Protective Roles of *N-trans*-feruloyltyramine Against Scopolamine-Induced Cholinergic Dysfunction on Cortex and Hippocampus of Rat Brains

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ABSTRACT

Objective: To study the protective effects of *N-trans*-feruloyltyramine (NTF) on scopolamine-induced cholinergic dysfunction, apoptosis, and inflammation in rat brains.

Materials and Methods: Treatments were administered intraperitoneally (i.p.). Wistar rats (8-week-old) were allocated into 4 groups (n = 3) as follows: scopolamine-only, NTF-only, NTF + scopolamine and control. Spatial cognition was evaluated by Morris water maze. ROS assay and Western blot analyses were conducted in 3 brain regions: the frontal cortex, hippocampus, and temporal cortex.

Results: NTF treatment inhibited scopolamine-induced memory impairment and significantly attenuated scopolamine-induced changes in the three brain regions. Investigated scopolamine-associated changes were as follows: increases in ROS production and BACE1 level, decrease in ChAT level, increases in inflammatory and apoptotic markers, and activation of signaling pathway kinases related to inflammation and apoptosis.

Conclusion: With its *in vivo* antioxidant, cholinergic-promoting, anti-apoptosis, and anti-inflammatory biological activities, NTF is a promising candidate to be further investigated as a potential treatment for Alzheimer's-associated neurodegeneration.

Keywords: Acetylcholine; Alzheimer's disease; antioxidant; Morris water maze; *N-trans*-feruloyltyramine (Siriraj Med J 2021; 73: 413-422)

INTRODUCTION

Alzheimer's disease (AD) causes progressive and irreversible deterioration of cognitive functions especially memory.[1] The major pathological characteristics of the human AD brain are extracellular aggregates of amyloid-β (Aβ) and intracellular aggregates of hyperphosphorylated tau, namely the senile plaques and the neurofibrillary tangles respectively.[2] The underlying mechanism of sporadic AD involves cholinergic dysfunction including degeneration of basal forebrain cholinergic neurons and loss of hippocampal cholinergic fibers.[3] Impaired cholinergic transmission affects learning and memory, cortical and hippocampal information processing, and ultimately behaviors.[6] Scopolamine, an antagonist of a muscarinic acetylcholine receptor,[4] induces cholinergic dysfunction and cognitive impairment through oxidative

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stress and neuroinflammation in animal models.[5] Intraperitoneal injection of scopolamine blocks cholinergic neurotransmission, dysregulates cholinergic system, and consequently impairs cognition in rodents.

N-trans-feruloyltyramine (NTF) was purified from *Polyalthia suberosa*, a shrubby tree found throughout southeast Asia and south China whose parts are used in Thai traditional medicine.[6] NTF has potent radical-scavenging antioxidant property, especially from phenolic hydroxyls. NTF and the other purified compound studied by our group, *N*-benzylcinnamide (PT-3), have been demonstrated to be effective in protecting rat cultured cortical neurons against Aβ-induced cytotoxicity by inhibiting ROS production, suppressing apoptotic caspase-3 and Bax, and in turn elevating anti-apoptotic Bcl-2.[7-8] As PT-3 that has elicited protective effects on primary cortical neurons also showed promising outcome in aged rat brain *in vivo*,[9] neuroprotective effects of NTF in vivo had become our next primary study target.

In this research study, the animals were used in the experiment, and they were calculated for the sample size in accordance with the ethical guideline to protect the unnecessary wastage of resources. Consistently, there are several studies that have demonstrated the statistically significant results with a small sample size (n = 3).[9-11] Meanwhile, our preliminary results showed the protective effects of NTF treatment against scopolamine-induced cholinergic dysfunction, including, ROS production, apoptosis, inflammation, and associated signaling pathways in several rat brain regions, specifically the frontal cortex, hippocampus and temporal cortex.

The frontal cortex integrity is correlated with the higher reservation of cognitive performance in aging populations.[12] The hippocampus and especially its associated cholinergic signaling pathway play a crucial role in memory formation.[13] The medial temporal lobe cortex or, in short, the temporal cortex is reciprocally interconnected to the hippocampus and it greatly involved in the hippocampus-associated cognitive processes and cognitive decline such as AD in humans[14] and ischemia-associated dementia in rats.[15] Furthermore, ChAT activity in the temporal cortex is positive correlated with cognitive preservation, but it is negatively correlated with AD pathologies.[16] Importantly, the frontal cortex, hippocampus, and temporal cortex are three of the regions heavily affected by scopolamine[17,18] and aging[9] in the rat brain.

Herein, we studied the neuroprotective effects of NTF treatment on scopolamine-associated cholinergic dysfunction, namely, ROS production, apoptosis, inflammation, and relevant signaling pathways in the rat frontal and temporal cortices and hippocampus.

MATERIALS AND METHODS

Reagents

Scopolamine (Sigma-Aldrich) and NTF were dissolved in a vehicle solution of (v/v) 40% dimethylsulfoxide, 59% phosphate-buffered saline (PBS), and 1% ethanol and diluted in PBS for animal administration. NTF was isolated from the acetone extract of *Polyalthia suberosa* stems as previously described.[6] The test drug NTF was in its form of pure compound. From 997.2 mg of the semi-solid fraction, 88.1 mg (8.83 %) of NTF could be purified by preparative layer chromatography.[6]

Animal experiments

All experimental procedures were approved by the Institute of Molecular Biosciences Animal Care and Use Committee (MB-ACUC) (COA.NO.MB-ACUC 2016/002).

Twelve 8-week male Wistar rats (250-300 g) from the National Laboratory Animal Center, Mahidol University, were individually cared for in cages under 12 h light/dark cycle, 22±1 °C temperature, 45-55 % humidity, and *ad libitum* water and diet. The 12 rats were separated into 4 groups, each with 3 animals, namely, scopolamine treatment only, NTF treatment only, scopolamine plus NTF treatment, and control. Following habituation for 5 days, animals were once per day injected intraperitoneally (i.p.) for 14 days.[19] In the control group, animals were injected with vehicle 1 h before the water maze test for 14 days. In the scopolamine-only group, animals were first injected for 7 days with vehicle followed by scopolamine treatment on day 8 to day 14 (3.0 mg/kg BW) 1 h before the water maze test. In the NTF treatment only group, animals were injected with 1 ml aliquot of NTF (1.5 mg/kg BW) for 14 days 1 h before the water maze test. In the scopolamine plus NTF treatment group, animals were administered with NTF as described above for 7 days and then together with n day 8 to day 14 (3.0 mg/kg BW) 1 h before the water maze test.

Rats were treated with NTF for 7 days prior to MWM training because we would like to investigate the neuroprotective effects of NTF against scopolamine. The NTF effects as a memory enhancer can still remain during the MWM training.[19] The dose of NTF was selected in accordance with our previous findings on the protective roles of N-benzylcinnamide (PT-3).[9] In animal studies the model of scopolamine has been performed using doses between 0.5,1 or 3 mg/kg IP). The effect of scopolamine to induce impairment of learning and memory is dose dependent.[19-20] The highest dose of scopolamine exhibits the greater severity of memory dysfunction. Scopolamine was administered via the injection 1 hr before MWM

training because the half-life of scopolamine is 2.4±1.4 h for its elimination.[21] Central pharmacodynamic effects of scopolamine peak between 1 and 3 h and disappear after 5–6 h.[22]

Morris water maze test

Water maze tests were conducted to evaluate the effects of NTF and scopolamine on the acquisition and retention of spatial memory.[18] Visible platform trial was performed on the day before the training trial. Animals have to associate the relative location of the visible platform with the provided visual cues. On the following training trials, the platform was submerged and invisible. The training trials were conducted over a period of 7 days as follows: day 1, visible platform test; days 2-6, hidden platform tests; and on the same day 6 after hidden platform, probe trial test. These consecutive 6 days correspond to the days 8-13 of the treatment period. Rats in each group were put in the water facing different quadrants, which were altered for each experiment, and the escape latency defined as time spent to find the submerged platform was recorded. In the probe trial, time spent in the target quadrant with the platform was recorded.

The experimental schedule was designed to investigate the protective effect of NTF as a memory enhancer in rats. Rats underwent habituation for 5 days. After that, NTF (1 ml, 1.5 mg/kg. i.p.) or vehicle was administrated once a day for 7 days. Then, 1 h prior to the first trial session, rats were administrated of veh, NTF, scopolamine (3.0 mg/kg) or NTF+Scop (i.p) every MWM training for 6 days, in accordance with the previously described protocol.[23]

ROS production assay

After animals were decapitated without prior anesthesia, the 3 regions of the rat brains – the frontal cortex, hippocampus, temporal cortex – were dissected and frozen at -80°C. For the frontal cortex, the frontal part of the brain was cut straight down coronally and attached subcortical brain regions were removed. For the hippocampus, the bilateral hippocampal tissues with all subsectors were collected. For the temporal cortex, all cortical regions encompassing the hippocampus or the medial temporal lobe were dissected. Reactive oxygen species (ROS) production was measured according to the protocol of our previous work.[24] In brief, 2.4-3.0 mg of brain tissue from each dissected region were homogenized in 80-100 µl of ice-cold Locke's buffer, then 10 µl of homogenate was adjusted to a concentration of 3 mg/ml tissue, incubated with 10 µM 2',7'-dichlorofluorescin diacetate (DCFH-DA) (stock solution in 100% ethanol) for 45 min at 37°C. Fluorescence of the oxidized product 2',7'-dichlorofluorescein (DCF) (485 nm excitation and 535 nm emission) was measured every 10 minutes for 4 times in total at 37°C in a DTX 880 multimode plate reader.

Western blot analysis

Rat brains were dissected into three regions: frontal cortex, hippocampus and temporal cortex).[25] The brain protein contents were extracted with the lysis buffer, centrifuged at 11,000 ×g for 15 min, and collected as a supernatant. The protein samples were loaded onto polyacrylamide gel. The proteins were separated by gel electrophoresis and transferred onto (PVDF) membranes where they were reacted to specific antibodies. On the PVDF membrane, proteins of interest were identified using a detection reagent. The protein bands were quantified and analyzed with the same loading sample groups of its own gel. PVDF membrane was incubated overnight at 4°C with primary rabbit anti-Bax, -Bcl-2, -activated caspase-3, -BACE1, -phospho-p38, -p38, -phospho-JNK, -JNK, -phospho-ERK1/2, -ERK1/2, -phospho-Akt, -Akt, -phospho-NF-κB p65 (Ser536), -NF-κB antibodies (1:1000 dilution) (Cell signaling), -IL-1β (1:1000) (Santa Cruz); and primary mouse anti-ChAT (1:1000) (Millipore) and -IL-6 (1:1000) (Santa Cruz); and rabbit anti-actin (1:2500) (Cell Signaling) for normalization of protein loading. Immunoreactive proteins were visualized by incubating PVDF membranes with secondary horseradish peroxidase-conjugated antibodies (1:1000) (Cell Signaling) at room temperature for 1 h and subsequently with enhanced chemical luminescence (ECL) reagents (Bio-Rad) before signal exposure to Hyperfilm™ (GE Healthcare). Intensities of the visualized protein bands were measured by scanning densitometry (Image J software). The optical density (OD) of protein bands on Hyperfilm was determined and normalized to the OD of β-actin and total phospho proteins. The results were expressed as the percentage of OD values by using the ImageJ software.

Each gel contains four sample groups (Control, Sco, NTF+sco, NTF). Each protein sample of three rats on different brain regions, including frontal cortex (3), hippocampus (3), and temporal cortex (3) was loaded separately on different polyacrylamide gel. Four sample groups of each region in each rat were underwent Western blotting. Each blot was repeated for at least 3 times from different batches of protein samples. The representative blots were shown in figures.

Statistical analysis

Statistical analyses were performed using Prism 6.0 (GraphPad). Differences in the escape latency from the training/hidden platform trials were analyzed using two-way analysis of variance (ANOVA). Differences between means of the 4 groups from all other experiments were determined using one-way ANOVA with pair-wise *p*-values corrected by Tukey's *posthoc* test. Results are deemed significant at $p < 0.05$. In all Western blot analyses, non-phospho proteins (ChAT, Bcl-2, Bax, Activated caspase-3, IL-6, and IL-1β) were quantified and normalized with β-Actin. Meanwhile, phosphoproteins (p-NFκB, p-ERK, p-JNK, p-p38, p-Akt) were quantified and normalized with the total protein counterparts.

RESULTS

Learning and memory in a Morris water maze test of rats administered scopolamine with and without NTF

Control rats became proficient at locating the submerged platform by day 5 of training compared to day 1 ($p < 0.05$) in a hidden platform test (Fig 1A). On day 5 of training scopolamine-treated rats perform significantly less well than controls ($p < 0.01$), but, interestingly, rats treated with scopolamine together NTF have proficiencies superior to those of controls, whereas NTF treatment alone was no more effective than non-treated control. In the probe trial tests, as expected, compared to control rats, those in the scopolamine plus NTF treatment group stay significantly longer in the platform quadrant. Those in the NTF only treatment group spent the same time. Lastly as expected, those in the scopolamine-only group stayed for a significantly shorter duration (Fig 1B).

ROS production in brain tissues of rats administered scopolamine with and without NTF

Scopolamine treatment for 7 days elevated ROS level 2 folds in all three types of brain tissues (frontal cortex, hippocampus, and temporal cortex) over control levels (Fig 2). ROS levels were restored to control levels by NTF administration (7 days prior and during the scopolamine treatment period), while NTF alone (for 7 days) had no effect on ROS levels in all three types of brain tissues.

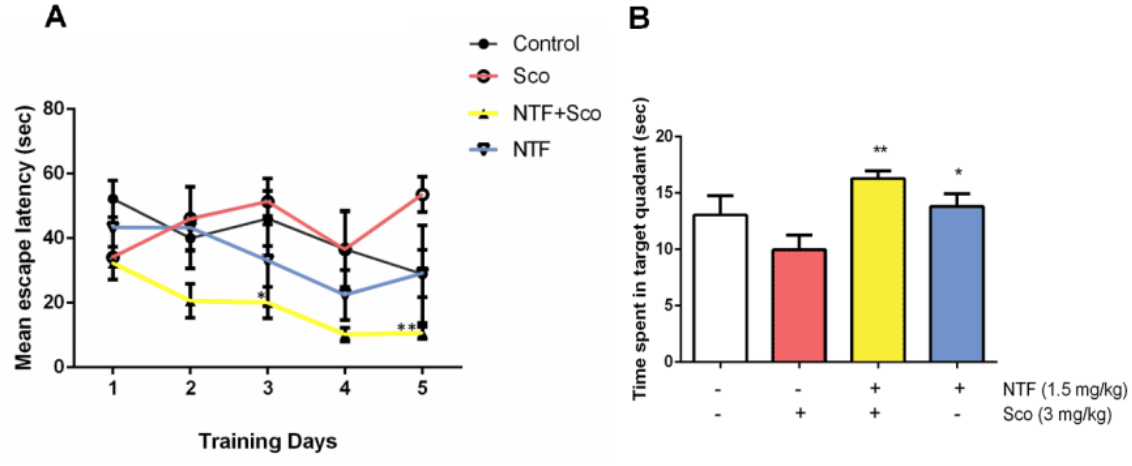

Fig 1. NTF (1.5 mg/kg) was injected i.p. daily to rats 7 days before and together with scopolamine (3.0 mg/kg BW i.p.) 1 h before the 7-day Morris water maze test; probe trial was conducted on day 7. Each data point (n=3) is expressed as mean ± SEM. A NTF prevented scopolamine from elevating escape latency. *p < 0.05 scopolamine-treated with NTF versus (vs) the scopolamine group on day 3, **p < 0.01 scopolamine-treated with NTF vs the scopolamine group on day 5. B NTF restored scopolamine-induced decrease in retention measured as time spent in the target quadrant. *p < 0.05 scopolamine vs NTF, **p < 0.01 scopolamine vs scopolamine with NTF treated group.

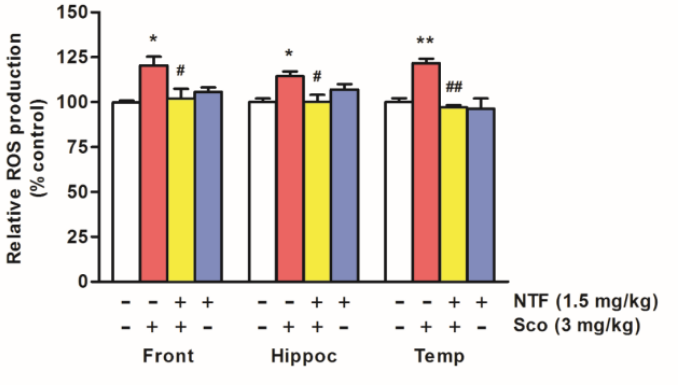

Fig 2. Effects of NTF treatment in scopolamine-treated rats on ROS. Production of ROS in brain homogenates were measured by fluorometric agent DCFH-DA. Each data bar (n=3) is expressed as mean ± SEM. *p < 0.05, **p < 0.01 vs control; #p < 0.05, ##p < 0.01 vs scopolamine-treated group.

Levels of cholinergic, apoptosis, and inflammatory proteins in brain tissues of rats administered scopolamine with and without NTF

Administration of scopolamine to rats significantly decreases ChAT and increases BACE1 levels in the 3 brain regions compared to control tissues (Fig 3A-C), and NTF cotreatment was able to ameliorate these alterations. However, NTF treatment alone had no effect on basal levels of the two proteins.

Exposure to scopolamine causes a significant increase in apoptotic proteins, Bax and activated caspase-3, and a corresponding decrease in anti-apoptotic Bcl-2 in the three types of brain tissues compared to controls with NTF treatment reversing these changes (Fig 4A-C). However, these relative changes in apoptosis-related proteins levels in the brain tissues of rats treated with NTF alone varied with tissue types and thus impacting the ability of NTF to restore the normal levels of apoptosis-related proteins in the scopolamine-treated animals.

Scopolamine significantly increased levels of cytokines – interleukin (IL)-6 and IL-1β – and of activated transcription factor – phosphorylated NF-κB – which were reversed by NTF. The extent of these phenomena relative to control levels also depended on brain tissue types (Fig 5A-C).

Activated cell signaling pathways in brain tissues of rats administered scopolamine with and without NTF

We investigated the activation indicated by the proportion of phosphorylated proteins of JNK, p38, ERK1/2, and Akt. In the frontal cortex (Fig 6A), scopolamine increased p-JNK and p-p38, decreased p-ERK1/2, and increased p-Akt. NTF downregulated p-ERK1/2 but upregulated p-Akt without affecting p-JNK or p-p38. A combination of scopolamine and NTF could only restore p-p38 to control level. In the hippocampus (Fig 6B), scopolamine elevated levels of p-JNK and p-p38, depressed p-ERK1/2 and had no effect on p-Akt. NTF had no effect on levels of p-JNK, increased p-p38, decreased p-ERK1/2, and had no effect on p-Akt. A combination of scopolamine and NTF could only restore p-ERK1/2 to control level and had no effect on the p-Akt level. In the temporal cortex (Fig 6C), scopolamine raised levels p-JNK and p-p38, lowered p-ERK1/2, and had no effect on p-Akt. NTF had no effect on levels of p-JNK, p-p38, or p-ERK1/2 but decreased p-Akt. A combination of scopolamine and NTF restored levels of p-JNK, p-p38 and p-ERK1/2, and had no effect on p-Akt. Table 1 shows the quantitative protein values as the percentage of change in expression relative to control groups in Western blot analysis.

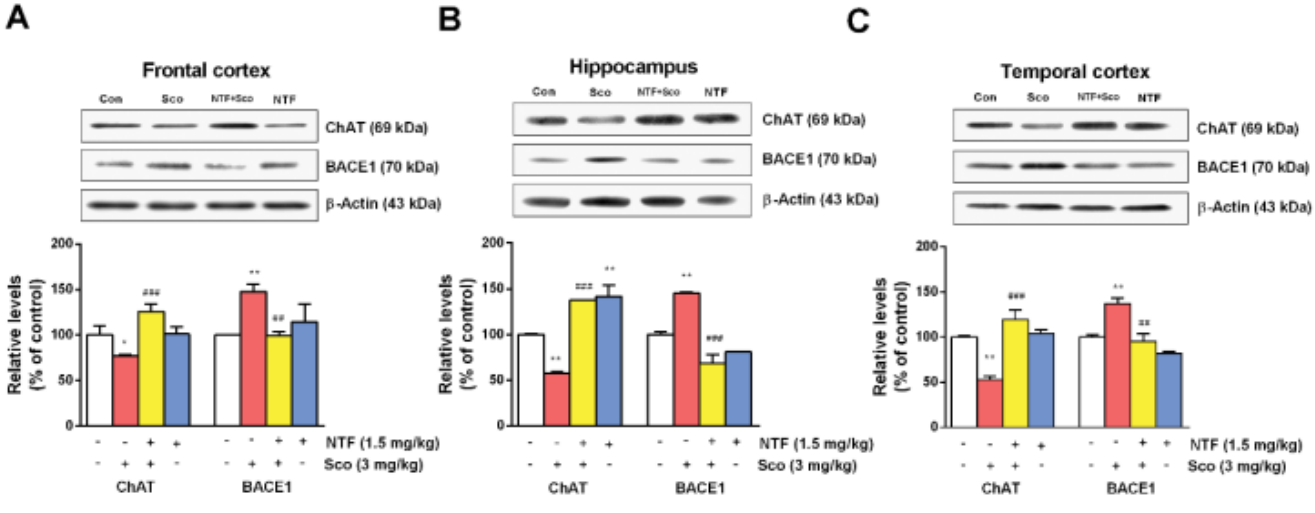

Fig 3. Effects of NTF on scopolamine-induced cholinergic dysfunction in rats on the levels of ChAT and BACE1 in: A frontal cortex, B hippocampus, and C temporal cortex. Each data bar (n=3) is expressed as mean ± SEM. *p < 0.05, **p < 0.01 vs control group; ##p < 0.01, ###p < 0.001 vs scopolamine-treated group

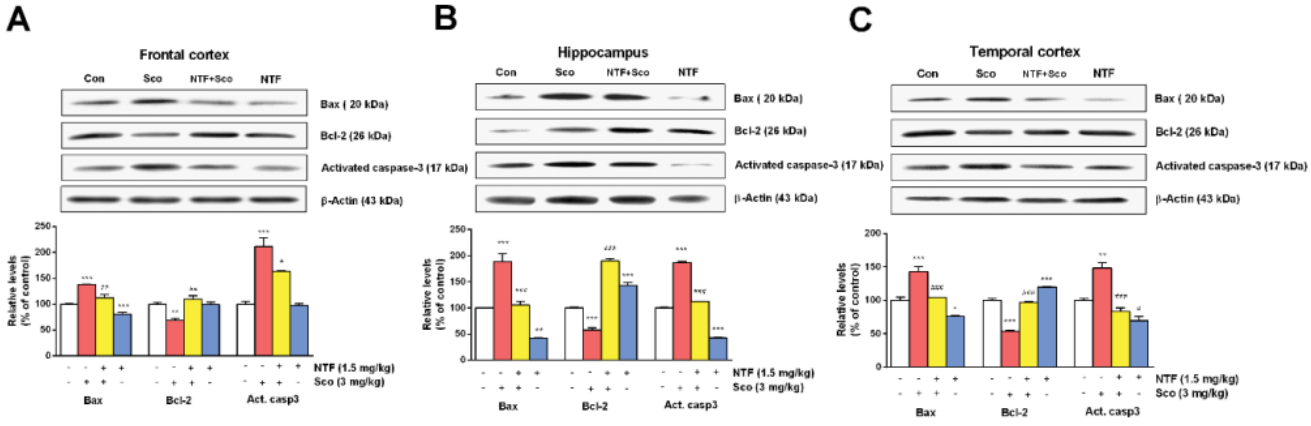

Fig 4. Effects of NTF on scopolamine-induced apoptosis represented by the levels of Bax, Bcl-2, and activated caspase-3 in: A frontal cortex, B hippocampus, and C temporal cortex. Each data bar (n=3) is expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 vs control group; #p < 0.05, ###p < 0.001 vs scopolamine-treated group.

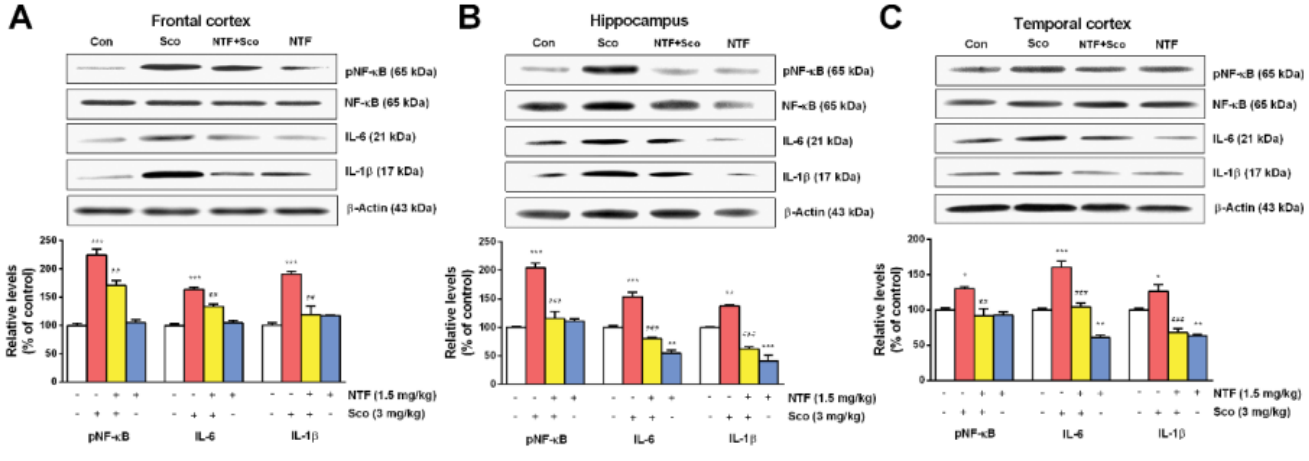

Fig 5. Effects of NTF on scopolamine-induced inflammatory responses indicated by the levels of p-NF-κB, IL-6, and IL-1β in: A frontal cortex, B hippocampus, and C temporal cortex. Each data bar (n=3) is expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 vs control group; #p < 0.05, ##p < 0.01, ###p < 0.001 vs scopolamine-treated group.

DISCUSSION

Scopolamine has been demonstrated to increase oxidative stress including ROS production in the mouse cortex and hippocampus, which could be reversed by pretreatment with sodium tanshinone IIA sulfonate, a radical-scavenging antioxidant.[26] The current study shows that administration of NTF was able to prevent scopolamine-augmented ROS production in the frontal and temporal cortices and hippocampus.

Oxidative stress contributes to the mechanisms of multiple neurodegenerative disorders including AD.[27] Both oxidative damage and cholinergic dysfunction have been implicated in cognitive impairment in rat dementia models. Treatment with antioxidant *Terminalia chebula* extract attenuated scopolamine-induced ROS generation and reduction in ChAT and ACh levels in mouse hippocampal tissue.[5] BACE1 is an enzyme necessary for the production of Aβ42 and Aβ40 peptides in AD.[28] Consistent with our finding, an antioxidant ipriflavone decreased BACE1 expression in the hippocampus of a scopolamine-treated rat.[29] Furthermore, NTF treatment alone significantly decreased apoptosis through downregulation of activated caspase-3 and Bax and upregulation of Bcl-2 in the hippocampus. Similarly, treatment with an antioxidant sodium tanshinone IIA sulfonate in a scopolamine-injected mouse significantly decreased Bax:Bcl-2 expression ratio and downregulated expression of activated caspase-3 in the hippocampus and cortex.[26]

Scopolamine also induces cytokines and NF-κB phosphorylation;[30] all of these changes were restored to normal levels in the rat cortex and hippocampus by NTF as described in the current study. Pretreatment

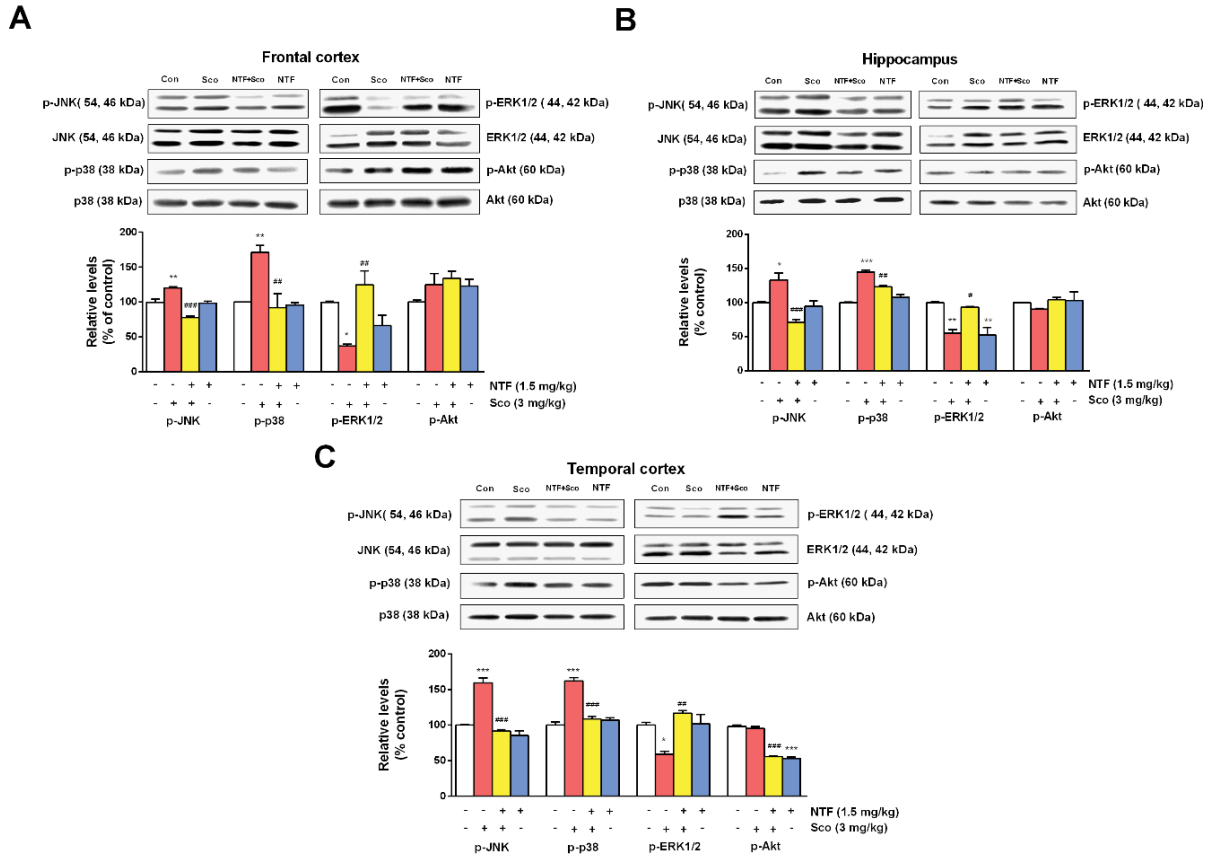

Fig 6. Effects of NTF on scopolamine-induced dysfunction in signaling pathways indicated by the levels of p-JNK, p-p38, p-ERK1/2 and p-Akt in: A frontal cortex, B hippocampus, and C temporal cortex. Each data bar (n=3) is expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 vs control group; ##p < 0.01, ###p < 0.001 vs scopolamine-treated group.

with a water-soluble ginseng oligosaccharide extract decreased scopolamine-induced expression of IL-1β and IL-6 mRNAs in the murine hippocampus and significantly prevented scopolamine from inducing spatial memory impairment during the water maze test.[31] In addition, antioxidant isoflavones from soybean-fermented food tempeh restored ACh level and suppressed scopolamine-induced IL-1β expression in the rat brains.[32]

The effects of scopolamine treatment on phosphorylation of the four signaling proteins (JNK, p38, ERK1/2, and Akt) were consistent across the three brain regions. Whether NTF could restore these changes could not be predicted based on the effects of NTF treatment alone in each brain tissue type. However, where scopolamine or NTF alone resulted in similar effects to a cell signaling protein, e.g. p-Akt (stimulated in the frontal cortex but unaffected in the temporal cortex and hippocampus), the results of their combination were as expected. In scopolamine-treated rats, NTF inhibited phosphorylation of JNK and p38 in these three brain regions. Previous studies have reported that JNK inhibitor SP600125 increased the number of surviving cells after ischemia in the rat hippocampus[33] and that SB202190 p38 inhibitor provided neuroprotection against cell apoptosis in mice.[34]

Scopolamine-decreased p-ERK1/2 levels in the three brain regions were accompanied by elevated BACE1 levels. Congruently, the expression and activity of BACE1 are negatively regulated by the ERK pathway.[35] Similar to our study, treatment of an antioxidant ipriflavone restored ERK1/2 phosphorylation and decreased BACE-1 expression and subsequent Aβ pathologies in a scopolamine-administered rat.[15] ERK1/2 inhibitor U0126 also blocked alleviating effects of amyrin, a triterpene with antioxidant activity, on scopolamine-induced memory deficits.[36]

CONCLUSION

This preliminary result demonstrates that NTF was able to attenuate scopolamine-induced impairment of the spatial cognitive performance, oxidative stress, inflammation, and apoptosis in the brain cortex and hippocampus, but its effects on cell signaling pathways in these brain tissues remained ambiguous. The utility of NTF as a potential preventive and therapeutic candidate for AD requires further experimentation. In a future study,

TABLE 1. Effects of NTF treatment on western blot protein expression in the frontal, hippocampus and temporal cortex in scopolamine-treated rats. Expression of target proteins is represented as the percentage of control. Results are expressed as mean ± SEM of three independent experiments.

	Frontal cortex			Hippocampus			Temporal cortex		
NTF (1.5 mg/kg)	-	+	+	-	+	+	-	+	+
Sco (3 mg/kg)	+	+	-	+	+	-	+	+	-
ChAT	76.9 ± 0.90*	125.9 ± 4.70###	101.1 ± 4.51	57.7 ± 1.41**	137.4 ± 0.73###	141.6 ± 12.38**	52.9 ± 3.78**	119.4 ± 10.98###	104.1 ± 4.11
BACE1	147.8 ± 4.45**	99.3 ± 2.43##	114.3 ± 11.36	145.0 ± 1.23**	68.6 ± 9.74###	80.7 ± 0.60	137.0 ± 6.40**	95.1 ± 8.43##	81.8 ± 2.13
Bax	137.7 ± 0.67***	112.33 ± 5.82##	81.1 ± 2.90***	188.5 ± 15.4***	105.6 ± 7.41###	42.3 ± 0.50**	143.0 ± 6.87***	104.2 ± 0.19###	76.2 ± 0.61*
Bcl-2	68.4 ± 4.11**	109.8 ± 6.23##	98.9 ± 5.14	58.2 ± 4.01***	189.7 ± 3.88###	143.5 ± 5.11***	54.0 ± 1.22***	96.6 ± 1.94###	119.8 ± 0.86***
Act. casp 3	211.2 ± 17.09***	163.5 ± 1.85#	97.2 ± 3.42	186.8 ± 2.32***	112.2 ± 0.92###	42.7 ± 0.83***	148.1 ± 8.3**	83.4 ± 5.29###	69.6 ± 5.92*
p-NFkB	224.8 ± 10.48***	170.2 ± 9.14##	105.3 ± 4.77	204.2 ± 7.93***	115.1 ± 12.8###	110.9 ± 3.90	130.1 ± 2.59*	91.1 ± 10.58##	92.2 ± 5.04
IL-6	163.5 ± 4.31***	133.7 ± 3.71##	104.8 ± 4.28	153.6 ± 7.63***	80.1 ± 2.50###	53.9 ± 6.29**	160.2 ± 8.74***	103.7 ± 6.28###	60.6 ± 4.00**
IL-1β	190.5 ± 5.52***	118.7 ± 15.4##	117.2 ± 0.79	137.5 ± 2.36**	62.2 ± 3.73###	40.7 ± 10.02***	126.2 ± 9.26*	67.6 ± 5.94###	63.4 ± 1.57**
p-JNK	120.2 ± 3.86**	77.8 ± 3.84###	98.5 ± 3.10	133.6 ± 9.39*	70.8 ± 4.67###	94.8 ± 7.94	159.7 ± 6.84***	91.3 ± 2.56###	85.6 ± 6.68
p38	171.2 ± 9.98**	91.9 ± 20.2##	96.1 ± 3.33	145.2 ± 2.44***	123.2 ± 2.26##	107.9 ± 4.02	162.0 ± 4.81***	109.0 ± 3.58###	107.1 ± 3.53
p-ERK	36.9 ± 2.68*	124.7 ± 20.27##	66.8 ± 14.29	55.1 ± 5.05**	93.4 ± 0.88#	52.3 ± 11.29**	58.8 ± 4.43*	116.9 ± 3.28##	101.7 ± 13.36
p-Akt	125.1 ± 16.05	134.0 ± 9.97	123.2 ± 9.56	90.3 ± 1.05	104.3 ± 3.20	103.2 ± 12.61	92.9 ± 4.52	55.5 ± 0.75###	54.4 ± 2.02***

*$p < 0.05$, **$p < 0.01$, *** $p < 0.001$ compared to control; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.01$ compared to scopolamine-treated group.

the effect of NTF on the altered activities of different cell signaling pathways and their mechanisms in rat brain tissues (frontal cortex, hippocampus, and temporal cortex) are needed to be investigated and the sample size should be expanded in order to acquire more conclusive results.

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Conflicts of Interest: All authors confirmed the absence of potential conflicts of interest as declared in the ICMJE form.

REFERENCES

1. Gold CA, Budson AE. Memory loss in Alzheimer's disease: implications for development of therapeutics. Expert Rev Neurother 2008;8:1879-91.
2. Querfurth HW, LaFerla FM. Alzheimer's disease. N Engl J Med 2010;362:329-44.
3. Schliebs R. Basal forebrain cholinergic dysfunction in Alzheimer's disease--interrelationship with β-amyloid, inflammation and neurotrophin signaling. Neurochem Res 2005;30:895-908.
4. Bajo R, Pusil S, López ME, Canuet L, Pereda E, Osipova D, et al. Scopolamine effects on functional brain connectivity: a pharmacological model of Alzheimer's disease. Sci Rep 2015;5: 9748.
5. Kim MS, Lee DY, Lee J, Kim HW, Sung SH, Han JS, Jeon WK. Terminalia chebula extract prevents scopolamine-induced amnesia via cholinergic modulation and anti-oxidative effects in mice. BMC Complement Altern Med 2018;18:136.
6. Tuchinda P, Pohmakotr M, Munyoo B, Reutrakul V, Santisuk T. An azaanthracene alkaloid from *Polyalthia suberosa*. Phytochemistry 2000;53:1079-82.
7. Thangnipon W, Suwanna N, Kitiyanant N, Soi-Ampornkul R, Tuchinda P, Munyoo B, et al. Protective role of N-trans-feruloyltyramine against β-amyloid peptide-induced neurotoxicity in rat cultured cortical neurons. Neurosci Lett 2012;513:229-32.
8. Thangnipon W, Puangmalai N, Chinchalongporn V, Jantrachotechatchawan C, Kitiyanant N, Soi-Ampornkul R, et al. N-benzylcinnamide protects rat cultured cortical neurons from β-amyloid peptide-induced neurotoxicity. Neurosci Lett 2013;556:20-25.
9. Thangnipon W, Suwanna N, Jantrachotechatchawan C, Ngampramuan S, Tuchinda P, Nobsathian S. Protective roles of N-benzylcinnamide on cortex and hippocampus of aged rat brains. Arch Pharm Res 2015;38:1380-8.
10. Chen W, Guo J, Guo H, Kong X, Bai J, Long P. Protective Effect of Vitamin C against Infancy Rat Corneal Injury Caused by Acute UVB Irradiation. Biomed Res Int 2020;2020: 8089273.
11. Noviana R, Ilmiawan MI, Handini M. Synergistic Protective Effect of Commercial Nigella Sativa Oil and Honey Combination against Cisplatin-induced Nephrotoxicity in Rats. Jurnal Biotek Medisiana Indonesia 2020;9:57-66.
12. Franzmeier N, Duering M, Weiner M, Dichgans M, Ewers M, Alzheimer's Disease Neuroimaging Initiative (ADNI). Left frontal cortex connectivity underlies cognitive reserve in prodromal Alzheimer's disease. Neurology 2017;88:1054-61.
13. Ballinger EC, Ananth M, Talmage DA, Role LW. Basal forebrain cholinergic circuits and signaling in cognition and cognitive decline. Neuron 2016;91:1199-218.
14. Alafuzoff I, Arzberger T, Al-Sarraj S, Bodi I, Bogdanovic N, Braak H, et al. Staging of neurofibrillary pathology in Alzheimer's disease: a study of the Brain Net Europe Consortium. Brain Pathol 2008;18:484-96.
15. Pluta R, Kocki J, Ulamek-Koziol M, Petniak A, Gil-Kulik P, Januszewski S, et al. Discrepancy in expression of β-secretase and amyloid-β protein precursor in Alzheimer-related genes in the rat medial temporal lobe cortex following transient global brain ischemia. J Alzheimer Dis 2016;51:1023-31.
16. DeKosky ST, Ikonomovic MD, Styren SD, Beckett L, Wisniewski S, Bennett DA, et al. Upregulation of choline acetyltransferase activity in hippocampus and frontal cortex of elderly subjects with mild cognitive impairment. Ann Neurol 2002;51:145-55.
17. Hirokawa S, Nose M, Ishige A, Amagaya S, Oyama T, Ogihara Y. Effect of Hachimi-jio-gan on scopolamine-induced memory impairment and on acetylcholine content in rat brain. J Ethnopharmacol 1996;50:77-84.
18. Piercey MF, Vogelsang GD, Franklin SR, Tang AH. Reversal of scopolamine-induced amnesia and alterations in energy metabolism by the nootropic piracetam: implications regarding identification of brain structures involved in consolidation of memory traces. Brain Res 1987;424:1-9.
19. Jang JY, Kim J, Shim J, Kim CY, Jang JH, Lee KW, Lee JH. Decaffeinated coffee prevents scopolamine-induced memory impairment in rats. Behav Brain Res 2013;245:113-9.
20. Rahimzadegan M, Soodi M. Comparison of Memory Impairment and Oxidative Stress Following Single or Repeated Doses Administration of Scopolamine in Rat Hippocampus. Basic and Clinical Neuroscience 2018;9:4-14.
21. Ali-Melkkila T, Kanto J, Iisalo E. Pharmacokinetics and related pharmacodynamics of anticholinergic drugs. Acta Anaesthesiol Scand 1993;37:633-42.
22. Ebert U, Oertel R, Wesnes KA, Kirch, W. Effects of physostigmine on scopolamine induced changes in quantitative electroencephalogram and cognitive performance. Hum Psychopharmacol 1998;13:199-210.
23. Semphuet T, Boongird A, Tantisira MH, Tiloksakulchai K, Tapechum S, Pakaprot N. The Neuroprotective effect of Bacopa monnieri against Pilocarpine-induced Status epilepticus in rats. Siriraj Med J 2017;69:345-50.
24. Sattayasai J, Chaonapan P, Arkaravichie T, Soi-Ampornkul R, Junnu S, Charoensilp P, et al. Protective effects of mangosteen extract on H2O2-induced cytotoxicity in SK-N-SH cells and scopolamine-induced memory impairment in mice. PLoS One 2013;8(12):e85053.
25. Katagiri T, Hatano N, Aihara M, Kawano H, Okamoto M, Liu Y, et al. Proteomic analysis of proteins expressing in regions of rat brain by a combination of SDS-PAGE with nano-liquid chromatography-quadrupole-time of flight tandem mass spectrometry. Proteome Sci 2010;8:41.
26. Xu QQ, Xu YJ, Yang C, Tang Y, Li L, Cai HB, et al. Sodium

Tanshinone IIA sulfonate attenuates scopolamine-induced cognitive dysfunctions via improving cholinergic system. Biomed Res Int 2016;2016:9852536.
27. Ancelin ML, Christen Y, Ritchie K. Is antioxidant therapy a viable alternative for mild cognitive impairment? Examination of the evidence. Dement Geriatr Cogn Disord 2007;24:1-19.
28. Ghosh AK, Osswald HL. BACE1 (β-secretase) inhibitors for the treatment of Alzheimer's disease. Chem Soc Rev 2014;43:6765-813.
29. Hafez HS, Ghareeb DA, Saleh SR, Abady MM, El Demellawy MA, Hussien H, Abdel-Monem N. Neuroprotective effect of ipriflavone against scopolamine-induced memory impairment in rats. Psychopharmacology (Berl) 2017;234:3037-53.
30. Liu T, Zhang L, Joo D, Sun SC. NF-κB signaling in inflammation. Signal Transduct Target Ther 2017;2:17023.
31. Xu T, Shen X, Yu H, Sun L, Lin W, Zhang C. Water-soluble ginseng oligosaccharides protect against scopolamine-induced cognitive impairment by functioning as an antineuroinflammatory agent. J Ginseng Res 2016;40:211-9.
32. Ahmad A, Ramasamy K, Jaafar SM, Majeed AB, Mani V. Total isoflavones from soybean and tempeh reversed scopolamine-induced amnesia, improved cholinergic activities and reduced neuroinflammation in brain. Food Chem Toxicol 2014;65:120-8.
33. Guan QH, Pei DS, Zhang QG, Hao ZB, Xu TL, Zhang GY. The neuroprotective action of SP600125, a new inhibitor of JNK, on transient brain ischemia/reperfusion-induced neuronal death in rat hippocampal CA1 via nuclear and non-nuclear pathways. Brain Res 2005;1035:51-59.
34. Hildesheim J, Awwad RT, Fornace AJ Jr. p38 Mitogen-activated protein kinase inhibitor protects the epidermis against the acute damaging effects of ultraviolet irradiation by blocking apoptosis and inflammatory responses. J Invest Dermatol 2004;122:497-502.
35. Tamagno E, Guglielmotto M, Giliberto L, Vitali A, Borghi R, Autelli R, et al. JNK and ERK1/2 pathways have a dual opposite effect on the expression of BACE1. Neurobiol Aging 2009;30:1563-73.
36. Park SJ, Ahn YJ, Oh SR, Lee Y, Kwon G, Woo H, et al. Amyrin attenuates scopolamine-induced cognitive impairment in mice. Biol Pharm Bull 2014;37:1207-13.