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In vivo evaluation of urokinase-loaded hollow nanogels for sonothrombolysis on suture embolization-induced acute ischemic stroke rat model

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ABSTRACT

The urokinase-type plasminogen activator (uPA) loaded hollow nanogels (nUK) were synthesized by a one-step reaction of glycol chitosan and aldehyde capped poly (ethylene oxide). The resultant formulation is sensitive to diagnostic ultrasound (US) of 2 MHz. Herein, we evaluated the in vivo sonothrombolysis performance of the nUK on acute ischemic stroke rat model which was established by suture embolization of middle cerebral artery (MCA). Via intravenous (i.v.) administration, the experimental data prove a controlled release of the therapeutic protein around the clots under ultrasound stimulation, leading to enhanced thrombolysis efficiency of the nUK, evidenced from smaller infarct volume and better clinical scores when compared to the i.v. dose of free uPA no matter with or without US intervention. Meanwhile, the preservation ability of the nanogels not only prolonged the circulation duration of the protein, but also resulted in the better blood-brain barrier protection of the nUK formulation, showing no increased risk on the hemorrhagic transformation than the controls. This work suggests that the nUK is a safe sonothrombolytic formulation for the treatment of acute ischemic stroke.

1. Introduction

Stroke is an important cause of death and disability around the world [1]. Thrombolysis and endovascular interventional therapy are both evidence-based therapy for vascular recanalization [2]. Except the recombinant tissue plasminogen activator (rt-PA), urokinase-type plasminogen activator (uPA) is the second thrombolytic agent used in clinical practice, especially in the primary stroke center in China [3,4]. For thrombolytic action, uPA enables to active the fibrinolytic system to catalyze the production of plasmin for fibrin-splitting while it can cause the degradation of fibrinogen, Factor V and Factor VIII to inhibit the occurrence of thrombin. Besides, it was also found that uPA is positive to increase the activity of ATPase, and hence hampers the ADP mediated platelet aggregation which decreases the tendency of thrombogenesis [4].

The treatment of cerebral ischemic stroke using rt-PA or uPA is

Abbreviation: SD, Sprague-Dawley; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; UK, urokinase; nUK, uPA-loaded nanogels; US, ultrasound; UK+US, ultrasound and free urokinase; nUK+US, ultrasound and uPA-loaded nanogels; TCD, Transcranial Doppler; MRI, magnetic resonance imaging; EB, evens blue; HT, hemorrhagic transformation; Hb, hemoglobin; BBB, blood-brain barrier; ELIP, echogenic liposomes; CCA, common carotid artery; TTC, 2,3,5-triphenyltetrazolium chloride.

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normally limited by the narrow time window as well as the risk of hemorrhage complication. As a protein, either rt-PA or uPA suffers from the shorter circulation duration due to the fast clearance by the proteinase in vivo. These facts could have led to the poor clinical outcome of patients with large artery occlusion, for example, a low recanalization level around 40% at twenty-four hour after an intravenous (i.v.) administration of the thrombolytic enzymes [5–7]. Furthermore, even the major occluded brain arteries were recanalized, patients may still fail to achieve clinical improvement since the ischemic tissue might not benefit from the recanalization due to the clots in the microcirculation and injuries to the endothelium [8,9], which makes microcirculation no-reflow, a phenomenon in which major vessel recanalization does not result in some part of microvascular reperfusion [9–11]. However, increasing the dose level would not be completely accepted in clinical application for better thrombolysis due to the neurotoxicity of the proteins [10], besides the concern of hemorrhage.

The improvement of specificity of the thrombolytic agents is a way to overcome the drawbacks of the rt-PA or uPA treatment. Many studies showed that entrapment of the proteins into a delivery system like liposomes could benefit the thrombolysis primarily resulting from the prolongation of circulation duration and the capacity of endowing passive or active targeting ability [12–20]. Among them, ultrasonic (US) induced delivery of thrombolytic proteins using microbubbles has demonstrated promising properties for site-specific release of payloads from the carrier through cavitational effects and acoustic radiation force on the carrier [21]. Besides, recent researches also showed that with microbubbles, ultrasound could not only recanalize the acute intravascular thrombi in large vessels [22–27], but also make the microcirculation recanalization [28–31], showing accelerated thrombolysis via the generation of localized mechanical stress on the thrombi [32]. Actually, the ability of ultrasound (US) energy to force enzymatic thrombolysis was already described in 1976 [33] and several experimental studies have confirmed this finding [34–36].

Nevertheless, to date, neither sonothrombolysis with nude intravenous thrombolytics nor with intravenous administrating formulations, e.g. thrombolytic loaded echogenic liposomes (ELIP), had acknowledged ultrasonic parameters and material consistency. Moreover, there are reports to suspect that with an ultrasonic frequency of 300 kHz, the thrombolysis process may take enhanced venture on intracerebral hemorrhage [37,38]. On the other hand, the utility of higher frequency ultrasound is considered safer [39–41]. In previous work, we synthesized hollow nanogels (diameter ~ 200 nm) for the loading of uPA [17,42]. The nanogels composed of glycol chitosan (GC) and benzaldehyde-capped polyethylene oxide (OHC-PEO-CHO) were fabricated via an one-step procedure for crosslinking through in situ Schiff's reaction. We found that the nanogels enabled to response the ultrasound at 2 MHz, a frequency being already used in diagnosis of intracranial artery stenosis. And different from microbubbles, the nanogels contains no gas. The responsibility was attributed to the vibration of the crosslinked polymer matrix driven by the ultrasonic energy. In vitro experiment has shown that under the ultrasonic mediation, the cargoes, i.e. the loaded uPA molecules, could be favorably released to enhance the thrombolysis of clots while in vivo tests proved that the half-life time of uPA in circulation system was significantly prolonged [17]. Although such characteristics indicate promising potentials for the uPA loaded nanogels to be used in stroke treatment, especially for safety issues, the test of real thrombolytic effect in animal model is still necessary. Therefore, in this study we established persistent middle cerebral artery occlusion (MCAO) model on Sprague-Dawley (SD) rats to investigate the efficiency and mechanism of combining uPA with ultrasound intervention by the hollow nanogel carrier for the thrombolysis of acute ischemic stroke.

2. Material and methods

2.1. Animal model

The animal studies were approved by the Animal Research Committee of Peking University First Hospital (Beijing, China), and the investigation conformed to the Guide for the Care and Use of Experiment Animals of Peking University First Hospital. Adult male SD rats weighing 270–300 g were obtained from the Vital River Company, China, and raised under normal conditions with free access to food and water. The animals were anesthetized with pentobarbital at a dose of 50 mg/kg through intraperitoneal injection and then subjected to permanent MCAO by use of the filament model as previously described [43,44]. Briefly, the right common carotid artery (CCA) were ligated permanently and a silicon-coated 6-0 surgical monofilament nylon suture 4 cm in length was advanced proximally until mild resistance was felt to occluding blood flow to the right MCA. The signs of a successful surgery included the following: flexion or less grasping ability of the left foreleg and spontaneous circling or toppling to the left. For transcranial ultrasound application, being similar to the previous studies [44,45], the animals were shaved on the scalp. And a plastic ring filled with ultrasonic coupling gel (diameter 3.5 mm, 10 mm) was placed on the midline of the animal's head. A diagnostic Transcranial Doppler (TCD) machine (VIASYS, UK) with the transducer was placed 5 mm above the skull to ascertain the full transmission of sound to the skull. The frequency and output intensity were set at 2 MHz and 530 mW/cm^2, respectively.

2.2. Preparation of the uPA-loaded hollow nanogels (nUK)

Benzaldehyde terminated poly(ethylene oxide) (OHC-PEO-CHO, MW = 600 Da) and uPA-loaded glycol chitosan (GC)/OHC-PEO-CHO hollow nanogels were synthesized according to our previous report [42]. GC, OHC-PEO-CHO and urokinase were dissolved in water at pH 5.0–5.5. The solution was pumped into a Sono-Tek ultrasonic nozzle with a peristaltic pump in a steady velocity. Ammonia was diluted by 50 times and sprayed through a bypath way in order to ensure a basic atmosphere around the fog drops. The fog drops were collected in a beaker and then transferred to a dialysis tube with a molecular weight cut-off of 12 kDa and dialyzed against $NaHCO_3$/NaOH solution (4.4 mM $NaHCO_3$, 1.4 mM NaOH, pH 8–8.5) at room temperature for 24 h with six changes, and then against distilled water with two changes in 8 h. The dialysate was freeze-dried to harvest white powder-like products with yields of 76 wt %, calculated from the mass of the product to the overall weight of feed reagents. The morphology of the nanogels was characterized using scanning electron microscope (SEM, HITACHI S-4300, Japan) and transmission electron microscopy (JEOL 1011, Japan). And the loading capacity of the uPA was measured using BCA protein quantitative kit. The uPA loaded nanogel (nUK) products were stored at −20 °C before use.

2.3. In vivo thrombolysis

The rats with signs of successful surgery were divided into five groups with each group of 42 rats as: group 1, permanent MCAO with saline treatment (denoted as MCAO); group 2, MCAO with UK treatment (UK); group 3, MCAO with nUK treatment (nUK); group 4, MCAO with US plus UK treatment (US+UK); group 5, MCAO with nUK plus US treatment (nUK+US). The active urokinase dose was 100,000 U/kg in the UK and nUK groups and was applied through intravenous tail injection 30 min after MCAO. And the 2 MHz US

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