Preparation of PLGA microspheres loaded with 10-hydroxycamptothecin and arsenic trioxide and their treatment for rabbit hepatocellular carcinoma

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Objective. This study aims to study the preparation method of arsenic trioxide (As_2O_3) polylactic-co-glyconlic acid (PLGA) microspheres and 10-hydroxycamptothecin (HCPT) PLGA microspheres and explore their therapeutic effects as embolic agents for VX2 hepatocellular carcinoma in rabbits.
Methods. As_2O_3 and HCPT PLGA microspheres were prepared by multiple emulsion solvent evaporation method. Scanning electron microscopy (SEM) and particle size distribution were used to analyze the morphology, the drug sustained release ability was observed by the release of microspheres in vitro. The rabbit model of VX2 hepatocellular carcinoma was established and the hepatocellular carcinoma was treated with combined microspheres. The therapeutic effects were detected by qPCR, western blotting, HE staining and immunohistochemical methods.
Results. The PLGA microspheres loaded with As_2O_3 and HCPT were successfully prepared by optimizing the ratio. The particle size was between 30 and 50 μm. In vitro release results showed that PLGA microspheres loaded with As_2O_3 released completely in 10 days and PLGA microspheres loaded with HCPT released completely in 12 days. Western blotting and qPCR results showed that the expression of ALDH1A1 and Nanog decreased significantly in treatment group. HE staining and immunohistochemical analysis showed that the expression of CD31, HIF and VEGF decreased significantly and the apoptosis of tissues was obvious.
Conclusion. The combination of As_2O_3 and HCPT PLGA microspheres as embolization for VX2 hepatocellular carcinoma in rabbits has significant therapeutic effect.

Key words: polylactic-co-glyconlic acid (PLGA), arsenic trioxide (As_2O_3), 10-hydroxycamptothecin (HCPT), western blotting; Immunohistochemistry

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INTRODUCTION

Primary liver cancer is one of the most common malignant tumors. Surgical resection is usually used in the early stage, but most patients with liver cancer are in the middle and advanced stage when they are diagnosed. At this time, non-surgical therapy is generally used[1,2]. Drug chemotherapy is a routine treatment for hepatocellular carcinoma. Many anticancer drugs have significant inhibitory effects on hepatocellular carcinoma cells, but they are easy to spread and have strong side effects on normal tissues, which limiting the development and application of drug therapy for hepatocellular carcinoma[3,4].

Arsenic trioxide (As_2O_3) was first used as an adjuvant drug in the treatment of malaria, tuberculosis and syphilis, it was later used in the treatment of acute promyelocytic leukemia and achieved certain therapeutic effects. Then it was found that As_2O_3 could also inhibit hepatocellular carcinoma and lung cancer, and As_2O_3 injection was developed for clinical treatment[5]. However, there are also many problems. Firstly, As_2O_3 has strong toxicity and side effects, it can kill tumor cells and damage normal cells at the same time. Secondly, As_2O_3 is less water-soluble and easy to precipitate when administered[6]. Hydroxycamptothecin (HCPT) is a kind of alkaloids extracted from plants with anti-cancer effect. Its disadvantage is that it is insoluble in water and slightly soluble in most organic solvents with short half-life. At present, the forms used in clinic are sodium salt injection, powder injection and capsule, but it is easy to oxidize and hydrolyze when exposed to light and heat, its curative effect is low[7].

In order to overcome these shortcomings, we developed a new treatment technology of hepatic artery embolization of hepatocellular carcinoma using polylactic-co-glyconlic acid (PLGA) microspheres as drug carriers to embolize As_2O_3 and HCPT. PLGA has good biocompatibility and biodegradability. It can be metabolized by human body, and eventually degraded to H_2O and CO_2. PLGA is widely used in drug carrier research. PLGA has the advantages of protecting the embedding drug, targeting the lesion, controlling drug release, prolonging drug action time, reducing drug toxicity and irritation[8-10]. Therefore, this study explored the preparation method of PLGA microspheres, and successfully embedded As_2O_3 and HCPT into PLGA microspheres. At the same time, the related characterization in vitro was car-

ried out. Animal model of liver cancer was established. The therapeutic effect was tested by embolization of PLGA microspheres combined with As_2O_3 and HCPT, and the possible mechanisms were analyzed.

MATERIALS AND METHODS

Experimental cells and animals

VX2 Rabbit anaplastic epidermal squamous cell carcinoma cell line was purchased from Beijing Beina Biological Technology Co., Ltd (BNCC100757). The cells were cultured in DMEM-H medium containing 10% FBS with 5% CO_2 at 37 °C.

New Zealand rabbits were purchased Nanchang Longping Rabbit Industry Co., Ltd. They were maintained in a temperature controlled room (18-22 °C) with 12-hr light /dark cycles, eat and drink freely. All animal experiments were conducted according to Principles of Laboratory Animal Care (National Society for Medical Research). This study was audited and approved by Animal Ethics Committee of Shenzhen Second People's Hospital.

Reagents and instruments

PLGA (aladdin P133293); As_2O_3 (Beijing double-aigrettes Pharmaceutical Co., Ltd, 20170701); 10-hydroxycamptothecin (Solarbio SH8220); PVA(aladdin P105126); Hematoxylin Stain (BOSTER AR11800-1); Eosin Y Staining Solution (BOSTER AR11800-2); Scott blue solution (Solarbio G1865); DMEM completely high sugar Medium (NanJing KeyGen Biotech Co.,Ltd. KGM12800S-500); Trypsin-EDTA digestive fluid (Solarbio T1300); Trizon Reagent(CW0580S, Beijing ComWin Biotech Co.,Ltd.);Ultrapure RNA extraction kit (CW0581M, Beijing ComWin Biotech Co.,Ltd.); HiFiScript cDNA synthesis Kit (CW2569M, Beijing ComWin Biotech Co.,Ltd.); UltraSYBR Mix(CW0957M, Beijing ComWin Biotech Co.,Ltd.); BCA Protein Assay kit (CW0014S, Beijing ComWin Biotech Co.,Ltd.);Mouse Monoclonal Anti-β actin (TA-09, Beijing ZSGB-BIO Co.,Ltd., 1:2000); Horseradish Enzyme Labeled Goat Anti-Rat IgG(H+L) (ZB-2305, Beijing ZSGB-BIO Co.,Ltd., 1:2000); Rabbit Polyclonal Anti-Nanog(bs-6552R, Bioss,1:1000); Rabbit Polyclonal Anti-ALDH1A1(bs-6509R, Bioss, 1:1000); Horseradish Enzyme Labeled Goat Anti-Rabbit IgG(H+L) (ZB-2301, Beijing ZSGB-BIO Co.,Ltd., 1:2000); Freeze dryer (Shanghai Bilon Instrument Co.,Ltd., FD-1A-50); Rotary evaporator (Shandong Biobase Co.,Ltd., RE-501); homogenizer (Ningbo Scientz Biotechnology Co.,L td., S10); Ultraviolet-Visible Spectrophotometer (Shanghai mapada Instrument Co.,Ltd., UV-1600PC); Inductively Coupled Plasma Mass Spectrometer (ICP-MS) (PE corporation, USA, optima 8000); Laser particle size analyzer (Malvern Zetasizer nano zs90); Fluorescent cell imaging instrument (Bio-Rad ZOETM); fluorescent quantitative PCR instrument (CFX Connect™, Bio-Rad Shanhhai Laboratories); Protein vertical electrophoresis instrument (DYY-6C, Beijing 61 instrument factory); Ultra High Sensitivity Chemiluminescence Imaging System (Chemi DocTM XRS+, Bio-Rad Shanhhai Laboratories).

Preparation of PLGA microspheres loaded with HCPT/As_2O_3

200 mg PLGA was dissolved in 10 mL dichloromethane solution by multiple emulsion solvent evaporation method, 10 mg As_2O_3 or 10 mg HCPT was added to the above solution, and the colostrum solution was formed by stirring 10 s at 5000 rpm with a hand-held homogenizer. Quickly pour colostrum solution into 10 mL 3% PVA, stir 10 000 rpm under ice bath for 10 s to form multiple emulsion solution.

The multiple emulsion solution was poured into 500 mL 0.5% PVA, stirred by magnetic force for 2 h at room temperature and distilled by vacuum distillation for 6 h to remove residual organic solvents. They were centrifuged at 5000 rpm for 15 min and washed with deionized water for three times, PLGA microspheres powder loaded HCPT or As_2O_3 can be obtained by freeze-drying precipitation. They were stored at 4 °C. The blank PLGA microsphere powder can be prepared using the same method without adding drugs.

Particle size determination of PLGA microspheres loaded with HCPT/ As_2O_3

PLGA microspheres (or PLGA microspheres loaded with HCPT/ As_2O_3) were weighed and dissolved in water containing 0.5% Tween80 to make the concentration of PLGA microspheres 1 mg/mL. The particle size was determined by laser particle size analyzer immediately after ultrasonic dispersion for 10 min.

Release of PLGA microspheres loaded with HCPT/ As_2O_3 in vitro

PLGA microspheres loaded with As2O3 were dissolved in 3 mL PBS and mixed, PLGA microspheres loaded with HCPT were dissolved in PBS containing 0.5% Tween80 and mixed, they shield from light with silver paper. They were cultured at 37 °C with shaking at 200 rpm. The supernatants were collected every 24 h continuously for 12 consecutive days and stored at 4 °C after they were centrifuged for 5 min at 3000 r/min. After continuous sampling, HCPT concentration was determined by ultraviolet spectrophotometer and standard curve was drawn. According to the standard curve, the release amount was determined and the release curve was drawn. As_2O_3 was determined by inductively coupled plasma mass spectrometry (ICP-MS), and the release curve was drawn.

Establishment of rabbit liver cancer model

VX2 Rabbit anaplastic epidermal squamous cell carcinoma cells in good condition were inoculated in the right hind leg of New Zealand rabbits and the tumors were formed after 1-2 weeks. The tumors were removed and cut into 2-3 mm^3 size granules. The rabbits were anaesthetized and opened about 2 cm below the xiphoid process, the liver exposed to visual field. Tumor granules were clamped with microforceps and embedded in the liver about 1 cm deep. The abdominal cavity should be closed

Table 1. Primers used in this study.

Primer name	Primer sequence (5'-3')	Primer length (bp)	Product length (bp)	Annealing temperature (°C)
Nanog F	CCAGCCTCCTGAACCTTAGC	20	122	60.2
Nanog R	GCACCCCTGAGTCACACTG	19		
Aldh1a1 F	CGTTTGCTCCCTGCTATGTG	20	225	59.1
Aldh1a1 R	CGTGCTCTGCAGTTATCGTC	20		
β-actin F	TCTCGACGAAACCTAACGGC	20	210	59.6
β-actin R	CAATCAAAGTCCTCGGCCAC	20		

and sutured until the liver is free of tumors and bleeding. The rabbits were randomly divided into Control group (Pseudo-embolization with normal saline), Empty group (Embolization with empty microspheres), Iodipin group (Embolization with lipiodol), As_2O_3 group (Embolization with PLGA microspheres loaded with As_2O_3), HCPT group (Embolization with PLGA microspheres loaded with HCPT), As_2O_3+HCPT group (Embolization with PLGA microspheres loaded with As_2O_3+HCPT).

qRT-PCR

Tissue samples were ground to powder in a mortar under liquid nitrogen. Total RNA was extracted using using a Trizol reagent kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA concentration and purity were detected using a Qubit Fluorometer (Thermo Fisher Scientific, Inc.). A total of 1 μg RNA was subjected to reverse transcription using a Prime Script Kit (Takara Bio Inc., Otsu, Japan). qPCR was performed using a SYBR Premix Ex Taq™ Kit (Takara Bio Inc.). The quantification method used was the 2-ΔΔCT method. The thermocycling conditions were as follows: Pre-degeneration at 95 °C for 10 min, followed by 40 cycles of 95 °C for 12 s and 62 °C for 40 sec, U6 and β-actin genes were used as an internal control. The primers used in this study are shown in Table 1.

Western blotting test

Total proteins were extracted and protein concentration was determined using BCA. Proteins (50 μg per lane) were separated using 12% SDS-PAGE, then they were electrotransferred to a PVDF membrane (Amersham Biosciences, Piscataway, NJ, USA). The PVDF membrane was rinsed with TBS for 10-15 min, placed in TBS/T blocking buffer containing 5% (w/v) skimmed milk powder. It was incubated at room temperature for 2 h following the addition of an appropriate dilution of primary antibodies. The membrane was then rinsed with TBST three times (5-10 min/wash) and then incubated at room temperature for 1 h with horseradish peroxidase-labeled secondary antibody (1:50,000; Abcam, Cambridge, UK; diluted with TBST containing 0.05% (w/v) skimmed milk powder). The membrane was then rinsed three times with TBST (5-10 min/wash). Protein bands were detected using an enhanced chemiluminescence kit (Perkin-Elmer Inc.) and quantified as the ratio to β-actin. Quantification was performed using Imagequant LAS4000 (GE Healthcare, Japan).

HE staining test

The tissues were taken and washed with PBS, then they were fixed with 4% paraformaldehyde solution and embedded in paraffin. They were cut into 5 μm slices and stained with HE using conventional method. Briefly, Deparaffinize sections, 2 changes of xylene, 10 min each; Re-hydrate in 2 changes of absolute alcohol, 5 min each; 95% alcohol for 2 min and 70% alcohol for 2 min; Wash briefly in distilled water; Stain in Harris hematoxylin solution for 8 min; Wash in running tap water for 5 min; Differentiate in 1% acid alcohol for 30 s; Wash running tap water for 1 min; Bluing in 0.2% ammonia water or saturated lithium carbonate solution for 30 s to 1 min; Wash in running tap water for 5 min; Rinse in 95% alcohol, 10 dips; Counterstain in eosin-phloxine solution for 30 s to 1 min; Dehydrate through 95% alcohol, 2 changes of absolute alcohol, 5 min each; Clear in 2 changes of xylene, 5 min each; Mount with xylene based mounting medium. They were observed under optical microscope.

Immunohistochemical test

Briefly, the tissues were embedded with paraffin using conventional method. They were cut into 5μm slices, and incubated with 0.3% endogenous peroxidase blocking solution for 20 min after dewaxing and hydrating. Then they were incubated at room temperature for 10 min with 3% hydrogen peroxide methanol solution, and washed with PBS for 3 times (3 min/time). Antigen retrieval was performed using citrate buffer (pH 6.0) at 121 °C for 2 min. After blocking with 5% BSA (Gibco; Thermo Fisher Scientific, Inc), they were incubated with a primary monoclonal antibody overnight at 4 °C. They were then incubated with goat anti-rabbit non-biotinylated regents (Zhongshanjinqiao, Beijing, China) according to the manual and mounted with epoxy resin. They were observed using Photo and Image Auto Analysis System (Image-Pro-Plus, China). Five visual fields were randomly selected for each slice.

Statistical analysis

The data were analyzed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). All results are presented as the mean ± standard deviation (SD) and t-test were used for comparison between groups. $P<0.05$ was considered to indicate a statistically significant difference.

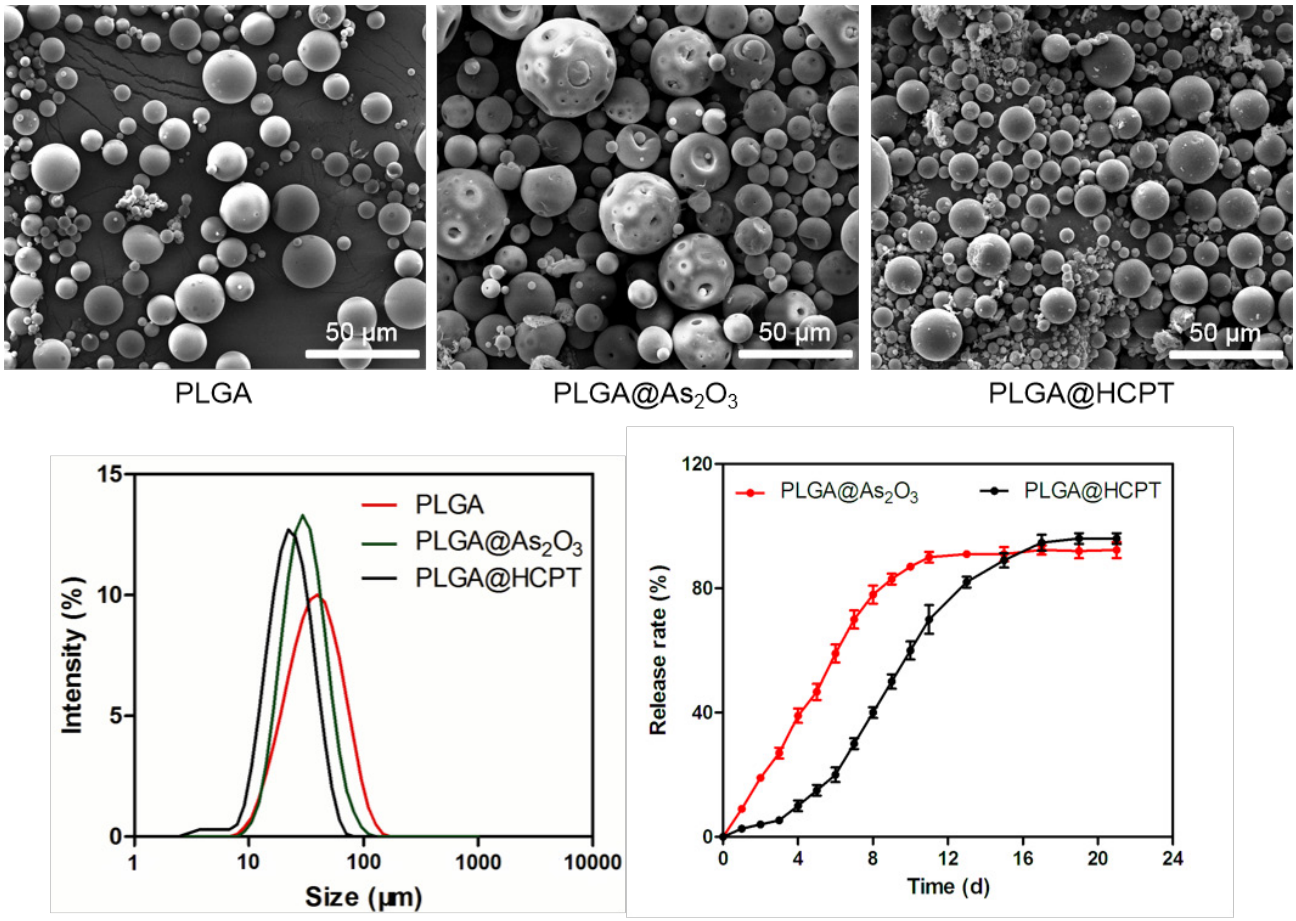

Fig. 1. Characterization and in vitro release of PLGA microspheres loaded with HCPT/ As_2O_3.

RESULTS

Characterization and in vitro release of PLGA microspheres loaded with HCPT/ As_2O_3

As shown in Fig. 1, the shape of HCPT/As_2O_3 PLGA microspheres and empty PLGA microspheres were uniform spherical, regular in size and shape. Only PLGA microspheres with As_2O_3 have some dents on the surface. The particle size distribution showed that the size of PLGA microspheres loaded with HCPT/As_2O_3 ranged from 30 to 50 μm and had no impurity peaks. PLGA microspheres loaded with As_2O_3 released rapidly and tended to release completely in 10 days, the PLGA microspheres loaded with HCPT tended to release completely in 12 days. Both of their respective release rates were above 90%.

ALDH1A1 and Nanog expression

The expression levels of ALDH1A1 and Nanog in HCPT+ As_2O_3 PLGA microspheres group were significantly lower than that in control group and single administration group, and the difference was significant (Fig. 2, $P<0.05$).

HE staining results of the effects of combined embolization of PLGA microspheres loaded with HCPT and As_2O_3 on liver tissues

As shown in Fig. 3, the HCC cells grew densely and ovally without necrosis or decay in Control group and Empty group. Large area of cell necrosis and apoptosis were observed in Iodipin group, As_2O_3 group and HCPT group. The apoptosis of HCC was more obvious, with nucleus cleavage and large area necrosis in As_2O_3+HCPT group. These results suggested that combined embolization had significant inhibitory effect on HCC cells.

Immunohistochemical results of the effects of combined embolization of PLGA microspheres loaded with HCPT and As_2O_3 on CD31, HIF and VEGF expression

The immunohistochemical results showed that CD31, HIF and VEGF expression levels significantly decreased in Iodipin group, As_2O_3 group, HCPT group and As_2O_3+HCPT group compared with that of control group (Fig. 4). The decline was more obvious at the 14th day. The results suggested that the HCC was controlled and the disease was alleviated after microsphere embolization.

DISCUSSION

As a new embolic agent, drug-loaded sustained-release microspheres have good drug treatment and block-

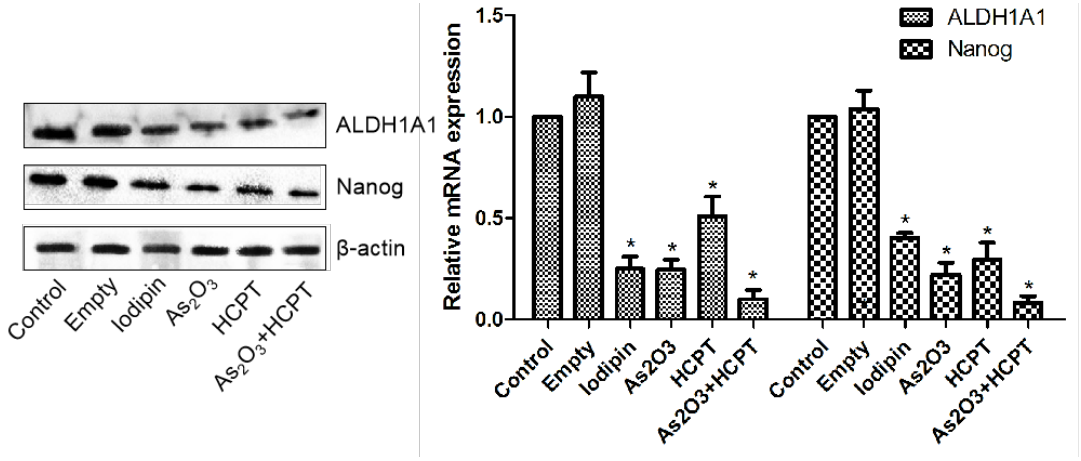

Fig. 2. ALDH1A1 and Nanog expression levels in different groups (*P<0.05 vs. Control).

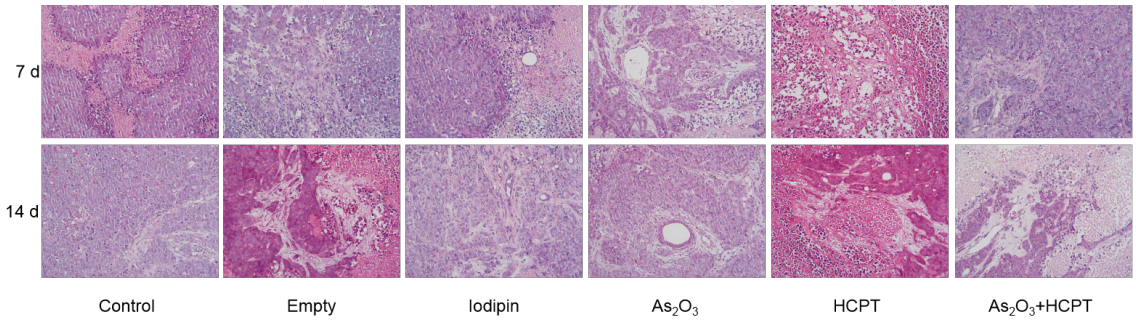

Fig. 3. HE staining detection of the effects of combined embolization of PLGA microspheres loaded with HCPT and As_2O_3 on liver tissues (200×).

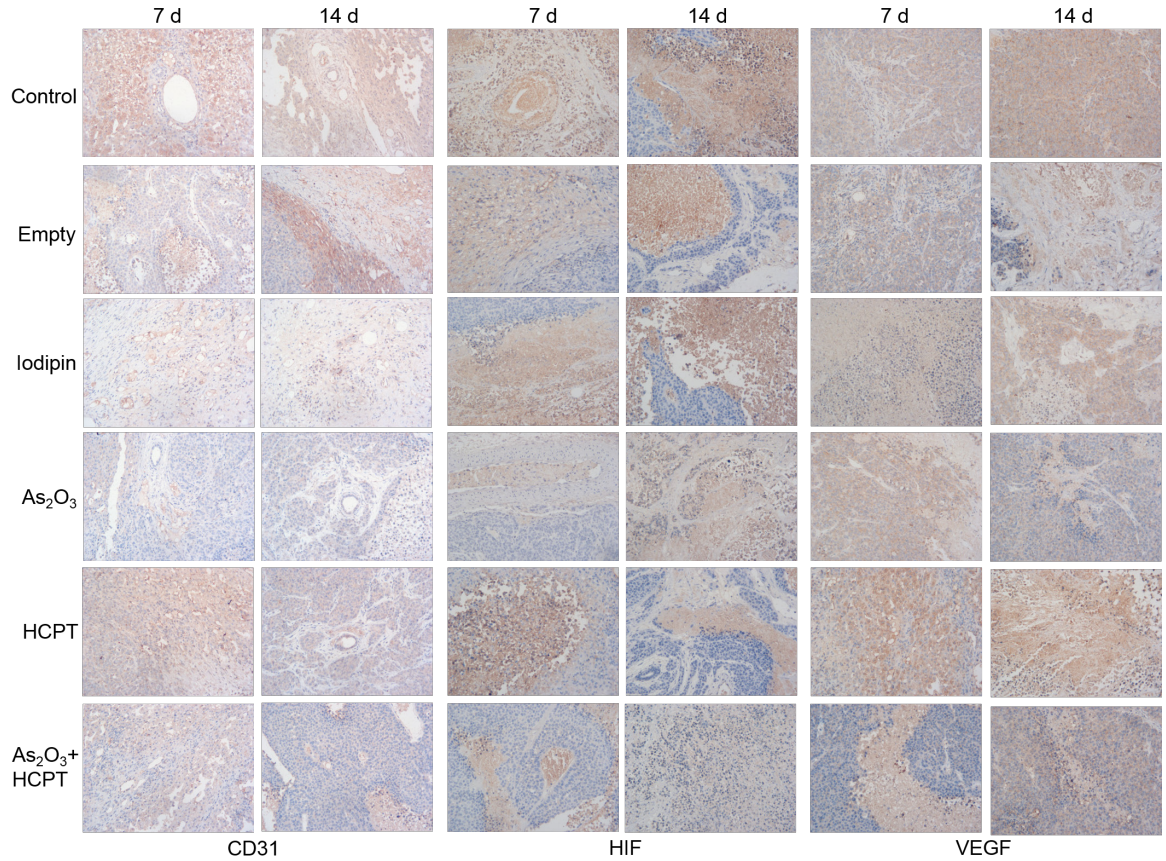

Fig. 4. Immunohistochemical detection of the effects of combined embolization of PLGA microspheres loaded with HCPT and As_2O_3 on CD31, HIF and VEGF expression (200×).

ing function of hepatic artery vessels, it has been widely used worldwide[11,12]. In this study, the characterization of HCPT/As_2O_3 PLGA microspheres was detected by scanning electron microscopy. The results showed that the shape of HCPT/As_2O_3 PLGA microspheres was not significantly different from that of PLGA microspheres without carrier. The particle size distribution showed that the microspheres were uniform and stable with good effect. There was no sudden or slow release and the process was smooth, which showed that PLGA microspheres had good drug carrier function.

Aldehyde dehydrogenases (ALDH1A1) A1 subtype is closely related to many kinds of cancers and overexpressed in cancer tissues[13]. Nanog expression in differentiated cells gradually decreased or lost, but it was abnormally activated and overexpressed in cancer cells. Nanog participated in the growth, metastasis and drug resistance of tumors by regulating the proliferation, migration and invasion of tumors, the dry nature of tumors and the immune escape of tumors[14,15]. In this study, western blotting and qPCR results showed that the expression of ALDH1A1 and Nanog decreased significantly in the control group and the single drug group. The combination drugs may inhibit the growth of cancer cells by inhibiting the expression of ALDH1A1 and Nanog through sustained release of drugs. HE staining results also showed the effect of combined embolization drugs. CD31, HIF and VEGF are angiogenesis factors[16-18]. Neovascularization is one of the conditions for tumorigenesis. Hepatocellular carcinoma (HCC) is a multivessel tumor. Compared with traditional lipiodol embolization, drug-loaded microspheres embolization for HCC has the advantage of not only blocking the existing hepatic artery[19], but also inhibiting the formation of new blood vessels by releasing drugs As_2O_3 and HCPT, which reduces the expression of CD31, HIF and VEGF.

It has been found that As_2O_3 can affect cell cycle, inhibit the proliferation and migration of cancer cells, and induce apoptosis of cancer cells. As_2O_3 can promote the apoptosis of tumor vascular endothelial cells, interfere with the interaction between endothelial cells and tumor cells, thus inducing endothelial cell apoptosis, inhibiting tumor angiogenesis, and indirectly inhibiting tumor growth[20,21]. HCPT mainly forms ternary complexes with DNA and topoisomerase I, inhibits angiogenesis, blockades DNA replication and leads to cell death, thus showing anti-tumor activity. HCPT acts on S phase of cell cycle and is a cell cycle specific drug. It can inhibit mitosis at high concentration and prevent cells from entering the mitotic phase[22]. Researchers who have different opinions on the mechanism of the two drugs for HCC have not formed a unified idea. In this study, we prefer that As_2O_3 and HCPT mainly act on blood vessel-related cells, inhibit the formation of new blood vessels and thus inhibit the growth of tumors.

The new PLGA drug-loaded microsphere embolization agent has incomparable advantages compared with traditional embolization agent, and has a broad application prospect in the treatment of HCC. This study provides a new choice for the application of drug-loaded microsphere embolization agent.

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Author contributions: YW: conceived and designed the experiments; DD, JC: execution of experiments; YW, DD: data analysis; YW, DD, CL: discussion of results; YW, DD: wrote and or critical reading of manuscript.

Conflict of interest statement: The authors declare that they have no competing interests to disclose.

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