Preparation of microspheres encapsulating sorafenib and catalase and their application in rabbit VX2 liver tumor

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Research

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Abstract

Background: Transcatheter arterial chemoembolization (TACE) is extensively used in the treatment of advanced hepatocellular carcinoma (HCC). However, the efficacy of TACE is usually limited to secondary tumor hypoxia and hypoxia-related tumor angiogenesis.

Methods: In this study, poly(lactic-co-glycolic acid) (PLGA) microspheres (SOR-CAT-PLGA MSs) encapsulating sorafenib (SOR) and catalase (CAT) were prepared by double-emulsion solvent diffusion method. Sorafenib inhibits tumor angiogenesis, and catalase decomposes hydrogen peroxide (H_2O_2) to generate oxygen in the tumor.

Results: In vitro and in vivo, SOR-CAT-PLGA MSs could significantly improve the efficacy of hepatic artery embolization in the treatment of rabbit VX2 liver tumors, regulate tumor hypoxia and immunosuppressive microenvironment, then achieved near-complete and rapid necrosis of liver tumors.

Conclusions: The application of new SOR-CAT-PLGA MSs in hepatic artery chemoembolization of rabbit VX2 liver tumor is a promising approach to improve the therapeutic effect of liver tumors and has a broad clinical application prospect.

Background

Hepatocellular carcinoma (HCC) is one of the most common cancers in East Asian countries. It is also the third leading cause of cancer-related death worldwide[1, 2]. Transcatheter arterial chemoembolization (TACE) can block tumor vessels by injecting embolic agents and chemotherapeutic drugs into hepatic arteries, ultimately leading to tumor necrosis[3, 4]. Consequently, TACE has become the preferred therapy for unresectable advanced HCC[5, 6]. The liver receives a dual blood supply from the portal vein (70%) and the hepatic artery (25%)[7], but the blood supply of most liver tumors comes from the hepatic artery. The embolic agent is injected into the hepatic artery, blocking the blood supply to the liver tumors without causing ischemia to the normal liver[8]. Therefore, hepatic artery embolization is especially suitable in the treatment of HCC. However, due to the heterogeneity of blood vessels in liver tumors, the embolization of tumor-feeding arteries usually leads to different degrees of hypoxia and ischemia in tumor tissues, which in turn enhances the transcriptional activity of hypoxia-inducible factor1 α (HIF-1 α) and promotes the progression of liver tumors[9]. If tumor cells cannot be completely killed after hepatic artery embolization, the tumor vascular system will regenerate, and the remaining tumor cells will recur and thrive[10].

Therefore, it is urgent to inhibit tumor angiogenesis in the treatment of TACE. Sorafenib (SOR), a multi-target tyrosine kinase inhibitor with the ability to inhibit angiogenesis and tumor proliferation, and is the only Food and Drug Administration (FDA) approved drug for the treatment of advanced HCC[11]. However, due to the lack of tumor specificity, oral-systemic administration of sorafenib often leads to high toxicity and severe side effects[12]. Therefore, local administration through TACE may reduce the side effects of sorafenib[13]. Catalase (CAT) is an enzyme that removes hydrogen peroxide (H_2O_2) and neutralizes the overexpression of H_2O_2 in tumor tissue to produce the oxygen[14]. At present, the union

application of SOR and CAT in TACE has not been reported, whether CAT can enhance the function of SOR in inhibiting tumor angiogenesis and improve tumor hypoxic microenvironment is worth further study.

Clinically available hepatic embolic agents include liquids, particles and hydrogels, etc.[10]. Lipiodol is a type of embolic agent extracted from poppy seeds. It has been applied in TACE for over 30 years[15]. Lipiodol can be combined with hydrophilic chemotherapy drugs through a water-in-oil (W/O) mode, but the drug release spectrum is short and the embolization is incomplete[16]. Recently, microspheres (MSs) composed of the biodegradable polymer poly(lactic-co-glycolic acid) (PLGA) have been developed for TACE in the treatment of liver tumors[17]. PLGA has excellent biocompatibility and biodegradability, and has been widely applied in bone tissue engineering[18]. As a drug carrier, PLGA has been approved by the FDA for some clinical treatments[19]. In this study, SOR and CAT were encapsulated in PLGA MSs, and the SOR-CAT-PLGA MS sustained-release system was established. SOR-CAT-PLGA MSs can effectively accumulate in tumor tissues *via* TACE.

The purpose of this study was to construct SOR-CAT-PLGA MSs containing sorafenib and catalase, then to study its application in cancer cells and rabbit VX2 liver tumor models through cell culture, Western blot analysis of protein expression, angiography analysis of the tumor vascular system, and immunohistochemical analysis. Meanwhile, the efficacy of hepatic artery embolization SOR-CAT-PLGA MSs in inhibiting tumor angiogenesis and improving tumor immunosuppressive microenvironment was evaluated.

Materials And Methods

Materials

The poly(lactic-co-glycolic acid) (PLGA, MW 7–17K), catalase (CAT) and Cell Counting Kit-8 (CCK-8) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sorafenib (SOR) was obtained from LC Laboratories (Woburn, MA, USA). Polyvinyl alcohol (PVA) was obtained from Aladdin (Shanghai, China). An Annexin-V-FLUOS Staining Kit was obtained from Roche Applied Science (Indianapolis, IN, USA). A Cell Death Detection Kit for the terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assay and a Ki-67 Cell Proliferation Detection Kit were purchased from Roche Applied Science (Indianapolis, IN, USA). DAPI (4',6-diamidino-2-phenylindole) was obtained from Beyotime Biotechnology (Wuhan, China). All other chemical reagents were analytically pure and were used directly without further purification.

New Zealand White rabbits (2.5–3 kg in weight, both sexes) were supplied by the Laboratory Animal Research Center, Medical School of Nanjing University. All procedures conformed to the provisions of the China National Animal Law on the use of laboratory animals (license no. SYXK-2018040002).

The preparation of microspheres (MSs)

SOR-CAT-PLGA MSs were prepared by the solid-in-oil-in-water (S/O/W) method. First, PLGA (40 mg) was dissolved in 0.8 ml of dichloromethane (DCM), and SOR (2 mg) was melted in 0.2 ml of dimethyl sulfoxide (DMSO). CAT (2 mg) was dissolved in 0.2 ml of PBS. Then, the SOR and CAT solutions were added to the PLGA solution and thoroughly mixed. The mixture was incubated at room temperature to form a uniformly distributed organic phase (O). Then, the emulsion was added to a 2% polyvinyl alcohol (PVA) solution to form an external aqueous phase. The emulsion became homogeneous at 500 rpm. After the organic phase was removed by stirring for 3 hours, the MSs were collected by centrifugation at 2,000 rpm for 10 minutes and stored at -20 °C after freeze-drying. The blank MSs, MSs containing only SOR and MSs containing only CAT were prepared via the same method.

The morphology of the SOR-CAT-PLGA MSs was analyzed by transmission electron microscopy (TEM) on a JEM-200CX electron microscope (JEOL Ltd., Tokyo, Japan) and by scanning electron microscopy (SEM) on an S-3400 scanning electron microscope (Hitachi Ltd., Tokyo, Japan). The size distribution of the SOR-CAT-PLGA MSs was determined using a laser particle size analyzer (Malvern Instruments Inc., Malvern, UK).

The encapsulation efficiency of SOR and CAT

The encapsulation efficiency of SOR and CAT in MSs was determined by the indirect method. After centrifugation, the concentration of SOR in the supernatant was measured at 265 nm by ultraviolet spectrophotometry on a Hitachi U4100 spectrophotometer. Micro CAT Assay kit was used to analyze the content of the unencapsulated catalase in the supernatant. The encapsulation efficiency was determined by dividing the actual amount of drug loaded by the total amount added to the emulsion preparation. All experiments were performed three times.

Release of SOR and CAT from MSs

We measured the release of SOR and CAT from MSs in 1.5-ml centrifuge tubes by ultraviolet spectrophotometry at 265 nm and 240 nm. Exactly 5 mg of MSs were resuspended in 5 ml of PBS. Triplicate samples were shaken at 37 °C for seven days. At the indicated time points, 1 ml of release buffer was collected and replaced with a new buffer. We also measured the oxygen generation of MSs in 0.5 mM H₂O₂ solution by the oxygen probe.

Cell viability and apoptosis assay

The cytotoxicity of the different MSs to HepG2 cells was measured by a CCK-8 assay. Cells (1 × 10⁴ cells/ml) were seeded in 96-well plates. The different MSs were added to the cells and cultured at 37 °C for 4 hours. Then, the culture medium was removed, and the cells were washed three times with PBS and cultured in fresh medium for twenty hours. The culture medium was aspirated, and the cells were incubated with fresh serum-free medium containing CCK-8 reagent at 37°C for two hours. Then, the viability of the cells was determined by assuming that the formazan dye produced by dehydrogenase activity in the cells was proportional to the number of viable cells, and the absorbance at 450 nm was

assessed on a Thermo Scientific Varioskan Flash multimode plate reader (Thermo Fisher Scientific, Waltham, MA, USA). All measurements were performed in triplicate.

An annexin V-FITC/PI apoptosis detection kit was used to detect cell apoptosis. Cells (1×10^5) were placed in 12-well plates and cultured. When the cells reached 80% confluence, different MSs were added and incubated for 24 hours. Annexin V-FITC-stained apoptotic cells were detected by flow cytometry following the manufacturer's instructions, and the data were analyzed using FlowJo software (Tree Star, Inc., San Carlos, CA, USA).

TACE therapy on VX2 liver tumors in rabbits

To evaluate the therapeutic effect of SOR-CAT-PLGA MSs, we established a rabbit VX2 liver tumor model. The flowchart of the experiment is shown in Fig. 1A. The rabbit VX2 liver tumor model was established following the method reported by Chen *et al.*[20]. Rabbits were anesthetized with an intravenous injection of pentobarbital sodium solution (2 wt%, 1 ml/kg) after fasting for 12 hours. After anesthesia, the abdominal wall of the rabbits was cut open, and VX2 tumor particles (1.0 mm^3) were implanted in the left liver lobe. The growth of the VX2 tumor in the liver lobe of the rabbits was evaluated by measuring the size of the rabbit liver tumors by computed tomography (CT) on a GE Discovery CT750 HD system (GE Healthcare, Medical Systems, Chicago, IL, USA). Perfusion CT imaging was performed with a 90 mA tube current, a 120 kVp tube voltage, a 1.25 mm slice thickness and a 25 cm field of view. The perfusion images were transferred to an Advantage imaging workstation (GE Healthcare Medical Systems) for perfusion analysis. The absolute values of blood volume (BV), blood flow (BF), permeability surface (PS) area product and mean transit time (MTT) were measured by CT perfusion software. TACE was performed 14 days after implantation of the VX2 tumors. Thirty rabbits were randomly divided into 5 groups of six rabbits per group. A radio-opaque short guidewire and sheath (Terumo Corp., Tokyo, Japan) were placed in the right femoral artery of the rabbits via an operative incision, and selective catheterization of the left hepatic artery feeding the VX2 liver tumor was accomplished using a 2.7 Fr Progreat microcatheter (Terumo Corp.) under fluoroscopy. Hepatic angiography was performed with a hand injection of 2 ml of Omnipaque 300 contrast medium (GE Healthcare), which was injected at a rate of approximately 0.5 ml/s. Subsequently, 50 mg of MSs were slowly injected into the tumor-feeding artery through a microcatheter at a speed of 0.5 ml/min; each group was injected as follows: group A (0.5 ml normal saline solution), group B (0.5 ml PLGA MSs solution), group C (0.5 ml SOR-PLGA MSs solution), group D (0.5 ml CAT-PLGA MSs solution) and group E (0.5 ml SOR-CAT-PLGA MSs solution). Digital subtraction angiography (DSA) images were recorded before and after embolization. After all images were acquired, all guides, sheaths and catheters were removed. The femoral artery was ligated, and the muscle, subcutaneous tissue and skin were sutured with absorbable sutures. Postoperative CT images were used to measure the degree of tumor necrosis and calculate the volume of liver tumors: $V = (\text{length} \times \text{width}^2) / 2$ [21]. Last, three rabbits were randomly chosen from each group and sacrificed on the 14th day after TACE for bioinformatics analysis and histopathological examination. The remaining three rabbits in each group were used for survival analysis.

Histopathology and immunohistochemistry

On the 14th day after the operation, liver tumors and various organs, such as the heart, lungs and kidneys, were harvested. Tissue was dissected and snap-frozen with optimum cutting temperature (O.C.T.) compound (Sakura Finetek USA Inc., Torrance, USA) for cryostat sectioning into 6- μ m slices. Slices were stained with hematoxylin and eosin (H&E) for routine microscopy examination and were stained with Ki-67 and subjected to a TUNEL assay to detect proliferation and apoptosis, respectively, following the manufacturer's guidelines. Ki-67-stained slides and slides from the TUNEL assay were analyzed by fluorescence microscopy using a Nikon TS100 fluorescence microscope (Nikon Corp., Tokyo, Japan).

Tumor specimens were fixed in acetone at -20°C for 5 minutes and washed 3 times with PBS after air-drying. Specimens were then placed in a 3% BSA PBS solution in a sealed container and incubated at room temperature for one hour. An anti-vascular endothelial growth factor (VEGF) antibody for VEGF staining was used at a 1:200 dilution, and an anti-HIF-1 α antibody for HIF-1 α staining was used at a 1:200 dilution. After incubation at room temperature for three hours, slides were washed three times. Alexa Fluor 488-conjugated goat anti-rat IgG and Alexa Fluor 555-conjugated goat anti-mouse IgG were added and incubated at room temperature for two hours, and slides were then washed and fixed. Fluorescence images were acquired by laser scanning confocal microscopy (LSCM) using a Zeiss LSM 710 fluorescence microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). PD-L1 and CD8 antibody were used to detect the expression of PD-L1 and CD8⁺ T cell infiltration in tumor tissues after TACE. In addition, serum interferon IFN- γ was detected by enzyme-linked immunosorbent assay (ELISA).

Biochemical Assay

Rabbit serum was collected before treatment and on days 1, 3, 7, and 14 after treatment for biochemical examination. The levels of alanine transaminase (ALT), aspartate aminotransferase (AST), creatinine (Cr), blood urea nitrogen (BUN) and total bilirubin (TBIL) were measured on an Olympus AU5421 automatic biochemical analyzer (Olympus Corp., Tokyo, Japan). TBIL is an indicator of hepatocyte damage, including damage due to hepatocellular carcinoma and hepatitis.

Statistical analysis

The experiments were repeated at least 3 times in each group, and all data are expressed as the means $\pm$ standard deviations (SDs). SPSS 19.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Data for different groups were compared by one-way ANOVA test, Student's t-test and Kaplan–Meier method. $P < 0.05$ was considered statistically significant.

Results

Preparation and characterization of SOR-CAT-PLGA MSs

We use an improved S/O/W emulsion method to prepare the SOR-CAT-PLGA MSs for TACE therapy (Fig. 1A). PLGA MSs, SOR-PLGA MSs and CAT-PLGA MSs were prepared by the same method. To evaluate the formation of the MSs, we examined the morphology of the MSs containing SOR and CAT by SEM and TEM (Fig. 1B (SEM) and 1C (TEM)). The images reveal that SOR-CAT-PLGA MSs have a smooth surface and regular spherical shape. In addition, the size of the MSs was relatively uniform. The particle size was determined using a laser particle size analyzer (Fig. 1D). The average diameter of the PLGA MSs and SOR-CAT-PLGA MSs was $75.7 \pm 7.9 \mu\text{m}$ and 73.5 ± 8.3 , respectively. Thus, the addition of SOR and CAT did not interfere with the formation of traditional PLGA MSs.

The drug encapsulation efficiency plays an important role in the drug delivery system, directly influencing the therapeutic effect of encapsulated drugs. We used ultraviolet-visible spectrophotometry to determine the entrapment efficiency of SOR and CAT. The encapsulation efficiency of SOR and CAT was 68.7% and 76.5%, respectively. Each mg of MSs contains approximately 22.5 μg SOR and 25.3 μg CAT.

The in vitro release of SOR-CAT-PLGA MSs was assessed in PBS at 37°C. As shown in Fig. 1E, SOR-CAT-PLGA MSs exhibited a two-phase release mode. In the first 12 hours, SOR and CAT were released rapidly from the MSs, and the initial release rate was $\sim 40\%$. After that, SOR showed relatively slow continuous release, and within 7 days, approximately 80% of the loaded SOR had been released into the surrounding medium. CAT also showed slow release; the release rate of CAT was 90% after 7 days. Patients with HCC usually receive TACE once every 1 or 2 months. Therefore, we anticipate that the degradation rate of the embolic agent will be slow and that the long-term release of the chemotherapeutic drugs carried by embolic agents is achievable. These results indicate that SOR-CAT-PLGA MSs may allow slow release of SOR and CAT.

As shown in Fig. 1F, PLGA MSs and SOR-PLGA MSs had no significant effect on the generation of O_2 in the H_2O_2 solution. However, when the CAT-PLGA MSs and SOR-CAT-PLGA MSs were incubated with H_2O_2 solution, O_2 was rapidly generated and the concentration of O_2 increased steadily over time. There was no significant difference in O_2 production between CAT-PLGA MSs and SOR-CAT-PLGA MSs, indicating that SOR loading did not affect the activity of CAT.

Antitumor activity of the SOR-CAT-PLGA MSs in vitro

The therapeutic effect of SOR-CAT-PLGA MSs was evaluated by the CCK-8 assay in vitro. HepG2 cells were incubated with PBS, PLGA MSs, SOR-PLGA MSs, CAT-PLGA MSs and SOR-CAT-PLGA MSs for 4 hours. Then, cells were incubated in regular medium for 20 hours, and cell viability was evaluated (Fig. 2A). As expected, the viability of HepG2 cells in the SOR-CAT-PLGA MSs group was found to be decreased as MSs concentration was increased. After 24 h, viability rates were 42% and 31% for 5 μg /well and 10 μg /well of SOR-CAT-PLGA MSs, whereas the cells remained viable in the negative control and PLGA MSs-treated wells.

The improved proapoptotic activity of SOR-CAT-PLGA MSs was further confirmed by annexin V staining. Necrotic cells, late apoptotic cells, viable cells and early apoptotic cells were represented by Q1, Q2, Q3

and Q4, respectively. The results showed that SOR combined with CAT had a significant proapoptotic synergistic effect on tumor cells (Fig. 2B and 2C). Compared with the control treatment, the SOR-CAT-PLGA MSs combined treatment significantly increased the apoptosis rate and reduced the percentage of viable cells. The percentage of apoptotic cells in the PBS, PLGA MSs, SOR-PLGA MSs, CAT-PLGA MSs and SOR-CAT-PLGA MSs groups were 0.9, 1, 18.5, 13 and 24.1%, respectively. In addition, the MSs developed in this study did not cause severe necrosis, which minimized peripheral cell damage.

TACE Treatment Process Using SOR-CAT-PLGA MSs

The TACE flowchart (Fig. 3) and intra-arterial injection of SOR-CAT-PLGA MSs via the hepatic artery are presented (Fig. 4A and 4B). The TACE process was performed using a digital subtraction angiography system (DSA, Philips, BV Pulsera) according to clinical regulations. The angiography showed that the tumor was surrounded by abundant blood capillaries, especially at the edge (Fig. 4C). Notably, blood vessels of its neighboring normal hepatic parenchyma were swollen after embolization, which indicates that MSs were deposited in the area of the lesion to a significant extent and consequently blocked the feeding routes (Fig. 4D). CT was used to measure the changes in the tumor volume before and after TACE. CT was also used to quantify the perfusion of tumors (Fig. 5A and 5B). The BF (blood flow), BV (blood volume), PS (permeability surface) area and MTT (mean transit time) values were determined before and after the operation. The BF, BV and PSA values of tumors in the SOR-PLGA MSs and CAT-PLGA MSs groups were significantly lower than control groups and PLGA MSs groups, but those in the SOR-CAT-PLGA MSs groups were the lowest. The MTT values of tumors in the SOR-CAT-PLGA MSs were also significantly higher than those in the other groups, which sufficiently indicated that the angiogenesis of tumors was significantly inhibited in the SOR-CAT-PLGA MSs groups.

Evaluation of the antitumor efficacy of SOR-CAT-PLGA MSs in vivo

Three rabbits were randomly selected from each group and sacrificed on the 14th day after TACE. The resected tumors were photographed and weighed. Analysis of the data confirmed that tumor growth inhibition was most effective in the SOR-CAT-PLGA MSs group (Fig. 6A). The images of the pathological specimens shown in Fig. 6A also reveals obvious necrosis of the left lobe tumors in the SOR-CAT-PLGA MSs, SOR-PLGA MSs and CAT-PLGA MSs groups. However, the extent of local necrosis at the center of the tumors in the control group and PLGA MSs group was small, and the cavernous vessels were obvious, which was closely related to the degree of malignancy of the tumors. We also evaluated the potential toxicity of SOR-CAT-PLGA MSs to the heart, kidneys and lungs of rabbits. The results revealed that there was no significant change in these organs after treatment, and lung metastasis was detected only in the untreated control group (Fig. 6A).

The volume of tumors in the control group increased significantly, from $1.69 \pm 0.24 \text{ cm}^3$ before treatment to $10.98 \pm 1.35 \text{ cm}^3$ after treatment (Fig. 6B). In contrast, the tumor volume in rabbits treated with SOR-CAT-PLGA MSs decreased significantly after treatment ($1.73 \pm 0.32 \text{ cm}^3$ vs. $0.62 \pm 0.13 \text{ cm}^3$; Fig. 6B), indicating that the tumors were significantly smaller after treatment. As shown in Fig. 6B, the growth rate

of tumors in the untreated control group was rapid, while SOR-PLGA MSs and CAT-PLGA MSs moderately inhibited the growth of tumors. We observed that SOR-CAT-PLGA MSs had the strongest antitumor effect. The synergistic effect of SOR and CAT significantly inhibited orthotopic tumor growth. The superiority of the SOR-CAT-PLGA MSs therapy further confirmed that the drug combination had better efficacy than single drug-containing microspheres.

There was a significant difference in the weight of tumors among the different treatment groups (Fig. 6C). The weight of tumors in the control group was significantly increased. In contrast, the weight of tumors in the SOR-CAT-PLGA MSs treatment group was significantly lower after treatment than before treatment, further confirming that the tumors were significantly reduced. SOR-PLGA MSs and CAT-PLGA MSs moderately inhibited the growth of tumors, but the growth rate of tumors in the untreated control group was increased.

In addition, histological examination of rabbit VX2 liver tumors was carried out to further assess the antitumor effect of various MSs combined with TACE. As shown in Fig. 7A, histopathological sections of rabbit VX2 liver tumors showed that SOR-CAT-PLGA MSs administered via TACE could thoroughly fill the tumor vessels. Additionally, infarction was observed in tumor tissues, accompanied by necrosis and degeneration of tumor cells. Compared with the control group, the SOR-CAT-PLGA MSs treated group showed nearly complete tumor necrosis, while the SOR-PLGA MSs and CAT-PLGA MSs groups showed moderate tumor necrosis. Notably, H&E staining of the main organs (heart, liver, kidneys, and lungs), as shown in Fig. 7A, revealed no clear histopathological abnormalities (tissue injury or inflammation).

We also studied the hypoxic status of tumors after different treatments. We analyzed the expression of HIF-1 α and VEGF using an immunohistochemical staining assay. In the SOR-CAT-PLGA MSs and CAT-PLGA MSs groups, the positive fluorescence staining of HIF-1 α in tumor tissue slices was significantly decreased, as shown in Fig. 7B, respectively. In addition, compared with the other groups, the SOR-CAT-PLGA MSs and SOR-PLGA MSs groups did not exhibit upregulated expression of VEGF, and that the expression of VEGF in SOR-CAT-PLGA MSs groups was the lowest, which further confirmed the synergistic inhibition of SOR and CAT on VEGF expression and angiogenesis.

We also investigated the role of SOR-CAT-PLGA MSs in the immunosuppressive microenvironment of tumor tissues after TACE. Hypoxia-mediated HIF-1 α upregulation is closely related to the expression of PD-L1, which mainly mediates immune escape. Our results showed that PD-L1 in the SOR-CAT-PLGA MSs group was significantly lower than that in the PLGA MSs group and control group (Fig. 7B), and significant CD8⁺T cell infiltration was observed (Fig. 7B). PD-L1 in the CAT-PLGA MSs group was also lower than that in the PLGA MSs group and control group, and the infiltration of CD8⁺T cells also increased. In addition, IFN- γ secreted by active CD8⁺T cells in the SOR-CAT-PLGA MSs group significantly increased compared with the PLGA MSs group and control group (Fig. 7D). IFN- γ also increased in the CAT-PLGA MSs group compared with the PLGA MSs group and control group.

The TUNEL assay and Ki-67 staining results further confirmed our previous findings (Fig. 7C). The results shown in Fig. 7E clearly reveals that SOR-CAT-PLGA MSs induced significant apoptosis and morphological changes, while tumors treated with CAT-PLGA MSs or SOR-PLGA MSs only showed moderate apoptosis. As shown in Fig. 7F, immunohistochemical staining of Ki-67 was used to evaluate the proliferation of tumor cells in each treatment group. The results revealed that the tumor cells in the control group were heteromorphous and of different cell types, had a significantly increased ratio of nucleus to plasma, and showed increased expression of Ki-67. In the SOR-CAT-PLGA MSs group, after treatment, the tumor cells showed necrosis and complete loss of nuclei, and Ki-67 expression was scant or even undetected. Moreover, the expression of Ki-67 decreased in the SOR-CAT-PLGA MSs treatment group, indicating that the proliferation of tumor cells decreased significantly after treatment. In the CAT-PLGA MSs and SOR-PLGA MSs groups, necrosis occurred mainly at the entrance to the tumors, and Ki-67 was mainly expressed in the periphery of the tumors. These findings further demonstrate that SOR-CAT-PLGA MSs induce significant apoptosis and almost completely inhibit the proliferation of tumor cells, which further indicates the synergistic antitumor effect of SOR and CAT.

We next evaluated the toxicity of SOR-CAT-PLGA MSs in rabbits. We analyzed the levels of various biochemical indicators in the blood of tumor-bearing rabbits at 1, 3, 7 and 14 days after TACE. As shown in Fig. 8A-E, there were no significant differences in liver and kidney function indices, such as blood parameters (white blood cell, red blood cell and platelet counts) and the levels of AST, ALT, Cr, BUN and TBIL. The results of these biochemical analyses showed that SOR-CAT-PLGA MSs had no significant MS-related, kidney or liver toxicities.

In addition, the Kaplan–Meier survival curve (Fig. 8F) showed that the survival time of rabbits in the SOR-CAT-PLGA MSs group was significantly prolonged (54 days) compared with that in the other groups, while the survival time of rabbits in the PLGA MSs group was only 31 days. Our findings further suggest that SOR-CAT-PLGA MSs may be a new approach to improve the efficacy of HCC treatment.

Discussion

TACE is the primary therapy for unresectable HCC, particularly for patients with multifocal HCC and normal liver function[22]. The embolization of the feeding arteries of HCC leads to tumor necrosis, and the clinical efficacy of this treatment has been widely recognized[23, 24]. TACE combined with cytostatic drugs (doxorubicin, mitomycin, cisplatin, 5-Fu, epirubicin, etc.) can inhibit the growth of tumors, but whether it can prolong the survival time is still uncertain[25]. The basic principle of this technique is that the combination of local ischemic necrosis and targeted chemotherapy can produce synergistic antitumor effects. However, some studies have shown that TACE can aggravate hypoxia in residual cancer cells and further enhance the drug resistance of cancer cells[26]. Besides, hypoxia can stabilize the level of HIF-1 α in cells, thereby increasing angiogenesis factor-induced angiogenesis[27, 28]. Thus, to overcome the limitations of TACE, we incorporated SOR, a multi-target tyrosine kinase inhibitor with the ability to inhibit angiogenesis[11]. We also added CAT, an enzyme that neutralizes the overexpression of H₂O₂ in tumor tissue to produce the oxygen[14].

Another limitation of the current clinical TACE therapy is that the commonly used lipiodol embolization of hepatic arteries is usually not durable. Chemotherapeutic drugs in lipiodol are released rapidly, and the blood flow of liver tumors is restored quickly[29]. This observation is supported by the finding that angiography at the second or subsequent embolization usually indicates the recanalization of the hepatic artery. To overcome the short half-life of the embolization agent, TACE is repeated clinically, but repeated TACE is likely to aggravate cirrhosis and eventually lead to liver failure[6]. Therefore, there is an urgent need for new hepatic embolic agents sustained-release drugs to improve the efficacy of TACE.

The experimental use of PLGA MSs for hepatic artery embolization has been reported[18]. PLGA is a copolymer that can be hydrolyzed into biocompatible substances, including lactic acid and glycolic acid monomers[30]. Lactic acid and glycolic acid are scavenged as water and carbon dioxide in the body through the Krebs cycle, which does not induce a robust immune response. The degradation time of PLGA is related to the monomer ratio, and its lifetime can be controlled to within a few days or months[31]. Encapsulating the drug in PLGA MSs is a common method of achieving controlled release. Some studies have shown that PLGA MSs releases drugs slowly into surrounding tissues through a diffusion mechanism to promote the complete release of the drug, thereby improving the treatment effect of drugs[32]. TACE combined with magnetic PLGA MSs significantly enhanced the delivery of cytotoxic drugs to rabbit liver tumors while reducing the systemic toxicity in the animals[18].

In our experiments, the addition of SOR and CAT did not interfere with the morphology and size of blank PLGA MSs. To make PLGA MSs embolize rabbit liver tumors, the size of the PLGA MSs should be adjusted according to the diameter of the capillaries and peripheral arteries in the rabbit liver. It has been reported that MSs with diameters less than 40 μm can be shunted to nontarget organs, such as the lungs, while MSs with diameters greater than 40 μm are mainly trapped in liver tumors and are seldom shunted to other organs[33]. According to the literature on rabbit hepatic tumor embolization and considering the diameter of rabbit capillaries and hepatic arterioles[34], we constructed PLGA MSs with an average diameter of 73 μm , which is suitable for rabbit hepatic artery embolization. In our study, SOR-CAT-PLGA MSs were not found in the lungs and did not cause a pulmonary embolism.

SOR and CAT were encapsulated into the SOR-CAT-PLGA MSs with moderate efficiency, and the SOR-CAT-PLGA MSs had uniform particle sizes and a stable drug release rate. Because of the low encapsulation efficiency of O/W emulsification, the S/O/W method was chosen to improve the encapsulation efficiency. Compared with the O/W method, the encapsulation efficiency of SOR and CAT was greatly improved by this method. In the in vitro drug release test, SOR-CAT-PLGA MSs exhibited a biphasic release mode with an initial explosive release. The drug release rate was approximately 50% in the first three days and was then relatively slowed in the next few days. The drug release rate was about 90% on the seventh day. A Relatively slow continuous release may maintain an adequate drug concentration in the surrounding environment, thus avoiding the limitation of short application time and enhancing the time of drug action. When SOR-CAT-PLGA MSs were incubated with H_2O_2 solution, oxygen was generated rapidly, indicating that it had sufficient catalytic capacity to produce oxygen.

In this study, we evaluated the antineoplastic efficacy of SOR-CAT-PLGA MSs in vitro and in vivo. The results showed that SOR-CAT-PLGA MSs effectively inhibited the growth of cultured HepG2 HCC cells in vitro, and also exhibited strong antitumor activity in the rabbit VX2 liver model. Compared with other treatments, SOR-CAT-PLGA MSs significantly promoted the apoptosis of HepG2 cancer cells. Flow cytometric analysis also showed that SOR-CAT-PLGA MSs increased the induction of early and late apoptosis in HepG2 cells. In vivo, SOR-CAT-PLGA MSs effectively inhibited the growth of VX2 tumors and reduced the volume of rabbit VX2 liver tumors by nearly 90%, and SOR-CAT-PLGA MSs were more effective than SOR-PLGA MSs and CAT-PLGA MSs. SOR-CAT-PLGA MSs successfully induced tumor necrosis and apoptosis in the targeted region of blood supply to rabbit VX2 liver tumors, and histopathological analysis of liver tumors showed a tumor necrosis rate of 90% after TACE treatment. In this study, the CT perfusion imaging was used to evaluate the perfusion parameters before and after SOR-CAT-PLGA MSs treatment, and the results showed that SOR-CAT-PLGA MSs acted directly on the blood vessels of liver tumors, resulting in decreased perfusion of VX2 liver tumors. Hypoxia is related to HIF-1 α expression and VEGF transcriptional activation[35]. There is much evidence that HIF-1 α and VEGF may contribute to tumor progression and metastasis, as well as poor patient survival[36]. We also found that the expression of HIF-1 α and VEGF was significantly decreased in the SOR-CAT-PLGA MSs group after TACE, which further proved that SOR inhibited VEGF expression and tumor angiogenesis, and CAT improved tumor hypoxia and inhibited HIF-1 α expression. The strong synergistic effect of SOR and CAT led to the inhibition of rabbit VX2 liver tumors.

It has been reported that tumor hypoxia leads to the upregulation of PD-L1, which further inhibits the function of CD8⁺T cells and induces immune escape[37]. Our results showed that SOR-CAT-PLGA MSs could down-regulate the expression of PD-L1, inhibit the apoptosis of CD8⁺T cells, and increase the release of IFN- γ secreted by activated CD8⁺T cells. The therapeutic effect may be attributed to the improvement of hypoxia in tumors after SOR-CAT-PLGA MSs treatment. The down-regulation of HIF-1 α significantly inhibited the expression of PD-L1, which further relieved the pressure on CD8⁺T cells and regulated the immunosuppressive microenvironment.

Rabbit lungs, kidneys, and hearts showed no noticeable toxic effect. The only side effects observed were a slight elevation of the serum ALT level 1 day after the operation, which recovered 3 days after treatment. Since the tumors began to necrotize one day after embolization, it was suggested that the elevated serum ALT level might be related to the necrosis of tumors after embolization. The survival curve based on the Kaplan-Meier method showed that the SOR-CAT-PLGA MSs group (average survival time, 59 days) had a significant survival advantage compared with the control group (average survival time, 31 days). TACE can induce tumor hypoxia and ischemia, while SOR and CAT can inhibit tumor ischemia and hypoxic injury. Our results further indicate that SOR-CAT-PLGA MSs can significantly improve the therapeutic effect on rabbit liver VX2 tumors.

Conclusion

In this study, SOR-CAT-PLGA MSs were synthesized, and their efficacy in TACE of rabbit VX2 liver tumors was verified. The release of SOR and CAT enhanced the antitumor activity of SOR-CAT-PLGA MSs in TACE of rabbit VX2 liver tumors in vivo. In the rabbit VX2 liver tumor model, the injection of SOR-CAT-PLGA MSs into the hepatic artery showed a clear embolization effect and had decreased systemic side effects. In conclusion, we successfully developed an embolization strategy combining drug-loaded MSs with TACE for the treatment of VX2 orthotopic tumors in rabbits. Our results suggest that combining SOR-CAT-PLGA MSs with TACE may be a promising strategy for HCC treatment.

Abbreviations

TACE: Transcatheter arterial chemoembolization; HCC: Hepatocellular carcinoma; PLGA: Poly(lactic-co-glycolic acid); SOR: Sorafenib; CAT: Catalase; H₂O₂: Hydrogen peroxide; HIF-1 α : Hypoxia-inducible factor-1 α ; FDA: Food and Drug Administration; MSs: Microspheres; CCK-8: Cell Counting Kit-8; PVA: Polyvinyl alcohol; TUNEL: Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling; DCM: Dichloromethane; DMSO: Dimethyl sulfoxide; TEM: Transmission electron microscopy; SEM: Scanning electron microscopy; BV: Blood volume; BF: Blood flow; PS: Permeability surface; MTT: Mean transit time; DSA: Digital subtraction angiography; H&E: Hematoxylin and eosin; VEGF: Vascular endothelial growth factor; LSCM: Laser scanning confocal microscopy; ALT: Alanine transaminase; AST: Aspartate aminotransferase; Cr: Creatinine; BUN: Blood urea nitrogen; TBIL: Total bilirubin.

Declarations

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Ethical approval and consent to participate

The authors declare that the manuscript was approved by the Experimental Animal Ethical Committee of Nanjing University.

Authors' contributions

Xihan Li and Xiaoping Zou designed the experiment. Hui Yu, Ying Huang, Yu Chen and Jialun Wang carried the experiment. Lei Xu and Feng Zhang analyzed the data. Xihan Li, Xiaoping Zou and Yuzheng Zhuge drafted the paper and revised it.

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Availability of data and materials

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

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Forner A, Llovet JM, Bruix J. Hepatocellular carcinoma. *LANCET* 2012 2012-03-31;379(9822):1245–1255.
2. Forner A, Gilabert M, Bruix J, Raoul JL. Treatment of intermediate-stage hepatocellular carcinoma. *NAT REV CLIN ONCOL* 2014 2014-09-01;11(9):525–535.
3. Lewis AL, Dreher MR. Locoregional drug delivery using image-guided intra-arterial drug eluting bead therapy. *J CONTROL RELEASE* 2012 2012-07-20;161(2):338–350.
4. Tsai M, Lu Z, Wientjes MG, Au JL. Paclitaxel-loaded polymeric microparticles: quantitative relationships between in vitro drug release rate and in vivo pharmacodynamics. *J CONTROL RELEASE* 2013 2013-12-28;172(3):737–744.
5. Wang Y, Benzina A, Molin DG, Akker N, Gagliardi M, Koole LH. Preparation and structure of drug-carrying biodegradable microspheres designed for transarterial chemoembolization therapy. *J Biomater Sci Polym Ed* 2015 2015-01-20;26(2):77–91.
6. Camma C, Schepis F, Orlando A, Albanese M, Shahied L, Trevisani F, et al. Transarterial chemoembolization for unresectable hepatocellular carcinoma: meta-analysis of randomized controlled trials. *RADIOLOGY* 2002 2002-07-01;224(1):47–54.
7. Sakr OS, Berndt S, Carpentier G, Cuendet M, Jordan O, Borchard G. Arming embolic beads with anti-VEGF antibodies and controlling their release using LbL technology. *J CONTROL RELEASE* 2016 2016-02-28;224:199–207.
8. Hsu MF, Tyan YS, Chien YC, Lee MW. Hyaluronic acid-based nano-sized drug carrier-containing Gellan gum microspheres as potential multifunctional embolic agent. *Sci Rep* 2018 2018-01-15;8(1):731.

9. Palazon A, Tyrakis PA, Macias D, Velica P, Rundqvist H, Fitzpatrick S, et al. An HIF-1alpha/VEGF-A Axis in Cytotoxic T Cells Regulates Tumor Progression. *CANCER CELL* 2017 2017-11-13;32(5):669–683.
10. Chao M, Wu H, Jin K, Li B, Wu J, Zhang G, et al. A nonrandomized cohort and a randomized study of local control of large hepatocarcinoma by targeting intratumoral lactic acidosis. *ELIFE* 2016 2016-08-02;5.
11. Park W, Chen J, Cho S, Park SJ, Larson AC, Na K, et al. Acidic pH-Triggered Drug-Eluting Nanocomposites for Magnetic Resonance Imaging-Monitored Intra-arterial Drug Delivery to Hepatocellular Carcinoma. *ACS Appl Mater Interfaces* 2016 2016-05-25;8(20):12711–12719.
12. Lencioni R, Kudo M, Ye SL, Bronowicki JP, Chen XP, Dagher L, et al. GIDEON (Global Investigation of therapeutic DEcisions in hepatocellular carcinoma and Of its treatment with sorafeNib): second interim analysis. *INT J CLIN PRACT* 2014 2014-05-01;68(5):609–617.
13. Chen J, White SB, Harris KR, Li W, Yap JW, Kim DH, et al. Poly(lactide-co-glycolide) microspheres for MRI-monitored delivery of sorafenib in a rabbit VX2 model. *BIOMATERIALS* 2015 2015-08-01;61:299–306.
14. Chen Q, Chen J, Yang Z, Xu J, Xu L, Liang C, et al. Nanoparticle-Enhanced Radiotherapy to Trigger Robust Cancer Immunotherapy. *ADV MATER* 2019 2019-03-01;31(10):e1802228.
15. Idee JM, Guieu B. Use of Lipiodol as a drug-delivery system for transcatheter arterial chemoembolization of hepatocellular carcinoma: a review. *Crit Rev Oncol Hematol* 2013 2013-12-01;88(3):530–549.
16. Choi JW, Cho HJ, Park JH, Baek SY, Chung JW, Kim DD, et al. Comparison of drug release and pharmacokinetics after transarterial chemoembolization using diverse lipiodol emulsions and drug-eluting beads. *PLOS ONE* 2014 2014-01-20;9(12):e115898.
17. Choi JW, Park JH, Baek SY, Kim DD, Kim HC, Cho HJ. Doxorubicin-loaded poly(lactic-co-glycolic acid) microspheres prepared using the solid-in-oil-in-water method for the transarterial chemoembolization of a liver tumor. *Colloids Surf B Biointerfaces* 2015 2015-08-01;132:305–312.
18. Liang YJ, Yu H, Feng G, Zhuang L, Xi W, Ma M, et al. High-Performance Poly(lactic-co-glycolic acid)-Magnetic Microspheres Prepared by Rotating Membrane Emulsification for Transcatheter Arterial Embolization and Magnetic Ablation in VX2 Liver Tumors. *ACS Appl Mater Interfaces* 2017 2017-12-20;9(50):43478–43489.
19. Shi X, Wang Y, Varshney RR, Ren L, Gong Y, Wang DA. Microsphere-based drug releasing scaffolds for inducing osteogenesis of human mesenchymal stem cells in vitro. *EUR J PHARM SCI* 2010 2010-01-31;39(1–3):59–67.
20. Chen JH, Lin YC, Huang YS, Chen TJ, Lin WY, Han KW. Induction of VX2 carcinoma in rabbit liver: comparison of two inoculation methods. *Lab Anim* 2004 2004-01-01;38(1):79–84.
21. Qian K, Ma Y, Wan J, Geng S, Li H, Fu Q, et al. The studies about doxorubicin-loaded p(N-isopropyl-acrylamide-co-butyl methylacrylate) temperature-sensitive nanogel dispersions on the application in TACE therapies for rabbit VX2 liver tumor. *J CONTROL RELEASE* 2015 2015-08-28;212:41–49.

22. Vogl TJ, Schroeder H, Trapp M, Straub R, Schuster A, Schuster M, et al. [Multi-sequential arterial chemoembolization of advanced hepatocellular carcinomas: computerized tomography follow-up parameters for evaluating effectiveness of therapy]. *Rofo* 2000 2000-01-01;172(1):43–50.
23. Chen MS, Li JQ, Zhang YQ, Lu LX, Zhang WZ, Yuan YF, et al. High-dose iodized oil transcatheter arterial chemoembolization for patients with large hepatocellular carcinoma. *World J Gastroenterol*. 2002;8(1):74–8. 2002-02-01.
24. Kamada K, Nakanishi T, Kitamoto M, Aikata H, Kawakami Y, Ito K, et al. Long-term prognosis of patients undergoing transcatheter arterial chemoembolization for unresectable hepatocellular carcinoma: comparison of cisplatin lipiodol suspension and doxorubicin hydrochloride emulsion. *J VASC INTERV RADIOL* 2001 2001-07-01;12(7):847–854.
25. Poyanli A, Rozanes I, Acunas B, Sencer S. Palliative treatment of hepatocellular carcinoma by chemoembolization. *ACTA RADIOL* 2001 2001-11-01;42(6):602–607.
26. Pleguezuelo M, Marelli L, Misseri M, Germani G, Calvaruso V, Xirouchakis E, et al. TACE versus TAE as therapy for hepatocellular carcinoma. *Expert Rev Anticancer Ther* 2008 2008-10-01;8(10):1623–1641.
27. Gilkes DM, Semenza GL, Wirtz D. Hypoxia and the extracellular matrix: drivers of tumour metastasis. *NAT REV CANCER* 2014 2014-06-01;14(6):430–439.
28. Li J, Xu Y, Long XD, Wang W, Jiao HK, Mei Z, et al. Cbx4 governs HIF-1alpha to potentiate angiogenesis of hepatocellular carcinoma by its SUMO E3 ligase activity. *CANCER CELL* 2014 2014-01-13;25(1):118–131.
29. Lin WH, Yeh SH, Yeh KH, Chen KW, Cheng YW, Su TH, et al. Hypoxia-activated cytotoxic agent tirapazamine enhances hepatic artery ligation-induced killing of liver tumor in HBx transgenic mice. *Proc Natl Acad Sci U S A* 2016 2016-10-18;113(42):11937–11942.
30. Zhang L, Zhang J, Ling Y, Chen C, Liang A, Peng Y, et al. Sustained release of melatonin from poly (lactic-co-glycolic acid) (PLGA) microspheres to induce osteogenesis of human mesenchymal stem cells in vitro. *J PINEAL RES* 2013 2013-01-01;54(1):24–32.
31. Hagiwara A, Sakakura C, Tsujimoto H, Imanishi T, Ohgaki M, Yamasaki J, et al. Selective delivery of 5-fluorouracil (5-FU) to i.p. tissues using 5-FU microspheres in rats. *Anticancer Drugs* 1997 1997-02-01;8(2):182–188.
32. Giunchedi P, Maestri M, Gavini E, Dionigi P, Rassu G. Transarterial chemoembolization of hepatocellular carcinoma—agents and drugs: an overview. Part 2. *Expert Opin Drug Deliv* 2013 2013-06-01;10(6):799–810.
33. Bastian P, Bartkowski R, Kohler H, Kissel T. Chemo-embolization of experimental liver metastases. Part I: distribution of biodegradable microspheres of different sizes in an animal model for the locoregional therapy. *EUR J PHARM BIOPHARM* 1998 1998-11-01;46(3):243–254.
34. Liu Q, Qian Y, Li P, Zhang S, Liu J, Sun X, et al. (131)I-Labeled Copper Sulfide-Loaded Microspheres to Treat Hepatic Tumors via Hepatic Artery Embolization. *THERANOSTICS* 2018 2018-01-20;8(3):785–799.

35. Giaccia A, Siim BG, Johnson RS. HIF-1 as a target for drug development. NAT REV DRUG DISCOV 2003 2003-10-01;2(10):803–811.
36. Ferrara N. VEGF and the quest for tumour angiogenesis factors. NAT REV CANCER 2002 2002-10-01;2(10):795–803.
37. Wu Q, Zhou W, Yin S, Zhou Y, Chen T, Qian J, et al. Blocking Triggering Receptor Expressed on Myeloid Cells-1-Positive Tumor-Associated Macrophages Induced by Hypoxia Reverses Immunosuppression and Anti-Programmed Cell Death Ligand 1 Resistance in Liver Cancer. HEPATOLOGY 2019 2019-07-01;70(1):198–214.

Figures

Figure 1

Fabrication process and particle characterization of SOR-CAT-PLGA MSs. (A) Schematic of the experimental rationale and fabrication process of SOR-CAT-PLGA MSs. (B) SEM and TEM images of SOR-CAT-PLGA MSs. (C) Size distribution of SOR-CAT-PLGA MSs. (D) Cumulative release of SOR and CAT from SOR-CAT-PLGA MSs. (E) Oxygen generation in H₂O₂ solutions with different microspheres. The data are presented as the means ± SDs (n = 3).

Figure 2

In vitro assessment of SOR-CAT-PLGA MS-mediated cell cytotoxicity. (A) HepG2 cell viability after incubation with different microspheres. (B) and (C) Flow cytogram presenting the results of the apoptosis assay based on Annexin V-FITC and PI staining of HepG2 cells after treatment with different microspheres. The results are presented as the means $\pm$ SDs ($n = 3$). The asterisks indicate significant differences (** $P < 0.01$).

Figure 3

Figure 4

Intraarterial injection of SOR-CAT-PLGA MSs via the hepatic artery and representative angiography of rabbit VX2 liver tumors before and after TACE. (A) The femoral artery was dissociated, and the distal femoral artery was ligated with one suture. (B) After the administration of the microspheres, the proximal femoral artery was ligated. (C) Selective placement of a 2.7F microcatheter (white arrow) and

hypervascular tumor staining (black arrow) in the left lobe of the liver before embolization. (D) Postembolization images showing that the feeding arteries of the tumor (black arrow) were successfully occluded by the microspheres.

Figure 5

Perfusion CT images of liver tumors in the control (sham operation), PLGA MSs, SOR-PLGA MSs, CAT-PLGA MSs and SOR-CAT-PLGA MSs groups before treatment and after treatment. (A) The red and black

circles in the perfusion CT images indicate liver tumors. The length of the scale bar on the right side is 1 cm. (B-E) Blood flow (BF), blood volume (BV), mean transit time (MTT) and permeability surface (PS) area product values in the different groups. The data are presented as the means $\pm$ SDs (n = 3). The asterisks indicate significant differences (*P < 0.05, **P < 0.01).

Figure 6

Therapeutic efficacy in the rabbit VX2 liver tumor model. (A) Photographs of the tumors dissected after different treatments. The red circles in the photographs of the dissected tumors indicate liver tumors. Notably, metastatic tumors in the lungs of the control group are indicated by the red arrows. (B) Tumor volumes in the different groups. (C) Tumor weights in the different groups. The data are presented as the means $\pm$ SDs ($n = 3$). The asterisks indicate significant differences (** $P < 0.01$, *** $P < 0.001$).

Figure 7

Histopathology and immunohistochemistry following different treatments. (A) H&E-stained images of dissected liver tumors, lungs, hearts and kidneys in the control, PLGA MSs, SOR-PLGA MSs, CAT-PLGA MSs and SOR-CAT-PLGA MSs groups. Microspheres were seen in tumor blood vessels and were surrounded by necrotic tissue; microspheres were not seen in normal liver tissues. Red arrows, microspheres. The scale bar represents 100 μm . (B) Representative immunofluorescence images of tumor slices from the various treatment groups stained with HIF-1 α , VEGF, PD-L1 and CD8 antibody. The scale bar represents 100 μm . (C) TUNEL assay and Ki-67 staining of sectioned tumor tissues collected after the completion of all doses in the different groups. The scale bar represents 100 μm . (D) Serum IFN- γ levels after different treatments determined by the ELISA assay. (E) The corresponding apoptosis index in the TUNEL assay of tumor tissues. (F) The proliferation index in the Ki-67 staining assay of tumor tissues. The data are expressed as the means $\pm$ SDs ($n = 3$). The asterisks indicate significant differences (** $P < 0.01$, *** $P < 0.001$).

Figure 8

Biochemical tests and Kaplan-Meier survival analysis of VX2 liver tumor-bearing rabbits during the experiment. (A) ALT levels. (B) AST levels. (C) TBIL levels. (D) CRE values. (E) BUN values. (F) Kaplan-Meier survival analysis. The data are expressed as the means $\pm$ SDs ($n = 3$).