Preparation and *in vitro* and *in vivo* evaluation of HupA PLGA Microsphere

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Abstract: Acetylcholinesterase inhibitors (AChEIs), including Huperzine A (HupA), have been the mainstay of treatment for Alzheimer's disease (AD). However, AChEIs can cause gastrointestinal side effects, which has been related to the high C_{max} and short t_{max} after oral administration. Clinical trials have verified that extended-release formulation with lower C_{max} and prolonged t_{max}, such as rivastigmine patch, could perform a similar efficacy with significantly improved tolerability compared with the oral formulations. In this study, we developed an extended-release microspheres formulation of HupA (called as HAM) with poly(lactide-co-glycolide) (PLGA) as drug carrier. HAM has showed the loading rate as 1.35% (w/w) and yielded 42% with mean particle size at 72.6 μm. *In vitro* and *in vivo* pharmacokinetics studies have showed that HAM produced a relatively smooth and continuous drug concentration in 14 days. Furthermore, *in vivo* pharmacokinetics data have demonstrated that the C_{max} was lower and the t_{max} was considerably later in single intramuscular administration of HAM (1,000 μg/kg) than the counterparts in single intragastric administration of HAT (75 μg/kg/d). Meanwhile, HAM has performed a continuous inhibition to brain AChE activity in normal rats and improvement of memory deficit in $A\beta_{1\text{-}40}$ i.c.v. infused AD rat model for 14 days. The results have suggested that HAM has performed good extended-release properties and good prolonged pharmacological efficacy *in vivo* in the 2-week period, and could exert a similar efficacy with significantly lowered gastrointestinal side effects as compared with oral formulation.

Keywords: Huperzine A; PLGA; Microspheres; Acetylcholinesterase inhibitor; extended-release

INTRODUCTION

Alzheimer's disease (AD) is a progressive neuro-degenerative disease with characteristics of a progressive loss of memory and cognitive functions (Sadowsky *et al.*, 2010). The cholinergic system plays a key role in learning and memory, and the severity of memory impairments parallels with loss of cholinergic function in AD (Coyle *et al.*, 1983). Acetylcholinesterase (AChE) inhibition in the brain could result in increase of the synaptic acetylcholine (ACh) level, which might relieve the cognitive dysfunction (Cummings and Mendez, 1997). AChE inhibitors (AChEIs), such as donepezil, galantamine, rivastigmine and huperzine A, have been on market for the treatment of AD (Little *et al.*, 2008). However, all AChEIs can cause gastrointestinal side effects by a rapid increase in ACh level in the brain, which is considered to related with higher C_{max} (maximum plasma concentration) and shorter t_{max} (time to C_{max}) after oral administration (Sadowsky *et al.*, 2010; Wilkinson and Roughan, 2007). In addition, these gastrointestinal side effects would interfere on patients' compliance and restrict access to higher therapeutic doses, which limits the therapeutic efficacy (Grossberg *et al.*, 2011). Rivastigmine patch (Exelon™) (Lefevre *et al.*, 2008), an extended-release formulation (once per day), has displayed continuous drug release with decreased fluctuations of plasma concentration, prolonged t_{max} and lowered C_{max}. Exelon™ has been proved to perform a similar efficacy with significantly improved tolerability in clinical trials, as compared with its capsules (Sadowsky *et al.*, 2010). Furthermore, due to the improved tolerability profile, Exelon™ may not only optimize treatment compliance, but also offer the opportunity to achieve high-dose efficacy for additional treatment benefits (Grossberg *et al.*, 2011). All these data indicated that extended-release formulations of AChEIs have been an important step in the continuing fight against AD (Alisky, 2003; Wilkinson and Roughan, 2007).

Huperzine A (HupA) (fig. 1) was found in the Chinese club moss *Huperzia serrata* in 1986 (Liu *et al.*, 1986), which is a potent, reversible, and selective AChEI. Its inhibition potency and duration to AChE is comparable to those of donepezil, rivastigmine and galanthamine (Liang and Tang, 2004; Wang and Tang, 1998a; Wang *et al.*, 1986; Zhao and Tang, 2002). HupA can improve the cognitive deficits in many animal models (Lu *et al.*, 1988; Wang *et al.*, 2006; Wang *et al.*, 2001; Wang and Tang, 1998b; Ye *et al.*, 1999; Ye *et al.*, 2000). It is used for AD treatment as a drug in China, (Jiang *et al.*, 2003; Sun *et al.*, 1999; Xu *et al.*, 1999) and is being used by some U.S. physicians to treat AD patients (Little *et al.*, 2008). But the pharmaceuticals forms of HupA on market now are

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tablet or capsule by oral administration with 2-3 times a day. The research results mentioned above have suggested us to develop an extended-release formulation which can produce a controllable and continuous delivery of HupA with a longer t_{max} and a lower C_{max}. In this study, we have developed an extended-release microspheres formulation containing HupA (also known as HAM) for intramuscular injection with poly(lactide-co-glycolide) (PLGA) as HupA carrier. And its physiochemical characteristics, including the loading rate, the encapsulation efficiency and the morphology, *in vitro* release, *in vivo* HupA concentration-time profiles in rat plasma, were determined, and its effects as an extended-release delivery system for AD treatment were evaluated.

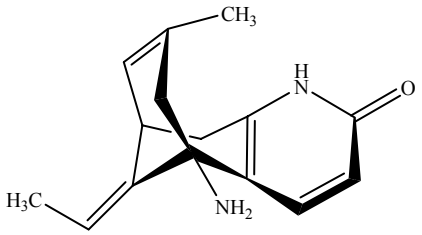

Fig. 1: Chemical structure of HupA

MATERIALS AND METHODS

PLGA RG502H (lactide/glycolide ratio, 50/50; Mn, 21,000) and PVA were purchased from Boehringer Ingelheim AG (Germany) and Sigma (USA), respectively. HupA were obtained from Joyline & Joysun Pharmaceutical Stock Co. Ltd. (China). HupA tablets (HAT, 50 μg/tablet) were purchased from Henan Zhulin Zhongsheng Pharmaceutical Limited Company (China). All other reagents were analytical grade.

Preparation of HAM

HAM was prepared with a modified O/W method according to the previous study (Chu *et al.*, 2006). Briefly, HupA (750 mg) and PLGA (1425 mg) were dissolved in 7.5 mL of dichloromethane. The solution was put into 750 mL 0.5% PVA solution under homogenization at 1600 rpm for 2 min, and then the microspheres were solidified at 300 rpm under mild mechanical stirring for 5 h at room temperature, followed by the solidified microspheres washed three times by sterile water and then freeze-dried (- 20 to - 25°C). Finally, they were stored at 4°C for the following use after the microspheres were filtered through a 150 μm sieve.

Characteristics of HAM

The drug loading rate, the encapsulation efficiency and the morphology of HAM were studied by the methods modified from the previous study (Liu *et al.*, 2005). In brief, 50 mg of HAM was dissolved in 2.5 ml acetone, and then the solution is top up with 0.02 M of HCl to 50 ml under vigorous stirring. To remove the precipitated polymer, the mixture was filtered using a 0.45 μm membrane and the supernatant was obtained to determinate HupA concentration by using HPLC (Agilent 1100, USA) connected with a UV detector. The liquid phase conditions: ODS C18 column (250×4.6 mm, 5-μm); 0.8 ml/ min of flow rate; the mixture of methanol and 0.2% H_3PO_4 (25 : 75) as mobile phase; 306 nm detection wavelength; 20 μl of injection volume every time. Then the encapsulation efficiency and drug loading were calculated.

Particle size was evaluated with a particle size analyzer (Mastersizer 2000, Malvern Instruments). 20 mg HAM was suspended in 20 ml of aqueous solution containing Tween 20 (0.1%) and vortex mixed before analysis. Morphology of HAM was observed by scanning electron microscopy (JSM-840, Japan).

In vitro release of HAM

5 mg of HAM was suspended in 3 ml of 0.01 M PBS (pH 7.4) containing 0.1% Tween 80 and 0.5% sodium azide in plastic tubes (n =3). Then the tubes were placed in a 37 °C shaker (HZS-H, China) at 40 rpm for 20 days. At the designed intervals, the tubes were centrifuged at 3,000 rpm for 30 min, and then 2 ml supernatant was collected, followed by 2 ml PBS was complemented into the tube. The HupA in the supernatant was detected using HPLC and the percent cumulative release was calculated.

Pharmacokinetics and pharmacodynamics studies

Animals

Male SD rats (Beijing Weitong Lihua Experimental Animal Centre) weighing 250 - 300 g were used to study *in vivo* pharmacokinetic and pharmacological characteristics of HAM. All animal experiments were in compliance with the requirements of the National Act on the use of experimental animals (China). The animals were raised under constant environmental conditions (22 ± 2°C, 40-70% relative humidity). The animals were free access to uniform food and sterile water.

Drugs

HAT solution was prepared by pestling and dissolving HAT in 0.9% NaCl. And HAM and placebo microspheres were suspended in sterile water containing 1% carboxymethyl cellulose sodium respectively.

Pharmacokinetics study

In order to know the *in vivo* release characteristics of HupA from HAM, HupA levels in rat plasma were measured. The rats were randomly divided into 2 groups. One group (n = 6) have intragastrically administrated a single dose of HAT (75 μg/kg). Blood samples (0.5 mL) were collected into heparinised tubes at the beginning and at 0.25 h, 0.5 h, 1 h, 2 h, 4 h, 8 h, 12 h, and 24 h after single dosing. Another group of rats (n=6) were intramuscularly injected with a single dose of HAM (1000 μg/kg) at the hind leg. Blood samples were collected with the same method at the beginning and at 1 h, 6 h, 12 h, 1 d,

2 d, 3 d, 4 d, 6 d, 8 d, 10 d, 12 d and 14 d after single dosing.

All plasma samples were centrifuged for 10 min at 3000g, separated and stored at -20 °C for followed analysis. HupA in plasma was detected by HPLC-MS (SCIEX API 4000 triple-quadrupole mass spectrometer) referring to the public data (Liu *et al.*, 2005). The plasma drug concentration-time curve was displayed as HupA concentration in ng/mL at various time points. The pharmacokinetic parameters such as C_{max} and t_{max} were calculated by Drug and Statistics (DAS) version 2.0 program (Anhui Provincial Centre for Drug Clinical Evaluation, China).

Activity of AChE

To study the effects of HAM on AChE activity in cerebral cortex, SD rats were randomly divided into six groups with the rats in each group randomly divided into three subgroups (6 rats/subgroup): Control, HAT (75 μg/kg/d), HAM (1,000 μg/kg).

HAM at the dose of 1,000 μg/kg was intramuscularly injected once at Day 0. The control subgroup received the same placebo microspheres by intramuscular injection. From Day 0 to Day 14, HAT at the dose of 75 μg/kg was intragastrically administered once per day.

The rats were killed by decapitation at 0d (1h), 2 d, 4 d, 7 d, 10 d and 14 d after receiving HAM or placebo microspheres. And the rats in HAT group were killed at 1h after dosing at the above time points. The cerebral cortex was rapidly taken out from the brains, followed by homogenization in ice-cold 0.9% NaCl, and then AChE activity of the homogenate supernatant was assayed referring to the protocol in the AChE Assay Kit (Nanjing Jiancheng Bioengineering Institution, China). And protein concentration in the homogenate supernatant was tested with BCA method. The inhibition percentage of AChE activity was calculated.

Effects of HAM on cognitive dysfunction

SD rats were divided randomly into four groups (9/group): Control, Model ($A\beta_{1-40}$), HAT ($A\beta_{1-40}$ + HAT 75 μg/kg/d) and HAM ($A\beta_{1-40}$ + HAM 1,000 μg/kg).

The $A\beta_{1-40}$ peptide (Sigma) was dissolved in sterile distilled water (1 mg/mL), which was incubated *in vitro* at 37 °C for 4 days before using. A single intra-cerebroventricular (i.c.v.) infusion of $A\beta_{1-40}$ peptide was performed according to the method in previous study (Wang *et al.*, 2001). In brief, the rats anesthetized with chloral hydrate (355 mg/kg, i.p.) were fixed on a stoelting stereotaxic instrument. By using a mini-pump, the solutions were stereotaxiclly injected with a Hamilton microsyringe with a 26-gauge needle. 10 μl of $A\beta_{1-40}$ solution was administered in 6 min into the left cerebral ventricle located at 1.4 mm to posterior, 0.9 mm lateral to bregma and 4.0 mm ventral to the skull surface (Wang *et al.*, 2001). Then the needle was remained at the position for an additional 8 min. Control animals were injected with sterile distilled water. Finally, the incision was sutured.

After the surgery, all animals were recovered for 3 days. The 4th day after surgery was defined as Day 0. HAM at the dose of 1,000 μg/kg was intramuscularly injected once on Day 0. The control and model animals received the same placebo microspheres as that of the HAM by intramuscular injection on Day 0. From Day 0 to Day 14, HAT at the dose of 75 μg/kg, was intragastrically administered once per day.

The Morris water maze trials were performed from Day 0 to Day 14 with a method modified from the references (Morris, 1984). The water maze apparatus were consisted of circular water tank (diameter 180 cm; height 60 cm) containing water at 23 ± 1 °C to a depth of 30 cm. The water was made opaque by adding black ink. The pool was averagely divided into four quadrants: northeast (NE), southeast (SE), northwest (NW) and southwest (SW). The platform was submerged about 1 cm below the water surface in the target quadrant (NE). One training trial was carried out every day, consecutively for six days. In each trial, each rat was put in the pool at the same starting location (SW). They could freely swim to find the hidden platform. The time taken to arrive onto the platform (escape latency) was recorded. The rats which had found the hidden platform were allowed to stay on the platform for 10 s. If a rat did not find the platform within 60 s, it would be guided to reach the platform and stays for 10 s. Each rat's swimming was recorded by a video camera and then analysed by an image analyzer system. At 1 hour after the end of the acquisition trial on Day 14, a probe trial was conducted after removing the platform and the rats were allowed to swim for 60 s. The swimming time and distance in the target quadrant were calculated as percentages of the totals. At 1 hour after the probe trial, six randomly selected rats in each group were killed, and AChE activities in cerebral cortex were determined according to the method mentioned above.

STATISTICAL ANALYSIS

All data were presented as Mean ± SD. Differences in mean values from pharmacological data were calculated with variance analysis ANOVA followed by post-hoc tests. $P < 0.05$ was considered as statistically significant.

RESULTS

Characteristics of HAM

To evaluate the character parameters of HAM, a series of experiments were carried out. The results have showed

that the loading rate of HupA was 1.35% (w/w), and the microencapsulation efficiency of HAM was 42%. The size analysis has showed a monodispersity of the microspheres with an average diameter of about 72.6 μm. Scanning electron microscopy analysis showed that there were a spherical morphology and non-porous surface in the microspheres (fig. 2).

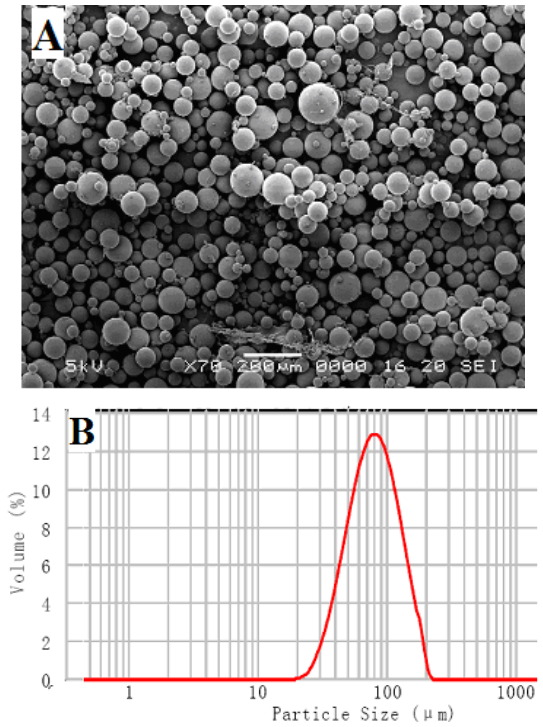

Fig. 2: Surface morphology (A) and particle size distribution (B) of HAM.

In vitro release of HAM

In the *in vitro* release experiment, the buffer solution of PBS (pH 7.4) was used as the release medium. The results (fig. 3.) have not shown any obvious initial burst with less than 10% of the total drug released within the first 24 h. An extended release of HupA was found in 2 week after drug injection. The average cumulative release rates were 90.9%, 93.2% and 94.4% on Day 14, Day 16 and Day 20, respectively.

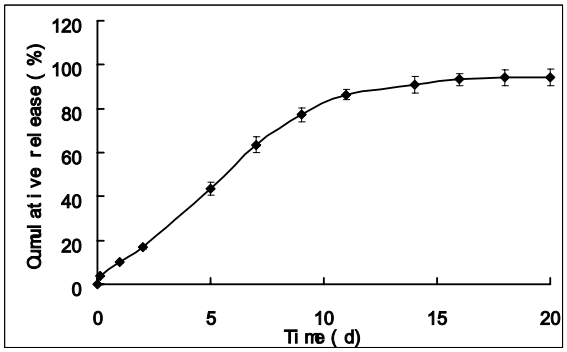

Fig. 3: *In vitro* release profiles of HAM in PBS (PH=7.4). Data were presented as mean ± S.D. (n=3).

Pharmacokinetics study

For pharmacokinetics study *in vivo*, the rats were administrated with HAT (75 μg/kg) or HAM (1000 μg/kg). HAM has provided significantly lower HupA C_{max} and longer t_{max} compared with HAT (C_{max} 0.56±0.20 vs. 1.53±0.21 ng/mL; t_{max} 60 h vs. 2 h). The average plasma drug concentration-time profiles indicated that HAM had a small initial release in 24 h after administration (fig. 4.). HAM reached its average maximum concentrations at 0.56 ± 0.20 ng/mL in Day 2-4, and then gradually decreased to the minimum concentrations at 0.15 ± 0.18 ng/mL by Day 14. The results have indicated that HAM provided a more controllable and continuous delivery of HupA than HAT did.

Activity of acetylcholinesterase

As shown in table 1, AChE activity was studied for 14 days after the administration of the test drugs. During the experiments, administration of HAT (75 μg/kg/d) or HAM (1000 μg/kg) have significantly inhibited AChE activity in the cerebral cortex from the tested rats compared with that in the rats from control group ($P < 0.05$). At each time points on Day 0, Day 2, Day 7 or Day 14, the AChE activities in the cerebral cortex from HAT group were significantly lower than those in HAM group ($P < 0.05$), respectively. Inhibition of AChE activity in HAT group was maintained at the level at about 23.4-26.2% in the cerebral cortex. However, In the HAM group, AChE was inhibited at the level between 8.5 and 18.3% in all preset time points within 14 days and maximum AChE inhibition in the cerebral cortex was noticed at the 4th day.

Effects of HAM on memory impairments after infusion of $A\beta_{1-40}$

In our study, the effects of HAM on cognitive impairment have been evaluated with a Morris water maze in rats received $A\beta_{1-40}$ (10 μg) i.c.v. infusion. In the acquisition trial as shown in Fig. 5A, the rats treated with $A\beta_{1-40}$ took longer time to find the hidden platform than did the rats from the control group from Day 1 to Day 14 ($P < 0.01$). This prolonged latency was shortened by HAT (75 μg/kg/day) through the intragastric application route ($P < 0.01$ vs. model group) or HAM (1000 μg/kg) by intramuscular injection ($P < 0.01$ vs. model group) from Day 2 to Day 14. Moreover, no significant difference was observed in escape latency between HAM group and HAT group ($P > 0.05$) at all the observed time points, indicating that HAM (1000 μg/kg) through intramuscular injection could reach the similar treatment effects as HAT (75 μg/kg/day) through the intragastric application route.

In the probe trial (fig. 5B), the swimming time and distance in the target quadrant was recorded. The results shown that the rats in control, HAT and HAM groups swam longer time in the target quadrant than did the rats in model group. HAT and HAM significantly enhanced

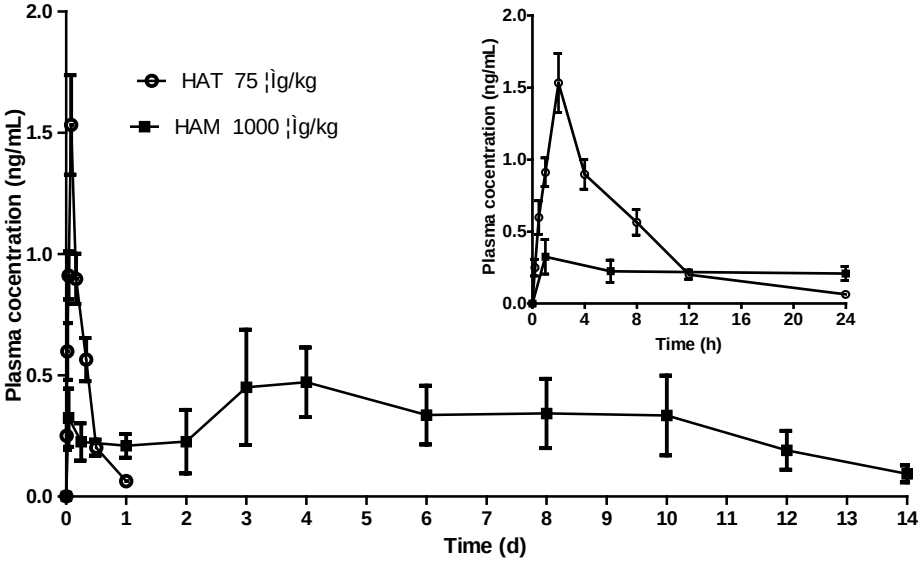

Fig. 4: Plasma drug concentration-time curves of HupA after single intragastric injection of HAT or single intramuscular injection of HAM in rats (mean ± S.D., n = 6).

Table 1: The AChE inhibition in cerebral cortex after intragastric administration of HAT (75 μg/kg/day) or intramuscular injection of HAM (1000 μg/kg) in rats

Groups	D0 (1h)		D2		D4		D7		D10		D14	
	AChE activity (U/mgprot)	IR (%)	AChE activity (U/mgprot)	IR (%)	AChE activity (U/mgprot)	IR (%)	AChE activity (U/mgprot)	IR (%)	AChE activity (U/mgprot)	IR (%)	AChE activity (U/mgprot)	IR (%)
Control	0.353 ± 0.009	—	0.361 ± 0.018	—	0.352 ± 0.022	—	0.339 ± 0.018	—	0.345 ± 0.033	—	0.335 ± 0.019	—
HAT	0.270 ± 0.011*	23.4	0.266 ± 0.027*	26.2	0.264 ± 0.021*	25.0	0.254 ± 0.027*	25.0	0.262 ± 0.016*	23.9	0.259 ± 0.028*	22.5
HAM	0.304 ± 0.013*△	14.0	0.316 ± 0.028*△	12.5	0.287 ± 0.022*	18.3	0.294 ± 0.017*△	13.2	0.295 ± 0.021*	14.4	0.306 ± 0.019△	8.5

Note: D0, D2, D4, D7, D10 and D14 represented 1hr, 48 h, 96 h, 168 h, 240 h and 336 h after treatment, respectively. Data were represented as mean ± SD (n = 6). *$P < 0.05$ vs. control group; □$P < 0.05$ vs. HAT group. 'IR' is short for inhibition rate.

the swimming time and distance in the target quadrant.

At 1 hour after the end of water maze experiment on Day 14, AChE activities in cerebral cortex from six randomly selected rats from each group were determined. The results have showed that AChE activity in model group was significantly higher than that in the control group (0.365 ± 0.012 vs. 0.331 ± 0.017 U/mgprot). The increases of AChE activities induced by $A\beta_{1-40}$ were significantly reduced by HAT or HAM with AChE activities at 0.283 ± 0.008 and 0.317 ± 0.019 U/mgprot, respectively.

DISCUSSION

To date, AChEIs are still the mainstay of treatments for AD. However, their gastrointestinal side effects have limited their clinical utility. To avoid those gastrointestinal side effects related to the high C_{max} and short t_{max} after oral administration, extended-release formulations of AChEIs have been a goal of drug development in the field of AD (Alisky, 2003; Wilkinson and Roughan, 2007). Rivastigmin patch (Lefevre *et al.*, 2008) has been successfully come into the market as once-daily extended-release formulation with good pharmacokinetic profiles of continuous drug delivery, which has contributed to reduce side effects and improve tolerability with similar efficacy compared with capsules counterparts (Sadowsky *et al.*, 2010).

Biodegradable PLGA polymers have been widely used for the extended release of various drugs, peptides and proteins with small molecular weights. Several drugs have become commercially available, such as Risperdal Consta, Lupron Depot, and Sandotalin LAR, *et al* (Dorati *et al.*, 2007). Safety of PLGA has been clinically demonstrated

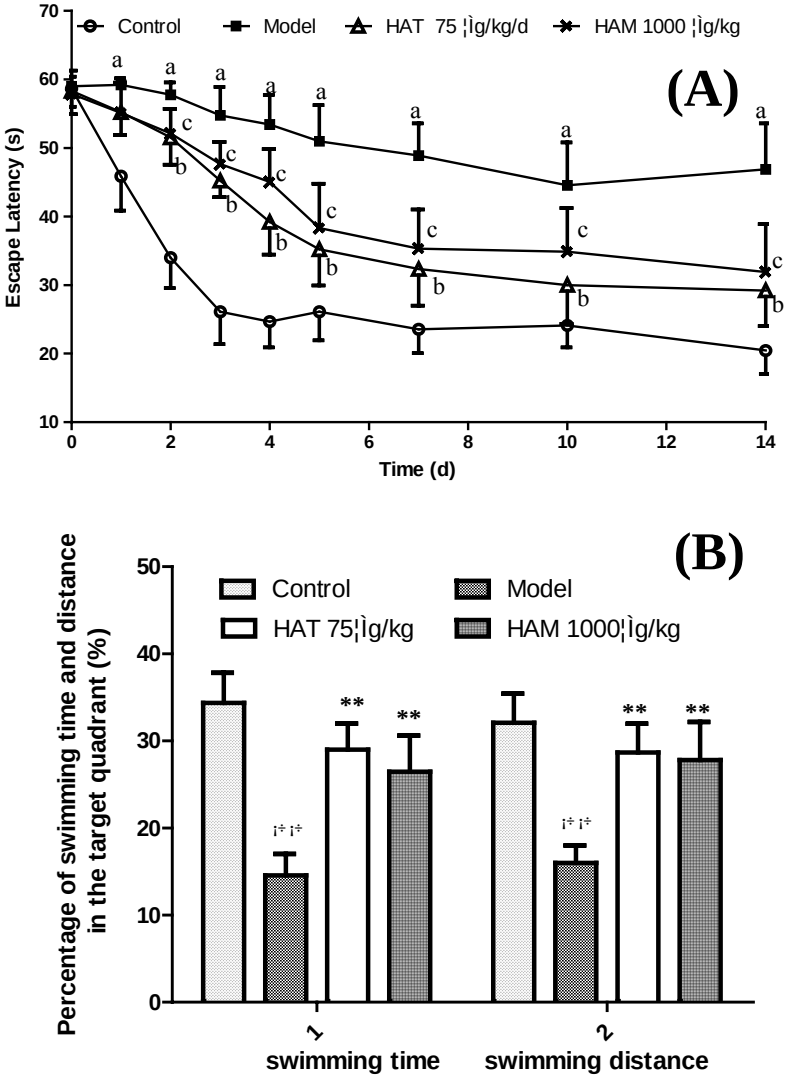

Fig. 5: HAM has improved the memory deficit induced by i.c.v. infusion of $A\beta_{1-40}$ in rats (n = 9 for each group). (A) Latencies to escape from the water onto the platform in the acquisition trial. Data were presented as mean ± S.D. $^{a}P<0.01$ vs. control group; $^{b}P<0.01$, $^{c}P<0.01$ vs. model group. (B) The swimming time and distance in the target quadrant in the probe trial. Data were expressed mean ± S.D. $^{\Delta\Delta}P<0.01$ vs. control group; $^{**}P<0.01$ vs. model group.

(Athanasiou *et al.*, 1996). In this study, HAM was successfully prepared with PLGA polymers of 502H as HupA carrier. Both *in vitro* and *in vivo* pharmacokinetics researches have showed that HAM produced a relatively controllable and continuous drug release in 14 days without obvious initial burst. Furthermore, *in vivo* pharmacokinetics data has also suggested that the C_{max} was lower (0.56 vs. 1.53 ng/mL) and t_{max} was considerably later (60h vs. 2h) by intramuscular administration of HAM than it was by intragastric administration of HAT.

According to the previous studies, AChE activity can be inhibited by HupA at cerebral cortex, hippocampus and striatum in rats, and cerebral cortex is the most sensitive area (Wang and Tang, 1998a). In this study, AChE inhibition in cerebral cortex was evaluated. Injection of HAM has performed a continuous inhibition of AChE activity for 14 days in rats, which was similar to its pharmacokinetics profile. But at some preset time points, HAT resulted a higher inhibition effects of AChE activity than HAM did, since HAT displayed higher plasma HupA concentration one hour after intragastric administration than HAM did after intramuscular administration.

Animal models of AD are very important in studying AD therapeutics. β-amyloid injection into the brain has been found to impair memory and HupA has been found to improve β-amyloid-induced deficit in learning and memory (Wang *et al.*, 2001). The increase of AChE

activity were also observed around and within amyloid plaques in AD brain (Ulrich *et al.*, 1990). In our study, HAT and HAM have showed the beneficial effects on relieving memory deficits from Day 2 to Day 14 in $A\beta_{1-40}$ i.c.v. infused AD rats, which was related to the continuous inhibition of AChE activity by HAT or HAM for 14 days in the brain of the rats. Furthermore, the enhancement of AChE activity induced by $A\beta_{1-40}$ in the brain was also noticed as compared with the control group in this experiment. The $A\beta_{1-40}$-induced increase of AChE activity was attenuated by HAT and HAM. These results indicated that memory impairment in $A\beta_{1-40}$-treated rats were relieved by increasing the cholinergic function with inhibition of AChE activity.

Pharmacokinetics results have suggested that controllable and continuous release of HupA from HAM with longer T_{max} and lower C_{max} will allow the probability to access a higher dose in order to optimise the therapeutic window with fewer side effects. Pharmacodynamics studies have showed that HAM had a prolonged pharmacological response in inhibition of AChE activity and good improvement in both learning and memory on memory dysfunction rats. These properties have made it possible to improve patients' compliance and long-term treatment effects. HAM has now been approved to carry out phase I clinical trials by the State Food and Drug Administration of China. The human pharmacokinetics and tolerability studies are going to be conducted during the clinical trials based on the above preclinical results.

In conclusion, pharmacokinetics study have suggested that the administration of HAM would provide controllable and continuous release of HupA with longer T_{max} and lower C_{max}, compared with the intragastric administration of HAT, which could lead to similar treatment efficacy with obvious improved gastrointestinal side effects as compared with oral formulation. Furthermore, HAM has performed good extended-release properties *in vivo* and good prolonged pharmacological efficacy *in vivo* in the 2-week period, and has been proved to be suitable for once-biweekly administration.

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