Doxorubicin-loaded zein in situ gel for interstitial chemotherapy of colorectal cancer

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Abstract The aim of this research was to evaluate doxorubicin (DOX)-loaded zein in situ gels, a new drug delivery system in which a liquid state drug can be transformed into semi-solid after intratumoral injection. \textit{In vitro} release of DOX-loaded zein was investigated and the pharmacokinetics, biodistribution and therapeutic efficacy of these DOX-loaded zein formulations were investigated using BALB/c nude tumor-bearing mice. \textit{In vitro} release of DOX from the gels extended up to 7 days. Efficient accumulation of DOX in the tumor with lower drug concentration in blood and normal organs was obtained resulting in effective inhibition of tumor growth and fewer off-target side effects. In conclusion, a DOX-loaded in situ gel was developed with sustained release, enhanced anti-cancer efficacy for colorectal cancer \textit{in vivo}, and especially with reduced off-target side effects.

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1. Introduction

Colorectal cancer (CRC) is one of the most frequent cancers worldwide and is a leading cause of cancer mortality. Doxorubicin (DOX) is widely used for the treatment of a number of cancers, but its usage has been limited due to cardiotoxic effects. Considerable effort has been put forth in the development of drug delivery systems (DDS) for DOX to change tissue selectivity and improve the toxicity profile.

The increased permeability of blood vessels and the lack of lymphatic vessels in tumors leads to high hydrostatic pressure in tumor stroma. Therefore, drugs cannot be effectively delivered to the tumor tissues by systemic administration. In order to eliminate these problems, efforts have been focused on development of the targeting DDS that achieves site-specific delivery and prolongs the exposure. During the last decade, injectable in situ forming gels, as polymeric drug carriers, have attracted considerable attention. For CRC, local injection of in situ gels can be applied by a Sigmoidoscope. Applications of the in situ gels provide a number of advantages, including easy application, localized delivery, prolonged delivery periods, decreased body drug dosage and improved patient compliance; however, the great majority of the polymers require artificial synthesis.

Zein, a major storage protein comprising about 45–50% of total protein in corn, was a promising biomaterial with good biocompatibility for tissue engineering. As indicated by SDS-PAGE in the present study, biochemically pure zein is mainly composed of two distinct bands with molecular weights of 23 and 21 kDa, with minor bands at 13 and 9.6 kDa. The high proportion of non-polar amino acids in zein determines its solubility behavior. The molecular structure is a helical wheel conformation where nine homologous repeating units are arranged in an anti-parallel form stabilized by hydrogen bonds. Zein has been used to form microspheres by cross-linking a zein solution containing the drug. In order to increase the drug concentration in tumor and hence to improve the antitumor potency of DOX, DOX-loaded zein in situ gel was developed in this study. In vitro release of DOX-loaded zein was investigated and the pharmacokinetics, biodistribution and therapeutic efficacy of these DOX-loaded zein formulations were investigated using BALB/c nude tumor-bearing mice.

2. Materials and methods

2.1. Materials and chemicals

DOX hydrochloride was obtained from Lingnan Pharmaceutical, Ltd. (Guangzhou, China). Zein was from Rixing Pharmaceutical Adjuvant Factory (Gaoyou, China). Glycerol formal (GF) was purchased from Tedia company (Fairfield, USA) and of HPLC or analytical grade.

2.2. Sample preparation

Zein was dissolved mixed with GF and 70% ethanol-water (3:7, v/v). The concentration of Zein was 15%, 20%, 25% for in vitro release and 20% for in vivo study. It was left overnight at room temperature to form a clear solution. DOX was added into the above solution and dissolved by stirring.

2.3. Tumor homograft

The HT-29 cells (human colorectal cancer cell line, Cell Bank of Peking Union Medical College) were harvested with 0.25% trypsin and made into suspension (1.0 × 10^7 cells/mL). The cell suspension was implanted subcutaneously into one foot of immunodeficient mice (BALB/c nude mice) (Vital River, China).

2.4. In vitro DOX release

Half a milliliter of the sample was added to 10 mL PBS (pH 7.4) containing NaNO3 (0.05%, w/v) and trypsin 1:250 (1.0%, w/v). The gels formed were shaken in a constant temperature oscillator at 50 rpm and 37°C. 5 mm of the buffer was collected at predetermined time intervals and replaced with fresh buffer. Drug concentration was analyzed by HPLC.

2.5. In vivo antitumor efficacy test

When the tumor volume reached approximately 100 mm^3 on the 7th day after cell injection, the mice were randomly divided into 4 groups (n=6): (1) normal saline (control), (2) aqueous solution of DOX, (3) blank gel only, (4) doxorubicin-loaded zein in situ gels (DOX-zein). The dosages of DOX for a mouse were set at 5 mg/kg. Gels or solutions were administered using a 22 G needle. The tumor diameters were measured in two dimensions every other day with the Somatom sensation 64 CT machine (Siemens, Germany) with automatic 3D reconstructions.

2.6. Plasma concentration and tissue distribution

When the tumor volume had reached about 100 mm^3, the mice were divided into two groups: (1) aqueous solution of DOX (i.v.) and (2) DOX-zein (intratumoral injection). The same dose of 5 mg/kg DOX was injected. At 1, 3, 5, 12, 24, 48, 72 h after injection the mice were killed. The heart, liver, spleen, lungs, kidneys and tumors were immediately removed, cleaned, weighed and homogenized with citric acid-buffered saline (CBS, pH 8.0, 0.5 mL/g tissue).

The drug was extracted with chloroform-methanol (4:1, v/v) from plasma diluted with CBS (pH 8.0) and tissues homogenized with the same buffer. The organic phase was dried under N_2 at room temperature. The residues were dissolved in methanol for HPLC analysis (Hitachi, L-2000, Japan). The AUCs of DOX in the plasma and tissues were calculated by DAS Ver1.0. The in vivo data was evaluated using Student’s t-test and P<0.05 was considered to be statistically significant.

2.7. HPLC method

The HPLC analysis was performed under the same conditions as for the release test and tissue and plasma concentration tests on a Hitachi HPLC system (L-2000, Japan) controlled by D-2000 workstation using a C18 column (Thermo Hypersil ODS, 5 μm, 200 mm x 4.6 mm). The mobile phase consisting of 0.01 M ammonium dihydrogen phosphate, methanol,
and glacial acetic acid (40:60:0.7, v/v/v) was set at a flow rate of 1.0 mL/min. Detection was at the wavelengths of maximum absorption of the doxorubicine in the mobile phase at 254 nm. The instrument room was maintained at 30 °C. The injection volume was 20 μL. The intra-day and inter-day coefficient of variations (CVs) of each tissue and plasma were less than 10%. The limit of quantitation (LOQ) was 0.031 μg/mL for plasma, 0.186 μg/mL for heart, 0.035 μg/mL for liver, 0.086 μg/mL for spleen, 0.064 μg/mL for lung, 0.036 μg/mL for kidney and 0.027 μg/mL for tumor.

3. Results and discussion

3.1. Preparation of an injectable in situ forming gel

The formulation of the in situ gel should have an optimum viscosity so that it may be easily injected as a liquid and undergo a rapid sol–gel transition. In addition, the formed gel should preserve its integrity to facilitate local sustained release of drugs rather than dissolve or erode quickly. Gel solutions containing DOX attained the gel state almost immediately after being injected into the subcutaneous dorsum of mice. The gel-forming ability of zein can be explained as follows: the molecular structure of zein was a helical wheel conformation in which nine homologous repeating units were arranged in an anti-parallel form stabilized by hydrogen bonds. After the zein in situ gel solution was injected into the tumor, the solvent composition at the aqueous interface was changed, GF diffused away from the depot and water infused in, resulting in increased concentration of zein at the aqueous interface. When the concentration of zein reached a critical value the proteins agglomerated and hydrogen bonding, disulfide bonding and hydrophobic interactions between protein molecules occurred. This maintained the meshwork and led to the formation of zein gel.

According to previous results from our laboratory18, gel viscosity (130–200 mPa · s) was favorable when the content of zein was below 30% (w/w) and the formulation was easily injectable through 26-gauge needles. The proposed content of macromolecular protein zein is 15–25%. The solvent GF can slightly increase the viscosity, resulting in a more stabilized system without sedimentation.

3.2. In vitro DOX release

The in vitro DOX release behavior of gels prepared with DOX at 0.2, 0.4, and 0.8 mg/mL was examined. Fig. 1 shows the release profiles for 7 days. The DOX was released rapidly on the first day followed by a sustained release for the following 6 days, and all formulations showed incomplete release on 7th day. Drug-loading had no significant effect on in vitro release. Fig. 2 indicates that higher protein concentration decreased burst release and extended in vitro release with higher viscosity of the gel depot. Considering the release profile and viscosity, a zein concentration of 20% (w/w) was chosen for in vivo study.

For a water-miscible solvent like ethanol, a relatively faster phase inversion was found, resulting in a porous, rubbery gel structure. Its release profile was characterized by an initial burst followed by a slow-release rate for a long period. However, as a whole, the burst effect was low and tolerable, because the Pharmacopoeia of the People’s Republic of China (2010. appendix XIX E)19 has given the limit of less than 40% release in first half an hour. On the other hand, solvents that have relatively low solvent affinity, such as GF, resulted in a relatively slower phase inversion and a less porous structure. A solvent system20 was composed of both hydrophilic (e.g., ethanol and benzyl alcohol) and hydrophobic (e.g., benzyl benzoate and sucrose acetate isobutyrate) components. The hydrophilic solvent can decrease the viscosity of gel, thus facilitating injection and resulting in a “coat” around the exterior of the depot. The hydrophobic solvent could slow down water penetration and therefore decreases the protein hydrolysis.
3.3. In vivo antitumor activity

The in vivo antitumor effect of DOX-zein was evaluated using BALB/c nude mice inoculated with human colon cancer cells. The tumor volume was measured by CT scanning 3D reconstruction. As shown in Fig. 3 in mice injected with saline or gel solution, the size of the tumor was steadily increased as a function of time, reaching 20 times of the original size after 15 days without a significant difference between these two groups (P>0.05).

Both single injection of DOX-zein and free DOX produced significantly inhibitory effects (P<0.05) against the tumor and decreased its propagation remarkably. Compared with free drugs, DOX-zein enhanced the antitumor effect (P<0.01) as shown in Fig. 3.

3.4. Plasma pharmacokinetics

The DOX solution was injected into the tail vein of a group of model mice (n=3) while the DOX-zein gel was injected into the tumor of another group (n=3). The plasma concentration vs. time profile of DOX is presented in Fig. 4. Not surprisingly, the drug concentration in blood of the DOX-zein group was less than that of the DOX solution group. The AUC of DOX-zein group and DOX solution group was 9.12 μg·mL⁻¹·h and 35.94 μg·mL⁻¹·h, respectively. The liquid state of the DOX-zein changed to solid state after injection and formed a drug reservoir, which reduced the chance that the drug entered the blood and resulted in a sustained release within the tumor.

3.5. Distribution study

To study the drug distribution in animal tissues, saline (control), blank gel, DOX solution and DOX-zein were injected into female BALB/c nude mice bearing tumors with

![Figure 4](image)

**Figure 4** Plasma concentration–time curves of DOX in plasma after single dose of 5 mg/kg DOX (n=3).

### Table 1

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Equation of standard curve</th>
<th>Linear range</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>y = 2 × 10⁻⁵x + 0.1772</td>
<td>0.5–5.0 μg/mg</td>
<td>0.9938</td>
</tr>
<tr>
<td>Liver</td>
<td>y = 5 × 10⁻⁵x + 0.1526</td>
<td>0.1–2.0 μg/mg</td>
<td>0.9920</td>
</tr>
<tr>
<td>Spleen</td>
<td>y = 8 × 10⁻⁵x – 0.0176</td>
<td>0.1–2.0 μg/mg</td>
<td>0.9956</td>
</tr>
<tr>
<td>Lung</td>
<td>y = 1 × 10⁻⁵x – 0.1591</td>
<td>0.1–3.6 μg/mg</td>
<td>0.9925</td>
</tr>
<tr>
<td>Kidney</td>
<td>y = 3 × 10⁻⁵x + 0.2415</td>
<td>0.05–2.0 μg/mg</td>
<td>0.9924</td>
</tr>
<tr>
<td>Tumor</td>
<td>y = 8 × 10⁻⁵x + 0.0852</td>
<td>0.1–5.0 μg/mg</td>
<td>0.9916</td>
</tr>
<tr>
<td>Plasma</td>
<td>y = 2 × 10⁻³x + 0.0079</td>
<td>0.1–3.0 μg/mL</td>
<td>0.9936</td>
</tr>
</tbody>
</table>

x represents peak area of the sample, y represents the concentration of DOX.

### Table 2

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>DOX</th>
<th>Heart (μg/g)</th>
<th>Liver (μg/g)</th>
<th>Spleen (μg/g)</th>
<th>Lung (μg/g)</th>
<th>Kidney (μg/g)</th>
<th>Tumor (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Solution</td>
<td>4.88±0.57</td>
<td>1.68±0.43</td>
<td>0.77±0.23</td>
<td>1.85±0.25</td>
<td>1.45±0.39</td>
<td>4.03±0.24</td>
</tr>
<tr>
<td></td>
<td>Zein</td>
<td>4.89±0.54</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>4.52±0.37</td>
</tr>
<tr>
<td>3</td>
<td>Solution</td>
<td>2.84±0.65</td>
<td>0.49±0.21</td>
<td>0.2±0.11</td>
<td>0.88±0.15</td>
<td>0.20±0.06</td>
<td>2.46±0.46</td>
</tr>
<tr>
<td></td>
<td>Zein</td>
<td>2.78±0.34</td>
<td>0.53±0.23</td>
<td>N.D.</td>
<td>0.77±0.18</td>
<td>0.44±0.17</td>
<td>3.51±0.46</td>
</tr>
<tr>
<td>5</td>
<td>Solution</td>
<td>1.73±0.35</td>
<td>0.88±0.15</td>
<td>0.72±0.32</td>
<td>1.69±0.37</td>
<td>0.72±0.16</td>
<td>1.05±0.26</td>
</tr>
<tr>
<td></td>
<td>Zein</td>
<td>1.33±0.51</td>
<td>0.60±0.15</td>
<td>1.40±0.17</td>
<td>N.D.</td>
<td>0.097±0.04</td>
<td>4.21±0.17</td>
</tr>
<tr>
<td>12</td>
<td>Solution</td>
<td>1.83±0.51</td>
<td>0.83±0.38</td>
<td>1.02±0.18</td>
<td>2.00±0.32</td>
<td>0.75±0.14</td>
<td>0.77±0.31</td>
</tr>
<tr>
<td></td>
<td>Zein</td>
<td>0.95±0.21</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>2.80±0.62</td>
</tr>
<tr>
<td>24</td>
<td>Solution</td>
<td>1.51±0.38</td>
<td>0.42±0.15</td>
<td>1.32±0.46</td>
<td>1.09±0.24</td>
<td>0.19±0.08</td>
<td>0.36±0.18</td>
</tr>
<tr>
<td></td>
<td>Zein</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>2.13±0.42</td>
</tr>
<tr>
<td>48</td>
<td>Solution</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.36±0.12</td>
<td>0.16±0.08</td>
<td>0.12±0.06</td>
<td>1.10±0.04</td>
</tr>
<tr>
<td></td>
<td>Zein</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.097±0.04</td>
<td>0.22±0.36</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>Solution</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.15±0.09</td>
<td>0.14±0.08</td>
<td>0.096±0.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Zein</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.12±0.08</td>
<td>1.12±0.15</td>
</tr>
</tbody>
</table>

N.D.: not detected.
diameter of 7–9 mm. DOX concentrations in tissues were determined by HPLC. A standard curve for each tissue was established by adding serial concentrations of DOX to tissue extracts from control group of mice (Table 1). As shown in Tables 2 and 3, the drug concentration of DOX solution group in tumor tissue was significantly higher than that of DOX-zein group \((P<0.05)\) at 3 h after the injection. However, the DOX-zein group maintained a relatively higher drug concentration for a longer time in the tumor than DOX solution group. The AUC of DOX-zein group and DOX solution group was 204.59 \(\mu g/mL\)-\(h\) and 18.71 \(\mu g/mL\)-\(h\), respectively.

In non-tumor tissues (heart and lungs), the drug concentration in DOX-zein group was lower than in the DOX solution group \((P<0.05)\). The AUC of DOX-zein group and DOX solution group in heart was 43.08 \(\mu g/mL\)-\(h\) and 57.40 \(\mu g/mL\)-\(h\), respectively. There were no significant differences in liver, spleen and kidney \((P>0.05)\). No DOX in DOX-zein group was examined in liver after 12 h of injection. In spleen, the \(C_{max}\) of the DOX-zein group was nearly as large as that of DOX solution group. In kidney, the drug concentration in DOX-zein group was higher than that of the DOX solution group after 3 h of injection, but the concentration of the DOX-zein group started decreasing thereafter. These results indicate that direct intratumoral injection significantly enhanced the distribution of DOX to the tumor and reduced the distribution to the other organs.

### 4. Conclusions

The present study describes a new drug delivery system of DOX-loaded Zein \(in\) \(situ\) gels, in which a liquid state was transformed into a semi-solid after intratumoral injection. \(In\) \(vitro\) release of DOX from the gels could be extended up to 7 days. Efficient accumulation of DOX in the tumor with lower drug concentration in blood and normal organs resulted in effective inhibition of tumor growth. This study demonstrated that a sustained-release DOX-loaded \(in\) \(situ\) gel was developed with enhanced anti-cancer efficacy for CRC \(in\) \(vivo\), especially with reduced off-target side effects.

### Acknowledgments

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