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Original Article

Anticancer effect of folic acid modified tumor-targeting quercetin lipid nanoparticle

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Abstract: Objective: This study aims to study the in-vitro and in-vivo anticancer effect folic acid modified tumor-targeting quercetin lipid nanoparticle. Methods: Self-assembly and a single-step nanoprecipitation method were used to prepare the folic acid modified quercetin lipid nanoparticle (FA-Quercetin/PLGA-Lipid) whose size was respectively detected by nanosizer, and encapsulation efficiency and releasing ratio of quercetin detected and calculated by ultraviolet(UV) spectrophotometer. Fluorochrome, Rhodamine, was used to label the nano lipid to observe its uptaking ratio and targeting property by confocal microscopy; CCK-8 method was used to detect the effect of FA-Quercetin/PLGA-Lipid on the cell viability of HepG2 cells; Lastly, a mouse subcutaneous tumor model was generated to verify the in-vivo anticancer effect of FA-Quercetin/PLGA-Lipid and its toxicity. Results: It was shown that FA-Quercetin/PLGA-Lipid prepared was round and about 85 nm in diameter, with encapsulation efficiency of the nanoparticle of 76.8 ± 2.3% and releasing rate after 24 h of 49.8 ± 1.9%. Quercetin/PLGA-Lipid was rarely uptaken by cells, while FA-Quercetin/PLGA-Lipid was uptaken significantly by target cells and therefore inhibited the viability of HepG2 cells greatly in vitro. Besides, the cell killing effect of quercetin encapsulated by nanoliposomes was more significant than that of single quercetin. Results of an in-vivo anticancer experiment indicated that the blank control and single nanoliposome had no inhibitive effect on tumor volume. Compared with Quercetin/PLGA-Lipid, FA-Quercetin/PLGA-Lipid remarkably inhibited the tumor volume after 12 days treatment until the tumor disappeared. Results of a toxicity test showed that FA-Quercetin/PLGA-Lipid had no evident toxicity within healthy mice. Conclusions: The FA-Quercetin/PLGA-Lipid nanoparticle, with nanoscale size, high encapsulation efficiency and great tumor cells targeting property, can inhibit the viability of target cells effectively. Moreover, its tumor inhibition effect in-vivo is better than Quercetin/PLGA-Lipid. Without any evident toxicity, it will be a potential nano drug for tumor therapy.

Keywords: Quercetin, folic acid, nanoliposome, targeting property, anticancer, liver cancer HepG2 cell

Introduction

In recent years, nano-sized lipid vesicles, as drug carriers, have been widely used for the treatment of diseases clinically, such as doxorubicin liposome injection and ursolic acid nano lipid promising to be used clinically [1-3]. As drug carriers, nano-sized lipid vesicles have such advantages as extremely high biocompatibility, sustained release property and lower dosage of administration which can improve drug stability, prolong the circulating time of drug in the blood and reduce drug toxicity, respectively [4, 5]. However, drugs encapsulated by regular nano-sized lipid vesicles are subject to be swallowed and destroyed by the reticuloendothelial system after entering into body's circulation system. Moreover, due to their poor targeting property, nano-sized lipid vesicles can only be transported to the lesion by enhancing permeability and retention effect (EPR). In this way, the effective concentration there will be so low that the effect of treatment cannot be achieved [6, 7]. Therefore, in recent years, researchers have been committed to preparing a drug encapsulated by nano-sized lipid vesicles with active targeting property.

The targeting property of nanoliposomes mainly can be classified into two types-passive tar-

geting and active targeting. The former primarily manage to carry little drugs to the lesion simply depending on the chemotaxis of lipids themselves and the EPR effect of the lesion like solid tumor, on condition that there is no modifying and targeting substance on the surface of lipids. By contrast, the latter, with some antibodies or ligands modifying the surface of nanoliposomes, can bind with some antigens or ligands actively with specificity, which enables it to be carried to the target site more precisely. In this way, the drug can accumulate at the site and play its therapeutic role [8, 9]. Nevertheless, antibodies, proteins in general, regardless of their good specificity, may be denatured during chemical synthesis of nanoliposomes so that their structure may change and they become invalid [10]. Ligands, instead, are highly favored by researchers owing to their stable structure. The most typical one is folic acid. Folic acid, also called pteroylglutamic acid, is composed of pteridine, paraaminobenzoic acid and L-glutamic acid. It belongs to water soluble vitamin B complex [11]. Being non-toxic with low cost, it has been widely used. The reason why folic acid can be used for preparing active targeting nanoliposomes can be explained by the following two aspects: Firstly, folic acid is a modifiable small molecule compound which can link with other molecules by amido bond and ester bond, etc. Secondly, folic acid can specifically bind with many folate receptors on the surface of tumor so as to achieve its active targeting effect [12]. Folate receptors, envelope glycoproteins linked by glycosylphosphatidylinositol, are tumor-associated antigens. Their expression in normal tissues is very low, while on the surface of malignant tumor (such as cervical cancer, ovarian cancer, breast cancer, kidney cancer and other cancers) cells, their expression is significantly higher [13, 14].

Quercetin, a traditional Chinese medicine extract, exists widely in many Chinese herbal medicinal ingredients, such as rutin, quercitrin, hyperoside and propolis flavone. It is of good effect in expelling phlegm and arresting coughing and can also relieve asthma to some extent. In recent years, it has been also found that it is effective in anti-inflammation, antioxidation, free radical scavenging, anticancer and other treatments [15, 16]. Moreover, Wang Gang et al. found that quercetin was also of good killing effect on neuroglioma C6 cells [17]. However, due to quercetin's extreme low water solubility, its absorption amount within human body is as low as 16 mg/day so that it is difficult to give full play to its effectiveness. This maximally restricted the clinical application of quercetin.

In this study, we prepared quercetin lipid nanoparticles targeting at folic acid receptors by encapsulating quercetin in nano-sized lipid vesicles and modifying on the surface by folic acid. Researchers showed that on the surface of liver cancer HepG2 cells, there were overexpression of folic acid receptors [18]. Therefore, we further investigated the in-vivo and in-vitro targeting property and killing effect of folic acid modified quercetinnanoliposomes for HepG2 cells. This study will provide a new method and theoretical foundation for the study of nano-sized liposome drugs and tumor targeting therapies.

Materials and methods

Experiment reagents and drugs

Poly (D, L-lactic acid-co-glycolic acid) (PLGA, Mw: 5000-15000), L-α-phosphatidylcholine (Soybean lecithin) with 90-95% lecithin, DSPE-PEG2k-FA and DSPE-PEG2k-COOH were purchased from Sigma (the US). Fetal calf serum, DMEM culture solution, Penicillin-Streptomycin Solution (double-antibody), Trypsin-EDTA digestive juices and phosphate buffer were all purchased from Gibco (the US). CCK-8 Assay Kit was purchased from Dojindo (Nippon). Rhodamine and 4', 6-diamidine base-2-phenylindole (DAPI) were purchased from Aladdin Industrial Corporation (Shanghai). Other chemical reagents were all analytically pure ones produced in China. Quercetin powder (purity > 98%) was manufactured by Hefei Bomei Biotechnology Co.,Ltd.

Main instruments

nano particle analyzer (Mastersizer 3000) from Malvern Instruments Ltd., confocal fluorescence microscope (FCFM, TCS SP5) from Leica (Germany), automatic microplate reader (DG-5033A) from Nanjing HuadongElectronics Group Medical Equipment Co., Ltd. and UV spectrophotometer (UV-2450) from Shimadzu Corporation.

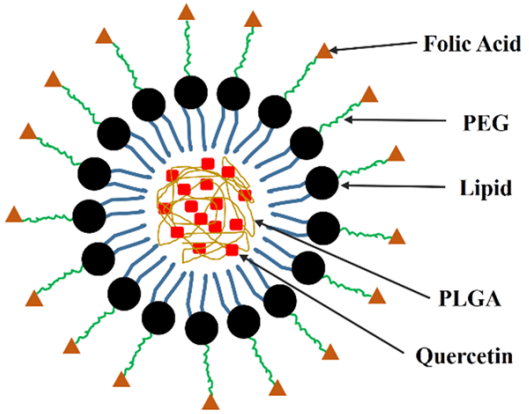

Figure 1. The scheme of FA-Quercetin/PLGA-Lipid.

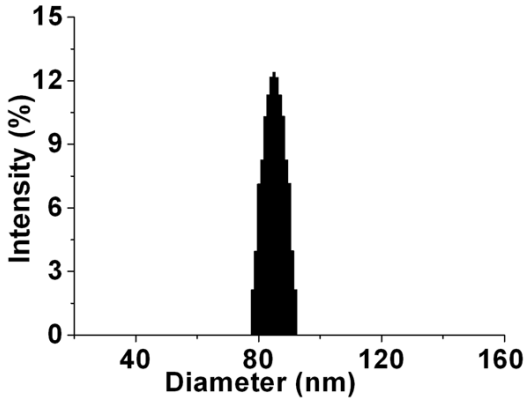

Figure 2. The average diameter distribution of FA-Quercetin/PLGA-Lipid.

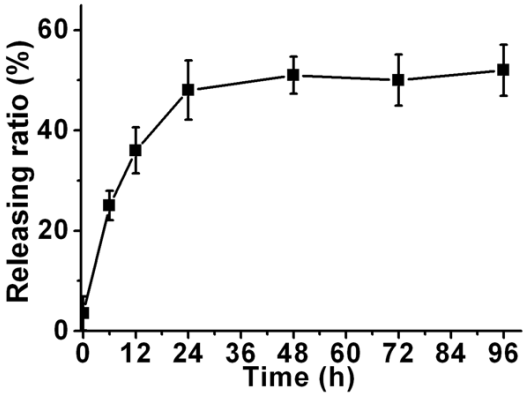

Figure 3. The releasing ratio of Quercetin in FA-Quercetin/PLGA-Lipid.

The preparation of FA-Quercetin/PLGA-Lipid

The folic acid modified quercetin lipid nanoparticle was prepared with PLGA, L-α-phosphatidylcholine and DSPE-PEG2k-FA by self-assembly through a single-step nanoparticle precipitation. Details were as follows. PLGA was dissolved in acetonitrile (1 mg/ml); L-α-phosphatidylcholine/DSPE-PEG2k-FA (4:1) was warmed at 60°C until it was assured to have become aqueous solution. Then, quercetin was added into the PLGA acetonitrile solution. After mixing for an hour, this mixed liquid was dripped into the pre-warmed L-α-phosphatidylcholine/DSPE-PEG2k-FA mixture, which was then stirred slowly. Then, the resultant solution was placed into a shaker and oscillated at room temperature for 3-5 hours at a low revolution. After completion, the solution was centrifuged at 6000 g/min for 30 min in a centrifugal ultrafiltration tube to remove redundant organic molecules and free quercetin. Finally, the folic acid modified quercetin lipid nanoparticle (FA-Quercetin/PLGA-Lipid) was obtained by re-suspending nanoliposomes with deionized water. The preparation process of quercetin lipid nanoparticle (Quercetin/PLGA-Lipid) was similar to those of FA-Quercetin/PLGA-Lipid expect that DSPE-PEG2k-FA was replaced by DSPE-PEG2k-COOH.

Characterization of FA-Quercetin/PLGA-Lipid

The particle size distribution by a nano particle analyzer and the encapsulation efficiency and releasing ratio of quercetin in lipidosomes by an ultraviolet spectrophotometer.

An in-vitro targeting study of nanoliposomes

Nanoliposomes were marked by fluorescent molecule rhodamine. During the preparation of nanoliposomes, rhodamine was enveloped within them. Thus, FA-Quercetin/PLGA-Lipid and Quercetin/PLGA-Lipid have fluorescent properties. Then, the marked nanoliposomes and culture solution were added into a confocal dish full of cells and cultivated for three hours. After that, the old culture solution was drained off. The dish was washed for three times with PBS. After addition of dye liquor DAPI, it was incubated with cells for 10-20 min. Again, it was washed for three times with PBS. At last, the fluorescence signal of rhodamine and DAPI in cells by confocal microscopy.

Cell culture and cell viability assay

HepG2 cells were purchased from the Cell Bank of Chinese Academy of Sciences in Shanghai.

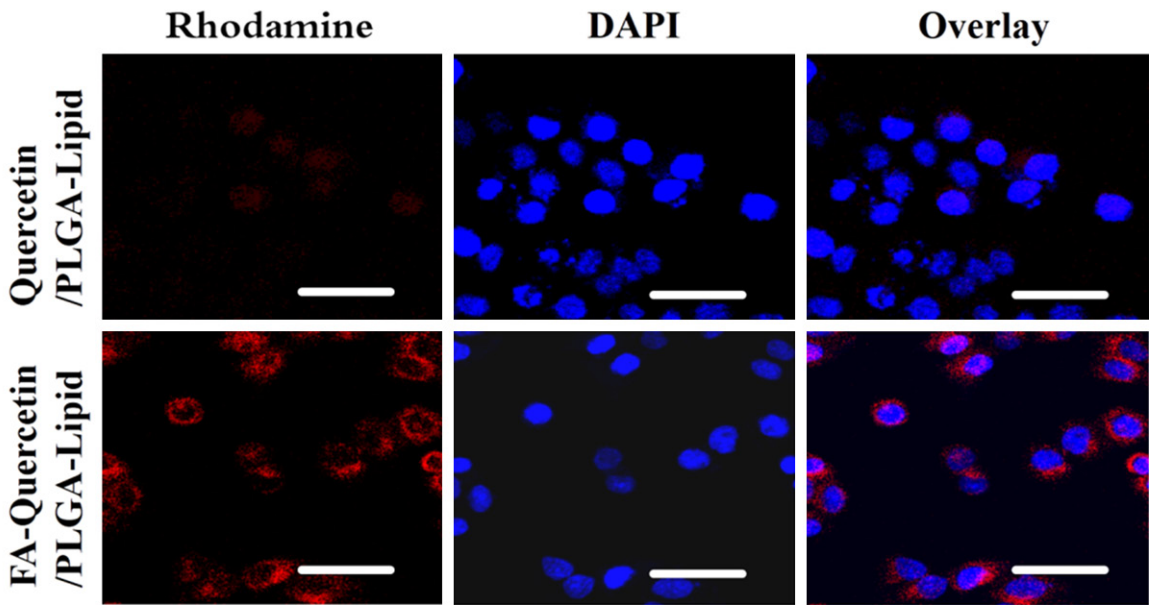

Figure 4. The confocal microscopy images of liver cancer cells. Scale bar = 20 μm.

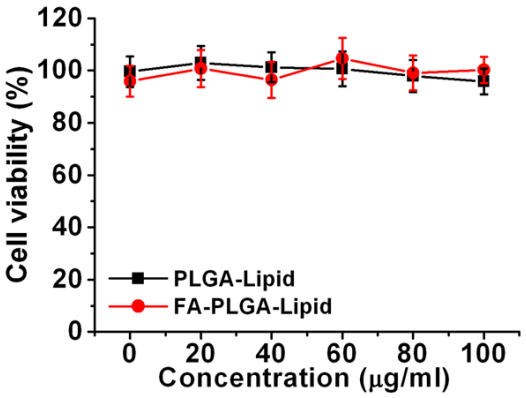

Figure 5. The cytotoxicity study of nano lipid carriers on HepG2 cells.

The cell culture fluid was DMEM culture solution containing 10% fetal bovine serum and 1% double antibiotic. Cells were cultured in a constant temperature incubator with 5% carbon dioxide at 37°C. After being digested by a digestive juice, these cells were homogeneously dispersed in culture solution (100000 cells/mL), which was then added into a 96-well plate (100 μL suspension/well) and cultured for 24 hours. Then, different concentrations of drugs were added and the resultant mixture was cultured for another 24 hours. After that, the old culture solution was removed and a new culture solution with 10% CCK-8 was added. Again, the plate was incubated for 20-30 min. Then, the plate was placed in a microplate reader for the detection of absorbance at 450 nm (OD450 nm). OD450 nm was proved to be proportional to cell viability.

The establishment of a mice tumor model and in-vivo cancer therapy

We bought 35 5 to 7-week-old nude mice of either sex. 150 mL cell suspensions (10^6 cells/ml) were injected subcutaneously at the right side of their lower back. When the gross tumor volume (GTV) reached 100 mm^3 (GTV = Length × $Width^2/2$), these mice were divided into 5 groups (7 mice/group) at random for the following assay of cancer therapy. These 5 groups were the blank control group (normal saline injected), FA-PLGA-Lipid group, quercetin group, Quercetin/PLGA-Lipid group, and FA-Quercetin/PLGA-Lipid group, respectively. The tumor volume and body weight of mice were measured every three days. After the completion of the treatment period of 35 days, mice were killed and major organs (their heart, liver, spleen, lung and kidney) were taken out to make pathological sections. Then, HE staining was performed to observe structural changes.

Statistical analysis

All data in this study was presented in mean ± SD and analyzed by a statistical software

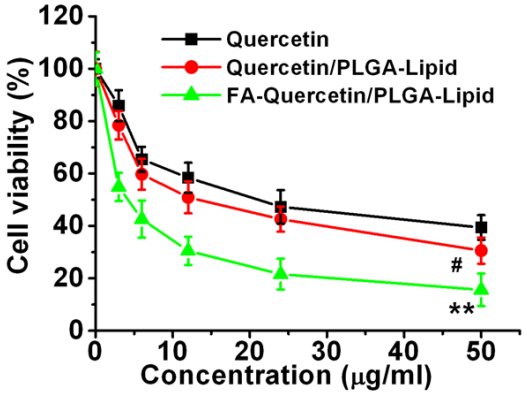

Figure 6. The effect of FA-Quercetin/PLGA-Lipid on cell viability. #P < 0.05, vs. the single Quercetin group; **P < 0.01, vs. the Quercetin/PLGA-Lipid group.

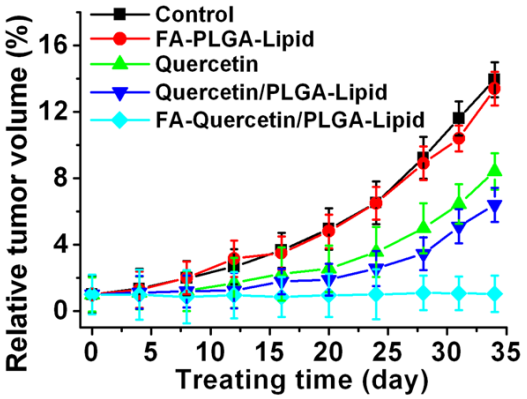

Figure 7. The curve of tumor volume change.

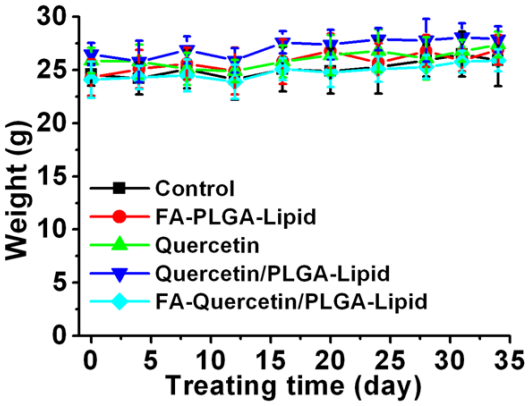

Figure 8. The weight change of mice.

SPSS13.0. Comparison among groups was conducted by using independent-samples T test. P < 0.05 indicated the presence of statistically significant difference.

Results

Synthesis and characterization of FA-quercetin/PLGA-lipid

In this study, FA-quercetin/PLGA-lipid was synthesized by self-assembly of liposomes and nanoprecipitation method. As shown in **Figure 1**, FA-quercetin/PLGA-lipid synthesized in this study was composed of five parts, namely, folic acid, PEG, lipid, PLGA and quercetin. Results of the nano particle analyzer indicated that the diameter of FA-Quercetin/PLGA-Lipid mainly ranged from 78 nm to 90 nm with its average value of 85 nm (**Figure 2**).

The encapsulation efficiency and releasing ratio of quercetin

The encapsulation efficiency of quercetin in FA-Quercetin/PLGA-Lipid was detected via measuring its absorbance at 374 nm by using an UV spectrophotometer (encapsulation efficiency = $W_{Encapsulated}/W_{Total}$ × 100%). The result was calculated to be 76.8 ± 2.3%. As shown in **Figure 3**, the releasing ratio of quercetin in FA-Quercetin/PLGA-Lipid was almost rising perpendicularly within 24 hours and rose slower after 24 hours. Until the 96th hour, the ratio was kept at 50% or so. Therefore, it clearly indicated that quercetin in FA-Quercetin/PLGA-Lipid was released slowly.

The in-vitro targeting study of FA-Quercetin/PLGA-Lipid

In this study, we incubated Quercetin/PLGA-Lipid and FA-Quercetin/PLGA-Lipid marked by rhodamine with liver cancer HepG2 cells for 3 hours. Then, we observed rhodamine fluorescence by confocal microscopy. DAPI, a dye used to stain nuclei, was used to locate nuclei. As shown in **Figure 4**, nuclei in both groups showed strong DAPI fluorescence. rhodamine fluorescence in the cytoplasm of Quercetin/PLGA-Lipid was weak, but strong in that of FA-Quercetin/PLGA-Lipid. This suggested that FA-Quercetin/PLGA-Lipid could target at tumor cells very well and be swallowed into the cytoplasm.

The cytotoxicity study of nanoliposomes

In this study, we investigated the cytotoxicity of pure nanoliposomes in the first place. 20, 40, 60, 80 and 100 μg/mLPLGA-Lipid and FA-PLGA-

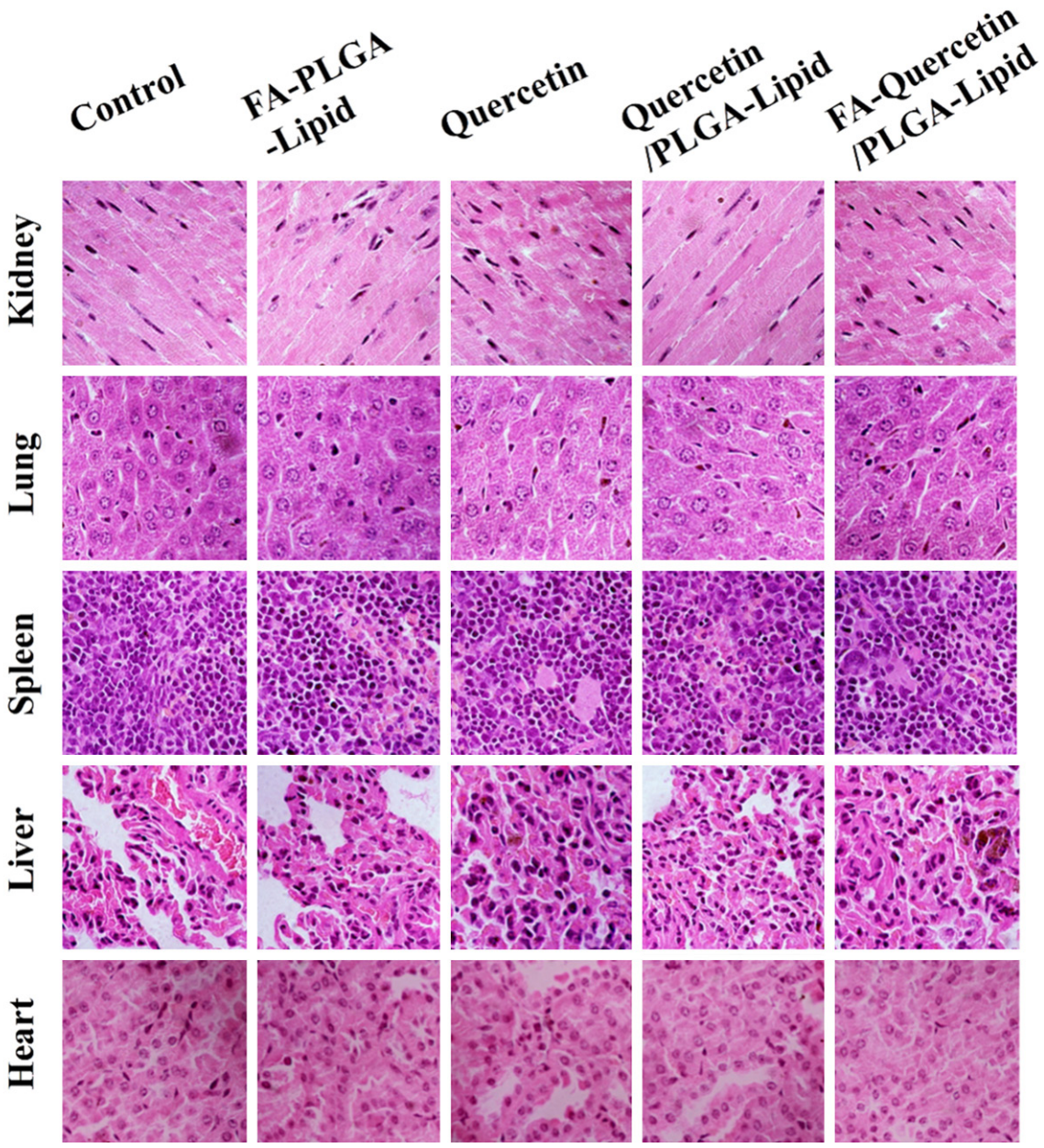

Figure 9. The HE staining images of heart, liver, spleen, lung and kidney.

Lipid were incubated with HepG2 cells for 24 hours, respectively. After that, the cytotoxicity was detected. As shown in **Figure 5**, different concentrations of both nanoliposomes had no significant effect on cell viability, indicating that nanoliposomes as carriers almost had no toxicity to cells. Next, we studied the toxicity of quercetin and quercetin encapsulated by nanoliposomes. 10, 20, 30, 40 and 50 μg/mL Quercetin, Quercetin/PLGA-Lipid and FA-Quercetin/PLGA-Lipid were incubated with tumor cells for 24 hours, respectively. As shown in **Figure 6**, these three drugs all had a concentration-dependent killing effect on cells. To the maximum extent, quercetin alone could inhibit 60.8 ± 2.1% of cell viability, Quercetin/PLGA-Lipid 68.5 ± 1.7% of cell viability, which was evidently higher than that of quercetin alone ($P < 0.05$). Moreover, FA-Quercetin/PLGA-Lipid could inhibit 80.9 ± 2.9% of cell viability, which was significantly higher than that of Quercetin/PLGA-Lipid ($P < 0.01$).

The in-vivo anticancer study of nanoliposomes

35 liver cancer bearing mice were randomized into five groups (7 mice per group), namely, normal saline group (blank control), FA-PLGA-Lipid group, quercetin group, Quercetin/PLGA-Lipid group and FA-Quercetin/PLGA-Lipid group. Samples were injected through caudal vein. From the date of administration, the tumor volume and body weight of mice were measured every three days. As shown in **Figure 7**, the tumor growth curve of FA-PLGA-Lipid group was similar to that of the blank control, so FA-PLGA-Lipid was considered to have no inhibitory effect on tumor. In the quercetin and Quercetin/PLGA-Lipid group, tumor growth was inhibited in the first 8 and 12 days, respectively. However, after that, tumor grew gradually. In the FA-Quercetin/PLGA-Lipid group, tumor growth was inhibited in the first 8 days. Moreover, during the following treatment, tumor didn't grow any further and was even eliminated. It was possible because FA-Quercetin/PLGA-Lipid was able to target at tumor cells and gathered around the tumor area, so as to release drugs slowly and treat the tumor effectively.

The in-vivo toxicity study of nanoliposomes

In this study, the in-vivo toxicity of FA-Quercetin/PLGA-Lipid was evaluated by measuring the body weight of mice and observing pathological sections of their major organs. During the entire treatment period, no significant decrease or increase of body weight was found in all groups (**Figure 8**), indicating that the samples had not influenced their metabolic function. HE staining of pathological sections suggested that their heart, liver, spleen, lung and kidney were not evidently damaged by all samples (**Figure 9**). These results indicated that FA-Quercetin/PLGA-Lipid prepared in this study had no significant toxicity in mice.

Discussion

Nanoliposomes, as one of the drug carriers, have been widely used in the development of new dosage forms and modification of drug's physical properties. In recent years, more and more nanoliposomal drugs have been explored to be applied or are being applied in clinical use [1, 3]. Nanoliposomes are nano-scale spherical particles with good biocompatibility. The center of these particles can load drugs or other functional molecules, while the surface can be modified by such functional molecules as targeting folic acid and antibody proteins through chemical approaches [7, 8]. In this way, they become multi-functional nanoliposomes. For ordinary nanoliposomes, the particle diameter generally ranges from 20 nm to 200 nm. After entering into the blood circulation system, their blood half-life is relatively long. Besides, they are of certain passive targeting property. Therefore, compared with drugs alone, nanoliposomal drugs have a better therapeutic effect. However, for some special lesions like solid tumor, ordinary nanoliposomal drugs have poor targeting efficiency. Hence, it is an essential process to modify the surface of nanoliposomes by targeting molecules [9].

In recent years, along with the technological development of extracts of traditional Chinese medicine (TCM), they have been widely used in experimental studies and clinical tests. Quercetin, as an efficient extract of TCM, has also been widely used in anticancer studies. Zhao Xinhan, et al. found that quercetin have certain killing effect on human cervical cancer cells [19]. However, as research continued, it was found that quercetin alone had some disadvantages, such as short blood half-life, low effective concentration at lesions, and significant toxicity of high-concentrations of drugs, etc. For this reason, researchers investigated some physiochemical modifications. Zhang Yang et al. encapsulated quercetin in nanoliposomes and the resultant products acted on tumor cells.

In this study, we also encapsulated quercetin in nanoliposomes. Moreover, we modified the surface of liposomes by the targeting molecule folic acid. This enabled nanoliposomal drugs to be more effective in targeting at tumors and thus more effective in anticancer treatments. This study found that Quercetin/PLGA-Lipid had certain targeting property compared with quercetin alone (**Figure 4**), but the in-vitro and in-vivo anticancer effect was still unsatisfactory (**Figures 6** and **7**). An in-vitro targeting experiment suggested that FA-Quercetin/PLGA-Lipid had good targeting property due to its specific binding with folate receptors on the surface of hepatoma cells. Furthermore, in-vitro and in-vivo anticancer studies also proved its anticancer efficacy. Therefore, it was confirmed in this

study that modification of quercetin enabled Quercetin/PLGA-Lipid to target at tumor cells. This kept relatively high-level drug concentration at tumor areas, achieving better killing effect.

To sum up, FA-Quercetin/PLGA-Lipid we prepared had good targeting property for liver cancer HepG2 cells and evident in-vitro and in-vivo anticancer effect without obvious toxicity in human body.

Disclosure of conflict of interest

None.

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