

## Nasal Administration of Quercetin Liposomes Modulate Cognitive Impairment and Inhibit Acetylcholinesterase Activity in Hippocampus

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**Abstract: Problem statement:** Oxidative stress is a major factor implicated in the degeneration of cholinergic neurons in Alzheimer's Disease (AD). Several reports have indicated that antioxidant intake is beneficial to delay or inhibit the progression of this disease. Presently, Acetylcholinesterase (AChE) inhibitors are the mainstay of therapy for AD. Quercetin, one of the flavonoids in fruits and vegetables, has a powerful antioxidant activity both *in vitro* and *in vivo*. However, the potential of quercetin as Acetylcholinesterase (AChE) inhibitors, an important aspect for neuroprotection, has not been properly investigated. **Approach:** This study was designed to evaluate the anti-cholinesterase activity and improves cognitive function of quercetin liposomes via nasal administration in a rat AF64A injection model of AD. Male Wistar rats were pretreated with quercetin liposomes, containing 0.5 mg of quercetin in 20  $\mu$ L via intranasal route once daily continually for 3 weeks. Learning and memory was evaluated using the Morris water maze test at 7 days after the lesioning and then the rats were sacrificed for determining the contents of Acetylcholinesterase (AChE), the activities of Superoxide Dismutase (SOD), Glutathione Peroxidase (GPx) and Malondialdehyde (MDA), a lipid peroxidation product in the hippocampus. **Results:** AF64A with nasal administration of free liposomes showed the loss of cognitive performance in Morris water maze test, increase in the markers of oxidative damage (MDA, SOD and GPX) and the AChE activity in the hippocampus. However, AF64A with nasal administration of quercetin liposomes reversed all the parameters significantly. **Conclusion:** Our studies demonstrated that quercetin liposomes via nasal administration may have a therapeutic importance in the clinical management of Alzheimer's disease.

**Key words:** Oxidative stress, Alzheimer's disease, Acetylcholinesterase (AChE) inhibitors, nasal administration, quercetin liposomes

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### INTRODUCTION

Nowadays, Alzheimer's Disease (AD) is becoming a greater medical and social problem. Acetylcholinesterase (AChE) inhibitors are used in the treatment of AD. Currently, the only effective treatment for AD targets the cholinergic system using anti-cholinesterase compounds (Lleo *et al.*, 2006). However, many of the existing drugs have adverse side effects such as bioavailability problems and gastrointestinal disturbance (Schulz, 2003; Small *et al.*, 1997). There is therefore a need to develop novel therapeutic approaches to treat this disease.

Currently, numerous studies have suggested that free radicals have been recognized to be one important factor contributing a crucial role on the pathogenesis of

AD (Ishige *et al.*, 2001). Interestingly, intake of polyphenols through diets rich in fruits, vegetables and beverages such as red wine was stated to reduce incidence of certain age related neurological disorders including macular degeneration and dementia (Bastianetto and Quirion, 2002; Commenges *et al.*, 2000). Therefore, these data suggest that high dietary or supplemental consumption of antioxidants in people may reduce the risk of AD. Thus, the market of herbal extracts possessing high antioxidant activity and improve memory continues to increase.

Quercetin, a member of the flavonoids family, is one of the most prominent dietary antioxidants. It is ubiquitously present in foods including vegetables, fruit, tea and wine as well as countless food supplements and is claimed to improve learning and

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memory ability (Lu *et al.*, 2006). However, poor absorption, rapid metabolism and limited ability to cross the Blood-Brain-Barrier (BBB) are obstacles to its use for treatment of neurological disorders (Manach *et al.*, 2004).

Liposomes have been used as an effective delivery system to the brain, because the particles entrap the compounds and prevent rapid degradation elimination or elimination as well as promote penetration through the BBB and distribution in the brain tissue (Krauze, 2006). Being entrapped in liposomes, a decrease in the dose of a compound to be administered is usually expected (Keller, 2001).

Nasal administration has been proposed as a non-invasive method to deliver bioactive substances to the brain. This route of administration has the potential of decreased hepatic metabolism and greater access to the brain tissue through the olfactory nerve compared to the oral route (Wang *et al.*, 2006) and could be a potential method of delivering quercetin to the Central Nervous System (CNS). Therefore, this provides more opportunity for quercetin to enter the CNS and then act on CNS to improve learning and memory with highly efficiency. However, the potential of quercetin as AChE inhibitors, an important aspect for neuroprotection, has not been properly investigated. Thus, the present study was undertaken to evaluate the effects of nasally administered quercetin liposomes on cognition and biochemical alterations, in the rat AF64A injection model of AD.

## MATERIALS AND METHODS

**Reagents:** High-purity egg L- $\alpha$ -phosphatidylcholine, type XVI-E (EPC), cholesterol (chol), quercetin dihydrate (98% HPLC purity) and polyethylene glycol 400 (PEG) were purchased from Sigma (Barcelona, Spain). Other reagents used were analytical grade such as chloroform, ethanol and methanol (HPLC and analytical grade) from BDH Laboratory Supplies (Poole, England), disodium hydrogen phosphate, perchloric acid and ortho phosphoric acids (Merck, Darmstadt, Germany), sodium dihydrogen phosphate (JT Baker Inc., Phillipsburg, New Jersey). All other chemicals were at least reagent grade and used as received.

**Preparation of quercetin liposomes:** Quercetin dehydrate (98%), high-purity egg L- $\alpha$ -phosphatidylcholine, Type XVI-E (EPC) and Cholesterol (CHOL) were prepared as quercetin liposomes. The method used was lipid thin film

formation and extrusion (Guo *et al.*, 2003; Liang *et al.*, 2005; Zu *et al.*, 2006).

**Animals:** Preparation, grouping and administration of liposomes: Adult male Wistar rats (n = 40, weighing 180-220 g, 8 weeks old) were obtained from the National Animal Center, Nakorn Pathom, Thailand. The rats were kept under standard laboratory conditions with food and water *ad libitum* and housed in standard metal cages (six per cage), temperature controlled at 20-22°C and relative humidity at 65%, on 12:12 h light-dark cycle. After acclimatization to the laboratory with free access to food and water for 2 weeks, the animals were divided into 5 groups of 8 each as following:

- Free liposomes + ACSF: The rats in this group were administered free liposomes via nasal route then they were administered artificial cerebrospinal fluid or ACSF bilaterally via intracerebroventricular route
- PEG + ACSF: The rats in this group were administered PEG which used as vehicle of quercetin liposomes via nasal route and subjected to ACSF administration
- Free liposomes + AF64A: All rats were administered free liposomes via nasal route then, they were administered AF64A bilaterally via intracerebroventricular route
- PEG + AF64A: The animals were administered PEG via nasal route then they were administered AF64A as mentioned in group 3
- QCL + AF64A: The rats in this group were administered quercetin liposomes via nasal route, then they were administered AF64A

All animals were administered freshly prepared quercetin liposomes, containing 0.5 mg of quercetin in 20  $\mu$ L (dose = 20  $\mu$ g), or liposomes without quercetin at the same volume via right nasal cavity with a micropipette at a period of 2 weeks before and 1 week after AF64A or Artificial Cerebrospinal Fluid (ACSF) administration.

The present experiment was conducted with attempts to minimize animal suffering in accordance with the legislation on the internationally accepted principles for the use and care of laboratory animals of the European Community (EEC Directive of 1986; 86/609/EEC) and approved by the respective university committee of animal experiments.

**AF64A administration:** After being anesthetized, the animals were bilaterally administered (2 nmol<sup>-2</sup>  $\mu$ L) AF64A which previously described by Fisher *et al.*

(1982) or ACSF via intracerebroventricular route according to the following co-ordinates: (from the bregma): Posterior 0.8 mm, lateral  $\pm 1.5$  mm and ventral (from dura) 3.6 mm.

**Assessment of cognitive function:** Learning and memory was evaluated using the Morris water maze test at 7 days after AF64A administration. The experimental apparatus consisted of a circular water tank (170 cm in diameter, 58 cm in height), containing water ( $25 \pm 1^\circ\text{C}$ ) to a depth of 40 cm, which was rendered opaque by adding talc. A platform (10 cm in diameter) was submerged 1 cm below the water surface and placed at the midpoint of one of the quadrants. The time for animals to climb on the hidden platform was recorded as escape latency or acquisition time. The next day, the rats were exposed to same test, except that the platform was removed and the retention time (time that the animal spent to swim around the previous location of platform) was recorded.

The results were expressed as mean  $\pm$  SEM and possible significant differences determined using one way ANOVA and Duncan's post hoc test.  $p < 0.05$  was considered significant. Any enhancement of cognition would be reflected by a decrease in escape latency and increase in retention time.

**Biochemical assays:**

**Acetylcholinesterase (AChE) activity:** Hippocampal homogenates were prepared in 0.1 M phosphate buffer solution (pH 8) and AChE activity determined as described by Robertson *et al.* (1988).

**Superoxide Dismutase (SOD), Glutathione Peroxidase (GPx) activities and Malondialdehyde (MDA) level:** Hippocampal homogenate was prepared in 50 mM phosphate buffer solution (pH 7) and determined SOD and GPx activities according to the methods of McCord and Fridovich (1969) and Ellman (1959) whereas were determined using Ohkawa *et al.* (1979). Protein content was measured by Lowry *et al.* (1951).

Results were expressed as mean  $\pm$  SEM and determined statistical significance using ANOVA, followed by Duncan's test. The significance was regarded at  $p$ -value  $< 0.05$ .

**RESULTS**

**Effect of quercetin liposomes on spatial learning and memory:** No significant change in acquisition and retention time in control, vehicle (PEG + ACSF) and free liposomes + ACSF group rats whereas rats given liposomes + AF64A and PEG + AF64A showed a significant increased in acquisition and decreased in

retention time in the Morris water maze test. Surprisingly, Rats that received AF64A and nasal administration of quercetin liposomes showed significantly decreased acquisition and increased retention time ( $p$ -value  $< 0.001$  all) as shown in Fig. 1 and 2 respectively, compared to both liposomes + AF64A and PEG + AF64A. This indicates that nasal administration of quercetin liposomes improves memory deficit induced by AF64A.

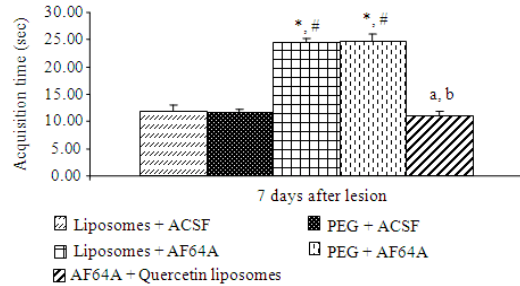


Fig. 1: Effect nasal administration of quercetin liposomes on the acquisition time of spatial learning in the Morris water maze test (N = 8). Results were expressed as mean  $\pm$  SEM. \*:  $p$ -value  $< 0.001$  compared with liposomes + ACSF treated group, #:  $p$ -value  $< 0.001$  compared with PEG+ACSF treated group, <sup>a</sup>:  $P$ -value  $< 0.001$  compared with liposomes + AF64A treated group, <sup>b</sup>:  $p$ -value  $< 0.001$  compared with PEG + AF64A treated group

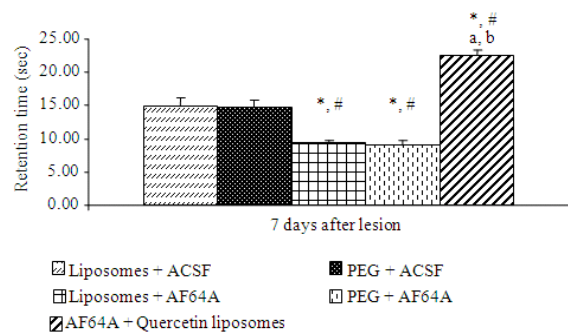


Fig. 2: Effect nasal administration of quercetin liposomes on the retention time of spatial learning in the Morris water maze test (N = 8). Results are expressed as mean  $\pm$  SEM. \*:  $p$ -value  $< 0.001$  compared with liposomes+ACSF treated group, #:  $p$ -value  $< 0.001$  compared with PEG+ACSF treated group, <sup>a</sup>:  $p$ -value  $< 0.001$  compared with liposomes + AF64A treated group, <sup>b</sup>:  $p$ -value  $< 0.001$  compared with PEG + AF64A treated group

Table 1: Effect nasal administration of quercetin liposomes on the activities of SOD, GPx and the levels of lipid peroxidation in animal model of AD induced by AF64A

Experimental conditions	SOD (U mg <sup>-1</sup> protein)	GPx (U mg <sup>-1</sup> protein)	MDA (U mg <sup>-1</sup> protein)
Liposomes + ACSF	16.80±0.13	14.44±0.12	1.45±0.11
PEG + ACSF	16.41±1.27	14.41±0.11	1.48±0.17
Liposomes + AF64A	11.20±0.17 <sup>#</sup>	11.20±0.47 <sup>#</sup>	2.48±0.27 <sup>#</sup>
PEG + AF64A	12.24±1.90 <sup>#</sup>	11.87±0.22 <sup>#</sup>	2.49±0.26 <sup>#</sup>
AF64A + Quercetin liposomes	22.50±0.12 <sup>*,a,b</sup>	18.31±0.33 <sup>*,a,b</sup>	1.19±0.13 <sup>*,a,b</sup>

Results are expressed as mean ± SEM <sup>\*</sup>: p-value<0.001 compared with liposomes + ACSF treated group, <sup>#</sup>: p-value<0.001 compared with PEG + ACSF treated group, <sup>a</sup>: p-value<0.001 compared with liposomes + AF64A treated group, <sup>b</sup>: p-value<0.001 compared with PEG + AF64A treated group

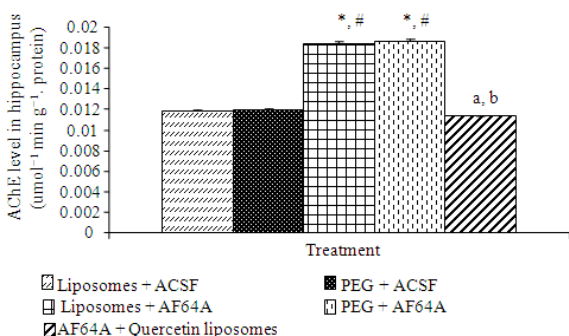


Fig. 3: Effect nasal administration of quercetin liposomes on the Acetylcholinesterase (AChE) level in the hippocampus (N = 8). Results are expressed as mean ± SEM <sup>\*</sup>: p-value<0.001 compared with liposomes + ACSF treated group, <sup>#</sup>: p-value<0.001 compared with PEG + ACSF treated group, <sup>a</sup>: p-value<0.001 compared with liposomes + AF64A treated group, <sup>b</sup>: p-value<0.001 compared with PEG + AF64A treated group

**Effect of quercetin liposomes on AChE activity:** The administration of AF64A in either free liposomes or PEG treated groups significantly increased the AChE activity in hippocampal homogenates, compared to both liposomes + ACSF and PEG + ACSF whereas quercetin liposomes administration could reverse these changes (p-value<0.001 all; compared to liposomes + AF64A and PEG + AF64A) as shown in Fig. 3.

**Effect of quercetin liposomes on MDA level, SOD and GPx activities:** In order to determine the possible underlying mechanism of quercetin liposomes, we had determined the effect of quercetin liposomes on oxidative stress indices including the level of Malondialdehyde (MDA), a product of lipid peroxidation product and the activities of scavenger enzymes including SOD and GPx in hippocampus as shown in Table 1. Our result showed that both liposomes + AF64A and PEG + AF64A groups

generated oxidative stress in rat brain, by increasing the lipid peroxidation, while decreasing the activities of SOD and GPx. But treatment with quercetin liposomes exerted complete protection to these enzymes and decreased the lipid peroxidation in rat brain changes (p-value<0.001 all; compared to liposomes + AF64A and PEG + AF64A).

## DISCUSSION

In this study, we investigate the neuroprotective effect of quercetin liposomes via nasal administration on cognitive dysfunctions and biochemical alterations in rats, has shown that quercetin liposomes significantly reversed the cognitive deficits and biochemical alterations seen in hippocampus induced by AF64A.

Neurochemical studies reported that one of the most prominent neurochemical changes in AD brain was a reduced concentration of Acetylcholine (ACh) in the hippocampus and neocortex, caused by degeneration of cholinergic neurons (Perry *et al.*, 1999). Therefore, inhibition of Acetylcholinesterase (AChE), the enzyme responsible for hydrolysis of ACh at the cholinergic synapse, is currently the most established approach to treating AD (Schneider, 1996).

On the other hand, oxidative stress, caused by Reactive Oxygen Species (ROS), is known to cause the oxidation of biomolecules leading to cellular damage. It was reported that oxidative stress is associated with the pathogenesis of AD and cellular characteristics of this disease are either causes or effects of oxidative stress (Mattson, 2004). Thus, many studies focused on the beneficial effects of supplement possessing a capability to improve cholinergic function and antioxidant activity were also considered to be a potential candidate for neuroprotective agent against AD.

Quercetin, a safe and dietary flavonoid, is found in onions, apples and other fruits and vegetables. It has been reported that quercetin prevents oxidant injury and cell death by several mechanisms including scavenging oxygen radicals, protecting against lipid peroxidation and chelating metal ions (Ishige *et al.*, 2001). However,

it was reported that quercetin was easily metabolized after absorption (Manach *et al.*, 2004). Our study found it worthwhile to use the vesicle mediated carrier system particularly liposomes to encapsulated the quercetin against Alzheimer's condition induced by AF64A as well as oxidative stress.

In order to mimic the cholinergic deficit and neurodegeneration in AD, a selective cholinotoxin or ethylcholine aziridinium (AF64A) had been applied as the tool to induce the neurodegeneration of the cholinergic system and resulting in memory deficit (Hanin, 1996). In addition, AF64A could increase oxidative stress in hippocampus, the areas contribution important role on learning leading to the memory impairment (Chrobak *et al.*, 1988) as those observed in AD. Based on the correspondence changes of behavior and neuropathology induced by AF64A and AD, this study used AF64A to develop animal model of AD.

In the present study, the results showed that both liposomes + AF64A and PEG + AF64A treated rats there was impairment of learning and memory as evidenced by significantly increased acquisition and decreased retention time in Morris water maze test, produced the oxidative stress and induced the level of AChE activity in the hippocampus. These results are in conformity with other workers who have demonstrated cognitive impairment after AF64A injection in rats (Chonpathompikunlert *et al.*, 2010). However, the rats was treated with quercetin liposomes via nasal route showed significantly decreased acquisition and increased retention time in Morris water maze test as compared to the liposomes + AF64A and PEG + AF64A treated animals, suggested its cognitive enhancing effect.

Extensive evidence exists for lipid peroxidation being an important mechanism of neurodegeneration in the AD (Liu *et al.*, 2003). Prophylactic treatment with nasal administration of quercetin liposomes significantly reversed the impact of oxidative alterations (MDA, SOD and GPx) and the elevation in AChE activity seen in Alzheimer's condition induced by AF64A. This suggests that the cognitive enhancing effect of quercetin liposomes might occur partly via the antioxidant property and the inhibition of AChE in hippocampus.

The mechanism how quercetin liposomes could decrease lipid peroxidation and improve its antioxidant systems against Alzheimer's condition induced by AF64A is not known, but it may be presumed that after entering the brain, the quercetin or its derivative might increase the activities of scavenger enzymes mentioned earlier and resulted in the decrease excess free radicals, which in turn decreased the lipid peroxidation process

leading to the reduction of MDA as shown in this study. These results are in accordance with the report of quercetin liposomes effect to decrease lipid peroxidation and increase scavenger enzymes activities in rat brain by the induction of cerebral ischemia and reperfusion (Sarkar and Das, 2006). However, the method to prepare the quercetin liposomes and the model to induce the oxidative damage were different. Moreover, quercetin liposomes acts as AChE inhibitors, this effect may be owed to its complex components, especially flavonoids. By the structural requirements of flavonoids as inhibitors of AChE *in vitro* and indicated that substitutions of OH at meta positions (positions 5 and 7) are beneficial to inhibiting AChE in a theoretical evaluation (Ji and Zhang, 2006).

Our results demonstrated that nasal administration of quercetin liposomes was a potential novel strategy to protect against AD. It could produce beneficial effect with very much low dose. Based on the previous findings, the decrease effective dose might be associated with many factors including the increase opportunity to transfer directly from the olfactory mucosa along the olfactory pathway to the CNS (Cho *et al.*, 2006) via bypassing BBB, which prevented some CNS-active drugs from reaching the brain (Behl *et al.*, 1998). In addition, it could also decrease the influence of the first pass metabolism (Krauze, 2006). However, the main proposed pathway of quercetin delivery via nasal administration was different. It was likely to permeate through the subarachnoid space through the olfactory epithelium and found in the CSF later, because the liposomes behaved as semilipophilic particles. Therefore, quercetin liposomes could rapidly absorb into the CSF.

## CONCLUSION

The present study clearly demonstrates that nasal administration of quercetin liposomes improves learning and memory deficits possibly by inhibiting the oxidative stress and reduces the level of AChE activity. However, further studies are still essential to understand the absorption and distribution after administered via nasal route of quercetin liposomes for the clinical management of AD.

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