

Research Article

Effect of a Drug Delivery System Made of Quercetin Formulated into PEGylation Liposomes on Cervical Carcinoma *In Vitro* and *In Vivo*

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Received 7 April 2021; Revised 5 July 2021; Accepted 26 July 2021; Published 14 August 2021

Academic Editor: Antonio Vassallo

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Quercetin is a well-known flavonoid for its potent antitumor and antiproliferative effects on a wide range of human cancer cell lines. However, the delivery of quercetin is challenging due to its extreme insolubility in water. The intention of this study was to evaluate the antitumor effect of quercetin-loaded PEGylated liposomes (PEG-Que-NLs) *in vitro* and *in vivo*. We first prepared PEG-Que-NLs by method of thin film hydration; further determined, the optimum ratios of quercetin to Soybean phosphatidylcholine (SPC), to cholesterol (CHL), and to PEG-4000 were 1:8, 1:2, and 1:2 (w/w), respectively, and the optimal hydration temperature was 55°C when the mean vesicle diameter and apparent Zeta potential of PEG-Que-NLs were found to be 171.3 ± 10.4 nm and -13.1 ± 2.1 mV, respectively; the encapsulation efficiency and the drug loading of PEG-Que-NLs were 81.25 ± 3.12 % and 8.5 ± 0.77%, respectively. Drug release study *in vitro* showed that PEG-Que-NLs exhibited a slow-release effect without significant burst effect. Furthermore, the inhibition effect of PEG-Que-NLs on HeLa cells was considerably higher than free quercetin (free-Que) and quercetin liposomes (Que-NLs). Intravenous injection of PEG-Que-NLs into U14 bearing mouse models inhibited the cervical carcinoma growth significantly, and the tumor inhibition prospects in the treatment of malignant tumors because of its tumor targeting, slow-release properties, and the solubility improvement of quercetin.

1. Introduction

Quercetin, as conjugated isoforms bound to alcohol and sugar, could be obtained by the human body through a regular daily diet of fruits and vegetables [1]. Among the identified flavonoids, quercetin has attracted increasing attention due to its strong antioxidant activity due to its extremely active oxygen species (O2' and ONOO') scavenging ability [2, 3]. However, its poor bioavailability, low aqueous solubility, and low stability limit its wide application in pharmaceutics [4]. Furthermore, quercetin tends to undergo a high level of enzymatic degradation in the gastrointestinal tract and has a short half-life time in the body [5]. In addition, because of its poor water solubility, sometimes quercetin needs to be dissolved in DMSO in clinical use. DMSO not only causes unpleasant odors but also exhibits certain toxicity to the liver and kidney [6–8]. Therefore, it is expected to achieve better therapeutic effect in clinical practice by encapsulating quercetin into an appropriate delivery system.

As a kind of self-assembled nanoparticles, liposomes are ideal carriers for the delivery of many drugs, including cytotoxic agents, antibiotics, and antifungal agents [9]. Additionally, liposomes showed excellent biocompatibility, biodegradability, low toxicity, and controlled release of the entrapped drug [10]. The size of vasculature endothelial pores around the solid tumor is 100-200 nm [11], which is much larger than those of normal tissues with discontinuous endothelium with pore sizes from 40 to 60 nm [12, 13]. However, liposomes may be removed by macrophages of the liver and spleen in circulation in the body, because the sinusoidal endothelium of the liver and spleen has pores up to 150 nm [12, 14]. Therefore, the optimal particle size distribution of the drug preparation is 150-200 nm, which is conducive to the accumulation of the drug in the tumor tissues.

Recent studies have shown that liposomes coated with inert; biocompatible polymers can evade the recognition of immune cells, so liposomes can be more effectively exposed to tumor tissue [15, 16]. Noteworthily, as a new drug carrier system, pegylated liposomes have been proved to be very successful, and the PEGylation of tumor targeting drugs based on EPR effect is superior to other surface modifications [17]. Although earlier studies including our previous studies have shown that some nanoparticle formulations of quercetin provide more beneficial *in vivo* behavior than free quercetin [18, 19], there is still little information about the therapeutic efficacy of quercetin-loaded PEGylated liposomes (PEG-Que-NLs) on cervical cancer mice models.

In this study, quercetin was encapsulated in the nonaqueous interior of PEGylated liposomes firstly; on the basis of systematic physical and chemical characterization, the anticervical cancer effects of PEG-Que-NLs were subsequently evaluated *in vitro* and *in vivo*. Our results showed that PEG-Que-NLs exhibit significant antitumor effects *in vitro* and *in vivo* for the improved solubility and bioavailability of natural quercetin. The main content of our study was illustrated in the Graphical Abstract.

2. Materials and Methods

2.1. Materials. Soybean phosphatidylcholine (SPC, AR) and cholesterol (CHL, AR) were obtained from A.V. T. Pharmaceutical Co., Ltd, and PEG-4000 was from Sigma-Aldrich. Quercetin (Que) (BR, purity \geq 98%) was got from Chengdu Breit Chemical Technology Co., Ltd, China. Other reagents were obtained from Tianjin Kai Chemicals Co. Ltd China.

2.2. Preparation of PEG-Que-NLs. Required quantity of SPC, CHL, PEG-4000, and Que was dissolved in chloroform/methanol (3:1, v/v), and then, the organic phase was removed by rotary evaporation (RE-301, Xi'an Yuanjian Instrument and Equipment Co., Ltd) to form a thin film of lipids on the wall of the eggplant-shaped bottle. After being filled with nitrogen for 5 minutes, the lipid film was hydrated with PBS at a specific temperature. After being ultrasonic by an ultrasonic cleaner (KQ5200, Kunshan Ultrasonic Instrument Co., China) for 15-30 minutes at a frequency of 40000 Hz, the resulting suspension was passed through filter membranes of $0.45 \,\mu\text{m}$ and $0.22 \,\mu\text{m}$ successively to remove the nonincorporated Que.

In this study, the ratios of Que to SPC (w/w), CHL (w/w), and PGE-4000 (w/w) and the hydration temperature were considered the most important factors for the entrapment efficiency (EE) of the formulation. Then, the orthogonal tests were designed for four factors and at three levels (Tables 1 and 2). At the same time, PEG-NLs, which were taken as blank control and Que-NLs, which were taken as non-PEGylated formulation control were prepared by our method reported previously [19].

2.3. Characterization of PEG-Que-NLs

2.3.1. Fourier Transform Infrared (FTIR) Spectroscopy. Que-NLs, physical mixture of Que, SPC, CHL and PEG-4000, and PEG-Que-NLs were mixed with KBr, and subsequently, pellets were prepared, respectively. The pellets were scanned over a wave number range of 4000-400 cm⁻¹ using FTIR spectroscopy (Alpha T, Bruker Led. Germany) [20].

2.3.2. X-Ray Diffraction (XRD) Scan. To verify the formulation of PEG-Que-NLs successfully, Que, PEG-4000, and PEG-Que-NLs were scanned from 5° to 60° diffraction angle (2 Θ) using X-ray diffractometer (Ultima-III, Regaku, Japan) [21].

2.3.3. TEM Observation. To observe the particle size and morphology of PEG-Que-NLs, the samples were visualized with TEM (HT-7700, Hitachi, Japan) after negative staining.

2.3.4. Particle Size and Zeta Potential Measurement. To verify the size and apparent Zeta potential of the PEG-Que-NLs, the samples were measured by Malvern Zetasizer (Nano ZS, Malvern Instruments Ltd., U.K.) further.

2.3.5. EE and Drug Lording Efficiency (DL) of PEG-Que-NL Measurement. EE and DL of PEG-Que-NLs were measured by the method of ultrafiltration combined with ultraviolet spectrometry by UV-visible spectrophotometer (UV-1800 PC, Hebei Yaoyang Instrument and Equipment Co., Ltd. China). Firstly, the regression curve of Que concentration vs. absorbance at 370 nm was obtained. Then, PEG-Que-NLs were put in ultrafiltration centrifugal tubes (10 KD, Millipore) and centrifuged to separate unentrapped querce-tin from PEG-Que-NLs (5000 rpm, 15 min). Subsequently, 1 mL of the resulting free quercetin solution was transferred into a new centrifuge tube, and 2 mL of methanol was added. Finally, the quercetin concentration was determined by the regression curve of above, and EE and DL of PEG-Que-NLs were calculated with the formula as follows [22].

$$EE\% = \frac{(W_T - W_F)}{W_{Tl}} \times 100,$$
 (1)

DL% =
$$\frac{(W_T - W_F)}{W_{L+P}} \times 100,$$
 (2)

TABLE 1: Levels of experimental factors.

Ratio of quercetin to SPC (<i>w</i> / <i>w</i>) A	Ratio of quercetin to CHL (<i>w</i> / <i>w</i>) B	Ratio of quercetin to PEG-4000 (<i>w/w</i>) C	Hydration temperature (°C) D
1:6	1:1	1:1	55
1:8	1:2	1:2	60
1:10	1:3	1:3	65

TABLE 2: Orthogonal test design and drug entrapment efficiency (%).

	А	В	С	D	EE (%)
1	1	1	1	1	65.2
2	1	2	2	2	81.2
3	1	3	3	3	67.4
4	2	1	2	3	72.3
5	2	2	3	1	69.7
6	2	3	1	2	80.7
7	3	1	3	2	68.2
8	3	2	1	3	79.7
9	3	3	2	1	74.6
K1	213.8	205.7	225.6	209.5	
K2	222.7	230.6	228.1	203.5	
K3	222.5	222.7	205.3	207.6	
K1	71.3	68.6	75.2	69.8	
K2	74.2	76.9	76.0	76.8	
K3	74.1	74.2	68.4	74.0	
R	10.8	21.7	6.6	3.1	

A: ratio of Que to SPC; B: ratio of Que to CHL; C: ratio of Que to PEG-4000; D: hydration temperature.

where W_T is the drug weight in the dispersions, W_F is the free drug weight in the supernatant, and W_{L+P} is the total weight of the lipids and PEG-4000 content.

2.3.6. Stability Study. Liposomes tend to adhere each other and fuse to form larger particles in the suspension and result in the change of the apparent Zeta potential subsequently. Therefore, during the process of storage, PEG-Que-NLs were evaluated for the stability in the storage condition at 4° C for 4 weeks. The liposome samples were taken out after 2 weeks and 4 weeks, respectively, and characterized by changes in their average particle size and Zeta potential [23].

2.3.7. In Vitro Drug Release Curve Drawing. The dialysis method was used to detect the release of Que from free quercetin, Que-NLs, and PEG-Que-NLs *in vitro*, respectively. 100 mL of pH 7.4 PBS was taken in a 250 mL beaker. 2 mL of liposomal suspension was put into a dialysis bag (molecular weight cut – off = 2000) and hung inside the beaker containing buffer solution. 2 mL of the samples was withdrawn at the specified time interval with replenish of the same volume of buffer solution. Concentration of free-Que was measured spectrophotometrically at 280 nm [21].

2.4. Cells and Animals. Both HeLa cells and U14 cells were purchased from Nanjing Kebai Biotechnology Co., Ltd., and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C incubator containing 5% CO₂. Female BALB/c mice (aged 4 weeks, 20 ± 2 g) were purchased from Beijing Vital River Laboratory Animal Technologies Co. Ltd (Beijing, China). The animals were provided with food and water ad libitum and maintained at 25°C with a relative humidity of 55%. All animal experiments were approved by the Institutional Animal Care and Use Committee of Yanshan University, China.

2.5. Cell Proliferation Inhibitory Effects of PEG-Que-NLs In Vitro. To evaluate the inhibitory effects of PEG-Que-NLs on cervical cancer cells, HeLa cells were seeded in 96-well culture plates at a density of 5×10^4 cells/mL and further cultured for 24 hours at 37°C in a 5% CO₂ atmosphere. Then, different concentrations of free-Que, Que-NLs, and PEG-Que-NLs were added into the culture plates. The final concentrations of Que in different formulas were 0.5, 1, 2, 4, 8, and 16 μ M, respectively. Blank culture medium was used as blank control, and PEG-NLs were chosen as the blank vector control. After incubation for 48 hours, the proliferation inhibition effects were measured by sulforhodamine B (SRB) staining assay [24]. Briefly, after removing the medium, the cells were fixed with 10% trichloroacetic acid (200 μ L) for 1 hour, then washed for 5 times, and stained with 0.4% SRB at room temperature. Finally, the absorbance was measured at 540 nm using a microplate reader. All experiments were performed in triplicate. Absorbance of untreated cells was considered 100% cell viability, and the treated cell viability was calculated by the following formula [25].

Survival% =
$$\left(\frac{A540_T}{A540_{BC}}\right) \times 100,$$
 (3)

where $A540_T$ is the absorbance value of treated cells at 540 nm and $A540_{BC}$ is the absorbance value of the blank control cells at 540 nm.

2.6. Animal Experiments

2.6.1. U14-Solid Tumor-Bearing Mouse Models' Establishment and Tumor Inhibition Determination of PEG-Que-NL Treatment. Female BALB/c mice were randomly divided into 5 groups with 8 mice in each group. U14 cells were diluted to about 1×10^7 cells/mL with sterile physiological saline and subcutaneous injected into the left axilla of each mouse (day 0). After 24 h of inoculation, the mice in the PEG-Que-NL, Que-NL, or free-Que groups were administrated daily by tail intravenous injection at a dose of 20 mg/kg (Que-NLs, PEG-Que-NLs $\approx 2 \text{ mg/kg}$ pure quercetin) or 2 mg/kg (free-Que), respectively, and the dose of these formula in our experiment depends on our preliminary experiment. The groups administrated with physiological saline and PEG-NLs alone were taken as the negative control and the blank vector control, respectively. All groups were treated once every two days for 14 days continuously, and all animals were fed with a standard

pellet diet and water ad libitum during the experiment time. The maximum diameter (a) and the minimum diameter (b) of transplanted tumors were measured after tumor formation; body weight and tumor volumes were recorded every two days. The tumor volume was expressed according to the following formula.

$$V = \frac{\left(a \times b^2\right)}{2},\tag{4}$$

where *V* stands for the volume of the measured tumor, *a* is the maximum diameter, and *b* is the minimum diameter of the measured tumor [26].

On the 16th day of the experiment, all mice were killed by ether anesthesia; tumors and other organs were dissected and weighed accurately. During the whole experimental stage, the health indicators of mice, such as behavior, feeding, hair status, and so on, were recorded.

2.6.2. Histopathology Study of Tumors Treated with PEG-Que-NLs. After the harvested tumors were fixed in formalin, embedded in paraffin, and cut in $4 \,\mu m$ sections, H. E staining and pathological observation were carried out further.

2.6.3. U14-Ascite Tumor-Bearing Mouse Models' Establishment and Survival Time Determination of PEG-Que-NL Treatment. To further explore the effect of PEG-Que-NLs on the survival time of mice bearing cervical cancer, we conducted another series of experiments. After injecting 2×10^6 U14 cells into the abdominal cavity of the five groups of experimental mice, the experiment mice were administrated according to the schedule of above. Then, the date of death was recorded to calculate the survival time of the mice in each group.

2.7. Statistical Analysis. All experiments were carried out three times in parallel, and the data were analyzed by the Graphpad Prism 8 software (GraphPad Software Inc., La Jolla and CA. USA). ANOVA was used for the comparison between different groups; multiple comparisons were performed using variance analysis; survival curves of tumor-bearing mice after different treatment were determined by the Kaplan-Meier method and considered P < 0.05 significant.

3. Results

3.1. Determination of the Optimum Conditions for the Preparation of PEG-Que-NLs. Soybean phosphatidylcholine and cholesterol are commonly used as liposomes forming materials with ideal biocompatibility; PEG-4000 can weaken the nonspecific protein adsorption capacity of drug loading systems and increase the long-term circulation of drug in vivo, so we choose Soybean phosphatidylcholine, cholesterol, and PEG-4000 to encapsulate quercetin to form PEG-Que-NLs. According to previous literatures, there were many factors that influence the EE of PEG-Que-NLs during the preparation process, especially the ratios of Que to SPC, CHL, and PEG-4000 (w/w), and the hydration temperatures are the main factors affecting the entrapment efficiency and morphology of liposoluble drugs. Therefore, we carried out the preexperiment of single factor experiments and then

designed the orthogonal experiment according to the experimental results (labeled as A, B, C, and D in Table 1). Furthermore, the four factors were investigated at three different levels regarding the EE as an important index. The L9 (3⁴) orthogonal design is shown in Tables 1 and 2. The results in Table 2 of the experiment revealed that the ranking of the four factors influencing EE was B > A > C > D, indicating that the ratio of quercetin to CHL was the most effective factor to EE; the ratio of Que to SPC also showed less influence. With regarding to the individual factors, the optimum ratios of quercetin to SPC, to CHL, and to PEG-4000 (w/w) were 1:8, 1:2, and 1:2, respectively, and the optimal hydration temperature was 55°C. According to the orthogonal test results, the optimal formulation should be $A_2B_2C_2D_1$.

Because of the lipid bilayer composition will have a significant impact on the particle size, EE, DL, and drug release from liposomes, we changed the ratios of Que to SPC, to CHL, and to PEG-4000 for further detecting the physical and chemical properties of liposomes. The ratio of Que to CHL from 1:1 to 1:2 considerably augmented the EE of liposomes might be because CHL could change the fluidity of the phospholipid membrane and increase the stability of vesicles by preventing the crystallization of the phosphatidyl chain and providing space resistance for its movement. While the ratio of them from 1:2 to 1:3, the EE deceased again may be owing to the fact that both Que and CHL prefer to stay associated in the hydrophobic region of the lipid bilayer, but the space is very limited, so they compete for the space which results lower EE [27–29].

3.2. Physical and Chemical Properties of PEG-Que-NLs. TEM results showed that the average particle size of PEG-Que-NLs was approximately 150 nm and displayed a spherical structure and complete morphology, and no significant aggregation was observed on representative TEM images (Figure 1(a)).

Figures 1(b) and 1(c) show the particle size, and the apparent Zeta potentials of PEG-QUE-NLs were approximately 171.3 ± 10.4 nm and -13.1 ± 2.1 mV, respectively, and the nanoparticles showed a narrow size distribution according the PDI value of 0.17 ± 0.026 .

To verify the connection of PEG to the Que-NLs, the PEG-Que-NLs were investigated by FTIR spectrometry further (Figure 2(a)). The typical absorption band at approximately 1120 cm⁻¹ is the characteristic C-O-C stretching vibration of the repeated $-OCH_2CH_2$ - units of the PEG backbone in the physical mixture and complexes, suggesting the successful link of PEG-4000 with Que-NLs (Figure 2(a) B and C vs. A). Furthermore, PEG-Que-NLs showed the minor change of some peaks compared to the physical mixture (Figure 2(a) B vs. C). These spectrum minor changes may be caused by the new hydrogen bonds, dipole moments, or van der Waals forces between phospholipid and drug polar functional groups. These interactions are also conducive to the formation of the vesicle shape, making it more stable and the drug slow-release effect more significant.

Important crystallographic reflections at different angles were observed in XRD patterns of quercetin and PEG-4000, which was due to their crystalline nature (Figure 2(b) A and B). However, in the XRD pattern of PEG-Que-NLs, these



FIGURE 1: Characterization of PEG-Que-NLs: (a) TEM images of PEG-Que-NLs; (b) size distribution of PEG-Que-NLs assessed by dynamic light scattering (DLS); (c) Zeta potential of PEG-Que-NLs assesses by DLS.



FIGURE 2: Confirmation of the formation of PEG-Que-NLs: (a) FITR spectra of (A) Que-NLs, (B) PEG-Que-NLs, and (C) mixture of quercetin and excipients; (b) XRD of (A) PEG-4000, (B) quercetin, and (C) PEG-Que-NLs.

peaks were disappeared (Figure 2(b) C). These results showed that PEG-4000 and quercetin were highly bound to or dissolved in the lipid system [30].

According to the formula above, the EE and DL of the PEG-Que-NLs were found to be $81.25 \pm 3.12\%$ and $8.5 \pm 0.77\%$, respectively. These high EE and DL are due to the fact that quercetin is a liposoluble compound that could be encapsulated in the bilayer membrane almost completely. Compared with hydrophilic compounds, lipophilic compounds tend to have higher EE and DL and show slower release [31].

We also completed stability studies with PEG-Que-NLs for 4 weeks and showed them to be stable with respect to the particle size and apparent Zeta potential for a minimum of 4 weeks at 4°C, and the result is shown in Figure 3(a). Quercetin released from the free-Que, Que-NL, and PEG-Que-NL formulations were conducted in the medium of plasma-containing PBS by dialysis method. As shown in Figure 3(b), free-Que, Que-NLs, and PEG-Que-NLs all showed rapid release at the beginning and slower release at the later stage. Furthermore, we can also observe that both



FIGURE 3: Stability analysis and quercetin release profile drawing: (a) stability of Que-NLs was stored at 4°C for 4 weeks; (b) the release profiles of quercetin from free-Que, Que-NLs, and PEG-Que-NLs. Values represented were means \pm SD (n = 3).



FIGURE 4: Proliferation inhibition effect of PEG-Que-NLs on HeLa cell line: (a) inhibition effects of PEG-NLs, free-Que, Que-NLs, and PEG-Que-NLs on HeLa cells in vitro; (b) IC50 value of free-Que, Que-NLs, and PEG-Que-NLs, respectively. Values represented were means \pm SD (n = 3), *P < 0.05 or ***P < 0.001, compared with the free-Que treatment group; ##P < 0.01, compared with the Que-NL treatment group.

PEG-Que-NLs and Que-NLs showed a slower release profile than free-Que. And PEG-Que-NLs exhibited the slowest release under the same conditions, suggesting that PEG-Que-NLs have excellent sustained release property compared with Que-NLs and free-Que.

3.3. Proliferation Inhibition Effects of PEG-Que-NLs on HeLa Cells. Figures 4(a) and 4(b) illustrate the proliferation inhibition effect and the IC 50 values of different types of formulations on HeLa cells. Regarding the survival rate, the HeLa cells exhibited different sensitivity to free-Que and different kinds of nanoliposomes. Results showed that even the con-

centration reached 16 μ M; the blank PEG-NLs showed little inhibition effect on HeLa cells. This result indicated that the inhibitory effect of Que-PEG-NLs on HeLa cells was mainly derived from the encapsulation of quercetin in nanoparticles. With free quercetin, the cell viability was higher than Que-NLs and showed lower antiproliferation activity. However, its cytotoxicity increased significantly when cells were treated with Que-NLs and PEG-Que-NLs, and the number of live cells decreased in a dose-dependent manner. These results demonstrated that the cell proliferation inhibition effect was increased when quercetin was encapsulated into nanoparticles. Furthermore, PEG surface-modified

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FIGURE 5: In vivo antitumor effect of PEG-Que-NLs. (a) Images of tumor sizes after the mice were treated with saline, PEG-NLs, free-Que, Que-NLs, or PEG-Que-NLs, respectively. (b) The evolution of the tumor volume with time after the mice was administered with saline, PEG-NLs, free-Que, Que-NLs, or PEG-Que-NLs. (c) Body weight changes with time of U14-solid tumor-bearing mice during the treatment period. Values represented were means \pm SD (n = 8), *P < 0.05 or ***P < 0.01, compared with the PEG-NL treatment group.

liposomes showed no stronger inhibition effect on HeLa cells than non-PEG modified nanoliposomes, and the IC50 values of free-Que, Que-NLs, and PEG-Que-NLs were 5.519, 3.322, and 3.033 μ M, respectively. Compared with Que-NLs, PEG-Que-NLs did not show further inhibitory effect on cell proliferation, which may be due to the fact that PEG surface modification did not increase the solubility of quercetin and the endocytosis of cells, and the long-term circulation of PEG in vivo was not shown in the environment of cultured tumor cells.

3.4. In Vivo Inhibitive Efficacy of PEG-Que-NLs on U14-Solid Tumor Growth in Mice. For further investigating the therapeutic efficacy of PEG-Que-NLs in vivo, we established U14-solid tumor-bearing mice models by subcutaneous inoculating U14 cells in the armpit of mice. Saline, PEG-NLs, free-Que (2 mg/kg), Que-NLs, and PEG-Que-NLs containing 2 mg/kg of quercetin were intravenously administrated in each group of mice. The tumor size and body weight of each animal were recorded every two days to evaluate the antitumor efficacy. As shown in Figures 5(a) and 5(b), there were no significant differences between saline and PEG-NL treatment groups for tumor growth (P > 0.05), which demonstrated that the drug carrier itself had no significant effect on tumor growth. Meanwhile, compared with the PEG-NL treatment group, the tumor growth in the free-Que group slowed down significantly (P < 0.05), indicating that free-Que showed antitumor activity in vivo. Furthermore, compared with the free-Que treatment group, both Que-NLs and PEG-Que-NLs showed significant inhibition effects on the growth of tumor and particularly; PEG-Que-NLs achieved an ideal treatment efficacy, which is much better than the Que-NL (P < 0.05), free-Que (P < 0.01), PEG-NL (P < 0.01), and saline groups (P < 0.01) (tumor volume was $530.8 \pm 43.31 \text{ mm}^3$ vs. 744.6 ± 39.37 , 921.3 ± 66.59 , 1279.0 ± 141.2 , and $1360.0 \pm 186.5 \text{ mm}^3$, respectively).

Meanwhile, the side effects of PEG-Que-NLs were paid special attention. Throughout the experiment, the mice were evaluated for any signs of toxicity. No obvious pathological



FIGURE 6: Effects of PEG-Que-NL treatment on the tumor tissue structure in mice (amplificatory rate 10×10) ((a) saline treatment control group; (b) PEG-NL treatment control group; (c) free-Que treatment group; (d) Que-NL treatment group; (e) PEG-Que-NL treatment group).



FIGURE 7: Effect of PEG-Que-NLs on ascites tumor-bearing mice. (a) Survival curve of the saline, PEG-NL, free-Que, Que-NL, and PEG-Que-NL treatment groups. Survival curves of tumor-bearing mice after different treatment, which were determined by the Kaplan-Meier method. (b) Effect of PEG-Que-NLs on survival time of ascites tumor-bearing mice. Values represented were means \pm SD (n = 8), *P < 0.05 or ***P < 0.001, compared with the PEG-NL treatment group; $^{#}P < 0.05$, compared with the Que-NL treatment group.

changes were found in the liver and kidney tissues (data not shown). In addition, any gross measures, such as weight loss (Figure 5(c)), fur wrinkles, or behavior, did not show any adverse consequences.

3.5. Effect of PEG-Que-NLs on Histopathology of Tumor Tissues. In saline- and PEG-NL-treated mice, the tumor tissues were liquefied and adjacent to the pleura. Some tumor tissues even invade skeletal muscle and adipose tissues, indicating diffuse growth and abundant blood vessels. Compared with those in PEG-NL mice, the tumor tissues in the Que, Que-NL, and PEG-Que-NL groups showed obvious boundaries with other parts. No skeletal muscle invasion was observed, and the tumor tissues were easy to peel off. Furthermore, the tumors in these groups were dense and firm in appearance, gray and white in color, and lack of blood vessels. Histopathological section photos of the tumor tissues are shown in Figure 6. In the saline and PEG-NL administrated groups, the tumor cells were round or polygonal in shape, and the nucleoli were round or oval with large volume and clear nuclear membrane (Figures 6(a) and 6(b)). Compared with the saline and PEG-NL treatment groups, the malignant degree of tumor tissue in the free-Que treatment group was lower, but there still existed a small amount of invasion to the skeletal muscles (Figure 6(c)). In tumor tissues treated with Que-NLs (Figure 6(d)) and PEG-Que-NLs (Figure 6(e)), rough chromatin cells appeared, which demonstrated that a variety of transformed tumor cells emerged in these two groups. Furthermore, a large number of apoptotic cells and necrotic areas appeared in the tumor tissues in the PEG-Que-NL treatment group, and the malignant degree of the tumor tissues was further reduced.

3.6. Effect of PEG-Que-NLs on Life Span in U14 Ascite Tumor-Bearing Mice. We also carried out another series of experiments to further evaluate the therapeutic effect of PEG-Que-NLs on malignant tumor through the effect on the survival time of mice with ascites tumor. Compared with the saline treatment group, the life span of the PEG-NL treatment group showed no significant differences (P > 0.05), which showed again that the carrier itself exhibited no significant effect on the growth of ascites tumor. The life span of ascites tumor-bearing mice increased significantly in the free-Que-, Que-NL-, and PEG-Que-NL-treated groups in comparison with the saline and PEG-NL control groups, respectively (P < 0.05, P < 0.01, and P < 0.001; Figures 7(a) and 7(b)). It is noteworthy that besides PEG-Que-NLs significantly prolonged the survival time of experimental animals, 25% of the tumor-harboring mice still survived by the end of our observation period (60 days). Apparently, PEG-Que-NLs achieved greatly enhanced therapeutic efficiency in comparison with free-Que and Que-NLs (P < 0.01 and P < 0.05, respectively).

4. Discussion

Cancer is one of the leading causes of death in the world. Cancer cases are increasing dramatically with the changes of environment and dietary habits. It was reported that by 2020, 10 million people will die of cancer every year in the world [32]. At present, chemotherapy is widely used as an effective treatment for malignant tumors, but its side effects are still the main challenge [33].

As a kind of flavonoid, quercetin has many biological activities such as antitumor, antiaging, and inhibition of glycolysis, etc., while it showed limitations in clinical application due to its poor water solubility [34–36].

Drug delivery systems (DDSs), which have been extensively studied as carriers to deliver small molecule chemo-drugs to tumors for cancer therapy, have received growing attention. Among all DDSs, lipid-based nanoparticles such as liposomes have emerged as a promising strategy for the treatment of cancer, and most of them are FDA approved [9, 37]. Increasing therapeutic efficacy was found in both Que-NL and PEG-Que-NL treatment groups in our study. These benefits derived from their targeting properties, slow-releasing potential, and high stability afforded by the liposomal carrier [16]. This may be especially important for water insolubility compounds from natural plants, such as quercetin.

Previous studies *in vitro* and *in vivo* identified that the surface PEGylation of liposomes helped particles evade premature clearance by the mononuclear phagocytic system because of their hydrated coverage and further prolong their half-life in serum [38, 39]. The binding of PEG to the liposome surface increased the biocompatibility and blood circulation time and reduced side effects of therapeutic agents. Additionally, hydrophilic PEG chains on the surface of the bilayers can adsorb more water molecules than those entrapped in the core. Thereby, the addition of PEG could enhance the EE and DL [40, 41]. In our study, the EE and DL of PEG-Que-NLs were found up to $81.25 \pm 3.12\%$ and $8.5 \pm 0.77\%$, respectively, which is similar to other previous reports [42].

The defects in tumor capillary endothelial cells of the tumor vasculature are usually in the range of 400-600 nm. Therefore, liposomes with diameter less than 400 nm can effectively infiltrate and accumulate in the tumor interstitial space [43]. Moreover, the tumor destroyed the lymphatic drainage, which made the exuded liposomal drugs have a long retention time in the tumor tissue [9]. In the present study, the diameters of PEG-Que-NLs were about 150~200 nm, which was beneficial to increase the concentration of quercetin in tumor tissue sites. Meanwhile, the drug sustained release caused by liposomes encapsulation prevented the rapid leakage of drugs during the process of transportation, which favored the accumulation of drugs in the tumor tissues further.

After preliminary studies to find the best formulation for PEG liposomes, an in vitro release study was carried out to evaluate the rate of leakage from the preparations. The *in vitro* release study results indicated that PEG-modified liposomes showed slower release and were more stable compared with free-Que and Que-NL formulations. This result showed that the configuration of PEG molecules on the surface of the liposome was important for steric repulsion *in vitro* because it determined the shielding efficiency, and this sustained release behavior may help with the antitumor progress, bringing about a continuous antitumor effect.

BALB/c mouse U14 allograft models were used to evaluate the therapeutic effects of PEG-Que-NLs on cervical cancer *in vivo*. Intravenous injection of PEG-Que-NLs exhibited the strongest inhibition effects on both solid tumor growth and ascitic tumor growth among different preparations and prolonged the life span of tumor-bearing models significantly (Figures 5(a), 5(b), 7(a), and 7(b)). Furthermore, cell apoptosis and necrosis in tumor tissues were also confirmed by subsequent pathological observation (Figure 6(e)). The enhanced antitumor efficacy of PEG-Que-NLs might be due to the following reasons: (i) The modification of PEG on the liposome surface made PEG-Que-NLs avoid the rapid clearance of reticuloendothelial system (RES), significantly prolonged the circulation time of this formulation, and further increased the aggregation in tumor site through EPR effect. (ii) The negative charge on the surface not only ensures their stability in serum but also ensures that serum albumin is not adsorbed in the circulation, which is not easy to be cleared by monocytes and macrophages. (iii) The particle size of the nanoparticles is around 170 nm, which has a significant EPR effect and ensures that the nanoparticles can be more detained in the vicinity of tumor tissues during the recycling process. (iv) Because of the good biocompatibility of liposomes, quercetin encapsulated in liposomes could significantly improve the solubility

and bioavailability of quercetin and maximize the antitumor

activity of quercetin. Compared with the previous reports about PEGylated liposomes of quercetin, the drug lording and the stability of PEG-Que-NLs that prepared in this study were enhanced markedly. Furthermore, the optimal preparation of PEG-Que-NLs with high entrapment efficiency was determined by orthogonal experiment. The entrapment efficiency and drug loading of quercetin were $81.25 \pm 3.12\%$ and $8.5 \pm$ 0.77%, respectively. The inhibitory activity of this drug delivery system in vivo and in vitro and the life prolongation rate of tumor cells were systematically discussed, and the safety of the drug was comprehensively evaluated.

Although the current research results showed that PEG-Que-NLs exhibited an obvious inhibitory effect on tumor growth in mice, it should be noted that according to the dosage and scheme of our experiment, PEG-Que-NL treatment on mice did not lead to the complete eradication of tumor cells. Clearly, the additional new formulation of quercetin needs to be further explored, and the detailed mechanisms and pharmacokinetics of PEG-Que-NLs were also being revealed in our laboratory.

5. Conclusion

In this study, a kind of PEG modified quercetin nanoliposomes were prepared for treating cervical cancer. PEG-Que-NLs with ideal physicochemical characteristics exhibit a significant sustained release effect. Studies on U14-bearing mice also demonstrated that the PEG-Que-NLs showed an enhanced antitumor effect and prolonged life time in tumor-bearing mice. In conclusion, PEG-Que-NLs provide a promising strategy for the treatment of cervical cancer.

Data Availability

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical Approval

All institutional and national guidelines for the care and use of laboratory animals were followed.

Consent

Written informed consent for publication was obtained from all participants.

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Conflicts of Interest

The authors declare that they have no direct financial relation with the commercial identities mentioned in this paper that might lead to a conflict of interests for any of the authors. Authors Li Jian, Li Zhen, Gao Yanting, Liu Shihe, Li Kun, Gao Liming, Shi Ming, Liu Zhiwei, Han Zengsheng, Qiu Yan, and Wang Shuai declared that they have no conflict of interest.

Authors' Contributions

J.L. and K.L. designed the experiments. S. W., Z.L., Y. G., S.L., and Y. Q. carried out the experiments. Z.H., L.G., and Z.L. analyzed the experimental results. J.L. and M. S. wrote the manuscript.

Acknowledgments

This work was supported by the Hebei Natural Science Foundation in China (grant number C2019203556), the Qinhuangdao Science and Technology Research and Development Plan in China (Numbers 201705B024, 201701B044, and 201801B035), Hebei Province Key Research and Development Projects in China (Number 19272401D), and Science and Technology Research Projects of Colleges and Universities in Hebei Province, China (Number ZD2018080).

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