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Reversal of Aging and Chronic Ethanol-induced Cognitive Dysfunction by Quercetin a Bioflavonoid

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Cognitive dysfunction, one of the most striking age-related impairments seen in human beings, has been correlated to the vulnerability of the brain to increased oxidative stress during aging process. Quercetin is a bioflavonoid with strong antioxidant properties. Experiments were performed to study the possible effects of quercetin on cognitive performance of young, aged or ethanol-intoxicated mice (an animal model for cognition dysfunction) using one trail step down type of passive avoidance and elevated plus maze tasks, respectively. Aged or chronic ethanol-treated mice showed poor retention of memory in step-down passive avoidance and in elevated plus-maze task. Chronic administration of quercetin (10, 25 and 50 mg/kg) for 30 days or its co-administration with ethanol (15% w/v, 2 g/kg per orally) for 24 days significantly reversed the age-related or chronic ethanol-induced retention deficits in both the test paradigms. However, in both memory paradigms chronic administration of quercetin failed to modulate the retention performance of young mice. Chronic quercetin administration for 30 days also reversed age associated increase in TBARS levels and decline in forebrain total glutathione (GSH), SOD and catalase levels. Chronic ethanol administration to young mice produced an increase in lipid peroxidation, and a decline in forebrain total glutathione (GSH), SOD and catalase levels, which was significantly reversed by the co-administration of quercetin (10, 25 and 50 mg/kg). The results of the present study showed that chronic quercetin treatment reverses cognitive deficits in aged and ethanol-intoxicated mice, which is associated with its antioxidant property.

Keywords: Oxidative stress; Aging; Dementia; Quercetin

INTRODUCTION

Oxidative stress due to increase in free radical generation or impaired endogenous antioxidant mechanisms has been implicated as an important factor in Alzheimer disease (AD) and cognitive deficits in elders.[1] Oxidative damage was considered a likely cause of age-associated brain dysfunction because the brain is believed to be particularly prone to oxidative stress due to a relatively high rate of oxygen free radical generation without commensurate levels of anti-oxidative defenses.[2] Chronic administration of antioxidants or diets rich in antioxidants is reported to alleviate age associated cognitive deficits as well as to reduce the incidence of certain types of cancer and cardiovascular disease.[1] Rats fed on antioxidant rich diets showed reverse of deficits in brain function, motor performance and learning and memory.[3]

Flavonoids are plant phenolic compounds with strong antioxidant properties. Quercetin (3,5,7,3′, 4′-pentahydroxyflavone) is a plant polyphenolic compound with potent antioxidant and iron chelating properties.[4,5] Quercetin and its metabolites are potent antioxidants[6] having oxygen radical scavenging properties and inhibited xanthine oxidase and lipid peroxidation *in vitro*. It is being considered as a putative active constituent in various phytopharmaceuticals. Quercetin is orally bioavailable and is reported to cross blood–brain barrier.[7] It is reported to have many beneficial effects on human health including cardioprotection, anticancer, antiulcer, anti-allergy, anti-inflammatory, antiviral activity and anti-cataract activities.[8,9]

In the present experiments, we studied the effect of chronic quercetin treatment on cognitive parameters of young, aged and ethanol-intoxicated

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mice. Learning and memory parameters were evaluated using one trial step-down type passive avoidance task and elevated plus maze. Previous studies have shown that aged mice performed poorly compared to young mice in both the animal models used.[10] Ethanol-intoxicated mice model has been used as an animal model for studying AD-associated dementia, since alcoholic dementia is reported to be very similar to AD as the mediator systems and the brain regions affected are the same.[11,12] Oxidative stress-induced depletion of reduced glutathione level, extent of lipid peroxidation, enzymes such as SOD and catalase were analyzed in the forebrains of animals following the behavioral paradigms.

MATERIALS AND METHODS

Animals

Male laka mice of 3 months (young) and 16 months old (aged), weighing 20–25 and 35–50 g respectively, bred in the Central Animal House facility of Panjab University were used. The animals were housed under standard laboratory conditions, maintained on natural 12-h light and dark cycle and had free access to food and water. Animals were acclimatized to the laboratory conditions prior to experimentation. Each animal was used only one time in the experiments. All the experiments were carried out between 09:00 and 15:00 h. The experimental protocols were approved by the Institutional Animal Ethics Committee and conducted according to the Indian National Science Academy Guidelines for the use and care of experimental animals.

Experimental Design and Drug Administration

The aged mice were randomly distributed into four groups. The first group of animals received only vehicle treatment (1 ml/100 g of 0.25% CMC) through per oral route for a period of 30 days. Subsequent groups of animals received varying doses of quercetin (10, 25 and 50 mg/kg p.o.) suspended in 0.25% CMC for a period of 30 days. Similar drug treatment was also performed in different groups of young mice. On the next day (day 31) animals were tested for their cognitive performances using one trial of passive avoidance and elevated plus-maze tasks. After recording the behavioral parameters, mice were sacrificed and their forebrain was immediately processed for estimating Total GSH, lipid peroxidation, SOD and catalase.

To study the possible effect of quercetin on chronic ethanol-induced decline in cognitive function, the following drug-regimen was employed. Young mice were randomly distributed into four groups. The first group of mice received 15%w/v ethanol (2 g/kg) once a day, per oral for a period of 24 days. The second, third and fourth groups of mice received varying doses of quercetin (10, 25 and 50 mg/kg p.o.) 30 min prior to ethanol administration for 24 consecutive days. After the treatment period, ethanol was withdrawn and 6 days of washout was allowed. The next day after the washout period, animals were tested for their cognitive performances using one trial of passive avoidance and elevated plus-maze tasks. After recording the behavioral parameters, mice were sacrificed and their forebrain was immediately processed for estimating total GSH, lipid peroxidation, SOD and catalase.

Passive Avoidance Learning

A one-trail step down type of passive avoidance task was used to examine the short-term working memory in mice based on negative reinforcement as described previously.[13] The apparatus consisted of an electric grid with a centrally located shock free zone (SFZ: $10 \times 7 \times 1.5\,cm^3$) and the entire grid having a perflex enclosure ($22 \times 22 \times 30\,cm^3$). Electric shocks (15 VDC) were delivered for a period of 15 s. The mouse was removed from the enclosure immediately after receiving the shock to their respective cages. The latency was recorded from the time the mouse was placed on the SFZ until it stepped down the SFZ. The retention test was carried out 24 h after training, in a similar manner, except the electric shocks were not applied to grid floor. Short latencies indicate poor retention compared to significant longer latencies.

Transfer Latency on Elevated Plus-maze

Cognitive behavior was evaluated by using the elevated plus-maze learning task, which measures spatial long-term memory. Transfer latency (TL, the time in which the animal moves from the open arm to the enclosed arm) was utilized as an index of learning and memory processes. The procedure was basically identical to that described by Itoh *et al.*,[14] which has been validated in our lab. The elevated maze consisted of two open arms ($16 \times 5\,cm^2$) and two enclosed arms ($16 \times 5 \times 12\,cm^3$) with an open roof. The maze was elevated to a height of 25 cm from the floor. The animals were placed individually at the end of either open arms and the TL was noted on the first day. To become acquainted with the maze, the animals were allowed to explore the plus maze for 20 s after reaching the enclosed arm. On the second day, 24 h after the first exposure, TL was noted again. A long latency period to reach enclosed arm indicates poor retention compared to significantly shorter latency.

Biochemical Estimations

The animals were sacrificed by decapitation immediately after behavioral assessments. The brains were removed (without cerebellum), and rinsed with cold isotonic saline and weighed. A 10% (w/v) tissue homogenate was prepared in 0.1 M phosphate buffer (pH 7.4). The post nuclear fraction for catalase assay was obtained by centrifugation of the homogenate at 1000*g* for 20 min, at 4°C and for other enzyme assays centrifuged at 12,000*g* for 60 min at 4°C.

Lipid Peroxidation Assay

The quantitative measurement of lipid peroxidation in brain was performed according to the method of Wills.[15] The lipid peroxidation products such as TBARS formed was measured by the reaction with thiobarbituric acid at 532 nm using Perkin Elmer lambda 20 spectrophotometer. The results were expressed as nmol of TBARS/mg protein using the molar extinction coefficient of chromophore ($1.56 \times 10^5\ M^{-1}\ cm^{-1}$).

Estimation of Reduced Glutathione

Reduced glutathione in the brain was estimated according to the method of Ellman.[16] A 0.75 ml of homogenate was precipitated with 0.75 ml of 4% sulphosalicylic acid. The samples were centrifuged at 1200*g* for 15 min at 4°C. The assay mixture contained 0.5 ml of supernatant and 4.5 ml of 0.01 M (in 0.1 M phosphate buffer, pH 8.0) DTNB (5-5′-Dithio Bis-(2-Nitrobenzoic acid)). The yellow color developed was read immediately at 412 nm spectrophotometrically. The results were expressed as nmol of GSH per mg protein.

Superoxide Dismutase Activity

Superoxide dismutase activity was assayed according to the method of Kono,[17] wherein the reduction of nitrazoblue tetrazolium (NBT) was inhibited by the superoxide dismutase measured at 560 nm spectrophotometrically. Briefly, the reaction was initiated by the addition of hydroxylamine hydrochloride to the reaction mixture containing NBT and post nuclear fraction of brain homogenate. The results were expressed as units/mg protein, where one unit of enzyme is defined as the amount of enzyme inhibiting the rate of reaction by 50%.

Catalase Activity

Catalase activity was assayed by the method of Luck,[18] wherein the breakdown of H_2O_2 being measured at 240 nm. Briefly, the assay mixture consisted of 3 ml of H_2O_2-phosphate buffer (1.25×10^{-2} H_2O_2 m) and 0.05 ml of supernatant of brain homogenate (10%) and the change in absorbance were recorded at 240 nm spectrophotometrically. Enzyme activity was calculated using the millimolar extinction coefficient of H_2O_2 (0.044). The results were expressed as μmol H_2O_2 decomposed/min/mg protein.

Protein Estimation

The protein content was measured according to the method of Lowry[19] using bovine serum albumin as standard.

Statistical Analysis

One specific group of rats was assigned to one specific drug treatment condition and each group comprised 6 rats ($n = 6$). All the values are expressed as means ± SEM. The data were analyzed by using analysis of variance (ANOVA) followed by Tukey's test. In all tests, the criterion for statistical significance was $P < 0.05$.

RESULTS

Effect of Chronic Treatment of Quercetin on Passive-avoidance Performance of the Young, Aged or Ethanol-intoxicated Mice

During the training session of step-down passive avoidance task, vehicle treated aged mice and chronic ethanol-treated young mice showed a similar step down latency (SDL) as that of young mice (data not shown). However, aged and ethanol-intoxicated young mice during the retention test performed 24 h after training session, showed a significant lower SDL as compared to that of vehicle-treated young mice (Fig. 1; comparison between vehicle treated young, aged and ethanol-intoxicated mice). Decrease in SDL indicates an impairment of memory retention

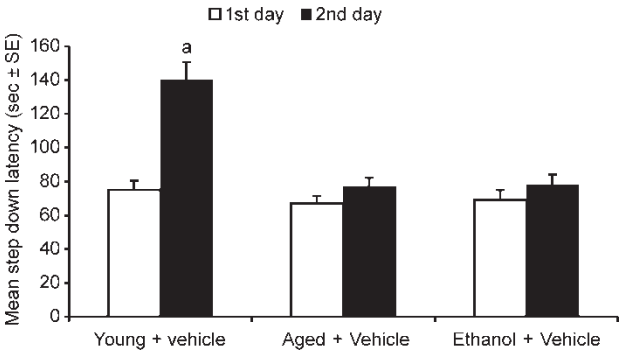

FIGURE 1 Performance of young, aged or chronic ethanol treated mice during retention test of passive avoidance. The SDL of each group of mice are expressed as mean ± SE ($n = 5–8$). [a]$P < 0.05$ as compared to the vehicle treated young mice.

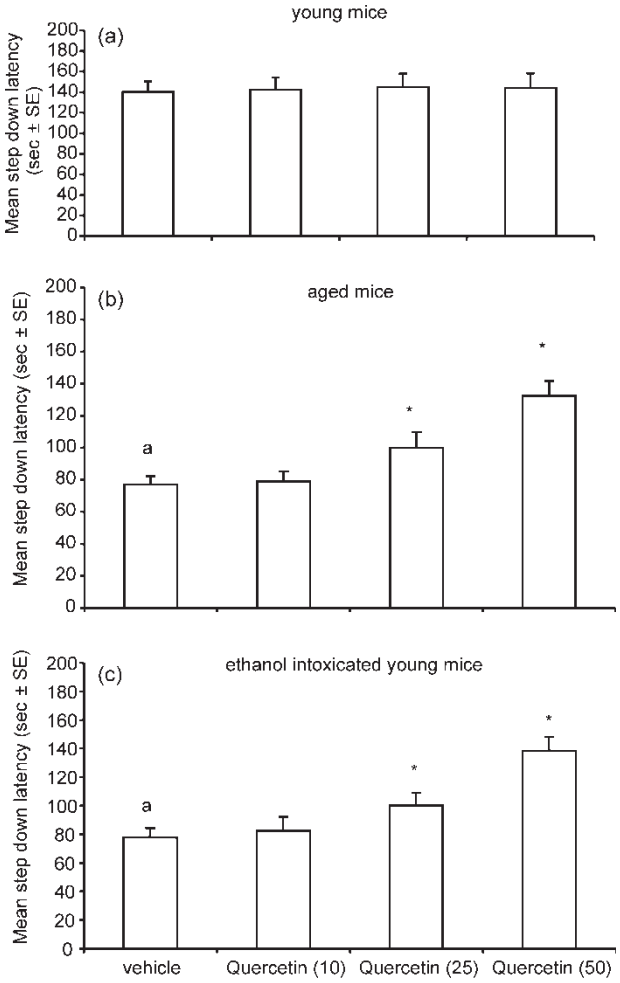

FIGURE 2 Effect of chronic administration of quercetin (10, 25 and 50 mg/kg) on the step down latency (SDL) of young (A), aged (B) or chronic ethanol (C) treated mice during retention test of passive avoidance. The SDL of each group of mice are expressed as mean ± SE ($n = 7 - 8$). $^{a}P < 0.05$ as compared to the vehicle treated young mice. $^{*}P < 0.05$ as compared to respective control groups.

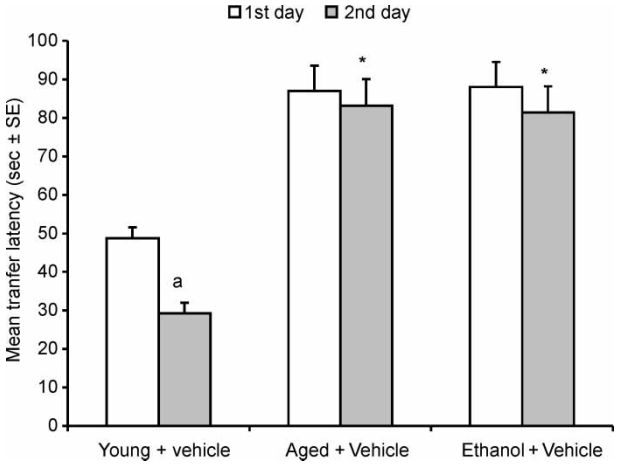

FIGURE 3 Performance of young, aged or chronic ethanol treated mice on elevated plus maze learning and memory task. Mean scores of first and second day transfer latency (TL) of each group of mice are expressed as mean ± SE ($n = 5–8$). $^{a}P < 0.05$ as compared to first day transfer latency and $^{*}P < 0.05$ as compared to the second day transfer latency of young mice.

of the passive avoidance task in aged and ethanol-intoxicated mice. As shown in Fig. 2A, chronic administration of quercetin (10, 25 and 50 mg/kg, for 30 days) to young mice had no effect on SDL during retention test of passive avoidance task as compared to vehicle treated young mice. However, quercetin at similar dose range significantly increased the SDL of aged, as well as ethanol-intoxicated mice in a dose dependent manner (Fig. 2B,C).

Effect of Chronic Treatment of Quercetin on the Elevated Plus-maze Performance of the Young, Aged or Ethanol-intoxicated Mice

As shown in Fig. 3, the TL measured on the second day in young mice was drastically shorter than that on the first day, indicating ability of the mice to recall the learned aspect in a lesser period of time. However, the TL of vehicle treated aged mice and that of chronic ethanol-treated young mice decreased on the second day, but it was found statistically insignificant, indicating poor retention ability of aged mice. Moreover, the TL of the vehicle-treated aged mice or chronic ethanol-treated mice recorded on the second day (retention test) was significantly higher as compared to vehicle-treated young mice, indicating an impairment of learning and memory of the elevated plus-maze task in aged and ethanol-intoxicated mice. Chronic treatment of quercetin (10, 25 and 50 mg/kg) had no effect on second-day (retention) TL of young mice in elevated plus-maze task. However, quercetin at similar dose range significantly and dose dependently shortened the second-day TL of aged mice to reach enclosed arm as compared to vehicle-treated age-matched control group. (Fig. 4A–C).

Effect of Chronic Treatment of Quercetin on the Whole Brain TBARS Level in Young, Aged or Ethanol-intoxicated Mice

There was a significant increase in the extent of lipid peroxidation in aged mice as compared to the vehicle treated young mice. Also, chronic quercetin (10–50 mg/kg for 30 days) treatment significantly and dose dependently reversed aged-induced increase in the forebrain TBARS levels as compared to vehicle treated aged mice. Chronic ethanol treatment for 24 days induced a significant raise in fore brain TBARS levels compared to vehicle treated mice. Co-administration of quercetin (10–50 mg/kg) along with ethanol significantly reversed the extent of lipid peroxidation as compared to ethanol-only-treated mice (Fig. 5A–C).

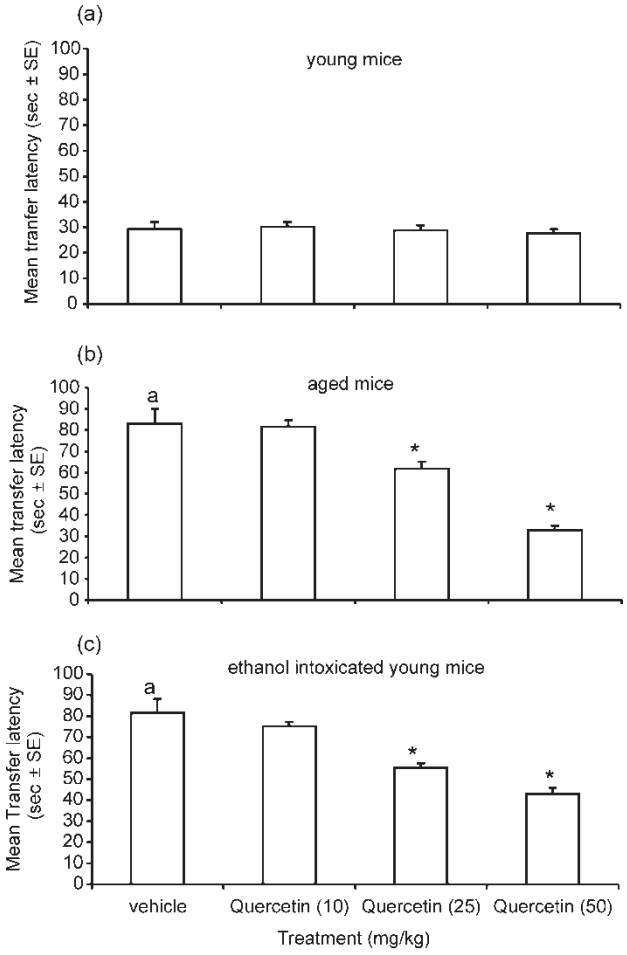

FIGURE 4 Effect of chronic administration of quercetin (10, 25 and 50 mg/kg) on the second day transfer latency (TL) of young (A), aged (B) or chronic ethanol (C) treated mice on elevated plus maze learning and memory task. Latency (TL) of each group of mice are expressed as mean ± SE ($n = 5–8$). $^{a}P < 0.05$ as compared to the vehicle treated young mice. $^{*}P < 0.05$ as compared to respective control groups.

Effect of Chronic Treatment of Quercetin on the Whole Brain GSH Level in Young, Aged or Ethanol-intoxicated Mice

There was a significant decline of GSH level observed in the aged mice as compared to young mice. Chronic quercetin (10–50 mg/kg) treatment for 30 days significantly reversed the aging-induced decrease in forebrain GSH. Statistical analysis revealed that, in young mice, exogenous administration of quercetin failed to increase forebrain GSH level as compared to vehicle treated young mice. Chronic ethanol treatment for 24 days induced a significant fall in the forebrain GSH level as compared to the control group of animals. Co-administration of quercetin (10–50 mg/kg) along with ethanol significantly reversed chronic ethanol-induced decrease in the forebrain GSH as compared to ethanol-only-treated mice (Fig. 6A–C).

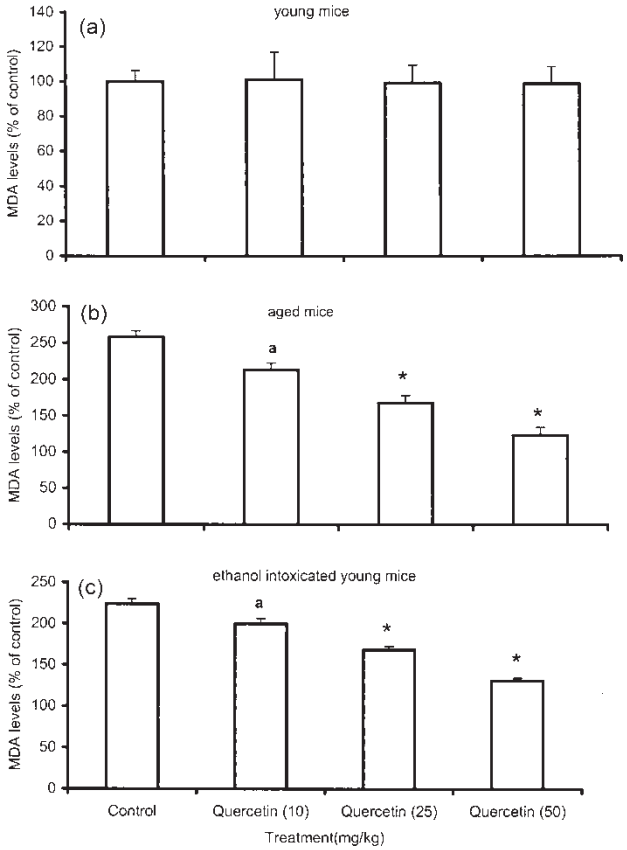

FIGURE 5 Effect of chronic administration of quercetin (10, 25 and 50 mg/kg) on whole brain TBARS levels in aged (A) or Ethanol intoxicated (B) mice. $^{a}P < 0.05$ as compared to the vehicle treated young mice. $^{*}P < 0.05$ as compared to respective vehicle treated aged or ethanol-intoxicated control groups.

Effect of Chronic Treatment of Quercetin on the Whole Brain SOD and Catalase Levels in Young, Aged or Ethanol-intoxicated Mice

There was a significant decline of SOD and catalase levels observed in the aged mice as compared to young mice. Chronic quercetin (10–50 mg/kg) treatment for 30 days significantly reversed the aging-induced decrease in forebrain SOD and catalase. Statistical analysis revealed that, in young mice, exogenous administration of quercetin failed to increase forebrain SOD and catalase levels as compared to vehicle treated young mice. Chronic ethanol treatment for 24 days induced a significant fall in the forebrain SOD and catalase levels as compared to the control group of animals. Co-administration of quercetin (10–50 mg/kg) along with ethanol significantly reversed chronic ethanol-induced decrease in the forebrain SOD and catalase levels as compared to ethanol-only-treated mice (Figs. 7, 8A–C).

DISCUSSION

The results of the this study clearly indicated that chronic administration of quercetin to aged mice,

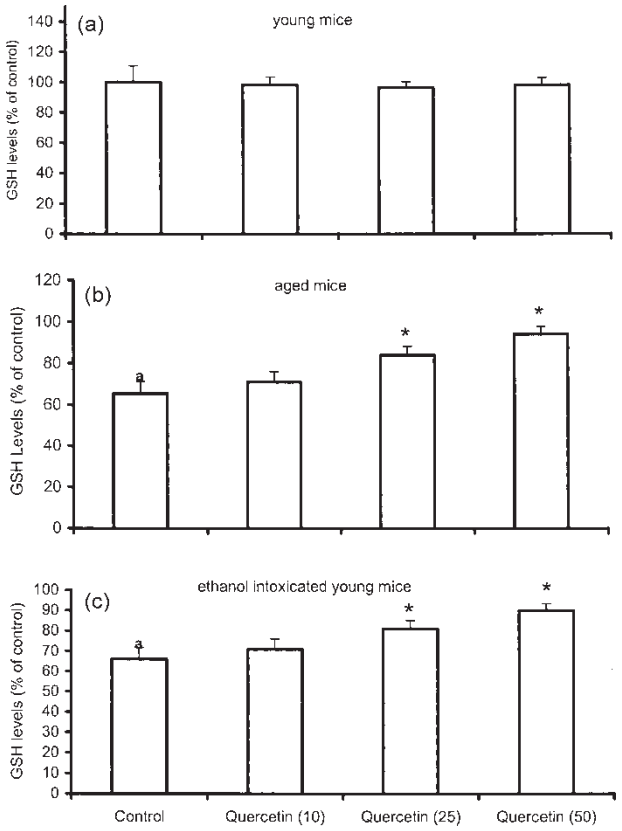

FIGURE 6 Effect of chronic administration of quercetin (10, 25 and 50 mg/kg) on whole brain GSH levels in young (A), aged (B) or chronic ethanol (C) treated mice. $^{a}P < 0.05$ as compared to the vehicle treated young mice. $^{*}P < 0.05$ as compared to respective vehicle treated aged or ethanol-intoxicated control groups.

or its chronic co-administration with ethanol to young mice reverses impairment of memory retention in one trail spatial and passive avoidance tasks. Furthermore, chronic administration of quercetin also protected the brain cells against increase in oxidative stress due to aging or by chronic ethanol treatment. Considering the fact that the amnesia is associated with increased oxidative stress during brain aging or after chronic ethanol treatment, and its reversal by antioxidants,[20–22] our results suggest that antiamnesic effect of quercetin observed in the present study could be due to its antioxidant mechanism.

Varying degrees of behavioral impairment is associated with aging and age associated neurodegenerative diseases.[23] Among the prime candidates responsible for producing the neuronal changes mediating this behavioral deficit appears to be free radicals and the oxidative stress they generate.[24] Oxidative stress refers to the cytotoxic consequences of oxygen radicals like superoxide anion, hydroxyl radical and hydrogen peroxide, which are generated as byproducts of normal aberrant metabolic process during ageing and other neurodegenerative diseases and act on polyunsaturated fatty acids (PUFA) in brain, thereby propagating the lipid peroxidation.[25] Cognitive deficits such as learning impairment and delayed amnesia are the debilitating consequence of aging.[26] The age-associated impairment of cognitive and motor functions have been hypothesized to be due to oxidative damage. The degree of age related impairment in spatial swim maze task in senescence mice was found to be positively correlated with oxidative molecular damage in the brain. Cognitive deficits such as learning impairment and delayed amnesia in mice were also observed in passive-avoidance and plus maze paradigms.[10] Normal aging is associated with decline in the endogenous antioxidant defense mechanism. Potential antioxidant therapy should include either natural antioxidant enzymes or agents, which are capable of augmenting the functions of these enzymes.[23] Earlier reports have shown that the natural drugs like *Ginkgo biloba* and *Withania somnifera* which improves cognition are also shown to have antioxidant properties.[27,28]

Thus in support of the evidences showing beneficial effects of antioxidants in reversing age-induced cognitive deficits in animals, chronic administration of quercetin (10, 25 and 50 mg/kg) for 30 days enhanced the memory retention of aged

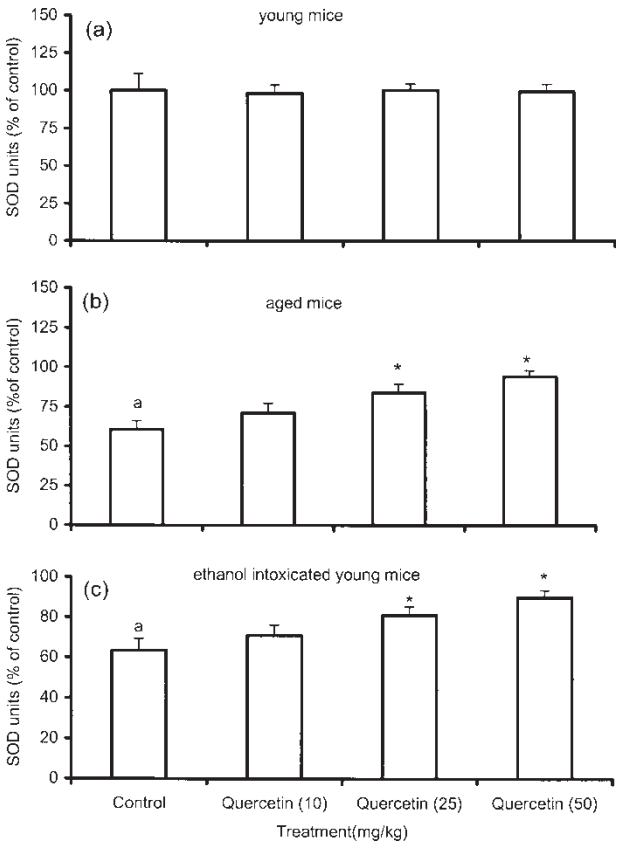

FIGURE 7 Effect of chronic administration of quercetin (10, 25 and 50 mg/kg) on whole brain SOD levels in aged young (A), aged (B) or chronic ethanol (C) treated mice. $^{a}P < 0.05$ as compared to the vehicle treated young mice. $^{*}P < 0.05$ as compared to respective vehicle treated aged or ethanol-intoxicated control groups.

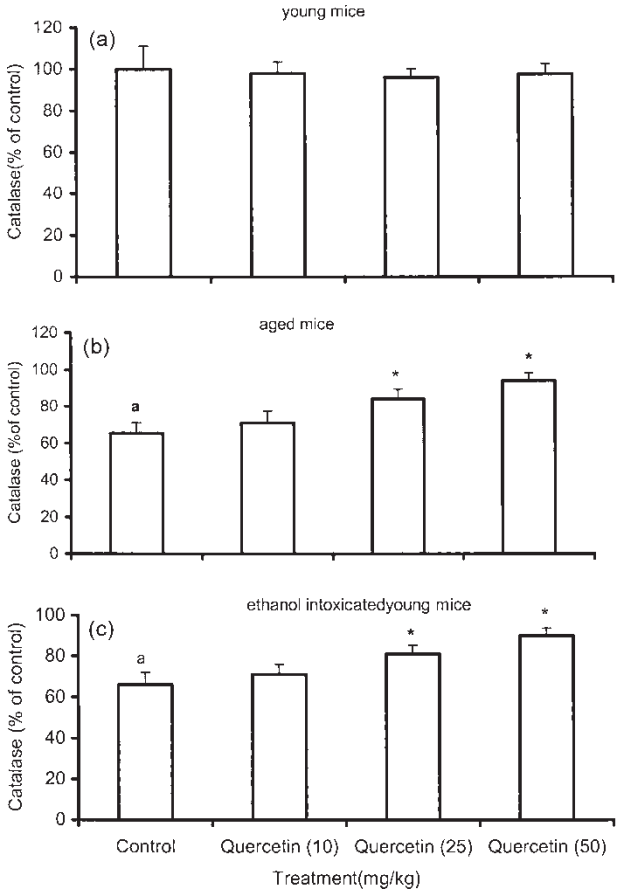

FIGURE 8 Effect of chronic administration of quercetin (10, 25 and 50 mg/kg) on whole brain catalase levels in young (A), aged (B) or chronic ethanol (C) treated mice. $^{a}P < 0.05$ as compared to the vehicle treated young mice. $^{*}P < 0.05$ as compared to respective vehicle treated aged or ethanol-intoxicated control groups.

mice in both passive avoidance and plus maze tasks. The present study showed a significant increase in the lipid peroxidation, decrease in GSH, SOD and catalase levels in aged mice as compared to young mice, which was reversed by chronic quercetin treatment. These results suggest that exogenous administration of quercetin to aged mice has a beneficial effect in reversing senescence-induced amnesia and oxidative stress.

Similar to β AP (A beta protein), a key factor in neurodegeneration seen in AD, chronic administration of ethanol induces oxidative stress, which leads to functional and biochemical alterations in the brain.[12] Most presenile dementia conditions seen in AD and alcoholism are characterized by common psychopathological phenomena such as memory impairment and deficits in sub cortical cholinergic projection systems. Also, animal studies showed that cognitive deficits observed in AD or during ethanol intoxication were sensitive to reversal by cholinomemitics.[29–32] Thus the alcoholic dementia is very similar to AD with regard to the influenced mediator systems and damaged brain regions.

Numerous studies have shown that quercetin is a potent antioxidant with free radical scavenging properties.[33] The antioxidant activity of quercetin might result from direct scavenging of free radicals and other oxidizing intermediates, or from the chelation of iron or copper ions and from inhibition of oxidases. Quercetin activate glutathione peroxidase enzyme[34] and prevent dehydroascorbic acid-induced glutathione depletion in red blood cells of rabbit.[35] Quercetin reversed the decreased levels of antioxidant defense enzymes glutathione peroxidase, glutathione reductase, catalase and superoxide dismutase induced by ultraviolet A.[36] Quercetin is reported to inhibit the H_2O_2-induced oxidative damage.[37,38]

Recently, it has been shown that the neuroprotective abilities of quercetin, resveratrol and catechin result from their antioxidant properties that favor protection against cardiovascular disease—the so-called "French paradox"—and possibly, central nervous system disorders such as AD and ischaemia.[39] Quercetin, may also act via PKC to produce its protective effects. In line with above findings, present results also showed that quercetin reduced the extent of lipid peroxidation and spared the GSH, SOD and catalase depletion induced by chronic ethanol treatment. Interestingly, quercetin also reversed the impairment of plus-maze and passive-avoidance memory studied in ethanol-intoxicated mice. Thus the improvement of cognitive performance by quercetin in ethanol-intoxicated mice may be due to its antioxidant mechanisms.

Endogenous thiol related antioxidant, in particular, GSH is involved in the protection of brain cells against oxidative damage. The ability of GSH, a tripeptide composed of L-glutamate, L-cysteine and glycine, to nonenzymatically scavenge both singlet oxygen and hydroxyl radicals provide the first line of antioxidant defense in the brain.[25] Previous studies have shown that the increased free radical generation during aging and in ethanol intoxication was associated with decreased cellular GSH levels, which can be reversed by antioxidants.[40,41] Alteration in glutathione levels may be involved in neurodegenerative disorders such as Parkinsonism and AD.[43] Moreover, it has been speculated that decreased GSH brain level coupled with oxidative stress may be responsible for the induction of age related cognitive deficits.[42] Quercetin due to its potent neuroprotective, antioxidant nature can spare the endogenous GSH depletion, and thereby can reverse cognitive deficits in aging and ethanol intoxication.

In conclusion, the present study has demonstrated that chronic quercetin treatment alleviates age-dependent and ethanol intoxication-induced cognitive deficit in mice which could be due to its antioxidant action.

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