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Original Article

Preparation of gemcitabine-loaded nanoliposomes and their lung adenocarcinoma-targeting treatment in vivo and in vitro

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Abstract: Objective: To investigate the preparation of gemcitabine (Gem)-loaded nanoliposomes (LPs) and their lung adenocarcinoma-targeting treatment *in-vivo* and *in-vitro*. Methods: Film dispersion method was used to prepare gemcitabine-loaded nanoliposomes which were modified with RGD molecule (RGD/Gem-LP). TEM was used to characterize the morphology of nanoliposomes and then a nanoparticle size analyzer was used to detect their diameter and zeta potential. Further, the encapsulation efficiency and release rate of gemcitabine were detected. FITC was used to label nanoliposomes and then the resultant substance was observed under confocal fluorescence microscopy for its targeting property and cellular uptake. CCK-8 Cell Counting Kit was used to detect the effect of single nano-lipid carriers, Gem-LPs and RGD/Gem-LPs on cell viability. The effect of Gem-LPs on tumor volume and their toxicity was detected by tail intravenous injection of Gem-LPs in a mice model bearing lung adenocarcinoma. Results: RGD/Gem-LPs were spherical particles whose diameter ranged from 95 nm to 115 nm, averaging about 105 nm. The mean diameter and zeta potential of RGD/Gem-LPs didn't showed significant fluctuation after standing for 1, 3, 5, 7 and 14 days. The encapsulation efficiency of Gem was about 69.5±2.3%, while the release rate increased with time and stabilized at 45.9±1.88% after 24 h. RGD/Gem-LPs targeted at lung adenocarcinoma A549 cells effectively, entered into these cells and located in the cytoplasm. The viability of A549 cells treated by 10-50 μg/mL single nano lipid carriers didn't showed significant decrease after both 24 and 48 hours. However, the viability of these cells treated by RGD/Gem-LPs showed concentration-dependent decrease. Moreover, RGD/Gem-LPs remarkably inhibited the growth of tumor almost without toxicity. Conclusion: RGD/Gem-LPs with diameter around 105 nm prepared in this study have a good targeting property to lung adenocarcinoma A549 cells. They can inhibit cell viability and the growth of tumor in mice effectively without obvious toxicity.

Keywords: RGD, gemcitabine, nanoliposomes, targeting property, lung adenocarcinoma A549 cells

Introduction

With its incidence increasing gradually nowadays, lung adenocarcinoma in bronchial epithelium has become a malignant tumor endangering life and health [1, 2]. It is reported that among malignant tumors existing in industrial cities in China, the incidence of lung adenocarcinoma is the highest in male and is increasing rapidly in female. By now, it has been one of three most frequently found malignant tumors in female [3]. Currently, clinical treatment methods for lung adenocarcinoma mainly include surgery, radiotherapy, chemotherapy and immunotherapy [4], among which surgery remains the top choice. However, surgery has a high risk of tumor cell metastasis, whereas radiotherapy and chemotherapy are cytotoxic. Therefore, it is imperative to find out an effective treatment method or discover a new drug with low toxicity [5].

Research findings show that multifunctional drug carriers can help to decrease the toxicity of chemotherapy and increase the effective therapeutic concentration of drugs. In recent years, liposomes have been used as common carriers of anti-cancer drugs [6-9]. Liposomes are double-layer spherical structures composed of phospholipids. They are of good biocompatibility. Lipophilic and hydrophilic drugs can be loaded in the core and multifunctional mole-

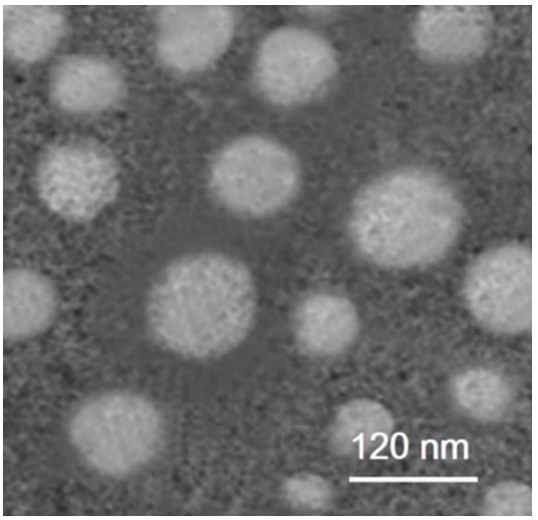

Figure 1. The TEM image of RGD/Gem-LPs.

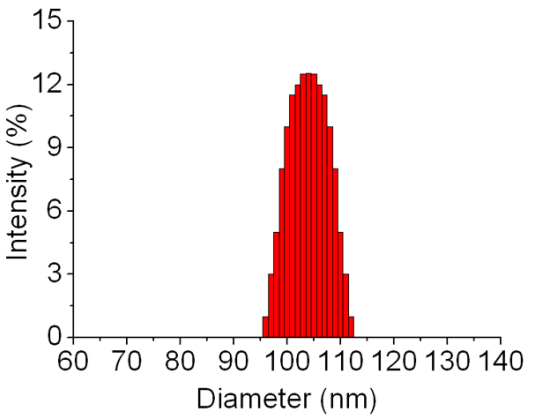

Figure 2. The diameter of RGD/Gem-LPs.

cules like targeting molecules can modify their surface which enables anti-cancer drugs to target actively [10, 11]. RGD is a polypeptide composed of arginine-glycine-aspartic acid which can bind with integrin cell-surface receptors specifically [12-14]. Studies indicate that integrin receptors are highly expressed by lung adenocarcinoma A549 cells [15, 16].

In this study, RGD-modified gemcitabine liposomes (RGD/Gem-LPs) was prepared. Its *in-vitro* and *in-vivo* tumor targeting property, anticancer effect and toxicity was observed in a mice model bearing lung adenocarcinoma A549 cells. This study will provide an experimental and theoretical basis for the clinical treatment of lung adenocarcinoma and the discovery of new drugs.

Materials and methods

Experimental drugs and materials

Soybean phospholipids, cholesterols and DSPE-PEG2000 were purchased from Sigma (the US), DSPE-PEG2000-RGD from China Peptides (Shanghai, China) and FITC (fluorescein isothiocyanate) from Alexis (The US). Other chemical reagents were analytically pure. Gemcitabine (GEM) powder (CAS No. 95058-81-4) was manufactured by MERCK (The US) with 99.9% purity. Fluorochrome DAPI (4',6-amidine base-2-phenyl indole) was purchased from Beyotime (China) and CCK-8 Cell Counting Kit from Dojindo Molecular Technologies, Inc. (Nippon).

Major instruments

Laser particle analyzer NanoSizer ZS90 and zeta potentiometric analyzer were purchased from Malvern Instruments (The UK), thermal-field emission scanning electron microscopy SU5000 from Hitachi (Nippon), ultraviolet spectrophotometer SP-756PC from Shanghai Spectrum Instruments Co., Ltd (SSI) and multimode reader SpectraMax i3x from Austria.

Cells and animals

A549 cells were epithelioid human lung adenocarcinoma cells provided by American type culture collection (ATCC). Test animals were Balb/c nude mice (SPF) aged from 3 to 5 weeks with body weight ranging from 20 g to 25 g provided by Vital River Laboratories (VRL) in Beijing.

Test methods

The preparation of and characterization of RGD/Gem-LP: In this study, film dispersion method was used to prepare gemcitabine-loaded nanoliposomes which were modified with RGD (RGD/Gem-LP). Detailed steps were as follows. Sufficient amount of Gem, DSPE-PEG2000-RGD and total phosphatide: cholesterol (with molar ratio of 73:27) was dissolved in chloroform, respectively. Then, the resultant substance was placed in a 50 mL begoon-shape flask. After film formation through rotary evaporation in a begoon-shape flask, it was put into a vacuum drying oven for 24 hours. Then, 3 ml PBS buffer solution (pH7.4) was added. Afterwards, the resultant substance was put into a shaker (200 rpm) at 37°C and hydrated

Table 1. Diameter and zeta potential of RGD/Gem-LPs after standing for different periods of time

	Time (days)				
	1	3	5	7	14
Diameter (nm)	104.2	105.1	104.8	103.5	105.6
Zeta potential (mV)	-16.8	-17.9	-15.6	-16.5	-16.1

Table 2. The releasing rate of Gem in RGD/Gem-LP

	Time (h)				
	0	6	12	24	48
Releasing rate (%)	1.9±1.3	24.9±1.9	37.9±2.1	45.9±1.88	46.9±2.3

for 20 minutes. At last, RGD/Gem-LPs were obtained after ultrasonic water bath for 5 to 10 minutes and ultrasonic treatment for 30 minutes. For an appropriate amount of RGD/Gem-LPs prepared, a transmission electron microscopy was used to observe the surface appearance and a laser particle analyzer was used to measure the diameter and zeta potential. By glucose gel column chromatography, liposomes were separated from unloaded Gem. Then, through methanol demulsification, the amount of Gem was detected by ultraviolet spectrophotometer at 269 nm. Encapsulation efficiency of Gem was calculated as follows: EE% = $W_{encapsulated}/W_{total} \times 100\%$, in which $W_{encapsulated}$ referred to the drug amount encapsulated in liposomes and W_{total} referred to the total drug amount used.

On the targeting property of RGD/Gem-LP to A549 cells: A549 cells in the logarithmic phase were inoculated in a confocal culture dish (10^5 cells per well) and cultured at 37°C for 24 hours. After that, Gem-LPs and RGD/Gem-LPs labeled by FITC were added, respectively. They were then put into an incubator and cultured for 3 hours. Afterwards, the culture solution was discarded and the rest remainder was washed with PBS for three times. Finally, a proper amount of PBS was added and the FITC-based fluorescence in cells was observed by confocal fluorescence microscopy.

Cytotoxicity test: A549 cells in the logarithmic phase were inoculated in a 96-well plate and cultured in an incubator for 24 hours. Then, different concentrations of liposomes, Gems, Gem-LPs and RGD/Gem-LPs were cultured for different periods of time. After that, the old culture solution was discarded and complete culture solution with 10% CCK-8 reagent was added. The mixture was later put back in the incubator for incubation for 30 minutes. Finally, its absorbance at 450 nm OD_{450nm} was detected by an automatic microplate reader.

The establishment of mice tumor model and RGD/Gem-LPs treatment: The A549 cell suspension was prepared at a density of 10^6 cells/ml. Each mouse received subcutaneous injection 200 μL cell suspension in the right lower back and was then fed for the observation of tumor size. When the tumor grew to be 300 mm^3, all nude mice were randomized into five groups (five mice for each group), each of which was injected with normal saline, LP, Gem, Gem-LP and RGD/Gem-LP by caudal vein. The day on which drug was injected was regarded as day 1 of treatment. From then on, tumor size (Tumor size = Length × $width^2/2$) and body weight of mice were measured every two days. One treatment cycle lasted for 28 days. All mice were sacrificed after one cycle. Major organs (heart, liver, spleen, lung and kidney) were taken out and sliced to pathological sections which were stained with HE. Then, structural changes were observed by microscopy.

Statistical methods

Experimental data was expressed as mean ± SD. Data analysis was conducted by statistical software SPSS10.0. Comparison among groups was performed by independent-samples t test. $P \leq 0.05$ indicated significant difference, while $P \leq 0.01$ indicated extremely significant difference.

Results

RGD/Gem-LP surface appearance and diameter

As shown in **Figure 1**, under transmission electron microscope (TEM), RGD/Gem-LPs were spherical particles evenly distributed. Their diameter measured by nanoparticle size analyzer ranged from 95 nm to 115 nm, averaging 105 nm (**Figure 2**). It indicated that liposomes prepared in this study were nano-scale particles.

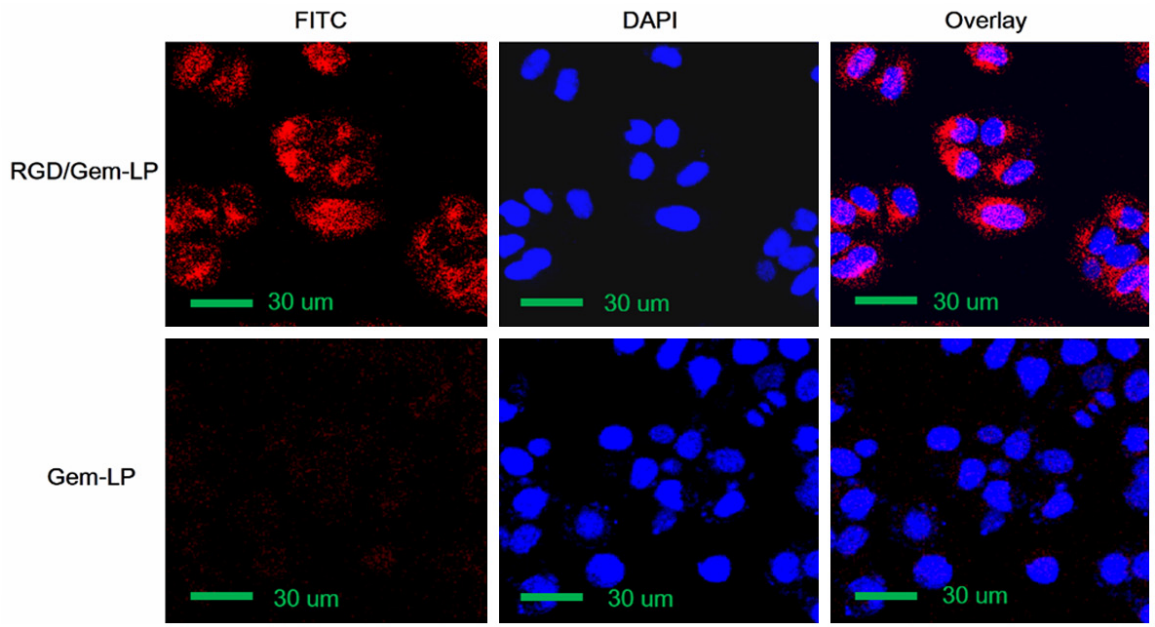

Figure 3. The cell uptake of RGD/Gem-LPs and Gem-LPs observed by confocal microscopy.

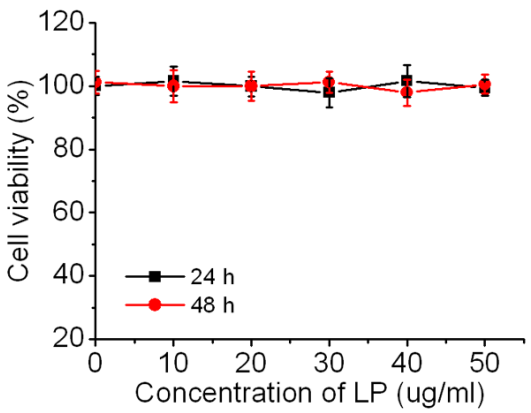

Figure 4. The cytotoxicity of LP.

Stability of RGD/Gem-LPs

RGD/Gem-LPs, after being placed at room temperature for different periods of time, were detected for their mean diameter and zeta potential. As shown in **Table 1**, after 1, 3, 5, 7 and 14 days, the mean diameter was about 105 nm and zeta potential about -16 mV. It indicated that RGD/Gem-LPs were of good water solubility and stability.

The encapsulation efficiency and release rate of Gem

The encapsulation efficiency of Gem was 69.5±2.3%, which showed that nanoliposomes can encapsulate Gem efficiently. After RGD/Gem-LPs stood for 0, 6, 12, 24 and 48 hours, the release rate of Gem was 1.9±1.3%, 24.9±1.9%, 37.9±2.1%, 45.9±1.88% and 46.9±2.3%, respectively, which suggested that the maximum value was reached after 24 hours (**Table 2**). It indicated that Gem released from liposomes slowly.

Test on the targeting property of RGD/Gem-LPs

Gem-LPs and RGD/Gem-LPs labeled by FITC were incubated with A549 cells for three hours. Then, as shown in **Figure 3**, the mixture was observed under a confocal fluorescence microscope. Plenty of RGD/Gem-LPs were found in cytoplasm, while little Gem-LPs were found there. It suggested that RGD/Gem-LPs could target at cell surface actively and effectively so as to be uptaken into cytoplasm.

Cytotoxicity test of RGD/Gem-LPs

First of all, the cytotoxity of drug carriers LP was detected. Different concentrations of LPs were incubated for 24 and 48 hours, respectively. Results showed that 0-50 μg/mL LP was of no obvious toxicity (**Figure 4**), which suggested that LP could be used as drug carriers. Next, cells were treated with different concentrations of Gem, Gem-LP and RGD/Gem-LP. After 24 hours, it was found that these three samples all

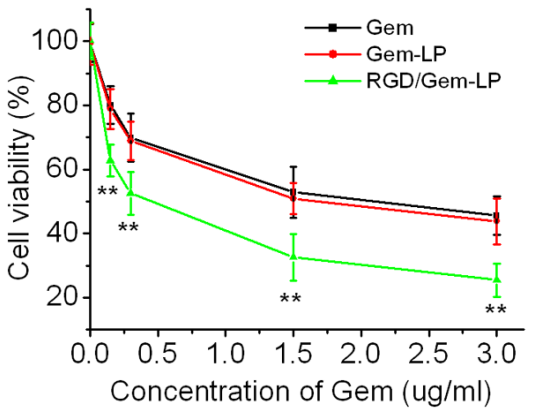

Figure 5. The cytotoxicity of RGD/Gem-LP.

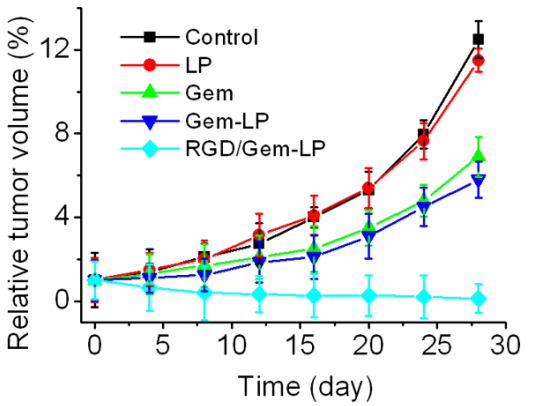

Figure 6. The effect of RGD/Gem-LP on the volume of tumor.

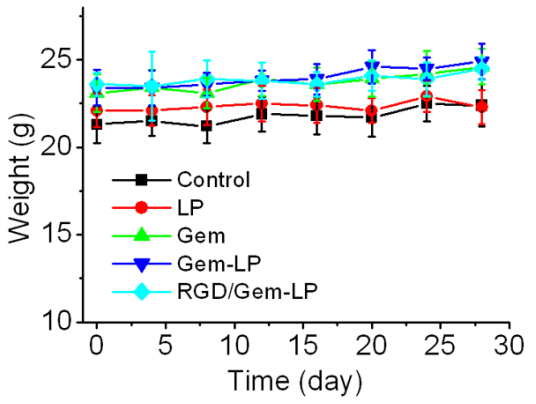

Figure 7. The body weight changes of mice.

had a killing effect on cells. As shown in **Figure 5**, cell death rate rose more and more significantly with increased concentration of Gem. However, compared with cells treated with Gem and Gem-LP, the decrease of cell viability was more evident in those treated with RGD/Gem-LP ($P<0.01$). It was possibly because targeting nanoliposomes led drugs to enter into cells.

In-vivo anti-tumor effect of RGD/Gem-LPs

As shown in **Figure 6**, within 30 days, tumor volume increased gradually in the control (normal saline) and LP group. Gem and Gem-LP inhibited the growth of tumor to a certain extent but the tumor volume still tended to increase. By contrast, RGD/Gem-LP inhibited the growth of tumor slowly from the beginning of injection. The tumor volume decreased with time and the tumor almost melted away after treatment for 12 days. Therefore, the effect of RGD/Gem-LP was obviously better than Gem-LP.

In-vivo toxicity of RGD/Gem-LPs

During treatment, the body weight of mice was measured every two days to observe the influence of drugs on metabolism. As shown in **Figure 7**, the body weight of mice almost had no obvious fluctuation within 30 days of treatment, which suggested that all drugs used had no significant influence on the metabolism of mice. HE staining images of tissue sections of major organs (**Figure 8**) also indicated that all drugs had no significant influence on the structure of their heart, liver, spleen, lung and kidney.

Discussion

Receptors on the surface of tumor and corresponding targeting molecules are key factors in the tumor-targeting drug delivery system. Integrins, a class of cell adhesion receptors, are widely expressed on the surface of karyocytes. In particular, integrin $\alpha_v\beta_3$ is highly expressed on the surface of tumor cells and endothelial cells associated with tumor in patients with neuroglioma, melanin, lung cancer and other neoplasms. It is closely related with vasculogenesis, tumor metastasis and anti-radiation therapy. Therefore, integrin $\alpha_v\beta_3$ is often used as specific target spots targeting at tumors [17-19]. Studies show that tripeptide arginine-glycine-aspartic acid (Arg-Gly-Asp, RGD) can recognize integrin family with α_v subunit specifically and has high affinity [20, 21].

In this study, RGD modified Gem liposomes (RGD/Gem-LPs) which could target A549 cell

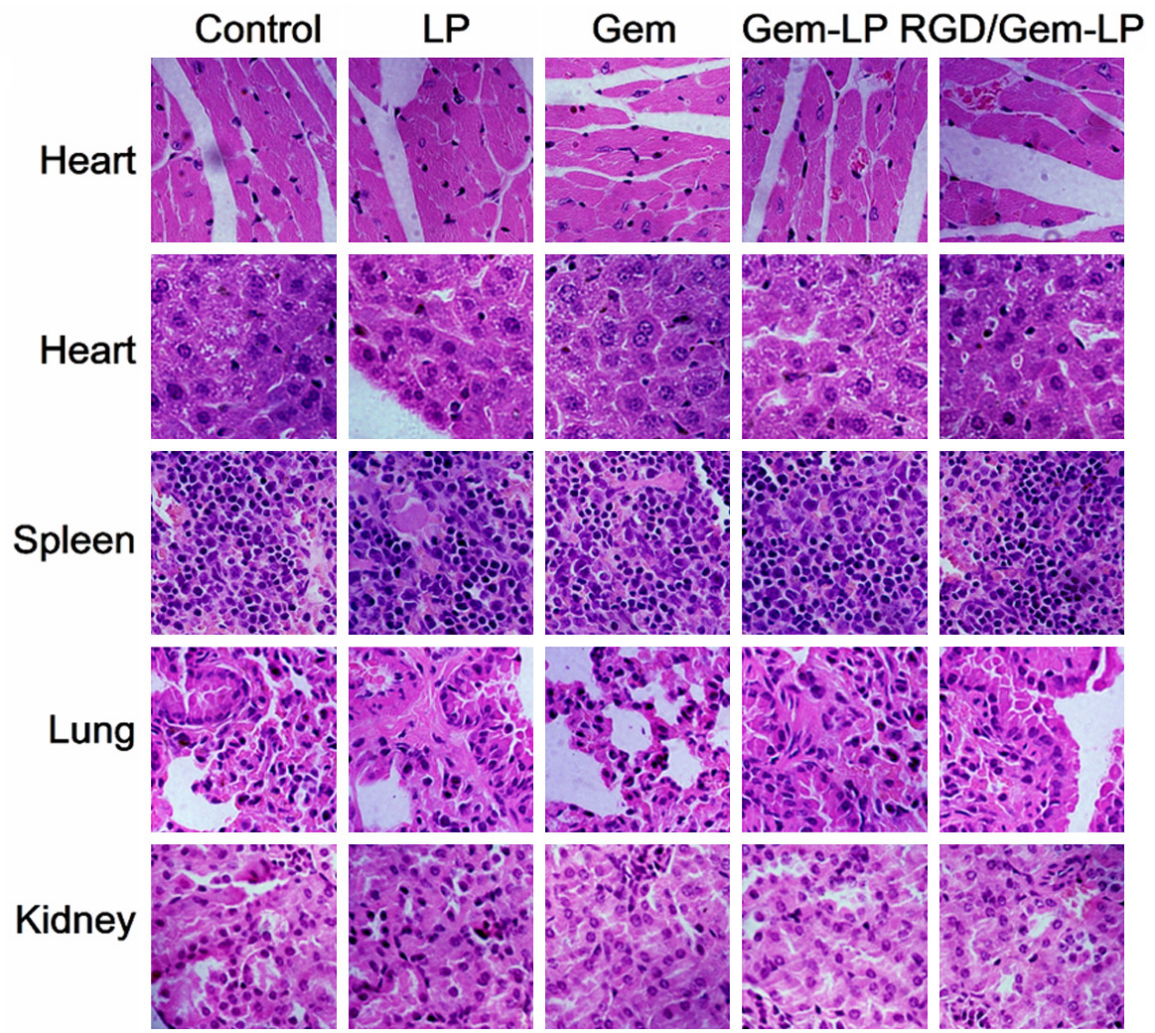

Figure 8. The HE staining images of heart, liver, spleen, lung and kidney.

surface effectively was prepared by using lung adenocarcinoma cells as a tumor cells model. RGD/Gem-LPs were nano-scale spherical particles, the diameter and high biocompatibility of which enabled them to be of good water solubility and stability. This laid a solid foundation for their application in treating tumors both *in-vivo* and *in-vitro*. The encapsulation efficiency of Gem was 69.5±2.3%. Being able to be released slowly, RGD/Gem-LPs were more effective than single Gem. A cytotoxicity test showed that LPs were of no obvious toxic effect on cells, while RGD/Gem-LPs were of higher cell-killing effect. It was partly because of the sustained-release effect of Gem and partly because of the targeting property of RGD which increased the amount of drugs entering into cells. In an *in-vivo* anti-tumor test, it was demonstrated that RGD/Gem-LPs had a good anti-tumor effect nearly without toxicity. It showed that RGD/Gem-LPs prepared in this study concentrated around tumor tissues and were specifically recognized and efficiently delivered into tumor cells. In this way, the therapeutical effect was maximized and side effects were reduced.

In conclusion, RGD/Gem-LPs prepared in this study, by targeting A549 cells, entering into cytoplasm and releasing drugs slowly, have a good *in-vivo* and *in-vitro* anti-tumor effect. This will provide an experimental and theoretical basis for the development of new drugs for lung adenocarcinoma.

Disclosure of conflict of interest

None.

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