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ROSMARINIC ACID AMELIORATES MEMORY DETERIORATION AND OXIDATIVE STRESS IN STREPTOZOTOCIN INDUCED DIABETIC RATS

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ABSTRACT

Diabetes induced memory dysfunction is found to be equivalent with Alzheimer's disease, disturbing normal physical, mental and social life of patients. Rosmarinic acid is flavonoid known to have pharmacological action against diabetes and diabetic complications. However, its effect in diabetic induced memory dysfunction and related oxidative stress has not been evaluated. Hence, present study was designed, conducted and analyzed for assessment of diabetes induced memory deterioration and oxidative changes in various parts of brain in Streptozotocin (STZ) induced diabetic rats. Diabetes was induced by streptozotocin (60 mg/kg, i.p.) in rats. After confirmation of diabetes treatment of Rosmarinic acid (12.5, 25, 50 mg/kg, p.o.) was started in separate group of animals. After 30 days diabetic rats showed significant memory deficits and also increased cholinesterase and MDA levels and depleted reduced glutathione levels in hippocampus and cerebral cortex. Moreover, chronic treatment of Rosmarinic acid had substantial influence on memory deficit in diabetic rats. Additionally, treatment of Rosmarinic acid significantly decreases blood glucose levels and MDA levels. Further, GSH levels found to be elevated in rosmarinic acid in diabetic rats. Also, cholinesterase levels are reduced which found to be elevated in diabetic rats. The result obtained from study points that treatment with Rosmarinic acid in diabetic rats ameliorates memory loss and oxidative stress.

KEYWORDS: Rosmarinic acid, Diabetes, Memory dysfunction, Acetylcholinesterase, oxidative stress, Novel object recognition test.

INTRODUCTION

Diabetes is a chronic carbohydrate metabolic disorder that occurred due to defective endocrine system. Diabetes is occurred due to deficiency of insulin which is either total or partial and/or inability of insulin receptor to free insulin i.e., insulin resistance. Prolonged elevation in blood glucose levels is responsible for diabetic complications as well as amplified oxidative stress. This collaborative upsets result in end organ damage and therefore impaired function of resulted organ.[1-3]

Diabetes leads to deterioration of Central Nervous System which is responsible for functional changes like diabetic depression, memory deficits, risk of Alzheimer's disease, cerebral ischemia. It is demonstrated that diabetic memory deficit might be a consequence of neurochemical, neurophysiological, neuronal death and structural abnormalities.[3-6] In clinical practice, the diabetics are found to have decreased performance at measurement of memory, cognitive flexibility, rapid information processing and psychomotor efficacy.[4,7,8] Different aspects that are responsible for diabetic memory deficit are hyperglycemia, elevated oxidative stress, increased cholinesterase levels, resistance to insulin, vascular diseases etc.[4, 9-13]

Flavonoid Rosmarinic acid is pharmacologically active phytochemical and natural antioxidant that have been investigated for its possible role in protection against and prevention of pathologies and various other activities such as diabetes, memory and diabetic complications etc.[14-19] Recently, it is reported to ameliorate spatial memory impairment and inhibition of pathophysiological mechanisms of it like inhibition of acetylcholine esterase, peroxynitrites, expression of senile plaques and anti-oxidant.[20-22]

MATERIALS AND METHODS

Male Sprague dawley rats (200- 250 gm.) were used in the present study. The animals were maintained under standard laboratory conditions at temperature 23 ± 2°C, relative humidity 55 ± 10% and 12:12 h light (08:00 – 20:00 h) /dark cycle maintained throughout the experiment. Animals had free access of water and standard laboratory feed ad libitum prior to the dietary manipulation. The animal studies were approved by the Institutional Animal Ethics Committee (IAEC/CPD/415/004), constituted for the purpose of

control and supervision of experimental animals by Ministry of Environment and Forests, Government of India, New Delhi, India. Animals were naive to drug treatments and experimentation at the beginning of all studies. All tests were conducted between 08:00 and 14:00 h.

Drugs and solutions

Rosmarinic acid (Sigma Aldrich), Streptozotocin (STZ) (Enzo Life sciences, UK) was used. STZ was dissolved in freshly prepared 0.1M citrate buffer (pH 4.4). Rosmarinic acid dissolved in saline. All drug solutions were prepared fresh. All other agents used were of analytical grade. The doses were selected as Rosmarinic acid [12.5, 25, 50 mg/kg, p.o.] were selected on the basis of previous reports.[16,23]

Induction of experimental diabetes

Diabetes was induced in rats by using streptozotocin (STZ).[4] In brief, STZ (60mg/kg, i.p.) was dissolved in 0.1 M citrate buffer of pH 4.4. After administration of STZ rats were provided with 5% glucose solution for next 24 h to avoid hypoglycemic shock. Blood samples were taken from the tail vein 48 h after STZ injection to measure blood glucose levels. Only animals with fasting blood glucose levels above 250 mg/dl were considered diabetic and used for the further study.

Treatment schedules

Treatment 1: Chronic treatment

As soon as diabetes was confirmed, separate groups of rats (n = 6) were administered orally with Rosmarinic acid (12.5, 25, 50 mg/kg, p.o.) or vehicle (1 ml/kg) twice daily (08:00–20:00 h) for next 30 days (day 1–30) and at the end of this treatment schedule rats were subjected to Object recognition test. Similar treatments were given to control (non-diabetic) rats.

Treatment 2: Acute treatment

In another set of experiment, 30 days after confirmation of diabetes, rats (n=6) were administered orally with Rosmarinic acid (12.5, 25, 50 mg/kg, p.o.) or vehicle (1 ml/kg) twice daily (08:00–20:00 h) for next 5 days (day 31–35) and at the end of this treatment schedule rats were subjected to Object recognition test. Similar treatments were given to control (non-diabetic) rats.

Assessment of behavioral paradigm

Novel object recognition test

After treatment with rosmarinic acid in respective treatment schedules, rats were tested for the novel object recognition test. Memory was evaluated at two retention intervals (30 min & 24 h) as described earlier.[4,24] Rats were transported from the animal vivarium to the testing laboratory and allowed to acclimatize to testing environment for at least 60 min before behavioral testing began. The test was performed in the open field arena 72×72×36 cm as previously described.[25] Each rat was exposed to three experimental conditions in the open field. In the initial trial, one object-stimulus (O1) was placed in one corner of the open field and the rat positioned in the opposite corner of the arena, and time spent exploring the object was measured. The session was terminated at cut-off time 10 min. During the second trial, performed at 30 min retention time interval following T1, a second object (O2) was introduced in the adjacent corner to that of the reference object. The time spent exploring the familiar (O1) and the novel (O2) objects was measured for a period of 10 min. In the final trial performed 24 h following T1, O2 was replaced by a new object (O3) and the time the rat spent exploring the reference (O1) and novel (O3) objects was measured for 10 min. After each trial, the objects and arena were cleaned with a 70% ethanol solution in order to remove or spread odor cues. The objects consisted of plastic toys heavy enough to prevent the animals from moving them. Raw data obtained in the object recognition test were transformed into a ratio, reflecting the preference of the animals for the novel versus the familiar object. The ratio formula was $[t_{novel}/ (t_{novel} + t_{familiar})]$, where $t_{familiar}$ is the time spent exploring the familiar object and t_{novel} is the time spent exploring the new object, in seconds. The closer this ratio gets to 1, the more the animal spent time exploring the novel object.

Locomotor activity

One hour after the last testing in the object recognition test same rats were transferred to an open-field apparatus, measuring 72×72×36 cm, with the floor divided into 12 squares. The open field session lasted for 5 min and during this time, an observer, recorded the number of crossings responses manually.

Assessment of biochemical parameters from cerebral cortex and hippocampus Supernatant preparation

After behavioral tests rats were sacrificed by spinal dislocation and brain structures were removed and separated cerebral cortex and hippocampus for the biochemical studies. Tissue were rinsed with ice cold saline (0.9% sodium chloride) and homogenized in chilled 50mM phosphate buffer (pH 7.4). The homogenates were centrifuged at 4600 rpm for 10 min at 4°C to separate the nuclear debris. The supernatant thus obtained was centrifuged at 15,000 rpm for 30 min at 4°C to get the post mitochondrial supernatant, which was used to assay cholinesterase activity.

Assessment of cholinesterase activity

Cholinergic dysfunction was assessed by measuring cholinesterase (ChE) levels, according to the Ellman's method with slight modifications.[4,26-27] The assay mixture contained 0.05 ml of supernatant, 3ml of 0.01M sodium phosphate buffer (pH 8.0), 0.10 ml of 0.75mM acetylthiocholine iodide (AcSCh) and 0.10 ml Ellman reagent (5´5 dithiobis [2-nitrobenzoic acid] 10mM, NaHCO3 15 mM). The change in absorbance was measured at 412 nm for 5 min. Results were calculated using molar extinction coefficient of chromophore (1.36×104M−1 cm−1). All samples were run in duplicate

or triplicate and the enzyme activity were expressed in µmol AcSCh/min/g of wet tissue.

Assessment of MDA levels

Malondialdehyde (MDA), a product of lipid peroxidation was measured by method described earlier with slight modification.[4,28] Briefly, the sample of 0.1 ml supernatant was taken and mixed with 0.2 ml 8.1% sodium dodecyl sulphate (SDS), 1.5 ml 20% glacial acetic acid and 1.5 ml of 0.8% thiobarbituric acid (TBA). Following these additions, tubes were mixed and heated at 95°C for 1 h on a water bath and cooled under tap water before mixing 1ml of distilled water and 5ml mixture of n-butanol and pyridine (15:1). The mixture was centrifuged at 4000 rpm for 10 min. The amount of MDA formed was measured by absorbance of upper organic layer at a wavelength of 532 nm using appropriate controls. A calibration curve was plotted using malondialdehyde bis-(dimethoxy acetyl) as a standard. The values were expressed as nmol/g of wet tissue.

Assessment of glutathione (GSH) levels

Glutathione (GSH) estimation was done according to the method described earlier.[29] Briefly, 160 µl of supernatant was added to 2 ml of Ellman's reagent (5´5 dithiobis [2-nitrobenzoic acid] 10mM, NaHCO3 15mM) and the mixture was incubated at room temperature for 5 min and absorbance was read at 412 nm. The values are expressed as nmol/gm of wet tissue.

STATISTICAL ANALYSIS

Data was analyzed using one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The results are expressed as mean ± SEM. P<0.05 was considered statistically significant in all the cases.

RESULTS

Influence of acute treatment of Rosmarinic acid on body weight and blood glucose levels in streptozotocin-induced diabetic rats

One-way ANOVA revealed that acute treatment with Rosmarinic acid significantly reduced the blood glucose levels [F(5, 30)= 237.6, P<0.0001, Fig. 1A] but not having any influence on changes in body weight [F(5, 30)=228.8, P<0.0001, Fig. 1B]. Post hoc test further revealed that long standing diabetes significantly increased the blood glucose levels and reduced the body weights compared to age matched normal control rats (P<0.001). Further, treatment with Rosmarinic acid (50 mg/kg, p.o.) significantly decreased blood glucose levels (P<0.01). But, there was no significant influence of acute treatment of Rosmarinic acid against decrease in body weight in any of doses compared with control diabetic group.

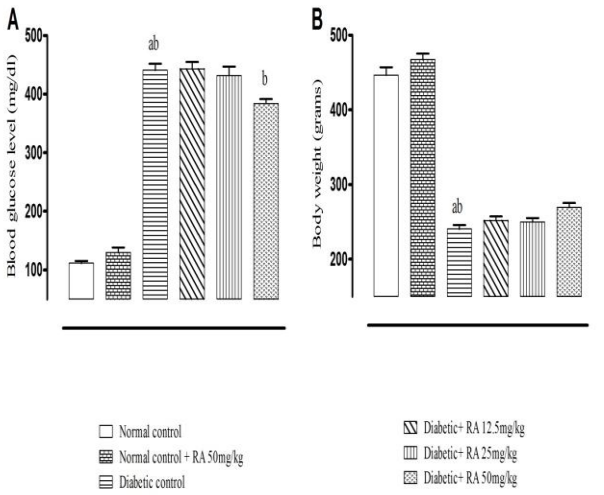

Fig 1 A & B: Effect of acute treatment of Rosmarinic acid on blood glucose level and body weight.

$^{ab}P< 0.001$ compared to normal control rat, *$^{b}P< 0.01$* compared to diabetic rats.

Influence of chronic treatment of Rosmarinic acid on body weight and blood glucose levels in streptozotocin-induced diabetic rats

One-way ANOVA revealed that chronic treatment with Rosmarinic acid significantly reduced the blood glucose levels [F(5, 30)= 248.3, P<0.0001, Fig. 2A] and ameliorates diabetes induced changes in body weight [F(5, 30)= 158.5, P<0.0001, Fig. 2B]. Post hoc test further revealed that long standing diabetes significantly increased the blood glucose levels and reduced the body weights compared to age matched normal control rats (P<0.001). Further, chronic treatment with Rosmarinic acid (12.5, 25, 50 mg/kg, p.o.) significantly reduced the blood glucose levels and also had influence against loss of body weight (P<0.05, P<0.001) compared to control diabetic group.

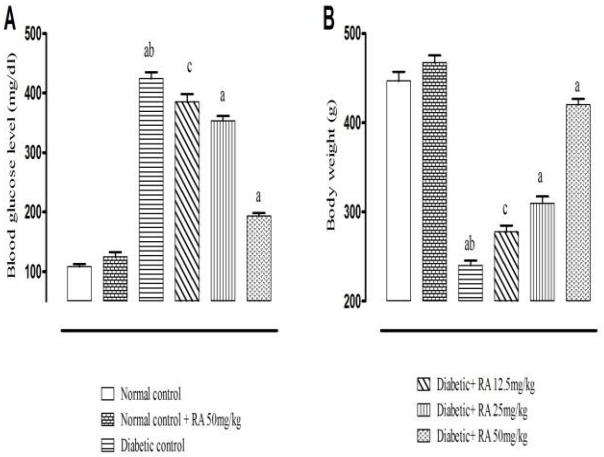

Fig 2 A & B: Effect of chronic treatment of Rosmarinic acid on blood glucose level and body weight.

[ab]P< 0.001 compared to normal control rat, [a]P< 0.001 compared to diabetic rats, [c]P< 0.05 compared to diabetic rats.

Influence of acute treatment of Rosmarinic acid on memory in Novel object recognition test

The object recognition test performance was measured in-terms of the investigation ratios for the two retention intervals (30 min and 24 h, respectively). One-way ANOVA revealed influence of Rosmarinic acid on memory deficits in short term 30 min retention interval [F (5, 30) = 170.2, P<0.0001, Fig. 3A] as well as at 24h retention trial [F (5, 30) = 146.3, P<0.0001, Fig. 3B]. Post hoc test revealed that STZ-induced diabetic rats explored significantly less to the novel object compared to control rats during both retention trials (P<0.001). Further, acute treatment with Rosmarinic acid (25, 50mg/kg, p.o.) had significant influence on investigation ratios (P<0.01 and P<0.001, respectively).

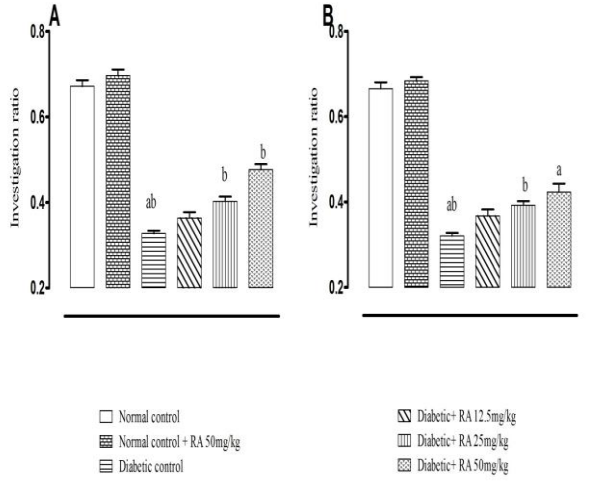

Fig 3 A & B: Effect of acute treatment of Rosmarinic acid on novel object recognition test at 30 min and 24 hr. retention time interval.

[ab]*P< 0.001* compared to normal control rat, [a]*P< 0.001* compared to diabetic rats, [b]*P< 0.01* compared to diabetic rats.

Influence of chronic treatment of Trigonelline on memory in Novel object recognition test in STZ-induced diabetic rats

The object recognition test performance was measured in-terms of the investigation ratios for the two retention intervals (30 min and 24 h, respectively). One-way ANOVA revealed influence of Rosmarinic acid on memory deficits in short term 30 min retention interval [F (5, 30) = 167.2, P<0.0001, Fig. 4A] as well as at 24h retention trial [F (5, 30) = 214.8, P<0.0001, Fig. 4B]. Post hoc test revealed that STZ-induced diabetic rats explored significantly less to the novel object compared to control rats during both retention trials (P<0.001). Similarly, shown significant decline in investigation ratio compared with vehicle treated diabetic rats (P<0.001). Further, chronic treatment of Rosmarinic acid (12.5, 25, 50mg/kg, p.o.) treatment had positive influence on investigation ratios (P<0.05, P<0.01, P<0.001).

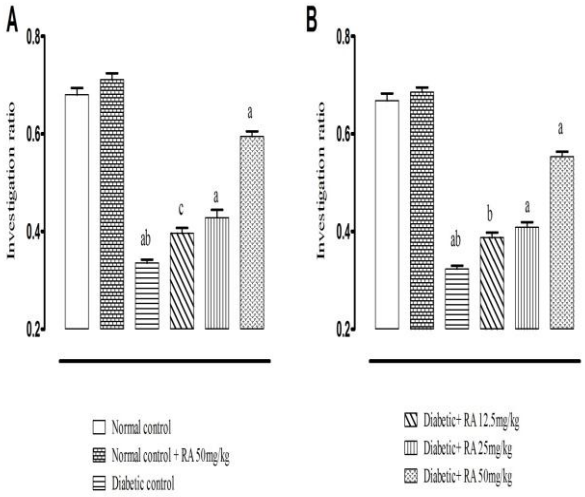

Fig 4 A & B: Effect of chronic treatment of Rosmarinic acid on novel object recognition test at 30 min and 24 hrs retention time interval.

[ab]*P< 0.001* compared to normal control rat, [a]*P< 0.001* compared to diabetic rats, [b]*P< 0.01* compared to diabetic rats, [c]*P< 0.05* compared to diabetic rats.

Influence of treatment of Rosmarinic acid on locomotor activity in open field test in STZ-induced diabetic rats

One-way ANOVA revealed that none of the acute and chronic treatments of Rosmarinic acid had significant influence on the locomotor activity in diabetic as well as non-diabetic rats [F (5, 30) = 0.1930, P=0.9629; F (5, 30) = 0.1986, P= 0.9605, Fig. 5].

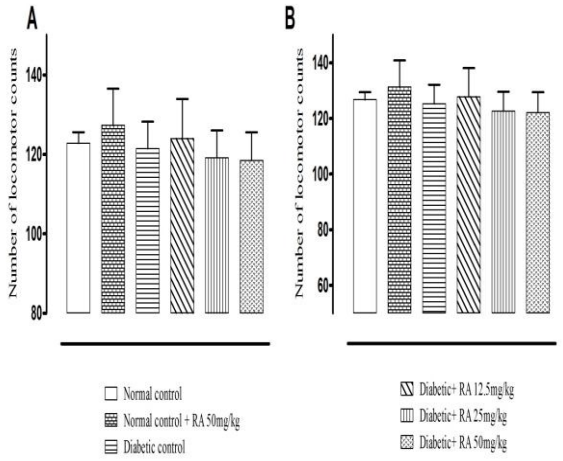

Fig 5: Effect of treatment of Rosmarinic acid on locomotor activity.

Influence of acute treatment of Rosmarinic acid on cholinesterase levels in hippocampus and cerebral cortex of STZ-induced diabetic rats

One way ANOVA revealed that there was significant change in cholinesterase levels in treatment groups from hippocampus [F(5, 30)= 34.14, P<0.0001, Fig. 6A] and cerebral cortex [F(5, 30)= 36.38 P<0.0001, Fig. 6B]. Post hoc test further revealed that treatment with Rosmarinic acid (25, 50 mg/kg, p.o.) significantly reduced hippocampal and cortical cholinesterase levels.

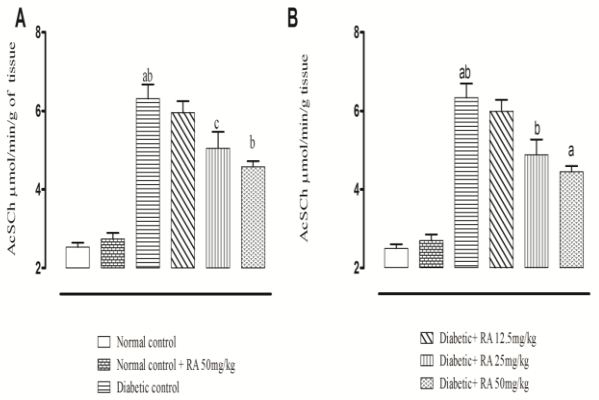

Fig 6 A & B: Influence of acute treatment of Rosmarinic acid on cholinesterase levels.

$^{ab}P< 0.001$ compared to normal control rat, $^{b}P< 0.01$ compared to diabetic rats, $^{c}P< 0.05$ compared to diabetic rats.

Influence of chronic treatment of Rosmarinic acid on cholinesterase levels in hippocampus and cerebral cortex of STZ-induced diabetic rats

One way ANOVA revealed that there was significant influence on cholinesterase levels in rosmarinic acid treatment groups in hippocampus [F(5, 30)= 35.63, P<0.0001, Fig. 7A] and cerebral cortex [F(5, 30)= 34.25, P<0.0001, Fig. 7B]. Post hoc test further revealed that treatment with Rosmarinic acid (25, 50 mg/kg, p.o.) had significant influence on hippocampal and cortical cholinesterase levels.

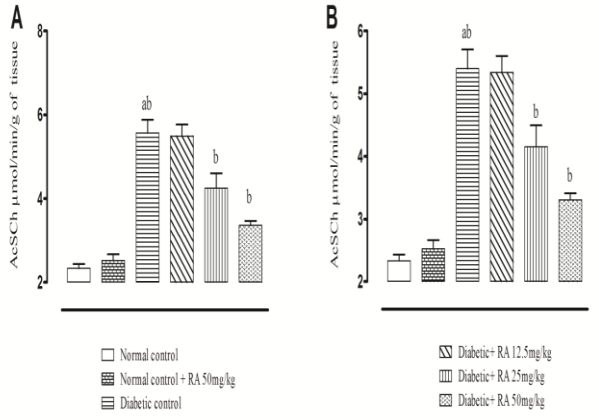

Fig 7 A & B: Influence of chronic treatment of Rosmarinic acid on cholinesterase levels.

$^{ab}P< 0.001$ compared to normal control rat, $^{a}P< 0.001$ compared to diabetic rats, $^{b}P< 0.01$ compared to diabetic rats.

Influence of acute treatment of Rosmarinic acid on MDA levels in hippocampus and cerebral cortex of STZ-induced diabetic rats

One way ANOVA revealed that there was significant change in MDA levels in treatment groups from hippocampus [F(5, 30)= 371.6, P<0.0001, Fig. 8A] and cerebral cortex [F(5, 30)= 193.3, P<0.0001, Fig. 8B]. Post hoc test further revealed that treatment with Rosmarinic acid (25, 50 mg/kg, p.o.) had significant influence on hippocampal and cortical MDA levels (P<0.01, P< 0.001).

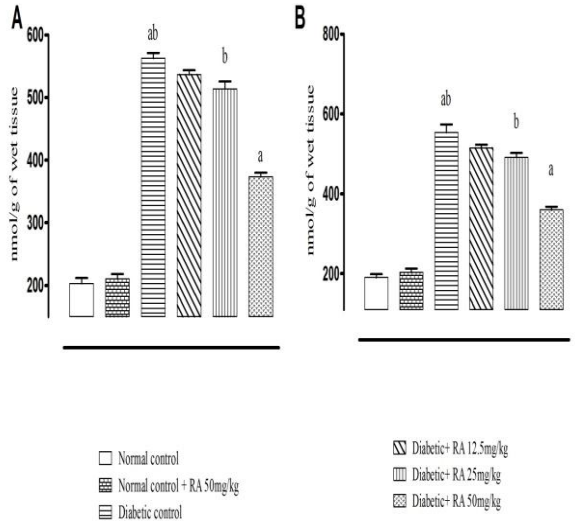

Fig 8 A & B: Influence of acute treatment of Rosmarinic acid on MDA levels.

$^{ab}P< 0.001$ compared to normal control rat, $^{a}P< 0.001$ compared to diabetic rats, $^{b}P< 0.01$ compared to diabetic rats.

Influence of chronic treatment of Rosmarinic acid on MDA levels in hippocampus and cerebral cortex of STZ-induced diabetic rats

One way ANOVA revealed that there was significant change in MDA levels in treatment groups from hippocampus [F(5, 30)= 364.9 P<0.0001, Fig. 9A] and cerebral cortex [F(5, 30)= 219.3, P<0.0001, Fig. 9B]. Post hoc test further revealed that treatment with Rosmarinic acid (25, 50 mg/kg, p.o.) has significant influence on hippocampal and cortical MDA levels (P< 0.001).

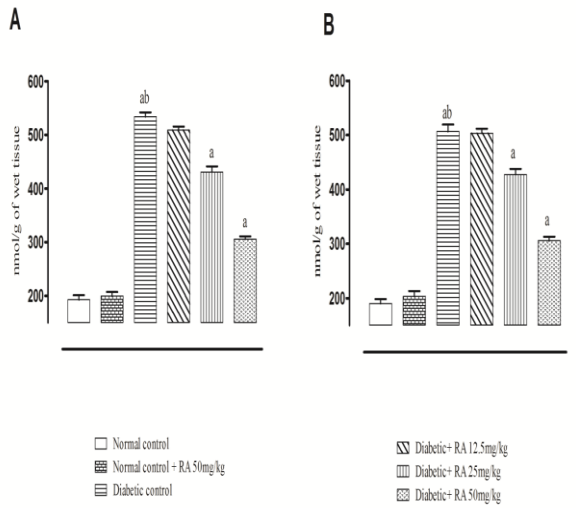

Fig 9 A & B: Influence of chronic treatment of Rosmarinic acid on MDA levels.
$^{ab}P< 0.001$ compared to normal control rat, $^{a}P< 0.001$ compared to diabetic rats.

Influence of acute treatment of Rosmarinic acid on GSH levels in hippocampus and cerebral cortex of STZ-induced diabetic rats

One way ANOVA revealed that there was significant change in GSH levels in treatment groups from hippocampus [F(5, 30)= 317.6, P<0.0001, Fig. 10A] and cerebral cortex [F(5, 30)= 216.4, P<0.0001, Fig. 10B]. Post hoc test further revealed that treatment with Rosmarinic acid (25, 50 mg/kg, p.o.) had significant influence on hippocampal and cortical GSH levels.

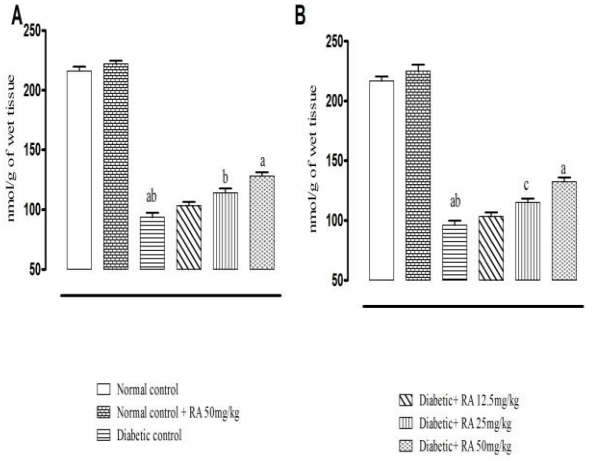

Fig 10 A & B: Influence of acute treatment of Rosmarinic acid on GSH levels.
$^{ab}P< 0.001$ compared to normal control rat, $^{a}P< 0.001$ compared to diabetic rats, $^{b}P< 0.01$ compared to diabetic rats, $^{c}P< 0.05$ compared to diabetic rats.

Influence of chronic treatment of Rosmarinic acid on GSH levels in hippocampus and cerebral cortex of STZ-induced diabetic rats

One way ANOVA revealed that there was significant change in GSH levels in treatment groups from hippocampus [F(5, 30)= 218.3, P<0.0001, Fig. 11A] and cerebral cortex [F(5, 30)= 146.2, P<0.0001, Fig. 11B]. Post hoc test revealed that treatment with Rosmarinic acid (25, 50 mg/kg, p.o.) significantly increased hippocampal and cortical GSH levels (P< 0.001).

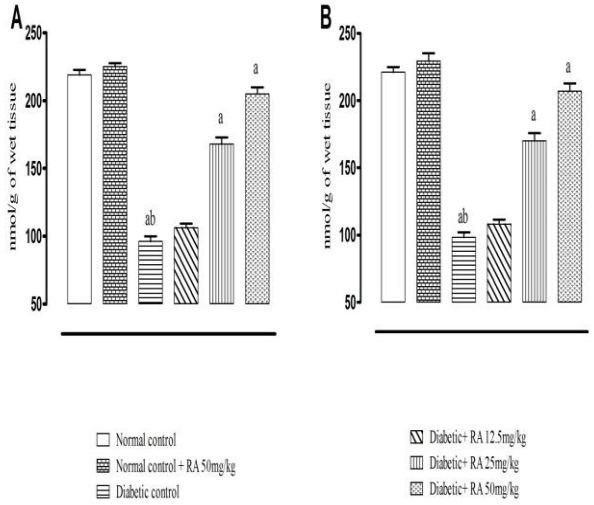

Fig 11 A & B: Influence of chronic treatment of Rosmarinic acid on GSH levels.
$^{ab}P< 0.001$ compared to normal control rat, $^{a}P< 0.001$ compared to diabetic rats.

DISCUSSION

The present study was designed, carried out and analyzed for the effect of Rosmarinic acid on diabetic dementia and physical and biochemical changes during this diabetic complication. STZ- induced diabetic rats is well documented model for study of diabetes and diabetic complications. The diabetic rats in this experiment showed marked reduction in body weight and higher levels of blood glucose levels. Further, diabetic rats showed decreased memory performance may be due to elevated oxidative stress. In present study, Novel object recognition task was used to evaluate learning and memory. The results of study suggest that treatment with Rosmarinic acid leas to reduction in elevated glucose levels and oxidative stress. Also, the impairment in memory was evaluated through investigation ratio studied by novel object recognition task. The results of the present study are in line that diabetic rats exhibit reduced memory.[4, 30-34] The results of present study indicate that treatment with Rosmarinic acid improves cognitive performance in novel object recognition task. Furthermore, all groups indicating unaffected motor performance which is confirmed by indifferent locomotor counts in open field in diabetic and non-diabetic rats.

Rosmarinic acid is well reported in streptozotocin-induced diabetic complications.[15,16,19,23,35] It also has ability to inhibit enzyme alpha-glucosidase inhibitor activity.[36] Also, Rosmarinic acid known to possesses acetylcholinesterase inhibitory activity.[35] It has significant activity in Morris water maze model of rats that used for evaluation of amnesic activity.[35] The results of this study also pointed in same direction. We found that, Rosmarinic acid reduced blood glucose levels, elevated diabetes induced reduction in body weight. Also, Rosmarinic acid significantly improved memory performance in diabetic rats.

Intracellular oxidation leads to glucotoxicity in the neurons.[37] This leads to synaptical damages in brain regions that involved in cognition and therefore contributes to cognitive impairment.[11,12,38] Confirming to this, we have also similar observation that diabetic rats exhibits elevated oxidative stress markers and reduced endogenous anti-oxidants level in hippocampus and cerebral cortex. Rosmarinic acid is known to possess strong antioxidant property.[39] The results of present study points towards that treatment with Rosmarinic acid reduces elevated MDA levels that found to be increased in hippocampus and cerebral cortex in diabetic rats. Also, it elevates the GSH levels that are depleted in hippocampus and cerebral cortex of diabetic rats. On the basis of earlier reports it is confirmed that these agents have substantial activity against lipid peroxidation and glutathione levels.[35]

Cholinergic neurotransmission is a vital process underlying memory and cognitive function. Cholinergic basal forebrain neurons in the nucleus basalis magnocellularis innervate the cerebral cortex, amygdaloid complex and hippocampus, and are essential for learning and memory formation.[40-41] One of the most important mechanisms responsible for correct cholinergic function is performed by enzyme cholinesterase.[42] Further, it is reported that cholinergic transmission is associated with performance of memory and cue detection.[43] Acetylcholinesterase is responsible for termination of acetylcholine induced responses and therefore use of acetylcholinesterase inhibitor is useful to improve memory. Several studies have found an increased ChE activity in brain is associated with cognitive impairments in diabetic rats.[4, 9-10, 44] Rosmarinic acid is polyphenolic compound obtained from Lamiaceaeous herbs[45-46], reported that polyphenolic compounds from Lamiaceaeous plants which includes rosmarinic acid possesses alpha-glucosidase inhibitory action. Also, these polyphenol compounds were tested and reported to possess angiotensin converting enzyme (ACE) inhibitory activity[19], reported that rosmarinic acid possess ameliorative effect against diabetic nephropathy. Further, rosmarinic acid reported to possess inhibitory action against pancreatic amylase which is known to modulate diabetes mellitus.[47] Also, it is reported that, rosmarinic acid regulates localization of the intestinal Na+/glucose cotransporter-1 (SGLT1), glucose transporter 2 and glucagon-like peptide-1 (GLP-1).[18] It was reported that rosmarinic acid possess protective action against β-amyloid induced neurotoxicity and therefore its role in treatment of Alzheimer's disease.[21] Rosmarinic acid is reported to improve cognitive and memory performance.[12,20] In current study we found similar kind of observations i.e., elevated cholinesterase levels in hippocampal and cerebral cortex region of diabetic brains. Further, treatment with Rosmarinic acid depletes elevated level of cholinesterase and therefore helps to improve memory performance.

In conclusion, the present study demonstrates that treatment with Rosmarnic acid prevents changes in oxidative stress and ChE activity and probably consequent memory impairment in diabetic animals.

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