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## Effects of tanshinone on neuropathological changes induced by amyloid $\beta$ -peptide<sub>1-40</sub> injection in rat hippocampus

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**KEY WORDS** tanshinone; amyloid beta-protein; acetylcholinesterase; nitric oxide synthase; hippocampus

### ABSTRACT

**AIM:** To investigate the effect of tanshinone (Tan) on the neuropathological changes induced by amyloid  $\beta$ -peptide<sub>1-40</sub> ( $A\beta_{1-40}$ ) injection in hippocampus in rats. **METHODS:**  $A\beta_{1-40}$  10  $\mu$ g was injected bilaterally into the dorsal blade of the dentate gyrus in the hippocampus. The level of acetylcholinesterase (AChE) in hippocampus was evaluated by histochemistry. The expressions of neuronal nitric oxide synthase (nNOS) and inducible form of NOS (iNOS) were detected by immunohistochemistry and Western blot.  $A\beta_{1-40}$ -injected rats were treated ig with Tan, the major active ingredient from *Salvia miltiorrhiza* of Chinese herb extract. **RESULTS:** The level of AChE positive fibers of each subfield in  $A\beta_{1-40}$ -injected hippocampus decreased significantly compared with those of control ( $P < 0.01$ ). The expression of nNOS was down-regulated whereas the iNOS was up-regulated. After treatment with Tan (50 mg/kg, ig), the changes mentioned above were significantly improved. Moreover, the correlation analysis revealed a significant negative correlation between the area percentage of AChE positive fibers and the number of iNOS positive neural cells in CA1, CA2 to CA3 (CA2-3), and dentate gyrus (DG) subfields ( $P < 0.01$ ). **CONCLUSION:** Tan can protect the neuropathological changes induced by  $A\beta_{1-40}$  injection in hippocampus.

### INTRODUCTION

Alzheimer's disease (AD) is characterized by neuronal loss and extracellular senile plaque, whose major constituent is amyloid  $\beta$ -peptide ( $A\beta$ ), a 39-43 amino acids peptide derived from amyloid precursor protein (APP). It is widely believed that the cellular actions of  $A\beta$  are responsible for the neuronal cell loss observed in AD and play a causal role in the pathogenesis of AD<sup>[1,2]</sup>.

Aggregated  $A\beta$  and its active fragments have been shown to have clear neurotoxic effect<sup>[3]</sup>. Intracerebral injection of  $A\beta$  or core protein from senile plaques from AD brain induces neuronal loss and memory impairment in rats<sup>[4,5]</sup>. Therefore, the rat with lesions induced by  $A\beta$  may represent an animal model of the pathogenic mechanism leading to AD. Although the neurotoxicity of  $A\beta$  has been proved, its precise role in the development of memory loss of AD has not been fully understood.

There are alterations at the level of various neurotransmitters in AD. The most severely affected neurotransmitter is acetylcholine (ACh), which is degraded by acetylcholinesterase (AChE). In hippocampus, there are rich cholinergic fibers, most of which rise from

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nucleus septi medialis and nucleus tractus diagonalis. The ACh in hippocampus plays a vital role in learning and memory process. A previous study showed that selective fimbria lesions hampered heavily the transportation of ACh, which impaired remarkably acquisition of working and reference memory of rats<sup>[6]</sup>. It was that nitric oxide (NO) and nitric oxide synthase (NOS) were associated with the neurotoxicity of A $\beta$ <sup>[7,8]</sup>. Tanshinone (Tan), the major active ingredient of *Salvia miltiorrhiza* extract, is a mixture of many kinds of analogue compounds<sup>[9]</sup>. Tan was reported to protect PC12 cells from all ischemia injury models including hypoxia, hypoglycose, oxidant injury, calcium overload, nitric oxide neurotoxicity, and glutamic acid injury effectively *in vitro* by inhibiting development of the primary period of ischemia injury and calcium overloading injury<sup>[10]</sup>. Tan IIA and IIB reduced the brain infarct volume in transient focal cerebral ischemia in mice and had potential neuroprotective effects<sup>[11]</sup>, and Tan improved the impaired learning and memory of mice with ischemia-reperfusion injury and scopolamine-caused spatial performance defects in mice<sup>[12]</sup>. In a recent study, we demonstrated that gastric administration of Tan at doses of 100, 50, and 25 mg/kg could ameliorate learning and memory impairment of rats induced by A $\beta$ <sub>1-40</sub> hippocampal injection, and further found that Tan (50 mg/kg) was the most effective<sup>[13]</sup>. In the present study, we trend to investigate the therapeutic effect of Tan on the neuropathological changes induced by hippocampal injection of A $\beta$ <sub>1-40</sub>.

## MATERIALS AND METHODS

**Animals** Male Sprague-Dawley rats (Grade II, Certificate No 19-053), aged 8-10 weeks and weighing 250-300 g at the beginning of the experiment, were supplied by the Experimental Animal Center of Tongji Medical College. Rats were housed in plastic cages, with free access to food and water, and maintained under a 12-h light/dark cycle at 23 °C temperature.

**Materials** A $\beta$ <sub>1-40</sub> (dissolved in sterile saline at a concentration of 10 g/L and incubated at 37 °C for 7 d before use), 3,3'-diaminobenzidine (DAB), SDS, Tris, and tetraisopropylphosphoramidate (ISO-OMPA) were purchased from Sigma Chemical Co, USA. Tan was kindly donated from the Department of Clinical Pharmacology, Tongji Medical College. The neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS) rabbit anti-rat polyclonal antibodies

were purchased from Santa Cruz Biotechnology Inc. Streptavidin/peroxidase (SP) biotinylated kit was obtained from Zhongshan Biotechnology Co (Beijing, China). HRP conjugated secondary goat anti-rabbit IgG was from Wuhan Boster Biotechnology Co, Ltd.

**A $\beta$  hippocampal injection** Injections of A $\beta$ <sub>1-40</sub> in hippocampus were performed as previously described<sup>[14]</sup>. Rats were anaesthetized with chloral hydrate (350 mg/kg, ip), and were set in a stereotaxic instrument. Peptide or saline solution 1  $\mu$ L was injected into hippocampus over 5 min through a 5  $\mu$ L microsyringe into rat hippocampus (coordinate: anterior-posterior (AP)=-3.5 mm, medial-lateral (ML)=2.0 mm, dorsal-ventral (DV)=2.7 mm from bregma determined by the atlas of Paxinos and Watson as a guide). The needle was left in place for 5 min after injection. A second injection was given at the same coordinate in the opposite hemisphere.

**Tan treatment** The rats were randomly assorted into the following groups: Tan treatment group were treated with Tan (dissolved in corn oil) at a dose of 50 mg/kg ig daily at 24 h after A $\beta$ <sub>1-40</sub> injection for 14 consecutive days; A $\beta$ <sub>1-40</sub> injected group (model group); saline controls were only administered with corn oil daily at 24 h after the injection.

**Histochemistry and immunohistochemistry** Two weeks after injection, rats were anesthetized deeply, transcardially perfused with 100 mL 0.9 % NaCl in 0.1 mol/L PB followed by 300 mL ice-cold fixative solution containing 4 % paraformaldehyde in 0.1 mol/L PB (pH 7.4). The brains were removed, post-fixed in the same fixative at 4 °C for at least 8 h, and then were sunk in 30 % sucrose phosphate buffer solution at 4 °C for 8 h to 2 d. Serial coronal sections (30  $\mu$ m) were cut in a cryostat. For AChE histochemistry, the sections were incubated in a solution prepared according to modified Karnovsky Roots method<sup>[15]</sup> at 37 °C for 2 h. The sections were then thoroughly washed several times with 50 mmol/L Tris-HCl buffer (pH 7.6). Labeling was visualized by incubating sections with 50 mmol/L Tris buffer, pH 7.6, containing 0.04 % DAB, 0.01 % nickel ammonium sulfate and 0.01 % hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Appropriate concentration of iso-OMPA was used to inhibit the activity of other cholinesterases. Immunohistochemistry was performed by SP detection method. After incubation with primary antibodies against nNOS (1 $\times$  100) and iNOS (1 $\times$ 100) at 4 °C for 72 h, the sections were stained with a SP kit according to the manufacture's instructions. Immunoreactivity

was visualized with 0.05 % DAB and 0.001 % H<sub>2</sub>O<sub>2</sub> and enhanced with 0.1 % ammonium nickel sulfate (negative controls were treated in the same way, except for incubation with primary antibodies). The area percentage of AChE positive fibers and the number of nNOS and iNOS immunoreactive neural cells as well as the optical density (OD) of each positive neural cell body and its processes or each fiber were made with the HPIAS-1000 image analyses computer program on images captured with video camera attached to the light microscope. All the sections containing needle tract were collected and photographed under light microscope (10×5). Five sections per animal in each group were randomly selected for measurement. In different subfields within CA1, CA2 to CA3 (CA2-3), and dentate gyrus (DG), the AChE positive fibers area and the whole area of the different subfields as well as nNOS, iNOS immunolabeled neural cells were computer-aided detected, counted, and averaged. In addition, the OD per fiber or neural cell in different subfields was measured and then averaged. The area percentage of AChE positive fibers was counted by formula as shown as below:

$$P_a = A_p / A_w$$

$P_a$ , area percentage of AChE positive fibers

$A_p$ , area occupied by AChE positive fibers

$A_w$ , the whole area of different subfield

**Western blot analysis** Two weeks after injection, rats were deeply anaesthetized and decapitated. The hippocampus was quickly dissected and homogenized with ice-cold homogenization buffer (50 mmol/L Tris-HCl buffer, pH 7.5, containing NaCl 0.15 mol/L, edetic acid 10 mmol/L, 0.1 % Tween-20, aprotinin 5 mg/L, leupeptin 5 mg/L, PMSF 0.1 mmol/L, and 0.1 % (v/v)  $\beta$ -mercaptoethanol). The concentration of total protein in the homogenate of hippocampus was determined using Bio-Rad protein assay reagent. Samples (20  $\mu$ g/lane) were subjected to 8 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and electrotransferred onto nitrocellulose membrane. Membranes were probed consecutively with the primary polyclonal antibody (rabbit anti-rat nNOS and iNOS, 1×1000) and the secondary antibody (goat anti-rabbit IgG conjugated to HRP, 1×500). The blots were visualized with DAB and H<sub>2</sub>O<sub>2</sub>. As an internal control,  $\beta$ -actin protein was blotted concurrently. The protein expression level was determined by calculating the ratio of density metric value from the target protein in relation to internal standard.

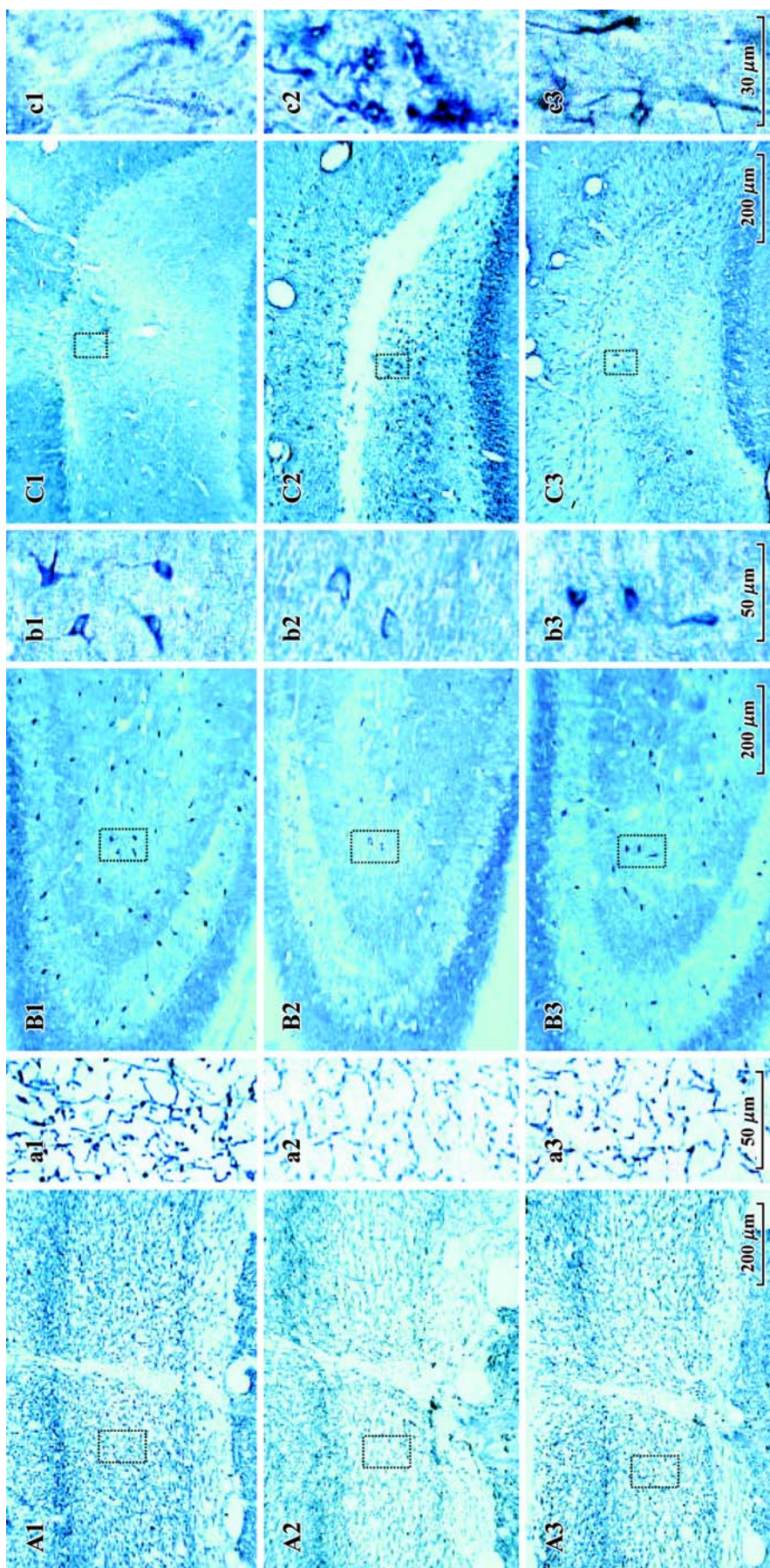
**Statistical analysis** Data were expressed as

mean±SD. Statistical analysis was carried out using SPSS 10.0 program for windows. The multiple comparisons were performed by ANOVA followed by *post hoc* test. Spearman rank correlation analysis was used for correlation between AChE and NOS studies.

## RESULTS

**Effect of Tan on AChE positive fibers in hippocampus of the A $\beta$ <sub>1-40</sub> injected rats** A large number of AChE positive fibers were seen in CA1, CA2-3, and DG subfields in the hippocampus of rats in controls. There were rich inflated bodies on most of the fibers. AChE positive fibers ran irregularly, but showed a well-organized laminar meshwork in the hippocampal formation (Fig 1A1). A few positive cells were located in the deepest portion of the granular layer in the DG. No clear change of AChE fibers was found in the area around the injection site in control rats. In contrast, a strong depletion of AChE positive fibers was observed around the injection site in the hippocampus of the A $\beta$ <sub>1-40</sub> injected rats, and the loss of AChE positive fibers was noted in the different subfields (CA1, CA2-3, and DG). The fibers became thinner, and the inflated bodies on the fibers declined (Fig 1A2). After 2 weeks treatment with Tan (50 mg/kg), the degree of the lesioned fibers in the different subfields of A $\beta$ <sub>1-40</sub> injected rats decreased remarkably (Fig 1A3), and area percentage of AChE positive fibers and its OD value also increased significantly (Tab 1).

**Effect of Tan on nNOS positive neurons and nNOS protein expression in hippocampus of the A $\beta$ <sub>1-40</sub> injected rats** In controls, the nNOS immunoreactive neurons were observed in every laminar structure in the hippocampus. The immunoreactive product was found in cytoplasm, but the nucleus was unstained (Fig 1, B1, b1). The immunoreactive neurons were most abundant in CA2-3, then CA1 and DG. In A $\beta$ <sub>1-40</sub> injected group, the lesion on neuronal cells was found around the injection site, in some cases, the whole granular layer in DG almost disappeared. In CA1, CA2-3 (Fig 1B2) and DG, the number of nNOS positive neurons was observed to decrease significantly compared with that of controls, and the changes were even more obvious in DG (Tab 1). Western blot analysis showed that the expression of nNOS protein significantly decreased in hippocampus of A $\beta$ <sub>1-40</sub> injected rats in comparison with that of saline controls (Fig 2). The decrease of the expression of nNOS positive neurons



**Fig 1. A)** Effect of Tan on the change of AChE positive fiber of CA1 subfield in hippocampus after  $A\beta_{1-40}$  hippocampal injection in rats. AChE histochemistry. A1: control group; A2: model ( $A\beta_{1-40}$  injected) group; A3: Tan treatment (50 mg/kg) group; A3 are magnification of A1, A2, and A3 respectively. **B)** Effect of Tan on nNOS positive neurons of CA2-3 subfield in hippocampus after  $A\beta_{1-40}$  hippocampal injection in rats. Immunohistochemistry. B1: control group; B2: model ( $A\beta_{1-40}$  injected) group; B3: Tan treatment (50 mg/kg) group; b1, b2, and b3 are magnification of B1, B2, and B3 respectively. **C)** Effect of Tan on iNOS positive neural cells of DG subfield in hippocampus after  $A\beta_{1-40}$  hippocampal injection in rats. Immunohistochemistry. C1: control group; C2: Tan treatment (50 mg/kg) group; C3: Tan treatment (50 mg/kg) group; c1, c2, and c3 are magnification of C1, C2, and C3 respectively.

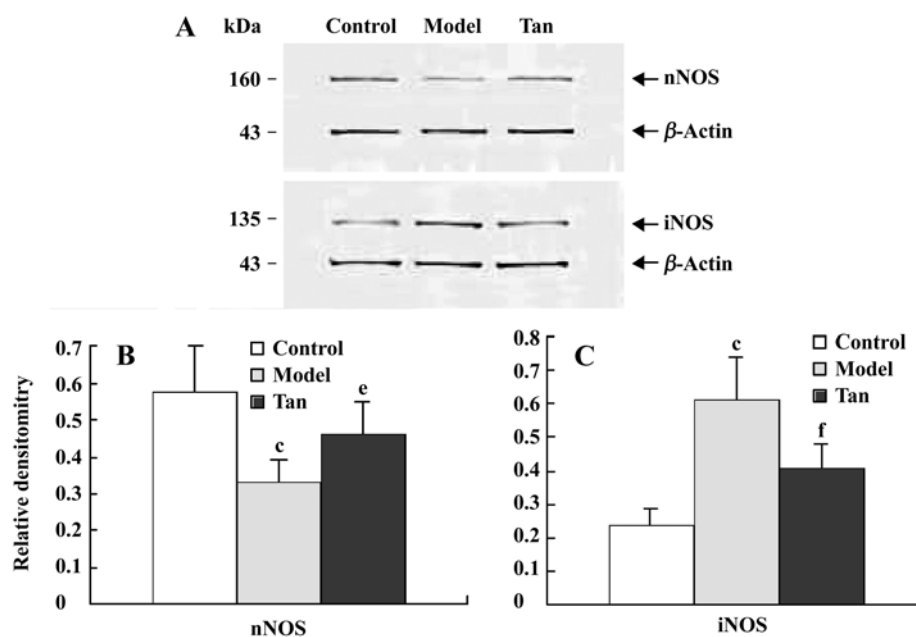
**Tab 1. Effects of Tan on AChE positive fibers and NOS (nNOS and iNOS) positive neural cells in hippocampus of the A $\beta_{1-40}$  injected rats.  $n=50$ . Mean $\pm$ SD. In same subfield,  $^cP<0.01$  vs control group.  $^eP<0.05$ ,  $^fP<0.01$  vs A $\beta_{1-40}$  injected or model group. ANOVA with multiple comparisons test.**

Group	Subfields	AChE positive fibers		nNOS positive neurons		iNOS positive neural cells	
		Area percentage/%	Optical density	Neurons count	Optical density	Cells count	Optical density
Control	CA1	19 $\pm$ 6	0.45 $\pm$ 0.08	35 $\pm$ 9	0.38 $\pm$ 0.07	11 $\pm$ 6	0.20 $\pm$ 0.03
	CA2-3	24 $\pm$ 6	0.46 $\pm$ 0.07	68 $\pm$ 12	0.41 $\pm$ 0.08	9 $\pm$ 5	0.18 $\pm$ 0.03
	DG	27 $\pm$ 61	0.48 $\pm$ 0.08	24 $\pm$ 7	0.37 $\pm$ 0.06	15 $\pm$ 6	0.23 $\pm$ 0.04
Model	CA1	13 $\pm$ 4 <sup>c</sup>	0.36 $\pm$ 0.05 <sup>c</sup>	21 $\pm$ 7 <sup>c</sup>	0.28 $\pm$ 0.04 <sup>c</sup>	140 $\pm$ 18 <sup>c</sup>	0.30 $\pm$ 0.05 <sup>c</sup>
	CA2-3	17 $\pm$ 4 <sup>c</sup>	0.37 $\pm$ 0.05 <sup>c</sup>	46 $\pm$ 10 <sup>c</sup>	0.32 $\pm$ 0.05 <sup>c</sup>	127 $\pm$ 17 <sup>c</sup>	0.29 $\pm$ 0.06 <sup>c</sup>
	DG	11 $\pm$ 4 <sup>c</sup>	0.38 $\pm$ 0.06 <sup>c</sup>	14 $\pm$ 4 <sup>c</sup>	0.25 $\pm$ 0.04 <sup>c</sup>	182 $\pm$ 26 <sup>c</sup>	0.33 $\pm$ 0.07 <sup>c</sup>
Tan	CA1	16 $\pm$ 5 <sup>f</sup>	0.40 $\pm$ 0.06 <sup>f</sup>	28 $\pm$ 8 <sup>f</sup>	0.32 $\pm$ 0.06 <sup>f</sup>	99 $\pm$ 13 <sup>f</sup>	0.26 $\pm$ 0.04 <sup>f</sup>
	CA2-3	20 $\pm$ 6 <sup>f</sup>	0.41 $\pm$ 0.06 <sup>f</sup>	54 $\pm$ 12 <sup>f</sup>	0.37 $\pm$ 0.06 <sup>f</sup>	90 $\pm$ 12 <sup>f</sup>	0.25 $\pm$ 0.05 <sup>f</sup>
	DG	14 $\pm$ 5 <sup>e</sup>	0.42 $\pm$ 0.07 <sup>e</sup>	17 $\pm$ 7 <sup>e</sup>	0.28 $\pm$ 0.05 <sup>e</sup>	133 $\pm$ 17 <sup>f</sup>	0.29 $\pm$ 0.06 <sup>f</sup>

(neuron number and optical density) in different subfields and down-regulation of nNOS protein expression in the hippocampus of A $\beta_{1-40}$  injected rats were inhibited after Tan treatment for 2 weeks (Fig 1B3, Tab 1, Fig 2).

#### Effect of Tan on iNOS positive neural cells and

**iNOS protein expression in the hippocampus of A $\beta_{1-40}$  injected rats** Only a few weak iNOS immunoreactive neural cells (neuron and glial cells) were noted around the saline injection site (Fig 1C1). In the CA1, CA2-3, and DG subfields of the hippocampus, the iNOS posi-



**Fig 2. Effects of Tan on the levels of nNOS and iNOS proteins in the hippocampus after A $\beta_{1-40}$  hippocampal injection in rats. A shows nNOS and iNOS protein expression in control group, model group and Tan treatment or 50 mg/kg group exposure by Western blot. Densitometric analysis of the levels of nNOS (B) and iNOS (C) proteins were detected by all Western analysis. Data were expressed as the ratio to the internal standard.  $n=6$ . Mean $\pm$ SD.  $^cP<0.01$  vs control group.  $^eP<0.05$ ,  $^fP<0.01$  vs A $\beta_{1-40}$  treated or model group. ANOVA with subsequent multiple comparisons test.**

tive neural cells showed very weak staining and sporadically scattered. In A $\beta_{1-40}$  injected rats, extensive neuronal degeneration presented surrounding the injection site. However, a large number of iNOS immunoreactive neural cells were detected in DG subfield (Fig 1C2). Most of these positive neural cells were morphologically similar to glial cells, their morphological appearances were diverse and the processes were obvious. The cytoplasm and processes were both stained with iNOS immunoreactive product. The iNOS positive neural cells were also seen in CA1, CA2-3 subfields. Western blot analysis revealed that the expression of iNOS protein significantly increased in hippocampus of A $\beta_{1-40}$  injected rats in comparison with that of saline controls (Fig 2). The increasing expression of iNOS positive neural cells (cell number and optical density) in different subfields and up-regulation of iNOS protein expression in the hippocampus of A $\beta_{1-40}$  injected rats were also inhibited after Tan treatment for two weeks (Fig 1C3, Tab 1, Fig 2).

**Correlations between AChE positive nerve fibers and iNOS positive neural cells in the hippocampus of rats** The area percentage of AChE positive fibers and the count of iNOS positive neural cells were detected with the methods mentioned in methods and materials. The mean value acquired from 5 sections of each rat represented the level of AChE and iNOS protein of the rat. Correlation analysis revealed a significant negative correlation between the area percentage of AChE positive fibers and iNOS neural cells count in CA1, CA2-3, and DG subfields (CA1:  $r_s = -0.843$ ,  $P < 0.01$ ; CA2-3:  $r_s = -0.826$ ,  $P < 0.01$ ; DG:  $r_s = -0.771$ ,  $P < 0.01$ ).

## DISCUSSION

In the present study, we demonstrated that a single acute injection of A $\beta_{1-40}$  in the hippocampus induced the marked cholinergic deficit in the rat hippocampus. Previous reports showed that intracerebroventricular injection of A $\beta_{25-35}$  induced a marked reduction in choline acetyltransferase (ChAT) activity in the medial septum and hippocampus<sup>[2]</sup> and chronic infusion of A $\beta_{1-40}$  into the cerebral ventricular caused performance impairment in the passive avoidance and water maze tasks and a decrease in the ChAT activity in the frontal cortex and hippocampus in the rat<sup>[16]</sup>. These results, together with our finding indicate that  $\beta$ -amyloid peptides may impair the function of cholinergic system. In contrast, it was demonstrated that administration of A $\beta_{25-35}$  was not neu-

rotoxic to the septo-hippocampal cholinergic system in rats<sup>[17]</sup>. The reason is not understood. One of explanations is that it may be related to the forms of A $\beta$  and its condition in solution. In the present study, the peptides were dissolved in sterile saline and incubated at 37 °C for one week before use, since this condition facilitated aggregation to increase the neurotoxic potency of the peptide.

NO is produced by NOS. The different isoforms of NOS such as nNOS, iNOS, and endothelium NOS (eNOS) have been identified in the brain. NO is considered as a messenger modulator and may play an important role in maintaining normal long-term potentiation (LTP). However, the overproduction of NO may cause neuronal damage<sup>[18]</sup>. The role of NO/NOS in AD is not clear and the studies have shown contradictory results regarding NO neurotoxicity/neuroprotection and NOS expression in AD. In recent years, various laboratories have shown that A $\beta$  can stimulate microglial and astrocytic NO production which may exert neurotoxic effect<sup>[19,20]</sup>. Evidence showed that astrocytes with elevated levels of iNOS were constantly seen in direct association with A $\beta$  deposits in AD and APP23 transgenic mice and were found in the vicinity of the lesion site in rat's cortex<sup>[21]</sup>. It is suggested that altered expression of iNOS being part of AD pathology is secondary to the amyloid pathology. Our results are in agreement with some previous reports. In the present study, we demonstrated that the level of iNOS immunoreactivity (expressed in OD) and the number of iNOS positive neural cells in CA1, CA2-3, and DG regions of hippocampus of A $\beta_{1-40}$  injected rats increased significantly and the neurons around the A $\beta_{1-40}$  injection site were heavily lesioned compared to that of saline controls. Western blot analysis also showed a significant increase in the total expression of iNOS protein in hippocampus in comparison with that of saline controls. With Tan administration (50 mg/kg, ig $\times$ 14 d), the changes mentioned above could be improved remarkably. These results indicate that Tan protects local neurons from lesioning at least in part by inhibiting the expression of iNOS. In the present study, a distinct loss of nNOS immunolabeled neurons in CA1, CA2-3, and DG in A $\beta_{1-40}$  injected rats was found. Western blot analysis also showed that the total expression of nNOS protein in hippocampus decreased after A $\beta_{1-40}$  hippocampal injection. These findings are partly consistent with existing data. A previous study revealed that semiquantitative assessment of numbers of nNOS expressing neu-

rons in different areas of the hippocampus and entorhinal cortex showed a remarkable loss of nNOS expressing neurons in the entorhinal cortex layer II and less severe loss in CA1 and CA3 of the hippocampus in patients with AD, in addition, double-immunolabeling studies revealed that nNOS was strongly associated with neurofibrillary tangles and plaques<sup>[22]</sup>. An *in vivo* study of  $\beta$  amyloid toxicity also revealed selective loss of nNOS neurons in rat striatum after  $\beta$ -amyloid injection<sup>[19]</sup>. On the other hand, some reports showed that the expression of nNOS increased in the brain of AD patients<sup>[7]</sup>, and several studies using antibodies against NADPH-d suggested increased<sup>[23]</sup>, decreased<sup>[24]</sup>, or unaffected<sup>[25]</sup> level of NOS immunoreactivity in different brain regions in AD. This contradiction might be due to different techniques and various antibodies that were used. These results, together with ours demonstrate that nNOS expressing neurons are affected in AD. These findings indicate that nNOS expressing neurons are highly susceptible to neurodegeneration and that nNOS might contribute to the pathogenesis of AD. Furthermore, nNOS expressing neurons in hippocampus play an important role in learning and memory and NO mediated by nNOS is a retrograde messenger in LTP, so the decrease of nNOS induced by  $A\beta_{1-40}$  might lead to memory deficit. In our study, Tan administration (50 mg/kg, ig $\times$ 14 d) could significantly increase the level of nNOS. The mechanism by which Tan improved the learning and memory dysfunction of  $A\beta_{1-40}$  injected rats might be related to protecting the nNOS neurons from lesioning.

In the present study, correlation analysis revealed a significant negative correlation between the changes of the area percentage of AChE positive fibers and iNOS positive neural cells. This indicates that the damage of AChE positive fiber may be related to the activation of glial cells which may produce large quantity of NO to induce a neurotoxic effect. These results also indicate that Tan protect the cholinergic system in hippocampus from lesioning, to a large extent, by inhibiting the expression of iNOS.

In summary, this study showed that in the hippocampus, expression of iNOS was up-regulated while nNOS was down-regulated, and the local cholinergic fibers were lesioned remarkably after aggregated  $A\beta_{1-40}$  hippocampal injection. Tan administration could improve the above changes respectively, which indicated an important therapeutic effect of Tan on amyloid-induced neural injury.

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