

Prevention of Local Liver Cancer Recurrence after Surgery Using Multilayered Cisplatin-loaded Polylactide Electrospun Nanofibers

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Abstract In this study a cisplatin-loaded, multilayered polylactide (PLA) electrospun nanofibers, with the structure of two layers of drug-loaded mat being sandwiched by three layers of blank mat, were designed for prolonged cisplatin release at surgical margin to prevent local cancer recurrence following surgical resection in a murine model. *In vivo* drug release and biodistribution study suggested that the multilayered fibrous mat displayed a slower cisplatin release behavior and a more stable drug retention in the local tissue within 24 h than that of single-layered fibrous mat. By covering the surgical site with the multilayered fibrous mat following resection of subcutaneous liver cancer in mice, retarded tumor recurrence, prolonged survival time and less systemic toxicity were observed compared with other treatment groups.

Keywords: Liver cancer; Local recurrence; Multilayered nanofibers; Cisplatin.

INTRODUCTION

Although the most effective treatment for patients with localized liver cancer is surgical resection, long-term survival is dismal if the disease recurs^[1, 2]. Even when recurrence is local, the majority of patients are not candidates for repeat surgical resection and the resultant 2-year survival is only 18%–24%^[3]. The risk of local recurrence in early-stage liver cancer is greater in patients receiving sublobar resection compared with lobectomy and is correlated with the distance between the surgical margin and the primary tumor^[4–7]. Residual microscopic tumor cells at the surgical margin have been identified postoperatively in 39% of patients following “curative” wedge resection, suggesting that local recurrence arises from nearby occult tumor cells that remain after surgery^[8].

Despite the benefit of systemic chemotherapy in later stage liver cancer, similar therapy has not decreased local recurrence or improved survival in patients with surgically resected early-stage disease^[9, 10]. Morbidity from common side-effects and the inability to predict which patients will develop local recurrence are particularly concerning for patients receiving drugs such as cisplatin which has severe dose-limiting systemic toxicities^[11]. Although cisplatin is a principal chemotherapeutic agent for the treatment of liver cancer, its accumulation within the liver has been shown to be poor after intravenous administration^[12]. Nearly half of

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systemically administered cisplatin is excreted in the first day and less than 0.5% of the total dose remains locally available to treat tumors within the liver.

In the case of solid tumors like hepatocellular carcinoma (HCC), a combination of surgical operation for removing the tumor with subsequent chemotherapy or radiation therapy is the normal procedure to reduce the probability of recurrence. To date, the best success at preventing local recurrence of liver cancer following sublobar resection has been with the application of ^{125}I brachytherapy seeds to the surgical margin following resection. Local recurrence in patients with stage I liver cancer was decreased from 18.6% to 2%, suggesting that better local disease control is possible following limited surgical resection^[13, 14]. However, radiation injury to nearby tissues, complexity of securing seeds to the resection line, radiation risk to personnel and patient, and concerns regarding impaired wound healing have limited widespread adoption of intraoperative brachytherapy, with only a few centers pursuing this operation in selected patients. Hence, it's worthy finding a new regional therapy for the prevention of local tumor recurrence after resection.

Polymer-based local drug delivery systems have been evaluated in several different malignancies as a means^[15, 16] in order to achieve a prolonged period of function, maximized efficiency, and minimized unwanted side effects to healthy tissues. A local drug-delivery system, aimed at delivering and retaining a sufficient amount of drug for an adequate period of time, consists of a formulation or a device that enables introduction of a therapeutic agent in the body and enhances its efficacy and safety by controlling the rate, time, and site of release within the body^[17].

Recently, electrospun nanofibers has been widely used in tissue regeneration and drug-delivery applications owing to their unique characteristics like the high surface area-to-volume ratio, high interconnected porosity with tunable pore size, possibilities for efficient surface functionalization, adjustable surface morphology and structural similarity to the extracellular matrix (ECM). A biodegradable, drug-loaded nanofiber mat not only provides high local dosage with incorporation of small amounts of the drug but also reduces the need for frequent administrations, and eliminates the need of removal of devices after the treatment, resulting in patient convenience^[18–21].

In consideration of the aqueous human body fluid, hydrophobic polymer materials and drugs can present an ideal drug-delivery system to achieve sustained drug release for a much longer period than that of hydrophilic polymer and drugs. Due to its oil-soluble nature and significant antiproliferative effect, paclitaxel is the most commonly used drug being incorporated into various hydrophobic polymers for post-surgery chemotherapy. Several studies have demonstrated the ability of paclitaxel-loaded polymer films or nanofibers to retard tumor growth and/or postpone the development of locally recurrent tumors^[22–24]. In our previous study, however, the murine hepatic carcinoma (H22) cells used in this study was not sensitive to paclitaxel (data not shown), thereby cisplatin with great cytotoxicity against H22 was utilized as model drug. However, lack of solubility of inorganic compound in the polymer solution causes just a dispersion of the drug inside the solution. During electrospinning of such drug dispersions, the drug molecules might migrate on or near the fiber surfaces, resulting in burst release. To retard the rapid release of cisplatin from fibrous mat, a multilayered mat was prepared with three layers of blank PLLA nanofibers served as the barrier.

In this study our objectives were to investigate the potential use of polymer nanofibers as an alternative local approach to addressing this unmet clinical need as mentioned above. Cisplatin-loaded, multilayered polylactide electrospun nanofiber mats were prepared for local cisplatin delivery following surgical resection of solid liver cancer in mice, aiming to minimize systemic toxicities by reducing the initial “burst” release of cisplatin to some extent, and improve tumor cytotoxicity by prolonging drug release locally.

EXPERIMENTAL

Materials

Poly(L-lactide) (PLLA) was synthesized from L-lactide in our laboratory. Its molecular weight (M_n) and polydispersity index (PDI) was determined by GPC were 13800 and 3.41, respectively. Cisplatin (purity 99%) was bought from Shandong Boyuan Chemical Company, China. All other chemicals and solvents were used

without further purification.

Preparation and Characterization of Multilayered Cisplatin-loaded PLLA Nanofibers

Multilayered cisplatin-loaded nanofiber mat were fabricated in following sequence (see Fig. 1a): (i) the top layer of blank PLLA nanofiber mat, (ii) PLLA nanofiber mat loaded with 7.5% cisplatin, (iii) blank PLLA nanofiber mat, (iv) PLLA nanofiber mat loaded with 7.5% cisplatin, (v) the bottom layer of blank nanofiber mat. The cisplatin-loaded nanofiber mat composed of five layers were coded as cis/M5.

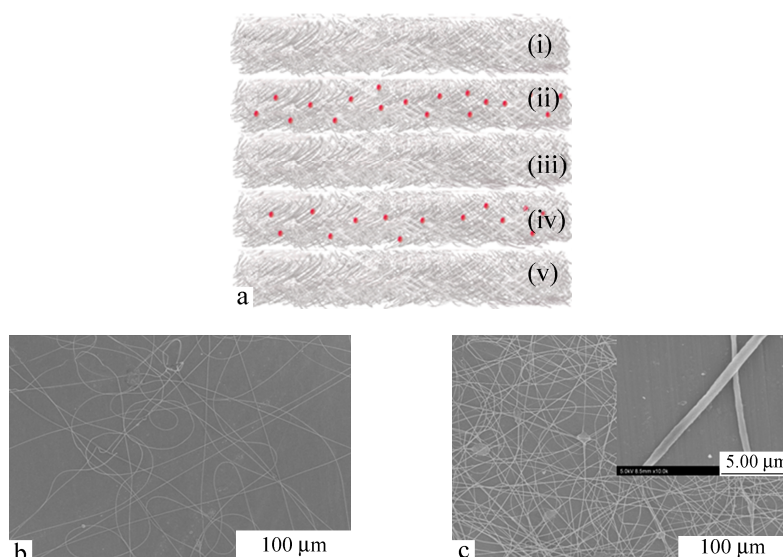


Fig. 1 (a) Graphical presentation of cis/M5 composed of (i) the top layer of blank PLLA nanofiber mat, (ii) PLLA nanofiber mat loaded with 7.5% cisplatin, (iii) blank PLLA nanofiber mat, (iv) PLLA nanofiber mat loaded with 7.5% cisplatin, (v) the bottom layer of blank nanofiber mat; (b) ESEM images of PLLA fiber and (c) ESEM images of cisplatin-loaded PLLA fiber

The layer of cisplatin-containing PLLA fibers was obtained by electrospinning of PLLA-cisplatin mixed solutions (7.5% cisplatin in weight percent of PLLA used) in chloroform-methanol-dimethyl sulfoxide (DMSO) (82/14/4, *V/V/V*) at a polymer concentration of 6 wt%. Electrospinning parameters were as follows: electric field strength: 1.8–2.0 kV·cm⁻¹; air gap distance: 15 cm; inner diameter of spinneret: 0.4 mm; flow rate of solution: 1–2 mL·h⁻¹ and all the experiments were conducted at room temperature in air.

The layer of blank PLLA fiber was prepared by electrospinning PLLA alone with the same mass as that utilized in cisplatin-loaded PLLA fibrous mat.

In order to remove the residual solvent, the fibrous mats collected were placed under vacuum for about 8 h at 25 °C.

As a control, single-layered cisplatin-loaded mat (coded as cis/M1) and unloaded mat (coded as PLA/M1) were prepared.

An environmental scanning electron microscope (ESEM, Model XL 30 ESEM FEG from Micro FEI Philips) was used to observe the morphology of blank PLLA fiber and cisplatin-loaded fiber.

Assessment of the Release Behavior of cis/M5 and Tissue Cisplatin Concentrations *in vivo*

Eighteen healthy Kunming (KM) male mice in a weight range of 20–25 g (8–12 weeks old) were obtained from Jilin University and used for the study of drug release profile of cis/M5 and cis/M1 *in vivo* and the tissue level of cisplatin. All animal studies were approved by the ethics committee of Jilin University (Jilin, China), and all animal care was performed humanely and in accordance with the designated and approved standards.

A subcutaneous pocket was made in the armpit of mice under ketamine/xylazine anesthesia into which

accurately weighed cis/M5 or cis/M1 of equivalent dose of cisplatin was placed. The animals were sacrificed at 1 h, 12 h, and 24 h after fibrous mat implantation and samples of residual fibrous mat, blood, underarm musculature and connective tissue from underneath the mat were harvested and analyzed for platinum concentration by inductively coupled plasma mass spectrometry (ICP-MS).

Prevention of Local Liver Cancer Recurrence in vivo by cis/M5

Subcutaneous liver cancer models were induced by injection of 5×10^6 H22 cells into the armpit of the mice. Tumor size was measured using digital calipers every 2 days. After the tumor volume reached 100–200 mm³, tumors were surgically removed by sharp dissection under ketamine and xylazine anesthesia through an incision adjacent to the tumor. All visible tumor tissue was removed before mice were randomized to one of five treatment groups in a blinded fashion: (1) implantation of cis/M5 (60 µg of cisplatin), (2) implantation of cis/M1 with equivalent dose of cisplatin to cis/M5, (3) implantation of PLA/M1, (4) three injections of cisplatin on day 0, 4, and 8 at the total dose of 60 µg *via* the tail vein for systemic administration, (5) only tumor resection. cis/M5, cis/M1 and PLA/M1 were implanted subcutaneously at the site of primary tumor resection. Mice were monitored every 2 days for weight loss and evidence of local recurrence. Time of local recurrence was defined as the detection of a palpable subcutaneous nodule (approximately 2 mm × 2 mm × 1 mm) in the armpit of mice.

Five animals separately from each group were sacrificed on the 4th day following local or systemic chemotherapy. Recurrent tumor tissue, underarm musculature and connective tissue were harvested and followed by paraffin sectioning and hematoxylin and eosin (H&E) staining.

The rest of mice were allowed to live until they succumbed to the tumor and survival days were recorded for each mouse.

Statistical Analysis

All data are expressed as mean ± SE. ANOVA with post-hoc analysis (Tukey's method) and pair-wise comparisons (Student's t-test), or nonparametric (Mantel-Cox) tests were applied where appropriate.

RESULTS AND DISCUSSION

Characterization of cis/M5

ESEM micrographs of both blank PLLA fibers (Fig. 1b) and cisplatin-loaded fibers (Fig. 1c) showed nanofibers with uniform diameter range and smooth surface. No drug crystals were detected in cisplatin-loaded fibers but obvious bead formation occurred due to increased surface tension resulted from the addition of DMSO.

Prolonged Cisplatin Release via cis/M5 in vivo

Cisplatin release rate *in vivo* was determined in healthy mice 1 h, 12 h and 24 h after implantation of cis/M5 or cis/M1 by using ICP-MS analysis. To be consistent with the tumor inoculation site, fibrous mat was subcutaneously placed into the armpit of mice. As shown in Fig. 2(a), cisplatin release rate of cis/M5 was 24%, 57% and 72% at 1 h, 12 h, and 24 h, respectively. As a comparison, cis/M1 demonstrated faster release kinetics, releasing 46%, 74% and 88% of the cisplatin payload at 1 h, 12 h and 24 h. The prolonged release behavior of cis/M5 was much likely due to its multilayered structure. Okuda *et al.* studied the release kinetic of a multilayered drug-loaded nanofiber mat *in vitro*, proving that the invasion depth of the release medium into the mat depends on the balance between the immersion pressure of the release medium and the surface hydrophobicity of each fiber inside the mat. The contact area of the release medium was restricted in a superficial region of the hydrophobic nanofiber mat and the release medium cannot reach the inner drug-loaded mat if the barrier mat is sufficiently thick to inhibit complete invasion of the release medium^[25]. Owing to the high hydrophobicity of polylactide and the two layers of barrier mat, prolonged cisplatin release *in vivo* from cis/M5 was achieved to a certain degree.

At the same time, the concentrations of cisplatin in plasma and underarm muscle tissue were measured (Figs. 2b and 2c). At 1 h, the cisplatin concentration in local tissue of cis/M1 was twice as high as that of cis/M5 (4.01 ± 0.98 versus 2.09 ± 0.65 µg·g⁻¹), while the ratio of cisplatin concentration between local tissue and blood

in cis/M5 was double that of cis/M1 (6.32 ± 2.31 versus 3.00 ± 0.95). Due to the faster release and clearance rate *in vivo*, however, the local tissue level of cis/M1 has been lower than that of cis/M5 at 24 h ($0.81 \pm 0.21 \mu\text{g}\cdot\text{g}^{-1}$ versus $1.28 \pm 0.75 \mu\text{g}\cdot\text{g}^{-1}$), and the ratio (tissue/blood) in cis/M5 remained much higher than that in cis/M1 (5.47 ± 2.11 versus 2.16 ± 0.93) all along, confirming the retarding release of cisplatin from multilayered fibrous mat to local implantation site and blood circulation. Hence, the prolonged exposure of target tissue to drug and decreased plasma drug concentration is much likely to assist in reducing the risk of tumor recurrence and systematic toxicity, at least during the initial postoperative period.

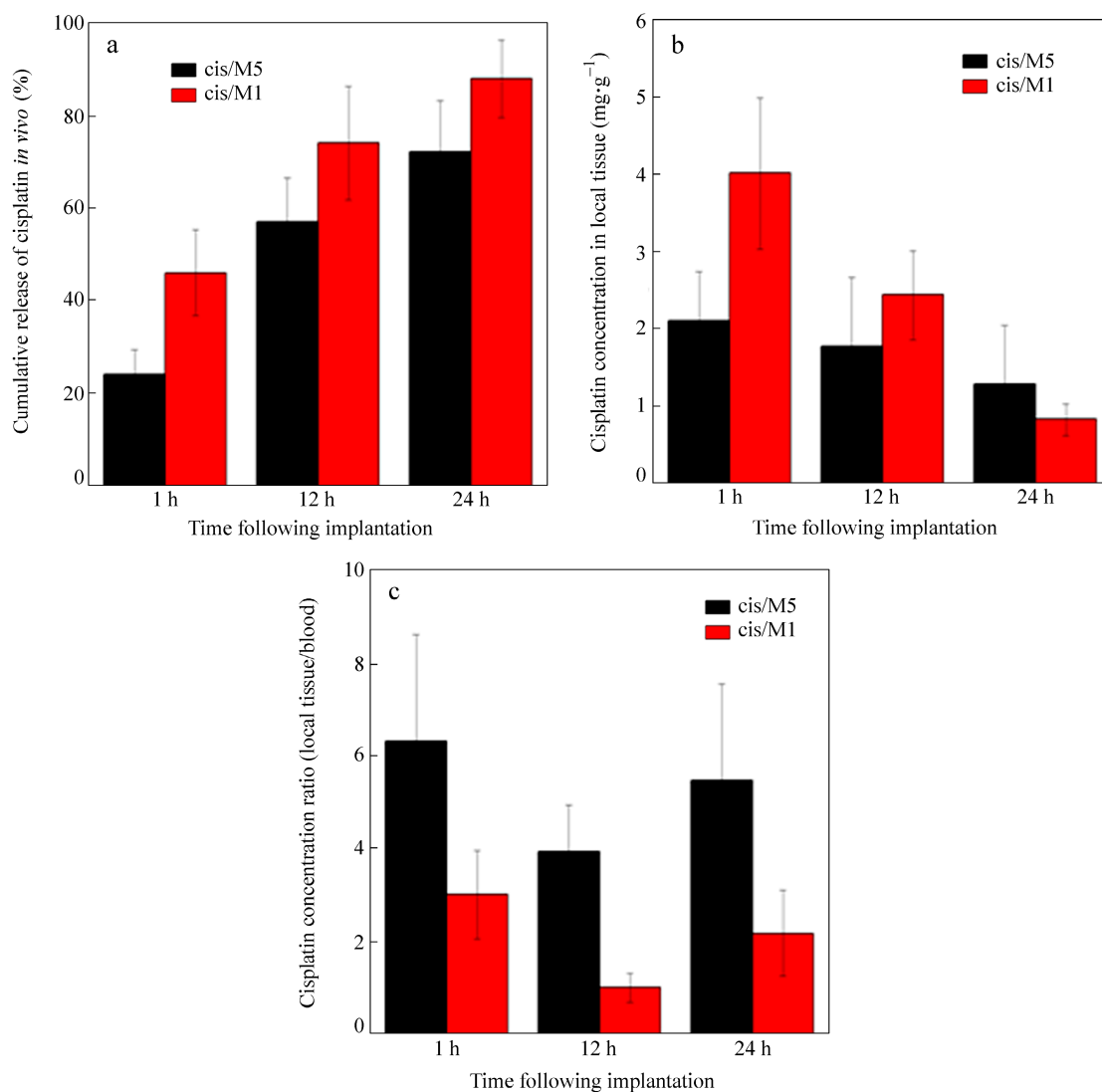


Fig. 2 (a) Cisplatin concentration in local tissue and (b) the ratio of cisplatin concentration between local tissue and blood and (c) cisplatin release kinetics *in vivo* from cis/M5 and cis/M1 at 1 h, 12 h, and 24 h following implantation

Cis/M5 Prevents Local Recurrence In Vivo

Since prolonged release behavior of cisplatin from cis/M5 *in vivo* has been observed, the prevention of local tumor recurrence by cis/M5 in animal model was further studied. H22, an aggressive murine liver cancer cell line, was utilized to establish the resection model. In order to assure 100% mice developing recurrent subcutaneous tumors following resection and screen out the possible “contribution” of tumor resection to

curative effect, the tumor inoculation site is the first and primary concern. In our preliminary experiment, we have ever tried injecting tumor cells subcutaneously on the dorsum of mice, a commonly used site adopted in nearly all reports on the prevention of tumor recurrence after surgery. Disappointingly, the formation rate of transplanted tumor was low and tumor growth was slow due to the lack of angiogenesis and nutrition. Hence, armpit, the most commonly used subcutaneous inoculation site with rich vascular circulation, was selected as the injection site of H22 cells but at the cost of the increasing difficulty in the surgery and subsequent treatment due to the fact that our tumor resection operation was virtually cytoreductive/palliative surgery rather than a complete resection. To date there have been no reports on the prevention of tumor recurrence with the armpit of animals as inoculation site.

As shown in Fig. 3, biodegradable polymer-based electrospun fibers loaded with anticancer drugs, such as cis/M5 with high flexibility which can be cut to almost any size and fabricated into other shapes using different target geometries for clinical applications, can be conveniently inserted to cover the formed cavity after surgical removal of the solid tumor.

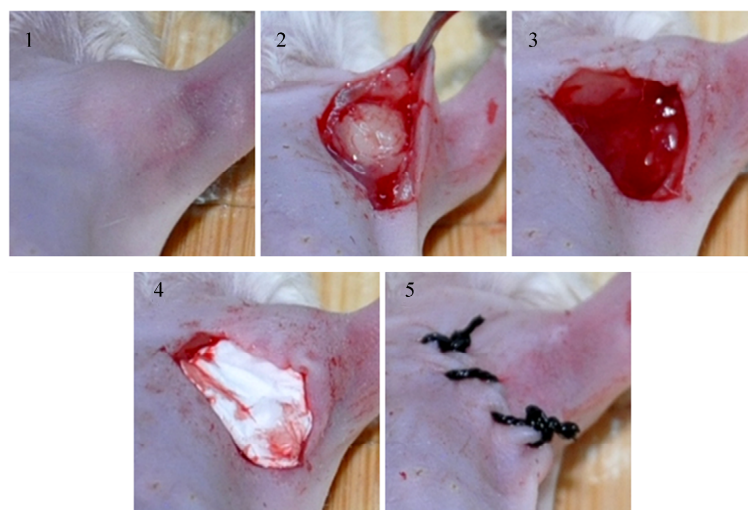


Fig. 3 Therapeutic process of cis/M5 in the treatment of mice: (1) anesthetization of mice, (2) exposure of the tumor, (3) surgical removal of the tumor, (4) direct placement of cis/M5 in the surgery site and (5) suturing

To determine *in vivo* efficacy of cis/M5, mice with primary H22 tumors underwent resection and were randomized in a blinded fashion to receive different treatments at time of surgery. The average estimated primary tumor volume ($127 \pm 57 \text{ mm}^3$) did not differ among the five experimental groups.

As shown in Fig. 4(a), on the 4th day post-surgery, the locoregional recurrence rate in the cis/M5, cis/M1, cis/i.v, PLA/M1, and control group was 0%, 50%, 20%, 80%, 100%, respectively. Median time to recurrence in these groups was 12, 4, 9, 4, and 4 days, respectively (cis/M5 versus cis/M1 $P = 0.291$, versus cis/i.v $P = 0.96$, versus PLA/M1 $P = 0.001$, and versus control $P = 0.005$; log-rank test). It would be specially mentioned that two of the eight mice in cis/M5 group remained freedom from local tumor recurrence up to five months; this is to say, they have been completely cured.

During the initial period after tumor resection, the growth of recurrent tumor was significantly inhibited in all cisplatin-treated groups while the average tumor volume in PLA/M1 and control group increased rapidly. There is no statistically significant difference among cis/M5, cis/M1 and cis/i.v group ($P > 0.05$).

Figure 4(c) showed that the survival time in cis/M5 group was greatly prolonged, with the median time to survival of 49 days compared with 33, 28, 30 and 23 days in cis/M1, cis/i.v, PLA/M1, or control group, respectively (cis/M5 versus cis/M1 $P = 0.279$, versus cis/i.v $P = 0.016$, versus PLA/M1 $P = 0.001$, and versus control $P = 0.001$; log-rank test).

As a typical index representative of systematic toxicity, relative body weights of the mice during the first 12 days of test were recorded. Among all test groups, cis/M1 and cis/i.v groups showed different degrees of weight loss, whereas the mice in cis/M5, PLA/M1 and control group kept steadily growing (Fig. 4d), indicating less systematic toxicity of the multilayered drug-loaded fiber mat.

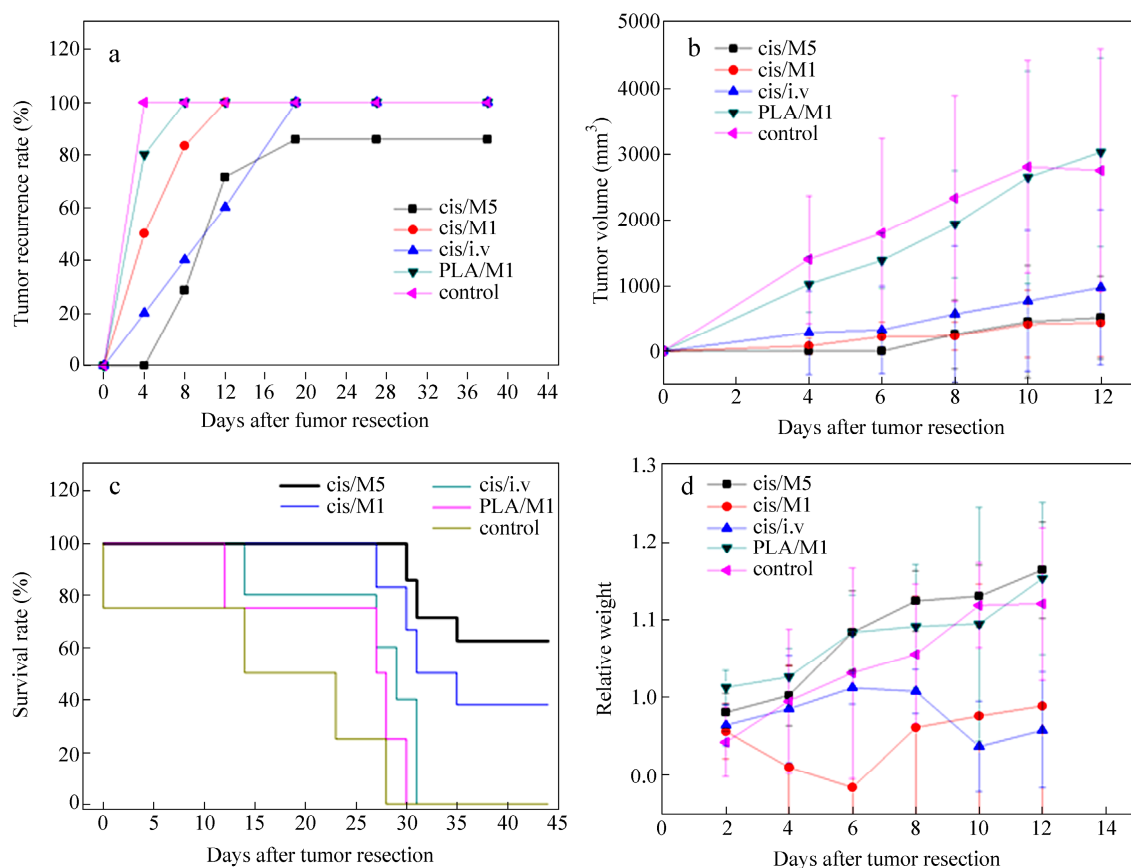


Fig. 4 (a) Tumor recurrence rate, (b) average tumor volume, (c) survival curves and (d) relative weight change of mice receiving different treatments after tumor resection

To comprehensively and objectively evaluate the inhibitory effect of cis/M5, a series of micrographs of H&E-stained sections were merged together to produce a panoramic scene. As shown in Fig. 5(a), the large

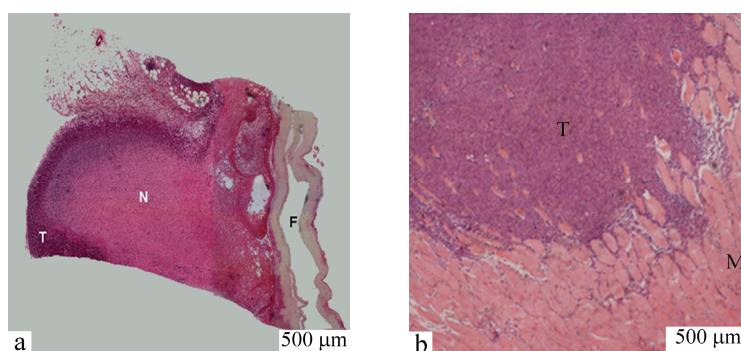


Fig. 5 Histopathological observation of local tissue samples in cis/M5 group (a) and control group (b) on the 4th day after tumor resection (F = cis/M5; N = necrosis or degeneration; T = viable tumor tissue; M = muscle)

tissue area beneath the cis/M5 fibrous mat was in the state of necrosis, with a thin layer of viable tumor remnants far away from the mat, whereas obvious tumor recurrence was observed in the control group. Meanwhile, different degrees of tumor recurrence occurred in cis/M1, PLA/M1, cis/i.v group (data not shown).

Based on the results and discussion above, we can draw the conclusion that the multilayered fibrous mat was more successful in retarding subcutaneous tumor recurrence, from the aspects of lower recurrence rate, prolonged survival time, and less systematic toxicity, compared to an equivalent dose of cisplatin delivered by single-layered fibrous mat or systemic injection. Obviously, these advantages are largely due to the improved release kinetics achieved by the multilayered fibrous structure.

CONCLUSIONS

The current study demonstrates that biocompatible multilayered cisplatin-loaded nanofibers have been designed to provide prolonged drug delivery and prevent local hepatocellular carcinoma recurrence following surgical resection while avoiding high systemic concentrations that lead to systemic toxicities. These results provide an encouraging prospect of using multilayered, drug-loaded electrospun nanofiber mats in local chemotherapy to prevent tumor recurrence following HCC surgery, alone or combined with presently employed treatment protocol.

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