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Serial MRI study of the enhanced therapeutic effects of liposome-encapsulated citicoline in cerebral ischemia

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ABSTRACT

Liposome encapsulation of active principles enhances their bioavailability to the brain. We investigated whether encapsulation of citicoline in liposomes increases its therapeutic effects in ischemia, performing a longitudinal MRI study of lesion volumes and edema in an animal model of stroke. Nineteen rats were submitted to permanent occlusion of the middle cerebral artery and treated with: (1) saline, (2) intraperitoneal citicoline (500 mg/kg), (3) intravenous citicoline (48 mg/kg), and (4) intravenous liposome-encapsulated citicoline (48 mg/kg). Lesion volumes were measured by MRI at days 0, 1, 3 and 7 following surgery. Encapsulation in liposomes increased the therapeutic effects of citicoline, as reflected by a 32% reduction of the infarct sizes at day 7, in contrast with controls where infarct sizes at day 7 increased by 39%, respect to values at day 0. Intravenously injected citicoline reduced infarct sizes by 9% while intraperitoneal citicoline resulted in an increase of infarct sizes by 10%. A slight (not significant) reduction of edema formation was observed for animals treated with citicoline, in all of its delivery forms. Liposome-encapsulated citicoline causes a noticeable reduction in lesion volumes as compared to free citicoline (either i.p. or i.v.) at days 1, 3 and 7 following permanent stroke.

1. Introduction

Ischemic stroke is a major cause of death and incapacity in developed countries, with an increasing incidence because of the progressive aging of the population in such countries. So far, the only treatment that has proven efficiency in clinical practice is thrombolysis, though it presents serious safety-related restrictions that limit its use to a reduced fraction of patients (<3%)(Kleindorfer et al., 2009). For the large number of patients where acute recanalization is not used, there is a need for neuroprotective and neuroreparative strategies to contain brain damage, and boost brain repair after the onset of ischemia. There is a hot debate on the stroke community about strategies to be followed to treat this disease. While neuroreparative therapies are under development, and seem to be far from being translated into the clinics, most neuroprotective strategies with impressive results in preclinical research have failed in the clinical setting, for different reasons(Dyken, 2010). In this regard, some authors claim that research should not be focused just on the development of new treatments for stroke, but must also consider how to deliver those agents efficiently to the stroke-stricken brain(Adibhatla et al., 2005; Pardridge, 2002).

A good example of this disjunctive is citicoline (CDP-choline, cytidine-5′-diphosphocholine) an essential intermediate in the synthesis of phosphatidylcholine (a major brain phospholipid)(Adibhatla and Hatcher, 2005), citicoline is believed to interfere in cell membrane damage, providing a benefit for disorders of the central nervous system, including stroke(Adibhatla and Hatcher, 2005). After the demonstration of efficiency in the preclinical field (Clark, 2009; Hurtado et al., 2005), citicoline has also been tested in several clinical trials with unclear outcome, and its usefulness for the treatment of stroke is under discussion(Adibhatla and Hatcher, 2005; Adibhatla et al., 2005; Clark, 2009; Davalos et al., 2002). One of the main reasons for the controversy on the efficiency of citicloine in the clinics is the different ways of administration used for the drug (oral versus intravenous), especially considering that only 0.2–2.0% of the administered citicoline ends up in the brain parenchyma, depending on the administration route(Adibhatla and Hatcher, 2005; Adibhatla et al., 2005; Clark, 2009; Fresta and Puglisi, 1996, 1997, 1999; Fresta et al., 1995; Puglisi et al., 1992). This fact is highly influenced by the polar nature of citicoline, which hampers the crossing of the drug through the blood–brain barrier (BBB). Therefore, the use of alternative ways of administration for citicoline, to increase its bioavailability in

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the brain parenchyma, would potentially enhance the therapeutic effects of this drug for the treatment of stroke.

We have performed a serial MRI study on an animal model of cerebral ischemia, comparing the therapeutic effects of liposome-encapsulated citicoline versus the free form of the drug, administered by two different routes (i.p. versus i.v.). Our intention was to investigate whether this well-known pharmacological approach to facilitate the trespassing of hydrophilic compounds through the BBB could become a feasible way to increase the therapeutic effects of citicoline in the clinics, bringing some light to the existing doubts of its possibilities for the treatment of stroke.

2. Materials and methods

2.1. Animal management

Nineteen (19) male Sprague–Dawley rats (Harlan) weighting 261 ± 12 g were kept at controlled conditions of temperature ($22 \pm 1\,^{\circ}C$) and humidity ($60 \pm 5\%$), with a 12/12 h light/dark cycle, and granting free access to food and water. One animal died during surgery and two died within the first 24 h after surgery. For surgery and MRI rats were anesthetized with sevoflurane (3% in 70% N_2O and 30% O_2). Rectal temperature was monitored and maintained at $37 \pm 1\,^{\circ}C$ with a feedback controlled heating system (1025 system, SA Instruments, NY, USA). Animals were sacrificed under deep anesthesia (8% sevoflurane). All procedures were performed under EU regulations (European Communities Council Directive of 24 November 1986 – 86/609/EEC), with the approval of our institution's ethics committee.

2.2. Middle cerebral artery occlusion

Permanent occlusion of the left middle cerebral artery (MCA) was performed by suture of the artery following the method of Shigeno et al. (1985). In brief, an incision was practiced along the temporal muscle of the rat. A small (3 mm) hole was drilled in the exposed skull and the MCA was proximally exposed, where the artery bifurcates in its frontal and parietal branches, and was carefully retracted from the brain using a Sinskey manipulation hook attached to a micromanipulator (both from World Precision Instruments Inc., Fl, USA). Then, the MCA was sutured with a 10-0 Ethilon (polyamide 6) surgical suture (Ethicon Inc., NJ, USA), the hook was retracted letting the artery to lay back over the brain, and the absence of blood flow was visually confirmed under the microscope. Finally, the temporal muscle was gently relocated over the skull and the skin of the animal was sutured. Brain surgery was performed after permanent ligation of the ipsilateral carotid artery.

2.3. Treatments and experimental groups

Citicoline (generous gift from Ferrer Internacional S.A., Spain) was solved in saline to prepare two stock solutions of different concentration (125 mg/ml, for i.p. injections, and 2 mg/ml, for i.v. injections). One hundred nm-sized DSPC:Cholesterol:PEG-DSPE (0.62/0.33/0.05 molar ratio) liposomes containing citicoline were prepared at the Photophysics and Photochemistry Laboratory of the University of Santiago de Compostela (Spain). Liposomes were obtained by the well-known method of lipid film rehydration using a mixture of methanol and $CHCl_3$ as organic solvent and HBS (Hepes buffer solution) 20 mM as rehydrating solution containing citicoline at a concentration of 40 mg/ml. After rehydration, liposomes were extruded at $60\,^{\circ}C$ 11 times through a 200 nm polycarbonate membrane, followed by another 11 extrusions through a polycarbonate membrane of 100 nm using a miniextruder from Avanti Lipids Inc., Alabama. Resulting liposomes were ultra-centrifuged twice (11,000 rpm, $4\,^{\circ}C$, 16 h) to separate free from encapsulated citicoline. Proper size of resulting liposomes was determined with a Zetasizer DLS system from Malvern Instruments Ltd (UK). The exact content of citicoline in the liposomes (2 mg/ml, 8%) was certified by fluorescence methods developed and performed at the laboratory of origin. Citicoline solutions and liposomes were used within 2 days from their preparation and kept protected from light at $4\,^{\circ}C$ until use.

Treatments where applied either intravenously (i.v.), injecting 1 ml of treatment in the jugular vein, or intraperitoneally (i.p.), injecting 1 ml of treatment in the abdomen of the animals. Rats were randomly assigned to the following groups: (1) control: receiving i.v. injections of saline at $t = 30$ min, 6 h, 12 h, 18 h, 24 h and 30 h post ischemia ($n = 4$); (2) citicoline IP: receiving one i.p. injection of citicoline in saline (1 ml, 125 mg/ml, total dose of 500 mg/kg) at $t = 30$ min post ischemia ($n = 4$); (3) citicoline IV: receiving i.v. injections of citicoline in saline (1 ml, 2 mg/ml, total dose of 48 mg/kg) at $t = 30$ min, 6 h, 12 h, 18 h, 24 h and 30 h post ischemia ($n = 4$), and (4) liposomes: receiving i.v. injections liposomes in saline (1 ml, 2 mg/ml of citicoline, total dose of 48 mg/kg) at $t = 30$ min, 6 h, 12 h, 18 h, 24 h and 30 h post ischemia ($n = 4$). The 500 mg/kg dose was chosen as the minimum one that has shown a therapeutic effect on the rat after i.p. administration(Hurtado et al., 2005). The 1/10th dose of 48 mg/kg of citicoline injected i.v. (either free of in liposomes) was adopted according to Adibhatla et al., who calculated that a dose ranging 7–40 mg/kg is equivalent to that administered to patients in the clinical setting (Adibhatla et al., 2005).

2.4. MR imaging

MRI explorations were performed 30 min and 1, 3 and 7 days following surgery. MR images were acquired at 9.4 T on a horizontal bore MR system Bruker Biospec USR94/20 (Bruker Bioespin, Ettlingen, Germany), and equipped with gradient coils of 440 mT/m and a transmitting RF "birdcage" coil and a receiving surface (4-channels) coil, working at 400 MHz. T2 weighted images (T2w) were obtained from a series of multi-slice multi-echo images (train of 16 echoes) acquired with a spin-echo sequence, using the following parameters: field-of-view: 19.2 mm × 19.2 mm; matrix size: 192 × 192 pixels (in-plane resolution of 100 μm); 14 consecutive coronal slices of 1 mm thickness, covering the whole brain from the rhinal fissure to the cerebellum; echo time: 9 ms (16 echoes); repetition time: 3000 ms; spectral bandwidth: 60,000 Hz. Diffusion weighted images (DWI) and apparent diffusion coefficient (ADC) maps were obtained using the Stejskal–Tanner model (Stejskal and Tanner, 1965) by acquiring a set of 3 DW images with the following parameters: diffusion-weighted echo-planar-imaging (DW-EPI) sequence; field-of-view: 28.8 mm × 28.8 mm; matrix size: 256 × 192 points, zero-filled to 256 × 256 points (in-plane resolution of 112.5 μm); echo time: 30 ms; repetition time: 7000 ms; 3*b* values of 0, 400 and 1000 s/mm^2; spectral bandwidth: 100.000 Hz.

2.5. Image analysis

All images were processed with custom-made applications for Image J (Rasband, 1997–2009). ADC maps were constructed by pixel-wise fitting of the sets of 3 DW Images to the Stejskal–Tanner equation (Stejskal and Tanner, 1965), using the Levenberg–Marquardt algorithm.

2.6. Statistical analysis

Data is presented as mean ± standard deviation. For the comparison of each individual group versus the control group, and between liposome-encapsulated group and free citicoline (i.p. and i.v.) groups, in an individual basis, a two-tailed Student's *t*-tests