Evaluation of neuroprotective effect of quercetin with donepezil in scopolamine-induced amnesia in rats

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Abstract:
Objective: The objective of this study was to evaluate the neuroprotective effect of quercetin with donepezil in scopolamine-induced amnesia in rats.

Materials and Methods: Five groups of adult male Wistar rats (12 months old) weighing 180–200 g (n = 6) were used. The normal control group received normal saline and test group animals were pretreated orally with quercetin (25 mg/kg), donepezil (3 mg/kg), and a combination of quercetin (25 mg/kg) with donepezil (3 mg/kg), respectively, dosed at every 24 h interval for 14 consecutive days, afterward amnesia was induced by scopolamine (3 mg/kg) on the 14th day through intraperitoneal route. Cognitive performance was assessed by the Morris water maze, elevated plus maze, and passive avoidance paradigm. Acetylcholinesterase enzyme (AchE) level, biochemical markers such as lipid peroxidase (LPO), glutathione (GSH), β amyloid level, and histopathological study of rat brain were estimated. Statistical analysis was done by one-way analysis of variance, followed by Dunnett's post hoc test. P > 0.05 was considered statistically significant.

Results: Pretreatment with quercetin, donepezil, and their combination showed a significant increase in escape latency, step-through latency, and decreased transfer latency in respective cognitive models of the Morris water maze, passive avoidance test, and elevated plus maze. Further coadministration significantly decreased AchE level, β amyloid level as compared to individual therapy. Biochemical markers such as elevated GSH, decreased LPO were observed, and histopathological studies revealed the reversal of neuronal damage in the treatment group (P < 0.05) as compared to scopolamine-treated control group.

Conclusion: Pretreatment with quercetin potentiates the action of donepezil in scopolamine-induced amnesia in rats. The improved cognitive memory could be due to the synergistic effect of the drugs by decreasing AchE level, β amyloid level, and antioxidant action in rat brain.

Key words:
Acetylcholinesterase enzyme, amnesia, donepezil, quercetin, scopolamine

Alzheimer’s disease (AD) is the most common form of dementia considered as progressive, neurodegenerative disease characterized by the presence of senile plaques rich in insoluble aggregate of beta-amyloid and neurofibrillary tangles in the brain. Factors such as decreased acetylcholine level, oxidative stress, and hypercholesterolemia have been reported in memory decline. Currently, 30 million people are affected worldwide by dementia with 4.6 million cases annually as per AD International analysis.

Many acetylcholinesterase inhibitor drugs are available in the market to treat, but they have modest benefits and associated with side effects; hence, different drugs are aiming at diverse targets which are expected to act better than single targeting agents to treat AD. Donepezil, a reversible noncompetitive cholinesterase enzyme inhibitor considered as first-line treatment in AD which is evident from recent report of “cholinergic anti-inflammation” pathway, is responsible for neuroprotective effect; however, its beneficial effect on memory is temporarily for 36 weeks and the progression of AD cannot be reversed or modified for long time; hence, noncholinergic therapies are also needed to overcome these problems.

Quercetin is flavonol, type of flavonoid abundantly found in apples, black tea, etc. Quercetin reported to show a promising effect in the treatment

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of AD and oxidative stress-related neurodegenerative disease by acting as an antioxidant.\[9\]

Scopolamine, a cholinergic antagonist, impairs learning and memory which is a characteristic manifestation of dementia.\[10\] hence, in this study, scopolamine-induced amnesia was used as an experimental model.

Literature survey indicates that there is a paucity of evidence regarding the synergistic effect of quercetin with AchE inhibitor donepezil to reverse the progression of AD in animal experimental model. Hence, the present study was conducted to evaluate the effect of quercetin with donepezil in scopolamine-induced amnesia in rats.

Materials and Methods

Animals
Healthy male Wistar rats (12 months old) weighing 180–200 g were used in this study. They were housed in cages in groups of six per cage and maintained under natural light and dark cycle and standard laboratory conditions. They were acclimatized for a week before the experiment in the departmental animal house. The rats were fed with standard rat chow pellet and water ad libitum. All the experimental procedures were carried out in accordance with the CPCSEA guidelines. The study was reviewed and approved by the Institutional Animal Ethics Committee, KLECOP/IAEC/Res. 20-09/08/2014, for conducting this study.

Experimental Protocol
Rats were divided into five groups (n = 6/group); Group I – normal control, Group II – disease control (scopolamine hydrobromide 3 mg/kg, i.p.), Group III – scopolamine + quercetin (25 mg/kg, p.o.), Group IV – standard treatment (scopolamine + donepezil hydrochloride 3 mg/kg, p.o.), and Group V – scopolamine + quercetin (25 mg/kg, p.o.) + donepezil (3 mg/kg, p.o.). Group III, IV, and V rats were dosed every 24 h interval with respective drugs for 14 consecutive days. The acquisition trial for Morris water maze, elevated plus maze, and passive avoidance test was conducted using the method of Pitchaimani et al.\[12\]. In acquisition trail, each rat was placed in the illuminated compartment, and 10 s later, the guillotine door was raised. Rat on entering the dark compartment, the door was closed, and a 50 Hz, 1 mA constant current shock was applied for 2 s. Each rat was retrained in the apparatus and received foot-shock if it entered the dark compartment in 120 s. Acquisition trial was terminated when the rat remained in the light compartment for 120 s. The retention trial was screened on the 15th day, by placing the animal into the light compartment and recording latency to enter the dark compartment. This escape latency served as a measure of retention performance of the step-through avoidance response.

Collection of Brain Sample
After behavioral testing (retrieval) by screening models of memory, the animals were sacrificed by cervical dislocation. The whole brain was carefully removed from the skull and weighed. 10% w/v brain homogenate was then prepared by homogenizing it in ice-chilled phosphate buffer (pH 8, 0.1M). The homogenate was subsequently centrifuged using a refrigerated centrifuge at 3000 rpm for 10 min, and the supernatant was separated and used for the biochemical estimations.

Estimation of Acetylcholinesterase Enzyme in Brain
AchE present in the brain was estimated using the method of Ellman et al.\[14\]. The rate of moles of substrate hydrolyzed per min per gram of tissue was calculated.

Estimation of Lipid Peroxidation in Rat Brain
LPO was determined by estimation of malondialdehyde (MDA) levels expressed as nanomoles of MDA/mg of protein, described by Ohkawa et al.\[15\].

Estimation of Reduced Glutathione
GSH level was measured by the method of Ellman and Lysko.\[16\]. GSH level was expressed in μmoles/mg of tissue.

Estimation of β–Amyloid (Aβ1-42)
The measurement of rat Aβ1-42 levels was conducted according to the manual of sandwich ELISA kit for rat Aβ1-42 (YH Bioresearch Laboratory, Shanghai, China). Rat brain was homogenized in phosphate buffer (pH 7.4) and centrifuged at 9500 g, and the supernatant was used for the detection of Aβ1-42. Standard curve analysis was run in parallel to test samples.

Elevated plus maze
Screening for index of improvement of memory in rat was carried out using elevated plus maze by the method of Vijayalakshmi et al.\[13\]. In acquisition trial, each rat was placed at the end of an open arm facing away from the center. The time taken to enter any one of the closed arms was recorded as transfer latency. Cutoff time allotted for each rat was 180 s, and the retention trial was carried out 24 h after the first trial, transfer latency was recorded in a similar manner as mentioned before. Shortened transfer latency was considered as an index of improvement of memory.

Passive shock avoidance test
The passive avoidance test was conducted using the method of Pitchaimani et al.\[12\]. In acquisition trial, each rat was placed in the illuminated compartment, and 10 s later, the guillotine door was raised. Rat on entering the dark compartment, the door was closed, and a 50 Hz, 1 mA constant current shock was applied for 2 s. Each rat was retrained in the apparatus and received foot-shock if it entered the dark compartment in 120 s. Acquisition trial was terminated when the rat remained in the light compartment for 120 s. The retention trial was screened on the 15th day, by placing the animal into the light compartment and recording latency to enter the dark compartment. This escape latency served as a measure of retention performance of the step-through avoidance response.

Screening Methods for Amnesia

Morris water maze
Screening model of Morris Water Maze was carried out by the method of Chakravarthi and Avadhan.\[11\] in which each rat was subjected to four acquisition trials per day for 4 consecutive days and their memory was tested on the 5th day. One day before the test, each rat was placed in the pool for 60 s; this free swim enabled the rat to become habituated to the training environment. If the rat did not find the platform in 120 s, it was manually placed on the platform for 30 s for rest. Whereas on day 5, time spent in the target quadrant (Q4) served as an index of retrieval or memory.
The absorbance was measured in the multi-scan spectrum spectrophotometer (Thermo Scientific, Multiskan GO) at optical density 450 nm. All the readings were performed in triplicate.

**Histopathological Studies**
Rats’ brains were collected after sacrifice and fixed in 10% neutral buffered formalin. Subsequently, brain tissues were further kept in 10% neutral buffered at 48°C. Then, the brains were routinely embedded in paraffin and stained with hematoxylin-eosin. The hippocampal lesions were assessed microscopically at ×40 magnification.[10]

**Statistical Analysis**
Results were analyzed by one-way analysis of variance, followed by Dunnett’s post hoc test using GraphPad Prisn software and expressed as mean ± standard error of mean (n = 6). P ≥ 0.05 was considered statistically significant.

**Results**

**Effect of Quercetin with Donepezil on Screening Models**
Quercetin (25 mg/kg) with donepezil (3 mg/kg) administered in scopolamine-induced amnesia in rats showed a significant reduction in transfer latency and a significant increase in escape latency and the time spent in target quadrant. All the treatment groups are compared with scopolamine-induced amnesia, normal control group [Table 1].

**Effect of Quercetin and Donepezil on Acetylcholinesterase Enzyme Level in Rat Brain Homogenate**
Scopolamine administration alone significantly increased the activity of AchE (7.98 ± 0.065; P < 0.001) when compared to the normal group (3.06 ± 0.296). Quercetin (3.46 ± 0.16; P < 0.001), donepezil (3.75 ± 0.530; P < 0.001), and a combination of quercetin with donepezil treatment groups (3.29 ± 0.14; P < 0.001) significantly decreased levels of AchE as compared to scopolamine-treated group [Figure 1].

**Biochemical Estimation**

**Antioxidant effects of quercetin and donepezil in scopolamine-induced amnesia in rats**
The animals treated with scopolamine reported a significant increase (34.61 ± 4.85; P < 0.01) in levels of MDA as compared to the normal group (12.82 ± 2.86). Simultaneously, the rats treated with quercetin (P < 0.001; 14.10 ± 1.95), donepezil (P < 0.001; 19.23 ± 1.65), and a combination of these two drugs (P < 0.001; 15.36 ± 1.28) significantly reduced the level of MDA as compared to scopolamine-treated group [Figure 2].

**Effect on reduced glutathione in rat brain**
The scopolamine-treated group showed significant decrease in reduced GSH level (P < 0.001; 0.1504 ± 0.03) as compared to the normal group (0.3906 ± 0.02). Quercetin (P < 0.01; 0.47 ± 0.05), donepezil (P < 0.001; 0.48 ± 0.06), and a combination of these two drugs (P < 0.01; 0.43 ± 0.05) significantly increased the level of reduced GSH as compared to scopolamine-treated group [Figure 3].

**Effect of quercetin and donepezil on β amyloid_{1-42} level in brain homogenerate**
The scopolamine-treated rats showed a significant increase in the concentration of Aβ_{1-42} (P < 0.001; 146.2 ± 1.74) as compared to the normal group (43.21 ± 3.46). The groups treated by quercetin (P < 0.01; 71.67 ± 17.57), donepezil (P < 0.01; 82.23 ± 6.02), and combination of these two drugs (P < 0.01; 76.44 ± 12.66) significantly decreased the concentration of Aβ_{1-42} as compared to scopolamine-treated group [Figure 4].

**Histopathology of rat brain (Hippocampus)**
In ×40 magnification, the scopolamine-treated group showed severe damage of the neurons along with hippocampal edema, pyknotic cells. The groups treated with quercetin and donepezil protected neurons and showed mild hippocampal edema as compared to diseased group [Figure 5].

**Discussion**
The present study evaluates the effect of quercetin (25 mg/kg) with donepezil (3 mg/kg) in scopolamine-induced amnesia in rats. Screening methods such as the Morris water maze, elevated plus maze, passive avoidance paradigm were performed to screen the effect of drugs. Furthermore, using rat brain homogenate AchE level, brain oxidative markers, and β amyloid_{1-42} level, histopathological studies were performed.

**Table 1: Effect of quercetin and donepezil alone and in combination on Morris water maze, elevated plus maze and passive avoidance test in scopolamine-induced amnesia in rats**

| Groups                        | Time spent in target quadrant (sec) | Transfer latency (sec) | Escape latency (sec) |
|-------------------------------|------------------------------------|------------------------|----------------------|
| Normal control                | 6.28±0.2682                        | 8.93±1.808             | 26.83±2.12           |
| Disease control Scopolamine (3 mg/kg) | 1.353±0.1370**                   | 24.83±3.349**          | 11.5±3.063**         |
| Quercetin (25 mg/kg)          | 7.515±0.2516***                    | 10.68±0.666***         | 24.67±2.108***       |
| Standard control Donepezil (3 mg/kg) | 7.627±0.2708***                   | 10.08±1.667***         | 19.33±1.333*         |
| Quercetin (25 mg/kg) + Donepezil (3 mg/kg) | 9.077±1.312***                     | 8.6±0.4914***          | 19±1.033*            |

Values represent in mean±SEM (n=6). *P<0.05, **P<0.01, ***P<0.001 as compared to disease control group. SEM=Standard error of mean.
In the screening models of learning and memory, the combined effect of quercetin with donepezil showed a significant decrease in transfer latency and increase in step-through latency; these results were correlated with the previous reports of Sonkusare et al.\(^1\)

In the Morris water maze, mean time spent by animals in the target quadrant was significantly increased as compared to individual therapy, which was correlated with the previous reports of Cachard-Chastel et al.\(^2\). These results suggest an improvement in the retrieval of memory in rats as compared to individual treatment.

One of the most promising therapies to treat a cognitive deficit in AD is to increase the cholinergic activity and inhibition of AchE enzyme.\(^3\) In this study, donepezil significantly reduced the level of AchE which, in turn, increased the availability of acetylcholine for improving memory, increased GSH level, and marked reduction in MDA level which were correlated with the previous reports of Zaki et al.\(^4\). Interestingly, coadministration of quercetin with donepezil potentiates the action of donepezil.

In neurodegenerative disease, brain is especially vulnerable to oxidative damage, due to the imbalance between the generation of oxygen free radicals and antioxidant defense system. In this study, MDA level was elevated while a decrease in GSH level was observed in scopolamine-treated group which indicated oxidative damage in the brain. On the other side, pretreatment with quercetin, donepezil, and their combination significantly reversed the endogenous antioxidant enzymes and decreased oxidative damage. These results were consistent with the previous reports of Singh et al.\(^5\).

Cognitive impairment is characterized by plaque deposition in extracellular spaces by aggregation of β amyloid protein in the brain tissue, and β amyloid\(_{142}\) is found predominantly in the brain. Extracellular deposition of β amyloid is considered as a biomarker for AD.\(^6\) Administration of quercetin with donepezil for 14 days showed a marked reduction in β amyloid\(_{142}\) level as compared to individual treatments. This result was consistent with the previous reports of Ansari et al.\(^7\). Hence, combination treatment may be used in delaying onset and reduce the severity of AD. Histopathological study of rat brain proved damaging in scopolamine-treated group which showed neuronal damage (neuronal death), hippocampal edema, pyknotic cells, and neural fibrillary tangles, while groups pretreated with quercetin, donepezil, and combination protected neurons by reversing the damage induced by scopolamine.

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**Figure 2:** Effect of quercetin and donepezil on malondialdehyde level in rat brain homogenate. Values represent the mean ± standard error of mean (n = 6). *P < 0.05, **P < 0.01, ***P < 0.001 as compared to disease control group

**Figure 3:** Effect of quercetin and donepezil on glutathione level in rat brain homogenate. Values represent the mean ± standard error of mean (n = 6). *P < 0.05, **P < 0.01, ***P < 0.001 as compared to disease control group

**Figure 4:** Effect of quercetin and donepezil on Aβ1-42 level in rat brain homogenate. Values represent the mean ± standard error of mean (n = 6). *P < 0.05, **P < 0.01, ***P < 0.001 as compared to disease control group

**Figure 5:** Histopathology of rat brain (hippocampus) (a) normal control, (b) disease control, (c) quercetin treatment, (d) donepezil treatment, (e) quercetin + donepezil treatment
Hence, these findings suggest that the pretreatment of quercetin with donepezil may act synergistically to enhance memory and learning in a scopolamine-induced amnesia model by reversing the oxidative stress, decreasing the AchE level and β amyloid_1-42 level in rat brain which may be the probable mode of actions for its beneficial effect as compared to the individual treatment. Thus, multi-drug therapy would be interesting to get the best response in the treatment of AD.

Conclusion

The present study concludes pretreatment with quercetin potentiates the action of donepezil in scopolamine-induced amnesia in rats. The observed neuroprotective and improved cognitive could be due to the synergistic effect of the drugs by decreasing the AchE level, β amyloid_1-42 level, and antioxidant action in rat brain; hence, this could be an adjuvant therapy for treatment of dementia. Further clinical evaluation is needed to establish therapeutic value in the treatment of dementia.

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Conflicts of Interest

There are no conflicts of interest.

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