ORIGINAL RESEARCH

Effect of tanshinone on the levels of nitric oxide synthase and acetylcholinesterase in the brain of Alzheimer's disease rat model

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Abstract

Purpose: To determine the influence of tanshinone on the levels of nitric oxide synthase (NOS) and acetylcholinesterase (AChE) in the brain of an Alzheimer Disease (AD) rat model and on its potential therapeutic mechanism.

Methods: 100 Male Sprague Dawley rats were divided into three groups: control group, model group and tanshinone treatment group. 10 μ g A β ₁₋₄₂ was injected bilaterally into the dorsal lateral region of the dentate gyrus in the hippocampus of rats in the model and tanshinone treatment groups to prepare the AD models. 24h after modeling, tanshinone, 50mg/kg, was administered by gastric perfusion to rats in the tanshinone treatment group. Later, immunohistochemical assay and Western blot analysis were used to detect expression of neuronal NOS (nNOS) and inducible NOS (iNOS) in the rat hippocampus. Activity of AChE in each subregion (CA1~CA4) of rats' hippocampus was determined by a histochemical technique.

Results: Expression of nNOS in the model group was down-regulated whereas iNOS was up-regulated. After A β ₁₋₄₂ injection, the number of AChE positive fibers in each subregion (CA1~CA4) of the hippocampus was decreased compared with controls. With tanshinone administration, the changes were improved to varying degrees.

Conclusion: Tanshinone modulates AChE and NOS proteins concentrations in the hippocampus of AD rats. This may have therapeutic potential in AD rats.

List of Abbreviations

NOS	nitric oxide synthase
nNOS	neuronal NOS
iNOS	inducible NOS
AChE	acetylcholinesterase
AD	Alzheimer's disease
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
NBT	NitroBlue Tetrazolium Tetrazolium
CNS	Central Neural System
A β	amyloid- β peptide

Alzheimer's disease (AD) is the most common form of dementia. Dementia is a disease of the nervous system

characterized by loss of certain mental abilities. The loss is severe enough to interfere with normal activities and lasts at least six months. AD is not present at birth but usually develops during old age. It is marked by a decline in mental functions such as memory, reasoning, and the ability to plan.¹ The more basic pathological abnormalities in AD are amyloid plaques, neurofibrillary tangles, and neuronal death.² Disorders of some neurotransmitters, to various degrees, occur in AD patients, whose level of acetylcholine (ACh) is decreased.³ In the past two decades, many attempts have been made to understand the molecular pathogenesis of AD, and to provide early diagnosis and therapeutic control. Cholinergic neurotransmission is the main target for effective treatment strategies. Acetylcholinesterase (AChE), the principal cholinesterase in the brain, causes hydrolysis of the endogenous neurotransmitter ACh.⁴ AChE inhibitors may ameliorate several symptoms of AD since a cholinergic deficit is characteristic of AD. Elevation of ACh leads to functional improvement of central cholinergic synapses, protection of neuronal degeneration, and modification of amyloid precursor protein and regional enhanced synthesis of neurotrophic molecules.⁵ Among the various AChE inhibitors, galantamine and huperzine A, isolated from plant extracts, have been used to treat the early symptoms of AD. This suggests that novel acetylcholinesterase inhibitors from plant sources could be valuable alternatives in AD treatment. *Salvia miltiorrhiza* Bunge (Lamiaceae), a well-known Chinese herbal plant, and its roots, known as Danshen in Chinese, are widely used for the treatment of menstrual disorders and blood circulation diseases, and for the prevention of inflammation.⁶ The chief bioactive ingredients of *S. miltiorrhiza* roots are the diterpenoid pigments, particularly the phenanthrofurane quinone derivatives known as tanshinones. Hairy root culture of *S. miltiorrhiza* has been established as a potential means for tanshinone production.⁷

Nitric oxide (NO) is a gaseous lipophilic free radical cellular messenger. It is synthesized from arginine by three distinct isoforms of nitric oxide synthases

(NOS), neuronal (nNOS), inducible (iNOS) and endothelial NOS (eNOS). It plays a role in neurotransmission and neuromodulation.⁸ However, overproduction of NO appears to be deleterious since it is associated with seizures and brain damage. On the other hand, the expression of NOS during development appears to be important for the final stages of neuronal differentiation. Although commonly localized to macrophage immune cells, iNOS is observed primarily in neurons and astrocytes in AD.⁹ The footprint of NO's past presence includes observation of nitrated proteins in AD brains, compared with normal age-matched brains, suggesting that NO plays a role in the disease process.¹⁰

In the present study, we investigate the influence of tanshinone on the levels of NOS and AChE in the brain of Alzheimer's disease rats to determine the potential therapeutic mechanism of tanshinone, to develop Alzheimer's disease therapeutics and to extend the use of Chinese Medicine all over the world.

Materials

Animals

Adult male Sprague-Dawley (SD) rats (n=100, 220~250g from the Experimental Animal Center of Chinese Second Military Medical University) were housed 6 per cage with free access to food and water, and kept in a constant environment ($22 \pm 2^\circ\text{C}$, $50 \pm 5\%$ humidity, 12h light/dark cycle). Experimental animals were overseen and approved by the Animal Care and Use Committee of Changzheng Hospital of Chinese Second Military Medical University before and during experiments.

Chemicals and reagents

Tanshinone was obtained from the National Institute for the standard substance of pharmaceutical and biological products, P.R. China. $\text{A}\beta_{1-42}$ was purchased from Sigma, St. Louis, MO, USA. Rabbit anti-nNOS, anti-iNOS polyclonal antibodies and Streptavidinbio-

tin peroxidase complex immunohistochemical assay kit (SABC) were obtained from a commercially available Vectastain Elite ABC kit obtained from Vector Laboratories, Burlingame, CA.

Chemicals were of analytical reagent grade. Before the experiment, all vessels and tips for pipetting were dipped in strong HNO₃ for 24 h and then washed with ultrapure water. The water used was purified in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Methods

Preparation of A β ₁₋₄₂-Infused Alzheimer's Disease Rat Model

A β ₁₋₄₂ was dissolved in sterile distilled water at a concentration of 5 μ g/ μ L, and incubated at 37°C for 7 days to obtain the aggregated form. Under anesthetization, 10 μ g (2 μ L) A β ₁₋₄₂ was injected bilaterally into the dorsal lateral of the dentate gyrus in the hippocampus of rats.

Groups

100 Male SD rats were divided into three groups: control group (30 rats), model group (35 rats) and tanshinone treatment group (35 rats). 24h after modeling, tanshinone, 50mg/kg, was administered by gastric perfusion to rats in the tanshinone treatment group, for 14 days, once per day. No medication was given in the model group and control groups.

Immunohistochemical assay

Animals were perfused through the heart under deep anaesthesia, chloral hydrate 0.35mL/100g, with 100mL PBS containing 10U/mL heparin followed by 150~200mL of paraformaldehyde 4% in PBS, pH=7.4. The brains were removed and immersed in PBS containing sucrose, pH=7.4, first at sucrose 20% for 24 h and then at 30% until sunk (2~5 days). Sections, 8 μ m, were cut on a cryostat and mounted in polylysine -coated slices. The number of nNOS and

iNOS positive cells around the injection point was analyzed immunohistochemically using an antibody against nNOS, dilution 1:50, and antibody against iNOS, dilution 1:50. Positive immunohistochemistry controls were used. routinely Negative control sections were incubated with PBS instead of the primary antibody. Reproducibility of staining was confirmed by re-immunostaining, using the same method, in multiple, randomly selected specimens. For visualization, under light microscopy, incubation with biotinylated rabbit/mouse anti-rat IgG and the immunoreaction was visualized using diaminobenzidine. Counting of nNOS and iNOS-expressing cells in subregions (CA1~ CA4) of each slides was done by an observer blind to the treatment status of the rats based on the visibility of a cell soma in 8 μ m-thick coronal sections in 400 $\times$ fields by Olysia Bioreport software (Olympus company, Japan). Five slides around the injection point were evaluated per animal.

Western blot analysis

The hippocampus tissue was homogenized with an Ultra-Turrax homogenizer in 20mM Tris-Buffer (pH=7.4), containing 0.25 sucrose, EDTA (1mM), PMSF (100 μ g/mL), aprotinin (10 μ g/mL), leupeptin (10 μ g/mL) and soyabean trypsin inhibitor (10 μ g/mL). After centrifugation at 7800g for 10min, the supernatants were collected and kept at -70°C until Western blot analysis was performed. The experiment was repeated three times. Protein (70 μ g) was loaded in each lane. Positive control aliquots were also loaded. We used mouse macrophage lysate for iNOS and rat pituitary lysate for nNOS. Samples were run on a 7.5% SDS-PAGE and transferred (overnight at 41°C, voltages: 40V) to a nitrocellulose membrane. The blots were incubated with a rabbit antiserum against iNOS and nNOS for 2h at room temperature. All the primary antibodies were used at final dilutions 1: 1000 in the blocking buffer. The blots were washed with wash buffer (10mM Tris, 100mM NaCl, 0.1% v/v Tween-20, pH=7.5) followed by an alkaline phosphatase-

conjugated anti-rabbit IgG as the secondary antibody and developed with 5-bromo-4-chloro-3-indolyl-phosphate BCIP toluidine salt and NBT NitroBlue Tetrazolium Tetrazolium. Molecular weight markers identified the protein bands. Pictures of the membranes were taken and were densitometrically scanned and analyzed using a Dekmate III scanner and Sigma Gel software package. In each lane, the concentration of protein loaded was measured by the Bradford method.¹¹

AChE histochemical staining assay

30µm sections of brain tissues were prepared according to the procedures in 'Immunohistochemical assay'. Sections were soaked into Karnovsky Roots medium at 37 °C for 2h and then the immunoreaction was visualized using diaminobenzidine. The percentage area with AChE positive fibers in each subregion (CA1~CA4) were detected by HPIAS-1000 photochromic pathological graphic analyzer and calculated according to the formula¹² as $P_{AChE} = S_{AChE} / S_{subregion} \times 100\%$.

Statistical Analysis

SPSS12.0 software for Windows (SPSS Inc, IL, USA) was used for analysis. Continuous variables were expressed as $\bar{X} \pm s$. Statistical analyses were performed by one-way ANOVA with post-hoc Tukey's test for paired comparisons. A difference between means was considered significant if the *P* value was < 0.05.

Results

Expression of nNOS and iNOS

Injection of Aβ₁₋₄₂ (10µg) in the hippocampus induced a decrease of nNOS and an increase of iNOS immunostaining (*P*<0.01), which were more significantly increased and decreased more in the tanshinone-treated rats, respectively (*P*<0.05) (Figure 1A~C, 2A~C).

The number of nNOS-positive cells decreased from 41.86 ± 4.61 (control group) to 25.07 ± 5.41 (model group) and increased to 34.91 ± 4.03 (tanshinone treatment group). The number of iNOS-positive cells increased from 10.81 ± 10.02 (control group) to 150.72 ± 12.16 (model group) and decreased to 103.71 ± 10.15 (tanshinone treatment group) (Figure 1D, 2D).

Western Blot analysis showed bands at 155 kDa and 130kDa corresponding to the size of nNOS and iNOS expressed at detectable levels in hippocampal homogenates of control animals. nNOS protein expression was reduced in the rats which had been injected by Aβ₁₋₄₂. This was augmented by *in vivo* administration of tanshinone, but it did not increase nNOS expression above control levels (Figure 3A). iNOS protein expression was augmented by the administration of Aβ₁₋₄₂.

Administration of tanshinone attenuated the augmentation of iNOS expression due to Aβ₁₋₄₂, but did not decrease iNOS expression under control levels (Figure 3B).

AChE positive fibers levels in hippocampus

AChE-positive neuron structures were found throughout the hippocampal formation of control brains. The positive nerve fibers, mostly of a varicose nature, formed a dense network with a laminar organization. Few positive cells were localized in the deepest portion of the granular layer in the dentate gyms. After administration of Aβ₁₋₄₂, damage of AChE-positive nerve fibers appeared and the number decreased (*P*<0.01, Table 1); A very dense accumulation of positive nerve fibers was seen in each subregion (CA1~CA4) of hippocampus after rats being treated by tanshinone (*P*<0.05, Table 1).

Discussion

The pathology of the brain in AD includes, in addition to neurodegeneration, deposits of insoluble amyloid, accumulation of abnormally phosphorylated cotton

FIGURE 1. Immunohistochemical evaluation of the effect of tanshinone on the expression of nNOS. (A) nNOS expression (black triangle) in the control rat hippocampus; (B) nNOS expression (black triangle) around the injection point in the model group. (C) nNOS expression (black triangle) around the injection point in the tanshinone treatment group. (D) the graph represents the number of nNOS positive cells around the injection point in 400 $\times$ fields. Values represent the $\bar{X} \pm s$ of rats in different groups. (a) $P < 0.01$ vs control group, (b) $P < 0.05$ vs model group. (A~C: 200 $\times$)

FIGURE 2. Immunohistochemical evaluation of the effect of tanshinone on the expression of iNOS. (A) iNOS expression (black triangle) in the control rat hippocampus; (B) iNOS expression (black triangle) around the injection point in the model group. (C) iNOS expression (black triangle) around the injection point in the tanshinone treatment group. (D) the graph represents the number of iNOS positive cells around the injection point in 400 $\times$ fields. Values represent the $\bar{X} \pm s$ of rats in different groups. (a) $P < 0.05$ vs control group, (b) $P < 0.05$ vs model group. (A~C: 200 $\times$)

FIGURE 3. Western blot analysis: using a monoclonal antibody against nNOS (A) and iNOS (B) from rat hippocampal preparations obtained from different groups. Densitometric analysis was done with Sigma Gel. (a) $P < 0.05$ vs control group, (b) $P < 0.05$ vs model group.

TABLE 1. Influence of tanshinone on the level of AChE in the hippocampal formation of the brains in different groups ($\bar{X} \pm s$)

Groups	Area of AChE positive nerve fibers	
	optical fiber density	percentage area of positive fibers (%)
Control (n=30)		
CA1	0.42 ± 0.03	18.81 ± 6.62
CA2~CA3	0.45 ± 0.06	23.13 ± 5.07
CA4	0.49 ± 0.04	29.27 ± 7.25
Model (n=35)		
CA1	$0.37 \pm 0.03^{\#}$	$12.43 \pm 4.07^{\#}$
CA2~CA3	$0.38 \pm 0.07^{\#}$	$17.06 \pm 4.21^{\#}$
CA4	$0.41 \pm 0.05^{\#}$	$10.25 \pm 4.91^{\#}$
Tanshinone treatment (n=35)		
CA1	$0.37 \pm 0.05^{**}$	$17.07 \pm 4.06^{**}$
CA2~CA3	$0.40 \pm 0.08^{**}$	$21.52 \pm 5.01^{**}$
CA4	$0.42 \pm 0.07^{*}$	$12.63 \pm 4.70^{*}$

* $P < 0.05$ and ** $P < 0.01$ refers to the comparison with the model group.

$^{\#}P < 0.01$ refers to the comparison with the control group.

and aggregated forms of microtubule binding proteins.¹³ AChE is the principal cholinesterase in the brain which causes hydrolysis of the endogenous neurotransmitter Ach.¹⁴ AChE inhibitors are given for the treatment of AD because a cholinergic deficit is characteristic of AD and elevation of ACh levels leads to functional improvement of central cholinergic synapses. This protects neuronal degeneration, modifies the amyloid precursor protein and enhances regional synthesis of neurotrophic molecules. Many plant extracts have been screened for activity against AChE. Galantamine and huperzine A have been introduced as drugs against the early symptoms of AD.¹⁵⁻¹⁶ It is likely that novel natural product AChE inhibitors exist, especially from traditional remedies for cognitive dysfunction. *S. miltiorhiza* contains diterpene quinines, known as tanshinone, which display several biological activities but there are no reports of anti-AD activity.

In the present study, we demonstrated that a single intracerebroventricular injection of A β ₁₋₄₂ induces a neuro-inflammatory response that is linked to a decline in learning and memory functions. In addition, by the use of Immunohistochemical Staining Assay and Western Blot Analysis, we have demonstrated a link between A β ₁₋₄₂, decrease of nNOS and increase of iNOS. Tanshinone might ameliorate the pathology of AD by increasing expression of nNOS and decreasing iNOS.

Nitric Oxide is an intra- and extra-cellular mediator of cell function.¹⁷ Constitutive NOS isoforms, especially nNOS and iNOS, make up approximately 80% of NOS activity in the adult brain of rats. The production of NO by iNOS plays an important role in inflammation, host defence and tissue repair. Brain oxidative stress seems to exert an important role in cognitive impairment observed in AD.¹⁸ iNOS generates NO and NO-derived reactive nitrogen species such as peroxynitrite. Accumulation of highly reactive molecules induces lipid peroxidation, tyrosine nitrosylation, DNA oxidative damage, and neuronal disruption, which are common characteristics of the

AD brain. The inflammatory reaction induced by A β in the CNS involves release of damaging factors, such as cytokines, NO and ROS that promote the activation of intracellular pathways, contributing to the progression of AD.¹⁹⁻²⁰

Our results are in agreement with previous reports. The level of iNOS immunoreactivity and the number of iNOS positive neural cells in the hippocampus of A β ₁₋₄₂ injected rats increased. Western blot analysis showed an increase in the total expression of iNOS protein in hippocampus compared with controls. Moreover, there was a distinct loss of nNOS immunolabeled neurons in A β ₁₋₄₂ injected rats. Western blot analysis that the total expression of nNOS protein in the hippocampus decreased after A β ₁₋₄₂ hippocampal injection. Furthermore, in the present study, tanshinone increased the level of nNOS and decreased the level of iNOS which were induced by AD.

Since the recognition of the cholinergic deficit in AD, AChE has been widely investigated in tissues involved in the disease. A decrease in the ratio of G4/G1 molecular forms of AChE may appear in selected regions of the AD brain.²¹ Most of the cortical AChE activity present in AD brain is associated with neuritic plaques, in which it is co-localized with A β deposits. This includes both pre-amyloid diffuse deposits and mature senile plaques. Treatment with AChEI has generally been beneficial in ameliorating global cognitive dysfunction and, more specifically, it is most effective in improving attention. In this study, we observed changes in the level of AChE after A β ₁₋₄₂ and tanshinone administration by AChE histochemistry.²² This allows, not only observation of detailed localization of AChE-containing structures with little background staining, but also simultaneous detection of neuron cell bodies and fibers. The technique is a modification of the direct thiocholine method of Karnovsky and Roots. Our results demonstrated that tanshinone, as an AChEI, could boost endogenous levels of AChE in the brain injected with A β ₁₋₄₂ and, thereby, enhance cholinergic neurotransmission.

Conclusion

This study has shown that tanshinone may be beneficial in AD by ameliorating spatial memory impairment, improving expression of inflammation factors and the clearance of senile plaques. The mechanism of action might be by the attenuation of the increase of iNOS, restoring nNOS expression and protection of the cholinergic system of AD rats. Further investigation should focus both on the mechanism of action of tanshinone and on its efficacy in the treatment of patients with AD.

References

1. Xiongwei Zhu, Mark A. Smith, Kazuhiro Honda, et al. Vascular oxidative stress in alzheimer disease. *J Neurol Sci* 2007; 257: 240–6.
2. Arias, E., Gallego-Sandin, S., Villarroja, M., Garcia, A.G., Lopez, M.G. Unequal neuroprotection afforded by the acetylcholinesterase inhibitors galanthamine, donepezil and rivastigmine in SH-SY5Y neuroblastoma cells: role of nicotinic receptors. *J Pharmacol Exp Ther* 2005; 315:1346–53.
3. Nathan C, Calingasan N, Nezezon J, et al. Protection from Alzheimer's-like disease in the mouse by genetic ablation of inducible nitric oxide synthase. *J Exp Med* 2005; 202: 1163–9.
4. Takatori YT, Kume T, Sugimoto M, Katsuki M, Sugimoto H, Akaike A. Acetylcholinesterase inhibitors used in treatment of Alzheimer's disease prevent glutamate neurotoxicity via nicotinic acetylcholine receptors and phosphatidylinositol 3-kinase cascade. *Neuropharmacol* 2006; 51:474–86.
5. Monsonego A. Immunotherapeutic Approaches to Alzheimer's Disease. *Science*. 2003;302:834.
6. Kim DH, Su Jeon J, Jung JW, et al. Tanshinone congeners improve memory impairments induced by scopolamine on passive avoidance tasks in mice. *Eur J Pharmacol* 2007;574:140–7.
7. Ge X, Wu J. Tanshinone production and isoprenoid pathways in *Salvia miltiorrhiza* hairy roots induced by Ag⁺ and yeast elicitor *Plant Science*.2005;168:487–91.
8. Colton CA, Vitek MP, Wink DA, et al. NO synthase 2 (NOS2) deletion promotes multiple pathologies in a mouse model of Alzheimer's disease. *PNAS*. 2006; 103:12867–72.
9. Law A, Gauthier S, Quirion S. NO to Alzheimer's disease: the putative between nitric oxide and dementia of the Alzheimer's type. *Brain Res Rev* 2001;35:73.
10. Lth HJ, Holzer N, Grtner U, et al. Expression of endothelial and inducible NOS isoforms is increased in Alzheimer's disease in APP23 transgenic mice and after experimental brain lesion in rat: evidence for an induction by amyloid pathology. *Brain Res*, 2001;913:57.
11. Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* 1976;72:246–54.
12. Li LX, Lin CY, Ru LQ. Mechanism of tanshinone in treatment of rat with Alzheimer's disease. *J Huazhong Univ Sci Tech [Health Sci]* 2003;23:19–24.
13. Ramírez BG, Blázquez C et al. Prevention of Alzheimer's disease pathology by cannabinoids: Neuroprotection mediated by blockade of microglial activation, *J Neurosci* 2005;25:1904–13.
14. Talesa VN. Acetylcholinesterase in Alzheimer's disease. *Mech Ageing Dev* 2001;122:1961–9.
15. Alvarez A, Alarcon R, Opazo C, et al. Stable complexes involving acetylcholinesterase and amyloid-beta peptide change the biochemical properties of the enzyme and increase the neurotoxicity of Alzheimer's fibrils. *J. Neurosci* 1998;18:3213–23.
16. Kihara T, Shimohama S, Sawada H, et al. Nicotinic receptor transduces signals to phosphatidylinositol 3-kinase to block Ab-amyloid induced neurotoxicity. *J Biol Chem* 2001;276:13541–6.
17. Kihara T, Shimohama S, Sawada H, et al. Protective effect of dopamine D2 agonists in cortical neurons via the phosphatidylinositol kinase cascade. *J Neurosci Res* 2002;70:274–82.
18. Etminan M, Gill S, Samii A. Effect of non-steroidal anti-inflammatory drugs on risk of Alzheimer's disease: systematic review and meta-analysis of observational studies. *BMJ* 2003;327:128.
19. Pan LR, Tang Q, Fu Q, Hu BR, Xiang JZ, Qian JQ. Roles of nitric oxide in protective effect of berberine in ethanol-induced gastric ulcer mice. *Acta Pharmacol Sin* 2005;26:1334–8.

20. Colton CA, Vitek MP, Wink DA, et al. NO synthase 2 (NOS2) deletion promotes multiple pathologies in a mouse model of Alzheimer's disease. *Proc Natl Acad Sci USA*.2006;103:12867-72.
21. Kwon YE, Park JY, No KT, et al. Synthesis, in vitro assay, and molecular modeling of new piperidine derivatives having dual inhibitory potency against acetylcholinesterase and A β 1-42 aggregation for Alzheimer's disease therapeutics. *Bioorg Chem* 2007;15:6596–607
22. Tago H, Kimura H, Maeda O. Visualization of detailed aAcetylcholinesterase fFiber and nNeuron sStaining in rRat bBrain by a sSensitive hHistochemical pProce-
dure. *The Journal of Histochemistry and Cytochemistry* 1986; 34: 1431-8.

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