

Cognitive-Enhancing and Antioxidant Activities of Quercetin Liposomes in Animal Model of Alzheimer's Disease

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Abstract: Problem statement: Recent findings demonstrated the crucial role of oxidative stress on the pathophysiology of Alzheimer's Disease (AD). Antioxidant intake is beneficial to delay or inhibit the progression of this disease. Quercetin, a bioflavonoid in fruits and vegetables, has a powerful antioxidant activity both *in vitro* and *in vivo*. Claims that quercetin has many biological activities. However, quercetin is rapidly metabolized and limited ability to cross the blood-brain-barrier are obstacles to its use for treatment of AD. Liposomes have been used as an effective delivery system to the brain. Advantages associated with the nasal administration over oral route include higher bioavailability due to no first pass hepatic metabolism and rapid absorption leading to shorter time to onset of effect. Based on this information, the effects of quercetin liposomes via nasal route on improving cognitive behavior and biochemical markers of oxidative stress, Superoxide Dismutase (SOD), catalase, glutathione and Malondialdehyde (MDA) in the hippocampus in animal model of AD were investigated. **Approach:** Male Wistar rats were pretreated with quercetin liposomes, containing 0.5 mg of quercetin in 20 μ L (dose = 20 μ g), via intranasal route once daily continually for 2 weeks before and 1 week after AF64A administration. Learning and memory was evaluated using the Morris water maze test at 7 days after the AF64A administration and then the rats were sacrificed for determining the content of MDA and the activities of SOD, catalase and glutathione in the hippocampus. **Results:** Quercetin liposomes via nasal administration significantly improved memory impairment by inhibiting the oxidative damage in hippocampus. The possible underlying mechanisms might be partly associated with the decrease the level of MDA whereas increase the activity of SOD, catalase and glutathione. **Conclusion:** Our studies demonstrated that quercetin liposomes via nasal administration may have a therapeutic importance in the clinical management of AD.

Key words: Quercetin liposomes, nasal administration, spatial memory, oxidative stress

INTRODUCTION

Reactive Oxygen Species (ROS), such as superoxide anion, hydroxyl radical and hydrogen peroxide, have a causal relationship with oxidative stress. Many studies have demonstrated that overproduction of ROS can further aggravate the oxidative stress and the result is the pathogenesis of Alzheimer's Disease (AD) (Markesbery, 1999; Bokov *et al.*, 2004). AD is a progressive and complex neurodegenerative disease, characterized by progressive decline in memory, language and other cognitive functions (Auld *et al.*, 2002). It has been observed that the use of antioxidants as well as dietary improvements with regard to the consumption of fruits and vegetables high in antioxidant activity and neuroprotective agents may decrease the risk of

memory deficits of the AD type (Weinstock and Shoham, 2004).

Quercetin (3,5,7,3',4'-pentahydroxyflavone), a bioflavonoid, frequently found in consumed foods including apples, berries, onions, tea and vegetables (Formica and Regelson, 1995). Indeed, quercetin has many beneficial effects on human health, including cardiovascular protection, anticancer activity, cataract prevention, antiviral activity and antiinflammatory effects (Nagata *et al.*, 1999). Despite extensive research on the beneficial effects of quercetin in various pathological conditions, the idea of applying quercetin as the agent to improve memory deficit in Alzheimer's condition has become attractive. In order to apply quercetin for this case, it is necessary to overcome the limitations of quercetin: Poor absorption and very low

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distribution to the brain after oral administration (Boer *et al.*, 2005) due to both rapid metabolism (Manach *et al.*, 1998) and difficulties in the penetration through the Blood-Brain Barrier (BBB) (Youdim *et al.*, 2004).

Liposomes have long been used as a Drug Delivery System (DDS) 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. In view of this, the present study was undertaken to investigate the effects of quercetin liposomes via nasal route on cognitive dysfunction and associated oxidative damage in animal model of AD.

MATERIALS AND METHODS

High-purity egg L- α -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- α -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: Adult male Wistar rats (180 \pm 20 g, 8 weeks old) were obtained from National Animal Center,

Salaya, Nakhon Pathom and they were housed in group of five per cage in standard metal cages at 22 \pm 2°C on 12:12 h light-dark cycle. All animals were given access to food and water *ad libitum*. The experiments were performed to minimize animal suffering in accordance with the internationally accepted principles for laboratory use and care of European Economic Community (EEC directive of 1986; 86/609/EEC).

The experimental protocols were approved by the Institutional Animal Care and Use Committee.

Experimental protocol: All rats were randomly assigned to five groups of eight animals each:

- 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 μ L (dose = 20 μ 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 animals were determined the spatial memory 1 week after AF64A administration then they were sacrificed for determined the activity of SOD, catalase, glutathione and the level of MDA in the hippocampus.

AF64A administration: AF64A was prepared as described previously by Hanin (1996). Briefly, an aqueous solution of acetyethylcholine mustard HCl (Sigma, St. Louis, MO) was adjusted to pH 11.3 with NaOH. After stirring for 30 min at room temperature, the pH was lowered to 7.4 with the gradual addition of HCl and stirred for 60 min. The amount of AF64A was

then adjusted either to 2 nmol/2 μL . The vehicle of AF64A was distilled water prepared in the same manner as the AF64A and recognized as ACSF. In order to administer AF64A bilaterally via intracerebroventricular (i.c.v.) route, the animals were anesthetized with the intraperitoneal injection of sodium pentobarbital at dose of 60 mg kg^{-1} BW. Then, AF64A (2 nmol/2 μL) was infused bilaterally via intracerebroventricular (i.c.v.) route with a 30-gauge needle inserted through a burr hole drilled into the skull into both the right and left lateral ventricles. Stereotaxic coordinates were (from the bregma): Posterior 0.8 mm, lateral ± 1.5 mm and ventral (from dura) 3.6 mm. The rate of infusion was 1.0 $\mu\text{L min}^{-1}$ and the needle was left in place for 5 min after infusion and then slowly withdrawn.

Behaviors evaluation:

Morris water maze test: The water maze consisted of a metal pool (170 cm in diameter \times 58 cm tall) filled with tap water (25°C, 40 cm deep) divided into four quadrants. In the center of one quadrant was a removable escape platform below the water level and covered with a nontoxic milk powder. The pool was divided into four quadrants (NE, NW, SE and SW) by two imaginary lines crossing the center of the pool. For each animal, the location of invisible platform was placed at the center of one quadrant and remained there throughout training. The rats must memorize the platform location in relation to various environmental cues because there was nothing directly shows the location of the escape platform in and outside the pool. Therefore, the placement of the water tank and platform were the same in all acquisition trials. Each rat was gently placed in the water facing the wall of the pool from one of the four starting points (N, E, S, or W) along the perimeter of the pool and the animal was allowed to swim until it found and climbed onto the platform. During training session, the subject was gently placed on the platform by the experimenter when it could not reach the platform in 60 sec. In either case, the subject was left on the platform for 15 sec and removed from the pool. The time for animals to climb on the hidden platform was recorded as escape latency or acquisition time. In addition to the acquisition test, the determination of retention memory was carried out on the next day. According to this test, the platform was removed and the animals were placed into the water maze for 60 sec. The retention of memory or the time that the animal spent to swim around the previous location of platform before removing the platform on the test occurring in the next day was also recorded. It has been postulated that if the spatial memory of the rat

for the trained platform location is accurate, the rat will swim to the platform location and search around the exact location. Therefore, the more accurate the spatial memory is, the greater the number of times rat swim across the trained platform. In each trial, the animal was quickly dried with towel before being returned to the cage. All test in Morris water maze tests were carried out within 30 min after the nasal administration of the substances.

Any enhancement of cognition would be reflected by a decrease in escape latency and increase in retention time.

Estimation of oxidative stress parameters:

Tissue preparation: Brain tissue samples were thawed and homogenized with 10 times (w/v) by homogenizer in ice-cold 0.1 M phosphate buffer (pH 7.4). Aliquots of homogenates from rat brain were separated and used to determine protein, MDA, SOD, catalase and glutathione. Whereas the remaining homogenates were centrifuged at 15,000 rpm for 60 min and supernatant was then used for enzyme assays. Catalase activity was determined immediately after sample preparation and SOD was determined within 24 h. Protein concentration was determined according to Lowry *et al.* (1951) using purified bovine serum albumin as standard.

Measurement of lipid peroxidation: MDA, which is a measure of lipid peroxidation, was measured spectrophotometrically (Ohkawa *et al.*, 1979). Briefly, brain tissues were homogenized with 10 times (w/v) 0.1 M sodium phosphate buffer (pH 7.4). The reagents acetic acid 1.5 mL (20%) pH 3.5, 1.5 mL thiobarbituric acid (0.8%) and 0.2 mL sodium dodecyl sulfate (8.1%) were added to 0.1 mL of processed tissue sample. The mixture was then heated at 100°C for 60 min. The mixture was cooled with tap water and 5 mL of n-butanol: Pyridine (15:1% v/v), 1 mL of distilled water was added. The mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was withdrawn and absorbance was measured at 532 nm using a spectrophotometer.

Measurement of glutathione: Glutathione was measured spectrophotometrically (Ellman, 1959). Briefly, brain tissues were homogenized with 10 times (w/v) 0.1 M sodium phosphate buffer (pH 7.4). This homogenate was then centrifuged with 5% trichloroacetic acid to centrifuge out the proteins. To 0.1 mL of this homogenate, 2 mL of phosphate buffer (pH 8.4), 0.5 mL of 505-Dithiobis (2-Nitrobenzoic acid) (DTNB) and 0.4 mL of double distilled water was added. The mixture was vortexed and the absorbance read at 412 nm within 15 min.

Measurement of catalase: Catalase activity was measured by the method of Goldblith and Proctor (1950). About 0.1 mL of supernatant was added to cuvette containing 1.9 mL of 50 mM phosphate buffer (pH 7.0). Reaction was started by the addition of 1.0 mL of freshly prepared 30 mM H₂O₂. The rate of decomposition of H₂O₂ was measured spectrophotometrically from changes in absorbance at 240 nm.

Measurement of SOD: SOD activity was measured according to the method described by McCord and Fridovich (1969). Solution A was prepared by mixing 100 mL of 50 mM PBS (pH 7.4) containing 0.1 mM EDTA and 2 µmol of cytochrome c with 10 mL of 0.001 N NaOH solution containing 5 µmol of xantine. Solution B included 0.2 U xantine oxidize mL⁻¹ and 0.1 mM EDTA 50 µL of a tissue supernatant were mixed with 2.9 mL of solution A and the reaction was started by adding 50 µL of solution B. change in absorbance at 550 nm was monitored.

Statistical analysis: Data are presented as mean ± standard error of mean (± SEM). One-way Analysis of Variance (ANOVA), followed by Duncan's post hoc test. A probability level less than 0.05 was accepted as significance.

RESULTS

Effect of quercetin liposomes on spatial memory in AF64A treated rats: Figure 1 and 2 shows that ACSF produced no significant changes on both escape latency and retention time in Morris water maze test. Intracerebroventricular administration of AF64A significantly increased escape latency but decreased retention time (p-value<0.001 all; compared to both liposomes + ACSF and PEG + ACSF). This indicated the memory impairment induced by AF64A. Rats that received AF64A and nasal administration of quercetin liposomes showed significantly decreased acquisition and increased retention time (p-value<0.001 all; compared to liposomes + ACSF and PEG + AF64A). Therefore, our data indicated that quercetin liposomes via nasal administration improved the memory deficit induced by AF64A.

Effect of quercetin liposomes on MDA level, SOD, catalase and glutathione activities in AF64A treated rats: 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, catalase and glutathione in hippocampus as shown in Table 1. Intracerebroventricular administration of ACSF had no effect on brain MDA levels or SOD catalase and glutathione, whereas AF64A administration caused a marked increase in free radical generation and a significant rise in brain MDA levels and depletion of SOD, catalase and glutathione (p-value<0.001 all; compared to both liposomes + ACSF and PEG + ACSF).

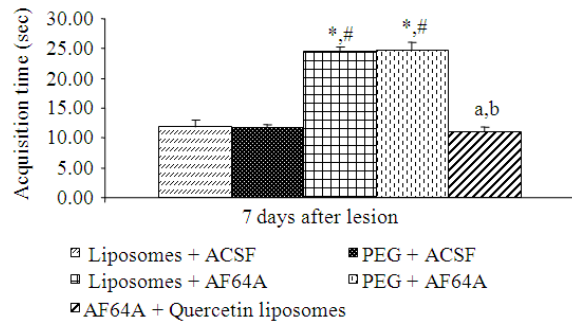


Fig. 1: Effect nasal administration of quercetin liposomes on the acquisition time of spatial learning in the Morris water maze test (N = 8). Results were expressed as mean ± SEM. *: p-value<0.001 compared with liposomes + ACSF treated group, #: p-value<0.001 compared with PEG + ACSF treated group, a: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

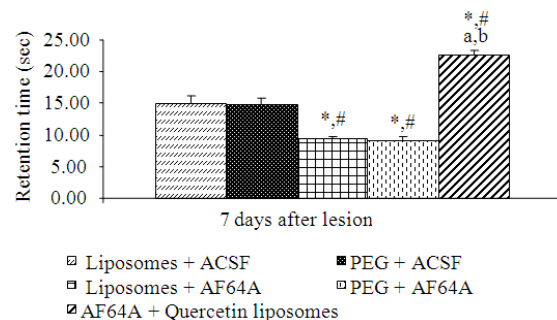


Fig. 2: Effect nasal administration of quercetin liposomes on the retention time of spatial learning in the Morris water maze test (N = 8). Results are expressed as mean ± SEM. *: p-value<0.001 compared with liposomes + ACSF treated group, #: p-value<0.001 compared with PEG + ACSF treated group, a: p-value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

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

Experimental conditions	SOD (U mg ⁻¹ protein)	Catalase (U mg ⁻¹ protein)	Glutathione (U mg ⁻¹ protein)	MDA (U mg ⁻¹ protein)
Liposomes + ACSF	16.80±0.13	18.10±4.55	14.44±0.12	1.45±0.11
PEG + ACSF	16.41±1.27	18.67±3.84	14.41±0.11	1.48±0.17
Liposomes + AF64A	11.20±0.17 [#]	16.14±1.48 [#]	11.20±0.47 [#]	2.48±0.27 [#]
PEG + AF64A	12.24±1.90 [#]	16.00±1.73 [#]	11.87±0.22 [#]	2.49±0.26 [#]
QCL + AF64A	22.50±0.12 ^{#,a,b}	19.80±3.43 ^{#,a,b}	18.31±0.33 ^{#,a,b}	1.19±0.13 ^{#,a,b}

Results are expressed as mean ± SEM; ^{*}: p-value<0.001 compared with liposomes + ACSF treated group; [#]: p-value<0.001 compared with PEG+ACSF treated group; ^a: p-value<0.001 compared with liposomes + AF64A treated group; ^b: p-value<0.001 compared with PEG + AF64A treated group

Surprisingly, 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

The present study examined the effect of quercetin liposomes via nasal administration on memory impairment and oxidative stress in animal model of Alzheimer's disease induced by AF64A.

Recently, numerous lines of evidence have demonstrated 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). During the last few years, antioxidant has received special attention as dietary supplements. Many studies have suggested that reversals in age-related memory declines might be accomplished by increasing the dietary intake possessing high antioxidant activity (Galli *et al.*, 2002; Andres-Lacueva *et al.*, 2005; Jabeen *et al.*, 2007) and this reversal effect has been claimed to be associated with the antioxidant activity (Raghavendra and Kulkarni, 2001). Thus, many researchers focused on the beneficial effects of supplement possessing a capability to improve antioxidant activity were considered to be a potential candidate for neuroprotective agent against AD.

Quercetin, a main flavonoid found in fruits, vegetables and beverages, was reported to possess antioxidant and cognition (Boots *et al.*, 2008; Reiter *et al.*, 2009). Previous studies confirm that quercetin supplementation improve memory deficit condition induced by reserpine in mice (Naumenko and Kulikov, 2006). 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.

Intracerebroventricular injection by AF64A has been described as an appropriate the substance to induce memory deficit via increase the oxidative stress in all area of hippocampus, the areas contribution important role on learning leading to the neurodegeneration in the mentioned areas resulting in learning and memory impairment as those observed in AD (Chrobak *et al.*, 1988). 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 show that both in the AF64A + free liposomes and AF64A + PEG treated groups, caused a persistent memory deficit as evidenced by a significant induction in acquisition time and reduction in retention time in Morris water maze. Moreover, our results show that in the AF64A with nasal administration of free liposomes and AF64A with nasal administration of PEG treated groups, there were the reduction of scavenging enzymes activities including SOD, catalase and glutathione, while enhanced the level of MDA, which is an indicative of lipid peroxidation were appear in these groups. These results are in conformity with other workers, who have demonstrated cognitive impairment after induced by AF64A in rats (McDonald and Overmier, 1998; Gulyaeva *et al.*, 1996).

No unexpected mortality of any animals occurred after nasal administration of quercetin liposomes used in the present study. As a result, vesicles were considered to be safe at the dosing schedule used. Interestingly, 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. Moreover, pretreatment with nasal administration of quercetin liposomes significantly reversed the impact of oxidative alterations (MDA, SOD, catalase and glutathione) seen in Alzheimer's condition induced by AF64A; this shows the antioxidant potential of quercetin liposomes via nasal administration.

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.

Our results demonstrated that nasal administration of quercetin encapsulated liposomes was a potential novel strategy to protect against neurodegeneration disorder such as AD. It was noticeable that the dose of quercetin required via nasal administration was very much low dose and rapid onset of cognitive enhancing effect. In this respect, quercetin could be delivered to the brain even though it was administered in nondissolved form in a liquid that was not a good candidate for delivery of this hydrophobic compound (Cho *et al.*, 2006). Some of the suspended particles of quercetin might not be absorbed after all. Our present study suggests that the enhanced delivery of quercetin in the form of liposomes to the brain could effectively reduce the dose, which would also reduce the potential of toxicity of the substance (Metodiewa *et al.*, 1999) and increase bioavailability (Jamal *et al.*, 2000). Moreover, it was also provided numerous benefits especially the direct nose-to-brain delivery, bypassing the blood brain barrier (Wu *et al.*, 2008) and avoid the first pass metabolism (Graf *et al.*, 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. Investigation of quercetin absorption and distribution after administered via nasal route would be of value for future studies.

CONCLUSION

Nasal administration of quercetin liposomes prevents AF64A-induced cognitive impairment and associated with oxidative stress. Further, the use of quercetin liposomes via nasal route is warrants

evaluation for the treatment of neurological disorders, which are associated with free radical generation and cognitive impairment such as AD.

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