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ORIGINAL ARTICLE

N-Acetyl Cysteine Together with Rutin Combats Oxidative Toxicity By Modulating Nrf2 Pathway in Inflammatory Brain–Liver Axis in Scopolamine-Administered Alzheimer's Disease Model in Rats

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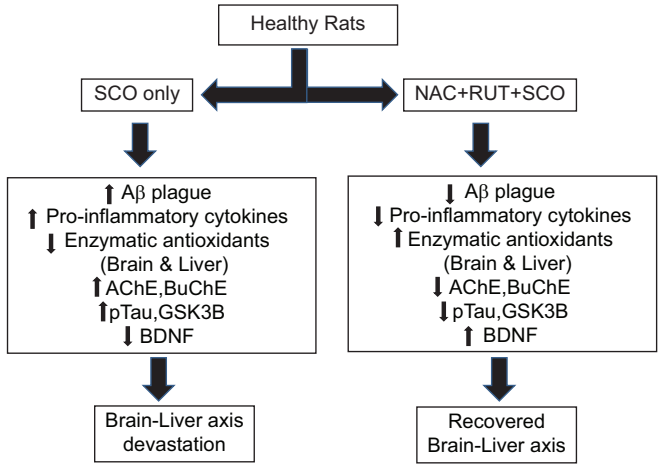
ABSTRACT

Background: Disruptive cholinergic neurotransmission and abnormal cognitive functions were the characteristics of Alzheimer's disease leading to abnormal brain–liver inflammatory responses. Oxidative stress was prominent in Alzheimer's disease-related neurodegeneration leading to inflammatory communication through altered cytokines between the brain and peripheral organs, mainly the liver. **Objectives:** Current study would demonstrate the healthier effect of N-acetyl cysteine and rutin against AD in rats. **Materials and Methods:** Experimental Wistar rats have been grouped appropriately for the administration of scopolamine and treatment using a combination of N-acetyl cysteine and rutin for 10 weeks. **Results:** In our study, scopolamine (2 mg/kg b.w.*i.p.*) administered Alzheimer's disease model in Wistar rats showed abnormality in behavioural changes (Morris water maze test), pro-inflammatory cytokines, decreased activities of enzymatic antioxidants, decreased reduced glutathione content, elevated brain oxidative stress markers, increased amyloid-beta, elevated acetylcholinesterase, elevated butyrl cholinesterase, increased phosphorylated tau protein, increased GSK-3β, BDNF, altered expressions of β-secretase, NADPH oxidase 2, and Nrf2 genes in brain, and augmented oxidative stress in liver affecting brain–liver axis. Oxidative stress in the liver was evident through decreased activities of enzymatic antioxidants, decreased glutathione content, and elevated liver oxidative stress markers. **Conclusion:** Treatment with N-acetyl cysteine with rutin showed protective efficacy by modulating the pathways related to Nrf2, NOX-2, and BACE1 genes in combating these abnormalities in rats. Modulation in the gene expressions in the brain tissue showed direct evidence of the drug's neuroprotective efficacy for future therapeutic strategies in scopolamine-induced Alzheimer's disease.

Key words: Alzheimer's disease, amyloid protein, BACE1, N-acetyl cysteine, Neurodegenerative disease, Nrf2, Rutin

SUMMARY

- Administration of scopolamine to Wistar rats produced a condition like Alzheimer's disease supported by elevated Aβ plague, extreme oxidative stress, decreased activities of enzymatic antioxidants in the brain and liver, and AD specific marker in the brain. Treatment with NAC + RUT was able to control these abnormalities and was able to restore the functions of brain-liver axis. NAC+RUT could be potential molecules to avoid AD-induced neurodegenerative changes.



Abbreviations used:
AD: Alzheimer's disease; AChE: Acetyl cholinesterase, BuChE: Butyl cholinesterase; APP: Amyloid beta precursor protein; NAC: N-Acetyl cysteine; RUT: Rutin; SCO: Scopolamine; ELISA: Enzyme linked immunosorbant assay; qPCR: Quantitative polymerase chain reaction; TNF: Tumor necrosis factor; IL6: Interleukin 6; GSK-3β: Glycogen synthase kinase 3β; BACE1: β-site APP cleavage enzyme 1; BDNF: Brain derived neurotrophic factor; CREB: cAMP response element binding protein; DEPC: Diethyl pyrocarbonate; MDA: Malondialdehyde; GSH: Reduced Glutathione; DTNB: 5,5′-Dithio bis-(2-nitrobenzoic acid); GSSG: Oxidized Glutathione; GST: Glutathione-S-transferase; CDNB: 1-chloro-2,4-dinitrobenzene; CAT: Catalase; GPx: Glutathione peroxidase; SOD: Superoxide dismutase

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INTRODUCTION

Dementia is one of the consequences of Alzheimer's disease (AD), and AD is a disease of concern worldwide. More than 43 million dementia patients have been reported all over the world, and this is expected to grow by three times by the year 2050.[1] In China, AD patients account for about 25% of the cases,[2] which burdens the economy.[3,4] This results in major policy-related changes for governments, the health care industry, AD patients, and family members. AD typically starts with episodic memory loss that then progresses to cognitive deficits and ultimately cognitive impairment.[5] AD is characterised by amyloid-beta (Aβ) protein accumulation as senile plaques, both inside and outside of the neuronal cells and neurofibrillary tangles produced by tau protein because of its hyperphosphorylation, leading to synaptic dysfunction. Several genetic and environmental factors significantly contribute to the pathophysiology of AD making it a multifactorial and highly complex disease. Acetylcholine deficiency was considered to be a major cause of AD that leads to the development of several different types of acetylcholinesterase (AChE) inhibitors and were important in maintaining the levels of acetylcholine, thus, alleviating the symptoms.[6]

In many neurodegenerative diseases, oxidative stress is considered as a major aetiological factor that can contribute extensively.[7] Studies have clearly demonstrated that the aetiology of AD is multifactorial and oxidative stress is considered to be a major pathophysiological factor.[8] Previously published reports confirmed that the production of Aβ can itself can contribute to the increase in oxidative stress and, thus, enhance oxidative stress in the brain of AD patients, which in turn may cause hyperphosphorylation of tau protein thus contributing to a feedback loop in the disease.[9] Aβ precursor protein (APP) is the precursor protein from which Aβ peptide is generated by the action of the enzymes β- and γ-secretase, and among them, Aβ40 and Aβ42 are well studied that exhibit greater toxicity, the stronger tendency to aggregate that results in higher neurotoxicity caused by these peptides.[10] Hence, these two peptides are recognised as important biomarkers for AD.[11,12] Oxidative stress being an independent pathophysiological process in AD, it is also associated with neuroinflammation that is widespread in diseases such as AD.[13] In general, chronic cytokine-induced neuroinflammation, particularly TNF and IL-6, along with oxidative stress was known to be involved in neurodegeneration. In the central nervous system, microglia play an important role in neuroinflammatory processes, and their activities in terms of inflammation and oxidative stress may be lethal for AD. Such pro-inflammatory conditions can trigger oxidative stress-induced neuronal death,[14] and there was sufficient evidence available to suggest the role of inflammation triggered by microglia in inducing chronic oxidative stress in the neurons.[15] Tau protein hyperphosphorylation is also considered as a hallmark of AD. This phosphorylation is mediated by a proline-directed ser/thr kinase enzyme called glycogen synthase kinase 3β (GSK-3β). This enzyme causes phosphorylation of tau protein on most of the ser/thr sites.[16,17] This enzyme is critical for the maintenance of cell physiological activities, including regulating the expression of a variety of gene expressions and apoptosis and this enzyme is also in turn regulated by phosphorylation of ser-9 residue. GSK-3β is generally well expressed in normal neuronal tissues and is associated with cytoskeletal proteins microtubules.[18] Studies have shown that GSK-3β overexpression in mice can cause hyperphosphorylation of tau protein,[19] and, hence, inhibitors of this enzyme can cause inhibition of tau hyperphosphorylation and reduce neurodegeneration mediated by hyperphosphorylated tau.[20] Nevertheless, the mechanism of GSK-3β up-regulation in the brain of patients with AD and its regulation remains unclear.

Currently, one of the major molecular targets in AD has been the pathways associated with the processing of APP, especially the secretase enzymes,[21] that are involved in the generation of amyloid peptides. β-site APP cleavage enzyme 1 (BACE1) is, thus, gaining prominence as a potential target in AD treatment. Although inhibitors of BACE1 were undergoing clinical trials, it is still unclear whether such an approach can affect cognitive abilities in patients with AD.[22] For the normal functioning of neurons, brain-derived neurotrophic factor (BDNF) is critical. It is an endogenous neurotrophin and is abundantly produced in the cerebral cortex and hippocampus regions of the brain.[23]This endogenous neurotrophic plays a critical role in neuronal cell survival as well as in learning and memory.[24]Activation of BDNF and its kinase TrkB signaling results in cAMP response element-binding protein (CREB) phosphorylation and gene expression changes.[25] Studies have indeed started to investigate agents that can activate neuronal BDNF expression that could prove to be beneficial in preventing neurodegeneration in AD.[26]

N-acetyl cysteine (NAC) is a thiol group-containing small molecule and is well known for its antioxidant properties and its major advantage is its ability to cross into the brain.[27] It is well known for the prevention of neuronal apoptosis and facilitates cell survival because of its antioxidant activities.[28] Rutin (RUT) is a natural flavonoid glycoside and is well known for its various biological effects, including cardioprotective, anti-carcinogenic, antimicrobial, and neuroprotective.[29] Many of these activities of RUT are because of its anti-inflammatory and antioxidant properties.[30] Studies indicate that RUT protects neurons by inhibiting cell death and oxidative stress in rodent models of Parkinson's disease.[31] RUT has also been found to be suitable in AD and several Aβ-related pathological changes in the brain.[30,32] Disruption of the brain–liver axis leading to oxidative stress and inflammatory responses was documented earlier.[8,9] Hepatoprotective agents could be useful, and NAC has been combined with RUT for a rationale for optimal protection of the brain–liver axis. Inline with these research data, a hypothesis of influencing the brain–liver axis in combating AD has been done in our lab. In the present study, we have made an attempt to assess the combined effect of NAC and RUT in the scopolamine (SCO)-induced AD model in the rat to explore the potential of this co-treatment in rescuing both biochemical and cognitive parameters of the brain and liver, associated with AD.

MATERIALS AND METHODS

Materials

NAC (A7250-50G; purity ≥99%), SCO (S1875-5G; purity ≥98%), and RUT (R5143-50G; purity ≥94%) were purchased from Sigma Company (Sigma-Aldrich Inc. St. Louis, USA) and all these were dissolved in physiological saline (0.9% NaCl). ELISA and RT-qPCR were performed using kits that were commercially available. All other reagents and chemicals used were of the highest purity and analytical grade.

Animals

Healthy male Wistar rats, aged 10 weeks and weighing 220 ± 10 g, were procured from an institutional animal facility, and this study protocol was reviewed and approved by the institutional animal care and use committee (IACUC; Approval number: CQMU2022-020ETH). Animals were maintained in standard cages and were provided with standard pelleted feed and water *ad libitum*. All animals were maintained at 23 ± 1°C and were acclimatised to laboratory conditions for at least 10 days before the start of the experiments. The total duration of the experiment was 10 weeks.

Experimental groups

The rats were divided into four groups with six animals each. Group 1: Control (normal saline); Group 2: NAC + RUT (200 mg NAC + 75 mg RUT/kg body weight each/oral/daily); Group 3: SCO alone (2 mg/kg b.w.*i.p.* for 10 weeks); and Group 4: NAC + RUT and SCO (co-treatment for 10 weeks). NAC + RUT dose was selected based on preliminary experiments performed in our laboratory and a minimum dose that exerted maximum efficiency was selected (data not shown). Stock solutions were prepared for each separately, and from this, daily dosage was freshly prepared by dilution using physiological saline.

Sample/Tissue preparation

Blood was collected after 5 and 10 weeks of experiments and serum was separated. For hippocampus and liver isolation, rats were euthanised at the end of the experimental period by cervical dislocation, tissues were isolated under aseptic conditions, immediately rinsed in ice-cold saline, and stored at −20°C. For RNA isolation, brain hippocampus tissue was dissected out, rinsed in ice-cold Diethyl pyrocarbonate (DEPC)-treated Milli Q water, and stored at −80°C until further analysis.

Determination of oxidative stress and antioxidant activity

SCO-induced oxidative stress and its effect on antioxidants were analysed in hippocampus and liver of experimental rats. Malondialdehyde (MDA) content was determined using the thiobarbituric acid method. Equal volumes of 0.67% thiobarbituric acid reagent were mixed with a sample from different treatment conditions, boiled (100°C, 10 min) and cooled. After cooling, the OD was measured at 532 nm. MDA content was calculated using a standard graph.[33]

Reduced Glutathione (GSH) was determined as previously published.[34] For 0.5 ml of final reaction mixture containing 100 mM phosphate buffer (pH 7.0), 1 mM EDTA, 0.24 mM NADPH, 0.0756 mM 5,5'-Dithio bis-(2-nitrobenzoic acid) (DTNB), 0.06 units glutathione reductase, and 100 µl of either sample or standard Oxidized Glutathione (GSSG) were added and OD was measured at 412 nm.

Glutathione-S-transferase (GST) activity was determined by incubating the sample with 100 mM phosphate buffer 9 (pH 6.5), 5.0 mM GSH, 2.0 mM 1-chloro-2,4-dinitrobenzene (CDNB) (final volume 1 ml), and the change in absorbance was monitored at 340 nm.[35]

Catalase (CAT) activity was measured as previously published.[36] To a final reaction mixture of 3 ml, containing 100 mM phosphate buffer (pH 7.0) and 10 mM H_2O_2, samples were added to initiate the reaction after 2.5 min and a decrease in absorbance because of H_2O_2 consumption was monitored at 240 nm.

Glutathione peroxidase (GPx) activity was measured as follows[37]: Samples were incubated with 0.05 M phosphate buffer (pH 7.0), 0.24 unit glutathione reductase, and 1 mM GSH for 10 min at 37°C, after which 20 µl NADPH (1.5 mM) was added and NADPH consumption was monitored at 340 nm, after initiating with hydroperoxide solution.

For Superoxide dismutase (SOD) activity, samples were incubated in a 0.2 ml reaction mixture containing 0.01 units/ml xanthine oxidase, 50 mM phosphate buffer (pH 7.4), 0.1 mM EDTA, 1 µM catalase, 0.05 mM xanthine, and 20 µM cytochrome C, and change in absorption was monitored at 550 nm.[38]

ELISA

In the present study, a series of biomarkers were assessed in serum, liver, and hippocampal regions. The biomarkers analysed were the following: Aβ 1–40 and 1–42 (Cloud-Clone Corp., Hubei 430056 P.R.C; Product No.CEA864Ra and CEA946Ra), phospho tau (pTau, Assay Genie, RTES00898, Dakewe Biotech Co., Ltd., China), GSK3β, BDNF, TNFα, and IL-6 (LS-F7877, LS-F24917, LS-F2558, and LS-F5113, respectively, from LS Bio, China). All ELISA assays were performed as per the manufacturer's instructions.

Hippocampal AChE and butyryl cholinesterase (BuChE) activity

AChE activity in the hippocampal regions of the brain was performed as per the published research.[39] Briefly, hippocampus tissue was homogenised (Homogenizer, Thermo Fischer Scientific Inc.) in 5 volumes of 20 mMTris–HCl buffer (pH 7.6) containing protease inhibitor cocktail (NacalaiTesque Inc.). About 100 µl of homogenate was added to 800 µl of 100 mM sodium phosphate buffer (pH 7.5) and then 50 µl of 10 mM DTNB was added. After initiating reaction with acetyl thiocholine iodide or butyrylthiocholine iodide, samples were incubated for 5 min, till yellow colour develops. OD of the samples was then measured at 405 nm in a plate reader.

RT-qPCR

Hippocampus tissue obtained from different treatment groups at the end of the study was used for RNA isolation and to analyse for gene expression. The following genes were estimated: Nrf2, NOX-2, and BACE1. Total RNA extraction was performed using All_Prep_DNA_RNA_miRNA Universal kit (Qiagen, MD 20874, USA) according to the manufacturer's instructions. Brain tissues were homogenised in the presence of a protease inhibitor cocktail (NacalaiTesque Inc.) and all assays were carried out with DEPC-treated Milli-Q water. RNA purity was assessed using a Nano-Drop instrument (Thermo Fischer Scientific Inc.) at 260 nm. Single-stranded cDNA was synthesised from 100 ng of total RNA using qScriptcDNASuperMixKit (Quantabio, MA, USA). RT-qPCR reactions were carried out using Fast SYBR Green Master Mix and using the manufacturer's protocol. *GAPDH* was used as a control gene against which all test samples were normalised. Fold change was calculated using the 2–ΔΔCT method for each gene. Customised and synthesised primers for the selected genes were used as follows: NRF2 gene Fwd-5'-CACATCCAGTCAGAAACCAG-3' and Rev-5'-GGAATGTCTGCGCCAAAAGC-3', NOX-2 gene Fwd-5'-CGACTGGACAGAGGGACTGT-3' and Rev-5'-CCAGGCATCTTGAAACTCCT-3', BACE1 gene Fwd-5'-TGGTGGACACGGGCAGTAGT-3' and Rev-5'-TCGGAGGTCTCGGTATGTAC-3', and GAPDH gene Fwd-5'-TGTGTCCGTCGTGGATCTGA-3' and Rev-5'-TTGCTGTTGAAGTCGCAGGAG-3'.

Morris water maze test

To analyse the effect of various treatment conditions on rat learning and memory, the Morris water maze test was performed as per the published research.[40] The water maze test was conducted in a round white tank with a water-filled halfway. The water was made opaque with powdered non-fat milk and the water temperature was maintained at 25 ± 1°C during the entire course of the experiment. An escape platform made of plexiglass was placed at one of the target quadrants and submerged 1 cm from the water surface. For initial training and trials, the escape platform was placed in the same position and later removed during the actual trial. Other visual cues were left unaltered throughout the course of the study. The escape latency of individual animals from different treatment groups was calculated and tabulated.

Statistical analyses

All results were expressed as mean ± S.D. for each assay. A significant difference between the treatment groups was analysed by one-way ANOVA with Tukey's *post hoc* analyses using Graphpad software

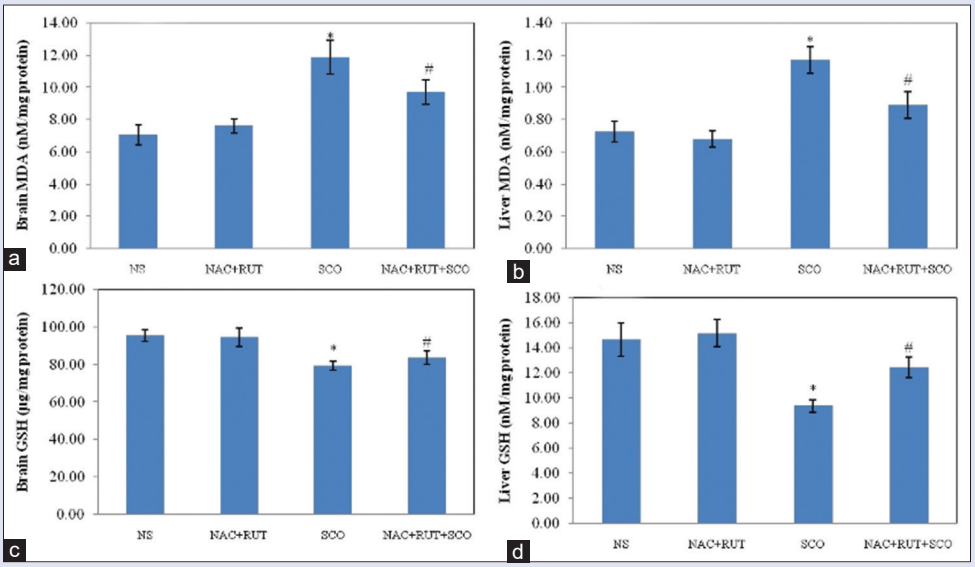

Figure 1: (a-d): Effect of NAC+RUT co-treatment on scopolamine induced MDA and GSH levels in the brain and liver of rats. Values are mean±S.D of 6 animals in each group. *indicates the significant difference with NS group and #indicates the significant difference with SCO group at $P<0.05$

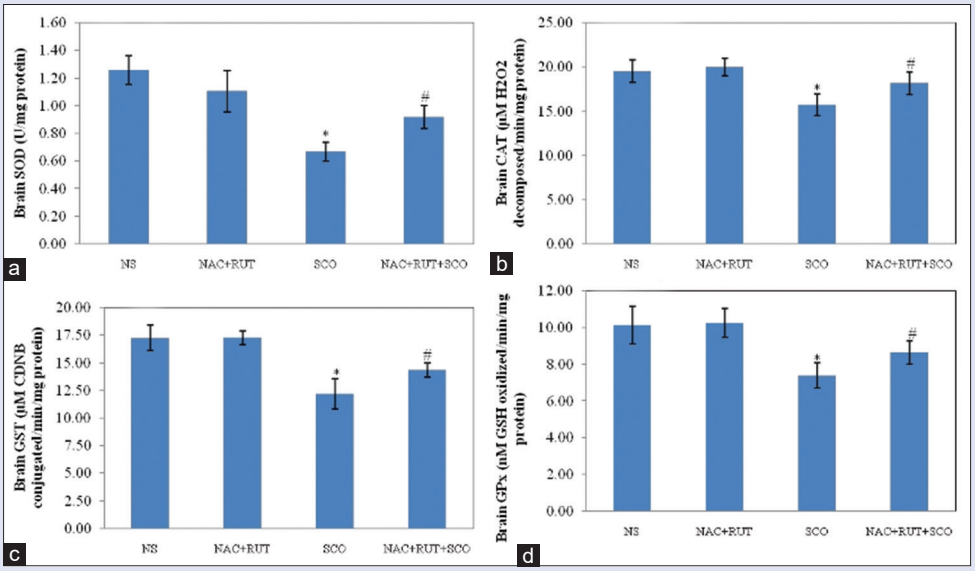

Figure 2: (a-d): Effect of NAC+RUT co-treatment on scopolamine induced antioxidant levels in the brain of rats. Values are mean±S.D of 6 animals in each group. *indicates the significant difference with NS group and #indicates the significant difference with SCO group at $P<0.05$

8.0 (GraphPad Software, Inc. USA). A *P* value less than or equal to 0.05 was considered statistically significant.

The ethical Committee approval was obtained from the Institutional Animal Ethics Committee on Aug 04, 2021.

RESULTS

NAC + RUT rescues rats from SCO-induced oxidative stress

SCO-induced oxidative stress is considered to be one of the major mechanisms of neuronal damage. In the present study, SCO administration was observed to induce a significant increase in MDA levels in both brain (68.8%) and liver (61%) samples, which was suggestive of severe oxidative stress in these tissues. The MDA levels were significantly higher compared to control brain and liver tissue. However, co-treatment of SCO rats with NAC + RUT resulted in a significant reduction in brain and liver MDA levels (18.3 and 23.9%, respectively), indicating the protective role of these compounds [Figure 1a and b].

Furthermore, we also analysed the activity of various antioxidants in these tissues to understand the effect of SCO on oxidative stress. As can be observed from the results, SCO administration resulted in a significant lowering in the activity of brain GSH (17.01%) [Figure 1c] and liver GSH (36.1%) [Figure 1d]. Also, in the brain, SOD (46.96%) [Figure 2a], CAT (19.45%) [Figure 2b], GST (29.37%) [Figure 2c], and GPx (27.14%) [Figure 2d] as well as in the liver, SOD (39.82) [Figure 3a], CAT (28.18%) [Figure 3b], GST (31.66%) [Figure 3c], and GPx (30.03%) [Figure 3d] than the

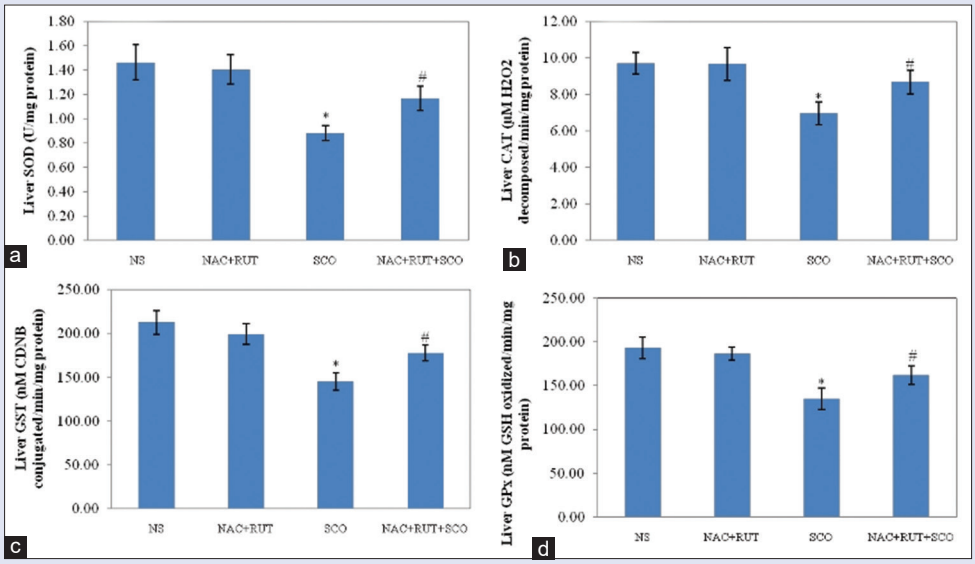

Figure 3: (a-d): Effect of NAC+RUT co-treatment on scopolamine induced antioxidant levels in the liver of rats. Values are mean±S.D of 6 animals in each group. *indicates the significant difference with NS group and #indicates the significant difference with SCO group at *P*<0.05

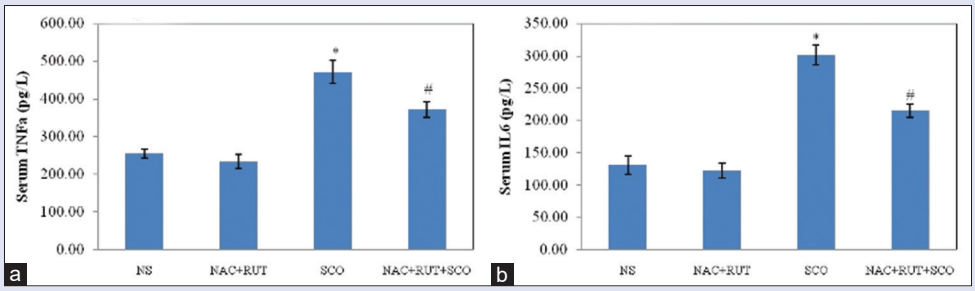

Figure 4: (a-b): Effect of NAC+RUT co-treatment on scopolamine induced inflammatory changes in the serum of rats. Values are mean±S.D of 6 animals in each group. *indicates the significant difference with NS group and #indicates the significant difference with SCO group at *P*<0.05

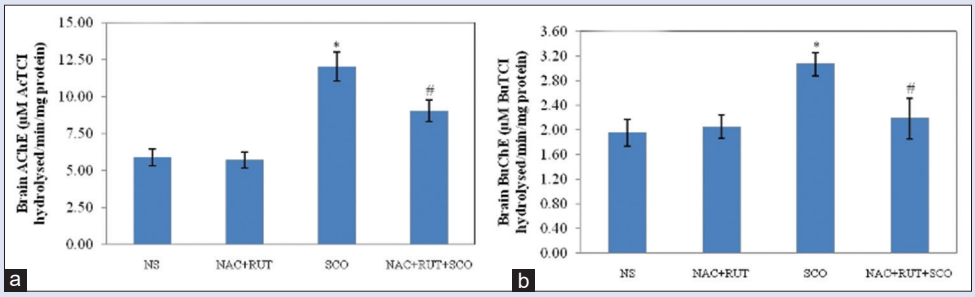

Figure 5: (a-b): Effect of NAC+RUT co-treatment on scopolamine induced changes in the activities of acetyl cholinesterase (AChE) and butyryl cholinesterase (BuChE) in the hippocampal regions of rats. Values are mean±S.D of 6 animals in each group. *indicates the significant difference with NS group and #indicates the significant difference with SCO group at *P*<0.05

respective controls. However, co-treatment with NAC + RUT rescued the activities of all these antioxidants in both brain (5.52, 37.41, 15.47, 17.78, and 16.93% for GSH, SOD, CAT, GST and GPx, respectively) and liver (32.68, 32.7, 24.64, 22.48, and 19.85% for GSH, SOD, CAT, GST, and GPx, respectively) compared to SCO-induced rats. Taken together, these results clearly showed the role of oxidative stress in SCO-induced cellular damage and its recovery during NAT + RUT treatment.

NAC + RUT reduces SCO-induced inflammation

An unregulated pro-inflammatory cytokine release is implicated in tissue inflammation and destruction. In the present study, the levels of both TNFα [Figure 4a] and IL-6 [Figure 4b] were found to be significantly ($P \leq 0.05$) elevated (84.7 and 129.61%, respectively) in the serum of SCO-administered rats. However, when these rats were co-treated with NAC + RUT, a significant reduction in both serum TNFα

and serum IL-6, 21.0 and 28.67%, respectively, were noticed, compared to the diseased group. These results clearly showed the anti-inflammatory potential of NAC + RUT during SCO-induced changes.

NAC + RUT alleviates hippocampal AChE and BuChE activity

Both AChE and BuChE activities are dysregulated in neurodegenerative diseases such as AD. As can be seen from the results, activities of both AChE [Figure 5a] and BuChE [Figure 5b] were found to be significantly ($P \leq 0.05$) enhanced (104.53 and 57.27%, respectively) in SCO-administered rats, compared to their respective controls, indicating dysregulation in cholinergic system. At the same time, treatment of SCO-administered rats with NAC + RUT resulted in a significant reduction in the activities of both AChE and BuChE, 24.79 and 28.8%, respectively, was noticed. This indicates the neuroprotective effect of NAC + RUT against SCO-induced adverse changes in the rat hippocampal region.

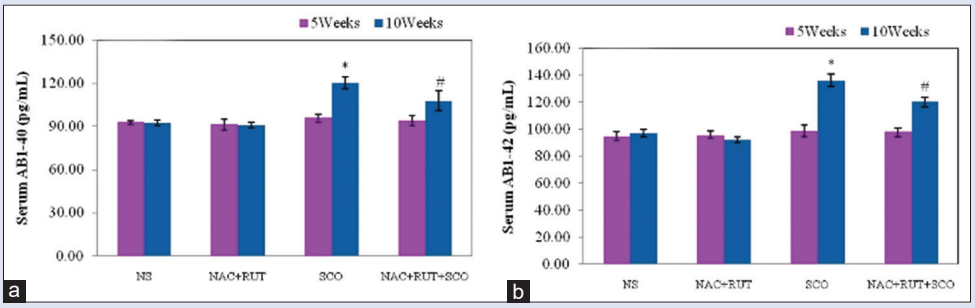

Figure 6: (a-b): Effect of NAC+RUT co-treatment on scopolamine induced amyloid β 1-40 and 1-42 peptide accumulation in the serum of rats. Values are mean±S.D of 6 animals in each group. *indicates the significant difference with NS group and #indicates the significant difference with SCO group at $P<0.05$

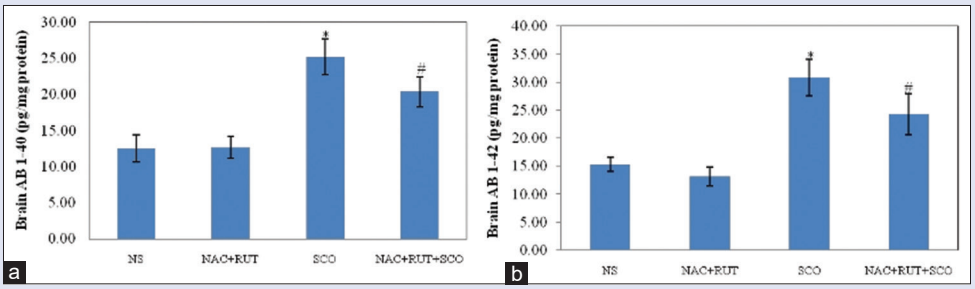

Figure 7: (a-b): Effect of NAC+RUT co-treatment on scopolamine induced amyloid β 1-42 peptide accumulation in the hippocampus of rats. Values are mean±S.D of 6 animals in each group. *indicates the significant difference with NS group and #indicates the significant difference with SCO group at $P<0.05$

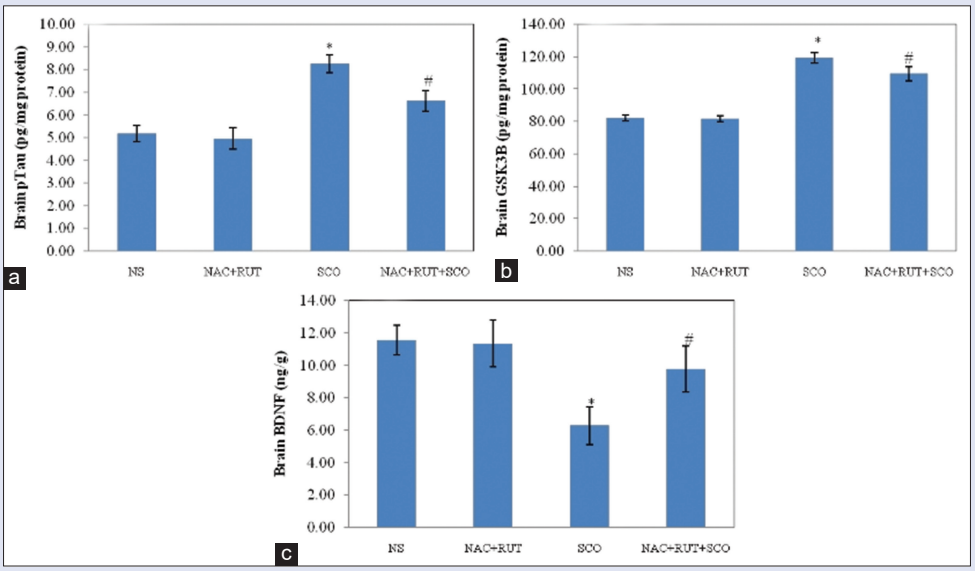

Figure 8: (a-c): Effect of NAC+RUT co-treatment on scopolamine induced changes in the levels of (A) pTau, (B) GSK3β and (C) BDNF in the hippocampus of rats. Values are mean±S.D of 6 animals in each group. *indicates the significant difference with NS group and #indicates the significant difference with SCO group at $P<0.05$. pTau: phosphorylated Tau; BDNF: brain derived neurotrophic factor; GSK3β: glycogen synthase kinase 3 beta

NAC + RUT treatment lowers primary markers of AD and confers neuroprotection

Aβ peptide fragments of length 40 and 42 are the pathological hallmarks of AD. Concentrations of these peptide fragments were assessed in the serum after 5th and 10th weeks of the study. As can be seen from Figure 6a and b, serum concentration of Aβ 1–40 was found to be significantly elevated in SCO-administered rats after 10 weeks (30.14%), compared to the control animals. Likewise, serum concentration of Aβ 1–42 was found to be significantly elevated in SCO-administered rats after 10 weeks (40.31%), compared to the control animals. However, treatment of SCO-administered rats with NAC + RUT for 10 weeks resulted in a significant reduction of Aβ 1–40 (10.4%) and Aβ 1–42 (11.74%) in serum. These results showed the protective effect of NAC + RUT against SCO-induced changes in amyloid formation. No significant changes were observed in 5 weeks of SCO toxicity and concurrent NAC + RUT treatment in the levels of Aβ 1–40 and Aβ 1–42 in serum. Similarly, concentrations of Aβ 1–40 and Aβ 1–42 peptides were also found to be elevated (101.33 and 101.09%, respectively) in the hippocampal regions [Figure 7a and b] of SCO-administered rats (10 weeks) compared to control rat hippocampus. However, when SCO-administered rats were treated with NAC + RUT, a significant reduction in hippocampal Aβ 1–40 and Aβ1–42 peptide concentrations (19.21 and 21.08%, respectively) was noticed.

Similar to amyloid, tau phosphorylation is also a major hallmark of AD neurodegeneration and, hence, in this study, we analysed the extent of tau phosphorylation in hippocampal regions of the brain. As can be seen from Figure 8a, SCO-administration led to significantly higher levels (59.16%) of phospho-Tau (pTau) in the hippocampal region, compared to control. But co-treatment of SCO-administered rats with NAC + RUT showed a significant reduction (19.8%) in hippocampal pTau levels, compared to disease control. Taken together with amyloid, these results showed the potential of NAC + RUT in preventing neurodegenerative changes. To understand the mechanism behind increased pTau in SCO-administered rats, we looked at the activities of GSK3β in the experimental groups. As can be seen from Figure 8b, in SCO-administered rats, a significant elevation (45.36%) in the levels of GSK3β in the hippocampus was noticed, compared to control rats. Interestingly, GSK3β levels were observed to be significantly reduced (8.19%) in the case of rat hippocampus that was subjected to co-treatment with NAC + RUT. Apart from this, SCO-administered

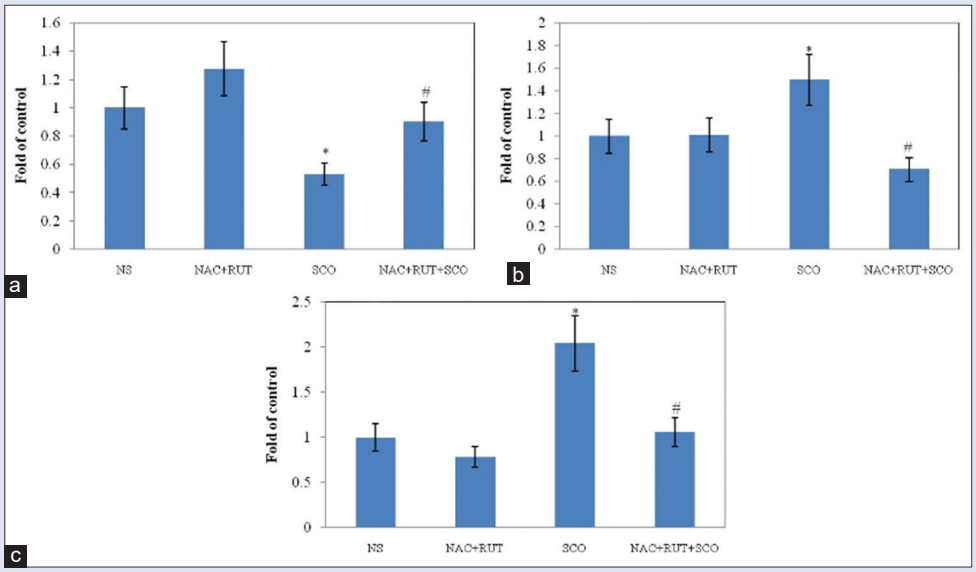

Figure 9: Effect of NAC+RUT co-treatment on scopolamine induced changes in the gene expression of (A) Nrf2, (B) NOX-2 and (C) BACE-1 in the hippocampus of rats. Values are mean±S.D of triplicate analysis in each group. *indicates the significant difference with NS group and #indicates the significant difference with SCO group at $P<0.05$. Nrf2: Nuclear factor erythroid 2-related factor 2; NOX-2: NADPH oxidase 2; BACE-1: β-site APP cleavage enzyme 1

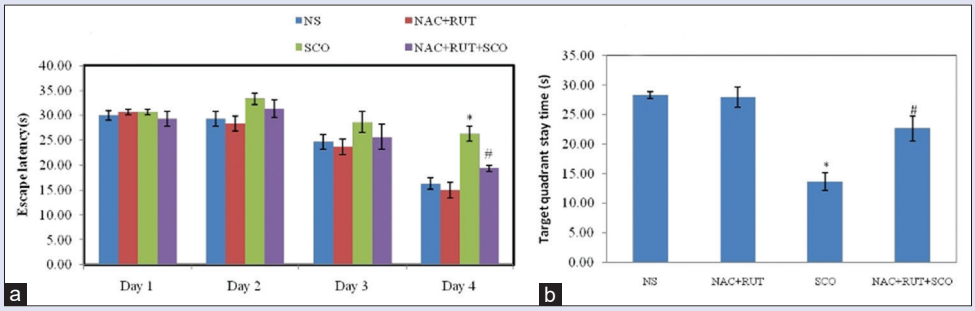

Figure 10: (a-b): Effect of NAC+RUT co-treatment on scopolamine induced memory and learning changes as assessed by Morris water maze test (A) and target quadrant stay time (B). Values are mean±S.D of 6 animals in each group. *indicates the significant difference with NS group and #indicates the significant difference with SCO group at $P<0.05$

rats also showed significantly reduced levels (45.74%) of BDNF in the hippocampus, compared to the control hippocampus. However, NAC + RUT co-treatment produced a significant increase towards normalization (56.12%) in the levels of BDNF in the hippocampus, suggesting the neuroprotective potential of NAC + RUT in the brain [Figure 8c].

NAC + RUT co-treatment stimulates antioxidant mechanisms and inhibits amyloid peptide generation

RT-qPCR results are given in Figure 9a–c. A series of different genes were analysed, including those responsible for antioxidative mechanisms and those primarily responsible for amyloid production. SCO administration lead to a significant reduction in the expression levels of Nrf2 (46.88%) [Figure 9a], compared to respective controls, indicating the onset of severe oxidative stress. At the same time, SCO administration lead to a significant elevation in the expression of NOX-2 (50.07%) [Figure 9b] and BACE1 (103.9%) [Figure 9c], compared to the respective controls. Interestingly, co-treatment of SCO-administered rats with NAC + RUT resulted in a significant increase in the expression of Nrf2 (69.84%) [Figure 9a], a significant decrease in the expression of NOX-2 (53.01%) [Figure 9b] and BACE1 (47.96%) [Figure 9c], compared to the SCO-administered disease controls. Taken together these results clearly show the potential of NAC + RUT in counteracting inflammation-induced neurodegenerative changes in rat hippocampus.

NAC + RUT decreases escape latency and increase quadrant stay time

Morris water maze test was performed to assess the effect of SCO-induced changes in rat learning and memory. As can be seen from Figure 10a, escape latency was observed to be significantly higher in animals that were subjected to SCO, for 4 days. However, in the case of control rats, escape latency was observed to be lesser from the day 1 of the experiment till day 4. Interestingly, escape latency in the case of SCO-induced and NAC + RUT-treated rats were found to decrease from day 1 to day 4 and in fact on day 4, the escape latency time was almost similar to that of control animals. At the same time, the ability of the SCO-induced rats to remain in the target quadrant [Figure 10b] was also significantly lower than that observed with control rats, whereas NAC + RUT treatment increased the target quadrant stay time and the values were quite near to control rats. All these results suggest that NAC + RUT co-treatment had a protective effect against SCO-induced neurodamage to the brain.

DISCUSSION

AD is a major neurodegenerative disease that affects a significant number of elderly populations worldwide,[27] which has both social and economic consequences. Neuronal death is the consequence of this disease that leads to learning and memory loss. A variety of mechanisms have been proposed that cause the pathogenesis of AD. Among them, amyloid plaque formation and deposition and oxidative stress are considered as the major pathophysiological changes.[41] Thus, any strategy targeting these pathological processes could prove to be an effective means of reducing disease severity. In the present study, we analysed the potential of NAC + RUT combination in preventing the pathological changes associated with SCO-induced AD.

Oxidative stress is considered to be a major pathophysiological mechanism associated with neurodegeneration,[41] and SCO administration resulted in excessive oxidative stress in both liver and hippocampus regions as seen with the increase in MDA levels and decrease in the activity of antioxidant enzymes. This increase in oxidative stress was counteracted by co-treatment with NAC + RUT that reversed all these changes. Oxidative stress can trigger neurodegeneration by directly altering membrane properties as well as molecules in the cell,[42] such as amyloid protein oxidation and aggregation.[43] Amyloid deposition has also been shown to trigger oxidative stress,[44] and, thus, this feedback loop can lead to the continuous deterioration of neurons and ultimately neurodegeneration. Inline with this information, in our study, treatment of NAC + RUT had inhibited the neurodegeneration in the brain of rats.

Apart from oxidative stress, neuroinflammation is also an important cause of neurodegeneration,[45] and in fact, inflammatory markers can be detected in the blood during AD.[46] If detected early, such inflammatory changes can be important for future diagnosis and prevention. Nevertheless, enhanced levels of inflammatory markers in the blood area consequence of neurodegeneration and in our study, serum levels of both TNFα and IL-6, were found to be significantly enhanced in SCO-administered group, and by contrast, these cytokines were found to be reduced on co-treatment with NAC + RUT. This suggests the anti-inflammatory potential of the treatment, which could reduce the severity of AD as published in the earlier research.[44]

Chronic oxidative stress and inflammation can trigger neurodegeneration associated with AD. This could be one of the factors for the excessive production of Aβ and its deposition as plaques outside the neuronal cells.[43] In fact, studies have clearly shown the neurotoxic effects of Aβ, wherein the treatment of neurons with high concentrations of Aβ has been shown to result in oxidative stress and cell death.[27] Aβ fragments of different lengths have been identified that have been associated with AD. However, Aβ 1–40/42 are the two well-studied peptides in AD.[47] In the present study, levels of both Aβ 1–40 and 42 peptides were found to be elevated in the serum of SCO-administered rats, and a similar increase in these peptides was observed in the hippocampal regions. However, co-treatment with NAC + RUT prevented Aβ 1–40 and 42 increase in the serum and Aβ 1–42 in the hippocampal region. This suggests the neuroprotective potential of these compounds. Thus, if Aβ levels can be reduced, then AD-induced neurodegenerative changes could also be minimised that correlated well with earlier reports.[43,47]

Another hallmark of AD is the hyperphosphorylation of tau.[48] Tau protein is critical for microtubule stability in neurons and hyperphosphorylated tau can thus cause loss of neuronal function.[49] As a consequence of SCO administration, we observed higher levels of pTau in the hippocampus regions, indicating AD pathogenesis. However, pTau levels in the hippocampal regions were significantly reduced on co-treatment with NAC + RUT, suggesting the neuroprotection conferred by these compounds. GSK3β is the enzyme that is associated with enhanced tau phosphorylation in AD,[50] and our results also showed an increase in GSK3β in the hippocampus of diseases rats. By contrast, this increase in GSK3β was reduced by co-treatment with NAC + RUT, suggesting that NAC + RUT inhibits tau hyperphosphorylation by directly causing reduced levels of pTau and lower AD pathology. Also, treatment with NAC + RUT was also able to provide neuroprotection by enhancing the levels of BDNF in the hippocampal regions. BDNF are crucial for the survival and normal functioning of hippocampus neurons,[51] and any deficiency in BDNF can trigger neuronal loss.[52] In fact, studies on AD have indeed shown an inverse relationship between BDNF and AD.[52] NAC + RUT treatment triggered an increase in hippocampal BDNF levels, and this could help prevent neurodegeneration and aid in neuronal survival.

Cholinergic neuron loss in AD is compensated for by anti-AChE and anti-BuChE treatment.[53] In fact, AChE activity has been positively correlated with plaque deposition in AD,[54] and this has been shown to

increase disease severity. In our study too, SCO administration resulted in enhancement in both AChE and BuChE activity in the hippocampal region, indicating AD pathogenicity. However, NAC + RUT co-treatment was able to inhibit their levels in diseased rats indicating the beneficial effect of these compounds in reducing the pathophysiological changes associated with AD. Thus, NAC + RUT could be potential anti-AChE and anti-BuChE compounds.

In addition to protein expression, analyses of gene expression of certain markers associated with AD were done. Among them, Nrf2 expression was found to be significantly reduced, whereas BACE1 and NOX-2 levels were found to be significantly elevated in SCO-administered diseased hippocampus. Co-treatment with NAC + RUT was found to reverse these changes that are well known to be connected with AD pathology. Nrf2 is a very critical transcription factor for antioxidant genes,[55] and is generally inhibited in AD-associated pathologies.[56] Nrf2 activates NQO-1 and HO-1 that facilitate oxidative detoxification mechanisms[55] and is important for preventing oxidation-induced vascular inflammation.[57] Rescue of these genes by NAC + RUT suggests the ability of these compounds in mounting an effective neuroprotective response in the hippocampus. BACE1 is essential for generating Aβ peptides in AD,[58] NOX-2 is responsible for increased superoxide anion generation in AD,[15] and NF-κB is a pro-inflammatory transcription factor that also has been shown to be involved in AD pathologies.[59] NAC + RUT co-treatment resulted in a lowering of NOX-2, indicating the ability of NAC + RUT not only to reduce oxidative stress and inflammation but also directly inhibit the enzyme responsible for Aβ formation in AD. Thus, by reducing inflammation and inducing neuroprotective genes, NAC + RUT facilitated neuronal cell survival in AD.

To correlate all these biochemical changes with physiological responses, we also performed behavioural experiments to assess for AD-induced changes in learning and memory. Our results clearly shown that NAC + RUT treatment was able to reduce escape latency and prolong target quadrant stay time in the treatment group of rats. This suggests that AD-induced neurodegeneration leads to alterations in animal learning and memory, and by preventing these behavioural changes, NAC + RUT was able to restrict AD-induced decline in cognitive abilities. This also shows that NAC + RUT not only prevented adverse biochemical and molecular changes in SCO-administered rats but such effects were also exemplified by positive behavioural responses in affected rats.

CONCLUSION

Because the treatment drug was able to control oxidative stress and inhibited Aβ plaque formation in the brain together with controlled oxidative stress through antioxidant system activation in the brain and liver, it could be a promising drug for future AD prognosis. Thus, NAC + RUT could be potential neuroprotective compounds for preventing AD-associated degenerative changes, which needs further extensive research.

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Data availability

All data generated or analysed during this study were included in this manuscript. Further enquiries can be directed to the corresponding author.

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Conflicts of interest

There are no conflicts of interest.

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