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RESEARCH ARTICLE

Evaluation of ameliorative effect of quercetin in experimental model of alcoholic neuropathy in rats

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Abstract
Objective The objective of the present investigation was to study the neuroprotective effect of the quercetin in alcohol induced neuropathy in rats.
Materials and methods Male Wistar rats were administered alcohol (10 gm/kg, 35% v/v, p.o. b.i.d.) for 10 weeks. Alpha tocopherol (vitamin E) was used as a standard drug. Vitamin E (100 mg/kg) and quercetin (10, 20 and 40 mg/kg) were co-administered 1 h after ethanol administration for 10 weeks. Behavioral assessment parameters, such as motor incoordination, tactile allodynia, mechanical and thermal hyperalgesia were recorded in all groups of animals. Meanwhile, motor nerve conduction velocity was also recorded. Biochemical parameters, such as nitric oxide (NO), Na^+–K^+-ATPase, malondialdehyde (MDA) and myeloperoxidase (MPO) were estimated in sciatic nerve. Apoptosis index was determined with help of DNA fragmentation in sciatic nerve.
Results and discussion Chronic ethanol administration for 10 weeks resulted in significant ($P < 0.001$) development of neuropathic pain. Chronic treatment with quercetin (20 and 40 mg/kg) for 10 weeks significantly ($P < 0.001$) attenuated allodynia, hyperalgesia as well as motor coordination and impaired nerve conduction velocity along with decreased level of membrane-bound Na^+–K^+-ATPase. It also significantly ($P < 0.001$) decreased elevated levels of MDA, MPO as well as pro-inflammatory mediators, such as NO. It also decreased the extent of DNA fragmentation. This alteration was more significant in vitamin E treated rats (100 mg/kg). Quercetin is a proven antioxidant that might have decreased the oxidative stress produced by chronic alcoholism.
Conclusion The present investigation elucidates neuroprotective effect of quercetin in alcohol induced neuropathy through modulation of membrane-bound inorganic phosphate enzyme and inhibition of release of oxido-inflammatory mediators, such as MDA, MPO and NO.

Keywords Alcoholic neuropathy · Allodynia · DNA fragmentation · Hyperalgesia · Malondialdehyde · Motor nerve conduction velocity · Myeloperoxidase · Na^+–K^+-ATPase · Nitric oxide

Introduction

The prevalence for neuropathy in alcoholics has been reported between 9 and 50% (Narita et al. 2007). Chronic alcohol consumption precipitated a phenomenon termed as "dying back" mechanism adversely affecting central and peripheral neurons. It has been documented that alcoholism is associated with decrease in motor and sensory nerve conduction velocity (Willer and Dehen 1997). It is characterized by the axonal degeneration of distal part of sensory axons affecting sensory axons as well as long and larger diameter axons followed by proximal degeneration of neurons (Juntunen et al. 1978; Cavanagh 1964). Ethanol neurotoxicity causes damage to the central and peripheral nervous system (Villalta et al. 1989; Monforte et al. 1995).

The behavioral characteristics observed during neuropathy in animal models are hyperalgesia (withdrawal reaction to a non-noxious heat stimulus) and allodynia (withdrawal in reaction to non-noxious tactile stimuli) and

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can be used to evaluate the progression of neuropathic pain (Zimmermann 2001). Chronic ethanol administration results in oxidative damage to proteins, lipids and DNA (Mansouri et al. 2001; McDonough 2003). Cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β) (Zelenka et al. 2005) and interleukin-6 (IL-6) (Arruda et al. 1998) play a pivotal role in the development and maintenance of neuropathic pain.

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is a major plant flavonoid has well-known antioxidant (Liu et al. 2010), antihistaminic, antiperoxidative, antiulcer (Kahraman et al. 2003), anti-inflammatory (Read 1995), anti-apoptotic (Ishikawa and Kitamura 2000) as well as neuroprotective potential (DokGo et al. 2003). It has been evaluated for an array of diseases conditions, such as cancer, osteoporosis, cardiovascular, pulmonary diseases and aging.

The objective of present investigation was to study the neuroprotective effect of quercetin against alcohol induced neuropathic pain in laboratory rats by assessing various behavioral, biochemical parameters and DNA fragmentation as a marker of apoptosis.

Materials and methods

Animals

Adult male Wistar rats (150–200 g) were procured from the National Institute of Biosciences, Pune (India) and housed in groups of six in solid bottom polypropylene cages. They were maintained at 24 ± 1°C, with relative humidity of 45–55% and 12:12 h dark/light cycle. The animals were acclimatized for a period of 2 weeks and were maintained under pathogen-free conditions. The animals had free access to standard pellet chow (Chakan Oil Mills, Sangli) and water throughout the experimental period. All experiments were carried out between 09:00 and 17:00 h. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Poona College of Pharmacy, Pune (CPCSEA/12/2010) and performed in accordance with the guidelines of Committee for Control and Supervision of Experimentation on Animals (CPCSEA), Government of India on animal experimentation.

Drugs and chemicals

Quercetin was purchased from Sigma-Aldrich, India. Ethanol, formalin, Tris buffer, sucrose, trichloroacetic acid, citric acid monohydrate, sodium nitrate, copper sulfate, sodium potassium tartrate, ethylene diamine tetra acetic acid disodium salt, sodium hydroxide, sodium carbonate, magnesium chloride, sodium carbonate, sodium bicarbonate, potassium chloride, calcium chloride, ether, hydrochloric acid and conc. sulfuric acid were purchased from S.D. Fine Chemicals, Mumbai, India. Sulfanilamides, naphthalamine diamine HCl, phosphoric acid were obtained from LobaChemi Pvt. Ltd., Mumbai, India.

Preparation of drug solutions

Quercetin was freshly prepared in double-distilled water after triturating with 5% CMC (carboxy methyl cellulose) in three different dosages (10, 20 and 40 mg/kg) and administered to rats orally for 10 weeks (Kumar and Goyal 2008; Jung et al. 2010). α-Tocopherol was freshly prepared in double-distilled water after triturating with 1% tween 80 (Tiwari et al. 2009).

Induction of alcoholic neuropathy and drug treatment schedule

Alcoholic neuropathy was induced by administration of 35% v/v ethanol (10 gm/kg, b.i.d., oral gavage) in double-distilled water for 10 weeks according to previously reported method (Tiwari et al. 2009).

The animals were divided into following group and each consisting of six rats.

[A] Animals without ethanol administration

Group I (N): normal group (10 gm/kg of distilled water, p.o., for 10 weeks).

Group II [QTN (40)]: quercetin (40 mg/kg), p.o., for 10 weeks.

[B] Animals with ethanol administration

Group III (EC): ethanol control (10 gm/kg of 35% v/v ethanol, b.i.d, p.o., for 10 weeks).

Group IV [E + α-T (100)]: ethanol (b.i.d) + α-tocopherol (100 mg/kg) (1 h before ethanol administration), p.o., for 10 weeks.

Group V [E + QTN (10)]: ethanol (b.i.d) + quercetin (10 mg/kg) (1 h before ethanol administration), p.o., for 10 weeks.

Group VI [E + QTN (20)]: ethanol (b.i.d) + quercetin (20 mg/kg) (1 h before ethanol administration), p.o., for 10 weeks.

Group VII [E + QTN (40)]: ethanol (b.i.d) + quercetin (40 mg/kg) (1 h before ethanol administration), p.o., for 10 weeks.

All the behavioral assays were performed by an observer blind to the drug administration on day 0, 6th, 8th and 10th week.

All animals were killed at the end of study, i.e. 10th week and sciatic nerve was collected and kept in chilled tris buffer (10 mM, pH 7.4) for the estimation of biochemical parameters.

Behavioral tests

Mechanical hyperalgesia (Randall–Selitto paw pressure test)

Mechanical nociceptive threshold, an index of mechanohyperalgesia, was assessed by method described by Randall and Selitto (1957). The nociceptive flexion reflex was quantified using the Randall–Selitto paw pressure device (UGO Basile SRL Biological Research Apparatus, Italy), which applies a linearly increasing mechanical force (g) to the dorsum of the rat's hind paw. The paw of the rat was placed under the tip and the progressive pressure applied until the rat vocalized. The nociceptive threshold was expressed in grams and measured three or four times to obtain two consecutive values that differed no >10%, and respecting an interval of at least 10 min between two measures. The withdrawal of hind paw was used to assess the nociceptive threshold.

Mechano-tactile allodynia (von-Frey hair test)

Mechano-tactile allodynia (non-noxious mechanical stimuli) was assessed as described by Chaplan et al. (1994). Rats were placed individually on an elevated mesh (1 cm^2 perforations) in a clear plastic cage and adapted to the testing environment for at least 15 min. von-Frey hairs (IITC, Woodland Hills, USA) with calibrated bending forces (g) of different intensities were used to deliver punctuates mechanical stimuli of varying intensity. von-Frey hairs were applied from below the mesh floor to the plantar surface of the hind paw, with sufficient force to cause slight bending against the paw, and held for 1 s. Each stimulation was applied five times with an inter-stimulus interval of 4–5 s. Care was taken to stimulate random locations on the plantar surface. A positive response was noted if the paw was robustly and immediately withdrawn. Paw withdrawal threshold was defined as the minimum pressure required to elicit withdrawal reflex of the paw, at least one time on the five trials.

Thermal hyperalgesia (tail immersion test)

Spinal thermal sensitivity was assessed by the tail immersion test as described by Necker and Hellon (1978). In this test, tail of rat was immersed in a water bath maintained at 55°C until tail withdrawal or signs of struggle were observed (cut-off time 15 s). The reaction time (i.e., the time necessary to observe the withdrawal of the tail from the bath) was measured 2–3 times to obtain two consecutive values that differed no >10%. The tail of the rat was immediately dried with a soft cellulose paper to avoid tail cooling between two measures. A shortened duration of tail withdrawal indicates thermal hyperalgesia.

Motor incoordination test (Rota-Rod test)

Motor incoordination was evaluated by a Rota-Rod device as described by Jones and Roberts (1968). Rats were placed for 1 min on the rotating rod of Rota-Rod apparatus (Techno Rotarod, Lucknow, India). The time taken for the falling of the rat from the rotating rod, during the period of 1 min was recorded.

Motor nerve conduction velocity (MNCV)

The recording of MNCV was performed in rats as described by Morani and Bodhankar (2008). Rats were anesthetized using thiopental sodium (50 mg/kg, i.p.) for electrophysiological recording. Dorsal sides of rats paw were shaved with hair removal cream and cleaned using moist cotton plug. Motor nerve conduction velocity was recorded by stimulating the sciatic and tibial nerves at sciatic and tibial notch, respectively, by 200 μs square wave pulse delivered through a pair of monopolar needle electrodes (1.0–1.5 mA, 2.0 mV/D) through a stimulator (Weltronics, India). Responses were recorded from the plantar muscles using data acquisition system (AD Instrument Pvt. Ltd. With software LABCHART 6, Bella Vista NSW 2153, Australia). The MNCV was determined using following formula:

$$\text{Motor nerve conduction velocity} = \{[\text{Distance between sciatic and tibial stimulation point (m)}]/[\text{latency for sciatic (s)} - \text{latency for tibial (s)}]\}$$

Biochemical estimations

Sciatic nerve homogenate preparation

A 50 mg sciatic nerve samples was rinsed with ice-cold saline (0.9% sodium chloride), minced and homogenized at 3,000 rpm in chilled tris buffer (10 mM, pH 7.4) and diluted up to 5 ml. The homogenates were centrifuged at 10,000*g* at 0°C for 20 min to obtain supernatant sample volume of 4.5 ml. It was divided into aliquot to determine, total protein estimation (0.1 ml), nitric oxide (NO) content (0.5 ml), membrane-bound inorganic phosphate enzyme (0.2 ml), malondialdehyde (MDA content) (2.0 ml), myeloperoxidase (MPO content) (2.0 ml) and DNA isolation (0.2 ml).

Estimation of total protein

Protein concentration was estimated according to the method of Lowry et al. (1951), using bovine serum albumin (BSA) as a standard.

Estimation of nitrite level

The NO level was estimated as nitrite by the acidic Griess reaction after reduction in nitrate to nitrite by vanadium trichloride according to the method described by Miranda et al. (2001). The Griess reaction relies on a simple colorimetric reaction between nitrite, sulfonamide and *N*-(1-naphthyl) ethylenediamine to produce a pink azo-product with maximum absorbance at 543 nm. The concentrations were determined using a standard curve of sodium nitrate and the results were expressed in μg/ml.

Estimation of membrane-bound inorganic phosphate (Na^+–K^+-ATPase)

Membrane-bound inorganic phosphate (Na^+–K^+-ATPase) was estimated in sciatic nerve as described by Bonting (1970). In brief, 1.0 ml of tris-hydrochloride buffer and 0.2 ml each of magnesium sulfate, sodium chloride, potassium chloride, EDTA, ATP were added to test tube containing 0.2 ml of sciatic nerve homogenate. The mixture was incubated at 36°C for 15 min. The reaction was arrested by the addition of 1.0 ml of 10% TCA, mixed well and centrifuged. The enzyme activity was expressed in μM of inorganic phosphorus liberated/mg protein/min.

Estimation of MDA content

Malondialdehyde (MDA) levels in the neural tissue were determined by the method of Slater and Sawyer (1971). The values were expressed in nanomoles of MDA/mg protein.

Estimation of MPO content

The MPO assay was assessed as a marker of neutrophil infiltration according to the method described by Krawisz et al. (1984). The values were expressed in units per grams (U/g).

Study of DNA fragmentation as an index of apoptosis

DNA isolation from neural tissue was performed according to standard phenol chloroform cetyl trimethyl ammonium bromide (CTAB) method mentioned by Tiwari et al. (2005). 10 μl of the DNA, isolated from the nerve homogenate was added to 3 μl of loading buffer (20 ml of glycerol 50%, 25 mg of bromophenol blue and three drops of 1 N NaOH) and subjected to 2D gel electrophoresis in 2% agarose gel. The gel was examined in gel documentation instrument (Alpha innotech) and gel image was captured.

Histopathological analysis

Samples of sciatic nerve were kept in the fixative solution (10% formalin), cut into 5 μm thickness and stained with hematoxylin and eosin as described by Yukari et al. (2004). Nerve sections were analyzed qualitatively under light microscope (400×) for axonal degeneration and various histopathological alterations.

Statistical analysis

Data were expressed as mean ± standard error mean (SEM). Data analysis was performed using GraphPad Prism 5.0 software (GraphPad, San Diego, CA). Data of behavioral tests were statistically analyzed using two-way repeated analysis of variance (ANOVA), Bonferroni's multiple range test was applied for post hoc analysis, while data of biochemical parameters were analyzed using one-way analysis of variance (ANOVA), Dunnett's multiple range test was applied for post hoc analysis. A value of $P < 0.05$ was considered to be statistically significant.

Results

Effect of quercetin on mechanical hyperalgesia in Randall Selitto paw pressure test

Before administration of the ethanol, mean paw withdrawal threshold in ethanol control rats (280.00 ± 13.22 g) on day 0 was not significantly different from that in normal rats (282.50 ± 7.15 g). Ethanol administration was associated with the development of mechanical hyperalgesia as reflected by decrease in the hind paw withdrawal threshold. When compared with normal rats, a significant decrease ($P < 0.001$) in mean paw withdrawal threshold was produced in the ethanol-administered rats after 6 weeks (122.50 ± 7.15 g) which gradually increased up to tenth week (127.50 ± 6.42). Chronic treatment with quercetin (20 and 40 mg/kg) for 10 weeks resulted significant increase ($P < 0.001$) in mean paw withdrawal threshold (180.00 ± 7.74 and 207.50 ± 11.88 g, respectively) as compared to ethanol control rats. However, this increase (215.00 ± 10.00 g) was more significant ($P < 0.001$) in rats treated with α-tocopherol (100 mg/kg) as compared to ethanol control rats. There was no significant change in the mean paw withdrawal

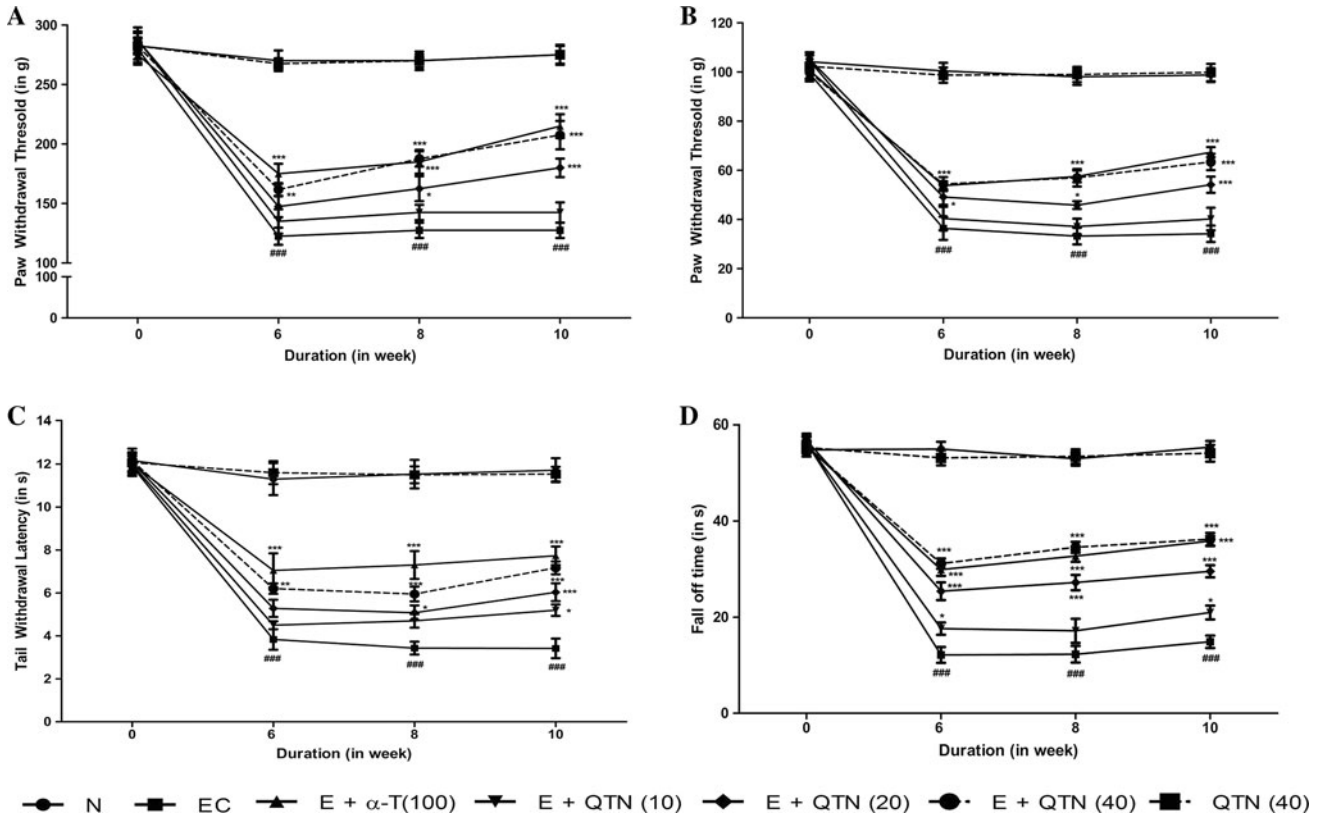

Fig. 1 Effect of chronic treatment of quercetin and α-tocopherol on mechanical hyperalgesia in paw pressure test (**a**), mechanical allodynia in von-Frey hair test (**b**), thermal hyperalgesia in tail immersion test (**c**), motor incoordination test (**d**). Data are expressed as mean ± SEM from five rats and analyzed by two-way ANOVA followed by Bonferroni's test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared to ethanol treated animals and $^{\#\#\#}P < 0.001$ as compared to normal animals ($n = 5$)

threshold of normal rats and per se group over the same time period (Fig. 1a).

Effect of quercetin on mechanical allodynia in von-Frey hair test

The mean paw withdrawal threshold in ethanol control rats before the administration of ethanol on day 0 was (99.43 ± 3.23 g) was not significantly different from that in normal rats (104.18 ± 2.61 g). Chronic ethanol administration for 6 weeks resulted in significant decrease ($P < 0.001$) in paw withdrawal threshold (36.41 ± 4.70 g) in ethanol control rats as compared to normal rats. The paw withdrawal threshold in ethanol control rats was gradually reduced (34.20 ± 3.35 g) up to tenth week. The treatment with quercetin (20 and 40 mg/kg) for 10 weeks significantly attenuated ($P < 0.001$) these decrease in paw withdrawal threshold (54.15 ± 3.28 and 63.41 ± 3.34 g, respectively) as compared to ethanol control rats. Rats treated with α-tocopherol (100 mg/kg) showed significant increase ($P < 0.001$) in paw withdrawal threshold (67.40 ± 2.04 g) as compared to ethanol control rats. There was no significant change in the mean paw withdrawal threshold of normal rats and per se group over the same time period (Fig. 1b).

Effect of quercetin thermal hyperalgesia in tail immersion test

Before administration of the ethanol, mean tail withdrawal latency in ethanol control rats (11.90 ± 0.45 s) was not significantly different from that in normal rats (12.16 ± 0.55 s). The tail withdrawal latency was significantly decreased ($P < 0.001$) in ethanol treated group (3.47 ± 0.45 s) after chronic administration of ethanol for 6 weeks and it was further reduced (3.41 ± 0.45 s) up to tenth week. The treatment with quercetin (10, 20, 40 mg/kg) for 10 weeks significantly ($P < 0.05$, $P < 0.001$ and $P < 0.001$, respectively) increased the tail withdrawal latency (5.20 ± 0.28, 6.03 ± 0.41 and 7.16 ± 0.30, respectively) as compared to ethanol control rats. However, α-tocopherol (100 mg/kg) treated rats more significantly increased mean tail withdrawal latency than quercetin (40 mg/kg). There was no significant change in the mean tail withdrawal latency of normal rats and per se group over the same time period (Fig. 1c).

Effect of quercetin on motor incoordination test

Mean fall off time in ethanol control rats (56.29 ± 1.36 s) before administration of ethanol on 0 day was not significantly different from that in normal rats (54.78 ± 1.38 s). Chronic administration of ethanol for 6 weeks significantly ($P < 0.001$) decreased the mean fall off time in ethanol control rats (12.12 ± 1.65 s) as compared to normal rats (54.93 ± 1.51 s). The treatment with quercetin (10, 20, 40 mg/kg) for 10 weeks significantly ($P < 0.05$, $P < 0.001$ and $P < 0.001$, respectively) restored this decrease in motor performance (20.94 ± 1.44, 29.51 ± 1.26 and 36.18 ± 1.30, respectively) as compared to ethanol control rats. Moreover, attenuation of decreased motor performance by α-tocopherol (100 mg/kg) was more significant than quercetin (40 mg/kg). There was no significant change in the mean fall off time of normal rats and per se group over the same time period (Fig. 1d).

Effect of quercetin on motor nerve conduction velocity

Before the administration of ethanol motor nerve conduction velocity on 0 day in ethanol control rats (41.43 ± 0.77 m/s) was not significantly different from that in normal rats (40.71 ± 0.75 m/s). Chronic administration of ethanol for 6 weeks significantly decreased ($P < 0.001$) motor nerve conduction velocity in ethanol control rats (16.12 ± 1.30 m/s) as compared to normal rats (40.56 ± 0.37 m/s) on sixth week. In ethanol control rats, motor nerve conduction velocity subsequently reduced up to 10 weeks and on tenth week, it was 12.35 ± 1.25 m/s. Chronic co-administration of quercetin (20 and 40 mg/kg) for 10 weeks significantly increased ($P < 0.001$) motor nerve conduction velocity (24.91 ± 0.98 and 28.92 35 ± 1.07 m/s respectively) as compared to ethanol control rats. Treatment with α-tocopherol (100 mg/kg) more significantly increased motor nerve conduction velocity than quercetin-(40 mg/kg) treated rats. There was no significant change in the motor nerve conduction velocity of normal rats and per se group over the same time period (Fig. 2).

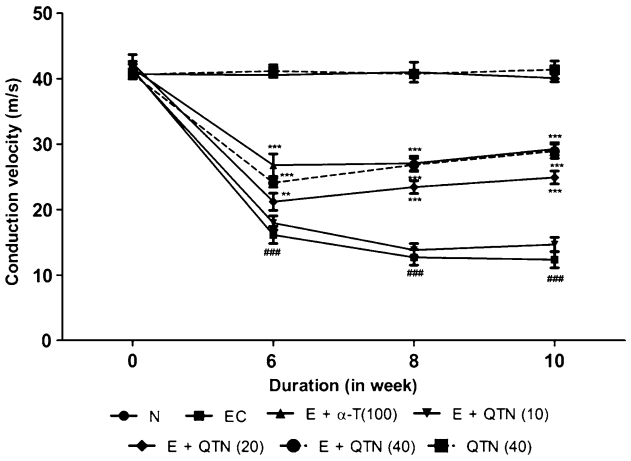

Fig. 2 Effect of chronic treatment of quercetin and α-tocopherol on nerve conduction velocity. Data are expressed as mean ± SEM from five rats and analyzed by two-way ANOVA followed by Bonferroni's test. $^{**}P < 0.01$, $^{***}P < 0.001$ as compared to ethanol treated animals and $^{\#\#\#}P < 0.001$ as compared to normal animals ($n = 5$)

Effect of quercetin on ethanol induced alterations in nitrosative stress

Chronic administration of ethanol for 10 weeks resulted in significant increase ($P < 0.001$) in level of neural nitrite in ethanol control rats (457.3 ± 15.08 μg/ml) as compared to normal rats (132.2 ± 13.62 μg/ml). Neural nitrite level of quercetin treated rats (20 and 40 mg/kg) was significantly decreased (355.8 ± 14.53 and 247.3 ± 20.42 μg/ml, respectively, $P < 0.001$) as compared to ethanol control rats, whereas α-tocopherol (100 mg/kg) more significantly attenuated this elevated level of neural nitrite when compared with quercetin (40 mg/kg). There was no significant change in the neural nitrite level of normal rats and per se group over the same time period (Table 1).

Effect of quercetin on ethanol induced alterations in membrane-bound inorganic phosphate enzyme (Na^+–K^+ATPase level)

The Na^+–K^+ATPase level of sciatic nerve in ethanol control rats (4.70 ± 0.32 μmol/mg of protein) was significantly decreased ($P < 0.001$) as compared to normal rats (10.56 ± 0.50 μmol/mg of protein) after chronic administration of ethanol for 10 weeks. Chronic co-administration of quercetin (40 mg/kg) for 10 weeks significantly elevated ($P < 0.001$) these decreased neural Na^+–K^+-ATPase level (8.32 ± 0.41 μmol/mg of protein) as compared to ethanol control rats (Table 1).

Effect of quercetin on ethanol induced alterations in malondialdehyde level

Chronic administration of ethanol for 10 weeks resulted in significant increase ($P < 0.001$) level of MDA in ethanol control rats (9.88 ± 0.36 nM/mg of protein) as compared to normal rats (2.55 ± 0.12 nM/mg of protein). Malondialdehyde level of quercetin treated rats (20 and 40 mg/kg) was significantly decreased (5.50 ± 0.42 and 4.12 ± 0.51 nM/mg of protein, respectively, $P < 0.001$) as compared to ethanol control rats. Moreover, this decrease in MDA level was more in rats treated with α-tocopherol (100 mg/kg) than quercetin (40 mg/kg). There was no

Table 1 Effect of quercetin on ethanol induced alterations in nitrite, Na^+–K^+ATPase, MDA and MPO levels

Treatment	NO (μg/ml)	Na^+–K^+-ATPase (μmol/mg of protein)	MDA (nM/mg of protein)	MPO (U/mg)
Normal	132.2 ± 13.62	10.56 ± 0.50	2.55 ± 0.12	0.48 ± 0.09
Ethanol control	457.3 ± 15.08$^{\#\#\#}$	4.70 ± 0.32$^{\#\#\#}$	9.88 ± 0.36$^{\#\#\#}$	1.80 ± 0.15$^{\#\#\#}$
E + α-T (100)	184.1 ± 14.02	9.07 ± 0.43	3.51 ± 0.61***	0.66 ± 0.09***
E + QTN (10)	432.2 ± 24.39	5.80 ± 0.47	8.12 ± 0.12	1.42 ± 0.12
E + QTN (20)	355.8 ± 14.53***	6.51 ± 0.72	5.50 ± 0.42***	1.02 ± 0.05**
E + QTN (40)	247.3 ± 20.42***	8.32 ± 0.41***	4.12 ± 0.51***	0.73 ± 0.08***
QTN (40)	152.4 ± 13.59	9.98 ± 0.40	2.81 ± 0.31	0.58 ± 0.07

Data are expressed as mean ± SEM from five rats and analyzed by one-way ANOVA followed by Dunnett's test

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared to ethanol treated animals and $^{\#\#\#}P < 0.001$ as compared to normal animals ($n = 5$)

significant change in the MDA level of normal rats and per se group over the same time period (Table 1).

Effect of quercetin on ethanol induced alterations in myeloperoxidase level

The MPO level of sciatic nerve in ethanol control rats (1.80 ± 0.15 U/mg) was significantly increased ($P < 0.001$) as compared to normal rats (0.48 ± 0.09 U/mg) after chronic administration of ethanol for 10 weeks. Chronic co-administration of quercetin (20 and 40 mg/kg) for 10 weeks significantly attenuated ($P < 0.01$ and $P < 0.001$, respectively) these increase in neural MPO level (1.02 ± 0.05 and 0.73 ± 0.08 U/mg, respectively) as compared to ethanol control rats. Rats treated with α-tocopherol (100 mg/kg) showed more reduced MPO level than quercetin (40 mg/kg) treated rats (Table 1).

Effect of quercetin on DNA fragmentation

Chronic administration of ethanol for 10 weeks resulted higher degree of apoptosis which was reflected by maximum fragmentation of DNA. Chronic co-administration of α-tocopherol (100 mg/kg) and quercetin (20 and 40 mg/kg) showed lower degree of DNA fragmentation which was not observed in normal and per se group (Fig. 3).

Histopathology

Chronic ethanol administration resulted in significant histopathological changes assessed in cross-sectional section of sciatic nerve. The swelling of nonmyelinated and myelinated nerve fibers along with decrease in number of myelinated fibers were observed in sciatic nerve of ethanol control rat. Chronic ethanol administration for 10 weeks resulted in neutrophilic infiltration (blue arrow), macrophages infiltration (green arrow), congestion (red arrow), edema (yellow arrow), necrosis (black arrow) and vacuolation (white arrow) in sciatic nerve. Chronic co-

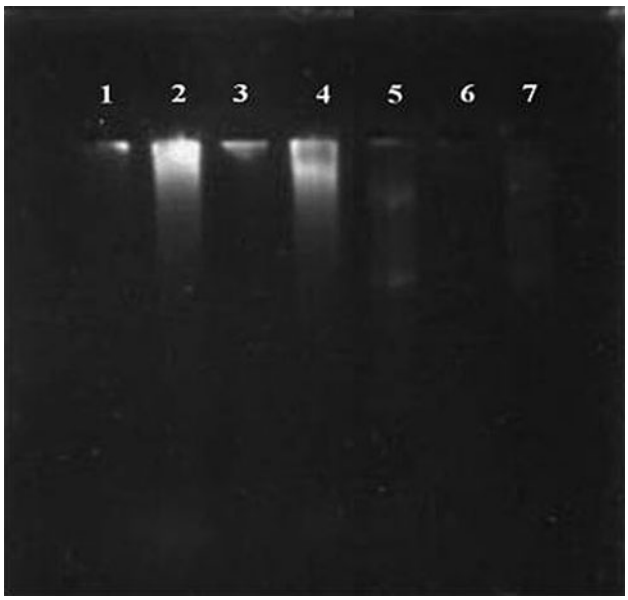

Fig. 3 Effect of chronic treatment of quercetin and α-tocopherol on DNA fragmentation in alcohol induced neuropathy. *Lane 1* normal, *lane 2* ethanol control, *lane 3* ethanol + α-tocopherol (100 mg/kg), *lane 4* ethanol + quercetin (10 mg/kg), *lane 5* ethanol + quercetin (20 mg/kg), *lane 6* ethanol + quercetin (40 mg/kg) and *lane 7* quercetin (40 mg/kg)

administration of α-tocopherol (100 mg/kg) and quercetin (40 mg/kg) attenuated the swelling of nonmyelinated and myelinated nerve fibers, axonal degeneration and histopathological alterations that were associated with the ethanol induced neuropathy (Fig. 4; Table 2).

Discussion

The present investigation revealed the neuroprotective effect of quercetin against in alcohol induced neuropathic pain in rats. Rats with long-term ethanol consumption have been proven to demonstrate characteristic tactile allodynia and mechanical hyperalgesia (Tiwari et al. 2009). According to the previous studies, hyperalgesia was

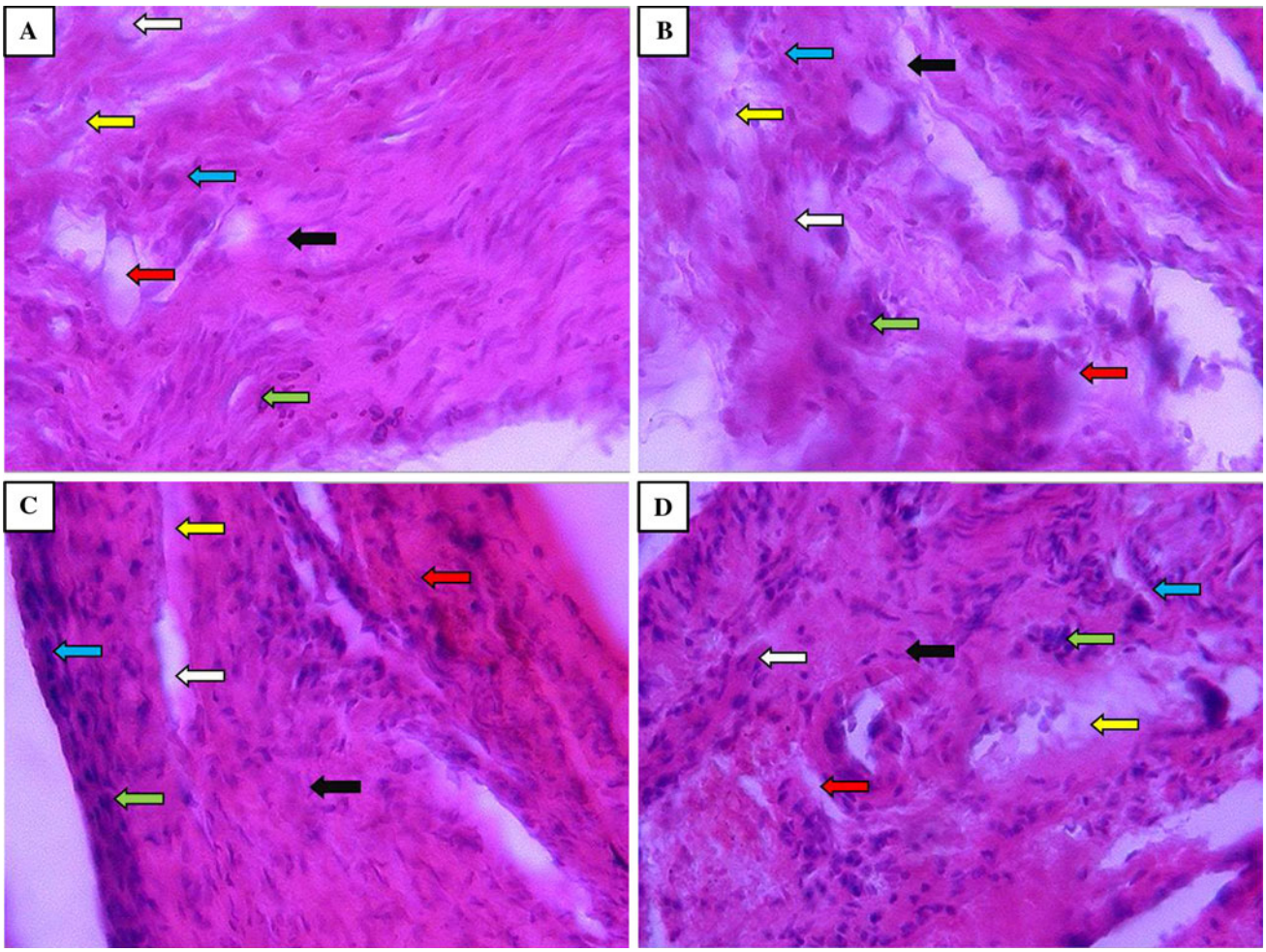

Fig. 4 Effect of chronic treatment of quercetin and α-tocopherol on ethanol induced axonal degeneration. Photomicrographs of sections of sciatic nerve from rats stained with H&E. Sciatic nerve microscopic image of **a** normal rat, **b** ethanol control rat, **c** α-tocopherol (100 mg/kg) and **d** quercetin (40 mg/kg) (microscopic examination under ×400 light microscopy)

Table 2 Effect of quercetin and α-tocopherol on pathological changes of rat sciatic nerve in alcohol induced neuropathy

Groups	Infiltration of neutrophils	Infiltration of macrophages	Congestion	Edema	Necrotic changes in nerve	Nerve cell vacuolation
Normal	+	+	+	–	–	+
Ethanol control	++++	+++	+++	+++	++++	+++
E + α-T (100)	+	+	+	+	+	+
E + QTN (40)	+	++	++	+	++	+

0 no abnormality detected, + damage/active changes up to <25%, ++ damage/active changes up to <50%, +++ damage/active changes up to <75%, ++++ damage/active changes up to >75%

evident at 6 weeks of ethanol consumption and consistently increased up to 10 weeks (Miyoshi et al. 2007). In present investigation, chronic treatment with quercetin increased the threshold for both tactile allodynia as well as mechanical hyperalgesia.

During nerve injury, release of inflammatory mediators, such as bradykinin, NGF, ATP or prostaglandin E_2 resulting in the activation of their respective receptors which in turn activates PKA, PKC and PLC. Activation of these intracellular messengers opens TRPV1 ion channel and resulted in the development of heat hyperalgesia (Michael and Priestley 1999; Amaya et al. 2003). Previous studies have shown that patients suffering from peripheral neuropathies due to chronic consumption of alcohol had impaired motor coordination. This might be due to the distinct morphological changes in the soleus and extensor

digitorum longus (EDL) muscles which were studied in rat (Torrejais et al. 2002). In present investigation, chronic treatment of quercetin improved the motor coordination.

Chronic ethanol administration is associated with decreased motor nerve conduction velocity (Tredici and Minazzi 1975). The mechanism is either segmental demyelination or axonal degeneration (Mawdsley and Mayer 1965). Acetaldehyde is one of the neurotoxic metabolite of the ethanol which increases the reactive oxygen species (Mantle and Preedy 1999). Previous studies have shown that increased oxidative stress leads to damage of protein, lipid and DNA (Mansouri et al. 2001; McDonough 2003). Injury to glial cells due to oxidative stress results in demyelination which causes decreased conduction velocity (Dobretsov et al. 2007). In the present investigation, quercetin improved the MNCV.

Oxidative stress and abnormalities of NO production in the endothelium of epi- and endoneurial blood vessels causes impaired endoneurial blood flow and endoneurial hypoxia (Dobretsov et al. 2007). Quercetin might have increased neural blood flow by its vasorelaxant activity (Cora and Cecil 1996).

Nitric oxide is associated with neuropathic pain (Levy and Zochodne 2004). It sensitizes spinal neurons as well as central neurons (Lin et al. 1999). Furthermore, upregulated production of NO react with deficient superoxide dismutase to produce peroxynitrite. In the present study, chronic treatment with quercetin resulted in decreased neural NO level by virtue of its free-radical scavenging property.

Decrease in membrane-bound inorganic phosphate enzyme Na–K-ATPase is associated with the failure of Na–K pump, which plays an important role in the maintenance of cellular electrolyte balance (Chiu 1993). Decreased Na–K-ATPase level is associated with decreased nerve conduction velocity. Chronic treatment with quercetin restored the decreased level of Na–K-ATPase indicating the role of Na–K-ATPase in nerve conduction velocity.

Increased MDA level is associated with decreased antioxidant defense enzymes in the neural tissues. Elevated level of MDA in sciatic nerve serves as a hall mark of lipid peroxidation (Ohkawa et al. 1979). Structural destruction of unsaturated fatty acids in lipid membrane causes tissue damage due to generation of reactive oxygen species (Cheesman 1993). Chronic alcohol administration resulted in increased level of MDA indicating a role of MDA in generation and maintenance of oxidative stress in the development of alcoholic neuropathy in rats (Tiwari et al. 2009). Quercetin was able to inhibit this elevated level of MDA through inhibition of generation of free radicals.

Elevated level of MPO is a marker of polymorphonuclear leukocyte accumulation (Bradley et al. 1982) which is widely used to detect the inflammatory processes in various tissues. The elevated level of MPO in sciatic nerve is positively correlated with the increase in levels of MDA indicating an increase in lipid peroxidation in alcohol induced neuropathic pain. Increased level of MPO as well as MDA can initiate a vicious cycle that generates reactive oxygen species that damage cellular antioxidants leads to further inflammation (Ohkawa et al. 1979; Bradley et al. 1982). The ability of quercetin to reduce granulocyte infiltration is indicated by decreased levels of MPO. The same trend was confirmed histologically.

There is evidence in literature regarding the role of vitamin E (α-tocopherol) in oxidative stress-mediated activation of PKC signaling which protect against alcohol induced neuropathic pain. It also inhibited elevated level of nitrosative stress as well as inflammatory cytokines, such as TNF-α and IL-1β (Tiwari et al. 2009).

Recently antioxidants like quercetin and vitamin E (α-tocopherol) have been screened in clinical trials for the treatment of neuropathic pain (Valensi et al. 2005; Pace et al. 2010). Hence, it can be concluded from the present investigation that quercetin elucidates its neuroprotective effect in alcohol induced neuropathic pain through modulation of membrane-bound inorganic phosphate enzyme and inhibition of release of oxido-inflammatory mediators, such as MDA, MPO and NO.

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