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Enhancement of nose-to-brain delivery of basic fibroblast growth factor for improving rat memory impairments induced by co-injection of β-amyloid and ibotenic acid into the bilateral hippocampus

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ABSTRACT

Basic fibroblast growth factor (bFGF) delivery to the brain of animals appears to be an emerging potential therapeutic approach to neurodegenerative diseases, such as Alzheimer's disease (AD). The intranasal route of administration could provide an alternative to intracerebroventricular infusion. A nasal spray of bFGF had been developed previously and the objective of the present study was to investigate whether bFGF nasal spray could enhance brain uptake of bFGF and ameliorate memory impairment induced by co-injection of β-amyloid$_{25-35}$ and ibotenic acid into bilateral hippocampus of rats. The results of brain uptake study showed that the $AUC_{0-12\,h}$ of bFGF nasal spray in olfactory bulb, cerebrum, cerebellum and hippocampus was respectively 2.47, 2.38, 2.56 and 2.19 times that of intravenous bFGF solution, and 1.11, 1.95, 1.40 and 1.93 times that of intranasal bFGF solution, indicating that intranasal administration of bFGF nasal spray was an effective means of delivering bFGF to the brain, especially to cerebrum and hippocampus. In Morris water maze tasks, intravenous administration of bFGF solution at high dose (40 μg/kg) showed little improvement on spatial memory impairment. In contrast, bFGF solution of the same dose following intranasal administration could significantly ameliorate spatial memory impairment. bFGF nasal spray obviously improved spatial memory impairment even at a dose half (20 μg/kg) of bFGF solution, recovered their acetylcholinesterase and choline acetyltransferase activity to the sham control level, and alleviated neuronal degeneration in rat hippocampus, indicating neuroprotective effects on the central nerve system. In a word, bFGF nasal spray may be a new formulation of great potential for treating AD.

1. Introduction

Basic fibroblast growth factor (bFGF), an important neurotrophic factor, is a cationic peptide composed of 154 amino acids with molecular weight of 16–18.5 kDa. Previous studies have demonstrated that bFGF enhanced cell survival and neurite outgrowth of dissociated neurons in culture (Morrison et al., 1986). Moreover, bFGF could promote axonal branch formation (Aoyagi et al., 1994), modulate synaptic transmission (Terlau and Seifert, 1990) and stimulate proliferation and differentiation of neural precursors (Sun et al., 2009; Ma et al., 2008). Therefore, bFGF is of great potential to treat the central nervous system (CNS) disorders based on its multiple effects mentioned above. Quite a few applications of bFGF in animal models to treat brain trauma (Sun et al., 2009; Yoshimura et al., 2003), ischemic stroke (Kawamata et al., 1997) and neurodegenerative diseases (Zechel et al., 2010) have already been reported, all of which definitely proved attractive therapeutic prospect of bFGF.

Alzheimer's disease (AD) is a progressive neurodegenerative disease of the CNS. Currently, there are several theories on the pathogenesis of AD. The commonly accepted β-amyloid protein (Aβ) hypothesis considered that senile plaques and neurofibrillary tangles in the cerebral cortex resulting from deposition of the Aβ protein were the main pathological changes. Because of its biological function, bFGF is effective in alleviating the pathological progress of AD in the following ways: competing with Aβ for the identical binding site in heparan sulfate proteoglycan receptor (low-affinity receptor of bFGF located on the cell surface or in extracellular matrix), thus reducing neurotoxicity signal transduction (Lindahl et al., 1999; Stopa et al., 1990); blocking nerve degeneration cascade reactions induced by Presenilins-1 (PS-1) gene mutation (Mattson, 1997); decreasing the secretion

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of Apolipoprotein E (ApoE) (Baskin et al., 1997) and inhibiting the apoptosis of neurons (Liu and Zhu, 1999; Tamatani et al., 1998). However, current researches on treating AD with bFGF mostly focus on intracerebroventricular injection, and this route is fully complex and not practical in humans. Therefore, the development of a less invasive method for bFGF delivery may significantly improve the prospects of bFGF clinical uses.

The biggest obstacle for peptide or protein drugs like bFGF to get an access into the brain is the blood–brain barrier (BBB) because of their hydrophility and high molecular weight. In recent years, intranasal (i.n.) administration has been proposed as an alternative strategy for CNS drug delivery (Illum, 2000), which could circumvent the BBB and to some extent avoid severe peripheral side effects following systemic administration (Thorne and Frey, 2001). It has recently been reported that i.n. administration of proteins and peptides, such as nerve growth factor (NGF) (Vaka et al., 2009), cholera toxin B subunit-NGF (Zhang et al., 2008), insulin (Djupesland, 2008; Henkin, 2010) and insulin-like growth factor-1 (Thorne et al., 2004; Liu et al., 2001), was able to enhance brain uptake and treat neurodegenerative diseases. However, because of low capacity of penetration through nasal mucosa, enzymatic degradation and rapid mucociliary clearance, the amount of those macromolecule drugs directly transported from nasal cavity into the brain is relatively low (Illum, 2000).

In order to enhance bFGF delivery into the brain, we previously developed a nasal spray of bFGF containing permeation enhancers, stabilizers, osmotic pressure regulator and antitack agent. In the present study, its brain delivery characteristic following i.n. administration to rats was further evaluated, in comparison with that of bFGF solution administrated intranasally or intravenously. The enzyme linked immunosorbent assay (ELISA) method was used to determine the concentration of bFGF in different brain regions following i.n. or intravenous (i.v.) administration of the above mentioned bFGF formulations.

The demonstration that the i.n. administration of bFGF nasal spray was effective in ameliorating memory impairment was achieved using the AD model rats. Some researches have reported that the co-injection of β-amyloid$_{25–35}$ (A$\beta_{25–35}$), the toxic fragments of Aβ, and ibotenic acid (IBO), a N-methyl-D-aspartate (NMDA) receptor selective agonist, into rat hippocampus, could simulate the pathological changes of AD, which provided a useful model for drug screening for AD treatment (Morimoto et al., 1998; Ito et al., 2003). Morris water maze task was conducted to evaluate the neuroprotective effect of bFGF formulations on the learning and memory impairment induced by co-injection of A$\beta_{25–35}$ and IBO. The enzyme activity of acetylcholinesterase (AChE) and choline acetyltransferase (ChAT) in rat hippocampus was determined to evaluate the protective effect of bFGF formulations on the central cholinergic neurons. In addition, haematoxylin–eosin (HE) staining was used to investigate the protective effect of bFGF formulations on hippocampus neurons.

2. Materials and methods

2.1. Materials

Recombinant human bFGF was obtained from Beijing SL Pharmaceutical Co. Ltd. (Beijing, China). Chitosan in the form of hydrochloride salt (83% N-deacetylated, viscosity 46 mPa s, Mw ~ 300 kDa) was a gift of Golden-Shell Biochemical Co. (Nanjing, China). Human bFGF ELISA development kit was purchased from Peprotech (NJ, USA). Ibotenic acid (IBO), β-amyloid$_{25–35}$ (A$\beta_{25–35}$), heparin sodium, mannitol and human serum albumin (HSA) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, US). Quantity Protein assay kit, ChAT and AChE activity assay kit was purchased from Jiancheng Bioengineering Institute (Nanjing, China). All other reagents were of analytical grade.

2.2. Animals

Male Sprague–Dawley (SD) rats weighing 200–230 g were obtained from Shanghai Sino-British Sippr/BK Lab Animal Ltd. (Shanghai, China), housed in animal holdings with fixed dark and light cycle of 12 h at a constant temperature (25 ± 1 °C). The studies were approved by the Animal Ethics Committee of Fudan University, and every effort was made to reduce the stress of animals.

2.3. Preparation of bFGF nasal spray

Based on previous studies, 0.5% (w/v) chitosan was ascertained as the optimal permeation enhancer for i.n. administration of bFGF. Heparin sodium and mannitol were chosen as stabilizing agent and osmotic pressure regulator, respectively, and HSA was added to reduce non-specific absorption. The bFGF nasal spray was prepared by dissolving chitosan in deionized water with moderate stirring, then adding heparin sodium, mannitol, HSA and bFGF in this solution, mixing well and adjusting pH between 6.0 and 6.5. The end concentration of bFGF was 200 or 400 μg/ml. The preparation was stored in 5 ml of spray device (Valois, Suzhou, China) at 4 °C before use.

2.4. Brain uptake study of bFGF formulations

2.4.1. Brain uptake experimental protocol

Eighty-four SD rats (200–230 g) were divided into three groups. The first group (i.v.-bFGF) received i.v. injection of bFGF solution (40 μg/kg) with 1% (w/v) HSA (to reduce non-specific adsorption); for the second group (i.n.-bFGF), bFGF solution with 1% HSA was administrated intranasally to each animal (40 μg/kg); the third group (i.n.-bFGF-NS) received i.n. administration of bFGF nasal spray (40 μg/kg).

Before administration, animals were anesthetized by intraperitoneal (i.p.) injection of chloral hydrate (5%, w/v, 350 mg/kg), and then fixed in a supine position. For i.n. solution, 20 μl of bFGF solution was given into the nostrils of each rat by a polyethylene 10 (PE 10) tube attached to a microlitre syringe (10 μl for each nostril). The nasal spray was given to rats same as the solution group since the nostrils of rats were too small to use the spray device. For i.v. group, 200 μl of dosing solution was injected into the femoral vein rapidly. Blood samples were obtained from the abdominal aorta at fixed intervals. Afterwards, animals were perfused by infusion pump (10 ml/min) with 50 ml cold saline. Immediately, the whole brain was removed and dissected with a scalpel carefully into four parts: cerebrum (CR), cerebellum (CL), hippocampus (HI) and olfactory bulb (OB), which were weighed and stored at −80 °C. Before assays, the brain tissues were homogenized in ice bath to generate a 10% (w/v) homogenate in saline, using tissue homogenizer (IKA). The suspension was centrifuged at 4000 rpm at 4 °C for 10 min. Blood samples were centrifuged at 10,000 rpm for 5 min to isolate serum. Then the serum and the supernatant of tissue homogenates were analyzed using a human bFGF ELISA development kit according to the manufacturer's instruction.

The optical density found in serum samples at zero time, which resulted from endogenous bFGF as well as the background due to non-specific binding, was subtracted from all detected values for each rat.

Brain tissues from eight control rats (without any treatment) were used to determine the endogenous level of bFGF in different brain regions. The blank brain tissues were divided into four parts and homogenized as described above, and analyzed by ELISA as well. In the rest part of this paper, the level of endogenous bFGF

has been subtracted from the drug concentration measured in brain tissues.

2.4.2. Recovery of bFGF from serum and brain tissues

Known amounts of bFGF were added to blank serum and brain tissue homogenates. The recovery of bFGF from serum and brain tissues was calculated by Eq. (1):

$$\text{Recovery } \frac{C_{\text{detected}} - C_{\text{endogenous}}}{C_{\text{added}}} \times 100\% \quad (1)$$

where C_{detected} represents the concentration of bFGF detected in the serum and brain tissues, $C_{\text{endogenous}}$ represents the level of endogenous bFGF, and C_{added} denotes the added concentration of bFGF.

2.4.3. Data analysis

Area under the serum or brain tissue concentration of bFGF versus time curve (AUC_{0-t}) was calculated by the trapezoidal method. The degree of bFGF targeting to the brain after i.n. administration was evaluated using drug targeting index (DTI) (Zhang et al., 2004a; Wang et al., 2003), which was calculated by Eq. (2):

$$\text{DTI} = \frac{(AUC_{\text{brain}}/AUC_{\text{serum}})_{\text{intranasal}}}{(AUC_{\text{brain}}/AUC_{\text{serum}})_{\text{intravenous}}} \quad (2)$$

2.5. Neuroprotective effect of bFGF formulations on the learning and memory impairment induced by co-injection of $A\beta_{25-35}$ and IBO

2.5.1. Surgical procedure

$A\beta_{25-35}$ dissolved in saline (5 mg/ml) was incubated for 7 days at 37 °C, while IBO was dissolved in PBS (10 mg/ml). Before surgery, four aliquots of $A\beta_{25-35}$ solution and one aliquot of IBO solution were mixed to a final concentration of $A\beta_{25-35}$ at 4 mg/ml and IBO at 2 mg/ml. Male SD rats (200–230 g, 8 weeks old) were anaesthetized with chloral hydrate following i.p. injection, and then placed in a David Krof stereotaxic instrument. 2.5 μl of the mixture solution of $A\beta_{25-35}$ and IBO was subsequently injected over 5 min through a microsyringe into rat bilateral hippocampus (3.5 mm posterior to the bregma, ±2.0 mm lateral to the midline and 2.7 mm ventral from the skull surface). After injection, the needle was left in place for additional 5 min before withdrawal. Sham-operated animals were injected with the same volume of saline.

2.5.2. Treatment with drugs

One week after co-injection of $A\beta_{25-35}$ and IBO, the rats were divided into six groups, respectively receiving i.n. administration of saline (AD control), bFGF solution with 1% HSA of two doses (20 μg/kg/day and 40 μg/kg/day) (i.n.-bFGF (20) and i.n.-bFGF (40)), bFGF nasal spray of two doses (20 μg/kg/day and 40 μg/kg/day) (i.n.-bFGF-NS (20) and i.n.-bFGF-NS (40)), and i.v. administration of bFGF solution diluted in 1% HSA saline (40 μg/kg/day) (i.v.-bFGF (40)) for 7 consecutive days. Sham control group received i.n. administration of saline. From the eighth day, Morris water maze tasks were carried out.

2.5.3. Morris water maze task

The effect of bFGF formulations on the learning impairment of rats induced by co-injection of $A\beta_{25-35}$ and IBO were studied in a Morris water maze. A circular pool (180 cm in diameter) was filled with water (30 cm deep) at 22 ± 1 °C, and a hidden platform (9 cm in diameter) was submerged 1–2 cm below the water surface in the center of one of the four quadrants of the maze. bFGF formulations or saline were administrated 60 min prior to the first trial of each day. In each trial, the rat was placed in the start location, i.e. one of the four cardinal points of perimeter of the pool, which was changed among trials in a random manner. If the rat found the platform within 60 s, it was allowed to remain there for 10 s. If the rat failed to find the platform within 60 s, it was then placed on the platform for 10 s guided by the experimenter. The movement of each rat and the latency of rat reaching the escape platform were monitored with a video tracking system. Rats received 4 trials each day at 60 s intertrial interval for 4 consecutive days.

A single probe trail was performed 1 h after the last training trial on the fourth day in order to determine whether the animals had remembered the position of the platform after training for 4 days. The escape platform was removed and each rat was allowed to swim for 30 s in the pool, with the start point at the diagonal of the platform. The number of times for each rat crossing the area where the platform had been located in the training trial was recorded.

2.5.4. Measurement of AChE and ChAT activity in rat hippocampus

In order to determine the protective effect of bFGF formulations on destroyed cholinergic nerves, AChE and ChAT activity in rat hippocampus were measured by using the method described by Li et al. and Jin et al. (Li et al., 2007; Jin et al., 2009). At the termination of the behavioral assays, the right hippocampus was dissected from the brain removed from rats under anesthesia by chloral hydrate (i.p.), and homogenized in ice-cold 20 mM sodium phosphate buffer (pH 7.4). After centrifugation at 4000 rpm for 30 min at 4 °C, the supernatant was assayed for AChE and ChAT activity according to the manufacturer's protocol. Protein concentrations were determined using the Quantity Protein assay kit.

2.5.5. Histology

In the end of behavioral studies, the rats ($n = 3$) were anaesthetized deeply, perfused with saline and then fixed with 10% formalin neutral buffer solution (pH 7.4). The whole brain was dissected and immersed in 10% formalin neutral buffer for 24 h, followed by ethanol and xylene treatment, and then embedded in paraffin for histological analysis. The brains were serially cut into 5-μm-thick sections from the injection site and stained with haematoxylin–eosin (HE).

2.6. Statistics analysis

All data were expressed as mean ± SD. For multiple-group comparison, one-way ANOVA was used followed by LSD post hoc test. Specific comparison between groups was carried out with an unpaired Student's t-test (two tailed). $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Brain uptake study of bFGF formulations

The concentration–time courses of intact bFGF in serum and different brain regions following i.v.-bFGF, i.n.-bFGF and i.n.-bFGF-NS administration were shown in Fig. 1. The recovery of added bFGF from serum and brain tissue homogenates were 86.95–103.71% and 74.42–96.20%, respectively.

Two minutes after i.v. injection, the concentration of bFGF in each brain region reached a plateau, followed by a steep decrease, dropping to the degree almost below the limit of detection in 2 h. The concentration–time curves of bFGF in olfactory bulb, cerebrum and hippocampus were similar, but the concentration in cerebellum was significantly lower than that in other brain regions. Additionally, bFGF concentration in serum was significantly higher than that in brain regions.

On the other hand, i.n. administration of bFGF solution produced a much different curve pattern. The concentration in serum reached

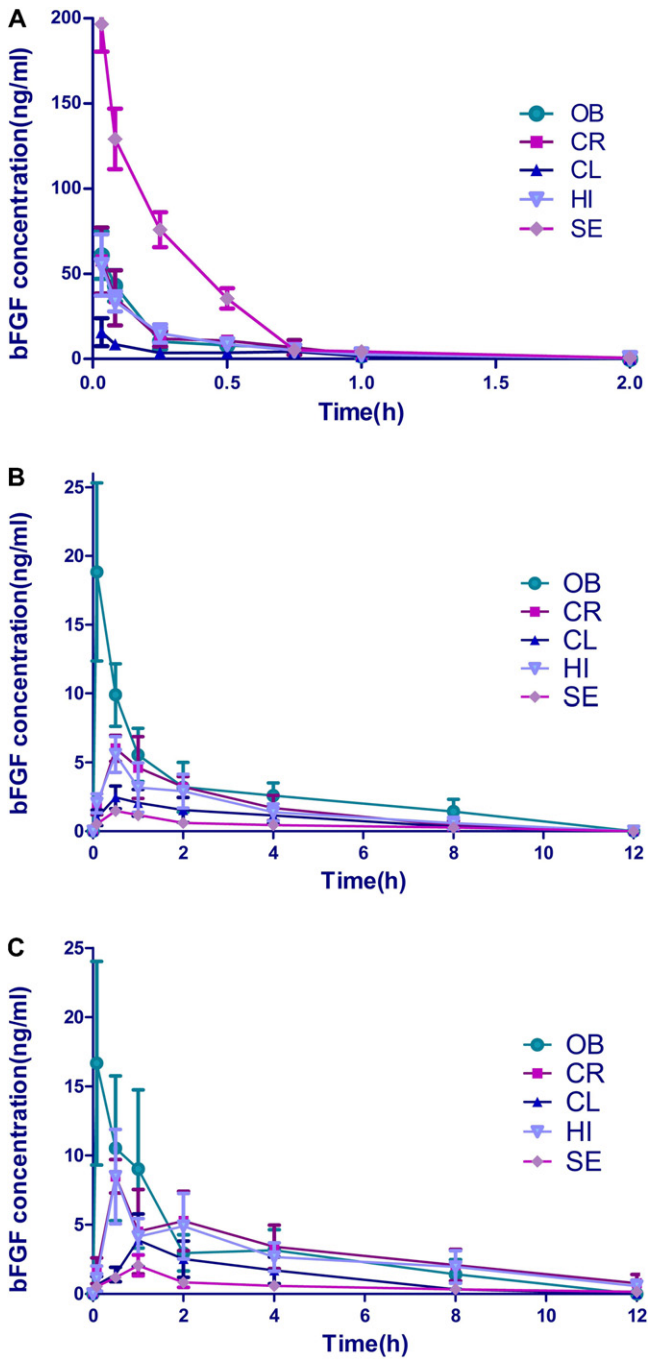

Fig. 1. Concentration–time curves of bFGF in serum and brain tissues following (A) intravenous administration of bFGF solution, (B) intranasal administration of bFGF solution and (C) intranasal administration of bFGF nasal spray (mean ± SD, $n = 4$). OB, olfactory bulb; CR, cerebrum; CL, cerebellum; HI, hippocampus; SE, serum.

peak at 30 min, and C_{max} was 1.49 ± 0.34 ng/ml. While t_{max} of bFGF in cerebrum, hippocampus and cerebellum was 30 min and C_{max} was 6.04 ± 0.94 ng/g, 5.60 ± 1.30 ng/g and 2.48 ± 1.02 ng/g respectively, the bFGF concentration in olfactory bulb reached a peak faster (5 min) and C_{max} was 18.84 ± 6.48 ng/g. The rank order of distribution of bFGF throughout the brain was as follows: olfactory bulb > cerebrum ≈ hippocampus > cerebellum, and the concentration in brain tissues was higher than that in serum.

Following i.n. administration of bFGF nasal spray, the intact bFGF was found in cerebrum and hippocampus even 12 h after administration. The bFGF concentration in olfactory bulb also reached a faster peak (5 min) and C_{max} was 16.67 ± 7.35 ng/g. bFGF nasal spray caused extended t_{max} (30–60 min) and higher C_{max} in cerebrum, hippocampus and cerebellum (8.50 ± 1.20 ng/g, 8.48 ± 3.41 ng/g and 3.88 ± 1.90 ng/g, respectively) than that of i.n.-bFGF.

As shown in Table 1, the absolute bioavailability of i.n.-bFGF-NS (13.50%) was 1.36-fold of i.n.-bFGF (9.96%). Uptake of intact bFGF increased significantly following i.n.-bFGF-NS administration by 2.47-, 2.38-, 2.56- and 2.19-fold in olfactory bulb, cerebrum, cerebellum and hippocampus respectively, in comparison with that of i.v.-bFGF, and 1.11, 1.95, 1.40 and 1.93 times, respectively, in comparison with that of i.n.-bFGF, suggesting that the nasal spray enhanced the drug delivery from nose to brain.

The AUC ratio of brain to serum and DTI values of each preparation were shown in Table 2. Drug concentrations in brain tissues were significantly higher than those in serum after i.n.-bFGF and i.n.-bFGF-NS, so the values of AUC_{brain}/AUC_{serum} were more than 1, especially in olfactory bulb, whose values were 6.23 and 5.12 for bFGF solution and nasal spray, respectively. Compared with i.v. injection, the DTI value of i.n.-bFGF and i.n.-bFGF-NS in different brain regions were all above 11, indicating that part of drug was directly transported into the brain after i.n. administration.

3.2. Neuroprotective effect of bFGF formulations on the learning and memory impairment induced by co-injection of $A\beta_{25-35}$ and IBO

3.2.1. Water maze tasks

The mean escape latency in Morris water maze tasks over 4 consecutive days was shown in Fig. 2. AD control group exhibited significantly longer latency than sham group. A two-way ANOVA between AD control and sham group showed significant main effects of both factors: group ($p < 0.01$) and day ($p < 0.05$), suggesting that the co-injection of $A\beta_{25-35}$ and IBO into rat bilateral hippocampus remarkably produced learning deficits. The animals of i.v.-bFGF (40) group prevented the decrease in the mean escape latency to some extent, but not significantly. bFGF solution following i.n. administration improved spatial memory in a dose-dependent manner: 40 μg/kg of bFGF solution apparently shortened the escape latency on days 3 and 4, while 20 μg/kg of bFGF solution showed no obvious effect (Fig. 2). bFGF nasal spray (20 μg/kg and 40 μg/kg) significantly shortened the escape latency on days 2, 3 and 4. Especially, the animals of i.n.-bFGF-NS (40) group were able to reach the platform within 10 s on the fourth day, indicating that they had learned the location of the platform completely.

In the probe test performed 1 h after the last training trial, the number of times for rats of AD control group crossing the area where the platform had been located in training trials was significantly fewer than that for those of sham group (Fig. 3A). The reduction in the number of times was ameliorated by i.n. administration of bFGF nasal spray dose-dependently, but in the case of. i.v.-bFGF (40), no significant improvement was observed even at the dose of 40 μg/kg. In addition, the groups given i.n.-bFGF (40) and i.n.-bFGF-NS (20) showed similar significant improvement.

The results were further confirmed by the mean time spent in the quadrant in which the platform had been placed in train trials but removed during the probe test, which were shown in Fig. 3B. The percentage of time spent in the target quadrant of AD control group (29.42%) was significantly shorter than that of sham group (43.75%). After treatment with i.n.-bFGF and i.v.-bFGF, there was no significant difference from AD control group in the time spent in the target quadrant. In contrast, bFGF nasal spray dose-dependently recovered the time spent in the target quadrant to an equivalent level of sham group, with the percentage of time significantly longer than that for AD control group.

Table 1
Area under the curve of intact bFGF in serum and brain tissues following intranasal administration of bFGF solution (i.n.-bFGF), bFGF nasal spray (i.n.-bFGF-NS) and intravenous injection of bFGF solution (i.v.-bFGF) (mean ± SD, $n = 28$).

Formulations	Regions	$AUC_{0-12\,h}$ (ng h/g)	Times to i.v.-bFGF	Times to i.n.-bFGF
i.v.-bFGF	SE	51.12 ± 1.97	1	–
	OB	14.27 ± 1.22	1	–
	CR	14.99 ± 1.57	1	–
	CL	5.45 ± 0.68	1	–
	HI	14.39 ± 1.13	1	–
i.n.-bFGF	SE	5.09 ± 0.52[b]	0.10	1
	OB	31.76 ± 3.34[b]	2.23	1
	CR	18.27 ± 2.15[b]	1.22	1
	CL	9.99 ± 1.48[b]	1.83	1
	HI	16.32 ± 2.02[b]	1.13	1
i.n.-bFGF-NS	SE	6.90 ± 0.88[a,b]	0.13	1.36
	OB	35.37 ± 4.80[b]	2.47	1.11
	CR	35.67 ± 4.49[a,b]	2.38	1.95
	CL	13.94 ± 2.29[a,b]	2.56	1.40
	HI	31.54 ± 4.02[a,b]	2.19	1.93

SE, serum; OB, olfactory bulb; CR, cerebrum; CL, cerebellum; HI, hippocampus.
[a] $p < 0.05$, significantly different from that of intranasal administration of bFGF solution.
[b] $p < 0.05$, significantly different from that of intravenous administration of bFGF solution.

Table 2
The AUC ratio of brain to serum and drug targeting index (DTI) following intranasal administration of bFGF solution (i.n.-bFGF), bFGF nasal spray (i.n.-bFGF-NS) and intravenous administration of bFGF solution (i.v.-bFGF) to rats (mean ± SD, $n = 28$).

Brain regions	AUC_{brain}/AUC_{serum}			DTI[a]	
	i.v.-bFGF	i.n.-bFGF	i.n.-bFGF-NS	i.n.-bFGF/i.v.-bFGF	i.n.-bFGF-NS/i.v.-bFGF
OB	0.28 ± 0.03	6.23 ± 0.91	5.12 ± 0.95	22.34 ± 4.12	18.34 ± 3.98
CR	0.29 ± 0.03	3.59 ± 0.56	5.16 ± 0.93	12.23 ± 2.34	17.61 ± 3.72
CL	0.11 ± 0.01	1.96 ± 0.35	2.02 ± 0.42	18.38 ± 3.89	18.93 ± 4.47
HI	0.28 ± 0.02	3.20 ± 0.51	4.57 ± 0.82	11.38 ± 2.22	16.22 ± 3.44

OB, olfactory bulb; CR, cerebrum; CL, cerebellum; HI, hippocampus.
[a] $DTI = (AUC_{brain}/AUC_{serum})_{intranasal}/(AUC_{brain}/AUC_{serum})_{intravenous}$.

3.2.2. AchE and ChAT activity in rat hippocampus

An important pathological change of AD patients is that the central acetylcholine level reduces significantly. Since acetylcholine is a necessity in the formation of short-term memory, thus inhibiting the central AChE activity to prevent the decomposition of acetylcholine is helpful for improving short-term memory by increasing and extending the activity of acetylcholine (Malkova et al., 2011). The results of AChE activity in rat hippocampus were shown in Fig. 4A, which revealed that the AChE activity of AD control group was significantly higher than that of sham control group. After administration of bFGF formulations, the AChE activity decreased to a level with no significant difference from sham group, indi-

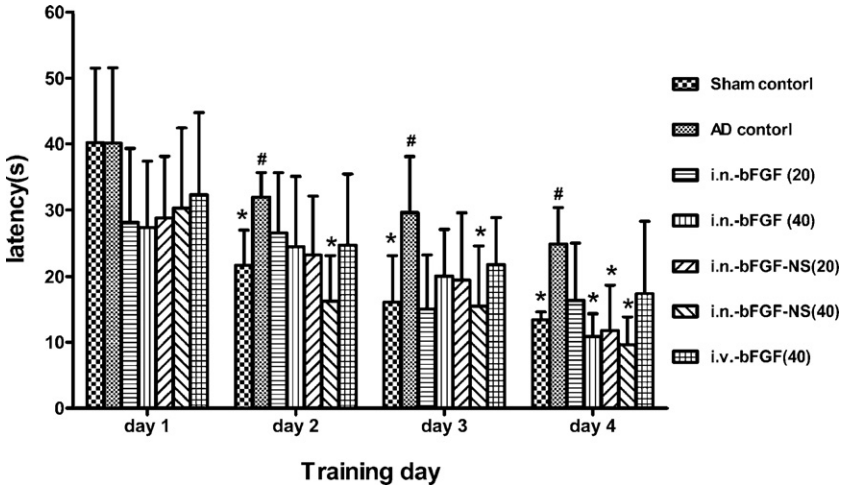

Fig. 2. Neuroprotective effect of intranasal administration of bFGF solution (i.n.-bFGF), bFGF nasal spray (i.n.-bFGF-NS) and intravenous administration of bFGF solution (i.v.-bFGF) on the impairment of water maze learning in rats with β-Amyloid$_{25-35}$ ($A\beta_{25-35}$) and ibotenic acid (IBO) lesions. Data represented the mean ± SD ($n = 10$). Sham control, given saline instead of $A\beta_{25-35}$ and IBO, which received daily nasal applied saline; AD control, daily nasal applied saline; i.n.-bFGF (20), nasal administration of bFGF solution with 1% HSA at the dose of 20 μg/kg; i.n.-bFGF (40), nasal administration of bFGF solution (40 μg/kg) with 1% HSA; i.n.-bFGF-NS (20), nasal administration of bFGF nasal spray (20 μg/kg); i.n.-bFGF-NS (40), nasal administration of bFGF nasal spray (40 μg/kg); i.v.-bFGF (40), intravenous administration of bFGF solution (40 μg/kg) with 1% HSA. $^{*}p < 0.05$, significantly different from AD control; $^{\#}p < 0.05$, significantly different from sham control.

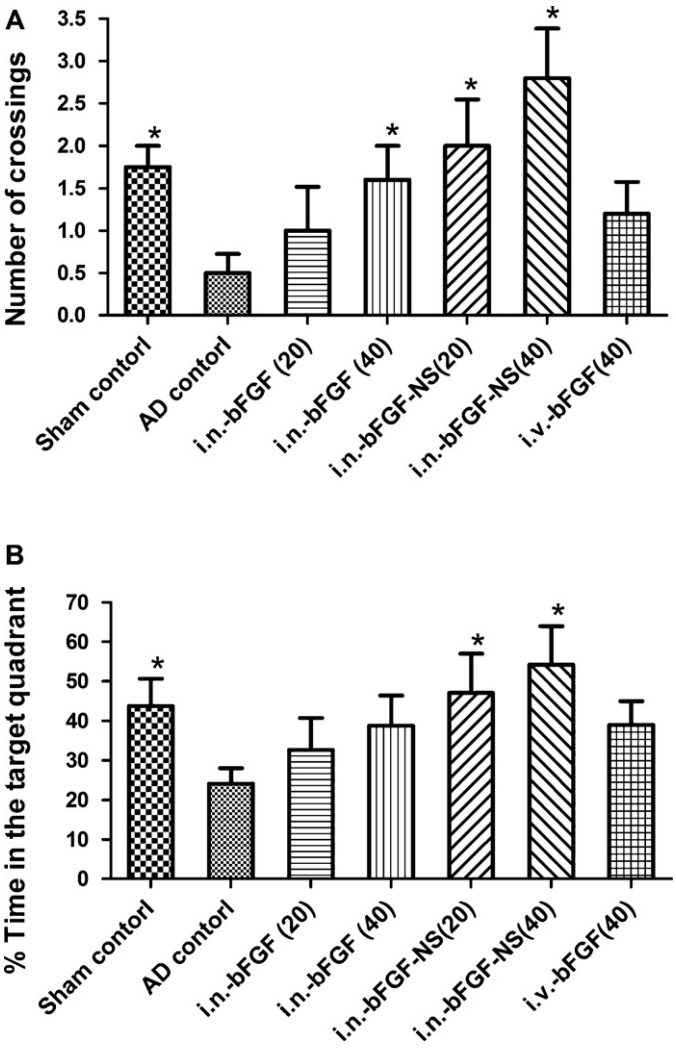

Fig. 3. Effects of intranasal administration of bFGF solution (i.n.-bFGF), bFGF nasal spray (i.n.-bFGF-NS) and intravenous administration of bFGF solution (i.v.-bFGF) on performance in Morris water maze probe test (A) the number of times crossed the area where the platform had been located; (B) the percent of (%) time in the targeted quadrant where the platform had been located. Data represented the mean ± SD ($n = 10$). $^{*}p < 0.05$, significantly different from AD control.

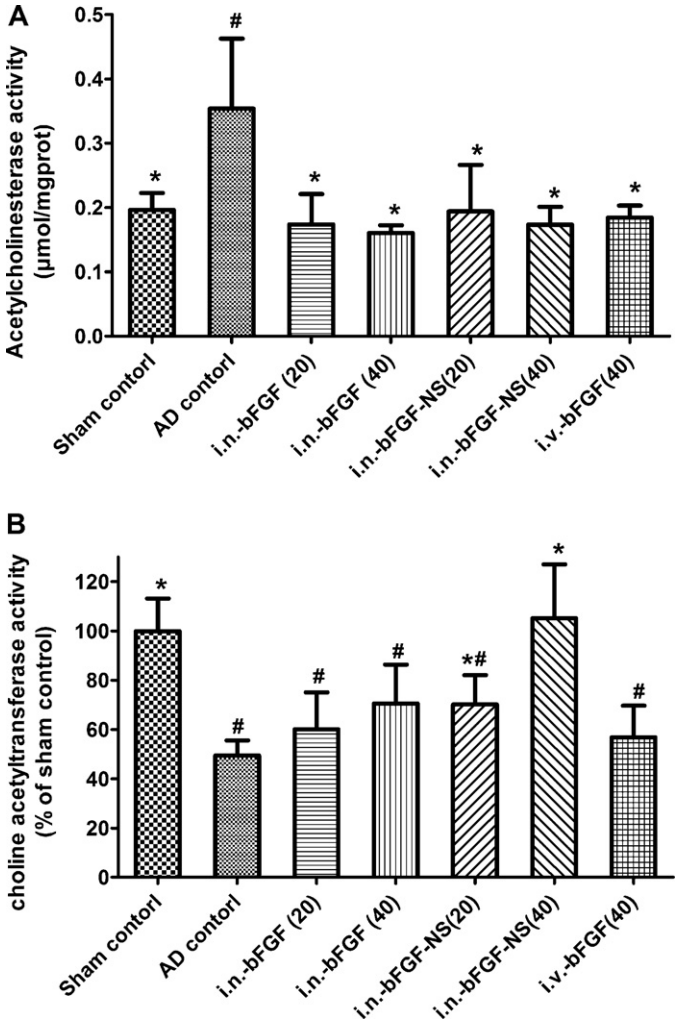

Fig. 4. Effects of intranasal administration of bFGF solution (i.n.-bFGF), bFGF nasal spray (i.n.-bFGF-NS) and intravenous administration of bFGF solution (i.v.-bFGF) on (A) acetylcholinesterase and (B) choline acetyltransferase activity in β-Amyloid$_{25–35}$ and ibotenic acid-treated rats. Data represented the mean ± SD ($n = 3$). $^{*}p < 0.05$, significantly different from AD control; $^{\#}p < 0.05$, significantly different from sham control.

cating that bFGF formulations displayed protective effects on the destroyed central cholinergic nerves.

ChAT was an enzyme for the synthesis of acetylcholine, and the content of ChAT in the CNS of AD patients was 90% less than normal. Therefore, increase of ChAT activity play an important role in AD treatment (De Rosa et al., 2005). As shown in Fig. 4B, the ChAT activity of AD control group was significantly lower than that of sham group. For i.v.-bFGF (40) and i.n.-bFGF (20) group, the ChAT activity did not change significantly; when i.n. dose increased to 40 μg/kg (i.n.-bFGF (40)), the ChAT activity increased to a certain degree, but still with no significance from AD control group. After administration of bFGF nasal spray (20 μg/kg and 40 μg/kg), the ChAT activity was significantly higher than that of AD control group. Especially for i.n.-bFGF-NS (40) group, the ChAT activity was 105.15 ± 21.86% that of sham group, ameliorated to normal levels, suggesting that the nasal spray could reverse the reduction of ChAT activity induced by A$\beta_{25–35}$ and IBO injection.

3.2.3. Histology

HE staining was used to evaluate the protective effect of bFGF formulations on hippocampus neurons (Fig. 5). Compared with sham control group (Fig. 5A–C), which did not show any neuronal change in the right hippocampus, AD control group (Fig. 5D–F) showed marked neuronal degeneration and loss in the region of CA1 and dentate gyrus (DG). Moderate neuronal damage was observed in the hippocampus of rats treated with i.v.-bFGF (40), but still less severe than AD control group (Fig. 5G–I). On the other hand, for i.n.-bFGF (40) and i.n.-bFGF-NS (20, 40) groups, no morphological change was observed in CA1 or DG region, and the hippocampus neurons were intensive and normal morphology (Fig. 5J–R). These results indicated that i.n. administration of bFGF formulations showed significant neuroprotective effect.

4. Discussion

bFGF, an important neurotrophic factor, has been proved to be effective in alleviating the pathological progress of AD by using intracerebroventricular administrations to animal models (Lindahl et al., 1999; Stopa et al., 1990; Mattson, 1997; Baskin et al., 1997; Liu and Zhu, 1999; Tamatani et al., 1998). However, from the standpoint of clinical applications for bFGF, it is important to establish drug delivery methods that are less invasive and CNS specific to administer bFGF. Drugs for AD therapy must be safely and

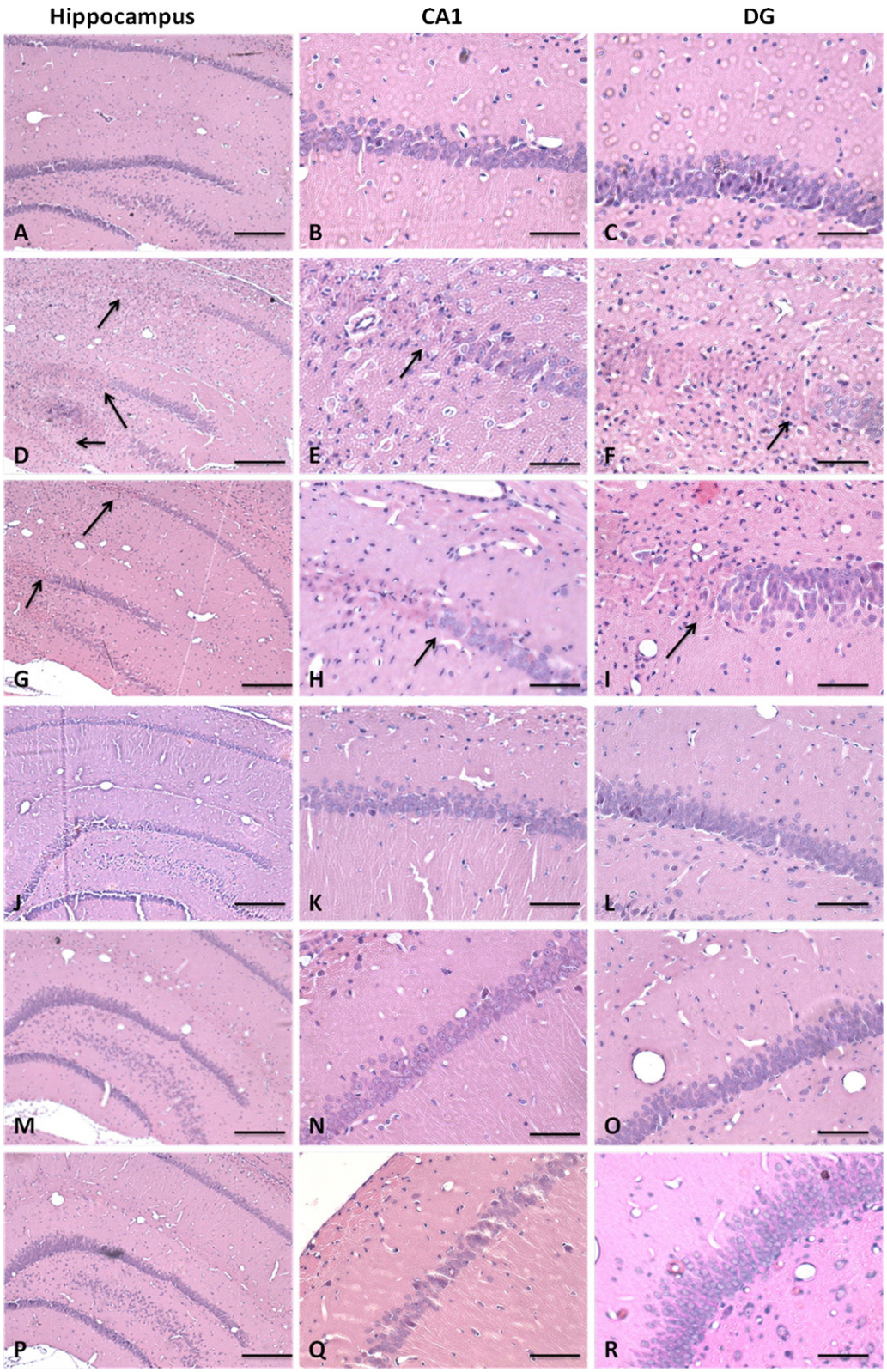

Fig. 5. HE staining in CA1 and dentate gyrus (DG) of the right hippocampus from rats treated with: (A–C) sham control; (D–F) AD control; (G–I) i.v.-bFGF (40); (J–L) i.n.-bFGF (40); (M–O) i.n.-bFGF-NS (20) and (P–R) i.n.-bFGF-NS (40). Scale bar = 200 μm (A, D, G, J, M, P); scale bar = 50 μm (B, C, E, F, H, I, K, L, N, O, Q, R). Arrows show cell loss in hippocampus in D, E, F, G, H and I.

continuously used over a long period, and intracerebroventricular or i.p. injection is not appropriate. In the present study bFGF was administrated through i.n. route which is a non-invasive method allowing drug delivery to CNS directly. The results showed that i.n. administration of bFGF resulted in higher brain delivery and better improvement on spatial memory as determined by the water maze behavioral test, in comparison with i.v. injection of bFGF. Meanwhile, the concentration of bFGF in serum following

i.n. administration was significantly lower than that following i.v. administration, which could reduce peripheral adverse effects related to i.v. administration. Those results certified that i.n. administration was more suitable than i.v. injection to treat CNS disorders, especially AD.

Vaka et al. (2009) measured NGF levels in the hippocampus after i.n. administration of NGF using a microdialysis method, and the result showed that the AUC value of 0.25% chitosan added group was 13 times more than that of NGF solution alone, demonstrating that chitosan as an permeation enhancer could significantly enhance the amount of NGF delivered from nose to the brain. Similar results were obtained in this study. The main mechanism of chitosan as nasal permeation enhancer involves in two aspects: it can reversibly open tight junctions to enhance the amount of nasal absorption; its characteristic of mucoadhesion can increase the viscosity of nasal formulations and therefore makes it difficult for them to be removed by nasal ciliary, thus lengthening the retention time of drugs in the nasal cavity (Illum et al., 1994). It is the superposition of these two effects that makes the amount of drug delivered into the brain increased significantly. In addition, compared with bFGF solution alone, stabilizer (heparin sodium) and osmotic pressure regulator (mannitol) were also added to prepare the bFGF nasal spray. Heparin sodium has been reported to protect basic and acidic FGF from inactivation (Gospodarowicz and Cheng, 1986), and mannitol, rich in hydroxyl group, is of high viscosity which can maintain the three dimensional structure and bioactivity of bFGF (Zhang et al., 2005). The addition of heparin sodium and mannitol increase the stability of bFGF solution. These probable reasons could contribute to the effective delivery of bFGF to brain in the form of nasal spray rather than solution.

Drugs could be absorbed into the brain via two routes after i.n. administration: for lipophilic drugs with relatively low molecular weight, they could be absorbed through respiratory mucosa into the blood, and then crossed BBB and immediately distributed throughout the CNS; for hydrophilic or macromolecular drugs, which were difficult to traverse through the BBB even if they had entered the systemic circulation, the olfactory pathway or the trigeminal nerve pathway was the main route for them to directly transport into the CNS (Mathison et al., 1998; Dhuria et al., 2010). Therefore, the physical and chemical properties of drugs, especially molecular weight and liposolubility, to some extent determine the drug delivery properties after i.n. administration. Previous work has shown that the AUC value in cerebrospinal fluid after i.n. administration of hydrophilic drug methotrexate was 13.8-fold higher than that of i.v. injection, and the DTI value was 21.7 (Wang et al., 2003); the DTI of lipophilic drug nimodipine following i.n. administration, compared with i.v. administration, was only about 1.26 (Zhang et al., 2004b). In this study, bFGF is a hydrophilic macromolecular drug, and the DTI value in different brain regions are all above 11, which is similar with that of hydrophilic drug methotrexate. Additionally, the $AUC_{0-12\,h}$ value in the olfactory bulb was significantly higher than that in other brain regions and the rank order of bFGF distribution throughout brain was olfactory bulb > cerebrum ≈ hippocampus > cerebellum, indicating that after i.n. administration, bFGF was absorbed through olfactory mucosa directly into olfactory bulb, the closest anatomical brain region of contact from the nasal cavity, then transported to the adjacent brain region – cerebrum, and subsequently spread to other distant brain regions like cerebellum.

In addition, the results of brain uptake and pharmacodynamics studies have good correlation with each other. First, i.v.-bFGF (40) showed mild improvement on spatial memory impairment induced by co-injection of $A\beta_{25-35}$ and IBO into the rat hippocampus. The probable reason is that intact bFGF was quickly degraded *in vivo* after i.v. injection of bFGF solution, resulting in little amount of bFGF gaining access into the CNS and therefore weak therapeutic effect being observed. In contrast, i.n.-bFGF (40), the same dose of i.v.-bFGF, could ameliorate spatial memory impairment obviously, which could be attributed to elevated amount of drug directly transported into the brain with the therapeutic effect lasting for a longer time, especially compared with i.v. administration. Following i.n. administration, in those brain regions closely related with AD (main regions of learning and memory) – cerebrum (cortex) and hippocampus, the $AUC_{0-12\,h}$ of bFGF nasal spray was nearly 2-fold that of bFGF solution. Consequently, bFGF nasal spray (20 (g/kg) could produce significantly improved effect on spatial memory impairment even at a dose half that of bFGF solution.

5. Conclusion

bFGF nasal spray was effective in delivering bFGF into the brain, especially to cerebrum and hippocampus and improved memory impairments induced by co-injection of $A\beta_{25-35}$ and ibotenic acid into the bilateral hippocampus in rats. These results definitely indicated that bFGF nasal spray was of great potential for treating neurodegenerative diseases, AD in particular, and undoubtedly set a good example for other peptide/protein drugs.

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