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Preparation of DHAQ-loaded PLA-PLL-RGD Nanoparticles and Comparison of Antitumor Efficacy to Hepatoma and Breast Carcinoma

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This study researches the synthesis of Arginine-Glycine-Asparti (RGD) peptide modification of copolymer: poly (lactic acid-*co*-$_{\text{L}}$-lysine) (PLA-PLL-RGD) and the preparation of mitoxantrone (DHAQ)-loaded PLA-PLL-RGD nanoparticles (DHAQ-NP), and evaluates antitumor efficacy of exercise and DHAQ-NP in hepatoma and breast carcinoma. The experimental findings demonstrate that DHAQ-NP group have more significant antitumor efficacy than DHAQ group in hepatoma-bearing mice and breast carcinoma-bearing mice. Compared with DHAQ-NP group, the antitumor efficacy of Exercise+DHAQ-NP group are significantly lower in breast carcinoma-bearing mice. However, the antitumor efficacy of Exercise+DHAQ-NP group have no significant change in hepatoma-bearing mice. This suggests that the same exercise intensity can cause different antitumor efficacies for different tumors undergoing targeted therapies.

Keywords: Nanoparticles, targeted therapy, antitumor efficacy

1 Introduction

Hepatocellular carcinoma and Breast cancer are prevalent cancers in the human population. Cancer therapy has become an issue of growing concern in both research and clinical practice. The efficacy of cancer therapy is often evaluated by the ability of inhibiting tumor growth without damaging normal tissue.

In the last decade, targeted drug that provides a novel mechanism to concentrate a drug within the tumor, thereby minimizing its toxic side effects on normal tissues, has emerged as a new field of tumor treatment and attracted many researchers' interest. The targeted drug carrier for encapsulating anticancer drugs are often facilitated by specific targeting ligands. This is because ligand-mediated targeting to target receptor expressed selectively or overexpressed on tumor cells is an effective strategy for improving the therapeutic efficacy of anticancer drugs and its systemic toxicity (1–3).

In the present experiment, we synthesize arginine-glycine-aspartic (RGD) peptide modification of copolymer: poly (lactic acid-*co*-L-lysine) (PLA-PLL-RGD), and prepare DHAQ-PLA-PLL-RGD nanoparticles (DHAQ-NP) (PLA-PLL-RGD nanoparticels for targeting delivery of antitumor agents with Mitoxantrone(DHAQ) as a model drug, which has excellent therapeutic effects against a wide spectrum of cancer). Owing to many researchers have been investigating the effects of exercise on cancer patients. Moreover, many researches have provided preliminary evidence for the safety, feasibility, and efficacy of exercise training as a supportive intervention for breast cancer patients undergoing conventional chemotherapy (4, 5). However, it is not determined that whether exercise can influence antitumor efficacy of different tumor in targeted therapies. So, we research the antitumor efficacy of exercise and DHAQ-NP for hepatoma and breast carcinoma, and make a comparison between them. To our knowledge, no study has examined the antitumor effects of exercise and targeted drug using nanoparticles for different cancer.

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2 Experimental

2.1 Materials

DHAQ (Mitoxantron) was purchased from Chongqing Carelife Pharmaceutical. (Chongqing, China). N^{ε}-(Carbonylbenzoxy)-$_{L}$-lysine, D-alanine and RGD (Arginine-Glycine-Asparti) peptides were purchased from GL biochem (Shanghai, China), $_{L,L}$-lactide was purchased from GLACO (Jinan, China). N.N-Diisopropylethylamine was purchased from J & K Chemical. N-hydroxylsuccinimide (NHS), dicyclohexy-lcarbodiimide (DCC) and Dimethylamino pyridine (DAMP) were purchased from Acros and used without further purification. All other chemical and reagents were analytical grade obtained commercially. Methanol with chromatographic grade were purchased from MERCK (Germany).

2.2 Preparation of PLA-PLL-RGD

The biodegradable copolymers PLA-PLL-RGD (RGD peptide modification of copolymer: poly (lactic acid-*co*-L-lysine)) were synthesized in four steps according to our previous literature (6). Briefly, I to prepare the monomer of 3-(N^{ε} -benzoxycarbonyl-$_{L}$-lysine)-6-$_{L}$-methyl-2,5-morpholinedione; II to prepare diblock copolymer poly (lactic acid-co-(Z)-L-lysine) (PLA-PLL(Z)) by ring-opening polymerization of monomer and $_{L,L}$-lactide with stannous octoate as initiator; III to prepare diblock copolymer PLA-PLL by deprotected the copolymer PLA-PLL(Z) in HBr/HoAc solution; IV the reaction between RGD and the primary ε -amine groups of PLA-PLL to form PLA-PLL-RGD.

2.3 Preparation of DHAQ-NP Nanoparticles

The DHAQ-loaded PLA-PLL-RGD nanoparticles (DHAQ-NP) were prepared by the "water-in-oil-in-water" solvent evaporation method (7). Briefly, a primary water-in-oil emulsion was prepared in the following way: 50 μL of a 5 mg/mL DHAQ aqueous solution were emulsified with a 1 ml mixture of methylene dichloride and acetone (3:2, v/v) containing 15 mg of polymers (PLA-PLL-RGD) with an ultrasonic processor (300 W, 10 × 4s) (JY92-II ultrasonic processor, Ningbo Scientz Biotechnology Co., Ltd., China). The second emulsion was performed by pouring the first emulsion into the aqueous solution of Pluronic F-68 (0.5%, w/v), using an ultrasonic processor in the same condition, and subsequently stirred at room temperature for 2 h to evaporate the organic phase. The resultant nanoparticles were purified and obtained by centrifugation at 10,000 rpm. Finally, the particles were lyophilized for 24 h.

2.4 Characterization of DHAQ-NP

2.4.1. *Morphology of DHAQ-NP*

The morphology of DHAQ-NP was performed by transmission electron microscope (TEM) (JEM-100SX Japan). The size and zeta potential of DHAQ-NP was measured by dynamic light scattering detector (NicompTM 380 ZLS, Santa Barbara, USA).

2.4.2. *Determination of the DHAQ-NP encapsulation efficiency*

The DHAQ-NP was centrifuged (12,000 × g for 20 min) and the supernatant was taken for measurement of the drug concentration using a Shimadzu HPLC (Kyoto, Japan). The encapsulation efficiency was calculated by the following equation: loading efficiency (wt%) = [(amount of drug in nanoparticles)/(initial amount of drug)] × 100%.

2.5 Antitumor Efficacy of DHAQ-NP in Tumor-Bearing Mice

2.5.1. *Animal*

Thirty healthy ICR female mice and thirty healthy female Balb/c nude mice (4–5 weeks, weight 18–22 g) supplied by the Animal Center of Shanghai Institutes for Biological Sciences (Shanghai, China), were housed 6/cage under standard 12 h light/dark circadian cycle condition at temperature-control (22 ± 1°C).

2.5.2. *Establishment of Hepatoma models*

The H_{22} murine hepatoma cells (1×10^6 cells/0.2 ml), supplied by the Cancer Institute of Shanghai Jiao Tong University, were hypothermically inoculated to the right oxter of each ICR mice.

2.5.3. *Establishment of Breast carcinoma models*

BCAP-37 breast carcinoma cells (1×10^6 cells/0.2 ml), supplied by the Cancer Institute of Shanghai Jiao Tong University, were hypothermically inoculated to the right flank of each Balb/c nude mice.

Animal welfare and experimental procedures were carried out in accordance with the Ethical Committee for animal care and use of the Cancer Institute of Shanghai Jiao Tong University.

2.5.4. *Experimental protocol*

Following the establishment of tumor model (6 days), thirty female hepatoma-bearing mice and thirty Breast carcinoma-bearing mice were randomly divided into five groups, respectively (n = 6 per group): (a) Control, (b) Exercise-only, (c) DHAQ-only, (d) DHAQ-NP, (e) Exercise+DHAQ-NP.

DHAQ-only, DHAQ-NP and Exercise+DHAQ-NP groups were given via weekly i.v. lateral tail vein injections at 2 mg/kg body weight for 13 days, respectively. Exercise-only and Exercise+DHAQ-NP groups

performed progressive treadmill running up to 13 m/min for 20 min, for 13 days. Exercise-only and Exercise+ DHAQ-NP groups began at 8 m/min, for 5 min for the first day, and were systematically increased until the scheduled exercise intensity was achieved. The mice were monitored continuously during the entire duration of exercise and achieved the designated exercise protocol.

After 13 days, all the mice were sacrificed. The tumor tissues were immediately dissected for the analysis of DHAQ concentration.

2.6 Analysis for the DHAQ (Mitoxantrone) Concentration in Tumor

500 μl homogenized tissues were mixed with 200 μl methanol and 200 μl formic acid, 100 μl 20% (w/v) trichloroactic acid and 400 μl chloroform in a polypropylene tube, vortexed for 3 min, centrifuged at 12,000 rpm for 10 min. Then, 15 μl clear supernatants were injected into a Shimadzu HPLC system (Kyoto, Japan) consisting of an LC-10AT pump and an SCL-10AVP UV-Vis spectrophotometer detector. The conditions for HPLC were: Chromatograph column (Kromasil 100-5$C_{18,}$ 250 mm $\times$ 4.6 mm ID, 5 μm), guard column (AUTO Science C. 270A, 4.0 mm $\times$ 2.0 mm), column temperature 35°C, mobile phase methanol-0.16M ammonium formate (48:52, pH2.7), flow rate 1.0 ml/min, wavelength for detection 610 nm.

2.7 Evaluation of Tumor Growth

Tumor volume and body weight of mice were measured every three days. Tumor volume and tumor inhibitory rate were calculated by the following formulas: tumor volume (mm^3) = [tumor length $\times$ (tumor width)^2]/2; Tumor inhibitory rate = (the average tumor weight of Control group—the average tumor weight of treatment groups)/the average tumor weight of Control group.

2.8 Statistical Analysis

Data are shown as mean $\pm$ standard deviation (SD) and SPSS software (version 13.0) was used for statistical analysis. The *p*-values less than 0.05 were considered significant.

3 Results and Discussion

3.1 Preparation of DHAQ-NP

Orthogonal design was applied to optimize the preparation technology on the basis of the single factor evaluation. The optimal conditions for preparation nanoparticle were as follows: 15 mg/ml was the concentration of PLA-PLL-RGD, the methylene dichloride /acetone ratio was 3:2 (v/v), the concentration of Pluronic F-68 was 0.5% and the volume ratio of O/W was 1/10 (v/v).

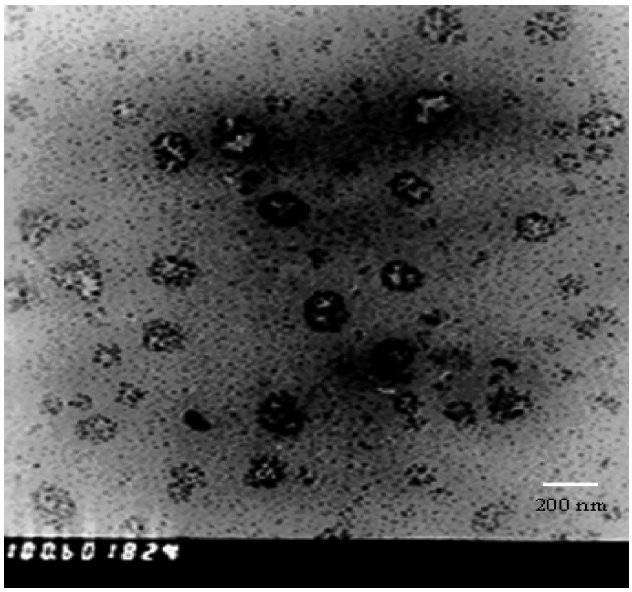

Fig. 1. TEM micrograph of DHAQ-loaded PLA- PLL-RGD nanoparticles.

3.2 Characterization of DHAQ-NP

The particle size is an important property of particles that affects its endocytosis by tumor cells and has a great effect on distribution of nanoparticles *in vivo*. The nanoparticles that are generally controlled under 200 nm can significantly target to tumor. Morphology of DHAQ-NP is shown in Figure 1. DHAQ-NP is spherical, discrete particles without aggregation, with a diameter of less than 200 nm. The encapsulation efficiency of DHAQ-NP is 82.5%.

Another characteristic of DHAQ-NP is zeta potential that plays an important role in determining solution stability, susceptibility to aggregation and cellular surface binding *in vivo*. In the case of charged particles, as the zeta potential increases, the repulsive interactions will be larger, which will lead to the formation of more stable particles with a more uniform sized distribution. A physically stable nano-suspension solely stabilized by electrostatic repulsion will have a minimum zeta potential of 6.30 mV (8). This stability is important in prevention aggregation. Zeta potential of DHAQ-NP is 5.4 mV, the negative zeta potential of DHAQ-NP is lowered, increasing the nanoparticles stablility.

3.3 DHAQ Concentration in Tumor

Figure 2 shows that compared with the DHAQ-only group, the DHAQ concentration of hepatoma and breast carcinoma in Exercise+DHAQ-NP and DHAQ-NP groups increase significantly ($P < 0.05$). This suggests that DHAQ-NP can target hepatoma and breast carcinoma more effectively than DHAQ-only, because RGD peptide that is used to modify DHAQ-NP is a targeting moiety and can

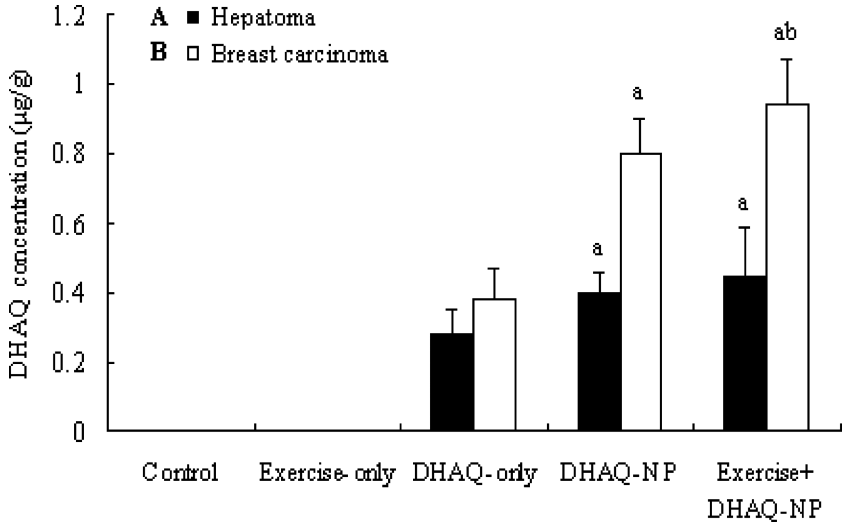

Fig. 2. The DHAQ concentration in hepatoma-bearing mice (A) and breast carcinoma-bearing mice (B). Data are represented as means ± SD (n = 6). $^{a}P < 0.05$, compared with DHAQ-only, $^{b}P < 0.05$, compared with DHAQ-NP.

effectively bind to α vβ 3 integrin which is significantly up-regulated in newly formed tumor vessels (9). Therefore, the DHAQ-NP has the potential to select targeting delivery to tumor. With the degradation of DHAQ-NP in tumor tissue, the DHAQ concentration also increases, respectively.

Although other authors have demonstrated that exercise can increase the drug concentration of tumor tissue, this is because of the blood flow decrease of tumor during exercises, the clearance of drug also decreases, respectively (10). However, the experimental results show that compared with the DHAQ-NP group, the DHAQ concentration of hepatoma in Exercise+DHAQ-NP group cannot increase significantly ($P > 0.05$) (Figure 2 A). But the DHAQ-NP concentration of breast carcinoma in Exercise+DHAQ-NP group can increase significantly as compared to the DHAQ-NP group ($P < 0.05$) (Figure 2 B). This suggests that exercise can lead to a different targeted effect for different tumor. Exercise is a potential pleiotropic intervention that influences a wide spectrum of biological processes which may potentially modulate the targeted therapies. But the potential interaction between exercise and targeted therapies need further research.

Table 1. The antitumor activity of DHAQ-NP in hepatoma-bearing mice

Groups	*Tumor weight (g)*	*Tumor inhibitory rate (%)*
Control	3.27 ± 0.36	—
Exercise-only	2.73 ± 0.23	16.5
DHAQ-only	2.68 ± 0.21^{a}	18.12
DHAQ-NP	2.15 ± 0.18^{a,c,d}	34.35
Exercise+DHAQ-NP	2.12 ± 0.12^{a,c,d}	35.16

3.4 Antitumor Efficacy

Figure 3 (A) and (B) shows the DHAQ-only group can inhibit the growth of tumor volumes as compared to Control group ($P < 0.05$). This finding shows the DHAQ has therapeutic effects against hepatoma and breast carcinoma. But Exercise+DHAQ-NP and DHAQ-NP groups can significantly inhibit the growth of tumor volumes as compared to Control, Exercise-only and DHAQ-only groups ($P < 0.05$). Table 1 and 2 also show the tumor weights in Exercise+DHAQ-NP and DHAQ-NP groups are significantly lower than Control, Exercise and DHAQ-only groups ($P < 0.05$). These experimental findings demonstrate that DHAQ-NP have more significant antitumor efficacy than DHAQ in hepatoma-bearing mice and breast carcinoma-bearing mice. Meanwhile, the findings also suggest that the copolymer PLA-PLL-RGD has the potential to be used as the carrier for targeted drug delivery system. Figure 3(B) and Table 2 show that the tumor volumes and tumor weight of Exercise+DHAQ-NP group are significantly lower as compared to DHAQ-NP groups in

Table 2. The antitumor activity of DHAQ-NP in Breast carcinoma-bearing mice

Groups	*Tumor weight (g)*	*Tumor inhibitory rate (%)*
Control	1.05 ± 0.33	—
Exercise-only	0.89 ± 0.28	15.24
DHAQ-only	0.78 ± 0.29^{a}	25.71
DHAQ-NP	0.66 ± 0.19^{a,c}	37.14
Exercise+DHAQ-NP	0.51 ± 0.21^{a,b,c,d}	51.43

Data are represented as means ± SD (n = 6). $^{a}P < 0.05$, compared with Control, $^{b}P < 0.05$, compared with DHAQ-NP, $^{c}P < 0.05$ compared with DHAQ-only, $^{d}P < 0.05$ compared with Exercise-only.

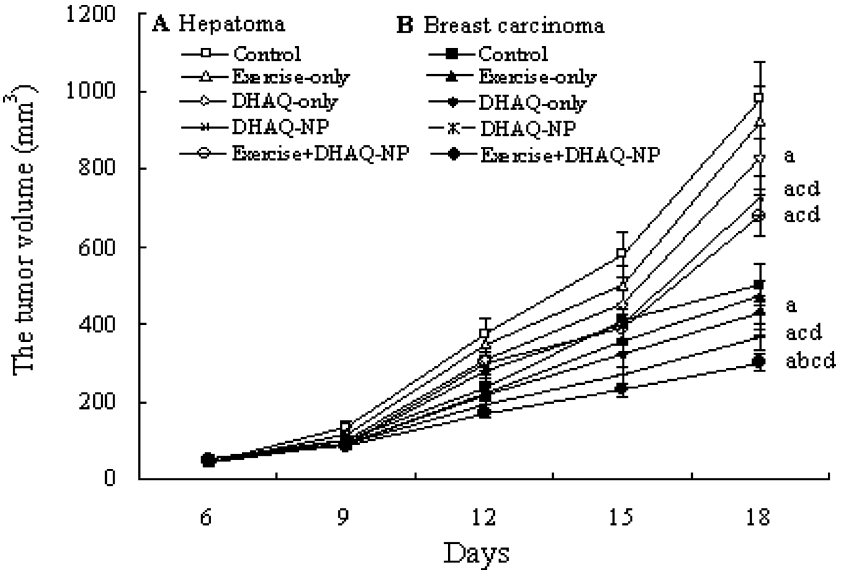

Fig. 3. The tumor growth curve of hepatoma-bearing mice (A) and breast carcinoma-bearing mice (B). Tumor volumes were measured every three days. Data are represented as means ± SD (n = 6). $^{a}P < 0.05$, compared with Control, $^{b}P < 0.05$, compared with DHAQ-NP, $^{c}P < 0.05$ compared with DHAQ-only, $^{d}P < 0.05$ compared with Exercise-only.

breast carcinoma-bearing mice. However, the tumor volumes and tumor weight of Exercise+DHAQ-NP group have no significant change as compared to DHAQ-NP groups in hepatoma-bearing mice (Figure 3(A) and Table 1). This suggests that the same exercise intensity can cause different antitumor efficacies for different tumors undergoing targeted therapies. At present, there is no consistent conclusion for the effect of exercise on antitumor efficacy. Many studies have reported both an inhibitory (11) and augmentary (12, 13) effect of exercise training on tumor growth and progression undergoing conventional chemotherapy.

The present results also show that the same intensity's exercise also can inhibit the growth of breast carcinoma undergoing targeted therapies, but it cannot inhibit the growth of hepatoma. This demonstrates that the same exercise intensity has different antitumor effects for different tumors undergoing targeted therapies and also suggests that the difference of tumors have an important impact for antitumor efficacy during exercises.

Taking into consideration the clinics, this experimental model is inadequate for evaluation on antitumor effect of exercise and DHAQ-NP. This is because the mouse model of hepatoma or breast carcinoma and patients with hepatoma or breast carcinoma have a difference in terms of hormonal and immune profile. Our hepatoma and breast carcinoma cell lines were implanted s.c rather than at the relevant orthotopic.

Moreover, there is also a difference between the exercise intensity of mouse and cancer patient. As far as cancer patients are concerned, not all the cancer patients are suitable to exercise training. Because the age and physical health status of patients have an obvious difference. So, exercise training may cause the different antitumor efficacy for cancer patients with different cancer. Thus, it is not suitable for extrapolating the present results to cancer patients undergoing targeted therapies. But the data of animal research can provide an important reference for clinical therapy in the future. So, such studies are very essential to fully understand that the application of exercise that can be served as a supportive intervention in targeted therapies.

4 Conclusions

We have successfully prepared the DHAQ-NP. Subsequently, we research the antitumor effects of exercise and DHAQ-NP. The present results demonstrate that DHAQ-NP can target tumor and inhibit tumor growth effectively. But the same intensity's exercise can cause different antitumor efficacies for different tumor undergoing targeted therapies.

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