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Pharmacokinetics and pharmacodynamics evaluation of a thermosensitive chitosan based hydrogel containing liposomal doxorubicin

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ABSTRACT

In situ gelling thermosensitive hydrogel formulation has been reported to effectively sustain the release of macromolecules for a long time. However, the low-molecular-weight hydrophilic drugs, such as doxorubicin (DOX), are not suitable for intratumoral injection because the release will complete within one day. In this study, liposomal doxorubicin (LipDOX) was added into the hydrogel to form a novel thermosensitive formulation which prolonged the sustained release of DOX. DOX + C/GP (doxorubicin in chitosan/β-glycerophosphate) was prepared to compare with LipDOX + C/GP (liposomal doxorubicin in chitosan/β-glycerophosphate hydrogel). The particle size of DOX-loaded liposome was 94.2 nm and the encapsulation efficiency of DOX was near 98%. *In vitro* release experiments, the release of DOX in both DOX + C/GP group and LipDOX + C/GP group increased along with the increasing pH of buffers. However, the LipDOX + C/GP group with lower initial burst release had a much longer releasing duration than DOX + C/GP group (21 days *vs.* 24 h). *In vitro* and *in vivo* antitumor experiments demonstrated that LipDOX + C/GP group had better antineoplastic effect and less toxicity than DOX + C/GP group. Pharmacokinetics study showed LipDOX + C/GP exhibited a higher AUC_{0-t} and longer MRT than DOX + C/GP in blood and tumor, which indicated that LipDOX + C/GP obtained an enhanced antitumor activity compared with DOX + C/GP. In addition, the lower distribution index (the ratio of AUC of normal tissue/AUC of tumor tissue) of the LipDOX + C/GP implied it had lower toxicity to normal tissues than DOX + C/GP. Therefore, the novel thermosensitive hydrogel formulation was potential for clinical application in cancer treatment.

1. Introduction

Hepatocellular carcinoma (HCC), the major histological subtype of primary liver cancer, is the third commonest cause of cancer-related death worldwide (Han, 2012). Surgical resection is the preferred treatment of HCC, but it is only applicable for 10–20% of HCC patients. The majority of HCC patients are not suitable for surgery partly because of hepatitis or cirrhosis, or extrahepatic metastases, or the lesion close to vital structures (Wedemeyer, 2014). If the HCC patients are not operable, chemotherapy is considered as an alternative option. Doxorubicin (DOX), one of the most commonly used anticancer agents, shows great anti-HCC potential (Lee et al., 2002). In general, the HCC patients are given the maximal tolerated dose of anticancer drugs aiming to effectively suppress the rapid dividing cancer cells (Finn et al., 2013). However, the dose should be restricted in clinical application of DOX since its severe dose-dependent toxicity, such as cardiotoxicity, nephrotoxicity and myelosuppression, and cardiotoxicity ranks the most prominent (Chen et al., 2007; Quiles et al., 2006). This is probably due to the fact that DOX non-specifically distributes to the tumor site and reaches high levels in any other parts of the body, hereby, it can suppress the growth of normal healthy cells. It is difficult to obtain sufficient concentration of DOX at the targeted tumor by improving systemic dose, but implantable delivery systems which belong to localized drug delivery system could overcome these problems in the treatment of HCC.

In recent years, implantable delivery systems have gained significant attention for attaining high concentration of drugs at the tumor site but with low systemic concentration (Zhou et al., 2014). Thermosensitive hydrogel formulation, one kind of implantable delivery systems, has the advantage of avoiding requirements for a surgical implantation, which improves the applicable range of patients (Eve and Lerou, 2004). The hydrogel formulations are directly injected into the targeted

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tumor site as a liquid, which rapidly changes into a solid gel at body temperature. The initial hydrogel solution with low viscosity can easily flow and fill the targeted tumor (Eve et al., 2000). The anticancer agents are released by the later formed solid gel in a sustained manner at the treatment site. Anita et al. has reported that short-term high concentrations of an anticancer agent are less effective than the sustained low concentrations (Lalloo et al., 2006). For normal drug solutions, in order to prevent the rapid metabolism of the drug, repeated injections are often required. Fortunately, since their sustained release of drugs, the hydrogel formulations can avoid multiple injections. Thermo-sensitive hydrogel formulation can be administrated by intratumoral injection, which will further enhance the drug deposition in tumors and decrease drug concentrations in normal organs and tissues. Hence, this will further strengthen the antitumor efficiency and reduce the systemic toxicity.

Admittedly, hydrogel formulations are generally not suitable for the controlled release of low molecular weight hydrophilic compound, such as DOX, since hydrogels have high hydrophilic content and large pores compared to the particle size of drugs, resulting in rapid release (Ruel-Gariepy et al., 2002). In order to achieve sustained release of anticancer drugs with low molecular weight, the selected drugs can initially be encapsulated into the microparticles, such as liposomes or microspheres, then the microparticles are embedded into the hydrogel (Ruel-Gariepy et al., 2002; Hosny, 2009; Mulik et al., 2009). In this case, no active connections exist between the microparticles and the hydrogel strands (Henriksen et al., 1994). The drug would experience two transport barriers which are combined by the network of microspheres and hydrogels, thus extending the time of release. It can also reduce the burst release, a large bolus of the drug released from the gel at the beginning brings in toxicity (Ruel-Gariepy et al., 2002). Among all microparticle formulations of DOX, liposomal DOX (LipDOX) has been used in this study for its approval by U.S. Food and Drug Administration (FDA), thus ensuring the safety of drug delivery system (Wu et al., 2006). In addition, LipDOX could increase intracellular drug accumulation and reduce the toxicity (Xu et al., 2008; Ren et al., 2014). However, if liposomes are directly injected into the tumor site alone, the majority of liposomes would be eliminated from the tumor in a short period of time, thus fail to provide sustained drug release (Tiwari et al., 2009). Fortunately, this problem can be solved by the incorporation of hydrogel. What's more, liposomes can avoid environmental stimuli if it is entrapped into the gel, so it will be more stable compared with bare liposomes in solution.

Chitosan (C) based thermosensitive hydrogel formulations have been evaluated by many researchers for the delivery of anticancer drugs due to its biocompatibility and biodegradability (Berger et al., 2004a, 2004b). Especially, the thermosensitive hydrogel prepared by neutralizing the chitosan solution with β-glycerophosphate (GP) attracts a lot of attention (Ruel-Gariepy et al., 2002; Ruel-Gariepy et al., 2000). Ruel-Gariepy et al. have incorporated liposomes into the C/GP hydrogel for controlled release of hydrophilic drugs, which shows that the release of drugs becomes more slowly and the initial burst is alleviated compared with free drugs in the C/GP (Ruel-Gariepy et al., 2002). Therefore, the C/GP hydrogel containing liposomes could be a promising drug delivery system to generate much higher local drug concentrations and minimize the toxic side effects.

In the present experiment, in order to investigate whether dual controlled-release system could enhance the antitumor efficiency and decrease systemic toxicity of anticancer drug, we developed *in situ* forming C/GP hydrogel containing LipDOX (LipDOX + C/GP) drug delivery systems for HCC treatment. C/GP hydrogel loaded with free DOX (DOX + C/GP) was also prepared to compare with LipDOX + C/GP hydrogel in both *in vitro* and *in vivo* study. The *in vitro* release profiles in phosphate buffer saline (PBS) with different pH values and cell toxicity of these two formulations were compared. The *in vivo* antitumor efficacy, pharmacokinetics and biodistribution were investigated in H22 tumor-bearing mice.

2. Materials and methods

2.1. Chemicals and reagents

Medical grade chitosan (deacetylation degree ~91%, $M_w = 200{,}000$), DOX and Phosphatidylcholine from soybean (90%), were purchased from Qingdao Jinke Biomedical Co., Ltd. (Qingdao, China), Hubei Huihua Biochemical Co., Ltd. (Hubei, China) and Shanghai Taiwei Pharmaceutical Co., Ltd. (Shanghai, China), respectively. Both β-glycerophosphate (98%) and cholesterol (95%) were obtained from Shanghai J&K Scientific Co., Ltd. (Shanghai, China). All other chemicals were analytic grade.

2.2. Cells and animals

Murine H22 hepatoma cells and human hepatoma cell lines (SMMC-7721) were received from China Institute of Cell Biology (Shanghai, China), cultured in RPMI 1640 medium containing 10% fetal bovine serum in air containing 5% CO2 at 37 °C. ICR mice were obtained from the experimental animal center of China Pharmaceutical University (Nanjing, China) and allowed to acclimate for a week. Complete food and water were provided for the mice during the whole study.

To generate the tumor model, firstly, 2×10^6 H22 hepatoma cells at logarithmic growth phase were injected into the mice abdominal cavity. Secondly, the mice were sacrificed after one week and the intraperitoneal tumor cells were collected by centrifugation. Lastly, 0.2 mL of saline containing 2×10^6 viable cells was subcutaneously injected into the mice weighing 18 ± 2 g on the dorsum. All protocols were evaluated and approved by the Ethics Committee for Animal Experimentation of China Pharmaceutical University which is in accordance with the Act for Use of Laboratory Animals of Jiangsu Province, China and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (No. 85-23, revised 1996). All efforts were made to minimize animal's discomfort and to reduce the number of animals used.

2.3. Preparation and characterization of liposome

LipDOX was prepared by the method of an ammonium sulfate gradient as previously described with slight modifications to achieve high drug loading (Ren et al., 2014). In brief, phosphatidylcholine and cholesterol were dissolved in methylene chloride and dried to form a thin lipid film. The film was placed in a cabin drier overnight to remove the residual methylene chloride and then hydrated with ammonium sulfate. Liposomes were prepared by probe sonication and homogenization under high pressure. To obtain a homogeneous liposome suspension, the liposomes were eluted through a size-exclusion polycarbonate membrane filters. The homogeneous liposome suspension was pre-equilibrated with physiological saline overnight and added to disodium hydrogen phosphate to adjust the pH. And then the final liposomes incubated with DOX to obtain active loading. The isolation of LipDOX from non-encapsulated doxorubicin was accomplished by centrifugation (3 circulations at 15,000 rpm for 40 min).

The encapsulation efficiency of the liposome was evaluated by passing through Sephadex G50 column, as described by Anita Lalloo et al. (Ruel-Gariepy et al., 2002) but with some modifications in which the liposome was lysed in acetonitrile to release the DOX.

DOX concentration was measured by a reverse-phase High Performance Liquid Chromatography (HPLC) system which included Shimadzu LC-10AD pump, SPD-10A UV detector and an Inertsic ODS-3 column. The mobile phase was constituted of methanol and water with 0.1% acetic acid (43:57, v/v). The flow rate was 1.0 mL/min with injection volume of 20 μL per sample and the wavelength of detection used for DOX was 480 nm. Laser light scattering with Zatasizer 3000HS (Malvern Instruments Ltd., UK) was used to determine the size of the prepared liposome.