NOOTROPIC ACTIVITY OF ISORHAMNETIN IN AMYLOID BETA 25-35 INDUCED COGNITIVE DYSFUNCTION AND ITS RELATED mRNA EXPRESSIONS IN ALZHEIMER’S DISEASE

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ABSTRACT: Oxidative stress appears to be an early event involved in the pathogenesis of Alzheimer’s disease. The present study was designed to investigate the neuroprotective effects of isorhamnetin (IRN) against amyloid beta 25-35 (Aβ 25-35)-induced memory impairment and oxidative damage in rats. Memory task was assessed using Y-arm maze and it revealed the impairment in spatial memory. The IRN treated rats showed improvement in memory task. Aβ 25-35 induced animals also exhibited increase in hydrogen peroxide (H₂O₂), Monoamine oxidase activity (MAO) and decrease in choline acetyltransferase (ChAT) activity. It also enhanced the expression of inducible nitric oxide synthase (iNOS) and proinflammatory cytokine, IL-β whereas all these abnormalities were reduced significantly in IRN treated rats showing the neuroprotective effect of IRN against Aβ 25-35 induced Alzheimer’s disease (AD). Thus, IRN may be a potential therapeutic agent for Alzheimer’s disease.

INTRODUCTION: The deposition of Aβ in the brain is assumed to initiate a pathological cascade that results in synaptic dysfunction, synaptic loss, neuronal death, and cognitive dysfunction. Amyloid beta (Aβ) is thought to be a major pathological cause of Alzheimer’s disease. The Aβ (25–35) is a partial fragment of Aβ that similarly forms a β-sheet structure and induces neuronal cell death, neurite atrophy, synaptic loss, and memory impairment. There are many reports showing that mice injected with Aβ (25–35) suffer from memory impairment and neurite and synaptic atrophy.

It has been confirmed that peroxynitrite-mediated damage contributes to Aβ-induced neuronal toxicity and cognitive deficits and is widespread in the brain of AD patients. In AD patients, learning and memory are impaired by the concomitant loss of the cholinergic marker enzyme, choline acetyltransferase (ChAT), in the cerebral cortex.

Flavonoids exert a multiplicity of neuroprotective actions within the brain, including a potential to protect neurons against injury induced by neurotoxins, an ability to suppress neuroinflammation, and the potential to promote memory, learning and cognitive function. Isorhamnetin is an abundant Flavonolaglycone in herbal medicinal plants, such as sea buckthorn (Hippophaerhamnoides L.) and Ginkgo biloba L., which are frequently used in the prevention and treatment of cardiovascular diseases. A number of studies suggested that isorhamnetin can
protect endothelial cells from injury caused by oxidized low-density lipoprotein, decrease blood pressure, and alleviate the damages of ischemia-reperfusion (I/R) to ventricular myocytes.

In the present study, to confirm the protective activity of isorhamnetin against Aβ-induced neurotoxicity, we investigated whether IRN prevents memory impairment in an Aβ2535-injected animal model of AD. Biochemical analysis of neurotransmitter enzyme was carried out to check the improvement in cognitive function. In addition, expressions of iNOS and IL-β mRNA in the hippocampus of Aβ2535-injected rats were analyzed using reverse transcriptase PCR.

MATERIALS AND METHODS:
Chemicals:
Aβ2535 and Isorhamnetin were purchased from Sigma-Aldrich. Acetyl coenzyme A, ethylene diamine tetra acetic acid (EDTA) and dithiothreitol (DTT) were purchased from Sisco Research Laboratories (SRL). Other chemicals were analytical grade.

Animals and Grouping:
Male albino rats weighing between 250–300 g bred in Central Animal House, Dr. ALMPGIBMS, University of Madras, Taramani campus, Chennai 113, Tamil Nadu, India were used. The animals were housed under standard laboratory conditions and maintained on natural light and dark cycle, and had free access to food and water. Animals were acclimatized to laboratory conditions before the experiment. The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) (IAEC NO. 01/05/2014) Dr. ALMPGIBMS, University of Madras, Taramani campus, Chennai 113, Tamil Nadu, India.

The rats were divided into six groups and six animals of each group. Group I: sham-operated control received 5μl of vehicle (PBS/DMSO) through intracerebroventricular injection. Group II: Rats were given Aβ 25-35 (10μg/rat) through intracerebroventricular injection on 1st day. Group III: Rats were given Aβ 25-35 (10μg/rat) through intracerebroventricular injection on 1st day (after one hour) followed by intraperitoneal administration of isorhamnetin (25 mg/kg in PBS/DMSO) for 21 days. Group IV: Rats were given Aβ 25-35 (10μg/rat) through intracerebroventricular injection on 1st day (after one hour) followed by intraperitoneal administration of isorhamnetin (50 mg/kg in PBS/DMSO) for 21 days. Group V: Rats were given isorhamnetin (25mg/kg in PBS/DMSO) intraperitoneally for 21 days. Group VI: Rats were given isorhamnetin (50 mg/kg in PBS/DMSO) intraperitoneally for 21 days.

Preparation of aggregated amyloid beta 25-35 and Intracerebroventricular injection:
Aβ2535 was “aged” by incubation at 37°C for 4 days as described previously. Rats were anesthetized by intraperitoneal (i.p.) injections of ketamine and xylazine and placed in a stereotaxic holder (Instruments and Chemicals, Ambala, New Delhi). A midline sagittal incision was made in the scalp and hole was drilled in the skull over the intracerebroventricle using the following coordinates: 0.8 mm posterior to Bregma, 1.5 mm lateral to the midline and 3.8 mm beneath the dura. All injections were made using a 10-μl Hamilton syringe equipped with a 26-gauge needle. The dura was perforated with the needle of the microsyringe. Animals were infused with 5μl of sterile distilled water (vehicle-treated), aggregated Aβ2535 (2μg/μl) into cerebral lateral ventricle at a rate of 1μl/min; the needle was left in place for an additional 5 min to permit sufficient diffusion and to avoid pressure induced damage. The scalp was then closed with a suture.

Postoperative Care:
Recovery of anesthesia took approximately 4–5 h. The rats were kept in a well-ventilated room at 25 ± 3°C in individual cages until they gained full consciousness. Food and water were kept inside the cages for the first week, allowing animals’ easy access, without physical trauma due to overhead injury. Animals were then treated normally; food, water, and the bedding of the cages were changed often.

Y-maze task:
Y-maze task is used to measure the spatial working through the spontaneous alternation of behaviour. The maze is made of black painted wood. Each arm is 40 cm long, 13 cm high, 3 cm wide at the
bottom, 10 cm wide at the top, and converges at an equal angle. Each mouse is placed at the end of one arm and allowed to move freely through the maze during an 8-min session. Rats tend to explore the maze systematically, entering each arm in turn. The ability to alternate requires that the rat know which arm they have already visited. The series of arm entries, including possible returns into the same arm, are recorded visually. Alternation is defined as the number of successive entries into the three arms, on overlapping triplet sets. The percentage of alternation is calculated as the ratio of actual alternations, defined as the total number of arm entries minus two, and multiplied by 100.

Dissection and Homogenization:
On day 22, animals were sacrificed for biochemical estimations. The animals were sacrificed and the brain was removed by decapitation. Hippocampus was separated from each isolated brain. A 10% (W/V) tissue homogenate were prepared in 0.1 M phosphate buffer (pH 7.4). The aliquots of supernatant were separated and used for biochemical estimations.

Biochemical Analysis:
Estimation of protein: The total protein content was measured according to the method of Lowry et al. (1951) using bovine serum albumin as standard.

Hydrogen Peroxide Assay:
The hydrogen peroxide (H$_2$O$_2$) generation was assayed by the method of Pick and Keisari. Horseradish peroxidase converts hydrogen peroxide in to water and oxygen. This causes oxidation of phenol redforms adduct with dextrose which has maximum absorbance at 610nm and can be recorded spectrophotometrically. Levels of H$_2$O$_2$ generation were expressed as nM of H$_2$O$_2$-generated/mg protein.

ChAT activity:
The choline acetyltransferase (ChAT) activity was determined spectrophotometrically according to Wolfgram(1972). The reaction mixture contained sodium phosphate buffer (pH 7.0), acetyl coenzyme A, chloride choline, physostigmine, NaCl, EDTA, hydrochloric creatinine and DTT. The mixture was preincubated at 37 °C for 5 min then mixed with the hippocampus homogenates, incubated at 37 °C for 20 min and finally stopped the reaction in boiling water. Sodium arsenate was added to each tube for precipitation. The supernatant was mixed with 3 nM 4-PDS and incubated at 25 °C for 15 min. Absorbances were read at 324 nm.

Monoamine oxidase: Monoamine oxidase (MAO) levels were measured by the method of Tabor et al. this method is based on measurement of benzaldehyde formed with benzyl-amine hydrochloride (0.1 M) used as substrate. The absorbance was read at 340 nm and expressed as nmolbenzaldehyde/min/mg protein.

Total RNA isolation and mRNA expression of iNos and IL-β by RT-PCR:
Total RNA was purified from freshly isolated brain tissue using 1 mL of the TRI reagent by the method of Chomczynski and Sacchi (1987). The RNA purity and concentration were determined using using Nano drop (Thermo Scientific) at A260/A280 nm. The purity of RNA obtained was 1.8–2.0. One microliter of total RNA was reverse transcribed by RT-PCR kit (Thermo scientific) according to the manufacturer’s instructions and further amplified by PCR. The details of the primer used, and size of the PCR amplified products are listed in Table 1. The PCR products were resolved by electrophoresis through 1.5% agarose gel and stained with ethidium bromide. The densities of PCR products in the agarose gel were scanned with a Gel Doc image scanner (Bio-Rad, USA) and quantified by Quantity One Software (Bio-Rad, USA).

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequences</th>
<th>Annealing temperature</th>
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<td>F: 5’TCAAGGCATAACAGGCTCATC 3’</td>
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<td>R:5’TCTGTCAGCCTCAAAGAACAGG 3’</td>
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<tr>
<td></td>
<td>R:5’TACTGGTCCATGCAGACAACC3’</td>
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</tbody>
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Statistical analysis:
The data was analyzed by using analysis of variance (ANOVA) followed by Tukey’s test. All the values are expressed as mean±S.D. In all tests, the criterion for statistical significance was \( P < 0.05 \).

Effect of isorhamnetin on \( \text{Aβ}_{25-35} \) induced impairment in spatial memory in Y-arm maze task:
\( \text{Aβ}_{25-35} \) injected group showed significant decrease \((P<0.01)\) in the percentage alternation in Y-arm task as compared to control group on 14\(^{th}\) and 21\(^{st}\) day (Fig.1), thereby showing significant impairment in spatial cognition of \( \text{Aβ}_{25-35} \) induced rats. Significant improvement in spatial cognition \((P<0.01)\) was noted in isorhamnetin \((25\text{mg/kg, b.wt.)}\) treated groups than the IRN 50\text{mg/kg, b.wt.} treated rats \((P<0.05)\). No significant change was found in the isorhamnetin \((25\text{mg/kg and 50 mg/kg, b.wt.)}\) per se group when compared to the control group.

Effect of isorhamnetin on \( \text{Aβ}_{25–35} \) induced changes in level of \( \text{H}_2\text{O}_2 \) in hippocampus of control and experimental rats:
There was significant \((P<0.01)\) increase in the \( \text{H}_2\text{O}_2 \) level in hippocampus of \( \text{Aβ}_{25–35} \) injected group as compared to control group (Fig.2). Isorhamnetin \((25\text{mg/kg b.wt.)}\) was able to counteract and reduced \( \text{H}_2\text{O}_2 \) level significantly \((P<0.01)\) as compared to the \( \text{Aβ}_{25–35} \) injected group whereas the rats treated with IRN 50\text{mg/kg, b.wt.} \((P<0.05)\) showed less effect than the Isorhamnetin 25\text{mg/kg b.wt.} treated rats. The group treated with isorhamnetin \((25 \text{ and 50 mg/kg, b.wt.)}\) alone did not show significant effect on level of \( \text{H}_2\text{O}_2 \) as compared to the control group.
Ameliorative effect of isorhamnetin on Aβ25–35 induced changes in activity of ChAT in hippocampus of control and experimental rats: ChAT activity was found lowered significantly in Aβ25–35 induced group (P<0.01) as compared to control animals (Fig.3). Treatment with isorhamnetin (25mg/kg b.wt.) significantly (P<0.01) increased the ChAT activity than the group treated with isorhamnetin (50mg/kg b.wt.) (P<0.05) as compared to the Aβ25–35 induced group. There was no significant change found on the activity of ChAT in isorhamnetin (25 and 50 mg/kg, b.wt) alone treated group while compared to the control group.

Attenuating effect of isorhamnetin on Aβ25–35 induced changes in the activity of MAO in hippocampus of control and experimental rats: Aβ25–35 injected group showed significant (P<0.01) increase in the activity of MAO in hippocampus compared to control group (Fig.4). Treatment with isorhamnetin (25mg/kg b.wt.) significantly (P<0.01) attenuated the abnormal increase in MAO activity and reverted it to normal when compared with Aβ25–35 induced group whereas the group treated with isorhamnetin (50mg/kg b.wt.) showed less significant (P<0.05) effect compared to the isorhamnetin 25mg/kg b.wt. treated rats. Treatment with isorhamnetin (25mg/kg b.wt. and 50mg/kg b.wt.) alone did not show significant change on activity of MAO when compared with the control group.

Ameliorative effect of isorhamnetin on Aβ25–35 induced changes in mRNA expression of iNOS in hippocampus of control and experimental rats: There was an abnormal (p<0.01) increase in iNOS mRNA expression in hippocampus of Aβ25–35 induced group as compared to control animals whereas isorhamnetin(25mg/kg b.wt.) treated group showed the significant decrease (p<0.05) in
abnormal expression of iNOS (Fig.5). The group treated with isorhamnetin (25mg/kg b.wt.) alone showed normal expressions of iNOS when compared with the control group.

Effect of isorhamnetin on Aβ25–35 induced changes in mRNA expression of IL-β in hippocampus of control and experimental rats: Aβ25–35 induced group showed an abnormal increase ($p<0.01$) in IL-β expression in hippocampus as compared to control animals (Fig.6). There was the significant decrease ($p<0.05$) in abnormal expression of IL-β in isorhamnetin (25mg/kg b.wt.) treated group. The group treated with isorhamnetin (25mg/kg b.wt.) alone showed normal expressions of IL-β when compared with the control group.
Lane M: DNA marker Lane.2. control group showing normal expression of IL-β. Lane.3. Increased expression of IL-β in Aβ25–35 induced group. Lane.3 Aβ25–35 + IRN 25mg/kg b.wt. treated group showing normal expression of IL-β(Lane.3). Lane.4. IRN alone treated group showing normal expression of IL-β. Data represents mean±SD of six rats in each group. Levels of mRNA were normalized to that of β actin. Statistical significance (P value): ap<0.01 significantly different from control group. b<0.05 different from Aβ25–35 induced group.

DISCUSSION: Aβ (25–35) is potential neurotoxic peptide for primary neuronal cortical cells which produces neurofibrillary tangles25-26 and the toxicity induced by Aβ (25–35) in rodents resembles to AD which is suitable for the evaluation of Alzheimer’s type of dementia. Isorhamnetin, which is an O-methylated metabolite formed in the small intestine and liver, and increased in the human brain, has a higher BBB permeability than aglycone by its apparent lipophilicity 27. The O-methylated metabolites of derived flavonoids have the potential to improve human memory and neuro-cognitive performance via their ability to protect vulnerable neurons and long-term potentiation, which is considered as major mechanism of memory in the brain.

In this study the Aβ (25–35) induce impairment in short term memory was significantly alleviated by the isorhamnetin treatment. It has been reported that Aβ induces the production of hydrogen peroxide and lipid peroxide in hippocampal neurons of the rat brain 28. There was an increase in the level of hydrogen peroxide indicating increase in oxidative stress and decrease in antioxidant activity in hippocampus. Isorhamnetin has been shown to alleviate the hydrogen peroxide induced oxidative stress in previous studies. In this study also IRN reduced the level of hydrogen peroxide via its antioxidant activity.

In AD patients, learning and memory are impaired by the concomitant loss of the cholinergic marker enzyme, choline acetyltransferase (ChAT), in the cerebral cortex 29. In this study, the Aβ (25–35) induced decrease in ChAT activity was reverted to normal by the IRN treatment and which may account for the improvement in the short term memory as assessed by y arm maze.

Aβ 25-35 has the potential to induced oxidative stress in the brain cholinergic hypo function, elevation of AChE and MAO 30-32. It is well accepted that oxidative stress (associated with MAO) contributes to the disturbance of neurotransmitters, including NE and the cholinergic system, which have a critical role in the cognitive impairment in AD 33-35. Elevations in MAOA in Alzheimer neurons have been linked to increase in neurotoxic metabolites and neuronal loss. Compelling studies have shown that the involvement of MAO in AD and neurodegenerative diseases plays an important role in several key pathophysiological mechanisms 36-37.

Elevated monoamine levels in the brain resulting from MAO induce changes in other neurotransmitter systems and lead to cognitive impairment. The isorhamnetin has been reported to inhibit MAO- A by 55% at 1.26 µM 38. Aβ 25-35 induced animals exhibited the increase in MAO activity whereas IRN reduced the abnormal increase in activity of MAO. It might be the reason for improving the spatial memory in Y-arm maze.

In this study, we found that IRN significantly inhibited the increase of iNOS mRNA in the hippocampus induced by Aβ25–35. It has been demonstrated in vitro that the stimulation of neuronal cell lines with TNF-α leads to increased expression of inducible nitricoxide synthase and subsequent apoptosis 39. We analyzed the expression of TNF-α in hippocampus of control and experimental rats. There was marked elevation in TNF-α expression which was reduced by IRN treatment (data not given). Hence, decrease in TNF-α would be the reason for reduction in the iNOS expression. In contrast to the NO synthesized by nNOS, which facilitates learning and memory formation under physiological conditions 40-43, the NO synthesized by iNOS is deleterious 44. The iNOS produced a large amount of NO, which may cause neurotoxicity in vitro, has been previously reported 45 46. It has been reported that isorhamnetin treatment down regulated the expression of iNOS, a key enzyme for catalyzing NO production47-48. IRN alleviated the oxidative...
stress in hippocampus by reducing the peroxinitrite formation via inhibiting iNOS expression.

TNF-α and IL-1β are involved in the inflammatory response, and they may also be toxic to neurons and glial cells. The increased level of pro-inflammatory cytokines in the central nervous system was shown to impair cognitive function and play a fundamental role in the pathogenesis of AD. In corroboration with previous report, there was an increase in the expression of IL-β in Aβ 25-35 induced rats. The current study reveals that Aβ 25-35 induced the expression of proinflammatory cytokine, IL-β which was significantly reduced by IRN treatment. Pro-inflammatory cytokine upregulation and excessive NO production play important roles in severe inflammatory diseases. Inhibition of pro-inflammatory cytokines and NO release in inflammatory cells could therefore beneficially attenuate excessive inflammatory response. In conclusion the IRN was able to revert the abnormal expression of iNOS and IL-β to normal, thereby improved the cognitive function and reduced the oxidative stress subsequently apoptosis.

CONCLUSION: In summary, this study highlights the potential neuroprotective mechanisms of IRN involved in improvement of cognitive function. Together, the results of the present study suggest that administration of isorhamnetin prevents Aβ 25-35 induced cognitive impairment and associated oxidative stress. Hence, neuroprotective activity of IRN may be mediated through free radical scavenging activity and inhibition of proinflammatory cytokine and iNOS.

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