflammatory effects in vivo (Murota et al., 2008; Fantone et al., 1983). However, until now, very little research has been done about the detailed molecular anti-inflammatory mechanism of PGE1 and the anti-inflammatory effects of new PGE1 delivery systems. Pro-inflammatory cytokines which are commonly found in vascular lesions (Ross, 1999) can lead to inflammatory cascades. The cell adhesion molecules also play an important role in several acute and chronic inflammatory diseases since the leukocyte adhesion to endothelial cells is crucial for extravasation of leukocytes to sites of inflammation (Walzog and Gaehtgens, 2000; Geng et al., 2004). In contrast with their requisite roles in host defense, aberrant interaction between the leukocyte and the endothelial cell results in the uncontrolled inflammation seen in tissue injury, thrombosis, atherosclerosis and other pathologic sequelae (McIntyre et al., 2003). Modulation of the inflammatory cytokines and the cell adhesion molecules have therefore been suggested as a new treatment strategy for inflammatory diseases. In this work, the inhibition effects of PLNs on the expression of inflammatory cytokines and the cell adhesion molecules were compared with that of free PGE1 to study whether or not PGE1 loaded into lipid nanoparticles could improve its anti-inflammatory effect.

3.3.1. TNF-α and IL-1β expression

Activated macrophages play an important role in inflammatory diseases via production of cytokines. Lipopolysaccharides (LPS) in the outer membrane of Gram-negative bacteria could elicit strong inflammatory reaction by the activation of immune cells (Raetz and Whitfield, 2002). Many previous reports have improved that LPS can induce macrophages to produce pro-inflammatory cytokines, such as TNF-α and IL-1β (Reddy and Reddanna, 2009), which are known to play a key role in the pathogenesis of many inflammatory diseases (Hsieh et al., 2007; Kim et al., 2004). In this work, TNF-α and IL-1β expression were investigated in LPS-induced response in a murine macrophage cell line, RAW264.7. The concentration of TNF-α and IL-1β released to the cultured supernatants after LPS stimulation were measured by ELISA kits. The TNF-α and IL-1β protein expression increased significantly after LPS stimulation (Fig. 2). Co-culture of RAW264.7 cells with LPS and free PGE1 or PLNs could significantly reduce TNF-α protein secretion, while only a weak suppression of IL-1β protein secretion was found. With drug concentration increasing, the inhibition of TNF-α protein secretion increased. PLNs exerted a stronger suppression effect than free PGE1 in the highest drug concentration (1 μM). Compared with free PGE1 without protection which would be inactivated quickly, the sustained release of PGE1 from the lipid nanoparticles could provide a sustaining effect on cell cultures. It demonstrated that PGE1 entrapped in lipid nanoparticles could improve the suppression of pro-inflammatory cytokine TNF-α and thus improve its anti-inflammatory activity.

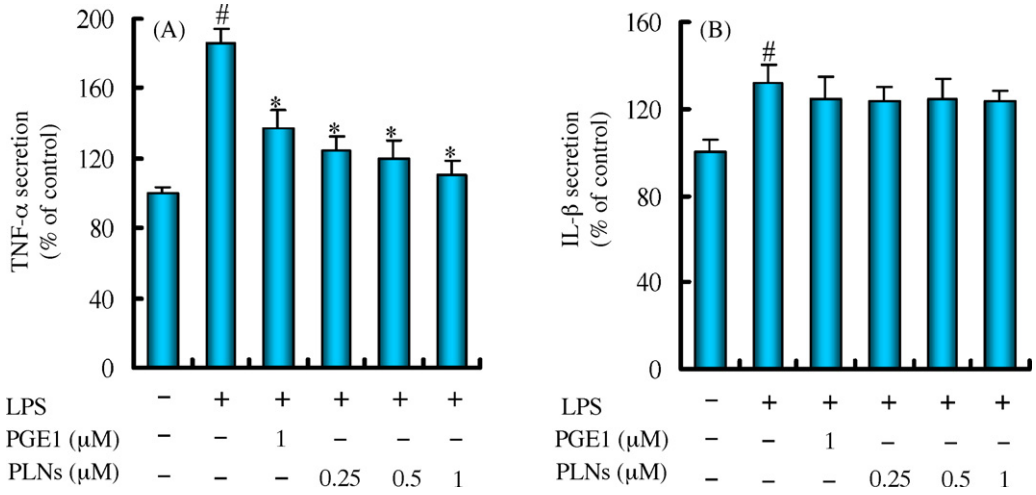

Fig. 2. Inhibition of LPS induced production of TNF-α (A) and IL-1β (B) by PLNs. For each concentration treatment, the level of TNF-α or IL-1β release was represented as a percentage of the control set at 100%. $^{\#}p < 0.05$ vs control group, $^{*}p < 0.05$ vs LPS treated alone group.

3.3.2. In vitro adhesion assay

A key event in the inflammatory response is the leukocyte recruitment by local production of chemoattractants and the adherence of leukocytes to endothelium by the expression of adhesion proteins (Rao et al., 2007). One of the effective ways to evaluate the anti-inflammatory effect of PLNs is to study the inhibition effect of PLNs on the interaction between the leukocyte and the endothelial cell. In this work, a static adhesion assay where human blood lymphocytes adhere to activated HUVEC was used to investigate the anti-adhesive property of PLNs. As shown in Fig. 3, with LPS stimulation, the adhesion of HL to HUVEC was one and a half times as much as to the control cells without LPS stimulation. The adhesion of HL to LPS-activated HUVEC was reduced by the addition of either free PGE1 or PLNs at all concentrations. The best inhibition effect of free PGE1 was achieved at 0.5 μM with cell adhesion of 114.1% of the control. Perhaps, rather unconventionally, PLNs showed significantly enhanced inhibition effects than free PGE1 at all three concentrations. At 1 μM, the cell adhesion was only 15.5% of the control, which demonstrated that PGE1 entrapped in lipid nanoparticles could increase the inhibition effects on the interaction between the leukocyte and the endothelial cell. It is suggested that PLNs could have great effect on the adherence of leukocytes to endothelium during the inflammatory response.

3.3.3. Flow cytometry analysis of adhesion molecule expression

On the endothelial cell membrane, the expression of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) which are members of immunoglobulin gene superfamily are known to increase significantly after exposure to stimuli such as endotoxin, interleukin-1 and TNF-α (Pu et al., 2002). Adhesion molecules are a prerequisite for leukocyte tethering and extravasation which is involved during the inflammatory response. It was reported that PGE1 could reduce circulating adhesion molecules (ICAM-1, VCAM-1) in peripheral vascular disease (Palumbo et al., 2000). So the effect of PLNs on the ICAM-1 expression on the membrane of HUVEC was first compared with that of free PGE1. The expression of ICAM-1 increased significantly on HUVEC membrane after LPS stimulation (Fig. 4). The addition of free PGE1 or PLNs to HUVEC could effectively reduce ICAM-1 expression.

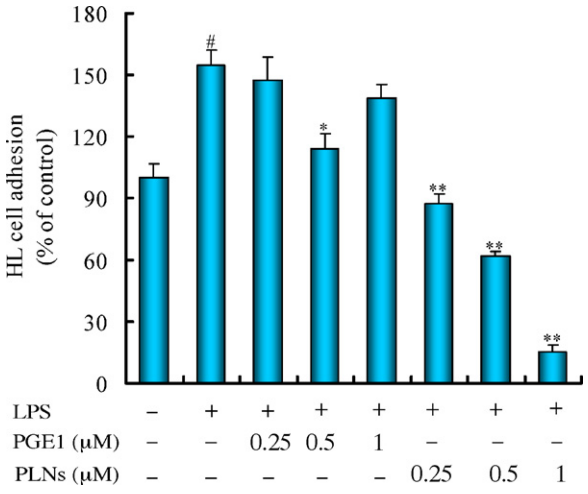

Fig. 3. Effects of PLNs on LPS induced adhesion of HL cells to HUVEC. For each concentration treatment, the HL cell adhesion to the HUVEC was represented as a percentage of the control set at 100%. $^{\#}p < 0.05$ vs control group, $^{*}p < 0.05$ vs LPS treated alone group, $^{**}p < 0.01$ vs LPS treated alone group.

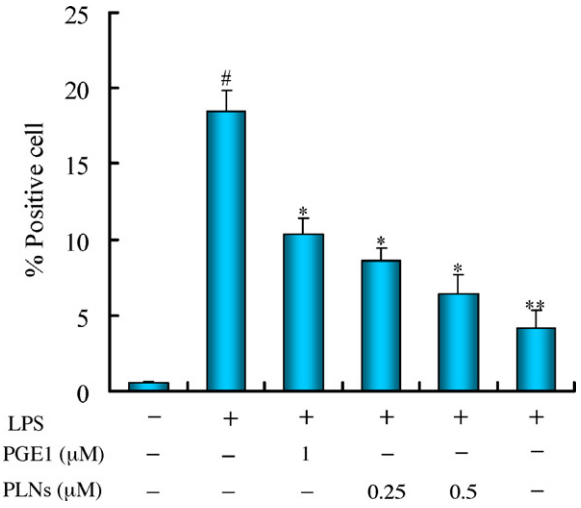

Fig. 4. Effects of PLNs on LPS induced ICAM-1 adhesion molecule expression on HUVEC membrane. The HUVEC monolayers were incubated with different concentration of PLNs for 8 h. After the incubation, ICAM-1 expression on HUVEC cell membrane was analyzed with PE labeled monoclonal antibodies by flow cytometry. $^{\#}p < 0.01$ vs control group, $^{*}p < 0.05$ vs LPS treated alone group, $^{**}p < 0.01$ vs LPS treated alone group.

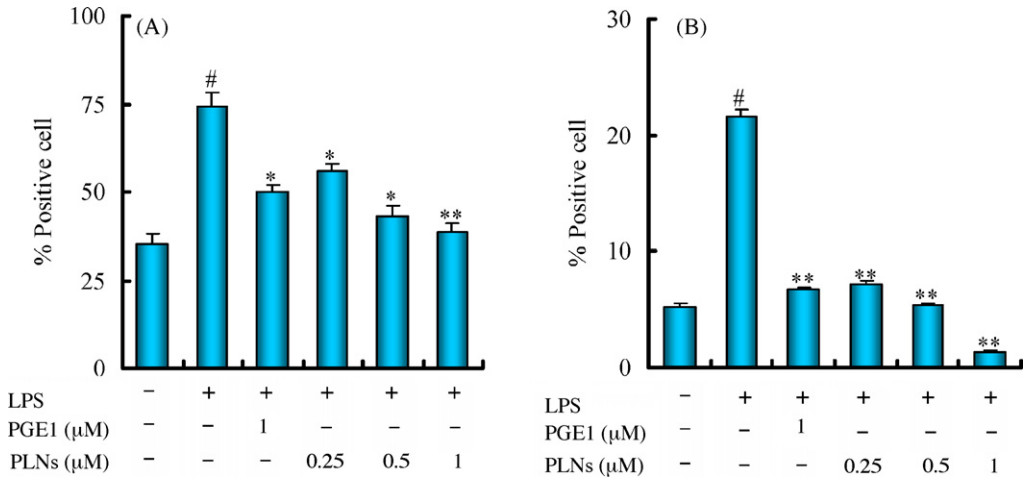

Fig. 5. Effects of PLNs on LPS induced ICAM-1 (A) and VCAM-1 (B) adhesion molecule expression on MDA-MB-468 cell membrane. The MDA-MB-468 cells were incubated with different concentration of PLNs for 8 h. After the incubation, ICAM-1 and VCAM-1 expression on cell membrane was analyzed with PE labeled monoclonal antibodies by flow cytometry. $^{\#}p < 0.01$ vs control group, $^{*}p < 0.05$ vs LPS treated alone group, $^{**}p < 0.01$ vs LPS treated alone group.

However, the ICAM-1 expression could not reduce to the control level. The inhibition effect of PLNs was much stronger than free PGE1 with a dose-response relationship. The inhibition effect of PLNs at 0.25 μM was comparable with that of free PGE1 at 1 μM.

As inflammation has recently been implicated in the genesis of many diseases including cancers, atherosclerosis and rheumatoid arthritis, and the mechanisms of action of PGE1 in the treatment of many diseases have not been fully elucidated. So a cancer cell line MDA-MB-468, which had been reported to constitutively express both ICAM-1 and VCAM-1 (Ali et al., 2000), was chosen and used to broaden the scope of clinical application of PGE1. In the present work, it was found for the first time that LPS could significantly increase both ICAM-1 and VCAM-1 on MDA-MB-468 cell membrane (Fig. 5). Either free PGE1 or PLNs could significantly reduce ICAM-1 or VCAM-1 expression on breast cancer membrane, and the adhesive molecules could be reduced to the control level. These results implied that PGE1 could have effect in cancer therapy. The inhibition effect of PLNs on either ICAM-1 or VCAM-1 was much stronger than free PGE1. The inhibition effects of PLNs increased with drug concentration. These results all demonstrated that PGE1 entrapped in lipid nanoparticles could improve its suppression of adhesion molecule expression on cell membrane.

3.4. Allergenicity test

In active systemic anaphylaxis test, the guinea pigs treated with PLNs at 0.86 μg/kg or 1.72 μg/kg showed no positive reaction such as scratch, tremor, dyspnea or cough. As control, 5% bovine serum albumin group showed strong reaction and no guinea pig survived after systemic anaphylaxis reaction was induced.

In passive cutaneous anaphylaxis (PCA) test, treated SD rats with two dose levels both showed no symptoms of anaphylaxis (Fig. 6). In contrast, the blue lesions on the skin of rats were very obvious

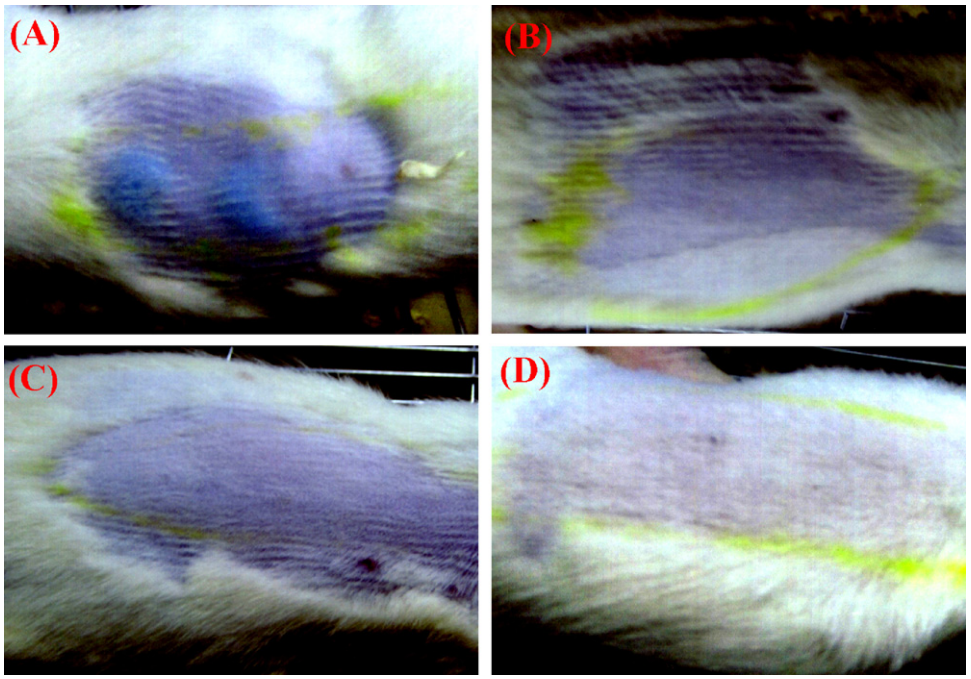

Fig. 6. Images of blue lesions on the skin of SD rats for the passive cutaneous anaphylaxis test. Representative rats from 5% bovine serum albumin group (A), saline solution group (B), PLNs treated group at doses of 0.86 μg/kg (C) and 1.72 μg/kg (D).

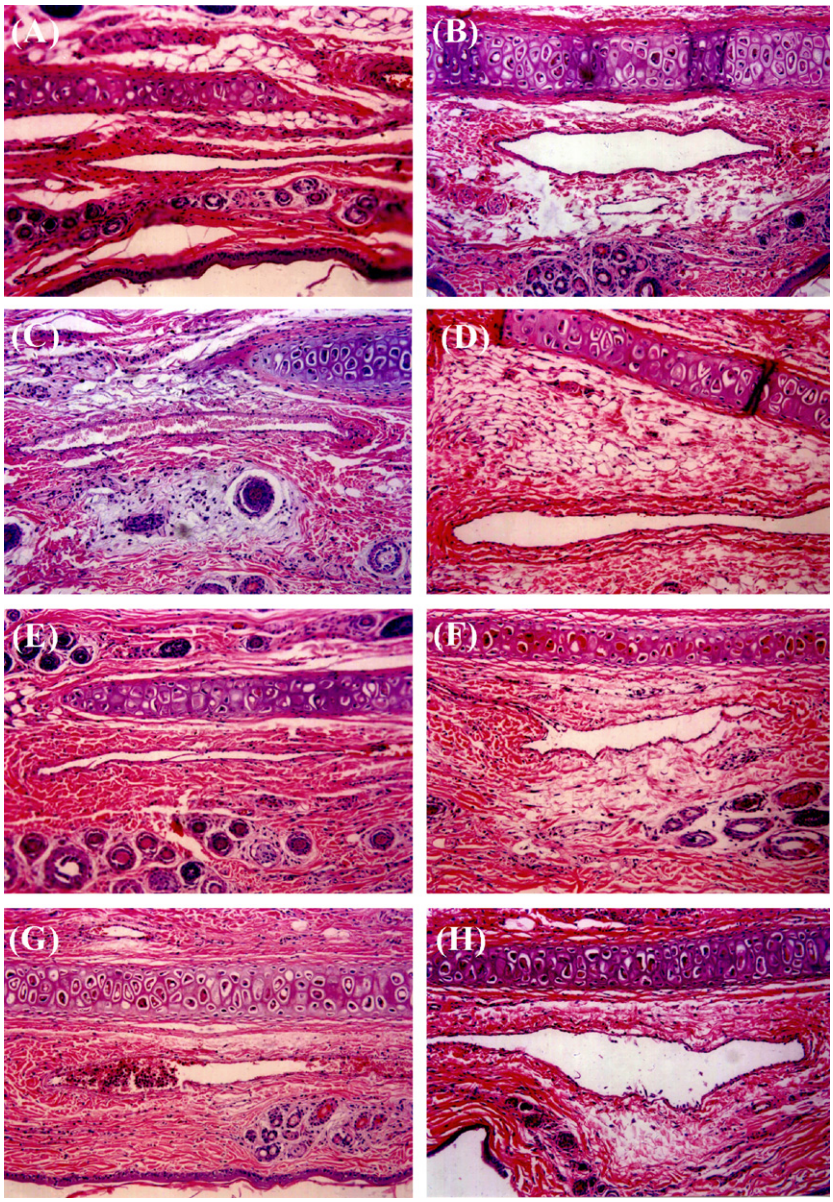

Fig. 7. Microscopic images of sections of auricular vein of treated rabbit, stained with hematoxylin-eosin. (A) Normal, treated with saline solution. (B) Treat with PLNs at dose of 0.47 μg/kg after successive three infusions. Treat with PLNs at dose of 0.94 μg/kg after the successive three infusions (C) and two days after the last infusion (D). Treat with Lipo-PGE1 at dose of 0.47 μg/kg after the successive three infusions (E) and two days after the last infusion (F). Treat with Lipo-PGE1 at dose of 0.94 μg/kg after the successive three infusions (G) and two days after the last infusion (H). Fibrin thrombus was found within the vessel lumen in image (G) and mild vascular congestion was found in image (F).

after infusion of 5% bovine serum albumin. These results implied that PLNs did not show any severe allergenicity.

3.5. Irritation test

It has been reported that PGE1 showed severe side effects in some patients (Schramek and Waldhauser, 1989). Among these side effects, local tissue irritation is the most common and serious because of the long-term infusion resulted from the rapid drug inactivation. Although PGE1 loaded into emulsions reduced the clinical dosage, the instability and rapid leakage of drug from emulsions still make Lipo-PGE1 induce side effects (Shen et al., 2005). In this work, PLNs were compared with the commercial available Lipo-PGE1 to study whether PLNs could cause less vascular or muscular tissue irritation than Lipo-PGE1.

The vascular irritation experiment was performed by infusion of PLNs or Lipo-PGE1 into the vein of the rabbit ear with dose of 0.47 μg/kg and 0.94 μg/kg. Representative histologic results were shown in Fig. 7. All rabbits treated with PLNs showed no vascular tissue injury after the successive infusion for three times. However, fibrin thrombus was found within the vessel lumen in one animal treated with high dose of Lipo-PGE1. Two days after the last infusion, one animal treated with low dose of Lipo-PGE1 was found mild vascular congestion. These results demonstrated that PLNs reduced vascular irritation compared with Lipo-PGE1.

As for muscle irritation, no noticeable irritating reactions were observed in animals treated with either saline solution or PLNs at two dose levels two days after the last injection on gross findings. Total score of 2 was recorded in animals treated with either saline solution or PLNs at two dose levels, which could be result from the mechanical injury by multiple punctures. Severe hemorrhage was found in half of the animals treated with Lipo-PGE1 at two dose levels, which demonstrated that Lipo-PGE1 could induce muscle irritation. Total scores of 8 were recorded in animals treated

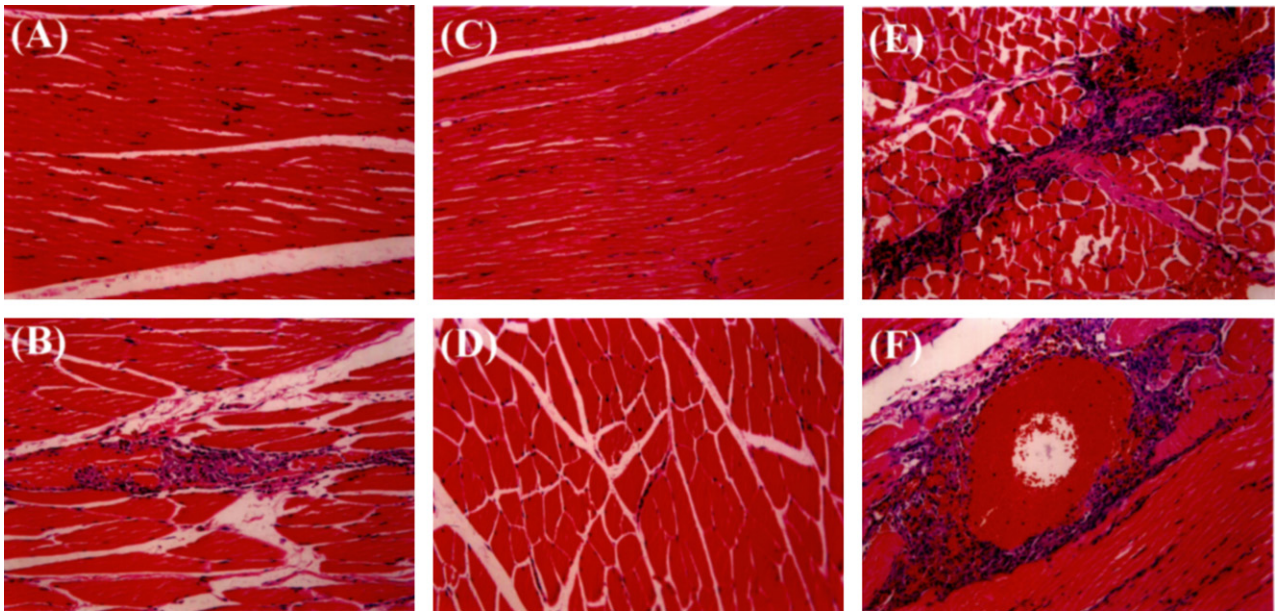

Fig. 8. Microscopic images of sections of quadriceps femoris muscle of treated rabbit, stained with hematoxylin-eosin. (A) Normal, treated with saline solution. (B) Treated with saline solution. Section of representative quadriceps femoris muscle with mechanical injury. (C) Treat with PLNs at dose of 0.47 μg/kg. (D) Treat with PLNs at dose of 0.94 μg/kg. (E) Treat with Lipo-PGE1 at dose of 0.47 μg/kg. (F) Treat with Lipo-PGE1 at dose of 0.94 μg/kg.

with Lipo-PGE1 at high dose level. With histological examination, the animals treated with PLNs at two dose levels showed no obvious changes on tissue structure compared with the animals treated with saline solution. Inflammatory cells infiltered into the stroma in some of the animals treated with saline solution or PLNs, which could be due to the mechanical injury (Fig. 8). Fig. 8E and F showed the typical micrographs corresponding to the lesions observed microscopically in animals treated with Lipo-PGE1. The muscle fibers lost their cross-striation and the sarcoplasms collapsed, which indicated the degeneration or necrosis of muscle fibers. Stromal oedema and hemorrhage were also observed. It also was found that the inflammatory cells such as polymorphs and phagocytes infiltered into the stroma in the surrounding portion of the lesions. These all demonstrated that PGE1 encapsulated into lipid nanoparticles could greatly improve its biocompatibility. The integrity structure of lipid nanoparticles could reduce the direct interaction between drug and vascular tissue or muscular tissue thus reduce the irritation of drug and reduce the local side effect.

4. Conclusion

Lipid nanoparticles loading PGE1 was successfully prepared by high pressure homogenization, and PLNs showed particle size 68.1 ± 4.7 nm, zeta potential -3.32 ± 0.37 mV and entrapment efficiency $92.1 \pm 1.3\%$. PLNs exhibited a sustained release with low burst drug release. Lipid nanoparticles could effectively protect PGE1 from degradation, and the sustained release of PLNs could provide a sustaining drug effect, both of which could result in the improved anti-inflammatory effects. No allergenicity, vascular and muscle irritation were induced by PLNs even at double of the highest concentration of clinical infusion. As a result, PLNs could be a more potential formulation in the treatment of inflammation-related diseases for PGE1.

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