Clinical and pathological benefit of Twendee X in Alzheimer's disease transgenic mice with chronic cerebral hypoperfusion

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Running headline: Neuroprotective effect of Twendee X.

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Abbreviations used: ameriod constrictors (ACs); Alzheimer's disease (AD); amyloid-β (Aβ); bilateral common carotid arteries (BCCAs); bovine serum albumin (BSA); cerebral blood flow (CBF); chronic cerebral hypoperfusion (CCH); cortex (CTX); dentate gyrus (DG); hippocampus (HI); months (M); phosphate-buffered saline (PBS); paraformaldehyde (PFA); subiculum (Sub); thalamus (TH); Twendee X (TwX); wild type (WT).

Abstract

Background: Multiple pathogeneses are involved in Alzheimer's disease, such as amyloid-β accumulation, neuroinflammation and oxidative stress. The pathological impact of chronic cerebral hypoperfusion on Alzheimer's disease is still poorly understood.

Methods: APP23 mice were implanted to bilateral common carotid arteries stenosis with ameroid constrictors for slowly progressive chronic cerebral hypoperfusion. The effects of the administration of Twendee X were evaluated by behavioral analysis, immunohistochemical analysis and immunofluorescent histochemistry.

Results: In the present study, chronic cerebral hypoperfusion, which is commonly found in aged Alzheimer's disease, significantly exacerbated motor dysfunction of APP23 mice from 5 months and cognitive deficit from 8 months of age, as well as neuronal loss, extracellular amyloid-β plaque and intracellular oligomer formations, and amyloid angiopathy at 12 months. Severe upregulations of oxidative markers and inflammatory markers were found in the cerebral cortex, hippocampus and thalamus at 12 months. Twendee X treatment (20 mg/kg/d, from 4.5 to 12 months) substantially rescued the cognitive deficit and reduced the above amyloid-β pathology and neuronal loss, alleviated neuroinflammation and oxidative stress.

Conclusions: The present findings suggested a potential therapeutic benefit of Twendee X for Alzheimer's disease with chronic cerebral hypoperfusion.

Introduction

Alzheimer's disease (AD) is the most common cause of dementia, accounting for 69% in all dementia among the people older than 75 years. [1] Although pathogenesis of AD is complex, [2-3] oxidative stress and inflammation are also considered to play important roles in the process of AD. [4-5] Chronic cerebral hypoperfusion (CCH) is a major cause of cognitive deficits and contributes to the progression of dementia. [6] Our recent studies showed that CCH strongly enhanced the AD pathology in mice, [7-8] which is appropriate to study the effects of CCH on cognitive impairments, AD pathology, neuroinflammatory and oxidative stress. Twendee X (TwX) is an anti-oxidant mixture that contains multiple ingredients, such as coenzyme Q10, ascorbic acid, L-glutamine and cystine. Our previous study demonstrated neuroprotective effects of TwX in an acute cerebral ischemia model in mice by reducing ischemic infarct and attenuating both oxidative stress and inflammatory markers. [9]

In the present study, therefore, we investigated a possible therapeutic effect of TwX on cognitive function, Aβ pathology, inflammatory and oxidative stresses in an AD mouse model with CCH.

Materials and Methods

Mouse model

All procedures were approved by the guidelines of the Animal Committee of the Graduate School of Medicine and Dentistry of Okayama University (OKU-2014-095). APP23 mice overexpress human APP751 isoform carrying the Swedish double mutation (KM670/671NL) under the control of the murine Thy1 promoter. [10] APP23 male mice were obtained from Dr. Takashi Saito (RIKEN Brain Science Institute, Saitama, Japan) and maintained as hemizygotes

by mating APP23 male mice with C57BL/6J female mice (CLEA Japan, Tokyo, Japan). The offspring were genotyped using a PCR assay with DNA obtained from tail tissue samples. Wild type (non-transgenic) littermates were used as controls. Mice were housed in 12:12-hour light-dark cycle with controlled temperature and free access to food and water.

Four groups of mice were used in this study: wild type mice (WT + sham surgery, n = 10), APP23 group (APP23 + sham surgery, n = 10), CCH group (APP23 + CCH, n = 12) and TwX group (APP23 + CCH + TwX, n = 13). Groups comprised approximately equal numbers of male and female mice.

For the CCH and TwX mice, ameriod constrictors (ACs) were applied to the bilateral common carotid arteries (BCCAs) at 4 months (M) of age, and cerebral blood flow (CBF) was measured with a laser-Doppler flowmeter (FLO-C1, Omegawave, Tokyo, Japan) before and 1, 3, 7, 14 and 28 d after surgery as our previously report. [8]

TwX is a mixture containing multiple antioxidants, [9] such as coenzyme Q10 (3.6 wt%; AQUA Q10 P40-NF, Nissin Pharmaceutical, Tokyo, Japan), niacin amid (0.7 wt%), L-cystine (18.2 wt%), ascorbic acid (34.2 wt%), succinic acid (3.6 wt%), fumaric acid (3.6 wt%), L-glutamine (34.6 wt%), and riboflavin (1.5 wt%; Bislase inj, Toa Eiyo, Tokyo, Japan). TwX was given to the mice in TwX group by oral gavage once daily 20 mg/kg per day from 4.5 M of age until sacrifice.

Behavioral analysis

Rotarod test was used to assess sensorimotor coordination of mice at ages of 2, 5, 7, 9 and 11 M, and evaluation criteria is the time that mice spent on the rotating rod (MK670;

Muromachi Kikai Co., Tokyo, Japan) before falling as our previous report. [11] In brief, the rotarod test began with mice trying to stay on a rod rotating at 4 rpm, the speed was then increased to 40 rpm in a period of 60 seconds, and then kept the speed of 40 rpm. The latency to fall (up to a maximum of 400 seconds) was recorded. Six trials were performed in each measurement, and the best result was recorded.

8-arm radial maze test was performed when mice aged 3, 6, 8, 10 and 12 M as our previous reports. [8, 12] Briefly, dietary restriction over 7 days was carried out which led to an 85% decrease of free-feeding body weight in all mice. Then mice were allowed to explore the baited arms of the maze for 10 min in 2 days habituation sessions. After adaptation, each mouse was left in the maze until either all pellets in 4 of the arms (1, 3, 4, and 7) were obtained or 5 min elapsed. Re-entry into the baited arms previously visited was recorded as a working memory error.

Tissue preparation

Mice aged 12 M were deeply anesthetized and then perfused with 20ml chilled phosphate-buffered saline (PBS, pH 7.4), followed by 4% paraformaldehyde (PFA) in PBS. After post-fixed in 4% PFA overnight, the brains were transferred into 10, 20 and 30% (wt/vol) sucrose in PBS for 24h, respectively. Coronal brain sections (20 μm) were cut on a cryostat at -20°C and mounted on silane-coated glass slides.

Histochemistry and immunohistochemistry

For Nissl staining, brain sections were incubated in 0.1% cresyl violet (CV) for 5 min at room temperature, dehydrated gradually in ethanol, and coverslipped with microcoverglass.

For single immunohistochemistry, after incubation in 0.3% hydrogen peroxide/methanol followed by 5% bovine serum albumin (BSA), the sections were strained overnight at 4°C with the following primary antibody: mouse anti-4G8 antibody (1:1000, Biolegend, San Diego, CA, USA), rabbit anti-A11 antibody (1:200, Invitrogen, Camarillo, CA, USA), goat anti-NLRP3 antibody (1:100, Abcam, Cambridge, MA, USA), mouse anti-caspase-1 antibody (1:200, Adipogen, San Diego, CA, USA), goat anti-IL-1β antibody (1:100, R&D Systems, Minneapolis, MN, USA), rabbit anti-Iba-1 antibody (1:1000, Abcam), rabbit anti-TNFα antibody (1:200, Abcam), mouse anti-4-HNE antibody (1:40, JaICA, Shizuoka, Japan), mouse anti-8-OHdG antibody (1:20, JaICA) and rabbit anti- Nitrotyrosine antibody (1:200, Sigma-Aldrich, St. Louis, MO, USA). After washed with PBS, brain sections were treated with suitable biotinylated secondary antibodies (1:500; Vector Laboratories) at room temperature for 2 h. Then the sections were incubated with the avidin–biotin–peroxidase complex (VECTASTAIN Elite ABC Kit; Vector Laboratories) for 30 min and visualized with 3,3′-diaminobenzidine (DAB). Negative control sections were stained in the same way as described above except for the primary antibodies.

3 sections per brain and 5 random selected regions were then analyzed with a light microscope (Olympus BX-51, Tokyo, Japan) for each measurement. Cerebral cortex (CTX), hippocampus (HI), and thalamus (TH) were measured for semiquantitative analysis of Nissl, A11, NLRP3, caspase-1, IL-1β, Iba-1, TNF-α, 4-HNE, 8-OHdG and nitrotyrosine staining intensity. The number of 4G8-positive plaques was expressed per 1 mm^2.

Double immunofluorescent histochemistry

After incubation in 5% BSA in PBS with 0.1% triton at room temperature for 1 h, the sections were incubated at 4 °C overnight with primary antibody. Antibodies were as follows: rabbit anti-A11 antibody (1:200, Invitrogen), goat anti-NLRP3 antibody (1:100, Abcam), mouse anti-Aβ40 antibody (1:100, Wako, Osaka, Japan), and mouse anti-αSMA antibody (1:100, Sigma-Aldrich). Following washes, sections were incubated with fluorescent secondary antibody, and the fluorescent signals were visualized by confocal microscope (LSM-780; Zeiss, Jena, Germany).

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistical comparison was performed using one-way ANOVA analysis followed by Tukey-Kramer test. $p < 0.05$ was considered significant.

<div align="center">

Results

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Changes of CBF with ameroid constrictors implantation

CBF gradually decreased in CCH and CCH + TwX groups from 1 d after surgery, reached a minimum at 7 d and then became stable until 28 d (Fig. 1B, [#] $p < 0.01$ vs APP23). There was no significant difference between CCH and TwX groups.

Behavioral analysis after CCH

CCH and CCH + TwX groups showed significant impairment of rotarod test at 5, 7, 9 and 11 M after surgery compared to WT and APP23 groups, and TwX treatment (CCH + TwX)

significantly improved the rotarod time at 9 M compared with CCH group (Fig. 1C, $^*p < 0.05$ and $^{**}p < 0.01$ vs WT; $^\#\,p < 0.05$ and $^{\#\#}p < 0.01$ vs APP23; $^\$p < 0.05$ and $^{\$\$}p < 0.01$ vs CCH). In 8-arm radial maze test, CCH + TwX group showed significant improvement in errors at 8, 10 and 12 M compared with CCH group (Fig. 1D, $^*p < 0.05$ and $^{**}p < 0.01$ vs WT; $^\#\,p < 0.05$ vs APP23; $^\$p < 0.05$ and $^{\$\$}p < 0.01$ vs CCH).

Neuronal loss after CCH

Nissl staining showed significant lower density of neurons in subiculum, CA1, dentate gyrus (DG) and TH regions of 3 APP23 groups than in WT mice (Fig. 2A and 2B). However, TwX treatment significantly suppressed these neuropathological changes in the hippocampus and TH (Fig. 2B, $^{**}p < 0.01$ vs WT; $^\#\,p < 0.05$ and $^{\#\#}\,p < 0.01$ vs APP23; $^\$p < 0.05$ and $^{\$\$}p < 0.01$ vs CCH).

Parenchymal and vascular Aβ deposits

The numbers of 4G8 positive-amyloid plaque (Fig. 3A, arrows) and A11-positive amyloid oligomer formation (Fig. 3A) were increased in CCH group, which were reduced by TwX treatment (Fig. 3A, 3D and 3E, $^*p < 0.05$ and $^{**}p < 0.01$ vs WT; $^\#\,p < 0.05$ and $^{\#\#}\,p < 0.01$ vs APP23; $^\$p < 0.05$ and $^{\$\$}p < 0.01$ vs CCH).

Immunofluorescent analysis showed a colocalization of Aβ oligomer with NLRP3 both in Aβ plaque and neural cell (Fig. 3B). Aβ was also accumulated in cerebral vessels in CCH group than APP23 group, which was attenuated by TwX treatment (Fig. 3C).

Neuroinflammation after CCH

NLRP3, caspase-1 and IL-1β were evidently increased in 3 APP23 groups (Fig. 4A, 5A

and 6A), and TwX treatment significantly reduced such enhanced expressions of NLRP3,

caspase-1 and IL-1β compared with CCH group (Fig. 4B, 5B and 6B, $^{*}p < 0.05$ and $^{**}p < 0.01$

vs WT; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs APP23; $^{\$}p < 0.05$ and $^{\$\$}p < 0.01$ vs CCH). The expression

of the microglial Iba-1 was also increased in APP23 and APP23 + CCH, especially in the

cerebral cortex, but was inhibited by TwX treatment (Fig. 7A and 7B, $^{*}p < 0.05$ and $^{**}p < 0.01$

vs WT; $^{\#}p < 0.05$ vs APP23; $^{\$}p < 0.05$ vs CCH). The expression of pro-inflammatory cytokine

marker TNF-α was increased significantly in the CTX, CA3 and TH, which was recovered by

TwX treatment (Fig. 8A and 8B, $^{*}p < 0.05$ and $^{**}p < 0.01$ vs WT; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs

APP23; $^{\$}p < 0.05$ and $^{\$\$}p < 0.01$ vs CCH).

Oxidative stress change after CCH

As shown in Fig. 9-11, 4-HNE, 8-OHdG and nitrotyrosine were increased in CTX and HI

in 3 APP23 groups (Fig. 9A, 10A and 11A). These oxidative stress markers were all attenuated

in CCH + TwX group compared with CCH group (Fig. 9B, 10B and 11B, $^{*}p < 0.05$ and $^{**}p <$

0.01 vs WT; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs APP23; $^{\$}p < 0.05$ and $^{\$\$}p < 0.01$ vs CCH).

Discussion

The present study showed that the administration of antioxidative mixture TwX improved

motor coordination and working memory (Fig. 1), rescued hippocampal neuron loss (Fig. 2),

decreased Aβ pathology (Fig. 3), and attenuated inflammatory reaction (Fig. 4-8) and oxidative

stress (Fig. 9-11) under CCH of APP23 mice. CCH is one major cause of vascular dementia, but is also an important risk factor of AD progression. [13] Our previous studies suggested that CCH enhanced neurodegenerative processes by promoting oxidative stress and neuroinflammation. [7-8]

Since hippocampal neurons are critically important for memory function, [14] the positive effect on working memory (Fig. 1D) was most likely a result of the protective mechanism of TwX against Aβ pathology under CCH (Fig. 2). In the present study, TwX significantly reduced amyloid plaques, Aβ oligomer formation, and CAA in the brain (Fig. 3). We also observed an anti-inflammatory effect of TwX by reducing the expression of these inflammatory markers (Fig. 4-8). Aβ oligomer induces the activation of the NLRP3 inflammasome, which then trigger downstream caspase-1, IL-1β, [15] microglia, [2] and TNF-α. [16] Thus the present results revealed the suppression of Aβ pathology based on the anti-inflammatory effects of TwX.

Oxidative stress is an early event in AD, and also plays a key role in the progression of AD pathology. [17-19] As was expected, TwX showed a positive effection on an early stage of Aβ pathology with anti-oxidative mechanism in reducing the 4-HNE, 8-OHdG and nitrotyrosine (Fig. 9-11). Several studies have reported that antioxidants have potential therapeutic roles against AD. [20-22] Compared to a single ascorbic acid, the multiple antioxidant TwX showed synergistic effects on attenuating oxidative stress in an irradiation mouse model. [23] Our previous study also showed the neuroprotective effects of TwX in an acute cerebral ischemia mouse model. [9]

In summary, the present study revealed that CCH enhanced primary AD pathology of APP23, and TwX improved motor and memory functions, reduced such primary AD pathology

with suppressing inflammatory and oxidative stresses.

Acknowledgements

Conflict of interest

The authors disclose no potential conflict of interests.

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Figure Legends

Fig. 1) Twendee X (TwX) improved behavioral deficits of Alzheimer's disease (AD) mice model with chronic cerebral hypoperfusion (CCH). (A) Surgical implantation of ameroid constrictors on bilateral common carotid arteries. (B) Temporal profile of the cerebral blood flow measured by laser-Doppler flowmeter. (C) Analysis of motor function by rotarod test, and (D) working memory by 8-arm radial maze test ([**]$p < 0.01$ versus WT; [#]$p < 0.05$ versus APP23, [##]$p < 0.01$ versus APP23; [$]$p < 0.05$ versus APP23+CCH, [$$]$p < 0.01$ versus APP23+CCH).

Fig. 2) (A) Nissl stainings in cerebral cortex (CTX), hippocampus (HI), and thalamus (TH) of AD mice model with CCH. (B) Quantitative analysis of Nissl staining intensity in the CTX, subiculum (Sub), CA1, dentate gyrus (DG), and TH ([**]$p < 0.01$ versus WT; [#]$p < 0.05$ versus APP23, [##]$p < 0.01$ versus APP23; [$]$p < 0.05$ versus APP23+CCH, [$$]$p < 0.01$ versus APP23+CCH. Scale bar = 50 μm).

Fig. 3) TwX ameliorated amyloid-β (Aβ) pathology in AD mice model with CCH. (A) 4G8 immunolabelling of Aβ plaque (arrows) in CTX and HI, A11 immunolabelling of amyloid oligomer in CTX and TH. (B) Double immunofluorescence staining for A11 plus NLRP3 in Aβ plaques and cells. (C) Double immunofluorescence staining for α-SMA plus Aβ40. (D) Quantitative analysis of amyloid plaques in CTX and HI, and (E) quantitative analysis of A11 staining intensity in CTX and TH ([*]$p < 0.05$ versus WT, [**]$p < 0.01$ versus WT; [#]$p < 0.05$ versus APP23, [##]$p < 0.01$ versus APP23; [$]$p < 0.05$ versus APP23+CCH, [$$]$p < 0.01$ versus APP23+CCH. Scale bar = 50 μm).

Fig. 4) Immunohistochemical staining of NLRP3 in AD mice model with CCH. (A) NLRP3 staining in CTX, HI and TH. (B) Quantitative analysis of NLRP3 ($^{*}p < 0.05$ versus WT, $^{**}p < 0.01$ versus WT; $^{#}p < 0.05$ versus APP23, $^{##}p < 0.01$ versus APP23; $^{$}p < 0.05$ versus APP23+CCH, $^{$$}p < 0.01$ versus APP23+CCH. Scale bar = 50 μm).

Fig. 5) Immunohistochemical staining of caspase-1 in AD mice model with CCH. (A) caspase-1 staining in CTX, HI and TH. (B) Quantitative analysis of caspase-1 ($^{*}p < 0.05$ versus WT, $^{**}p < 0.01$ versus WT; $^{#}p < 0.05$ versus APP23; $^{$}p < 0.05$ versus APP23+CCH, $^{$$}p < 0.01$ versus APP23+CCH. Scale bar = 50 μm).

Fig. 6) Immunohistochemical staining of IL-1β in AD mice model with CCH. (A) IL-1β staining in CTX, HI and TH. (B) Quantitative analysis of IL-1β ($^{*}p < 0.05$ versus WT, $^{**}p < 0.01$ versus WT; $^{#}p < 0.05$ versus APP23, $^{##}p < 0.01$ versus APP23; $^{$}p < 0.05$ versus APP23+CCH, $^{$$}p < 0.01$ versus APP23+CCH. Scale bar = 50 μm).

Fig. 7) Immunohistochemical staining of Iba-1 in AD mice model with CCH. (A) Iba-1 staining in CTX, HI and TH. (B) Quantitative analysis of Iba-1 ($^{*}p < 0.05$ versus WT, $^{**}p < 0.01$ versus WT; $^{#}p < 0.05$ versus APP23; $^{$}p < 0.05$ versus APP23+CCH. Scale bar = 50 μm).

Fig. 8) Immunohistochemical staining of TNF-α in AD mice model with CCH. (A) TNF-α staining in CTX, HI and TH. (B) Quantitative analysis of TNF-α ($^{*}p < 0.05$ versus WT, $^{**}p <$

0.01 versus WT; $^{\#}p < 0.05$ versus APP23, $^{\#\#}p < 0.01$ versus APP23; $^{\$}p < 0.05$ versus APP23+CCH, $^{\$\$}p < 0.01$ versus APP23+CCH. Scale bar = 50 μm).

Fig. 9) Immunohistochemical staining of 4-HNE in AD mice model with CCH. (A) 4-HNE staining in CTX, HI and TH. (B) Quantitative analysis of 4-HNE ($^{**}p < 0.01$ versus WT; $^{\#}\,p < 0.05$ versus APP23; $^{\$}p < 0.05$ versus APP23+CCH, $^{\$\$}p < 0.01$ versus APP23+CCH. Scale bar = 50 μm).

Fig. 10) Immunohistochemical staining of 8-OHdG in AD mice model with CCH. (A) 8-OHdG staining in CTX, HI and TH. (B) Quantitative analysis of 8-OHdG ($^{*}p < 0.05$ versus WT, $^{**}p < 0.01$ versus WT; $^{\#}p < 0.05$ versus APP23, $^{\#\#}p < 0.01$ versus APP23; $^{\$}p < 0.05$ versus APP23+CCH, $^{\$\$}p < 0.01$ versus APP23+CCH. Scale bar = 50 μm).

Fig. 11) Immunohistochemical staining of nitrotyrosine in AD mice model with CCH. (A) Nitrotyrosine staining in CTX, HI and TH. (B) Quantitative analysis of nitrotyrosine ($^{*}p < 0.05$ versus WT, $^{**}p < 0.01$ versus WT; $^{\#}p < 0.05$ versus APP23, $^{\#\#}p < 0.01$ versus APP23; $^{\$}p < 0.05$ versus APP23+CCH, $^{\$\$}p < 0.01$ versus APP23+CCH. Scale bar = 50 μm).