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In vitro and in vivo evaluation of donepezil-sustained release microparticles for the treatment of Alzheimer's disease

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Abstract

The purpose of this work is to prepare donepezil microparticles (DM) and evaluate its advantage as a sustained release delivery system with subcutaneous injection once a month. DM was prepared using poly (D,L-lactide-*co*-glycolide) (PLGA) by an oil-water emulsion solvent evaporation technique. DM showed the loading ratio 13.2±2.1% (w/w) and yield 54.8±0.8% with mean particle size about 75 μm. In vitro release of DM showed that donepezil completely released within 28 days in water, but the cumulative release percentages up to day 30 were 98.4% and 49.1% for phosphate buffer saline (PBS, pH 5.8) and PBS (pH 7.4), respectively. The in vivo experiment demonstrated that DM (90 mg/kg) produced a sustained release process in rats, and reached steady-state concentration at day 8 and maintained until day 27 with steady-state levels of donepezil between 130.3±7.8 and 121±9.8 ng/ml, which was accordance with that of free donepezil by oral application route (3 mg/kg day). DM (90 mg/kg) by subcutaneous infusion in rats produced the same pharmacological role as free donepezil (3 mg/kg day) by oral application route. These results implicated that DM as a sustained release delivery strategy could substitute for its oral formulation for therapy of AD and come true its administration once a month.

Keywords: Donepezil; PLGA; Microparticles; Acetylcholinesterase inhibitor; Sustained release

1. Introduction

Alzheimer's disease (AD) is a degenerative disorder, in which there is a progressive deteriorative of intellectual and social functions, memory loss, personality changes and inability for self care, and has become the fourth leading cause of death in developed countries [1,2]. Although the exact pathogenesis of neuronal degeneration and cognitive impairment in AD remains to be unclear, increasing one of pharmacological and neurochemical evidences associated with AD is a deficit in central cholinergic neurotransmission [3,4]. It has been known that the activity of choline acetyltransferase (ChAT), which synthesizes acetylcholine (ACh), is markedly reduced in cortex and hippocampus in the AD brain, and the depletion of ChAT is to correlate with the severity of cognitive disturbance [5]. The cholinesterase inhibitors (ChEI) can prevent the hydrolysis of ACh and elevate ACh concentration in the synaptic cleft, which results in cholinergic transmission increasing. So far, many attempts have been made to reverse the cognitive impairment using cholinergic agents, but only several ChEI such as tacrine (Cognex), donepezil (Aricept), rivastigmine (Exelon), galantamine (Reminyl) and huperzine A have shown efficacy and have been approved for the treatment of AD [1].

Donepezil is a reversible and non-competitive cholinesterase inhibitor, and a far more selective inhibitor of acetylcholinesterase (AChE) than of butyrylcholinesterase. It produces obvious and long-lasting inhibition of brain cholinesterase without marked effects on cholinesterase in peripheral tissues and increases the brain content of acetylcholine in vivo. Moreover, donepezil significantly ameliorates performance deficits in several learning and memory tasks including 8-arm radial maze impairments after scopolamine, and passive avoidance deficits produced by lesions of the nucleus basalis magnocellularis in rats [6–8]. Double blind, placebo-controlled clinical tries showed that donepezil produces a significant improvement

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of cognition and global function in patients with mild to moderately severe AD and shows an excellent tolerability and safety profile [7]. Donepezil is available currently in the market as once a daily tablet or capsule (5 mg or 10 mg/day) [9]. Though this daily repeated oral administration is convenient for some of patients, it is very difficult for AD's patient who suffers memory disorder not to miss scheduled self-medication. In addition, donepezil also showed the gastrointestinal side effects such as diarrhea, nausea, anorexia and muscle convulsion etc. As a result, it is very important to develop a long-term, non-gastrointestinal delivery system of donepezil for treatment of AD.

We are interested in developing a sustained-release formulation for donepezil with inexpensive, biocompatible and convenient administration by direct subcutaneous injection. In this work, we selected poly (D,L-lactide-*co*-glycolide) (PLGA) microparticles as donepezil carrier because of its excellent tissue compatibility, biodegradable property and safety profile [10]. The purpose of this work was to prepare donepezil microparticles (DM), determine its physicochemical characteristics including the loading ratio, thermal profile, in vitro release, in vivo donepezil levels in rat plasma, and assess the effect of DM as a sustained-release delivery system for treatment of AD.

2. Materials and methods

2.1. Materials

PLGA (75:25, $M_W = 15{,}000$) and polyvinyl alcohol (PVA, 98% hydrolyzed, $M_W = 16{,}000$) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Donepezil (98.5%) was offered by Sangtian Pharmaceutical Co. LTD (Chongqing, China). Dichloromethane (DCM) of HPLC grade was used in this experiment. All other reagents and buffer components were analytical grade.

2.2. Animals

Sprague-Dawley (SD) rats (Grade II, 300 ± 20 g) were supplied by Shanghai Experimental Animal Center, Chinese Academy of Sciences. The animals were acclimatized at a temperature of 25 ± 2 °C and a relative humidity of 75 ± 5% under natural light-dark cycle (7:00–19:00) for 1 week before dosing. They obtained food and water was available freely.

2.3. DM preparation

The DM was prepared by an oil-water emulsion solvent evaporation technique [11]. Briefly, donepezil (100 mg) and PLGA (400 mg) were dissolved in 8 ml of DCM by sonification (Sonifier 250, Branson, output = 10 units) for 5 min. Then, the oil phase was injected into aqueous phase (150 ml) containing PVA (2%, w/v) through a fine needle under slightly stirring at 500 rpm (Stirrer, H97-A, Shanghai) to form emulsion (O/W). After 10 min, 300 ml distilled water was added to the emulsion and the stirring speed was decreased to 300 rpm for 2 h to evaporate DCM. DM was collected by centrifugation at 3000 rpm (Centrifuge, KA-1000, Shanghai) for 10 min and washed 3 times with distilled water. Finally, DM was dried and stored at 4 °C until use. The percentage yield was calculated according to the amount of microparticles to solid materials used in the dispersed phase.

2.4. Determination of the loading ratio

To measure loading ratio, 3.5 mg of DM was dissolved in 20 ml of DCM to gain a solution containing donepezil within the range of standard concentration. Donepezil was determined using UV–Visible Recording Spectrophotometer (UV-2450 PC, Shimadzu, Japan) at 268 nm. The calibration curve for the quantification of donepezil was drawn in advance. The loading ratio of donepezil was calculated by the difference between the amount of donepezil loaded in microparticles and the weight of microparticles.

2.5. Physicochemical characteristics of DM

The particle size distribution of DM was measured by the laser light scattering technique (AccuSizer 780 model, Particle Sizing Systems Inc., Santa Barbara, CA, USA). Briefly, 15 mg of microparticles was suspended in PVA solution (30 ml, 2%, w/v) and dispersed to obtain homogeneous suspension for determination of the particle size distribution. To observe the morphology, microparticles were visualized using optical microscope (Olympus BX51 Motorized system, Japan). Thermal characteristics of microparticles were performed with a Mettler TA 4000 system with a differential scanning calorimeter that equipped with a computerized data system (Mettler DSC 822, Mettler-Toledo AG, Switzerland). Sample (5 mg) was placed in sealed aluminum pans. The equipment was calibrated with indium and the samples were scanned at 10 °C/min from 25 to 325 °C. All the determination was performed in triplicate.

2.6. In vitro release experiment

The release of donepezil from microparticles was determined by suspending DM (200 mg) in 15 ml different medium at 37 °C under horizontal shaking (100 rpm, Shaker, HZ-81B, Shanghai). The medium included PBS (pH5.8), PBS (pH7.4) and distilled water. At predetermined intervals, the suspension of microparticles was centrifuged at 3000 rpm (Centrifuge, KA-1000, Shanghai) for 10 min, and the supernatant was used for further quantitative analysis of donepezil by method as described in above determination of the loading ratio of donepezil. Then, the microparticles were suspended in the same volume of fresh medium and incubated again under the same condition with horizontal shaking at 100 rpm (Shaker, HZ-81B, Shanghai). In vitro release profiles of donepezil were drawn according to cumulate release amount of donepezil in each time point.

2.7. In vivo experiment

2.7.1. Measurement of donepezil levels in rat plasma

In order to know in vivo release behavior of donepezil from microparticles, we measured donepezil levels in rat plasma. The rats were divided into 2 groups, one group ($n = 12$) received free donepezil in solution consisting of carboxymethylcellulose sodium (CMC-Na, 0.5%), mannitol (5%) and Tween 80 (0.1%) at a dose of 3 mg/kg day by oral application route. Another group rats ($n = 12$) received donepezil microspheres in suspension prepared using sterile CMC-Na (0.5%), mannitol (5%) and Tween 80 (0.1%) at a dose of 90 mg/kg by subcutaneous infusion on the back of each animal. At predetermined interval, blood sample (0.2 ml) was collected via caudal vein with heparinized tubes. Then, 10 μl of 0.1 mM HCl and 10 μl/ml of internal standard solution (1.1 nmol/ml of (R,S)-1-benzyl-4-[2-[(5,6-dimethoxy-1-indanon)-2-yl]-ethyl] piperidine hydrochloride) were added to 0.2 ml of plasma sample. Next, 0.5 ml of ethyl acetate was added, and the samples were shaken for 10 min. After centrifugation (2000*g*, 5 min, 4 °C), the organic phase was collected. Then, 0.5 ml of ethyl acetate was added to the samples again, and the above manipulations were repeated. The organic phase was combined and dried by blowing nitrogen at 40 °C. The dried residue was dissolved in 0.1 ml of 0.1 M HCl, and this solution was injected into the LC-MS/MS system (Finnigan LTQ-FT, Germany). The spectrometer was set to admit the

protonated molecules $[M+H]^+$ at m/z 380 (donepezil) and m/z 394 (internal standard) with monitoring of the product ions at m/z 91 (donepezil) and m/z 91 (internal standard).

2.7.2. Morris water maze test

For surgical procedures, the rats were anesthetized with chloral hydrate (350 mg/kg, i.p.). Bilateral common carotid arteries were exposed through a midline neck incision, double ligated with 4–0 type surgical silk, and cut between ligation in ischemia rats. During ischemia, body temperature was maintained at $37\pm0.5\,^{\circ}C$ by means of a heating lamp until the rats recovered thermal homeostasis.

Four groups of SD rats were used. The first group ($n=10$) as sham-operated control, rats received the same operation except ligation. The second group ($n=12$) as ischemia control, rats received the same operation including ligation. The third group ($n=12$), rats received free donepezil in solution consisting of CMC-Na (0.5%), mannitol (5%) and Tween 80 (0.1%) at a dose of 3 mg/kg day orally since one day before surgical operation. Final group ($n=12$), rats received DM in suspension prepared using sterile CMC-Na (0.5%), mannitol (5%) and Tween 80 (0.1%) at a dose of 90 mg/kg by subcutaneous infusion on the back of each animal at 1 day before surgical operation.

A Morris water maze was constructed as described in Ref. [12]. A circular pool (150 cm in diameter and 45 cm deep) with walls and floor painted in blank was filled with water ($23\pm1\,^{\circ}C$). A hidden circular platform (28 cm high, 12 cm in diameter, 2 cm below the water surface, fixed position) was located in the pool away from the pool wall. The pool was conceptually divided into four equal quadrants: NW, NE, SW and SE. Rats were given two trials per day at 1-min intervals for 5 consecutive days (total of 10 trials). A rat was placed in the water facing the pool wall at one of the 4 quadrants at a different place everyday, and allowed to swim for a maximum of 90 s to find the hidden platform where it was allowed to stay for 10 s. If the rat did not find the platform in 90 s, it was placed on the platform by hand and remained there for 10 s. The time to reach the platform (escape latency) was measured with a stopwatch.

2.7.3. Activity of AChE

After the last Morris water maze test, rats were killed by decapitation, the frontal cortex and hippocampus were removed and homogenized in 49 vol of sodium phosphate buffer (75 mM, pH 7.4, 4 °C), respectively. For the assay of AChE activity, a 4-ml reaction mixture that contained acetylthiocholine iodide (0.3 mM), sodium phosphate buffer (0.1 mM pH 7.4) 1 ml and homogenate 0.1–0.2 ml was incubated at 37 °C for 8 min. The reaction was terminated by adding 1 ml of 3% sodium lauryl sulfate, then 1 ml of 0.2% 5,5′-dithiobis(2-nitrobenzoic acid) to produce the yellow anion of 5-thio-2-nitrobenzoic acid. The color production was measured spectrophotometrically at 440 nm. All samples were assayed in duplicate. ChE activity was calculated as optical density (OD) value/mg protein for AChE. Protein concentrations were determined with the Coomassie blue protein-binding methods [13] using bovine serum albumin as standard.

2.7.4. Statistics

Analysis of variance (ANVOA) followed by Duncan's multiple-range test was used for data obtained in the behavioral tests. The data from the biochemical studies were presented as mean ± SD. Statistical analysis was performed by applying the Student's t-test.

3. Results and discussion

3.1. Yield and loading efficiency

In order to prepare satisfied DM, PLGA (75:25, $M_W=15$ kDa) was used because this polymer could fully degrade and continuously release drug as long as about 30 days [14]. To incorporate donepezil, the solvent evaporation technique was used because donepezil showed good solubility in DCM or $CHCl_3$. However, influential factors on the characteristics of DM with this technique were very complicated. To find out the optimal values of main independent variables including the amount of PLGA or donepezil, stirring speed, and volume of organic and aqueous phase, the Uniform Design was used as our previous other work after the influence of every factor on particle size and loading ratio had been investigated (data not shown) [15]. It has been reported that the Uniform Design shows many advantages, for example, only one experiment is needed for each level of every factor, and experimental results can be dealt with on a computer easily and accurately, in particular, the design can quantitatively analyze the influence of each experimental factor, and calculate optimal condition and range of each factor [16]. The DM was prepared according to the optimal value of each experimental factor as shown in above materials and methods (Section 2.3). The result showed that the loading ratio of donepezil was $13.2\pm2.1\%$ (w/w), and the yield of DM was $54.8\pm0.8\%$. The loading efficiency was a key criteria for evaluating microencapsulation processes. The low loading efficiency of DM could be due to its solubility in water because the drug leaked into water phase easily during preparation of microparticles.

3.2. Physicochemical characteristics of DM

The microparticles were observed by optical microscopy (Fig. 1a). From these micrographs, it was seen that DM was spherical in shape. The particle size distribution of DM was shown in Fig. 1b. The mean particle size of microparticles was about 75 μm. DSC thermograms of pure donepezil, blank PLGA microparticles, physical mixture of donepezil and PLGA, and DM were shown in Fig. 2. The glass transition temperatures (T_g) of PLGA was 37.4 °C (Fig. 2, line a), which was in accordance with our previous report [17]. No peak appeared during temperature rising from 50 to 325 °C for blank PLGA microparticles (Fig. 2, line a) and DM (Fig. 2, line b), however, the physical mixture of donepezil and PLGA showed an endothermic peak around 220 °C (Fig. 2, line c). This result suggested the presence of some crystalline donepezil. The melting endotherm of pure donepezil was 230 °C (Fig. 2, line d). The physical mixture of donepezil and PLGA showed an endothermic peak temperature lower than pure donepezil, which may be due to the effect of the melted PLGA because donepezil molecules were gradually detaching from solid form from 205 to 230 °C [18]. According to above results, the only two opportunities of donepezil could occur: (1) the drug was finally dissolved in polymer, and formed a solid solution (SS); (2) donepezil remained molecularly dispersed state in polymer.

3.3. In vitro release experiment

We prepared DM using PLGA (75:25) as vector to make drug release controlled about 30 days. We hoped to know the

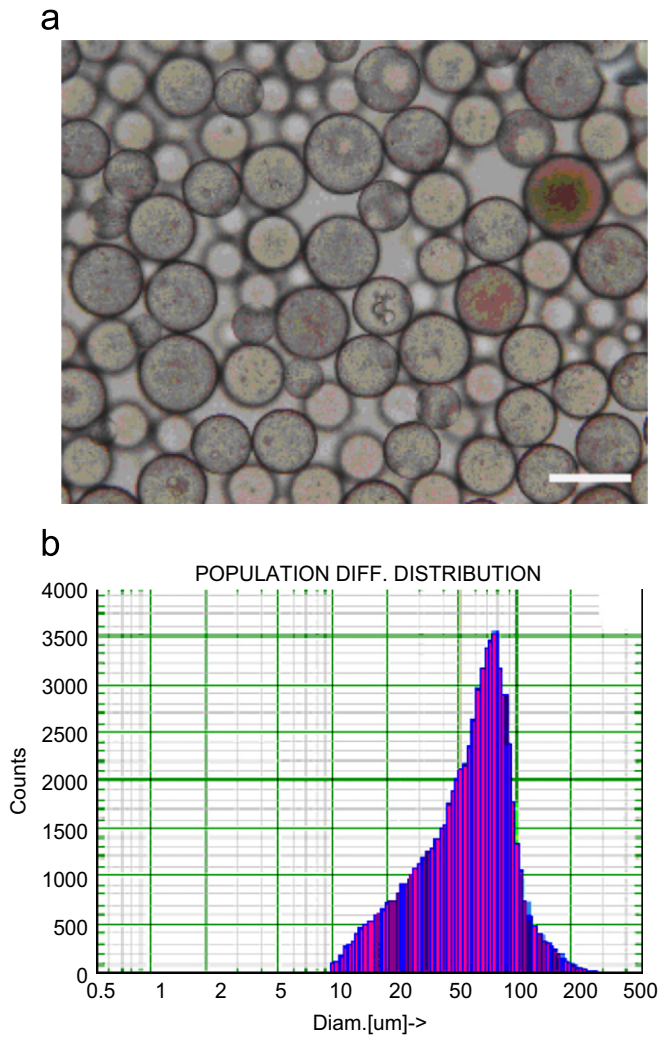

Fig. 1. The optical microscopy photograph (a, bar = 100 μm) and particle size distribution (b) of DM.

release behavior of DM in different medium, and considered that pH and osmolarity of the release medium might have an effect on the release rate of donepezil. As a result, we selected three types of medium for in vitro release experiment of DM. The in vitro release profiles of donepezil from microparticles were obtained by representing the percentage of donepezil release with respect to the amount of donepezil encapsulated (Fig. 3). DM showed no burst release effect, and the release was slow within 10 days. It could be due to, at least partially, the lack of donepezil absorption on the surface of microparticles. The microparticles with less porous at the beginning became more and more porous with the degradation of PLGA. In water, donepezil completely released in 28 days, but the cumulative release percentages up to day 30 were 98.4% and 49.1% for PBS (pH5.8) and PBS (pH7.4), respectively. This difference may be explained from following

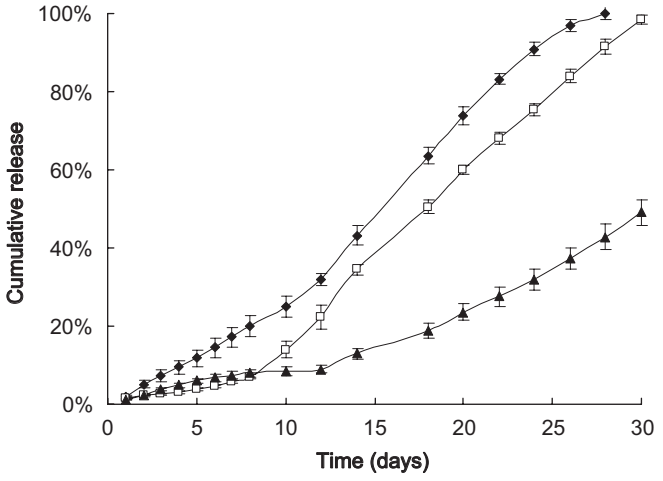

Fig. 3. In vitro release profiles of DM: water (♦); PBS (pH 5.8) (□); PBS (pH7.4) (▲).

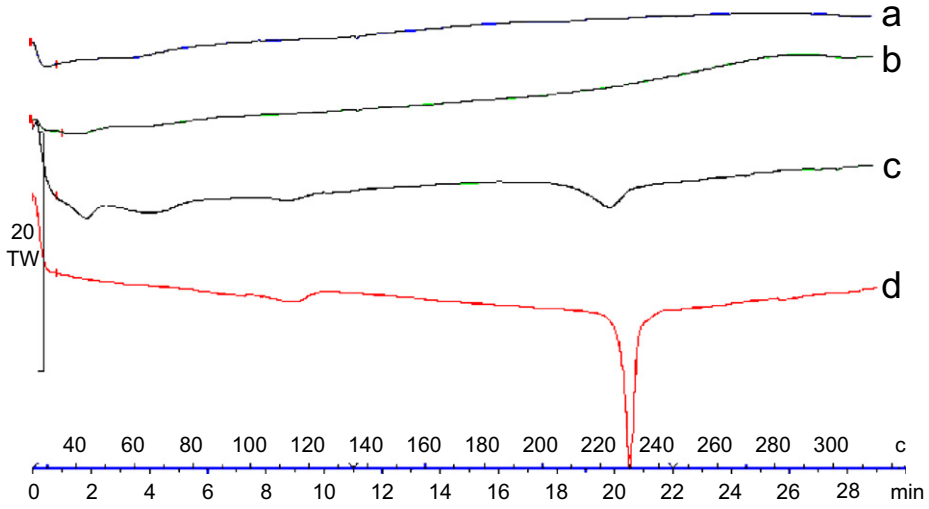

Fig. 2. DSC thermogram of PLGA (a), DM (b), physical mixture of donepezil and PLGA (c), and pure donepezil (d).

reasons: (1) low pH made the glass transition temperature (T_g) of PLGA below 37 °C, and the polymer was much more mobile in rubber state [19]; (2) the pure water can penetrated into microparticles more easily than PBS (pH 5.8) due to its low osmolarity.

3.4. In vivo experiment

3.4.1. Donepezil levels in plasma

In order to indirectly know some information about in vivo release of DM, rats ($n = 12$) received DM at a dose of 90 mg/kg by subcutaneous infusion on the back of each animal. As control, another group rats ($n = 12$) received free donepezil at a dose of 3 mg/kg day by oral application route. The experimental results demonstrated that DM produced a sustained release process, and reached steady-state concentration at day 8 and maintained steady-state values until day 27. The steady-state concentration levels of donepezil in rat plasma changed between 130.3 ± 7.8 and 121 ± 9.8 ng/ml (Fig. 4) with a small peak-valley situation, which offered us satisfying experimental evidence that DM showed a typical sustained release in rats. The control group maintained steady-state values from days 12 to 30 with the donepezil levels between 128.5 ± 8.1 and 116.3 ± 7.4 ng/ml, which was accordance with that of DM in Fig. 4. The DM group reached steady-state concentration earlier than free donepezil group, which could be due to more donepezil released from microparticles at beginning phase. The donepezil was released from microparticles at a relatively steady speed until the drug was exhausted at day 33 because drug concentration decreased according to the same ratio as free donepezil after day 33 (Fig. 4). However, drug release from microparticles in rats was not accordance with in vitro release result with the cumulative release percentages 49.1% for PBS (pH 7.4) up to day 30 (Fig. 3). This difference showed that in vivo release of drug was a very complicated process, and in vivo release of donepezil could mainly depend upon PLGA degradation ratio as observed in our previous work [17].

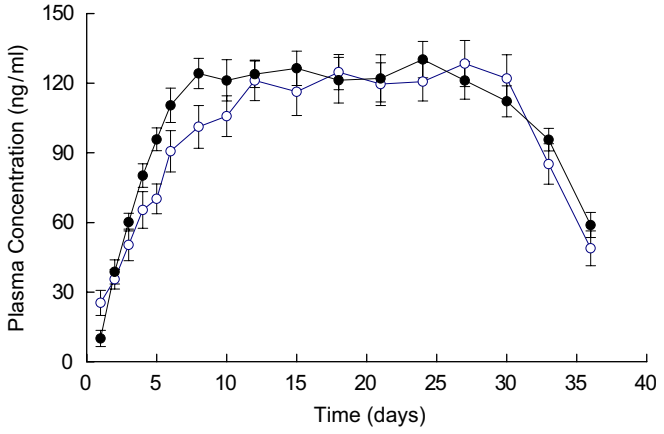

Fig. 4. Donepezil concentration curve in plasma in rats ($n = 7$–9). DM by subcutaneous infusion on the back of each animal (●); free donepezil by oral application route (○).

3.4.2. Effects of DM on water maze learning

It has been reported that the permanent bilateral ligation of the common carotid arteries in rats is a chronic cerebral hypoperfusion model, which results in a significant reduction of cerebral blood flow and causes learning and memory impairments and neuronal damage resembling those in cerebrovascular disease [20,21], and chronic cerebral ischemia-induced marked amnesic effects along with signs of neurodegeneration including: (1) spatial learning and memory deficits shown by longer escape latency and shorter time spent in the target quadrant; (2) significant neuronal loss and nuclei condensation in cortex and hippocampus especially in CA1 region [22]. In the present study, we evaluated the effect of DM on permanent bilateral ligation of the common carotid arteries resulting in significant reduction of cerebral blood flow and memory impairment using a Morris water maze. The rats with chronic hypoperfusion took longer to find the platform than did sham-operated rats from day 2 ($P < 0.01$, Fig. 5). This prolongation of latency was shortened by DM (90 mg/kg) by subcutaneous infusion on the back of each animal or free donepezil (3 mg/kg day) by oral application route from day 3 ($P < 0.01$, Fig. 5). In addition, there was no obvious difference between DM group and free donepezil group ($P > 0.05$, Fig. 5). It showed that DM (90 mg/kg) by subcutaneous infusion on the back of each animal could reach the same effect as free donepezil (3 mg/kg day) by oral application route.

3.4.3. Activity of AChE

Currently, the cholinergic deficiency is considered one of the main reasons of dementia and cognitive deficits in AD. Based on this hypothesis, many attempts have been made to reverse cognitive deficits by increasing brain cholinergic activity through the cholinomimetic use of AChE inhibitors, ACh precursors and cholinergic agonizts. In present

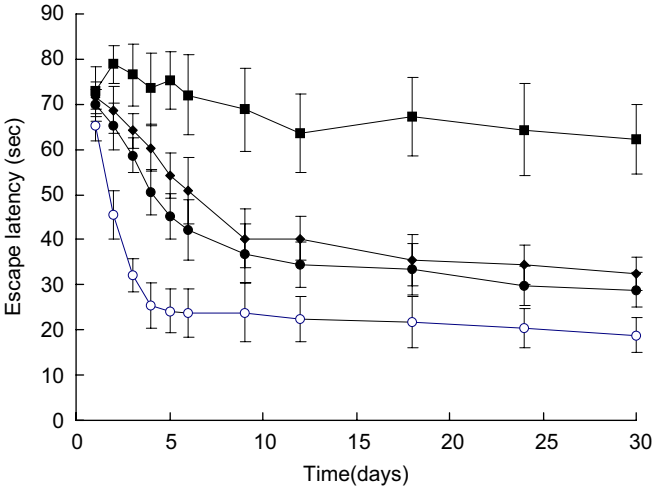

Fig. 5. Effects of DM on water maze learning. Data are mean ± S.E. *$P < 0.01$ ($n = 6$–8). Sham-operation group (○); ischemia group (■); free donepezil group by oral application route (●) and DM by subcutaneous infusion on the back of each animal (♦).

Table 1
Activity of AChE in different brain regions

	Cortex	Hippocampus
Sham-operation	1.53±0.08	1.78±0.09
Ischemia	1.07±0.07[a]	0.89±0.06[b]
Free donepezil	0.75±0.03[c]	0.62±0.04[c]
Donepezil microparticles	0.76±0.04[c]	0.59±0.05[c]

Data represent means±SEM ($n = 6$–8 animals each group) expressed as OD values/mg protein for activity of AChE.
[a] $P<0.05$ vs. sham-operation group.
[b] $P<0.01$ vs. sham-operation group.
[c] $P<0.05$ vs. ischemia group.

work, to two donepezil formulations, activities of AChE in cortex and hippocampus were determined after water maze experiment, and activity of AChE was expressed as OD values/mg protein in Table 1. Compared with sham-operation group, AChE activity of ischemia group showed an obvious reduction in cortex ($P<0.05$) and hippocampus ($P<0.01$), which meant that ischemia had impaired cholinergic function, and animal model was built successfully by the permanent bilateral ligation of the common carotid arteries in rats [22]. When compared with ischemia group, AChE activities of free drug group and DM group also showed an obvious decrease in cortex ($P<0.05$) and hippocampus ($P<0.05$), which meant that free donepezil or microparticles could further inhibit AChE activities in ischemia model in rats. It has been reported that the concentration of ACh will rise with reduction of AChE activity under normal conditions, but both the concentration of ACh and AChE activity will reduce under ischemia due to the permanent bilateral ligation of the common carotid arteries in rats [23]. Our experimental result showed that, in free donepezil or microparticles group, drug inhibited AChE effectively, and the reduction of AChE concentration resulted in slower degradation of ACh. Therefore, the concentration of ACh rose in brain in rats, and cholinergic system could reache a new equilibrium between AChE and ACh, which improved memory and cognitive deficits of rats under ischemia.

4. Conclusions

The DM showed the loading ratio 13.2±2.1% (w/w) and yield 54.8±0.8% with mean particle size about 75 μm. In vitro release of DM showed that donepezil completely released within 28 days in water, but a slow release in PBS (pH7.4). DM (90 mg/kg) by subcutaneous infusion produced a sustained release process in rats, reached steady-state concentration that was accordance with that of free donepezil (3 mg/kg day) by oral application route, and showed the same pharmacological role as free donepezil (3 mg/kg day) orally. These results offered us useful information that DM could substitute for its oral formulation for therapy of AD.

Acknowledgments

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