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Antifungal efficacy of amphotericin B encapsulated fibrin microsphere for treating *Cryptococcus neoformans* infection in Swiss albino mice

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ABSTRACT

A natural and biocompatible fibrin microsphere is one of the most promising dual delivery vehicle as compared to other traditionally designed delivery modalities. It represents sustained delivery of encapsulated drug and is easily biodegradable in the blood circulation. In the present study, we evaluated the systemic augmentation of the antifungal activity of amphotericin B loaded in fibrin microsphere (AMB-fibrin microsphere) against cryptococcosis in Swiss albino mice. Mice infected with *Cryptococcus neoformans* were treated with 0.5 mg/kg AMB-fibrin microsphere that was given alternately for 7 days. The antifungal potential of AMB-fibrin microsphere was assessed on the basis of reduction of cfu count in the systemic circulation and various vital organs of infected mice. The formulation was found to be highly effective in reducing intracellular pathogen from the experimental animals where fibrin microsphere significantly controlled the release of amphotericin B for longer time duration. The AMB-fibrin microsphere chemotherapy was significantly more effective than free amphotericin B in reducing the fungal burden and showed better survival efficacy ($p < 0.05$). The current study demonstrating the use of novel amphotericin B loaded fibrin microsphere not only imparts protection to the encapsulated amphotericin B but also offers an effective strategy to decrease the drug associated toxicities.

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Introduction

Cryptococcus neoformans, a medically important opportunistic fungal pathogen showing multi-organ involvement, has been isolated from various natural sources like soil and rotten vegetables.[1] In human beings, *C. neoformans* is capable of causing pneumonia, meningitis and disseminated cryptococcosis in the presence of impaired cell mediated immunity.[2,3] Experimental studies have shown partial protection and delayed-type hypersensitivity in mice after vaccination with various protein preparations.[4–6] Prolonged survival and reduced fungal burden responses have also been observed after passive administration of anti-cryptococcal mAbs.[7] In

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recent years, *Cryptococcus* infection has shown tremendous increase in its frequency of occurrence across the globe.[8] Despite the emerging new trend of this pathogen no vaccine or immunotherapy has been found to avail complete protection against recurrence of *C. neoformans* challenge. Hence, the development of new chemotherapeutic strategies will be helpful in conjunction with traditional drug delivery system to combat intracellular pathogens.

Amphotericin B (AMB) is the most effective antifungal antibiotic and is recommended as treatment of choice for disseminated cryptococcal infections.[8–10] A major factor limiting the use of drug is poor accessibility to the intracellular pathogens. AMB is not used usually because of high toxicity and various side effects at high doses.[9,11,12] The in-house predesigned delivery system is a strategy to combat *C. neoformans* with improved delivery of the drug to the target site. Various traditional drug delivery vehicles have the capability of intracellular delivery of the antifungal or antibacterial agents and control its release over a prolonged period.[4,13] Several workers have shown the encapsulation of AMB into the multilamillar or unilamellar liposomes that release the drug thereby steadily reducing its toxicity.[14–16]

In the recent past, plasma beads have been used for delivery of various biological components. The plasma beads are composed of recipient own blood plasma and are highly biocompatible and biodegradable material.[17] They can entrap both low and high molecular weight drugs and have an ability to stay longer in blood circulation. In fact, plasma beads could have an extra edge over other methods thus becoming a potential and safe drug-delivery vehicle. On the other hand, poly-lactide co-glycolide (PLGA) microsphere based system has been designed to use constant delivery of various biological components, including drugs, antibiotics and proteins, etc.[18,19] The in vitro designed PLGA microspheres have revealed excellent biocompatibility. Concomitantly, due to non-toxicity, biodegradation, biocompatibility, and physiochemical properties, PLGA microspheres have a broad scope in being a potent chemotherapeutic delivery system.[20]

Materials and methods

Chemicals

Amphotericin B, poly-lactide co-glycolide (PLGA) and polyvinyl alcohol (PVA) were purchased from Sigma–Aldrich, USA. All other chemicals used in this study were of highest purity.

Experimental animals

Pathogen free Swiss albino female mice with an average weight of 18–22 g were used throughout the study. The animals were fed rodent feed and filtered water ad libitum. The animals were housed in propylene cages on wood powder bedding under standard atmospheric conditions (22 ± 1 °C temperature; 12 h light/12 dark photoperiod and 50–60% humidity). All experiments were performed according to the Guide for Experimental Animal Care Review Board, College of Pharmacy, King Saud University.

Experimental strain Cryptococcus neoformans

C. neoformans (ATCC 24067) strain was procured from American Type Culture Collection, USA. The strain was recovered from glycerol stocks and maintained in yeast extract peptone dextrose (YPD) media while shaking at 37 °C for 48 h. The cell suspension was centrifuged at 5000 × *g* for 15 min at 4 °C and washed thrice with sterile normal saline. After quantifying viable cell count the mice were infected intravenously with *C. neoformans* (1×10^6 cfu/mouse).

Preparation of AMB suspension

AMB was diluted firstly in 5% glucose and subsequently in phosphate buffered saline (PBS) and was given intraperitoneally (*ip*).

Preparation of plasma beads

For isolation of plasma, blood was collected through retro-orbital puncture from healthy mice with a heparinized capillary. Blood in heparin was centrifuged at 400 × *g* at 4 °C and plasma (supernatant) was isolated. An aliquot of plasma (250 μL) was immediately used for preparation of plasma beads whereas rest of the plasma was stored at −20 °C for further use. Plasma beads were prepared according to the published protocol.[21]

Preparation of AMB encapsulated PLGA microsphere

Preparation of PLGA was based on water in-oil-in-water (W/O/W) system. Microspheres were prepared by solvent evaporation technique according to the published protocol.[22] For the preparation of AMB encapsulated PLGA emulsion: 5 mg of AMB in 5% glucose was mixed with 190 mg PLGA dissolved in dichloromethane, 10% PVA was added to primary emulsion formed by sonication. After homogenization the resulting O/W emulsion was stirred for 18 h after which the formulation of AMB-PLGA microsphere was centrifuged and washed. The final purified formulation was kept in dessication after lyophilyzation at 4 °C.

Preparation of fibrin microsphere

The fibrin microsphere based dual delivery system was prepared by encapsulating AMB in PLGA microsphere and further entrapped in fibrin beads according to the published protocol.[4] Plasma (250 μL) was mixed with the suspension of AMB encapsulated PLGA microsphere in PBS. To the mixture, calcium chloride (40 mM) was also added. Aliquots (3 μL) of the mixture were transferred as droplets over a parafilm covered glass slide which were then incubated at 37 °C for 40 min. The beads after collecting from the parafilm were washed with normal saline and then with PBS.

Characterization of AMB-fibrin microsphere

The particle size and surface morphology

Scanning electron microscopy (SEM; Zeiss EVO 40; Carl Zeiss SMTAG, Oberkochen, Germany) of AMB-PLGA microsphere

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