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RESEARCH ARTICLE

A clinical study on regional lymphatic chemotherapy using an activated carbon nanoparticle–epirubicin in patients with breast cancer

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Abstract The purpose of this study was to understand the short-term therapeutic effects of an activated carbon nanoparticle–epirubicin suspension for regional lymphatic chemotherapy in patients with breast cancer. One hundred and twenty patients with stage I, II, or III primary breast cancer were randomly divided into three groups: the lymphatic chemotherapy group using the activated carbon nanoparticle–epirubicin suspension, the epirubicin control group, and the activated carbon control group. Each group of 40 patients was further divided into two subgroups with the drug injected either 24 or 48 h before surgery. The terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate–biotin nick end labeling (TUNEL) assay was used to determine cancer cell apoptotic indices in metastatic lymph nodes. The epirubicin concentration in the black-stained lymph nodes in the lymphatic chemotherapy treatment group was 4,144.64± 2,426.44 ng/g, which is significantly higher than in the epirubicin control group (335.87±212.82 ng/g, $P<0.001$). The plasma epirubicin concentrations at 0.5, 1.5, and 24 h postinjection in the regional lymphatic chemotherapy treatment group were significantly lower than in the epirubicin control group ($P<0.001$). Tolerable mild pain was observed at the injection area after administration of the epirubicin–activated carbon nanoparticle suspension. No regional necrosis or adverse effects were found. The TUNEL assay demonstrated that there was no significant difference in the apoptotic indices in the metastatic lymph nodes from the three groups. Performing lymphatic chemotherapy by regionally injecting the epirubicin–activated carbon nanoparticle suspension could significantly enhance the drug concentration in the stained lymph nodes and lower the plasma drug concentration. The epirubicin–activated carbon nanoparticle suspension has the ability to release the drug slowly in the lymph nodes and, as a result, can prolong the chemotherapeutic effects.

Keywords Breast cancer · Regional lymphatic chemotherapy · Carbon nanoparticle–epirubicin

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Introduction

Breast cancer is one of the malignant tumors that severely threatens women's physical and mental health. Recently, the incidence of breast cancer has increased and become the primary malignant tumor in some developed countries and regions [1, 2]. Lymph node metastasis is an important clinical prognostic factor in patients with breast cancer [3–6]. Currently, surgical excision of the involved lymph nodes is the primary method to treat metastatic lymph nodes. However, there is always a risk that residual cancer cells present in other small lymph nodes may be left behind [7]. Additionally, both the most commonly used current surgery, the level I and II axillary lymph node dissection, and the increasingly used sentinel lymph node biopsy cannot solve the problem of residual cancer cells remaining in the lymph nodes. Currently, intravenous chemotherapy is the most common approach to treat metastatic breast cancer. However, a systemically administered drug traveling through the bloodstream cannot be highly concentrated in the regional lymph nodes [8]. Therefore, the maximum therapeutic effect cannot be achieved in the regional metastatic lymph nodes.

Moreover, the high plasma drug concentration could easily cause severe side effects. Although preventive adjuvant radiotherapy after surgery can reduce the risk of regional tumor recurrence [9–12], severe complications may occur during therapy because important physiological structures, such as the brachial plexus, lung, or heart, are in the target radiation field. Methods to increase the chemotherapeutic dose and, thereby, reduce residual metastatic disease in the regional lymph nodes constitute a key area for further study. In this study, we explored the therapeutic value of an activated carbon nanoparticle–epirubicin suspension for regional lymphatic chemotherapy in patients with breast cancer.

Patients and methods

Patients

One hundred and twenty female patients with invasive breast cancer who were surgically treated in our hospital from March 2010 to October 2010 were selected based on our study criteria. Subgroups of six eligible individuals were randomly assigned into three groups until a total of 40 patients were reached in each group. Based on the duration of drug activity in vivo postinjection, each group was further divided into the 24- and 48-h presurgery subgroups. The activated carbon nanoparticle–epirubicin suspension, the epirubicin solution, or the activated carbon nanoparticle suspension was injected subcutaneously adjacent to the cancer and areola 24 or 48 h before the surgery. Regional and systemic reactions were monitored after treatment. At the end of the injection, the pain was also evaluated with a visual analog scale (VAS) within 6 h. The VAS consisted of a nongraduated 10-cm line ranging from "no pain" to "pain as bad as it could be." It was recorded low (VAS score ≤3), mild (VAS scores >3 and <6), or high (VAT score ≥6). A consent form was signed by each participant. As shown in Table 1, no significant differences were observed in the clinicopathological characteristics of the patients in each group.

Inclusion criteria Patients who were 18–70 years old with invasive breast cancer confirmed by core needle or excisional biopsy presurgery; who have no regional lymph node aspirations or excisional biopsies performed presurgery; no evidence of remote metastasis; no history of chemotherapy presurgery; and whose routine examinations of the heart, lung, and kidney were normal were included in the study

Exclusion criteria Patients who were treated with neoadjuvant chemotherapy or neoadjuvant endocrine therapy, who were allergic to the study drug, had axillary lymph node aspirations or excisional biopsies performed before the surgery, and were not able or willing to receive the regional injection were excluded from the study.

Main chemicals and reagents

Epirubicin hydrochloride (epirubicin, size 10 mg, lot number 09-11-110) was purchased from Pfizer Pharmaceuticals, Ltd. (Wuxi, China). The activated carbon suspension (size 1 mL, 50 mg, lot number 0910010) was purchased from Chongqing Lummy Pharmaceutical Co., Ltd. (Chongqing, China).

Main instruments and operating conditions

The LC-10AVP HPLC and the API3000 MS/MS instruments were purchased from Shimadzu, Japan and AB, USA, respectively. A Luna 5 μM C18 (150×4.6 mm, 5 μm) chromatographic column was purchased from Phenomenex. The column was used at a temperature of 40 °C. The mobile phase was a 60:40 ratio of 10 mmol/L ammonium acetate and acetonitrile (pH= 6.0) containing 0.05 % methanoic acid with a flow rate of 0.4 mL/min. The samples were analyzed using electrospray ionization mass spectrometry with multiple reaction monitoring of positive ions. The ions monitored were 554.3/397.3. The chromatographic conditions were as follows: collision voltage (CE)=18 V, curtain gas (CUR)=11, collision-active dissociation (CAD)=7, ion spray voltage (IS)=5,300 V, dry temperature=500 °C, declustering potential voltage (DP)=60 V, focusing potential voltage (FP)=130 V, and Q_0 entrance potential voltage (EP)=8 V.

Drug preparation and administration

Lymphatic chemotherapy group Three milliliters of activated carbon nanoparticle–epirubicin suspension was obtained by adding 1 mL of an activated carbon nanoparticle suspension to 2 mL of epirubicin (10 mg/mL). After mixing manually, the suspension was placed on an orbital shaker (120 rpm) at 37 °C for 1 h to let the epirubicin saturate the pores of the activated carbon nanoparticles. The 3-mL suspension was administrated subcutaneously adjacent to the tumor and areola 24 or 48 h before the surgery. Regional and systemic reactions were monitored following treatment.

Epirubicin control group In 100 mL of saline, 20 mg of epirubicin was dissolved to obtain the aqueous epirubicin solution. The solution was administrated intravenously for 18–20 min at 24 or 48 h before the surgery. Systemic reactions were monitored after the injection.

Activated carbon control group One milliliter of activated carbon nanoparticle suspension was administrated subcutaneously adjacent to tumor and areola. Regional and systemic reactions were monitored.

Table 1 Clinical and pathological characteristics of the patients in each group

Clinical and pathological characteristics	Lymphatic chemotherapy group	Epirubicin control group	Activated carbon control group	*P* value
Age (median)	33–69 (47)	30–70 (44)	31–70 (48)	0.268
Menopause				
No	27	31	24	0.241
Yes	13	9	16	
Pathological type				
Ductal carcinoma	38	38	40	0.544
Others	2	2	0	
Pathological stage				
Stage I	3	3	1	0.078
Stage II	11	20	12	
Stage III	24	15	27	
Undefinable stage	2	2	0	
Lymphovascular invasion				
No	35	35	39	0.225
Yes	5	5	1	
Primary cancer location				
The outer upper quadrant	17	21	19	0.821
The inner upper quadrant	8	10	6	
The outer downer quadrant	6	5	5	
The inner downer quadrant	4	2	4	
Center[a]	5	2	6	
T stage				
T1	14	16	18	0.929
T2	25	23	21	
T3	1	1	1	
N stage				
N0	22	23	23	0.723
N1	11	13	10	
N2	4	4	4	
N3	3	0	3	
Estrogen receptor (ER)				
–	10	9	14	0.416
+	30	31	26	
Progesterone receptor (PR)				
–	11	9	18	0.076
+	29	31	22	
C-erbB-2				
–	16	22	13	0.333
+ to ++	14	12	17	
+++	10	6	10	

T tumor, *N* node

[a] The tumor was located at the deep layer of the areola

Testing methods

Blood sample collection In the lymphatic chemotherapy and epirubicin control groups, 3–5 mL venous blood samples were collected 0.5, 1.5, and 24 h after the regional or intravenous injection. The plasma epirubicin concentration was determined using an HPLC–mass spectrometry (MS) assay.

Surgery and lymph node sample collection The modified or traditional radical mastectomy was performed for each patient in the three groups at the designated time. One to two

black-stained and/or nonstained lymph nodes were randomly excised. Each lymph node was bisected, and the outside fat and capsule were removed. Using an electronic scale, 0.1 g of lymph node tissue from each sample was obtained and homogenized in 2 mL of saline solution for the HPLC-MS assay. The other half of each lymph node was used for hematoxylin and eosin (HE) staining and analysis of apoptosis.

HE staining After lymph node excision, half of each black-stained and/or nonstained sample was immediately fixed in 10 % paraformaldehyde. HE staining was performed to observe the morphological changes in the lymph nodes in each group.

Determination of the apoptotic index of the cancer cells in the positive metastatic lymph nodes using the terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate–biotin nick end labeling (TUNEL) assay: The previously described fixed samples were confirmed to be metastatic lymph nodes by pathological examination of the tissue. The TUNEL assay was performed to determine the cancer cell apoptotic indices in the lymph nodes. The kit was purchased from Roche, Inc. (Mannheim, Germany). Briefly, paraffin-embedded tissue sections were deparaffinized and rinsed under running water. Proteinase K treatment was performed at 37 °C for 20 min followed by three phosphate-buffered saline (PBS) washes for 3 min each. The TUNEL reaction mixture was added in the dark at 37 °C for 60 min followed by three PBS washes for 3 min each. The ratio of the number 1 and number 2 solutions in the TUNEL reaction mixture was 1:9, where the number 1 solution is the enzyme solution from the kit and the number 2 solution is the label solution from the kit. Next, the number 3 solution (Coverter-AP) from the kit was added and incubated in the dark at 37 °C for 30 min followed by three PBS washes for 3 min each. The alkaline phosphatase substrate, AP-Red chromogenic reagent, was added in the dark for 10 min at room temperature and rinsed with running tap water to terminate the reaction. The samples were washed with ddH_2O before the HE staining was performed to double stain the cell nuclei. The samples were rinsed with running tap water and then ddH_2O. An aqueous-based mounting medium was used to cover the stained tissue section. A negative control was also performed. The cells that had red nuclei were defined as positive apoptotic cells. Avoiding lymphocyte-rich and necrotic areas, 1,000 cells in 10 nonrepeat fields were observed to calculate the number of positive cells. Apoptotic index (AI)=apoptotic cells/total cells×100 %.

Statistical analysis

SPSS17.0 software was used to perform the statistical analysis of the data. A χ^2 test was performed on the count and measurement data, and the results are presented as the mean ± standard deviation. *t* tests and a repeated measures analysis of variance (ANOVA) were used for comparisons between each group. Two-sided *P* values <0.05 were considered statistically significant.

Results

Regional and systemic toxicity

Only mild pain (VAS scores >3 and <6) in the injection area was observed in the activated carbon control group 24 and 48 h after the regional injection. Similarly, only mild pain was observed in the lymphatic chemotherapy group. However, 6 out of 20 subjects in the 48-h subgroup of the lymphatic chemotherapy group were also found to have regional mild swelling, which did not require additional treatment. No skin necrosis or ulcers were found in patients from these two groups. No gastrointestinal side effects, such as nausea and vomiting, were observed in patients from any group. Routine blood and biochemical examinations before and after surgery indicated that no adverse effects, such as myelosuppression or lung and kidney damage, were observed in patients from any group; 37, 38, and 37 patients underwent modified radical mastectomy in the lymphatic chemotherapy group, the epirubicin control group, and the activated carbon control group, respectively, and 3, 2, and 3 patients underwent traditional radical mastectomy in the lymphatic chemotherapy group, the epirubicin control group, and the activated carbon control group, respectively.

Drug concentration

Comparison of epirubicin concentrations in the black-stained and nonstained lymph nodes showed that the drug concentrations in the black-stained and nonstained lymph nodes in the lymphatic chemotherapy group were 4,144.64±2,426.44 and 254.33±235.58 ng/g, respectively. The average drug concentration in the lymph nodes from the intravenous epirubicin control group was 335.87±212.8 2 ng/g. The drug concentration in the stained lymph nodes in the lymphatic chemotherapy group was significantly higher than in the intravenous epirubicin control group ($P<0.001$). There were no significant differences between the drug concentrations in nonstained lymph nodes in the lymphatic chemotherapy group and the epirubicin control group ($P=0.108$).

The drug concentrations in the black-stained lymph nodes in the 24- and 48-h subgroups of the lymphatic chemotherapy group were 3,965.4362±1,867.59462 and 4,323.834±2,920.54748 ng/g, respectively. The differences were not statistically significant ($P=0.646$). The drug concentration in the 48-h subgroup (419.89±235.17 ng/g) was significantly higher than in the 24-h subgroup (251.846± 151.01 ng/g, $P=0.011$) in the epirubicin control group.

Table 2 Comparison of the plasma epirubicin concentrations

Group		30 min	90 min	24 min	Drug distribution time	Group
Lymphatic chemotherapy group	Drug given 24 h before surgery	11.83±2.93	4.46±1.94	0.67±0.35	F=0.035, P=0.851	F=167.481, P<0.001
	Drug given 48 h before surgery	9.43±2.13	3.17±1.30	0.50±0.29		
	Total	10.63±2.81	3.81±1.76	0.59±0.33		
Epirubicin control group	Drug given 24 h before surgery	49.16±21.13	6.25±1.26	2.67±1.12		
	Drug given 48 h before surgery	54.50±21.32	6.17±1.25	2.61±0.88		
	Total	51.83±21.13	6.21±1.24	2.64±0.99		

Plasma epirubicin concentrations

The drug concentrations in the peripheral venous blood collected 0.5, 1.5, and 24 h after regional administration in the lymphatic chemotherapy group were 10.6, 3.8, and 0.6 ng/mL, respectively. The drug concentrations in the peripheral venous blood collected 0.5, 1.5, and 24 h after intravenous administration in the epirubicin control group were 51.8, 6.2, and 2.6 ng/mL, respectively. The drug concentrations 0.5, 1.5, and 24 h after regional administration in the lymphatic chemotherapy group were significantly lower than in the epirubicin control group (F=167.481, P<0.001); however, there was no significant difference between the 24- and 48-h subgroups (F=0.035, P=0.851; Table 2).

Morphological changes in the lymph nodes observed by HE staining

HE staining of the lymph nodes showed no nuclear degenerative changes, such as pyknotic and nuclear fragmentation;

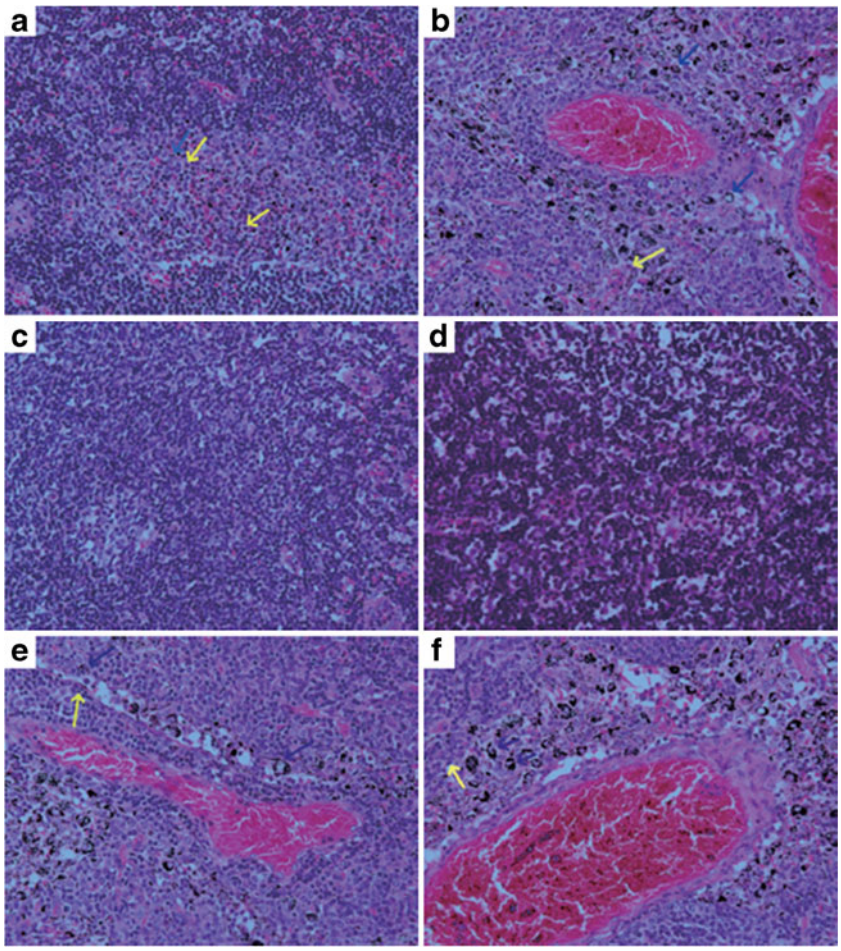

Fig. 1 Lymph node morphology in the 24-h subgroup (**a**) and the 48-h subgroup (**b**) of the lymphatic chemotherapy group, in the 24-h subgroup (**c**) and the 48-h subgroup (**d**) of the epirubicin control group, and in the 24-h subgroup (**e**) and the 48-h subgroup (**f**) of the activated carbon control group. The *yellow arrow* indicates neutrophils; the *blue arrow* indicates macrophages

no cytoplasmic blebbing; and no typical cell morphological changes, such as intercellular fibrosis, after chemotherapy. Acute inflammatory reactions, such as neutrophil aggregation, macrophage phagocytosis, and capillary hyperemia, were observed in the lymph nodes of the lymphatic chemotherapy and activated carbon control groups independent of metastasis (Fig. 1).

Apoptotic rates of cancer cells in the positive metastatic lymph nodes

Metastatic lymph nodes from patients among the three groups were selected for the TUNEL assay to determine the cancer cell apoptotic indices. Apoptotic cancer cells were observed in the metastatic lymph nodes of all three groups (Fig. 2). The apoptotic rates in the lymphatic chemotherapy, epirubicin control, and activated carbon control groups were 2.7722±0.8295, 2.7765±0.9497, and 2.6294±0.8068 (in percent), respectively. There were no significant differences in the apoptotic indices among the three groups as determined using one-way ANOVA analysis (P=0.852).

Discussion

Activated carbon has an extraordinarily rich pore structure, large surface area, and high adsorption capacity, which enable it to easily bind anticancer drugs. Moreover, it has a strong

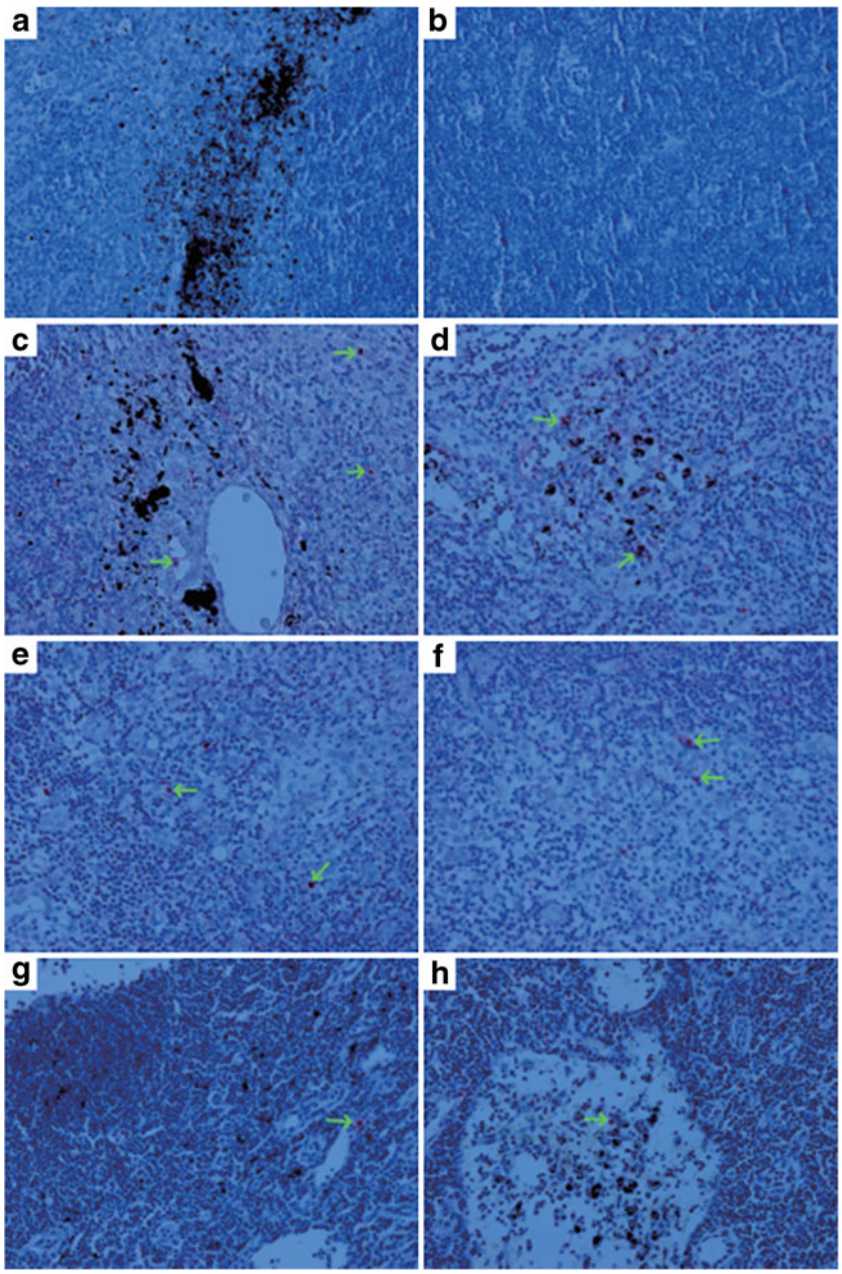

Fig. 2 Negative control (**a**, **b**). The 24-h subgroup (**c**) and 48-h subgroup (**d**) of the lymphatic chemotherapy group, the 24-h subgroup (**e**) and 48-h subgroup (**f**) of the epirubicin control group, and the 24-h subgroup (**g**) and 48-h subgroup (**h**) of the activated carbon control group. The *green arrow* indicates apoptotic cells

affinity for the lymphatic system. Additionally, due to the easy preparation process and the affordable price, it is often used as a carbonaceous adsorbent in industry, agriculture, national defense, environmental hygiene, and biomedical fields. After reprocessing, the activated carbon nanoparticles, which become smaller and have more specific surface area, maintain their high adsorption capacity and slow-release capabilities, in addition to becoming better lymph node tracers [13–16]. These characteristics make activated carbon a potential drug delivery vehicle for lymphatic chemotherapies. Regional lymphatic chemotherapy using activated carbon as a vehicle to deliver anticancer drugs can increase the drug concentration in the lymph nodes, lower the plasma drug concentration, and, as a result, lower systemic toxicities [8, 17]. Being an affordable method, this treatment does not require any special instruments, and the procedure is easy to perform. Additionally, the activated carbon can blacken the lymph nodes, which makes it easier to remove the lymph nodes during surgery. Therefore, it is an ideal method to control the recurrence of regional residual metastatic lymph nodes.

Chen et al. demonstrated that the drug concentration in axillary lymph nodes was improved markedly by regional injection of an activated carbon–carboplatin suspension in patients with breast cancer [8]. However, there are only a few studies on breast cancer lymphatic chemotherapy. Epirubicin, a non-cell-cycle-specific drug, is one of the most effective and commonly used drugs in breast cancer chemotherapy [18]. It is possible to enhance the curative effect if epirubicin can be applied using lymphatic chemotherapy for breast cancer. Therefore, we conducted preliminary studies to confirm the feasibility and safety of using activated carbon nanoparticles containing epirubicin in regional lymphatic chemotherapy by testing the maximum binding capacity of the activated carbon nanoparticles with epirubicin, determining the structural stability of epirubicin after binding to the activated carbon nanoparticles, and performing animal studies using regional injections with an activated carbon nanoparticle–epirubicin suspension, epirubicin alone, or activated carbon nanoparticles alone.

Our study demonstrated that activated carbon nanoparticles can bind epirubicin and deliver the drug to lymph nodes. The drug concentration in the lymph nodes was significantly higher than in the blood. Additionally, the drug concentration in the lymph nodes from the lymphatic chemotherapy group was higher than it was in the intravenous epirubicin control group, demonstrating a satisfactory ability to deliver the drug to lymph nodes. Moreover, a higher drug concentration was detected in the stained lymph nodes from the 48-h subgroup relative to the 24-h subgroup. This finding confirmed the slow drug release properties of lymphatic chemotherapy, which can be expected to enhance the effect on regional therapy.

However, the lymph node samples taken from each of the three groups did not show the morphological changes that are often observed after neoadjuvant chemotherapy. Additionally, there were no statistical differences in the apoptotic rates of the cancer cells in the metastatic lymph nodes in the three groups. These results may imply that the cancer cells had not been damaged or killed by the drug at the time we collected the lymph nodes, which can be explained by the following potential reasons: (1) the duration of drug activity in vivo was not long enough, as it takes a certain amount of time for the drugs to reach the cancer cells via the lymphatic pathway to initiate its pharmacological effects and (2) due to the slow-release properties, the activated carbon nanoparticles may not have had enough time to release the drug to observe a pharmacological effect. Therefore, we may need to increase the treatment duration or frequency to achieve the best anticancer therapeutic effect in the lymph nodes.

Currently, there are only a few studies on regional lymphatic chemotherapy using activated carbon nanoparticles. Consequently, additional information is greatly needed. For example, the chemical structure of lymphatic chemotherapeutic compounds, the administration method, and the duration of treatment need further study. Additional comparative studies are needed to evaluate the efficacy of anticancer drugs in the lymphatic system, the regional recurrence rate of lymphatic chemotherapy in breast cancer patients after surgery, the effect on the long-term survival rate, and the regional safety issues following regional chemotherapy, which all require further investigation. Moreover, because activated carbon nanoparticles are not a biodegradable material that can be degraded inside the body, additional research is needed to determine whether long-term side effects will occur.

Conflicts of interest None.

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