

Original Article

Effects of interventional therapy with norcantharidin-loaded gelatin microspheres on hepatocellular carcinoma in mice and its mechanism

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Abstract: The object of this study was to investigate the effects of interventional therapy with norcantharidin-loaded gelatin microspheres (NCTD/GMSs) via hepatic artery on hepatoma in mice. NCTD/GMSs were prepared by an emulsification-coacervation method. Tumor-bearing mice were divided into three groups and infused from the proper hepatic artery, which were control group receiving physiological saline, NCTD and NCTD/GMSs group. The concentration of the released NCTD was measured by UV-vis spectroscopy. Tumor volumes and weight were detected pre-injection and 1 to 5 weeks after NCTD/GMSs or NCTD treatment. MTT assay was used to detect the cell viability in HepG2 cells. Flow cytometry analysis was employed to measure apoptosis of HepG2 cells. Bcl-2, Bax, caspase-3 and caspase-9 levels were analyzed by Western blot. In vitro drug release kinetics indicated that there was an initial burst release followed by a slow and sustained release of NCTD from GMSs. The tumor volume of the mice in the NCTD/GMSs group was smaller than those in NCTD group and control group. Importantly, NCTD/GMSs promoted HepG2 cell death by triggering apoptosis and inhibited the ability of HepG2 cell invasion. In addition, NCTD/GMSs induced the reduction of Bcl-2 and increased Bax, caspase-3 and caspase-9 in HepG2 cells. Interventional therapy with NCTD/GMSs could yield preferable therapeutic effects on HCC in mice. This anti-tumor efficacy may be associated with the sustained releasing of NCTD. The mechanisms of NCTD antitumor effects seem to be mediated by inducing a caspase-3-dependent apoptosis.

Keywords: Interventional therapy, norcantharidin-loaded gelatin microspheres, hepatocellular carcinoma, apoptosis

Introduction

Hepatocellular carcinoma (HCC) is a major health problem worldwide. It is the sixth most common cancer and the third most common cause of cancer-related death [1]. Additionally, HCC is highly fatal, with an overall ratio of mortality to incidence of 0.93 [2]. HCC is the leading cause of death among patients with liver cirrhosis [3, 4]. Transcatheter arterial embolization (TAE) is one of the therapeutic procedures of choice for HCC, which is done using a catheter inserted into the hepatic artery that injects embolizing agents to block blood flow to the tumor without infusion of chemotherapeutic agents [5]. A variety of embolic agents have been developed to improve the therapeutic effect of TAE, such as Gelfoam powder, polyvi-

nyl alcohol (PVA) and super absorbent polymer (SAP) microsphere and so on [6]. These materials facilitate the sustained release of anticancer agents.

Natural hydropolymers are perhaps the best choice available as the encapsulating material. Gelatin is a nature polymer that is biodegradable and biocompatible, which makes gelatin-based nanoparticles a promising carrier system for drug delivery [7]. For example, using gelatin sheets conjugated to cisplatin, Konishi et al. found that gelatin degradation correlated with cisplatin elution [8, 9]. Moreover, Nitta et al. have already applied the GMSs in clinical cases and have reported their utility and good anticancer effects for various tumors [10, 11]. Thus, repeated TAE using GMSs could be performed for HCC.

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Norcantharidin (NCTD, exo-7-oxabicyclo-[2.2.1] heptane-2,3-dicarboxylic anhydride) is synthesized from cantharidin, which is an active constituent obtained from the dried body of the Chinese blister beetle (mylabris), commonly used in traditional Chinese medicine [7]. It was found that NCTD possesses anti-tumor properties and has the advantage of inducing the production of leucocytes [12]. The reported anti-cancer effects of NCTD include interruption of DNA synthesis, retardation of cell cycle progression, and induction of apoptosis via regulating p53 and Bcl-2 gene expression [13, 14]. Clinical studies showed that NCTD was effective against primary liver cancer. However, the significant side-effect of NCTD is irritation to the urinary organs, thereby limiting its use [15]. Norihisa Nitta demonstrated that the side-effect of cisplatin could be overcome by encapsulating the drug in GMSs and had few adverse impacts on hepatic and renal function [11]. Therefore, it is very important to find a new drug delivery system for NCTD to reduce these side effects. The object of this study was to confirm prolonged NCTD release from NCTD-loaded GMSs and their improved anticancer efficacy *in vitro* and *in vivo*.

Materials and methods

Materials

Acidic gelatin (molecular weight, 99 kDa) with an isoelectric point of 5.0 was supplied by Nitta Gelatin Co., Ltd. (Osaka, Japan). NCTD (exo-7-oxabicyclo-[2.2.1] heptane-2,3-dicarboxylic anhydride) was purchased from Nanjing Zelang Medical Technology Co., Ltd (Nanjing, China), and was dissolved in DMEM with a stock concentration of 2 mM. All other chemicals used were of the highest purity available commercially.

All BALB/c nude mice were purchased from Shanghai Laboratory Animal Center (SLAC) for pharmacodynamic study. All animals were housed in a temperature-controlled facility and provided with standard diet and water *ad libitum*. All experiments followed the recommendations of the local animal protection legislation and approval (SYXK 2005-0008) was obtained from the Ethics Committee of The First Affiliated Hospital of Zhengzhou University for conducting the animal study.

Human hepatoblastoma cell lines HepG2 was purchased from Cell Bank of the Chinese Academy of Sciences Shanghai Institute of Cell Biology (Shanghai, China) and maintained in high-glucose DMEM (HyClone/Thermo Fisher Scientific, Beijing, China) supplemented with 10% heat inactivated fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd, Hangzhou, China). Cells were incubated in a humidified 5% CO₂ atmosphere at 37°C.

Preparation of GMSs and NCTD-loaded GMSs

GMSs were prepared according to the method of Tabata and Ikada with some modifications [16]. The size of GMSs was from 50 to 100 µm. The GMSs were cross-linked by a thermal dehydration method in an oven (140°C, 12 h, 0 atm). The cross-linked GMSs that were used in this experiment were designed to degrade completely in the extravascular tissue within 14 days. The GMSs (1 mg) were immersed in 50 ml of the NCTD solution at 38°C to allow conjugation to NCTD. The sample was then repeatedly washed in double-distilled water (DDW) and centrifuged (seven times) to remove the uncombined cisplatin from the GMSs before freeze-drying.

Drug release *in vitro*

An *in vitro* release test measuring NCTD release from NCTD-conjugated GMSs was performed according to the method of Jinlong Ma with some modifications [17]. In brief, samples equivalent to about 4 mg NCTD were directly added to dialysis tubes (MWCO 3500, Shanghai Green Bird Science and Technology Development Co., Ltd. Shanghai, China), which were then immersed in dissolution medium (pH 7.4 PBS phosphate buffer saline, 150 ml) in a flask with continuous stirring at 100 rpm. The temperature was maintained at 37°C in a water bath. At predetermined intervals (0, 1, 2, 3, 6, 12, 24, 48, 120, 240 and 360 hours), 1 ml aliquots were withdrawn and replaced with fresh medium. The sample solutions were passed through a 0.22 µm polyamides filtermembrane and then analyzed by HPLC. Each sample was analyzed in triplicate.

Drug content determination *in vivo*

The NCTD concentration within liver parenchyma was measured using the atomic absorption

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spectrophotometry method (NAC Co., Ltd., Tokyo, Japan). For measurement of the NCTD concentration in the livers, five mice from each group were sacrificed immediately after the drug administration on days 1, 2, 3, and 7 using an intracardial overdose of pentobarbital (Nembutal; Dainippon Sumitomo Pharma Co., Ltd., Tokyo, Japan). Subsequently livers were surgically removed, homogenized and conditioned for assessment.

Immunohistochemical staining

Control untreated, NCTD treated and NCTD/GMSs treated mice were sacrificed to collect the tissue samples after the seven-day treatment. Tissues were post-fixed in 10% formalin (Hangzhou Norming Biological Technology Co., Ltd., Hangzhou, China) for 15 min, washed twice in phosphate-buffered saline and then processed for immunostaining with rabbit anti-cleaved caspase-3 polyclonal antibody (1:1,000; Millipore, Germany). This was followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3,000; cat. no. ta140003; OriGene Technologies, Inc., Beijing, China) and then 3, 3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis, MO, USA). Subsequently, the sections were visualized under a light microscope (LSM-510; Carl Zeiss AG, Oberkochen, Germany).

In vivo therapeutic study

All BALB/c nude mice aged 6-7 weeks and weighing 20-22 g were transplanted with hepatoma (HepG2 cells) in accordance with the technique previously described by Xiong et al. [18]. The mice were divided into three groups of 75. With the aid of a binocular microscope (YZ-20T4, Suzhou Medical Device, China) for laparotomy, a silastic microcatheter (ID 0.25 mm, OD 0.4 mm) was retrogradely inserted into the gastroduodenal artery leading to the hepatic artery. The 3 groups were administered with normal saline, NCTD and NCTD/GMSs, respectively, by injection through the microcatheter. Each experimental group consisted of 25 mice. Animals were sacrificed and tumor tissues were resected at different time intervals. The tumor weight was measured and the tumor volume was calculated according to the formula: Tumour volume (mm^3) = $(wh^2)/2$, where w is the

longest axis (mm) and h is the shortest axis (mm) [19].

MTT viability assay

Cell viability was measured by 3-[4,5-dimethylthiazol-2-thiazolyl]-2,5-diphenyl-tetrazolium bromide (MTT) assay, based on mitochondrial conversion of MTT from soluble tetrazolium salt into an insoluble colored formazan precipitate, which was dissolved in DMSO and quantified by spectrophotometry (ThermoMultiskan MK3; ThermoLabsystems, Shanghai, China) to obtain optical density (OD) values. HCC cells were plated in 96-well culture clusters (Costar, Cambridge, MA) at a density of 5000-6000 cells/well in 100 μL medium. Serial dilutions were made from stock solution of drugs to desired concentrations. All experimental concentrations were replicated in triplicate. Four hours before desired time points, 10 μL of 10 mg/mL MTT was added. After incubation for 4 h, the plates were depleted and 100 μL DMSO was added. The percentages of absorbance relative to those of untreated control samples were plotted as a linear function of drug concentration. Inhibition of cell viability was measured by percentage of viable cells relative to the control: % inhibition = $100\% \times \text{ODT}/\text{ODC}$, where ODT is the average OD value of the treated samples and ODC is the average OD value of the control samples.

Flow cytometry apoptosis assays

Apoptosis in cells was examined by flow cytometry using a commercial TUNEL kit (APO-DIRECT; BD Biosciences) according to the manufacturer's instructions. Cells treated with physiological saline, NCTD alone or NCTD/GMSs were collected, washed, centrifuged, and resuspended in 1% (w/v) paraformaldehyde in PBS (pH 7.4). Cells were then kept on ice for 60 min, washed in PBS, centrifuged, and fixed in 70% (v/v) ice-cold ethanol at -20°C for 12 to 18 h, before being stained with terminal deoxynucleotidyltransferase (TdT) and fluorescein isothiocyanate (FITC)-labeled dUTP (FITCdUTP) for 2 h at 37°C . After washing with PBS, cells were resuspended in 0.5 mL of propidium iodide (PI) and RNase containing buffer (5 $\mu\text{g}/\text{mL}$ PI, 200 $\mu\text{g}/\text{mL}$ RNase). Cells were then incubated in the dark for 30 min at room temperature prior

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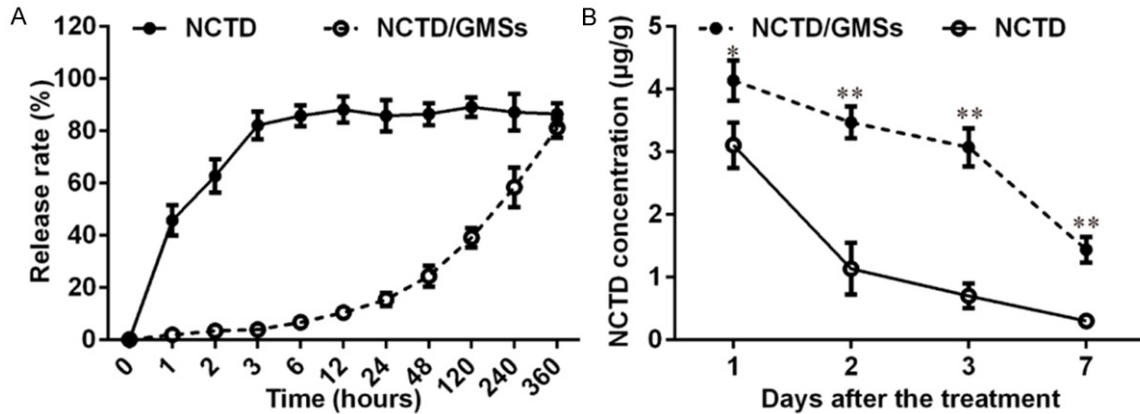


Figure 1. Release rate from the norcantharidin-conjugated gelatin microspheres (GMSs). A: Release profiles of microspheres: NCTD/GMSs and NCTD. Most NCTD in the GMSs was not released in the first 24 h until 48 h. In fact, most of the NCTD bonded to the GMSs strongly throughout the entire 360-h period. B: In vivo assessment of NCTD concentrations in the liver parenchyma measured with atomic absorption spectrophotometry method after treatment with NCTD-loaded GMSs. All values are presented as mean \pm standard error based on at least three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. NCTD group.

to analysis by flow cytometry. The percentage of TdT-mediated fluorescent cells was measured by flow cytometry using band pass filters of 525 ± 25 nm for FITC and 610 ± 25 nm for PI (7, 32) in a BD LSR II system (BD Biosciences) equipped with the DiVA software (version 4.1.2; BD Biosciences).

Tumor cell invasion assay

Cell invasion was assayed using a Millicell-transwell chamber (Millipore, USA), with or without Matrigel (BD Biosciences, USA). For the invasion assay, a transwell chamber was placed into a 24-well plate that was precoated with a 5 mL mixture of BD Matrigel and DMEM (1:1, v/v) and was incubated for 40 min at 37°C . In transwell assays, 2×10^4 tumor cells in 0.1 mL of media without FBS were plated in the upper chamber. In the lower chamber, 0.6 mL of the medium with 10% FBS was added. After 48 h of incubation, cells on the upper surface of the Millicell chambers, noninvasive cells, were scraped with a cotton swab. Tumor cells on the bottom surface of the membrane were fixed in 90% ethanol for 20 min and stained with 0.1% crystal violet for 15 min.

Statistical analysis

Data are reported as mean \pm standard deviation (SD). Statistical significance was determined using Double-sided Student's t test. Multiple groups were analyzed using ANOVA. A

p value of less than 0.05 was considered to be significant.

Results

Drug release of NCTD in vitro and assessment of NCTD concentration in vivo

To exert the therapeutic effect, the encapsulated NCTD within GMSs has to be released. It can be seen that bulk drug of NCTD was released quickly and completely in 3 h. NCTD/GMSs released nearly 60% of its total amount of drug in 10 days, and then reached its equilibrium in 15 days (Figure 1A). The release pot of NCTD from NCTD/GMSs indicates that the NCTD/GMSs were released in a sustained manner and slowly.

The concentrations of NCTD in liver parenchymal tissues after administration of NCTD/GMSs and NCTD are shown in Figure 1B. Our results with the measurement over time of the liver parenchymal NCTD concentrations prove a completely different distribution of the NCTD/GMSs compared to the simple NCTD treatment group. Embolization leads to a much longer local persistence of the drug-loaded GMSs in the liver parenchyma. The slow decrease of NCTD concentration over time suggests indirectly that the GMSs are also slowly degraded and in parallel release the active principle NCTD. Our findings of the in vivo therapeutic study regarding the anti-cancer efficacy of the

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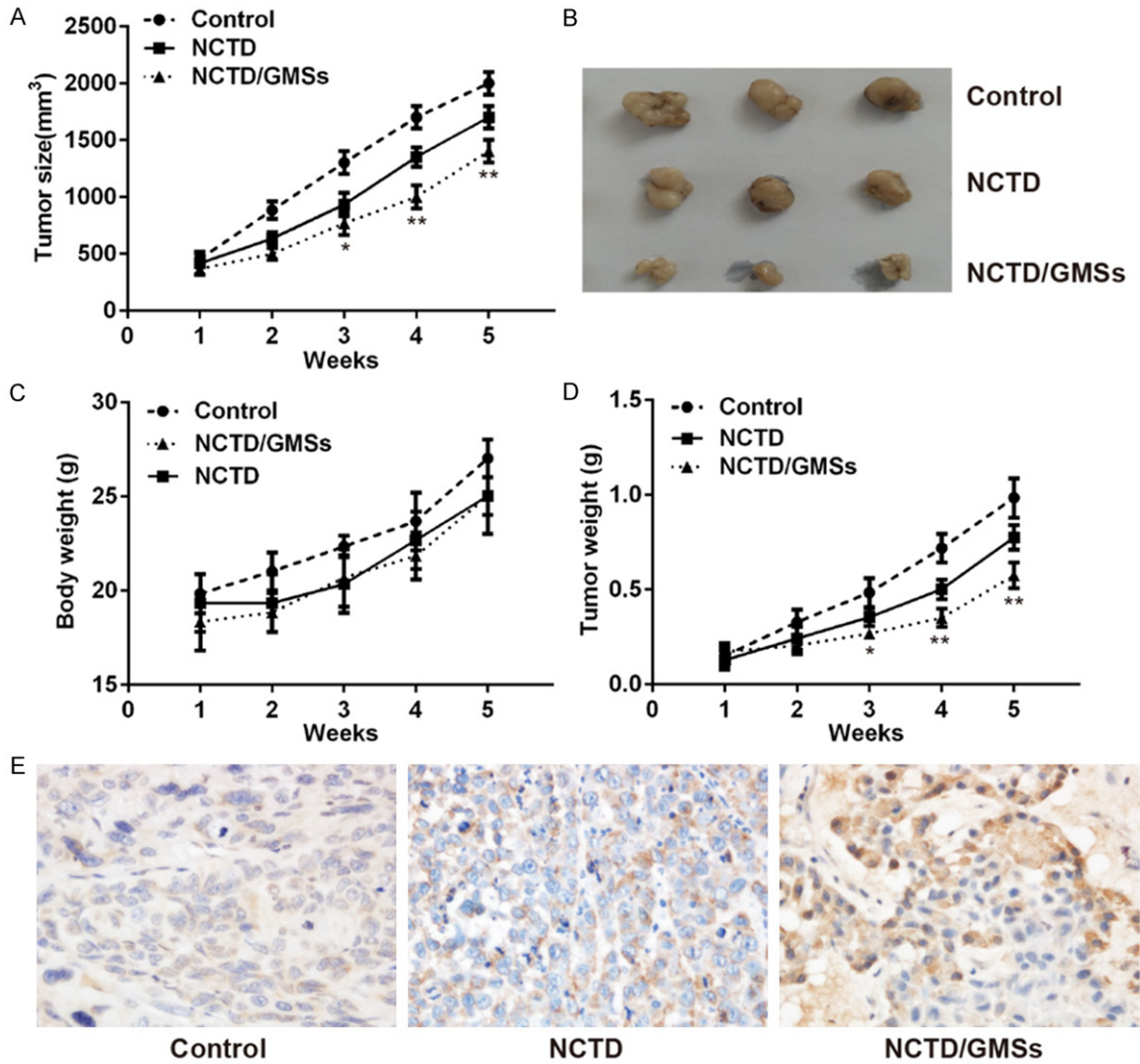


Figure 2. NCTD/GMSs inhibits HCC cell growth in vivo. All BALB/c nude mice aged 6-7 weeks and weighing 20-22 g were randomized into 3 different groups (normal saline, NCTD and NCTD/GMSs) of 25 mice each group prior to transplant with 2×10^6 hepatoma (HepG2 cells) in a volume 0.1 ml culture medium. A: The volume of subcutaneous tumors (in mm³, recorded every week) in these three groups was calculated and compared. B: Representative images of the extracted tumors after treatment with NCTD and NCTD/GMSs. C: The weekly body weights of the mice in these three groups were measured. D: Tumor weight. The mice in each group were sacrificed every week after treatment to collect the tissue samples. Freshly resected specimens were collected and the weight of the tumors was measured. E: The expression of apoptosis associated protein, cleaved caspase-3, was observed by immunohistochemistry. Data were expressed as mean \pm SD. *P < 0.05, **P < 0.01 vs. Control group.

NCTD/GMSs are certainly a preliminary proof of hypothesis regarding biodegradation and drug-release over time.

NCTD inhibits HepG2 cell in vivo growth

To examine the influence of tumor growth after embolization, we used mice HepG2 cell xenograft model. Results in **Figure 2A, 2B** demonstrated that the xenograft volume in NCTD or

NCTD/GMSs group was markedly decreased compared to control group, and more obvious tumor inhibition in NCTD/GMSs group in comparison with control or NCTD group. In this work, no significant body weight variation was found between the mice in NCTD treatment group, NCTD/GMSs group and control group (**Figure 2C**). To assess the anti-tumor activity of the NCTD/GMSs treatments, tumor weight were measured. The tumor weight in NCTD or

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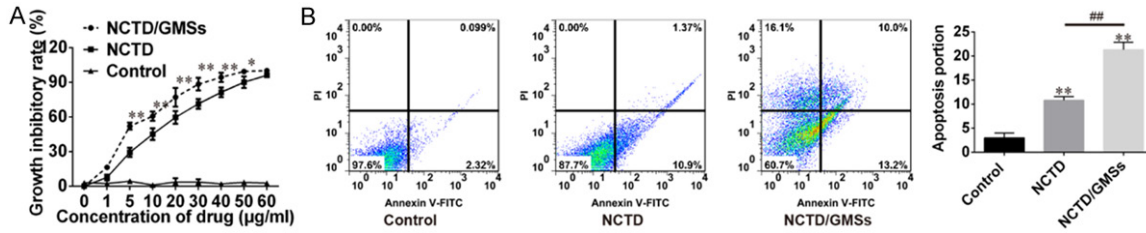


Figure 3. Viability and apoptosis of HepG2 cells upon NCTD/GMSs and NCTD treatment. Cells were treated with negative control and different concentration of NCTD/GMSs and NCTD (1-60 µg/ml). A: Cell viability was observed by MTT assay. B: Apoptosis was examined by flow cytometry. All values are presented as mean ± standard error based on at least three independent experiments. *P < 0.05, **P < 0.01 vs. NCTD group.

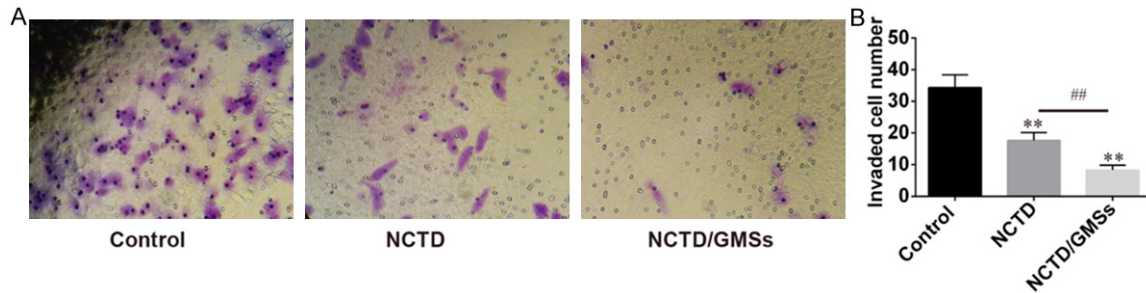


Figure 4. Inhibitory effect of NCTD/GMSs on invasion of HCC cells in vitro. A: Representative histomorphological of HepG2 cells (original magnification, × 200) with crystal violet staining under an optic microscope. B: The invaded number of HepG2 cells in control group, NCTD/GMSs group and NCTD group. The invaded number of HepG2 cells in NCTD/GMSs or NCTD group was much less than that of control group, and NCTD/GMSs significantly inhibited significantly invasion of HepG2 cells compared to NCTD treatment alone. All values are presented as mean ± standard error based on at least three independent experiments. **P < 0.01 vs. control group, ##P < 0.01 vs. NCTD group.

NCTD/GMSs group was significantly lower than that of the control group (**Figure 2D**).

To investigate the anti-apoptotic effects of NCTD/GMSs, we detected expression of cleaved-caspase 3 in tumors using immunohistochemical staining. As shown in **Figure 2E**, a significantly higher level of cleaved-caspase-3 was observed in NCTD/GMSs-treated mice compared with the NCTD treatment group or the control group, indicating the anti-tumor effect of NCTD/GMSs is partly induced by apoptosis. Thus, we believed that NCTD/GMSs inhibited growth of the xenografts effectively and safely in vivo.

NCTD/GMSs enhances viability inhibition and apoptosis induction in HCC cells

We next tested the potential cytotoxicity of NCTD against HCC cells using an MTT assay. As shown in **Figure 3A**, the cell viability of HepG2 was significantly inhibited by NCTD/GMSs in a time dependent manner. Further, NCTD/GMSs

showed significantly enhanced cytotoxicity when compared to NCTD treatment alone.

To determine whether NCTD enhanced cell viability inhibition was mediated through apoptotic cell death induction, we next performed flow cytometry assays in HepG2 cell lines. We found that the apoptosis of cells treated with NCTD alone was markedly decreased compared with control group. However, exposure to NCTD/GMSs for 48 h resulted in massive induction of apoptosis in HepG2 cells (**Figure 3B**).

NCTD/GMSs inhibits invasion of HepG2 cells in vitro

The transwell plates were used to measure the in vitro ability of HepG2 cells to invade a basement membrane matrix. We found that HepG2 cells in control group passed more of the Transwell membrane and had more invasive capability than NCTD or NCTD/GMSs group in vitro (**Figure 4A**); the number of invaded tumor cells in NCTD or NCTD/GMSs group markedly

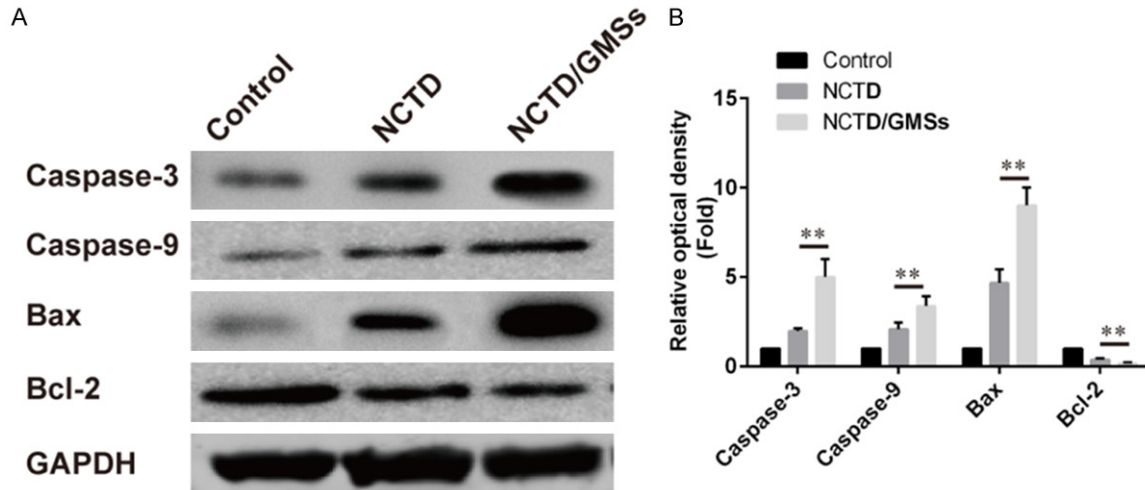


Figure 5. Expression of apoptosis-related proteins in HepG2 cells treated with NCTD/GMSs or NCTD. A, B: After treatment with NCTD and NCTD/GMSs in HepG2 cells, the expression levels of Bax, caspase-3 and caspase-9 proteins were increased significantly, with decreased expression of Bcl-2. Representative images (left) and quantification (right) of western blot analysis of apoptosis-related proteins in HepG2 cell lines. All values are presented as mean \pm SD based on at least three independent experiments. **P < 0.01 vs. Control.

decreased (**Figure 4B**). Further, NCTD/GMSs significantly inhibited invasion of HepG2 cells in vitro when compared to NCTD treatment alone.

NCTD/GMSs induces apoptosis in cultured HCC cells

Great progress has been made in research into the genetic regulation of apoptosis in recent years. Apoptosis-related genes and their regulatory molecules regulate the apoptosis of cells. Thus, changing the genetic regulation of apoptosis may be an important mechanism by which many anti-cancer agents inhibit tumor growth. After treatment with NCTD or NCTD/GMSs, the expression of the anti-apoptotic protein Bcl-2 was significantly decreased, with increased expression of caspase-9 and caspase-3 and Bax when compared with the NCTD group or control group in HepG2 cells (**Figure 5A, 5B**). This may be one of the mechanisms by which NCTD/GMSs induces apoptosis of the HCC cells in vitro.

Discussion

In the present study, NCTD/GMSs were prepared by coacervation process and studied its anticancer efficacy in vivo and vitro. We found that NCTD/GMSs were released in a sustained manner and slowly. Furthermore, we demonstrated that NCTD/GMSs inhibited significantly

proliferation, invasion of HepG2 cells in vitro and then caused down-regulation of Bcl-2, up-regulation of Bax and activation of caspase-3 and caspase-9, and finally induced the apoptosis. More importantly, we found that NCTD/GMSs inhibited tumor xenografts' growth. Based on these results, it can be concluded that the GMSs is an ideal way to deliver NCTD because of its high loading efficiency and superior efficacy in cancer cell line and animal model.

Several reports have demonstrated that NCTD inhibited the proliferation and growth of a variety of human tumor cells and is used in clinic to treat human cancers, e.g., hepatic, gastric, colorectal and ovarian carcinoma because of its effective anticancer activity, fewer side effects and leukocytosis [7, 12, 20-26]. However, the significant side-effects of NCTD, including cardiac and renal damage, limit its use in clinical situations. To obtain a safer and more effective NCTD treatment, many new alternative formulations have been studied to improve the targeted delivery, such as N-trimethyl chitosan nanoparticles [27], lipid microspheres, polycaprolactone microspheres [28], NCTD-polymer conjugates [29] and gelatin microspheres (GMSs) [30]. However, most of these dosage forms have been found to be clinically unsuccessful because of lower encapsulation efficiency and poor physicochemical sta-

bility. Among them, Gelatin is a biodegradable polymer that exhibits excellent biocompatibility, plasticity, and adhesiveness, which have been used for the effective delivery of salmon calcitonin to the pulmonary system [31]. Hence, in this study, GMSs was combined with NCTD to form microspheres for use in chemoembolization. In the present study, GMSs were generated as drug carriers (NCTD) to be administered via injection for the treatment of liver cancer. We found that the release of NCTD from the GMSs was initially burst followed in a sustained manner. GMSs lead to a much longer local persistence of the drug-loaded GMSs in the liver vessels and parenchyma. The slow decrease of NCTD concentration over time suggests indirectly that the GMSs are also slowly degraded and in parallel release the active principle NCTD. Similar to this finding, Konishi et al. reported that degradation of gelatin is related to sustained cisplatin release [9]. Our findings of the in vivo therapeutic study regarding the anti-cancer efficacy of the NCTD-loaded GMSs are certainly a preliminary proof of hypothesis regarding biodegradation and drug-release over time. Nevertheless, this needs to be confirmed in the future with the help of radiotracer labelled GMSs and NCTD.

Recent experimental studies have revealed that NCTD inhibits the growth of the human tumor cell lines HepG2, HT29, CT26, K562, and HL-60 by inducing apoptosis [12, 14, 22, 32, 33]. In the present study, both NCTD and NCTD/GMSs treatment showed cytotoxicity on HepG2 cells in a concentration dependent manner. Further, NCTD/GMSs showed significantly enhanced cytotoxicity when compared to NCTD treatment alone. In addition, apoptosis assays indicated that the cytotoxicity of NCTD/GMSs on HepG2 cells is induced by apoptosis. This is also consistent with the report about the inducement of apoptosis in tumor cells by NCTD. It has been reported that the cell apoptosis is regulated by cell apoptosis-related genes and proteins. Bcl-2 and Bax are apoptosis-related proteins [34]. In addition, caspases and especially caspase-3, a family of cysteine proteases that are activated during the apoptotic processes, are crucial mediators in the apoptotic pathway [35]. In this study, after treatment with NCTD/GMSs, the expression of Bcl-2 proteins of the tumor cells were decreased significantly, with increased expression of Bax

when compared with the control group, as measured by Western blot. Furthermore, we also found that NCTD/GMSs accelerates the activity of caspase-9 and caspase-3. This may be one of the mechanisms by which NCTD induces apoptosis of the HCC cells in vitro.

In summary, NCTD-loaded GMSs administered into the liver artery allow achieving more pronounced and prolonged local parenchymal NCTD concentrations offering the advantage of an increased anti-cancer effect with reduced toxicity compared to NCTD alone or controls. Indirectly the breakdown of the GMSs and slow-release of NCTD could thus be demonstrated. We still need to ascertain, however, whether NCTD released from NCTD/GMSs is an effective anticancer agent.

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Disclosure of conflict of interest

None.

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