



The effect of nobiletin on hippocampal acetylcholinesterase activity and apoptosis in amyloid beta-induced rat model of Alzheimer's disease

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Abstract

Background and Objective: Alzheimer's disease (AD) is the most common neurodegenerative disease. Nobiletin could ameliorate acetylcholinesterase activity and apoptosis. The present study aimed to investigate the effects of nobiletin on acetylcholinesterase (AChE) activity and cell death in amyloid beta-induced model of AD in the rat.

Materials and Methods: 32 male Wistar rats were divided into four different groups as follows: sham, sham plus nobiletin, amyloid beta, and amyloid beta plus nobiletin. Rats were injected amyloid beta into the CA1 region of the hippocampus through stereotaxic surgery. Nobiletin was administered 10 mg/kg daily one hour after surgery for one week via gavage feeding. Then, apoptosis parameter (DNA fragmentation) and AChE activity were measured via separate kits in hippocampal homogenate.

Results: Our findings showed that amyloid beta group had significantly elevated level of AChE activity and DNA fragmentation as an apoptotic biomarker versus sham group ($p < 0.05$). However, treatment of amyloid beta-microinjected rats with nobiletin non-significantly lowered hippocampal AChE activity and DNA fragmentation.

Conclusion: According to the findings of this study, AChE activity and apoptosis are increased in amyloid beta-induced model of AD and with no significant ameliorating effect of nobiletin on these indices.

Keywords: Alzheimer's disease, Amyloid beta, Nobiletin, Acetylcholinesterase, Apoptosis

1. Introduction

AD is characterized by progressive dementia associated with production in the brain of histopathological hallmarks as insoluble extracellular plaques mainly consisting of amyloid beta ($A\beta$) and eventually forms senile plaques. These deposits are present mainly in the hippocampal and cortical regions and of intracellular neurofibrillary tangles (1, 2). AD results in the loss of neuron

function and causes synaptic damage, as well as inducing cholinergic neurotransmission dysfunction. This occurs from a reduction in acetylcholine (ACh) levels in the synapses, as well as from a decrease in the number of nicotinic and muscarinic receptors, It is well known that the cholinergic system can be negatively affected by the accumulation of β -amyloid ($A\beta$) peptides (3) because the three main known Alzheimer's disease risk factors, advancing age, female gender and APOE4, have been linked to a high apolipoprotein-E and accumulation of the

acetylcholine degrading enzyme, butyrylcholinesterase in cerebrospinal fluids of patients (4). Apoptosis is a selective cell deletion process which requires the involvement of a specific cell death program. Inappropriate apoptosis control is implicated in many human diseases including neurodegenerative disorders such as Alzheimer's disease (AD) (5). The hippocampal stereotaxic infusion of oligomeric A β in rats is a method involving the direct infusion of A β oligomeric species into the brain parenchyma. Usually, the infusion target is the CA1 subregion of the hippocampus (6), since this brain region is one of the areas most affected by neurodegeneration in AD; alternatively, this peptide can be infused into the neocortex (7), according to the experimental aim. The infusion of oligomeric A β into the brain of wild-type rat provides an excellent *in vivo* model which replicates the amyloidopathy and consequent neuronal cell death (8). Interactions between AB and surface neuronal cell receptors may induce ROS production and expression of caspases and proapoptotic genes like p53/p35 and determine an increase in mitochondrial membrane permeability. AB may also stimulate the extrinsic apoptotic pathway through its proinflammatory action, able to activate astrocytes and microglia and trigger the release of proinflammatory mediators such as TNF α (9).

Behavioral data obtained after an acute intracerebroventricular injection of A β 1-40 to mice also showed a significant cognitive decline, which was supported by a biochemical analysis showing an increase in AChE expression in the hippocampus, and a decrease in hippocampal and cortical ChAT activity and an increase in apoptosis (10).

Nobiletin is a bioactive polymethoxylated flavone (5,6,7,8,3',4'-hexamethoxyflavone), that has been isolated from the peel of citrus fruits. The beneficial effects of dietary flavonoids for health due to their bioactivities including antioxidant action, anti-inflammation, and neuroprotection (11). In a report, nobiletin attenuated memory decline in AD model rats by restoring β amyloid-impaired cAMP response element binding protein phosphorylation (12). Moreover, other studies have shown that nobiletin restored cognitive deficits in animal models of AD (13). Nowadays natural herbal supplements are considered more by the general public since the increasing incidence of degenerative diseases. As one of these agents, nobiletin have been biologically and medically emphasized and can be expected to be addressed as a topic for future studies, medical trends and pharmacology (14). The present study aimed to investigate the effects of nobiletin on acetylcholinesterase (AChE) activity and cell death in amyloid beta-induced model of AD in the rat.

2. Materials and Methods

2.1. Animals

32 healthy male Wistar rats (250-290 g) at about 10 weeks of age were allocated for the pharmacological screening in the present study. The animals were housed at temperature-controlled room, 12 h light/dark cycle. Rats were feed with standard diet and water.

2.2. Experimental Groups and Treatments

Rats were randomly divided to four different groups having 8 rats in each group as: sham, sham plus nobiletin, amyloid beta, and amyloid beta plus nobiletin. Rats were anesthetized by IP injection of a combination of ketamine and xylazine (100 and 5 mg/kg, respectively) and then the rats were operated by using stereotaxic apparatus (NARISHIGE, Japan). Paxinos and Watson stereotaxic atlas was used for the surgery, rats scalp washed by using iodine solution, incised on midline and a hole was drilled through the skull 0.8 mm post bregma, 1.4 mm lateral from midsagittal line and 3.4 mm below the dura. The amyloid-beta, and amyloid-beta plus nobiletin groups received bilateral ICV injection of amyloid beta (3 mg/kg body weight). Nobiletin was administered 10 mg/kg daily one hour after surgery for one week via gavage feeding.

2.3. Hippocampal Acetylcholinesterase Assessment

At the end of week 3 post-amyloid-beta injection, animals were decapitated, under overdose of diethyl ether and the brains were quickly removed. Hippocampal tissue (n=6-8 from each group) was separately dissected out and 10% homogenate was prepared in ice-cold normal saline containing 0.1% Triton X100 and protease inhibitor cocktail (containing AEBSF, aprotinin, bestatin, E-64, leupeptin and EDTA; SigmaAldrich, USA) and the obtained supernatant was aliquoted and stored at -70 °C for the following experiments. The acetylcholinesterase activity was determined on the basis of degradation of acetylthiocholine iodide into a product that binds to 5, 5'-dithiobis-2-nitrobenzoic acid and turns yellow (15). The kinetics of the reaction was followed spectrophotometrically over 5 min at 412 nm. Acetylcholinesterase activity was expressed as mM of substrate hydrolyzed/min/g protein.

2.4. Determination of DNA Fragmentation (Apoptosis)

In this experiment, the determination of histone-associated DNA fragments was performed using the Cell Death Detection ELISA kit (Roche Diagnostics, Germany) as an indicator of apoptosis according to the protocol from the company and the procedure as described before (16). The assay is based on a quantitative sandwich-enzyme-immunoassay principle

using mouse monoclonal antibodies directed against DNA and histones, respectively. This allows the specific determination of mono- and oligonucleosomes (histone-associated DNA fragments) in the fraction of tissue lysates. The amount of nucleosomes demonstrating DNA degradation was quantified by peroxidase (POD) retained in the immunocomplex. POD was determined photometrically at 405 nm with 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) as a substrate by microplate reader (BioTek, USA) after 15 min of substrate reaction time. Values were expressed as the optical density (OD).

2.5. Statistical analysis

Statistical analysis and graphical representation were performed using SigmaStat Software (version 3.5,

2006). Results were expressed as mean \pm standard error of the mean (SEM). Statistical analyses were performed using one-way analysis of variance (ANOVA) method and if a difference was found to be significant, followed by Tukey test with a p value <0.05 considered statistically significant.

3. Results

3.1. Hippocampal Acetylcholinesterase Activity

Fig. 1 shows acetylcholinesterase activity in hippocampal lysate. This activity was significantly greater in amyloid beta group compared with control group ($P<0.05$) and there were no significant changes between amyloid beta and nobiletin treated amyloid beta group.

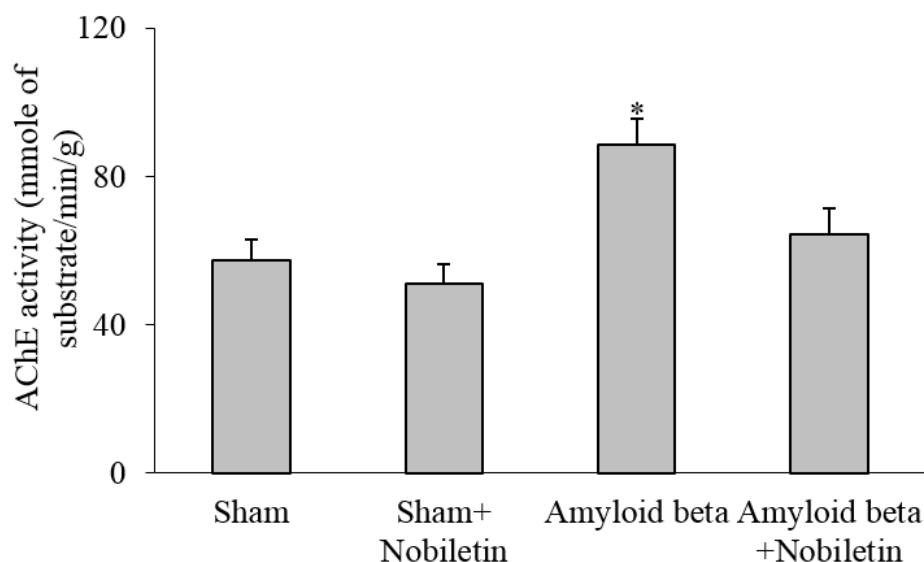


Figure 1. Animals were divided into four groups, one of which was designated as the sham group. The second was normal group which received nobiletin. The third group received amyloid-beta and was thus denoted the amyloid beta group. The last group, nobiletin-treated amyloid-beta group was injected first with amyloid-beta followed by administration nobiletin via gavage feeding (10 mg/kg daily one hour after surgery for one week). Animals brains (n = 8) in each group were obtained and homogenized as 10% homogenate in 0.1% Triton X100, then centrifuged, and supernatants were used in the AChE Assay kit. Statistical analyses of mean AChE activity were performed using one-way analysis of variance (ANOVA) followed by the Tukey test, whereby each value was presented as mean \pm standard error of the mean (SEM). For each group, * $p<0.05$ (as compared to control)

3.2. Hippocampal DNA Fragmentation (Apoptosis) Assessment

Fig. 2 shows assessment of DNA fragmentation as a valid index of apoptosis. DNA fragmentation was

significantly greater in amyloid beta group compared with control group ($P<0.05$) and there were no significant differences between amyloid beta and nobiletin treated amyloid beta group.

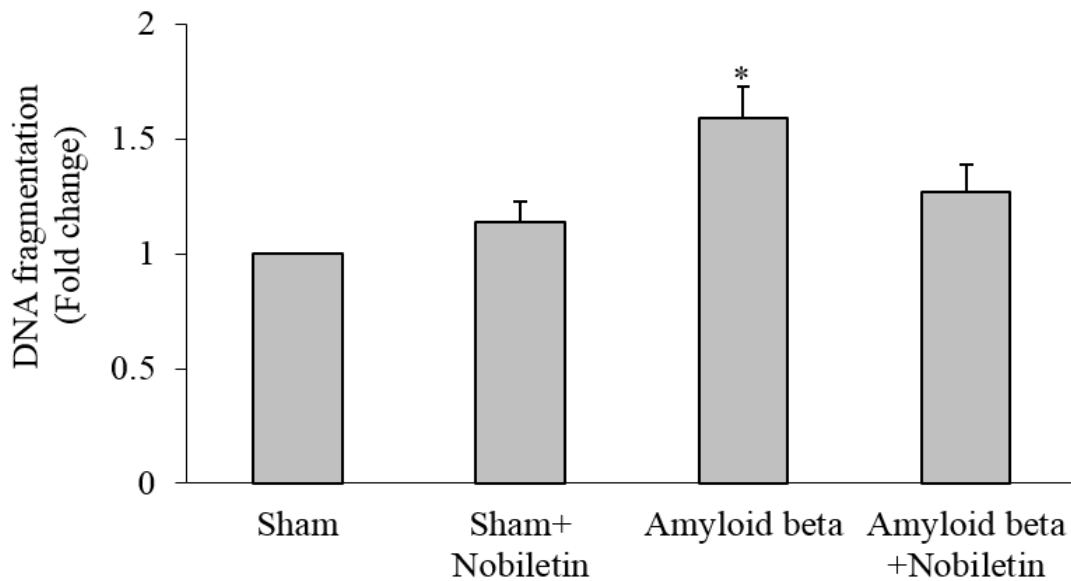


Figure 2. Animals were divided into four groups, one of which was designated as the sham group. The second was normal group which received nobiletin. The third group received amyloid-beta and was thus denoted the amyloid beta group. The last group, nobiletin-treated amyloid-beta group was injected first with amyloid-beta followed by administration nobiletin via gavage feeding (10 mg/kg daily one hour after surgery for one week). Animals brains (n = 8) in each group were obtained and homogenized as 10% homogenate in 0.1% Triton X100, then centrifuged, and supernatants were used in the DNA fragmentation Assay kit. Statistical analyses of mean DNA fragmentation were performed using one-way analysis of variance (ANOVA) followed by the Tukey test, whereby each value was presented as mean \pm standard error of the mean (SEM). For each group, * $p < 0.05$ (as compared to control).

4. Discussion

This present study was designed to evaluate the effect of nobiletin, a bioactive polymethoxylated flavone, that has been isolated from the peel of citrus fruits a major polyphenolic component of many plants and beverages, on amyloid beta induced model of Alzheimer's disease in rats (11). The hippocampal stereotaxic infusion of oligomeric A β in rats is a method involving the direct infusion of A β oligomeric species into the brain parenchyma (7). In this study we measured the levels of acetylcholinesterase enzyme in the hippocampal homogeneous tissue. The enzyme hydrolyzes the neurotransmitter acetylcholine in the synapse and is responsible for balancing the cholinergic system. The main function of this enzyme is the memory and the learning and organization of the cortical movements. Acetylcholin is an important neurotransmitter that facilitates learning and reduces in Alzheimer's disease (17). In a study conducted by Kissalari et al in 2017, acetylcholinesterase levels increased in Alzheimer's disease (18). In our study, beta-amyloid injection increased acetylcholinesterase in the beta-amyloid group compared with the sham group, which was consistent with the above study.

In the study of Nakajima and his colleagues in 2014, Nobiletin has been promoting a cholinergic system and reducing the density of acetylcholinesterase-containing strands (19). Orhan and colleagues in a 2007 study on several types of flavone reported only

one species called quercetin, which has a high levels of acetylcholine esterase inhibitor activity, and other flavonoids have shown this less (20). In our study, Nobiletin treatment reduced acetylcholinesterase, but this was not significant. In the study of Nakajima Nobiletin was administered 50 mg/kg orally t for 11 days, but in our study, were given 10 mg / kg and 7 days orally, for the reason The drug did not have a proper volume distribution. According to Orhan, who said that only one species of flavonoids had anti-acetylcholine esterase properties, and the rest did not show this. We conclude that our research was in line with Orhan.

Amyloid beta strands are very toxic to neurons and cause complete death of neurons within 24 hours of exposure to them. The mechanism of death in these cells seems to be due to apoptosis and is due to oxidative and inflammatory effects of amyloid beta (5). In a study conducted by Kamat et al in 2016, they survey the mechanism of oxidative stress and synaptic disorder in the pathogenesis of Alzheimer's disease, and found that Alzheimer's disease caused large amounts enter of calcium to the cell due to over-stimulation of NMDAR receptors. This excess Ca²⁺ + defects in mitochondria And cytochrome C disorder then activates caspase 9, and then starts caspase 3 and directs the cell to apoptosis (21) . To determine the apoptosis in Alzheimer's hippocampal cells, DNA fragmentation was used. In our study, In the beta-amyloid group, there was a significant increase in this

factor in comparison with the sham group, which was consistent with the study.

In 2015, Cho and colleagues explored the role of neuroprotective effect of nobiletin in preventing oxidative stress in hippocampal neuronal cells. It was concluded that Nobiletin prevents cell death from hydrogen peroxide through the MAPK and apoptosis pathways (22). Bi and colleagues examined the effects of nobiletin on isoflurane induced cognitive impairment and showed that nobiletin had antiapoptotic activity via activation of phosphorylated Akt activity, and a reduction in the level of Bax cells (23). In our study, reductions in DNA fragmentation was not significant. The reason for the difference with the study of Cho is that he has used cell culture to

study the effects of nobiletin, but in the living system certainly other factors are involved. In the study of Bi, nobiletin was administered 200 mg/kg orally for 11 days before surgery, but in our study, rats were given 10 mg/kg and 7 days orally after surgery. For this reason, the drug did not have a proper volume distribution. However, pretreatment groups are needed and effective concentration should be higher.

Conclusion

AChE activity and apoptosis are increased in amyloid beta-induced model of AD and with no significant ameliorating effect of nobiletin on these indices.

References

- Gulyaeva NV, Bobkova NV, Kolosova NG, Samokhin AN, Stepanichev MY, Stefanova NA. Molecular and Cellular Mechanisms of Sporadic Alzheimer's Disease: Studies on Rodent Models in vivo. *Biochemistry. Biokhimiia* 2017; 82(10):1088-102.
- Facchinetti R, Bronzuoli MR, Scuderi C. An Animal Model of Alzheimer Disease Based on the Intrahippocampal Injection of Amyloid beta-Peptide (1-42). *Methods in Molecular Biology* (Clifton, N.J.) 2018; 1727:343-52.
- Loizzo MR, Tundis R, Bonesi M, Menichini F, De Luca D, Colica C, et al. Evaluation of Citrus aurantifolia peel and leaves extracts for their chemical composition, antioxidant and anti-cholinesterase activities. *Journal of the Science of Food and Agriculture* 2012; 92(15):2960-7.
- Kumar R, Nordberg A, Darreh-Shori T. Amyloid-beta peptides act as allosteric modulators of cholinergic signalling through formation of soluble BAbetaACs. *Brain* 2016; 139(Pt 1):174-92.
- Obulesu M, Lakshmi MJ. Apoptosis in Alzheimer's disease: an understanding of the physiology, pathology and therapeutic avenues. *Neurochemical Research* 2014; 39(12):2301-12.
- Jean YY, Baleriola J, Fa M, Hengst U, Troy CM 48 Stereotaxic Infusion of Oligomeric Amyloid-beta into the Mouse Hippocampus. *Journal of Visualized Experiments: Journal of Visualize Experiments* 2015(100):e52805.
- Bolmont T, Clavaguera F, Meyer-Luehmann M, Herzog MC, Radde R, Staufenbiel M, et al. Induction of tau pathology by intracerebral infusion of amyloid-beta -containing brain extract and by amyloid-beta deposition in APP x Tau transgenic mice. *The American Journal of Pathology* 2007; 171(6):2012-20.
- Sotthibundhu A, Sykes AM, Fox B, Underwood CK, Thangnipon W, Coulson EJ. Beta-amyloid (1-42) induces neuronal death through the p75 neurotrophin receptor. *The Journal of Neuroscience* 2008; 28(15):3941-6.
- Davydov V, Hansen LA, Shackelford DA. Is DNA repair compromised in Alzheimer's disease? *Neurobiology of Aging* 2003; 24(7):953-68.
- Schmid S, Jungwirth B, Gehlert V, Blobner M, Schneider G, Kratzer S, et al. Intracerebroventricular injection of beta-amyloid in mice is associated with long-term cognitive impairment in the modified hole-board test. *Behavioural Brain Research* 2017; 324:15-20.
- Braidy N, Behzad S, Habtemariam S, Ahmed T, Daglia M, Nabavi SM, et al. Neuroprotective Effects of Citrus Fruit-Derived Flavonoids, Nobiletin and Tangeretin in Alzheimer's and Parkinson's Disease. *CNS & Neurological Disorders Drug Targets* 2017; 16(4):387-97.
- Matsuzaki K, Yamakuni T, Hashimoto M, Haque AM, Shido O, Mimaki Y, et al. Nobiletin restoring beta-amyloid-impaired CREB phosphorylation rescues memory deterioration in Alzheimer's disease model rats. *Neuroscience Letters* 2006; 400(3):230-4.
- Nakajima A, Aoyama Y, Shin EJ, Nam Y, Kim HC, Nagai T, et al. Nobiletin, a citrus flavonoid, improves cognitive impairment and reduces soluble A β levels in a triple transgenic mouse model of Alzheimer's disease (3XTg-AD). *Behavioural Brain Research* 2015; 289:69-77.
- Moosavi F, Hosseini R, Saso L, Firuzi O. Modulation of neurotrophic signaling pathways

- by polyphenols. *Drug Design, Development and Therapy* 2016; 10:23-42.
15. Ellman GL, Courtney KD, Andres V, Jr., Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology* 1961; 7:88-95.
 16. Afshin-Majd S, Khalili M, Roghani M, Mehranmehr N, Baluchnejadmojarad T. Carnosine exerts neuroprotective effect against 6-hydroxydopamine toxicity in hemiparkinsonian rat. *Molecular Neurobiology* 2015; 51(3):1064-70.
 17. Tan JL, Li QX, Ciccotosto GD, Crouch PJ, Culvenor JG, White AR, et al. Mild oxidative stress induces redistribution of BACE1 in non-apoptotic conditions and promotes the amyloidogenic processing of Alzheimer's disease amyloid precursor protein. *PloS one* 2013; 8(4):e61246.
 18. Kiasalari Z, Heydarifard R, Khalili M, Afshin-Majd S, Baluchnejadmojarad T, Zahedi E, et al. Ellagic acid ameliorates learning and memory deficits in a rat model of Alzheimer's disease: an exploration of underlying mechanisms. *Psychopharmacology* 2017; 234(12):1841-52.
 19. Nakajima A, Yamakuni T, Haraguchi M, Omae N, Song SY, Kato C, et al. Nobiletin, a citrus flavonoid that improves memory impairment, rescues bullectomy-induced cholinergic neurodegeneration in mice. *Journal of Pharmacological Sciences* 2007; 105(1):122-6.
 20. Orhan I, Kartal M, Tosun F, Sener B. Screening of various phenolic acids and flavonoid derivatives for their anticholinesterase potential. *Zeitschrift fur Naturforschung. C, Journal of Biosciences* 2007; 62(11-12):829-32.
 21. Kamat PK, Kalani A, Rai S, Swarnkar S, Tota S, Nath C, et al. Mechanism of Oxidative Stress and Synapse Dysfunction in the Pathogenesis of Alzheimer's disease: Understanding the Therapeutics Strategies. *Molecular Neurobiology* 2016; 53(1):648-61.
 22. Cho HW, Jung SY, Lee GH, Cho JH, Choi IY. Neuroprotective effect of Citrus unshiu immature peel and nobiletin inhibiting hydrogen peroxide-induced oxidative stress in HT22 murine hippocampal neuronal cells. *Pharmacognosy Magazine* 2015; 11(Suppl 2):S284-9.
 23. Bi J, Zhang H, Lu J, Lei W. Nobiletin ameliorates isoflurane-induced cognitive impairment via antioxidant, anti-inflammatory and anti-apoptotic effects in aging rats. *Molecular Medicine Reports* 2016; 14(6):5408-14.