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CDP-choline liposomes provide significant reduction in infarction over free CDP-choline in stroke

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Abstract

Cytidine-5'-diphosphocholine (CDP-choline, Citicoline, Somazina) is in clinical use (intravenous administration) for stroke treatment in Europe and Japan, while USA phase III stroke clinical trials (oral administration) were disappointing. Others showed that CDP-choline liposomes significantly increased brain uptake over the free drug in cerebral ischemia models. Liposomes were formulated as DPPC, DPPS, cholesterol, GM₁ ganglioside; 7/4/7/1.57 molar ratio or 35.8/20.4/35.8/8.0 mol%. GM₁ ganglioside confers long-circulating properties to the liposomes by suppressing phagocytosis. CDP-choline liposomes deliver the agent intact to the brain, circumventing the rate-limiting, cytidine triphosphate:phosphocholine cytidylyltransferase in phosphatidylcholine synthesis. Our data show that CDP-choline liposomes significantly ($P < 0.01$) decreased cerebral infarction (by 62%) compared to the equivalent dose of free CDP-choline (by 26%) after 1 h focal cerebral ischemia and 24 h reperfusion in spontaneously hypertensive rats. Beneficial effects of CDP-choline liposomes in stroke may derive from a synergistic effect between the phospholipid components of the liposomes and the encapsulated CDP-choline.

Keywords

Blood-brain barrier; cytidine triphosphate:phosphocholine cytidylyltransferase; Focal cerebral ischemia; CNS injury; Cytidine-5'-diphosphocholine; Citicoline; Ischemic injury volume; Somazina

Cytidine-5'-diphosphocholine (CDP-choline, citicoline or Somazina) is an important drug which is commercially available as a treatment of stroke in over 70 countries including France, Germany, Italy and Spain. CDP-choline has shown beneficial effects in transient cerebral ischemia [1,2,4,10,25,26] and has undergone clinical trials for stroke treatment [1,2,4,14,28]. Clinical trials in Japan and Europe showed that CDP-choline improved recovery, while USA trials [11–13,30] have provided ambiguous results. Our review [2] evaluated several factors including route of administration that might have hindered efficacy of CDP-choline in USA clinical trials. In the light of recent clinical evaluations [14], experimental data in cerebral ischemia, excellent safety nature of this drug [22] and realization that oral route used in USA trials was less effective, new methods of administration such as liposome encapsulation of CDP-choline [18] are warranted. Exogenous CDP-choline is rapidly hydrolyzed and absorbed as cytidine and choline [28]. CDP-choline has to be re-synthesized from phosphocholine and cytidine triphosphate (CTP) by CTP:phosphocholine cytidylyltransferase (CCT) [21] that is

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expressed in brain [33]. In animal studies, brain uptake is 2% when CDP-choline is administered i.v. [18] compared to only 0.5% uptake by oral route [7]. Liposome encapsulation of CDP-choline (i.v.) provides more efficient delivery to the ischemic brain and thus increases brain uptake [18]. Liposomes remain in circulation for more than 24 h [18], thereby providing a sustained release of the drug. Previous studies showed that liposome encapsulated CDP-choline increased the survival rate and decreased lipid peroxidation after transient global cerebral ischemia in Wistar rats [18]. To the best of our knowledge, this is the first report showing that liposome encapsulated CDP-choline significantly ($P < 0.01$) reduced infarction compared to equivalent dose of free CDP-choline (not in liposomes) after 1 h transient middle cerebral artery occlusion (tMCAO) and 24 h reperfusion in spontaneously hypertensive rats.

The following materials were obtained from the indicated supplier: dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylserine (DPPS), cholesterol, GM₁ ganglioside (Avanti Polar Lipids, Alabaster, AL); CDP-choline (BioMol, Plymouth Meeting, PA); other chemicals and Nucleopore filters (Fisher Scientific); 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma, St. Louis, MO).

Focal cerebral ischemia [15,16]

All surgical procedures were conducted according to the animal welfare guidelines set forth in the *Guide for the Care and Use of Laboratory Animals* (National Academy Press Washington, D.C. 1996) and were approved by the animal care and use committee of the University of Wisconsin-Madison. Male spontaneously hypertensive rats (SHR, 250–275 g) were subjected to 1 h MCAO. Under halothane anesthesia (induction: 2%; maintenance: 1.2%) in an O₂ and N₂O (50:50) mixture, the left common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were exposed through a ventral midline incision. The upper region of the left temporal muscle was dissected to expose the temporal bone. A 1 mm burr hole, 2 mm posterior to the bregma and 11–12 mm left of the midline, was drilled, leaving the inner layer of the temporal bone intact. A laser Doppler flow meter probe (model PD-434; Vasamedics, LLC, St. Paul, MN) connected to a laser flow meter (LaserFlo blood perfusion monitor BPM 403A; Vasamedics, LLC, St. Paul, MN) was fixed over the bore hole for continuous monitoring of rCBF. A 3–0 monofilament nylon suture with a rounded tip was introduced into the ECA lumen and gently advanced to ICA until slight resistance was felt and a reduction in rCBF was seen. After 1 h occlusion, the suture was withdrawn to restore the blood flow (confirmed by laser Doppler flowmetry). The left femoral artery was cannulated for continuous arterial blood pressure monitoring and to obtain measurements of pH, PaO₂, hemoglobin and blood glucose concentration. Mean arterial blood pressure, blood gases PaO₂ and PaCO₂ were monitored and regulated. Body temperature was maintained at 37–38 °C throughout the procedure. Arterial blood gases were maintained within normal limits (PaO₂ 100–140 mm Hg; PaCO₂ 30–40 mm Hg) [15]. Treatment with free CDP-choline (i.p. or i.v.) or liposome encapsulated CDP-choline (i.v.) did not affect the physiological variables and did not cause any hypothermia [26,27].

Preparation of CDP-choline liposomes [18]

The liposome system consisted of DPPC, DPPS, cholesterol and GM₁ ganglioside (7/4/7/1.57 molar ratio or 35.8/20.4/35.8/8.0 mol%). CHCl₃:MeOH solutions of the lipids were combined and evaporated to dryness to form a thin lipid film in a round-bottom flask using a Buchi rotary evaporator. The lipid film was rehydrated at 60 °C to a concentration of 50 mg lipid/mL with a solution of CDP-choline (40 mg/mL) in sterile saline. The resulting vesicles underwent eight freeze/thaw cycles by freezing in liquid N₂ and warming in a 70 °C water bath. The resulting suspension was extruded at 70 °C (above the phase transition temperature) successively through 400 nm, 200 nm and 50 nm Nucleopore polycarbonate filters (six passages each) using

a Lipex™ stainless steel Thermobarrel extruder pressurized with argon (Northern Lipids, Vancouver, British Columbia, Canada). Free CDP-choline was separated from liposomes containing CDP-choline by Sephadex G-50 gel permeation chromatography. CDP-choline content of the liposomes (eluted in the void volume) was measured by UV ($E_{282\text{ nm}}$ 12800) against a CDP-choline standard curve and determined to be 9 mg/mL (encapsulation efficiency was 23%). Particle size was measured using a Nicomp 380 particle size analyzer (Particle Sizing Systems, Santa Barbara, CA). Control liposomes were similarly prepared, substituting sterile saline for the CDP-choline solution.

CDP-choline treatment

Intravenous injections were performed via a catheter inserted into the femoral vein. CDP-choline liposomes were administered i.v. at the onset of reperfusion and at 3 h. Control rats received a corresponding volume of control liposomes (liposomes without CDP-choline) i.v. Free CDP-choline was dissolved in saline to give the desired dose (18 mg/kg or 500 mg/kg) and administered either i.v. or i.p. at 0 and 3 h reperfusion. Control rats received saline at 0 and 3 h reperfusion. Rats received injections of 0.5 mL/250 g body weight.

Ischemic injury volume (infarction) [15]

After 24 h reperfusion, anesthetized rats were decapitated. Brains were placed in a -70°C freezer for 4.5 min, and then 2 mm coronal slices were cut using a rat brain matrix (Ted Pella, Inc., Redding, CA) and stained with 2% TTC. Brain sections were scanned, and the ischemic injury volumes were computed by the numeric integration of data from individual slices using the NIH Image program. To compensate for brain swelling in the ischemic hemisphere, corrected infarct volumes were calculated as: corrected infarct volume = right hemisphere volume – (left hemisphere volume – measured infarct volume) [29].

Statistical analyses

Infarct volumes (mm^3) were presented as mean $\pm$ SD and analyzed by ANOVA followed by Bonferroni's multi-group comparisons post-test using Prism software (GraphPad, San Diego, CA). A value of $P < 0.05$ was considered significant.

Liposome encapsulated CDP-choline significantly attenuated infarction compared to equivalent dose of free CDP-choline

Representative TTC-stained brain sections are presented in Fig. 1. Two doses of CDP-choline liposomes (CDP-choline 18 mg/kg each dose) at 0 and 3 h reperfusion significantly ($P < 0.01$) reduced the infarction by 62% compared to saline group (Table 1). CDP-choline liposome treatment also significantly ($P < 0.01$) reduced the infarction compared to a corresponding dose of free CDP-choline (26% reduction, $P < 0.01$ compared to saline group). Control liposomes reduced the infarction by 11% (not significant compared to saline group). Higher dose of free CDP-choline (500 mg/kg i.p. or i.v. at 0 and 3 h reperfusion) also reduced the infarction by 50–55% ($P < 0.01$ compared to saline group). There was no significant difference between 500 mg/kg i.p. and i.v. treatments. Free CDP-choline at 100 and 250 mg/kg at 0 and 3 h reperfusion provided intermediate protection (32% and 41%, respectively, $P < 0.01$ compared to saline group, Table 1).

In our studies, free CDP-choline (18 mg/kg i.v.) at 0 and 3 h reperfusion provided a small but significant reduction (26%, Table 1) in infarction. This is in contrast to other studies reporting that free CDP-choline at 100 and 250 mg/kg was ineffective in tMCAO in Sprague–Dawley rats [26,27]. In 48 h permanent MCAO in Fischer rats, free CDP-choline 500 mg/kg did not

reduce cortical infarction [20]. These differences may be due to the longer ischemia period and the use of different strains (Wistar or Fischer) of rats [15].

In the clinical setting, CDP-choline has been administered orally at doses of 500 to 2000 mg per patient irrespective of their body weight. For patient body weights of 50–70 kg, these doses are equivalent to 7 to 40 mg/kg. This is in the lower dose range of free CDP-choline used in our studies (18 mg/kg at 0 and 3 h, total dose 36 mg/kg) which provided modest benefit. However, similar dose of CDP-choline encapsulated in liposomes provided more robust protection even compared to 500 mg/kg free CDP-choline (i.p. or i.v., Table 1). Other studies have also demonstrated that free CDP-choline (500 mg/kg) significantly reduced total infarct volume in tMCAO [26] and striatal infarct volume in permanent MCAO [20].

GM₁ ganglioside confers long-circulating time to liposomes

The liposome formulation used in the current study incorporating phosphatidylserine and GM₁ ganglioside gave the highest encapsulation efficiency for CDP-choline and provided the longest circulation time [17,18]. Incorporation of GM₁ ganglioside and anionic lipids such as phosphatidylserine into liposomes causes prolonged circulation time in vivo since complement-dependent phagocytosis of the liposomes is almost completely suppressed [19, 31,32]. The presence of GM₁ ganglioside inhibits uptake by phagocytes similar to PEGylation of liposomes [9]. Circulation half-lives of liposomes also increase with decreasing size, such as the 50 nm liposomes used in the current study [8].

There are several advantages to using CDP-choline liposomes: (1) effective at low doses, (2) the presence of ganglioside GM₁ in the liposome formulation prolongs the vesicle circulation time for up to 24 h with approximately 30% of the liposomes still in circulation [18], providing a sustained delivery of the agent, (3) brain uptake of the drug is increased to ~23% of the injected dose [18] compared to 0.5–2.0% by oral or i.v. administration of free CDP-choline, thus the bioavailability of the agent is substantially augmented. During ischemia/reperfusion, a hyper-permeability of blood–brain barrier results, allowing the passage of lipid vesicles <100 nm directly through such a compromised vasculature.

We have previously demonstrated increases in phospholipase A₂ activity [3,5] and levels of lyso-phosphatidylcholine (an inhibitor of CCT) and loss of CCT activity following transient cerebral ischemia [6]. Recent data suggest that mRNA and protein levels of secretory phospholipase A₂ group IIA showed significant increases after 1 h transient MCAO at 24 h reperfusion which were attenuated by CDP-choline. An additional and important benefit of liposome encapsulation of CDP-choline is that the liposomes protect exogenous CDP-choline from hydrolysis by liver, and the intact drug will be delivered to the brain without going through the rate-limiting CCT step [6,21]. Thus, the status of CCT activity during ischemia and reperfusion may not be a limiting factor for CDP-choline availability in the brain.

The slight reduction in infarction by control liposomes may be due to the presence of GM₁ ganglioside [18]. Another factor that may have contributed to the enhanced therapeutic efficiency of CDP-choline liposomes is the phenomenon of passive targeting due to the presence of DPPS in the liposomes. The CDP-choline liposome neuro-protection may come from a synergistic effect between the phospholipid components of the liposomal delivery system and the encapsulated CDP-choline.

CDP-choline use in stroke treatment in USA may still be achievable by efficient delivery methods and warrants additional clinical trials. This study also serves as a reminder that development of effective treatments for stroke requires not only the discovery of neuroprotective agents but also insight in how to deliver those agents efficiently to the stroke-stricken brain [23,24].

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Fig 1.

Effects of control liposome, free CDP-choline and CDP-choline liposome treatments on infarction after 1 h tMCAO and 24 h reperfusion in SHR. (A) Saline; (B) control liposomes, 0.5 mL i.v. at 0 and at 3 h reperfusion; (C) free CDP-choline (500 mg/kg i.v. at 0 and 3 h reperfusion); (D) free CDP-choline (18 mg/kg i.v. at 0 and 3 h reperfusion); and (E) CDP-choline liposomes (0.5 mL i.v. at 0 and 3 h reperfusion, delivering CDP-choline 18 mg/kg). Liposomes consisted of DPPC, DPPS, cholesterol and GM₁ ganglioside (7/4/7/1.57 molar ratio or 35.8/20.4/35.8/8.0 mol%). Details of the liposome preparation are given under Preparation of CDP-choline liposomes section. Infarction was determined by TTC staining. $n = 5$ per each treatment group.

Table 1

Effect of saline, control liposomes and different doses of free CDP-choline (two doses of 18– 500 mg/kg i.v.) and CDP-choline liposome treatment on infarction after 1 h MCAO and 24 reperfusion in SHR ($n = 5$ per group)

Treatment	Infarction, mm ³	% decrease in infarction
Saline (Fig. 1A)	270 ± 38	–
Control liposomes 0.5 mL i.v. (Fig. 1B)	240 ± 20	11%
Free CDP-choline (500 mg/kg, i.p.)	135 ± 10 ^a	50%
Free CDP-choline (500 mg/kg, i.v., Fig. 1C)	121 ± 12 ^a	55%
Free CDP-choline (250 mg/kg, i.v.)	159 ± 13 ^a	41%
Free CDP-choline (100 mg/kg, i.v.)	183 ± 15 ^a	32%
Free CDP-choline (18 mg/kg, i.v., Fig. 1D)	200 ± 18 ^a	26%
CDP-choline liposomes (0.5 mL i.v., delivering CDP-choline 18 mg/kg, Fig. 1E)	102 ± 11, ^{abc}	62%

Liposomes consisted of DPPC, DPPS, cholesterol and GM1 ganglioside (7/4/7/1.57 molar ratio or 35.8/20.4/35.8/8.0 mol%). Details of the liposome preparation are given under Preparation of CDP-choline liposomes section. Each treatment was given at 0 and 3 h reperfusion. Infarction was determined by TTC staining. Reduction in infarction was calculated in comparison to saline group.

^a $P < 0.01$ compared to saline group.

^b $P < .01$ compared to control liposomes.

^c $P < 0.01$ compared to free CDP-choline (18 mg/kg, i.v.).