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Effects of Quercetin Encapsulated Liposomes via Nasal Administration: A Novel Cognitive Enhancer

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Abstract: Problem statement: Demand for cognitive-enhancing drugs is growing. Numerous medicinal plants possessing antioxidant activity have received much attention as food supplement to improve cognitive function. Quercetin is a potent free radical scavenger and antioxidant. However, the limitations of quercetin: Rapidly metabolized is an obstacle to its use for a cognitive enhancer. In addition, the burden of blood brain barrier can be overcome by nasal administration and liposomes. In the present study, we investigated whether nasal administration of quercetin liposomes could improve spatial memory in healthy adult rats. **Approach:** Male Wistar rats were pretreated with quercetin liposomes, containing 0.5 mg of quercetin in 20 μL (dose = 20 μg), via right nasal cavity once daily continually for 4 weeks. Evaluation of rodent learning and memory was assessed by Morris water maze test and then all rats were sacrificed for determining the survival and cholinergic neurons densities in hippocampus. **Results:** Quercetin liposomes via nasal route treated rats exhibited a significant improvement in cognitive performance. In addition, nasal administration of quercetin liposomes also resulted in induced the densities of survival and cholinergic neurons in hippocampus. However, further researches about the precise underlying mechanism are still required. **Conclusion:** Our studies demonstrate that quercetin liposomes via nasal administration may have a candidate for cognitive enhancer in the future.

Key words: Quercetin liposomes, nasal administration, antioxidant, blood brain barrier, cognitive enhancer

INTRODUCTION

Memory is a complex process requiring the coordination of many different regions of brain and many neurotransmitter systems. Among the various neurotransmitter systems, the cholinergic system has attracted special attention because lesions leading to loss of cholinergic neurons in the basal forebrain can cause defects of memory (Leanza *et al*., 1996; Zhang *et al*., 1996), muscarinic acetylcholine receptor antagonists when given to animals can induce memory deficit (Introini-Collison and McGaugh, 1988) and agonists (Schwarz *et al*., 1999) as well as cholinesterase inhibitors (Lyketsos *et al*., 2004) when given to memory impaired animals or dementia patients can improve the memory.

At present, numerous lines of evidence have demonstrated that many antioxidants can improve cognitive function (Emilien *et al*., 2000; Kontush and Schekatolina, 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 (Youdim *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, a large list of herbal extracts possessing high antioxidant activity and improve memory is now available in the market.

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 anti-inflammatory effects (Nagata *et al*., 1999). Despite extensive research on the beneficial effects of quercetin in various

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pathological conditions, the idea of applying quercetin as the cognitive enhancer has become attractive and challenge. In order to apply quercetin for this case, it is necessary to overcome the limitations of quercetin: poor absorption and very low 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 drug delivery has now been recognized as a very promising route for delivery of therapeutic compounds including biopharmaceuticals. 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 (Wang *et al*., 2006). Here, we investigated whether nasal administration of quercetin liposomes could improve spatial memory in healthy adult rats.

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±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±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 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 four groups of eight animals each:

Group 1: Naive intact control
Group 2: Free liposomes: The rats in this group were administered free liposomes via nasal route
Group 3: Liposomes + PEG: The rats in this group were administered PEG which used as vehicle of quercetin liposomes via nasal route
Group 4: Liposomes + Quercetin (QCL): The rats in this group were administered quercetin liposomes via nasal route

All animals were administered freshly prepared quercetin liposomes, containing 0.5 mg of quercetin in 20 μL (dose = 20 μg), being drawn into a micropipette, were administered to the right nasal cavity of each rat which was then holding still for 20 sec to ensure the flow and contact of the quercetin liposomes to the nasal cavity whereas the control group received liposomes without quercetin or liposomes + PEG at the same volume once daily for 4 weeks.

Behaviors evaluation: The rats were divided into various groups as mentioned earlier. The behavioral profiles were assessed both after the single dose and repetitive administration of the substance as following: 1-4 weeks of treatment. All animals were submitted to the following behavior tasks (a) Open field test (b) Morris water maze test.

Open field test: In order to assure that cognitive enhancing effect which determined by Morris water maze test was not false positive due to the effect of quercetin liposomes on motor behavior, we also determined the effect of quercetin liposomes on the spontaneous locomotor activity by open field test (Kulkarni and Dandiya, 1973). The open field apparatus was an arena 80 cm in diameter with a white, opaque wall 30 cm high. Rats were individually placed in the center of the arena and locomotor activity including the number of grooming, licking and rearing were scored within 5 min.

Morris water maze test: The water maze consisted of a metal pool (170 cm in diameter×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 20 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.

Histological procedure: Following anesthesia with sodium pentobarbital (60 mg kg^{-1} BW), fixation of the brain was carried out by transcardial perfusion with fixative solution containing 4% par formaldehyde in 0.1 M phosphate buffer pH 7.3. The brains were removed after perfusion and stored over a night in a fixative solution that used for perfusion. Then, they were infiltrated with 30% sucrose solution for approximately 4°C. The specimens were frozen rapidly and 30 μM thick sections were cut on cryostat. They were rinsed in the phosphate buffer and picked up on slides coated with 0.01% of aqueous solution of a high molecular weight poly L-lysine.

Nissl staining: The duplicate coronal sections of brains were stained with 0.75% cresyl violet, dehydrated through graded alcohols (70, 95, 100% 2×), placed in xylene and cover-slipped using DPX mountant.

Choline acetyltransferase and immunohistochemistry: A series of section containing hippocampus from each groups were reacted in parallel experiments using a mouse monoclonal antibody detected against Choline Acetyltransferase (ChAT) (Chemicon Internation, Inc., CA, USA) and a modification of a previously described protocol employing the DAKO Strept ABC Complex/HRP duet kit. In brief, the sections were eliminated endogenous peroxidase activity by 0.5% H_2O_2 in methanol. Sections were washed in running tap water and distilled water for 1 min each, then rinsed in KPBS and KPBS-BT for 5 min per each process. Excess was removed and then incubated for 30 min in a blocking solution composed of 5% normal horse serum in KPBS-BT. The sections were then incubated in mouse primary antibody against ChAT diluted 1:100 in KPBS-BT at room temperature for 2 h and then incubated at 4°C for 48 h. The tissue was rinsed in KPBS-BT (two washes × 7 min), incubated for 4 h in biotinylated goat antimouse IgG antibody, rinsed in KPBS-BT (two washes × 7 min) and then incubated in Strep ABC Complex/HRP for 4 h. In preparation for visualization step, sections were rinsed in KPBS-BT (1 min) and KPBS (two washes×10 min). ChAT immunoreactivity was visualized using 0.025% 3, 3' Diaminobenzidine (DAB, Sigma) and 0.01% H_2O_2. Finally, sections were rinsed in running tap water, air dried and cover-slipped using permount.

Morphological analysis: Five coronal sections of each rat in each group were studied quantitatively. Neuronal counts in hippocampus were performed by eye using a 400×magnification with final field 255 μm^2 according to the following stereotaxic coordinates: AP-4.8 mm, lateral ±2.4-6 mm, depth 3-8 mm. The observer was blind to the treatment at the time of analysis. Viable stained neurons were identified on the basis of a stained soma with at least two visible processes. Counts were made in five adjacent fields and the mean number extrapolated to give total number of neurons per 255 μm^2. All data are represented as number of neurons per 255 μm^2.

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 locomotor activity: The effects of quercetin liposomes via nasal administration on spontaneous motor behaviors including grooming, rearing and licking behaviors were demonstrated in Fig. 1-3. The results showed that the spontaneous behavior as mention earlier did not differ significantly between the control, vehicle (PEG), free liposomes treated group and quercetin liposomes throughout the experimental period.

Effect of quercetin liposomes on spatial memory: Hippocampus-dependent spatial memory was assessed with the Morris water maze test. The results showed that vehicle (PEG) and free liposomes treated rats did not differ from their controls either in the escape latency for finding the platform and the retention time. After 20 min of single administration, quercetin liposomes via nasal administration significantly decreased acquisition and increased retention time ($p<0.05$ all) as shown in Fig. 4 and 5. In addition, this phenomenon was still observed when the treatment duration was increased to 28 days. This indicates that nasal administration of quercetin liposomes could produce cognitive enhancing effect.

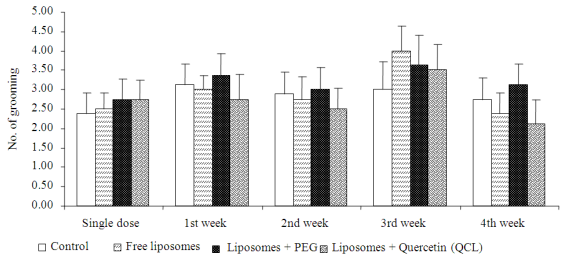

Fig. 1: Effect nasal administration of quercetin liposomes on grooming behaviors (N = 8)

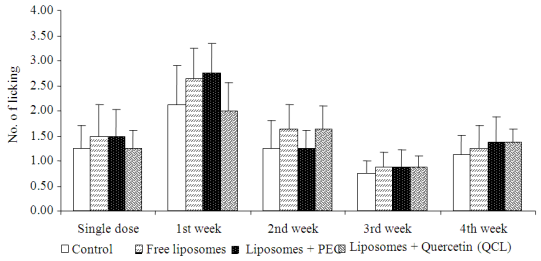

Fig. 2: Effect na + sal administration of quercetin liposomes on licking behaviors (N = 8)

Neuroprotective effect of quercetin liposomes via nasal administration: Accumulating data demonstrated that learning and memory particularly spatial memory were tightly associated with the function of various brain areas, which in turn depended on the density of survival neurons particularly cholinergic neurons (Katzman, 1986). Therefore, this study also determined the effect of quercetin liposomes via nasal administration on the alteration of the survival and cholinergic neurons densities in various subregions of hippocampus, the area which played an important role on learning and memory. It was found that the mean ± SEM survival neurons density in various subregions of hippocampus in vehicle (PEG) and free liposomes treated group were not significantly different from that of control group while the mean ± SEM survival neurons density in these areas was significantly ($p<0.05$ all) higher in quercetin liposomes treatment as shown in Fig. 6.

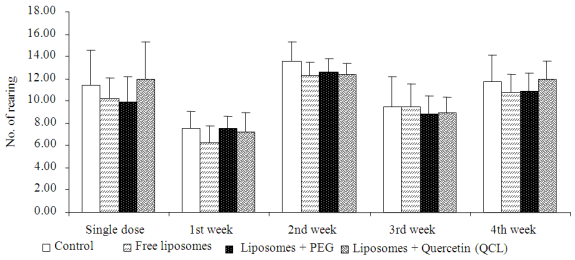

Fig. 3: Effect nasal administration of quercetin liposomes on rearing behaviors (N = 8)

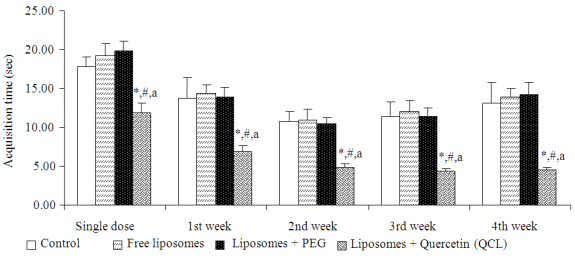

Fig. 4: Effect nasal administration of quercetin liposomes on the acquisition time in the Morris water maze test (N = 8). Results were expressed as mean ± SEM; *: p-value<0.05 compared with control treated group; $^{\#}$: p-value<0.05 compared with free liposomes treated group; ^{a}: p-value<0.05 compared with liposomes + PEG treated group

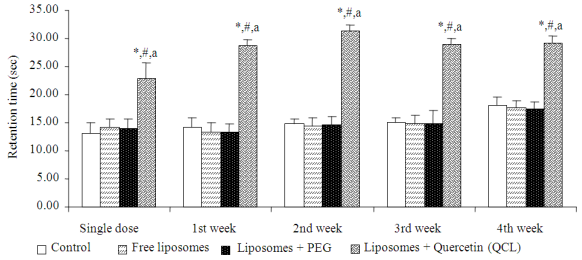

Fig. 5: Effect nasal administration of quercetin liposomes on the retention time in the Morris water maze test (N = 8). Results were expressed as mean ± SEM. *: p-value<0.05 compared with control treated group; #: p-value<0.05 compared with free liposomes treated group; a: p-value<0.05 compared with liposomes + PEG treated group

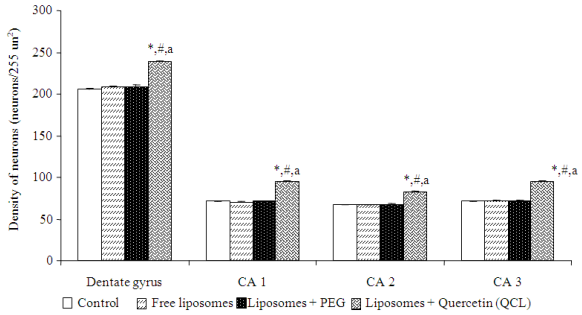

Fig. 6: Effect nasal administration of quercetin liposomes on the survival neurons density in hippocampus. Data were presented as mean ± SEM. (N = 8/group); *: p-value<0.05 compared with control treated group; #: p-value<0.05 compared with free liposomes treated group; a: p-value<0.05 compared with liposomes + PEG treated group

In addition, statistical analysis revealed that there were no significant differences in the number of cholinergic neurons density in various subregions of hippocampus in control, free liposomes and vehicle (PEG) treated group while there was a significant induction in cholinergic neurons density of quercetin liposomes treated group in areas as mentioned earlier ($p<0.05$ all) as shown in Fig. 7, reflecting the cognitive enhancement via increase in the cholinergic neurons density after nasal administration of quercetin liposomes.

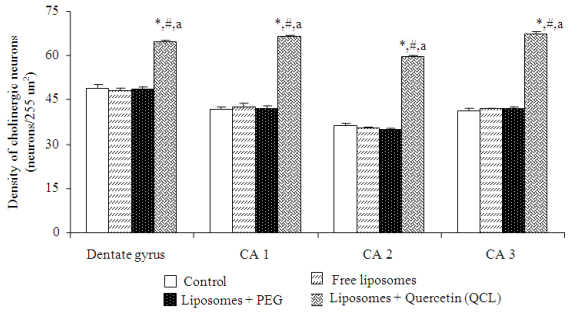

Fig. 7: Effect nasal administration of quercetin liposomes on the cholinergic neurons density in hippocampus. Data were presented as mean ± SEM. (N = 8/group). *: p-value<0.05 compared with control treated group; #: p-value<0.05 compared with free liposomes treated group; a: p-value<0.05 compared with liposomes + PEG treated group

DISCUSSION

Here we show that nasal administration of quercetin liposomes could induce the densities of survival and cholinergic neurons in hippocampus resulted in the enhancement of learning and memory in healthy rats.

Recently, a pile of evidence suggested that the prime candidate responsible for producing the neuronal changes mediating the cognitive deficits appeared to be free radicals and oxidative stress generated (Mattson, 2004; Cantuti *et al*., 2000). Thus, numerous medicinal plants or herbs possessing antioxidant activity have received much attention as food supplement to improve cognitive function. Moreover, treatment with herbal agents is generally cheap and relatively free of side effects (Howes *et al*., 2003).

Quercetin is a well-known flavonoid distributed ubiquitously in fruits, vegetables and herbs (Havsteen, 1983). Owing to its polyphenolic hydroxyl groups, quercetin exhibits its antioxidant property and is known as a strong free radical scavenger (Rodriguez *et al*., 2001). 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). Clinical studies investigating different programs of administration of quercetin have been limited by its poor water solubility. Many researchers have attempted to improve its solubility by adding Dimethylsulfoxide (DMSO) (Ader *et al*., 2000). In addition, the safety of the higher DMSO is questionable due to risk of

vasoconstrictor effect and neurological toxicity (Windrum *et al*., 2005). It is necessary to develop a strategy, which could provide an elevated pool of antioxidants in the brain region for complete protection of neuronal cells and improve cognitive function. Our study found it worthwhile to use the vesicle mediated carrier system particularly liposomes to deliver flavonoidal antioxidant quercetin to rat brain 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.

The Morris water maze is commonly used to assess hippocampal-dependent spatial memory in rodents (Morris, 1984). In this task, the rats was treated with quercetin liposomes via nasal route showed significantly decreased acquisition and increased retention time as compared to the control, free liposomes and vehicle (PEG) treated animals, suggested its cognitive enhancing effect.

Moreover, pretreatment with nasal administration of quercetin liposomes significantly increased the density of survival neurons and cholinergic neurons in all areas of hippocampus as compared to the control, free liposomes and vehicle (PEG) treated animals. Therefore, the increased neuron density in hippocampus might involve its neurotrophic action resulted in the neurogenesis.

The neurogenesis could occur throughout adulthood especially in hippocampus and sub ventricular zone of lateral ventricle (Hagg, 2005). Numerous factors had been reported to be regulators of the adult neurogenesis. They also included various neurotransmitters such as dopamine, acetylcholine, serotonin, glutamate and nor epinephrine. All the mentioned neurotransmitters could stimulate the proliferation process (Nacher *et al*., 2001; Kulkarni *et al*., 2002; Baker *et al*., 2005). Recent findings also proposed that the new neuron occurring from the neurogenesis also contributed the important role on learning and memory (Bruel-Jungerman *et al*., 2007; Kitabatake *et al*., 2007). However, further study particularly BrdU staining is essential in order to elucidate the neurotrophic effect of quercetin liposomes on neurogenesis in hippocampus resulting in memory improvement.

Our results demonstrated that nasal administration of quercetin encapsulated liposomes was a potential novel cognitive enhancer. It was noticeable that the dose of quercetin required via nasal administration was very much low dose and also produced the rapid onset of cognitive enhancing effect. 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 Central Nervous System (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 Cerebro Spinal Fluid (CSF) later, because the liposomes behaved as semilipophilic particles. Therefore, quercetin liposomes could rapidly absorb into the CSF.

CONCLUSION

Quercetin liposomes via nasal administration to healthy adult rats could increase the densities of survival and cholinergic neurons in the hippocampus. These effects were associated with the enhancement in performance in the Morris water maze behavioral test. Thus, our study, stresses the cognitive enhancement of quercetin liposomes. Despite the fact that the mechanisms underlying these effects are still unknown and require more pharmacological, neurochemical and pharmacokinetic research to establish any therapeutic advantage, quercetin liposomes seem to have the potential to benefit healthy people and contribute to prevention of cognitive decline during aging and neurodegenerative disease.

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