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Cationic Liposomes as Carriers for Aerosolized Formulations of an Anionic Drug:

Safety and Efficacy Study

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Abstract

This study tests the hypothesis that pegylated cationic liposomes are a viable carrier for inhalable formulations of low molecular weight heparin, an anionic drug. Cationic liposomal formulations of low molecular weight heparin were prepared by the hydration method using 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt), cholesterol and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000]. The formulations were characterized for particle size, entrapment efficiency, pulmonary absorption and pharmacological efficacy. For absorption studies, the formulations were administered to anesthetized male Sprague-Dawley rats via the pulmonary route and drug absorption was monitored by measuring plasma anti-factor Xa activity. The pharmacological efficacy of the formulations was studied in rodent models of pulmonary embolism and deep vein thrombosis. The mean particle size of the liposomes was 104.8 ± 20.7 nm and the drug entrapment efficiency was $90.3 \pm 0.1\%$. The half-life of the cationic liposomal formulation was 10.6 ± 0.2 h, a 2.2-fold increase compared to low molecular weight heparin formulated in saline, and the relative bioavailability was $\sim 73.4 \pm 19.1\%$ when compared to subcutaneously administered drug. A once-every-other-day inhaled dose of the formulation showed similar efficacy in reducing thrombus weight as a once-daily dose of subcutaneously administered drug. Likewise, cationic liposomal formulations administered via the pulmonary route 6 h prior to embolization in the lungs showed a thrombolytic effect comparable to that of low molecular weight heparin administered subcutaneously 2 h before embolization. Histological examination of lung tissue and measurement of injury markers in bronchoalveolar lavage fluid suggest that the formulations did not produce extensive damage. The results demonstrate that pegylated cationic liposomes could be a viable carrier for an inhalable formulation of low molecular weight heparin.

Keywords

Cationic liposome; Low molecular weight heparin; Pulmonary delivery; Jugular vein thrombosis; Pulmonary embolism; Lung toxicity

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1. Introduction

Liposomes prepared with cationic lipids—cationic liposomes—have traditionally been investigated as non-viral vectors for gene delivery (Audouy et al., 2002; Lv et al., 2006) because this type of liposome, with its positive surface charge, offers several advantages over its charge-neutral counterparts. Positively charged liposomes can entrap and condense a relatively large amount of negatively charged DNA molecules by electrostatic interactions. The condensed and positively charged DNA-liposome complex can facilitate binding and endocytosis with the negatively charged plasma membrane (Pedroso de Lima et al., 2001). Furthermore, cationic liposomes can protect DNA from degradation by DNases. Because of the high payload of intact DNA in cationic liposomes and the positive surface charge of DNA lipoplexes, enhanced gene transfection efficiency is observed with these non-viral vectors.

Despite being a promising delivery approach, the use of cationic liposomes has been limited to non-viral gene-delivery research over the past two decades. There are little or no data on the use of these lipidic carriers in the delivery of drug molecules having electrostatic properties similar to that of DNA. Low Molecular Weight Heparin (LMWH) is one drug molecule that resembles DNA in its high negative surface charge. LMWH is a highly-sulfated glycosaminoglycan with a relatively large molecular weight (average MW, 5 kDa). LMWHs have been widely used for the treatment of vascular thromboembolism, including deep vein thrombosis (DVT) and pulmonary embolism (PE) (Segal et al., 2007). However, there are some important limitations that preclude the use of LMWH as a maintenance therapy. Because of the high negative surface charge, LMWHs are not absorbed via the oral route, thus requiring that they be administered as a subcutaneous injection. Furthermore, LMWHs are quickly metabolized in the liver and have a relatively short duration of action; therefore, the drug needs to be injected once or twice daily (Weitz, 1997).

A more patient-friendly delivery approach would be administration of the drug via non-invasive routes, including the oral, nasal and pulmonary routes. In a series of studies, it has been shown that it is feasible to administer LMWH via the oral, nasal and pulmonary routes (Bai et al., 2007; Qi et al., 2004; Yang et al., 2005; Yang et al., 2006; Yang et al., 2004a; Yang et al., 2004b). Moreover, we showed that nasal and pulmonary absorption of LMWH can be increased by formulating the drug with absorption enhancers (Mustafa et al., 2003). More recently, we also established that positively charged polymeric carriers, including polyethylenimines and dendrimers, can increase pulmonary absorption of LMWH by neutralizing the negative surface charge or by acting as cationic absorption promoters (Yang et al., 2006; Bai et al., 2008). However, previous studies failed to address the limitations associated with the short half-life of the current therapy with LMWHs.

Based on the similarity between the ionic properties of DNA and LMWH, we hypothesize that the limitations associated with the poor absorption, the invasive means of administration, and the short half-life of LMWH can be overcome by developing an aerosolized pegylated cationic liposome-based formulation of the drug. Therefore, this study was designed to investigate the feasibility of pegylated cationic liposomes as a carrier for pulmonary delivery of LMWH. Toward this end, we studied pulmonary absorption, circulation time, and the efficacy of cationic liposomal formulations of LMWH in preventing the thromboembolic disorders DVT and PE in rat models.

2. Materials and Methods

2.1. Materials

LMWH (average molecular weight and anti-factor Xa activity are 4494 Da and 61 U/mg, respectively) was purchased from Celsus Laboratories (Cincinnati, Ohio, USA).

Phosphatidylcholine (PC) and cholesterol were purchased from Spectrum Chemical Corp. (New Brunswick, New Jersey, USA). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (DSPE-PEG-2000) and 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP) were purchased from Avanti Polar Lipids Inc. (Alabaster, Alabama, USA). ¹²⁵I-labeled fibrinogen was obtained from Amersham (GE Healthcare Bio-Sciences Corp., Piscataway, New Jersey, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

2.2. Preparation of PEG-cationic Liposomal Formulation of LMWH

Cationic liposomes of LMWH were prepared with DOTAP, cholesterol and DSPE-PEG-2000. The molar ratio of DOTAP: cholesterol: DSPE-PEG-2000 was 6:3:0.8 and the total amount of lipid was 15 mg. Liposomes were prepared as described by Szoka and Papahadjopoulos (1978). Briefly, lipid mixtures were dissolved in an organic solvent, and a dry thin lipid film was prepared in a round-bottom flask using a Buchi R-114 Rotavapor (Buchi Laboratories AG, Postfach, Switzerland). Subsequently, the flask was kept under vacuum for 2 h to ensure complete removal of residual solvent. The dry lipid film was hydrated with a phosphate-buffered solution of LMWH at 50±2°C. The dispersion thus obtained was vortexed and subjected to ultrasound for 2 min. The final formulation was extruded through 0.4, 0.2, and 0.1 µm polycarbonate membrane filters (Costar Nucleopore, Cambridge, Massachusetts, USA). All formulations were prepared to contain 300 U of LMWH in each 1 ml.

2.3. Determination of Particle Size and Entrapment Efficiency

For particle size determination, LMWH liposomal sample solutions (~300 µl) were dispensed into disposable tubes and particle size measurements were performed in triplicate using a NICOMP™ 380 ZLS, PSS-Nicomp particle sizing system (Santa Barbara, California, USA). LMWH entrapment efficiency in the formulations was determined by separating the liposomes from the dispersion medium by ultracentrifugation after minor modifications of a previously published procedure (Heeremans et al., 1995). An aliquot (200 µl) of LMWH liposomal formulation was mixed with 20 ml of water and centrifuged in a Optima™ LE-80K ultracentrifuge (Beckman Coulter Inc., Fullerton, California, USA) at 15°C, 40,000g for 30 min. LMWH content in the supernatant was determined using an azure A assay as described by Yang et al. (2006). The entrapment efficiency was calculated using the equation: $[(T-C)/T] \times 100 (\%)$, where *T* is the total amount of LMWH in the liposomal dispersion, and *C* is the amount of LMWH detected only in the supernatant.

2.4. Pulmonary Absorption Studies in Rats

Optimized cationic liposomes containing LMWH (100 U/kg; ~0.4 mL/kg) were administered intratracheally to anesthetized adult male Sprague-Dawley (SD) rats weighing 200-250 g, as described in previous studies (Bai et al., 2007; Yang et al., 2004b). Briefly, after laryngoscopic visualization of the trachea, the formulations were administered directly to the lungs by using a Microsprayer® (Model IA-1B; Penn Century Inc., Philadelphia, Pennsylvania, USA). For bioavailability studies, formulations were administered subcutaneously (50 U/kg) as a single injection under the back skin. After both pulmonary and subcutaneous administration, blood samples (300 µl) were collected from the tip of the rat-tail at 0, 0.5, 1, 2, 4, 8, and 12 h in citrated microcentrifuge tubes and placed on ice. Subsequently, the plasma was treated and analyzed for antifactor Xa activity as described in previous studies (Bai et al., 2007; Yang et al., 2004b).

2.5. PEG-cationic liposomes of LMWH in the Treatment of Deep Vein Thrombosis

A DVT model was developed according to our previously published procedure (Bai et al., 2007). Three groups of rats with a thrombus received the following formulations via the

pulmonary route for eight days: (i) 100 U/kg of LMWH plus saline, once daily; (ii) 100 U/kg of LMWH plus saline, once every 48 h; (iii) cationic liposomes containing 100 U/kg of LMWH, once every 48 h. Two additional treatment groups were used as controls: (i) a once-daily dose of saline with no drug administered via the pulmonary route as a negative control, and (ii) 50 U/kg subcutaneous LMWH administered once daily as a positive control. On the eighth day of the treatment, the jugular vein was excised and the thrombus was meticulously extracted from the vein and weighed. After extraction of the thrombus, the animals were euthanized.

2.6. PEG-cationic liposomes of LMWH in the Treatment of Pulmonary Embolism

A rat model of PE described by Stassen et al. (1991) was used to study the efficacy of the formulations for the treatment of PE. First, a blood clot was prepared *in vitro* by mixing 1 ml of freshly collected rat blood, 40 μ l of 125 I-labeled fibrinogen (198 μ Ci/mg, GE Healthcare of BioSciences Corp., Piscataway, New Jersey, USA), 5 μ l of 4 U thrombin and 0.5 M CaCl_2 . The resulting solution was aspirated into a silicone catheter (I.D.= 0.64 mm, Silastic, Dow Corning, Midland, Michigan, USA) and the clot was then allowed to form overnight at 37°C. After aging, the radiolabeled clot was withdrawn from the tubing, and washed in warm saline for 30 min to remove radioactive fibrinogen that was on the clot surface. After washing, the clot in the capillary tube was cut into a 1-cm length and the radioactivity in the clot was measured in a Packard Cobra II Gamma Counter (GMI Inc., Ramsey, Michigan, USA). The clot was then aspirated into micro medical tubing (I.D. = 0.68 mm, Scientific Commodities Inc., Lake Havasu City, Arizona, USA) filled with warm saline. Immediately afterwards, the catheter containing the blood clot was introduced into the left auxiliary vein via the left external jugular vein of an anesthetized rat. After careful insertion of the catheter into the jugular vein, the clot was flushed into the venous circulation with 0.3 ml of warm saline and thereby embolized in the lungs.

The rats were pretreated with the formulations at 2 or 6 h prior to embolization and received the following formulations via the pulmonary route: (i) 100 U/kg of LMWH plus saline administered 2 h prior to embolization; (ii) 100 U/kg of LMWH in cationic liposomes, 6 h before formation of embolus. Two additional groups that received (i) 50 U/kg of LMWH via the subcutaneous route and (ii) saline with no drug via the pulmonary route 2 h prior to embolization were used as positive and negative controls, respectively. Blood samples were collected 60 min after embolization and the radioactivity in the blood was measured in a gamma counter. The rats were then euthanized with an overdose of anesthetic drug, the lungs and heart were dissected, and the radioactivity in the lungs and heart was measured in a gamma counter. The efficacy of the formulations was evaluated from the ratio of radioactivity of the thrombus in the lungs or blood to the initial radioactivity of the injected thrombus.

2.7. Bronchoalveolar Lavage Studies

Bronchoalveolar lavage (BAL) studies were performed as reported previously (Bai et al., 2007). Rats were divided into three groups to receive: (i) saline; (ii) LMWH in PEG-cationic liposomes; or (iii) 0.1 μ g/ml lipopolysaccharide (LPS) as a positive control. The animals received 100 μ l of the test or control formulations via the pulmonary route. The lungs were surgically removed 12 h after administration of the formulations and the BAL fluid was collected according to our previously published studies (Bai et al., 2007). Before collection of BAL, lungs were weighed to determine wet lung weights. The collected BAL fluid was analyzed to determine the concentration of alkaline phosphatase (ALP) in the lavage fluid using commercial assay kits. Enzyme activities are expressed in U/L.

2.8. Histopathology of the Lungs

The pathological changes that may occur in the lungs due to administration of cationic liposomes were further evaluated by histological staining of the lungs according to a previously

published report with minor modifications (Darien et al., 1997). Similar to the BAL studies, the rats were divided into three groups to receive different treatments: (i) saline; (ii) LMWH in cationic liposomes; and (iii) 0.1 µg/ml LPS as a positive control. Lungs were isolated 12 h after administration of the formulations. After freezing at -80°C with isopentane, the left lung tissue was sliced into 10-µm-thick transverse sections by a Shandon Cryotome® SME Cryostat (Shandon Scientific LTD., Cheshire, England) and the sections were collected onto the glass slides. Tissue slides were stained with hematoxylin-eosin following a published method (Darien et al., 1997)). The slides were then evaluated by the investigators under a light microscope (×40).

All animal studies were approved by the Texas Tech University Health Sciences Center (TTUHSC) Animal Care and Use Committee and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

2.9. Data Analysis

Standard noncompartmental analysis was performed for LMWH absorption profiles using Kinetica, Version 4.0 (Innaphase Corp., Philadelphia, Pennsylvania, USA). The area under the plasma concentration versus time curve ($AUC_{0 \rightarrow \infty}$) was calculated by the trapezoidal method. The relative bioavailability was estimated by comparing the $AUC_{0 \rightarrow \infty}$ for LMWH after pulmonary delivery with that of subcutaneously administered LMWH. All values are expressed as the mean±S.D. One-way ANOVA was used to compare the data. When the differences in the means were significant, post-hoc pair wise comparisons were conducted using Newman-Keuls multiple comparison (GraphPad Prism, version 3.03, GraphPad Software, San Diego, CA). Differences in *p*-values less than 0.05 were considered statistically significant.

3. Results and Discussion

3.1. Characterization of LMWH PEG-cationic Liposomes

Characterization of PEG-cationic liposomal formulations of LMWH was performed by evaluating particle size and drug entrapment efficiency. As shown in Fig. 1, the particle size of the cationic liposomes was 104.8 ± 20.7 nm and the LMWH entrapment efficiency was about $90.3 \pm 0.1\%$. The particle size and entrapment efficiency can provide important information regarding the circulation time of the particles and the dose of the formulation, respectively. In fact, particle size of particulate drug carriers plays an important role in the uptake of carriers by cells of the reticuloendothelial system (RES) (Gabizon and Papahadjopoulos, 1988; Woodle and Lasic, 1992). An increase in circulation time results from the fact that small liposomes (<200 nm) can circumvent RES uptake due to their reduced recognition by circulating opsonin (Woodle and Lasic, 1992). Thus, liposomes with an average particle size of 104 nm should show a long-circulating effect. A marked increase in LMWH entrapment efficiency (EE) was observed (90%) with the cationic liposomes; in fact, EE was three times higher than that reported for neutral liposomes of LMWH (Song and Kim, 2006). The increase in EE may be attributed to the electrostatic interactions between negatively charged LMWH and the positively charged lipid, DOTAP. This ionic interaction may increase loading of LMWH both in the inner core of the liposomes and on the liposomal surface. Pegylated cationic liposomes with increased LMWH entrapment and adequate particle size may lead to favorable pharmacokinetics in *in-vivo* pulmonary absorption studies.

3.2. Pulmonary Absorption Studies in Rats

The efficacy of cationic liposomes in extending circulation time and enhancing pulmonary absorption of LMWH was investigated in a rodent model (Fig. 2, Table 1). Upon pulmonary administration of PEG-cationic liposomes of LMWH in adult male SD rats, appreciable increases in half-life, C_{max} , and relative bioavailability were observed (Fig. 2, Table 1).

Compared to plain LMWH administered via the pulmonary or subcutaneous route, an approximately twofold increase in the half-life of LMWH encapsulated in PEG-cationic liposomes was observed: the $t_{1/2}$ of LMWH in PEG-cationic liposomes was 10.6 ± 0.2 h, whereas it was 4.9 ± 3.3 and 6.5 ± 1.8 h for plain LMWH in saline administered as an aerosol and by injection, respectively ($p < 0.05$). Similarly, $C_{\max}$ of the liposomal formulation (0.41 ± 0.03 U/ml) was higher compared to that of plain drug (0.36 ± 0.03 U/ml). In fact, the $C_{\max}$ of the drug in PEG-cationic liposomes was significantly higher than that of LMWH in PEGylated neutral liposomes (data not shown). Moreover, compared to a plain aerosol formulation of LMWH ($F_{\text{relative}} = 21.7 \pm 4.9\%$), a 3.4-times increase in relative bioavailability was observed for LMWH formulated in PEG-cationic liposomes ($F_{\text{relative}} = 73.4 \pm 19.1\%$). Furthermore, the anti-factor Xa levels became subtherapeutic (less than 0.2 U/ml) 3 h after administration of plain LMWH as an aerosol or by injection. However, for the PEG-cationic liposomal formulations of LMWH, the anti-factor Xa levels were above therapeutic levels (more than 0.2 U/ml) for about 6 h. This difference in the anti-factor Xa levels of liposomal LMWH and plain LMWH suggests that PEG-cationic liposomes produced a long-acting effect of the drug, which would be beneficial for the treatment of DVT and PE. Overall, the pulmonary absorption profiles presented in Fig. 2 and Table 1 suggest that cationic liposomes of LMWH were efficacious in increasing the pulmonary absorption and extending the half-life of LMWH *in vivo*. Furthermore, the increase in bioavailability of LMWH suggests that biologically active LMWH was absorbed after pulmonary delivery.

The favorable pharmacokinetic profiles of the formulation may stem from several factors, including the long-circulating property of pegylated lipids, high drug-loading capacity of cationic lipids, and ability of lipids to facilitate transport across the respiratory epithelium. Potential applications of long-circulating liposomes have broadened considerably since the development of PEG-coated liposomes almost 17 years ago (Woodle and Lasic, 1992). Polymer coating of lipids resulted in increased circulation times for liposomes. The mechanism proposed for the increased circulation time suggests that a steric stabilization effect conferred by the PEG polymer causes reduced recognition of PEG liposomes by cells of the RES, and thus produces a reduced rate of clearance for the liposomes (Bradley and Devine, 1998; Harashima et al., 1994; Yan et al., 2005). Because of their opposite surface charges, cationic liposomes can entrap and condense negatively charged LMWH. Moreover, the nano-size of the LMWH-cationic complex and positive surface charge can facilitate binding and endocytosis by the negatively charged plasma membrane. The reduction in negative surface density can also play a role in increasing drug absorption because negative surface charge is a major barrier to LMWH absorption. Unlike for anionic liposomes, the resulting LMWH-cationic complexes are believed to be endocytosed, as was observed in the case of DNA encapsulated in cationic liposomes, which provides more potential to facilitate intracellular delivery of macromolecules (Hafez et al., 2000). Successful pulmonary delivery of LMWH in cationic liposomes may be because of high drug-entrapment efficacy and favorable physicochemical characteristics of lipoplexes, which facilitate more drug transportation to the blood.

3.3. Therapeutic Efficacy of LMWH formulated in PEG-cationic Liposomes in the Treatment of Deep Vein Thrombosis

An overall reduction in thrombus weight was observed in rats that received LMWH compared to rats that received only saline ($p < 0.05$). When LMWH was administered via the pulmonary route at a dose of 100 U/kg once daily, a significant decrease in thrombus weight was observed as compared to that of saline-treated rats (Fig. 3). The thrombus weight in saline-treated rats was 5.3 ± 0.8 mg whereas that for rats that received pulmonary LMWH was 0.8 ± 0.8 mg. When LMWH was administered subcutaneously at a dose of 50 U/kg, the thrombus weight was decreased to 0.5 ± 0.5 mg ($p < 0.05$) (Fig. 3). There were no statistically significant differences

between the thrombus weights in rats treated with subcutaneous LMWH and those treated with pulmonary LMWH in either saline or PEG-cationic liposomes ($p>0.05$). However, the thrombus weight in rats that received pulmonary LMWH plus saline once every 48 h was significantly higher compared to that of rats treated once daily subcutaneously or that received plain LMWH via the pulmonary route ($p<0.05$). Nevertheless, an overall decrease in thrombus weight (3.5 ± 0.6 mg) was observed as compared to rats that received no drug.

However, there were no significant differences between the thrombus weight in rats that received a once-every-48-h dose of pulmonary PEG-cationic liposomes containing LMWH (0.4 ± 0.2 mg) and those received a once-daily dose of subcutaneous LMWH ($p>0.05$). Although the LMWH plus saline formulation administered once daily produced an acceptable efficacy in reducing the thrombus weight, it failed to keep the same level of efficacy when given once every other day. These data agree with the pharmacokinetic profiles presented in Fig. 2, which show that the drug half-life was shorter for plain LMWH compared to liposomal LMWH, and that therapeutic anti-factor Xa level produced by the conventional liposomes disappeared after 3 h following pulmonary administration of plain LMWH. The pulmonary formulation of cationic liposomes of LMWH administered once every 48 h was as efficacious in preventing DVT as the once-daily dose of subcutaneous LMWH.

3.4. Therapeutic Efficacy of PEG-cationic Liposomes containing LMWH in the Treatment of Pulmonary Embolism

The effect of the formulations in preventing propagation of a clot formed in the lungs was studied in a rodent PE model. Toward this end, we measured both the amount of radioactive clot remaining in the lungs and that dissolved in the blood. The data presented in Fig. 4A show that $88.5\pm 7.9\%$ of the clot was present in the lungs of rats that received no treatment. However, the radioactivity in the lungs was reduced from 88% to 60% in rats treated with plain LMWH or liposomal LMWH 2 h before formation of the clot. There was practically no difference between the amounts of radioactivity in the lungs of rats that received LMWH as a subcutaneous injection versus those that received plain LMWH as an aerosol. A similar reduction in the amount of clot in the lungs was observed when PEG-cationic liposomal formulations were administered 6 h prior to formation of the clot. The data suggest that, when delivered by the pulmonary route, plain LMWH and PEG-cationic liposomes containing LMWH are as efficacious as subcutaneous LMWH in preventing further propagation of the clot. In contrast to the amount of radioactivity in the lungs, a reduced amount of radioactivity was observed in the blood of rats that received no treatment (Fig. 4). The amount of radioactivity was increased in the blood of rats that were treated with either subcutaneous or pulmonary plain LMWH. Radioactivity in the blood was also increased when the cationic liposomal formulation of LMWH was administered 6 h post-embolization. The presence of a relatively large amount of radioactivity in the blood suggests that LMWH administered via the pulmonary route was efficacious in dissolving the clot by preventing its propagation in the lungs. Overall, the data presented in Figs. 3 and 4 clearly suggest that the cationic liposomes were efficacious in prolonging the half-life of LMWH and also in maintaining therapeutic anti-factor Xa levels. The data also suggest that PEG-cationic liposomes containing LMWH were efficacious in preventing DVT and PE in rodent models.

3.5. Bronchoalveolar Lavage Studies

Wet lung weights were recorded 12 h after administration of the formulations to adult male SD rats. The lung weights of rats treated with liposomal formulations did not differ significantly from those treated with either saline or liposomal formulations of LMWH ($p>0.05$), but were significantly lower compared to those of the LPS-treated group ($p<0.05$) (Fig. 5A). This lack of change in lung weights suggests that PEG-cationic liposomes have little or no toxic effects on the lungs and are significantly less toxic than LPS. The lung weight measurements were

followed by analysis of BAL fluid for the presence of lung-injury markers. High ALP levels in BAL fluid indicate lung-cell damage (Hickey and Garcia-Contreras, 2001). Treatment with cationic liposomes showed no significant difference in ALP level from that produced by saline ($p < 0.05$) (Fig. 5B). However, the ALP levels produced by the liposomal formulation and the saline solution were significantly lower than that produced by LPS ($p < 0.05$). The data suggest that acute administration of cationic liposomes did not produce an increase in the levels of injury markers compared to saline-treated animals. However, as these data were obtained after a single dose of the formulation, a more extensive toxicological study is required to determine the safety of cationic liposomes as carriers for pulmonary drug delivery.

3.6. Histological Studies of Lung

Hematoxylin and eosin (HE) are the most commonly used stains in histopathology studies. Hematoxylin stains the nuclei blue and eosin stains the cytoplasm pink (Darien et al., 1997). Upon staining of tissue samples, any injury or inflammation can be observed and analyzed under a microscope. Lung inflammation can be characterized by extensive inflammatory infiltration by neutrophils, lymphocytes and plasma cells, and by fibrosis and granulomas in the perivascular region. Examination of saline-treated samples showed no cell infiltration or tissue fibrosis. Normal morphology of alveoli was clearly observed (Fig. 6A). However, histopathological examination of the lungs of rats treated with LPS showed thickening of the alveolar walls (Fig. 6C). Compared to normal tissue, rat lungs exposed to LPS, showed cell accumulation in the interalveolar septa. As a result of cell invasion, the interalveolar septae were thickened, the alveolar surface area became smaller, and the alveoli were irregularly organized. There were no noticeable changes in rat lungs treated with neutral or cationic liposomes compared to the saline control (Fig. 6B). The alveolar walls remained intact and normal in both treatment groups. Thus the data suggest that inhaled LMWH liposomal formulations did not produce major damage to the lungs.

In summary, the above study is the first to demonstrate that cationic liposomes can be used as carriers for pulmonary delivery of a relatively large molecular weight anionic drug. Cationic liposomes are highly effective in enhancing EE and pulmonary absorption of LMWH. Liposomes with positive surface charge possibly formed a compacted complex with the linear polymeric LMWH, thereby facilitating endocytosis-mediated absorption.

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Fig. 1. Particle size and entrapment efficiency of PEG-cationic liposomes. Data represent mean $\pm$ S.D., n=3.

Fig. 2. Changes in anti-factor Xa activity after subcutaneous and pulmonary administration of LMWH in saline or formulated in liposomes. Data represent mean $\pm$ S.D., n=3-5.

Fig. 3. Changes in thrombus weight after administration of LMWH formulations as aerosols or subcutaneous injections. Data represent mean $\pm$ S.D., n=3-6. *Results are significantly different, $p < 0.05$.

Fig. 4. Percent initial radioactivity as quantitated from the clot (A) remaining in the lungs and (B) dissolved in the blood after pulmonary or subcutaneous administration of different formulations. Data represent mean $\pm$ S.D., n=3-6. *Results are significantly different, $p < 0.05$.

Fig. 5. Corrected wet lung weights (A) and alkaline phosphatase (ALP) levels (B) at 12 h after administration of different formulations. Data represent mean $\pm$ S.D., n=3-5. *Results are significantly different, $p < 0.05$.

Fig. 6. Photographs of HE-stained lung tissue from rats that received (A) saline control, (B) PEG-cationic liposomes of LMWH, and (C) lipopolysaccharide (LPS) as a positive control.

Table 1

Pharmacokinetic parameters following pulmonary delivery of PEG-cationic liposomes of LMWH. The subcutaneous dose administered was 50 U/kg and the pulmonary dose delivered was 100 U/kg. Data represent the mean $\pm$ S.D., n=3-5

Formulations	C _{max} (U/ml)	T _{1/2} (h)	AUC _{0→∞} (U·h/ml)	F _{relative} (%)
LMWH plus saline	0.36 $\pm$ 0.03	4.9 $\pm$ 3.3	2.0 $\pm$ 0.5	21.7 $\pm$ 4.9
LWMH cationic liposome	0.41 $\pm$ 0.03 [*]	10.6 $\pm$ 0.2 [*]	6.9 $\pm$ 1.8 [*]	73.4 $\pm$ 19.1 [*]
Subcutaneous LMWH	0.32 $\pm$ 0.08	6.5 $\pm$ 1.8	4.7 $\pm$ 1.3	---

^{*} $p < 0.05$ compared to LMWH saline group.