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Pulmonary delivery of insulin by liposomal carriers

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Abstract

Growing attention has been given to the potential of a pulmonary route as a non-invasive administration for systemic delivery of therapeutic agents (mainly peptides and proteins). The lungs provide a large absorptive surface area, extremely thin absorptive mucosal membrane, and good blood supply. The non-invasive nature of this pathway makes it especially valuable for the delivery of large molecular protein. However, pulmonary delivery of peptides and proteins is complicated by the complexity of the anatomic structure of the human respiratory system and the effect of disposition exerted by the respiration process. In this study, novel nebulizer-compatible liposomal carrier for aerosol pulmonary drug delivery of insulin was developed and characterized. Experimental results showed that insulin could be efficiently encapsulated into liposomes by preformed vesicles and detergent dialyzing method. The optimal encapsulation efficiency was achieved when 40% ethanol was used. The particle size of liposomal aerosols from ultrasonic nebulizer approximated to 1 μm. Insulin was stable in the liposomal solution. Animal studies showed that plasma glucose level was effectively reduced when liposomal insulin was delivered by inhalation route of using aerosolized insulin-encapsulated liposomes. Including fluorescent probe (phosphatidylethanolamine-rhodamine) into liposome, we found that the liposomal carriers were effectively and homogeneously distributed in the lung aveolar. Liposome-mediated pulmonary drug delivery promotes an increase in drug retention-time in the lungs, and more importantly, a reduction in extrapulmonary side-effects which invariably results in enhanced therapeutic efficacies.

Keywords: Pulmonary delivery; Insulin; Liposomes; Aerosol

1. Introduction

Peptides and/or proteins are becoming more important in medication. When taken orally, peptides and/or proteins are degraded by the proteolytic enzymes in the gastrointestinal tract, and might be impermeable to the intestinal mucosa due to their hydrophilicity and large molecular size. As a result, systemic delivery of these macromolecular drugs and other therapeutic and diagnostic agents has been limited to the parenteral route. Repeated injections are required due to the short half-lives of peptide/protein drugs. In order to find alternative ways of administration other than injection, many efforts have been devoted to investigating the feasibility of nasal, transdermal, buccal, or other routes for drug administration. In the search for non-invasive delivery of biologics, it was discovered that the large and highly absorptive surface area of the lungs (~100 m^2) could be used for systemic delivery of peptides/proteins such as insulin, calcitonin etc. Even drugs with small molecular weight that are administered by injection are tested via the inhalation route either to provide non-invasively rapid onset of action, or improve the therapeutic ratio for drugs acting in the lungs [1]. Pulmonary delivery offers a number of advantages such as providing a large absorptive area, extensive vasculature, easily permeable membrane, and low extracellular and intracellular enzyme activity [2,3]. Another benefit is the direct targeting of drugs to the lungs for treatment of pulmonary diseases [4,5].

To convey a sufficient dose of drug to the lungs, suitable drug carriers are required. These can be either liquid [2,4–9], solid [9,10] or gaseous excipients. Incorporation of liposomes into pulmonary drug delivery systems provides an attractive means for sustained [2,11], non-invasive delivery to facilitate the treatments of chronic diseases such as asthma and diabetes.

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Liposome-mediated pulmonary drug delivery may increase in drug retention-time in the lungs, and more importantly, a reduction in extrapulmonary side-effects which invariably results in enhanced therapeutic efficacies. Metered dose inhaler (MDI) and nebulizers are the common devices frequently used in the pulmonary drug administration.

The formulation of liposomal protein for pulmonary delivery as an aerosol must not cause adverse pulmonary reactions such as cough or brochoconstriction. The formulation must also stabilize the protein sufficiently to ensure that the protein survives the rigors of the aerosol generation process. Formulation of proteins and peptides is often more challenging than formulation of small molecules because of the important role of protein conformation as well as the potential for chemical degradation pathways [12]. The engineering of an effective liposomal drug formulation for inhalation therapy must take into consideration the leakage problem associated with the nebulization process, vesicle stability and release kinetics within the pulmonary milieu, and the altered pharmacokinetics of the encapsulated drugs [13]. In some cases, the formulation components should not interfere with the generation of the aerosol.

Liu et al. [2] studied the effects of the formulation of a mixed liposomal formulation (dipalmitoylphosphatidyl choline:cholesterol, 7:2) on the enhancement of the pulmonary bioavailability of insulin. The results showed no difference in bioavailability between liposomally entrapped insulin and insulin administered with blank liposomes. In a recent study [9], interleukin 2 (IL-2)-encapsulated liposomes were administered as aerosol to individuals with immune deficiency. Patient acceptance, safety, toxicity and immune effects of IL-2 liposomes were studied. No significant changes in chest X-ray or pulmonary function tests were noticed after following administration of the liposomes. In recent year, Pfizer/Aventis/Neketar have launched a clinical trial for an inhaled, non-liposomal insulin, Exubera, through pulmonary routes [14,15]. However, more studies are needed in this related field, especially, the concerns about the drug's long-term pulmonary safety [16,17].

In this study, a novel nebulizer-compatible liposomal carrier for aerosol pulmonary drug delivery of insulin was developed. The drug was encapsulated into liposomes by preformed vesicles and detergent dialyzing method. The encapsulation efficiency, particle size, lung disposition, and the effect of reducing glucose concentration were investigated. The bioavailability of the formulations was verified by in vivo animal studies using inhalation of insulin-encapsulated liposomes administered directly into the lungs.

2. Materials and experimental methods

2.1. Materials

Hydrogenated egg yolk PC (HPC, Type I-EH, highly purified egg lecithin hydrogenated in the presence of a platinum catalyst, 99% phosphatidylcholine), egg yolk PC (Type X-E, 60% phosphatidylcholine), cholesterol (Chol), insulin and phosphate buffer saline were purchased from Sigma Chemical Company (St. Louis, MO, USA). PEG-DPPE (*N*-methoxypolyethyleneglycol succinoyl-2-*N*-dipalmitoylphosphatidyl ethanolamine, PEG Mw = 5000) was purchased from NOF Corporation (Hyogo, Japan). Chloroform, methanol, *n*-butanol and other chemicals used in the experiments and analysis were reagent grade (Wako Pure Chemicals, Japan). Low melting point agarose was purchased from Gibco Life Technologies (Grand Island, NY, USA). Phosphatidylethanolamine-rhodamine (lissamine rhodamine B) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA).

2.2. Encapsulating insulin into liposomes

Liposomal insulin was prepared by conventional film shaking method and membrane destabilizing/detergent dialyzing method. In film shaking method, lipid film was first formed at the bottom of a rotary vacuum evaporator. Aqueous citric buffer (pH 4.0) containing insulin was used to hydrate the thin film. Multilamellar vesicles were formed after hydration and mechanical shaking. The isoelectric point (pI) of insulin is 5.6. At pH < pI, insulin is a polycation and with good solubility. The membrane destabilizing/detergent dialyzing method was described previously [18]. Briefly, unilamellar or multilamellar vesicles were formed by slow addition of the lipids dissolved in ethanol (0.4 ml) into aqueous citrate buffer (0.6 ml, pH 4.0). Large unilamellar liposomes were formed by extrusion through 2 stacked 200 nm filters (5–10 passes). The compositions of lipids in liposomes were HPC/Chol (70/30 mol%) and HPC/Chol/PEG-DPPE (70/30/1 mol%). The final ethanol concentration was 40% [*v*/*v*]. Insulin in citric buffer was slowly added under vortexing to the ethanolic liposome dispersion, which typically contained 10 mg/ml lipid at room temperature. It was subsequently incubated at the appropriate temperature for 1 h, dialyzed for 2 h against a 1000-fold volume excess of citrate buffer to remove most of the ethanol and twice against a 1000-fold volume excess of Hepes buffered saline (HBS: 20 mM HEPES/145 mM NaCl, pH 7.5). These dialysis steps ensure complete removal of ethanol (0.01% as determined with the alcohol dehydrogenase assay kit from Sigma). Unencapsulated insulin was removed by gel chromatography through sepharose CL-4B gel column. Then we used Triton-X to break down the insulin-encapsulated liposomes and used the conventional BCA kits to assay the insulin content. Different liposome compositions were used to evaluate their physical and chemical stability.

Particle size and size distributions of liposome were measured by quasi-elastic light scattering (QELS). QELS measurements employed a Coulter Model N4 Plus sub-micro particle analyzer (Hialeah, FL, USA).

2.3. In vivo animal studies

BALB/c mice, 25–30 g, were induced to be diabetes by consecutive injection of alloxan for three days, 5, 5, and 2.5 mg respectively. In vivo animal inhalation studies were conducted in a flow chamber. Aerosols containing insulin-encapsulated

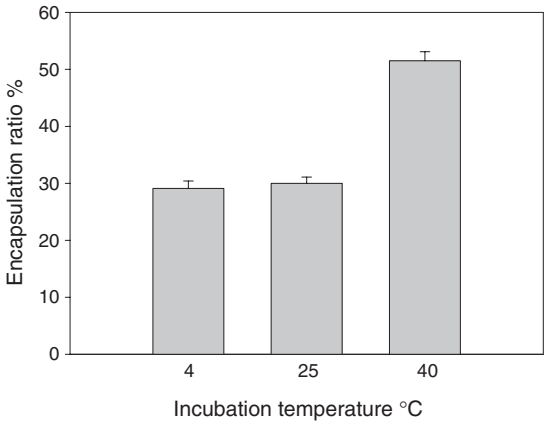

Fig. 1. The effect of incubation temperature on the insulin encapsulation ratio. Liposome composition: PC/Chol (70/30 mol%).

liposomes were generated by ultrasonic nebulizer (DEVILBISS Ultra-Neb 99). Particle size of aerosols was measured by aerodynamic particle sizer spectrometer, TSI 3320 (St Paul, MN, USA). The particle size of the liposomal aerosols from the nebulizer was approximately 1 μm. Before the experiment, the mice were fasted for 12 h. The diabetic mice were put in a chamber consciously. The chamber was connected to the nebulizer. The mice moved and breathed freely in the chamber. During the experiments, liposomal insulin was aerosolized and directed to the chamber for mice inhalation. Each mouse was exposed to the aerosols for 30 min. Blood was drawn from the eye socket. Glucose concentration in the blood was determined by a glucose analyzer, YSI 2300 (Yellow Springs Instrument, Ohio, USA).

2.4. Agarose inflation of the mouse lungs

To label the liposomal lung deposition fluorescently, aerosols of liposomal insulin containing phosphatidylethanolamine-rhodamine were introduced to the experimental mice. Inflation of the mouse lungs was achieved by a protocol developed by McLean et al. [19] and Uyechi et al. [20]. In summary, at the end of inhalation experiments, the mice were anesthetized with an intrapertioneal injection of ketamine (200 mg/kg) and xylazine (10 mg/kg) followed by inhalation with ethyl ether. A midline incision was opened to visualize the trachea, followed by intubation with an 18 G blunted needle. Solution of 3% low melting point agarose in PBS were prepared and were warmed to 42 °C before perfusion. The mouse thoracic cavity was cut open, exposing the lungs and heart. A volume of 0.8 ml of 3% low melting point agarose in PBS was instilled into the lungs. Warm PBS was used as a lavage in the open chest cavity and was important to keep agarose solution flowing into the entire lung. An ice-cold PBS lavage was then used to solidify the agarose. The lungs and trachea were removed, and stored in fixative (10% formalin in PBS) until tissue sectioning. Lung lobes were dissected and set in 7% low melting point agarose, and subsequently sectioned at 200 μm with a with a microtome (Sledge, Microtome IVS-400, Sakura, Tokyo, Japan).

3. Results and discussion

Formulation of proteins and peptides is more challenging than formulation of small molecules because of protein conformation and the potential for chemical degradation during preparation. In this study, we found that insulin could be efficiently encapsulated into preformed vesicles by using membrane destabilizing method and detergent dialyzing procedures without losing the biological activities. The optimal encapsulation efficiency was achieved when 40% ethanol was used. Because ethanol was a solvent of lipid and a kind of surfactants, it can make the lipid membrane become more flexible. The encapsulation efficiency was as high as 52% when liposomes were made up of HPC/Chol (70/30 mol%). A direct correlation between the encapsulation efficiency and the membrane destabilizing effect of ethanol was observed. Increasing the proportion of cholesterol to HPC or adding PEG-DPPE, the encapsulation efficiency decreased. As the stability of lipid bilayer was strengthened by increasing the cholesterol ratio, the lipid bilayer became more rigid, the fluidity was reduced, and the encapsulation efficiency was lowered. Adding PEG-DPPE, which was inserted into lipid bilayer and formed three-dimensional steric hindrance around the liposomes, kept insulin from entering the inside of vesicles and then decreased the encapsulation efficiency. However, liposomes made up of HPC/Chol (70/30 mol%) aggregated easily. Incorporating a small fraction of PEG-DPPE, less than one mole percent, would dramatically reduce the aggregation and increase the physical stability.

Fig. 1 shows the effect of incubation temperature on the encapsulation efficiency. When the incubation temperature was increased to 40 °C, the encapsulation efficiency increased. At higher temperature, the structure of lipid membrane became more irregular and looser, and the permeability of the lipid bilayer increased. Insulin would pass through the bilayer easily, and as a result, the encapsulation efficiency was higher. The optimal incubation time was 1 to 2 h. The encapsulated insulin amount increased as insulin loading amount increased, while the encapsulation efficiency decreased. The particle size and size distribution of the liposomes prepared in this study is

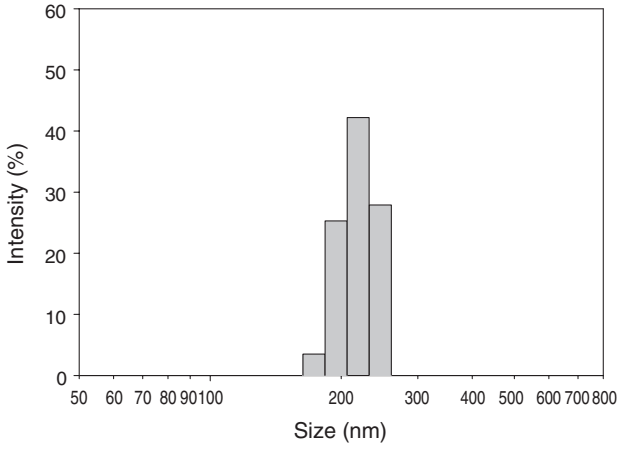

Fig. 2. The particle size distribution of liposomal insulin.

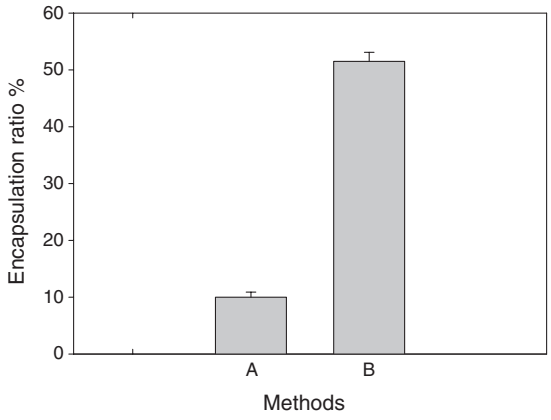

Fig. 3. Comparison of encapsulation efficiency between (A) conventional film shaking method and (B) membrane destabilizing method. Liposome composition: PC/Chol (70/30 mol%).

shown in Fig. 2. Mean particle sizes of (203.5±20.5) nm was obtained. Using membrane destabilizing/detergent dialyzing method can avoid the use of toxic organic solvent such as chloroform which may result in residual solvent problem, can reduce the possibility of insulin degradation during encapsulation process, and can increase the encapsulation efficiency.

Compared to the conventional film shaking method, the dripping ethanol method and the detergent-dialyzing procedures could increase encapsulation efficiency 4 to 5 times, as shown in Fig. 3. Long incubation time and high reaction temperature promote the insulin to be encapsulated into liposomes and increase the encapsulation efficiency.

The particle number of aerosols generated by ultrasonic nebulizer depends on the liposome concentration, power intensity of nebulizer, and the composition of phospholipid in the liposome. The particle number of aerosols was higher if the power intensity of nebulizer was larger, as shown in Fig. 4. The particle number of aerosols was proportional to the applied ultrasonic intensity. Changing the applied intensity did not cause the particle size to change. The particle size of the aerosols was independent with the ultrasonic intensity. From the capillary wave theory, the particle size of the aerosols depends only on the frequency of ultrasound applied to the nebulizer, the

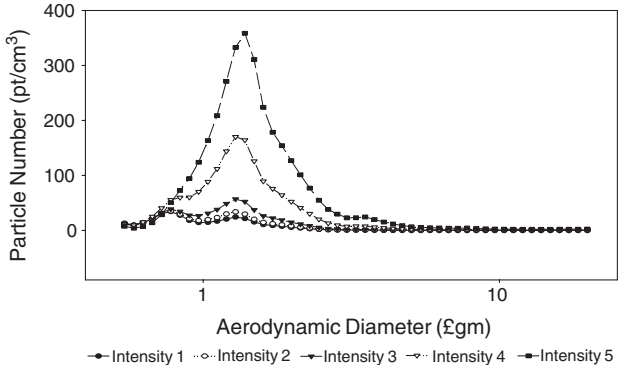

Fig. 4. The particle size of liposomal aerosols from ultrasonic nebulizer. Liposome composition: PC/Chol (70/30 mol%).

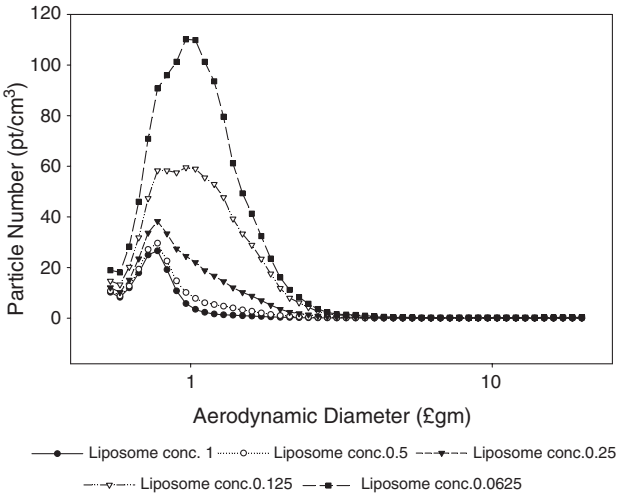

Fig. 5. The particle size of liposomal aerosols from ultrasonic nebulizer. Lipid composition HPC/Chol/PEG-DPPE (70/30/1 mol%).

density and the surface tension of the solution. The particle size of liposomal aerosols from the nebulizer was approximately 1 μm. It was suitable for therapeutic biologics to pass through into the alveolar of the lungs.

The particle size of liposomal aerosols from ultrasonic nebulizer was strongly affected by the amount of liposome in the solution. Fig. 5 shows that the particle size and particle size distribution of liposomal aerosols with different contents of liposome from the ultrasonic nebulizer. Phosphatidylcholine is a mixture of lipids, being considered as a surfactant. The liposomal solution containing higher contents of liposome possesses lower surface tension. Therefore, the particle size of aerosols nebulized from the liposomal solution containing higher contents of liposome was smaller than the particle size of the liposomal solution containing lower contents of liposome. Liposomal solution containing higher contents of liposome also shows higher viscosity. As a result, the total number of aerosols generated from the nebulizer is lower. The number of aerosols was inversely proportional to the liposome contents in the solution. Incorporating the PEG-DPPE would dramatically reduce the numbers of aerosols they formed.

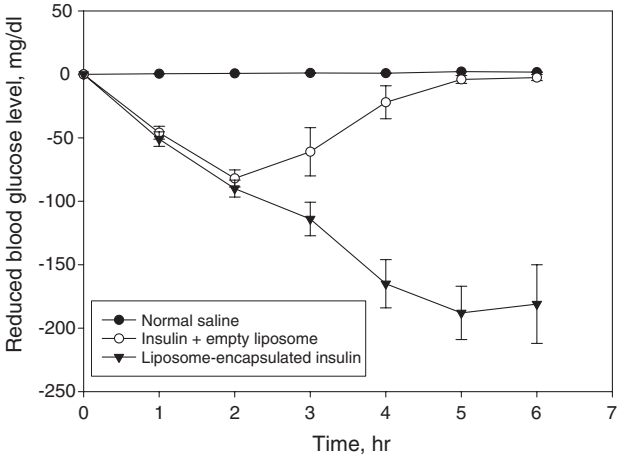

Fig. 6. Result of blood glucose level reduction in diabetic mice, (n=9).

In in vivo mice inhalation tests, aerosols of liposomal insulin were generated from normal saline solution containing 20% liposomal insulin (6 ml), approximating 0.5 IU/ml, by ultrasonic nebulizer. The animal experimental results showed that plasma glucose level could be reduced efficiently in diabetic mice by inhaling aerosolized insulin-encapsulated liposomes. The reduced glucose levels were shown in Fig. 6. Comparing to those inhaling normal saline and insulin with empty liposomes, the effect of reducing blood glucose level by liposome-encapsulated insulin was significantly better than the others. Pulmonary delivery of insulin not only provides a non-invasive method but also provides sustained release. Coadministering of insulin with empty liposome also showed the enhancement of insulin adsorption. Pulmonary delivery of liposomal insulin may provide valuable solutions to the currently unmet medical needs.

Fig. 7 shows that the liposomal insulin was homogeneously deposited on the mice alveoli after inhaling liposomal insulin freely. The mechanism of absorption enhancement by liposomes may be attributed to the presence of surfactants on alveolar surface. The addition of exogenous liposome molecules accelerates the surfactant recycling process in the alveolar cells, leading to an enhanced uptake of the protein molecule into the systemic circulation. The use of liposomal systems offers clear advantages such as high loading capacity and the possibility of controlling size and permeability. Thus, the release kinetics of the drugs from the carrier systems can be controlled. This system makes it possible to use a relatively small number of vector molecules to deliver substantial amounts of drugs to the target.

To assay the immunoreaction of the lungs after receiving aerosolized insulin-encapsulated liposomes, we extracted the alveolar lavage fluid from the mice and analyzed these after receiving aerosolized liposmal insulin for a 12 h period. Aerosolized liposmal insulin did not provoke any immunoreaction since there was no obvious symptom of hyperplasia or inflammation in the alveolar lavage fluid, as shown in Fig. 8. Compared to the control group that did not receive the aerosolized liposmal insulin, the amount of leukocytes in the alveolar lavage fluid of the experimental group did not increase significantly, and the types of leukocytes and morphology of cells in the lavage fluid were almost identical. From the experimental results, we found that the inhalation of aerosolized liposmal insulin in mice caused no virulent effect, inflammation and immunoreaction on the lungs.

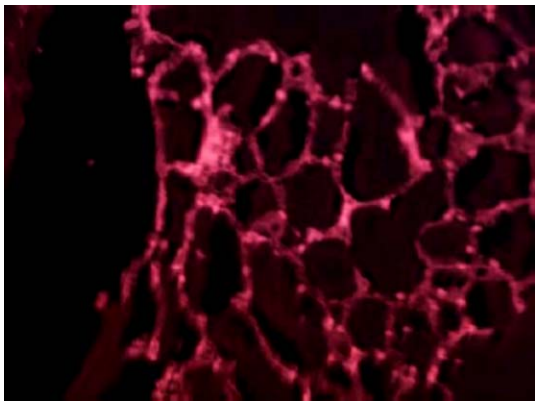

Fig. 7. The lung deposition of liposomal insulin in the mice.

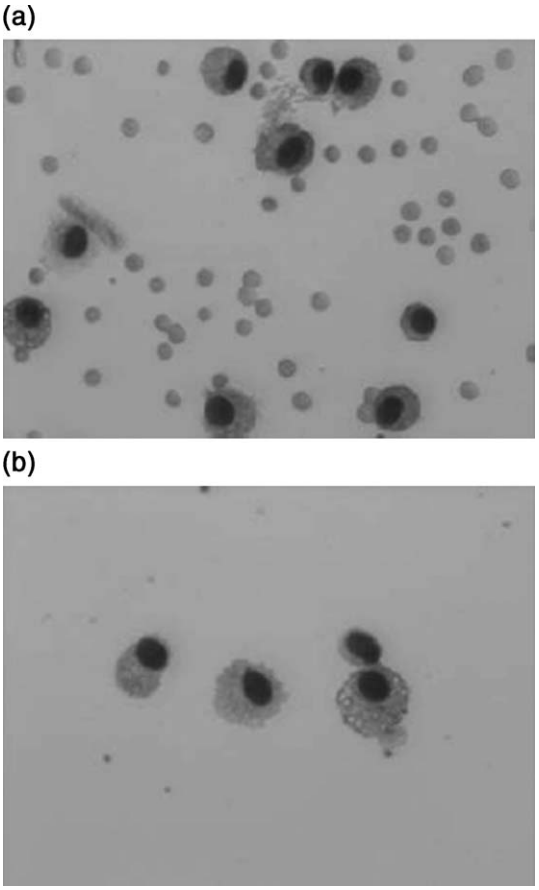

Fig. 8. The types of leukocytes and morphology of cells in the alveolar lavage fluid of experimental mice. (a) Control group: without treatment; (b) experimental group after inhaling aerosolized liposomal insulin for 12 h.

4. Conclusions

Liposomal carrier showed promising properties in formulating the insulin for pulmonary delivery. Insulin could be efficiently encapsulated into liposomal carriers. The optimal encapsulation efficiency was achieved when 40% ethanol were used. The particle size of liposomal aerosols from the ultrasounic nebulizer was approximately 1 μm. It was suitable for therapeutic biologics to pass through into the alveoli of the lungs. In vivo animal studies showed plasma glucose level effectively reduced when insulin was administered by inhalation route using liposomes as carriers. Liposomal carriers were effectively homogeneously distributed in the lung alveoli. Administering the insulin by liposome-mediated pulmonary delivery promotes an increase in the drug retention-time in the lungs and more importantly, a reduction in extrapulmonary side-effects which invariably results in enhanced therapeutic efficacies. Alveolar lavage fluid evaluation showed that inhalation of aerosolized liposomal insulin in mice caused neither virulent effect nor inflammation or immunoreaction on the lungs.

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