Rivastigmine attenuates the Alzheimer's disease related protein degradation and apoptotic neuronal death signalling

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Abstract
Rivastigmine is clinical drug for patients of Alzheimer’s disease (AD) exerting its inhibitory effect on acetylcholinesterase activity however, its effect on other disease related pathological mechanisms are not yet known. This study was conducted to evaluate the effect of rivastigmine on protein aggregation and degradation related mechanisms employing streptozotocin (STZ) induced experimental rat model. The known inhibitory effect of rivastigmine on cognition and acetylcholinesterase activity was observed in both cortex and hippocampus and further its effect on tau level, amyloid aggregation, biochemical alterations, endoplasmic reticulum (ER) stress, calcium homeostasis, proteasome activity and apoptosis was estimated. STZ administration in rat brain caused significant cognitive impairment, augmented acetylcholinesterase activity, tau phosphorylation and amyloid aggregation which were significantly inhibited with rivastigmine treatment. STZ also caused significant biochemical alterations which were attenuated with rivastigmine treatment. Since AD pathology is related to protein aggregation and we have found disease related amyloid aggregation, further the investigation was done to decipher the ER functionality and apoptotic signalling. STZ caused significantly altered level of ER stress related markers (GRP78, GADD153 and caspase-12) which were significantly inhibited with rivastigmine treatment. Further the effect of rivastigmine was estimated on proteasome activity in both regions. Rivastigmine treatment significantly enhances the proteasome activity and may contributes in removal of amyloid aggregation. In conclusion, findings suggested that along with inhibitory effect of rivastigmine on acetylcholinesterase activity and up to some extent on cognition, it has significant effect on disease related biochemical alterations, ER functionality, protein degradation machinery and neuronal apoptosis.

Keywords- Alzheimer’s disease Pathology; Streptozotocin; Neuroprotective; Antioxidative; Antiapoptotic; Rivastigmine; Endoplasmic Reticulum Stress; Antinitrosative.

Introduction
Alzheimer’s disease (AD) is the most common age related progressive neurocognitive disorder leading to cognitive impairment, disorientation and hallucination [1]. AD pathology includes the extracellular aggregation of amyloid beta and intracellular aggregation of hyperphosphorylated tau protein which initiate the neurofibrillary tangles formation and these are considered as pathological hallmarks of disease [1]. These extracellular deposits of amyloid beta are also called as senile or neurite plaques. The other hypothesis which is considered for AD pathology is the depletion of neurotransmitter acetylcholine due to loss of cholinergic neurons. It has been reported that the imbalance of acetylcholinesterase (AChE) and butyrylcholonesterase (BuChE) enzymes causes cholinergic deficit in the brain and thus,
deficiency of neurotransmitter acetylcholine is one of the reason for interrupted neuronal communication [2]. Presently, the cholinesterase inhibitors which could inhibit both AChE and BuChE, are donepezil, rivastigmine and galantamine (FDA approved) which are being used clinically and offer therapeutic benefits to AD patients [3]. However, these inhibitors are unable to prevent the disease progression but significantly offer symptomatic improvement for considerable duration after diagnosis of disease in patients. To date, it is uncertain rather unexplored the effect of these cholinesterase inhibitors on other AD related pathological mechanisms. If such information will be available at scientific platform, it will surely assist the scientific community to provide the better therapeutic molecule and effectual use of available drugs for the cure of AD patients. In this context the recent study by Ray et al (2020) [4] showed that rivastigmine can modify the levels of various shedding proteins and directs the processing of APP towards non-amyloidogenic pathway employing cellular, transgenic models as well as in primary cultures suggesting the mechanistic effects of rivastigmine in addition to its reported symptomatic effects in AD patients. Another study also showed that rivastigmine offer neuroprotection through signalling pathways like PI3K-Akt in neuronal SHSY5Y cells [5]. However, the lacunae still exist regarding effect of rivastigmine in neurodegenerative signalling pathways. The present study was conducted to investigate the effect of rivastigmine on AD related pathological signalling mechanisms except its effect on AChE activity. Rivastigmine inhibits the acetylcholine esterase enzyme thus prevent the degradation of acetylcholine and maintain its appropriate level in synaptic clefts. In this direction previously we have reported the effect of drug donepezil, which is also AChE inhibitor on the AD related pathological markers and mechanisms. We had reported the protective effect of donepezil on AD related augmented AChE activity, tau phosphorylation, glucose uptake and mitochondrial functionality and neuronal apoptosis [6]. The present study was done by employing streptozotocin (STZ) induced experimental rat model of AD utilizing both cortical and hippocampal regions of rat brain. Intracerebroventricular administration of STZ induces AD pathology involving significant occurrence of AD related pathological markers like increased AChE activity, phosphorylation of tau and deficit of cholinergic neurons which has already been reported by us and others previously [7–11] therefore, being utilized in this study. First, the known inhibitory effect of rivastigmine on AChE activity was confirmed utilizing both cortical and hippocampal regions of rat brain. Afterwards, the effect of rivastigmine on various disease related parameters like antioxidant capacity of brain, nitrosative stress, endoplasmic reticulum (ER) stress, proteasome activity and on neuronal morphology along with its effect on astrocytes reactivity was evaluated in both cortical and hippocampal regions of rat brain.

Material and methods

**Biochemicals**

Laboratory chemicals used were bovine serum albumin, copper sulphate, glucose, ethylene diamine tetra acetic acid (EDTA), folin ciocalteu reagent, potassium chloride, potassium phosphate dibasic anhydrous, sodium bicarbonate, 4-(2hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium chloride, sodium dihydrogen phosphate, sodium hydroxide and sodium potassium tartarate were procured from Sisco research laboratory (SRL), India. Acetylthiocholine iodide, 5,5′-dithio-bis (2-nitrobenzoic acid (DTNB), Congo red dye, pepstatin, PMSF, protease inhibitor cocktail, streptozotocin (STZ), Tris buffer, mouse monoclonal anti β-actin antibody (Ab), mouse monoclonal anti caspase-12, anti-GFAP Ab and anti-rabbit and mouse HRP secondary Ab were procured from Sigma (St. Louis, MI, USA). Rabbit polyclonal anti pTau Ab (phosphorylated at T231), GRP-78 Ab, GADD 153 Ab, iNOS Ab, goat polyclonal anti tau Ab and mouse monoclonal anti caspase-3.
mouse monoclonal anti β-amyloid Ab were purchased from Santa Cruz (Dallas, Texas USA). PVDF membrane, luminata substrate and Flurojade C stain was brought from Millipore (USA).

**Animals**

Adult male Sprague–Dawley rats of 7-8 weeks old (180–200 g) were procured from the National Laboratory Animal Centre of Central Drug Research Institute and experiments were done at CSIR- Central Drug Research Institute animal house facility according to internationally followed ethical standards and approved by the Institutional animal ethics committee of CSIR – Central Drug Research Institute (IAEC/2018/88/Renew-0/Dated-14/06/2018). Two animals per polycrylic cage were housed with access to food and water ad libitum and were maintained in standard housing conditions i.e. room temperature 23±1°C and humidity 60–65% with 12 h light and dark cycle.

**Animal groups and treatment:**

Animals were treated with Rivastigmine (2 mg/kg, oral) 30 minutes prior to streptozotocin (STZ, dissolved in ACSF) administration and continued till sacrifice at difference of each 24 h [12–14] . After dosing the rats were anesthetized and mounted on stereotaxic platform (Stoelting, USA). STZ was administered in intracerebroventricular (ICV) region of rat brain bilaterally (3mg/kg) on day 1 and 3 [6]. The coordinates for ICV region were AP– 0.8 mm, L– 1.6 mm and DV– 3.5 mm, from the bregma point [15]. Proper postoperative care was done till the animals recovered completely. After 21 days the rats were anesthetized by combination of ketamine 80mg/kg and xylazine 10mg/kg administered via intraperitoneal (i.p.) injection followed by intra-cardiac perfusion with saline and after decapitation whole brain was removed. The brain regions cortex (C) and hippocampus (HP) were isolated [16] and processed for various estimations. The animal groups taken were control, per se rivastigmine, STZ, STZ + rivastigmine. Three to four experimental repeats per parameter were taken for study. For behavioural and biochemical estimations 10 animals/group were taken while for assessment of protein level of various signalling factors and histopathological alterations 6 animals/group were taken.

**Memory function by Morris water maze test:**

To assess the spatial learning and memory function in STZ administered rats, morris water maze test was performed. The morris water maze consist of a large circular black pool (120 cm diameter and 50 cm height) filled to a depth of 30 cm with water coloured with black nontoxic dye. The temperature in pool was maintained at 25 ± 2 °C. Four equally spaced points around the edge of the pool were designed as all four directions. A black coloured round platform of 8 cm diameter was placed 1 cm below the surface of water. The rats were given a maximum time of 120 seconds (cut-off time) to find the hidden platform and were allowed to stay on it for 30 seconds. Rats who failed to locate the platform within 120 seconds were put on platform only in the first session (acquisition trial) [17]. The rats were given a daily session of four trials from the 17th to 20th day after the STZ administrations. At 21st day the escape latency time to reach the platform, speed of the rat and the path length covered by the rat to reach to the platform was recorded in each trial. There is no test performed without platform. The mean ± SEM of latency time (seconds) of the test is shown in the results.
Acetylcholinesterase (AChE) activity:
After 21 day of treatment the rats were sacrificed, brain was isolated, dissected and both cortex and hippocampal regions were collected. Brain homogenate of both regions were prepared separately in 0.1 M phosphate buffer (PB, pH 7.0) with 1% triton-X 100 and centrifuged at 20,000 rpm for 30 minutes at 4°C. AChE activity was estimated according to Ellman’s method [18]. The reaction mixture was prepared consisting of supernatant (10 µl), 0.1mM 5,5′-dithio-bis (2-nitrobenzoic acid (DTNB) (50 µl), 0.1 M phosphate buffer (90µl) and the reaction was initiated by addition of 150 µl of 2mM acetylthiocholine iodide. The kinetic profile of enzymatic activity was measured at 412 nm for 2 minutes at 15seconds interval by spectrophotometer (Eon, BioTek). The specific activity of AChE was calculated and represented as μmoles / min / mg of protein.

Estimation of nitrite level:
Nitrite level was estimated based on protocol reported by [19] Esposito et al. (2008). Briefly, the brain homogenate was prepared in 10% w/v tris-HCl buffer using homogenizer (Cole Parmer). Centrifugation of brain homogenate was done at 10,000xg at 4°C for 20minutes. The nitrite level was estimated in supernatant by using Griess reagent. Hundred microliter of Griess reagent [(0.1% (w/v) napthylethylenediamine HCl and 1% (w/v) sulphanilamide in 5% (v/v) phosphoric acid (vol:1:1)] was added to 100 µl of brain homogenate for reaction mixture. The reaction mixture was incubated for 20 minutes in dark and the absorbance was measured using a microplate reader (EON, BIOTEK, USA) at 550 nm wavelength. Nitrite concentrations (mM) were extrapolated from the standard curve of sodium nitrite.

Glutathione (GSH) estimation:
Reduced glutathione (GSH) assay was done to assess the oxidative stress in both rat brain regions. Homogenates (10% (w/v) of brain regions was prepared in sodium phosphate buffer (30 mmol/l, pH 7.0) using homogenizer (Cole-Parmer LabGEN 7B, USA). Equal volume of rat brain homogenates and 1% trichloroacetic acid (TCA) 1:1 ratio was taken and incubated for 20 minutes at 4°C for deproteinization. After centrifugation at 5000xrpm for 5 minutes at 4°C, 10µl of supernatant was taken for GSH estimation in 96 wells plate. Then 140µl of potassium phosphate buffer (0.1M, pH 8.4) and 50µl of 5-5′-dithiobis[2-nitrobenzoic acid] (5mM) was added to each sample [20]. The reaction mixture yielded yellow colour and readings were immediately taken at 412 nm using ELISA plate reader (BIO-TEK Instruments). Data are expressed as GSH μg/mg protein.

Lipid peroxidation:
Lipid peroxidation was measured by estimating MDA levels [21]. The reaction mixture was prepared using 500µl brain homogenate with 30%(w/v) TCA and 5N HCl, followed by 2% (w/v) thiobarbituric acid in 0.5 N sodium hydroxide then incubated on a water bath at 90°C for 45 minutes. After cooling at room temperature the mixture was centrifuged at 12000rpm for 10 minutes. The supernatant was collected and the absorbance was measured at 532 nm, using ELISA plate reader (BIO-TEK Instruments). The MDA concentration in the samples was extrapolated from the standard curve obtained by plotting the optical density of the tetraethoxypropane. Results are expressed as MDA nmol/mg protein.

Estimation of Calcium level:
Calcium level was estimated [22] in both regions of rat brain, the regions were separately homogenised in phosphate buffered saline (PBS). In a 96 well plate 40µl of homogenate was diluted with 160µl of PBS buffer and incubated with 5µM of Fluo-3AM dye for 1h at 37°C in
dark. The fluorescence was read in fluorimeter (Varian, Cary Eclipse) at 506 nm/530 nm excitation/emission.

Sample preparation for western blot:
Preparation of total lysate:
The rats were decapitated and brains were removed, cortex and hippocampus were dissected out. Both isolated brain tissues were taken separately and homogenized in lysis buffer (10% w/v) containing HEPES 200 mM (pH 7.4), sucrose 250mM, KCl 10 mM, MgCl₂ 1.5 mM, EDTA 1mM (pH-7.4), EGTA 1Mm (pH-7.4), Dithiotheritol (DTT) 1mM, protease inhibitor cocktail, pepstatin-A 1 mM, PMSF 1 mM and Nonidet P-40 (0.05%). The homogenate was centrifuged at 12,000 x rpm for 20 minutes at 4°C. Supernatant was collected and protein concentration was determined by Lowry’s method [18].

Western blot:
Protein was loaded on SDS polyacrylamide gel and blots were transferred on polyvinylidene fluoride membrane as reported previously [23]. The membrane was blocked with 5% BSA for 2 h at room temperature. After washing with PBS, the membranes were incubated with anti GRP-78 (1:10000), anti p-TAU (1: 2000) antibody, anti tau(1:500), anti cleaved caspase-3 (1:500)antibody, anti-β-amyloid (1:500) anti–GADD153 (1:500), anti-iNOS(1:1000), cleaved caspase 12 (1:1000) and anti β-actin (1:10000) separately at 4˚Cover night. After incubation the membrane was washed and incubated with appropriate HRP conjugated secondary antibodies at room temperature for 2 h. Signals were developed by femtoLUCENT plus-HPR chemiluminescent substrate (G-biosciences, USA) and detected under ChemiDoc HRS+ (Bio-Rad). Integrated density of bands was determined and normalized by β-actin using Image J software (NIH, USA) [24].

Estimation of protein content:
To estimate the protein concentration in the rat brain samples, the brain homogenates of different brain regions was estimated by method reported by [25]. The protein concentration in the samples was extrapolated from the standard curve obtained by plotting the optical density of the known concentrations of bovine serum albumin.

Histopathology:
Tissue block preparation and sectioning
Anaesthetised rats were transcardially perfused with saline and 4% paraformaldehyde (PFA). Brain was removed by decapitation and kept in 4% PFA for fixation. After fixation, brain was removed and then proceeded for the wax block preparation after dehydration in graduated isopropanol series and xylene as reported previously [26] by using Shandon Histocentre 2 (Minnesota USA). The brain sections of thickness 5μm were cut by microtome (Leica, USA) and placed on albumin coated slides.

Immunohistochemistry:
The tissue sections were deparaffinised by two washes of xylene (5 minutes each). The sections were then rehydrated with 100%, 90%, 70%, and 50% ethanol (5 minutes each). Then, slides were washed in PBS (1.3 M NaCl, 70 mM Na₂HPO₄,30mM NaH₂PO₄) for 15 minutes. Quenching of endogenous peroxidase activity was done using quenching buffer (4 ml PBS+1 ml 30 % H₂O₂) for 10 minutes at room temperature in dark. After washing, the sections were blocked for 2 h in 5 % BSA prepared in PBS-T (PBS containing 0.3 % Triton X-100). After blocking, the sections were incubated with primary antibodies at the dilution of
1:250 for 24 h at 4\(^{0}\)C. After that, the sections were washed with PBS-T and incubated for 1 h with appropriate alexa fluor. Sections were washed with PBS-T and mounted on slides with antifade solution and visualized under fluorescence microscope (Nikon eclipse E200, Japan). Images of the cortex and hippocampus regions of the brain sections were captured at 40X magnification [21].

**Cresyl violet (CV) staining:**
To assess the neuronal shrinkage CV staining was done in brain sections of rat brain [23]. Tissue sections were deparaffinised and rehydrated in xylene and graduated alcohol series. After that the slides were kept in distilled water for 1 minute, following 5 minutes incubation in CV dye. Slides were then given quick dip in distilled water followed by 95% alcohol. Then, slides were cleared in alcohol: xylene solution (1:1) and mounted with DPX. Images were captured in Nikon Eclipse E200 microscope. Quantification of images were done using Leica Qwin software.

**Flurojade C staining:**
Degenerating neurons were observed by Flurojade C dye as reported previously [26]. Tissue sections were deparaffinised and rehydrated in xylene and graduated alcohol series. After that, the slides were kept in distilled water for 2 minutes, incubated in 0.06% potassium permanganate solution for 10 minutes and again rinsed for 2 minutes in distilled water. Slides were then transferred in 0.0001 % solution of Flurojade C dissolved in 0.1 % acetic acid for 40 minutes. Finally, slides were rinsed in distilled water, cleared in xylene for 1 minute and mounted with DPX.

**Congo red staining:**
Amyloid aggregation in cortex& hippocampus regions was assessed by congo red staining [27]. Tissue sections were deparaffinised and rehydrated in xylene and graduated alcohol series. The slides were incubated for 20 minutes in alkaline saturated NaCl solution in 80% ethanol and then incubated in filtered 1% alkaline congo red solution for 30 minutes. After incubation the slides were given quick dips in 95% and 100% ethanol, kept in xylene for 5 minutes and finally mounted with DPX. The slides were observed and images were taken in Nikon Eclipse E200 microscope.

**Proteasomal Activity:**
After 21 day of treatment, the rats were decapitated and brains were removed. Brain tissues of cortex & hippocampus (were taken separately and homogenized in assay buffer (10% w/v) containing HEPES 50 mM (pH-7.8),sucrose 250mM, NaCl 10 mM, MgCl\(_2\) 1.5 mM, EDTA 1mM (pH-7.4), EGTA 1Mm (pH-7.4), Dithiotheritol (DTT) 5mM as reported previously [28]. The homogenate was centrifuged at 16,000 x g for 10 minutes at 4° C. Supernatant was collected and protein concentration was determined by Lowry’s method. This supernatant was used as the sample for both chymotrypsin and trypsin activity. For trypsin activity the reaction mixture was prepared with addition of 40μg of protein with the 2mM ATP and 100μM of trypsin enzyme substrate Boc-LRR-AMC. The reaction mixture was incubated in dark for the 60 minutes at 37° C. The released fluorogenic product was measured at 360nm excitation and 460nm emission using fluorimeter (Varian, Cary Eclipse). Chymotrypsin activity was estimated similarly except the difference of substrate that is Suc-LLVY-AMC.

**Statistics analysis:**
Data are expressed as the mean±S.E.M and analysed by one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison post-hoc. Newman - Keuls
multiple comparisons was used for comparison between control vs. STZ and STZ vs. STZ + rivastigmine treatment. P value less than 0.05 was considered statistically significant.

Results

Effect of rivastigmine on cognition:
Morris water maze assay was done to estimate the cognitive deficit. The escape latency (time taken to reach to the hidden platform in seconds) in STZ administered rats was significantly (p<0.001) more than the control. In rats of rivastigmine per se group, the latency time was approximately same as observed in control rats. Rivastigmine treatment significantly (p<0.01) inhibited the STZ induced increased latency time. Path length travelled by rats was also significantly (p<0.05) higher in STZ administered rats in comparison to path travelled by control rats (Fig1A). There was no significant difference observed in the swimming speed of all the group of the rat. STZ administration induced augmented path length was significantly (p<0.01) inhibited with rivastigmine treatment.

Effect of rivastigmine on AD related pathological hallmark:
Since rivastigmine is clinically used drug, first we have assessed its effects on AD related known pathological hallmarks like AChE activity. Further, its effects on tau phosphorylation and amyloid aggregation were estimated. AChE activity was significantly (p<0.001) higher in both cortex & hippocampus regions of rat brain of STZ treated rats in comparison to control and rivastigmine per se groups. The STZ induced augmented AChE activity was significantly (p<0.001) inhibited with rivastigmine treatment (Fig.1B).

STZ administered rats also exhibited the significant increase in protein level of phosphorylated tau (p<0.01), beta amyloid (p<0.001) in comparison to respective control rat brain regions which were significantly (p<0.01) inhibited with rivastigmine treatment (Fig1C). The protein level of tau was not altered after any treatment. Rat brain sections of STZ administered rats showed the considerable aggregation of amyloid in comparison to control and rivastigmine per se rats, as observed through congo red staining in rat brain sections. Rivastigmine treatment considerably inhibited this aggregation (Fig.1D).

Effect of rivastigmine on nitrosative stress:
To assess the effect of rivastigmine on nitrosative stress the estimation of nitrite level and protein level of iNOS was done. Protein level of iNOS was significantly increased in both cortex (p<0.001) and hippocampus (p<0.01) regions of STZ administered rat brain (Fig. 2A). Rivastigmine treatment offered significant (p<0.01) protection against STZ induced augmented iNOS level.

The nitrite level was also significantly (p<0.001) increased in both cortex and hippocampus regions of STZ administered rats. Rivastigmine treatment offered significant (p<0.01) protection against STZ induced augmented nitrite level (Fig.2B). Rivastigmine per se treatment did not offer any alteration in nitrite level and iNOS level in comparison to control rat brain regions.

Effect of rivastigmine on oxidative stress:
To assess the oxidative stress, the level of GSH and MDA was estimated in both cortex and hippocampus regions of rat brain. GSH level was significantly (p<0.01) decreased in both cortex & hippocampus regions of STZ administered rats which was significantly (p<0.05) inhibited with rivastigmine treatment (Fig.2C). Similarly, STZ administered rat brain, cortex and hippocampus regions exhibited the significant (p<0.001) lipid peroxidation as assessed
through estimation of MDA level. STZ induced increased MDA level was significantly (p<0.001) inhibited with rivastigmine treatment (Fig.2D)

Effect of rivastigmine on endoplasmic reticulum (ER) stress:
The ER stress was evaluated by estimating the intracellular calcium level and protein levels of ER stress related markers like GRP78, GADD153 and caspase12. STZ administration caused significant (p<0.001) increase in the calcium level in both cortex and hippocampus regions in comparison to respective control rat brain regions. Rivastigmine per se group of rat brains had no effect on calcium level as the levels were almost similar to control groups in both the cortex and hippocampus regions. STZ induced increased level of calcium was significantly (p<0.001) inhibited with rivastigmine treatment (Fig.3A).

STZ administration in rat brain caused significantly increased level of GRP78 (p<0.001), GADD153 (p<0.01) and cleaved caspase12 (p<0.01) in both studied rat brain regions, which were significantly (p<0.01) inhibited with rivastigmine treatment. Per se rivastigmine treatment did not alter the level of the studied ER stress markers in comparison to levels of control rat brain (Fig.3B).

Effect of rivastigmine on proteasome activity:
To assess the effect of rivastigmine on cellular protein degradation machinery, the proteasome activity (both trypsin and chymotrypsin) was estimated in both cortex and hippocampus regions of rats of all groups. Trypsin activity was significantly increased in both cortex (p<0.001) & hippocampus (p<0.01) region of STZ administered rat brain in comparison to respective control rat brain regions (Fig.4). However, Rivastigmine treatment further enhanced the trypsin activity. Chymotrypsin activity was not significantly altered after STZ administration in both studied brain regions, in comparison to respective control rat brain regions. However, rivastigmine treatment further enhanced the chymotrypsin activity significantly (p<0.001).

Effect of rivastigmine on neuronal degeneration and apoptosis:
Neuronal degeneration was assessed through staining of rat brain sections with florojade stain, while neuronal apoptosis was evaluated through estimating the protein level of cleaved caspase 3. STZ administered rat brain sections showed the considerable neuronal degeneration which was inhibited with rivastigmine treatment with no per se effect of rivastigmine (Fig.5A).

STZ administration caused significant (p<0.001) increase in level of cleaved caspase 3 in both cortical and hippocampal regions which was notably (p<0.001) inhibited with rivastigmine treatment (Fig.5B).

Effect of rivastigmine on neuronal morphology and astrocytes activation:
To verify the morphological alterations in the neuronal cells, the brain sections were stained with the cresyl violet stain. STZ administration caused significant (p<0.001) increase in the number of shrunken neurons in both cortex and hippocampus regions. Whereas, there were no change in the morphology of neurons of control and rivastigmine per se groups. Rivastigmine treatment exhibited the significant protection (p<0.05) to morphological alterations in neurons (Fig.5C).

STZ administration also caused considerable astrocytes activation in both studied rat brain regions which was inhibited with rivastigmine treatment. In rats of rivastigmine per se group, the GFAP reactivity was approximately similar to respective control rat brain regions.
suggesting no per se effect of rivastigmine. Astrocytes and neurons were individually labelled with markers protein GFAP and NeuN and images were captured and quantified (Fig.6).

Discussion

Rivastigmine is FDA approved clinically being used anti AD drug which provide symptomatic improvement in AD patients however, it could not modify the disease status significantly. The mechanistic evaluation of rivastigmine may unfold the new dimensions and clues in search of potent therapeutic affordable molecule for AD patients in comparatively less duration. Findings of the present study validated the known inhibitory effect of rivastigmine against increased AChE activity. Along with this, we have also observed the significant inhibitory effect of rivastigmine on tau phosphorylation and amyloid deposits however, more sensitive technique may provide better understanding regarding effect if rivastigmine on amyloid deposition. Earlier, we have reported the effect of donepezil on STZ induced energy crisis and significant alterations in ER stress related markers involving chaperon GRP78, GADD153 and caspase12 employing STZ induced experimental model of AD [11]. This study is suggesting the varied responses of rivastigmine in AD related neurodegenerative mechanisms. Previous studies have suggested the close association of oxidative stress, nitrosative stress and unfolded protein responses during AD pathology [29,30]therefore, the evaluations were done to investigate the effect of rivastigmine on these suggested mechanisms. Previously, we and others have observed the direct or indirect involvement of energy crisis in cholinergic neurodegeneration during disease along with role of nitric oxide and mitochondrial function [11][31,32]. In this study first the evaluation of rivastigmine on nitrosative stress was done through assessing the level of nitrite as well as protein level of iNOS. Findings have shown that rivastigmine treatment significantly inhibits the STZ induced augmented level of iNOS and nitrite in both cortical and hippocampal regions of rat brain, suggesting its inhibitory effect against nitrosative stress during AD pathology. No alterations were observed for neuronal nitric oxide synthase in any of the treatment (data not shown).

Since neurons are particularly more vulnerable to oxidative damage due to their high content of polyunsaturated fatty acids in their membrane and their high oxygen consumption with their weak antioxidant defence [33–36], the effect of rivastigmine on oxidative stress was also estimated. The considerable involvement of oxidative stress has also been suggested and reviewed recently in AD pathology [37, 38]. Findings of this study has shown that rivastigmine treatment significantly inhibited the STZ induced depleted level of glutathione and increased level of lipid peroxidation (MDA). In accordance to our this finding the recent report has suggested that STZ induced depleted level of GSH and increased MDA could be inhibited with nanoparticles of hesperetin [39]. Another report by [40] Kumar et al, (2017) has also shown that STZ administration caused the depletion of GSH level and inflammatory responses which could be inhibited with caffeic acid. Our findings are in concordance to these reports and showed that rivastigmine contains antioxidative properties.

Both nitrosative stress and oxidative stress may initiate or contribute in implication of ER stress either directly or indirectly [41, 42]. For few other diseases, the direct correlation among them is establish [43–45] but for neurodegenerative diseases the exclusive information is not available. It has been reported that NO may regulate the ER stress through modifications of its chaperon proteins [41] however, lacunae exist. Among numerous chaperon the protein disulfide isomerase is most studied [46]. Besides this, NO could also nitrosylate the ER membrane located sensors of ER stress (PERK1, IRE1 and ATF6) [47, 48]. In AD pathology, the correlation of NO and PDI has well suggested through complexities of unfolded protein responses [49]. In 2019, the emerging role of various
Electrophiles including NO has also been suggested as regulator of ER stress in various diseases [46]. Oxidative stress is correlated with impairment of mitochondrial functions and thus afflicted cellular physiology which could lead to the activation of chaperons and may subsequently initiate the ER stress and decide the cellular fate. In this regard, [50] Cao and Kaufman (2014) have reviewed the various evidences and detail may be read there. Numerous reports have been published showing the critical implication of oxidative stress and energy metabolism in AD pathology [31]. In view of such discussed association, further we have evaluated the effect of rivastigmine on AD related ER stress. Findings have suggested that rivastigmine treatment significantly prevent the STZ induced alteration in ER stress markers which are associated with initiation of unfolded protein responses (dissociation of GRP78), DNA damage (GADD153) and neuronal apoptosis (caspase12). In addition to this, we have also observed the increased level of intracellular calcium which was also inhibited with rivastigmine treatment. This finding further validates the occurrence of STZ induced ER stress with involvement of altered calcium homeostasis. Previously, the association of calcium homeostasis and ER stress has been reviewed thoroughly [51].

Observed ER stress reflects the accumulation of misfolded proteins, as we have observed the aggregates of amyloid beta which may initiate the protein quality control system like unfolded protein responses (UPR) and ER associated degradation (ERAD). These both UPR and ERAD interacts in a coordinated manner with the UPS [52,53]. Since we have observed the STZ induced aggregates of amyloid beta as well as ER stress in both cortical and hippocampus regions, which were inhibited with rivastigmine treatment, further we intend to evaluate the effect of rivastigmine on proteasome activity. STZ administration caused significant increase in trypsin activity in both studied brain regions, however the chymotrypsin activity was not significantly altered. Interestingly the rivastigmine treatment further enhances both trypsin and chymotrypsin activity in both studied brain regions which may be one of the contributor in observed depleted level of amyloid aggregates in STZ+rivastigmine treated rats. In accordance to this finding recent study by Ray et al (2020) [4] has suggested the novel property of rivastigmine in augmented level of soluble APP in PC12 cells, in 3xTg mice and as well as in post-mortem AD brain and this property in combination of findings of this study may be further exploited in therapeutics of AD.

Further to evaluate the neuronal apoptosis, the protein level of cleaved caspase3 was estimated in both regions. STZ administration caused significantly upregulated level of cleaved caspase 3 which were inhibited with rivastigmine treatment. Concomitantly, the florojade staining of rat brain section has also shown the neuronal degeneration in both brain regions which was considerably inhibited with rivastigmine treatment.

Significant pathological as well as therapeutic implication of astrocytes activation has been suggested in AD patients and in experimental models [54, 55]. Therefore, concomitantly the effect of rivastigmine on astrocytes activation has also been evaluated. STZ administration caused significantly increased astrocytes reactivity which was inhibited with rivastigmine treatment. STZ induced considerably altered neuronal morphology was also observed in both brain regions which was inhibited with rivastigmine treatment.

In conclusion, findings suggested that STZ administration caused the significant occurrence of AD related pathological markers which were inhibited with rivastigmine treatment offering its antinitrosative, antioxidative and antiapoptotic activity along with its inhibitory effect on astrocytes reactivity and reflecting that rivastigmine may provide better curing effect than donepezil at early phase of disease after disease diagnosis (Fig.7).
Conflict of interest- Authors has no conflicts of interest.

Ethical approval- Informed animals were used after approval by the Institutional animal ethics committee of CSIR – Central Drug Research Institute (IAEC/2018/88/Renew-0/Dated-14/06/2018).

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Fig. 1 (A) Effect of rivastigmine on cognition: Image illustrate the paths swum by rats in different groups. Bar diagram showing the effect of rivastigmine on mean escape latency, path length and swimming speed in STZ induced AD rat model. (n=10) (B) Bar diagram showing the effect of rivastigmine in Ache activity (n=6) Abbreviations: Con – control; R - Rivastigmine, STZ- streptozotocin. Data are presented as mean± SEM, Control vs STZ - *p<0.05, **P<0.01; STZ vs STZ+R- $SP<0.01

Fig. 1(C) Blots showing the protein levels of pTau, Tau, β-amyloid & respective bar diagrams represent the quantification of pTAU and β-amyloid abundance with respect to loading control β-Actin in both cortical and hippocampal regions of rat brain. No significant alterations were observed in protein level of tau after treatment. (n=6) (D) Bright field images (40X) showing the stained amyloid deposition by congo red in cortex & Hippocampus regions of rat brain sections. (n=6) Abbreviations: Con/C – control; R - Rivastigmine, STZ- streptozotocin. Data are presented as mean± SEM analyzed by one way ANOVA post hoc Newman Keuls multiple comparison test, Control vs STZ - *p<0.05, **P<0.01, ***P<0.001; STZ vs STZ+R, $P<0.05 ,$SP<0.01,$$$P<0.001.
Fig-2 Effect of rivastigmine on nitrosative stress: (A) Images showing the increased level of iNOS protein & bar diagram represents the quantification of iNOS abundance with respect to loading control β-Actin. (n=6) (B) Graphical representation of nitrite level in both cortex & hippocampus regions of rat brain. (n=6)

Abbreviations: Con – control; R – Rivastigmine, STZ- streptozotocin. Data are presented as mean± SEM analyzed by one way ANOVA post hoc Newman Keuls multiple comparison test, Control vs STZ - *p<0.05, **p<0.01, ***P<0.001; STZ vs STZ+R- $P<0.05, $$P<0.01, $$$P<0.001.

Fig-2 Effect of rivastigmine on oxidative stress: Bar diagram represents (C) GSH (D) MDA levels in both cortex & hippocampus regions of rat brain. Abbreviations: Con – control; R – Rivastigmine, STZ- streptozotocin. (n=6) Data are presented as mean± SEM analyzed by one way ANOVA post hoc Newman Keuls multiple comparison test, Control vs STZ - **P<0.01; STZ vs STZ+R- $P<0.05
Fig-3 Effect of rivastigmine on endoplasmic reticulum stress: (A) Bar diagram represents calcium level estimation by fluora-3AM (n=6) (B) Blots showing the level of ER stress marker proteins GRP-78, GADD153 & cleaved caspase-12 in both cortex & hippocampus regions of rat brain. (n=6) Abbreviations: Con – control; R - Rivastigmine, STZ- streptozotocin. Data are presented as mean± SEM analyzed by one way ANOVA post hoc Newman Keuls multiple comparison test, Control vs STZ - ***P<0.001; STZ vs STZ+R- $$$$P<0.001.

Fig- 3B Effect of rivastigmine on endoplasmic reticulum stress: Bar diagram represents the quantification of GRP-78,GADD153 & cleaved caspase-12 abundance with respect to loading control β-Actin in both cortex & hippocampus regions of rat brain. (n=6) Abbreviations: Con – control; R - Rivastigmine, STZ- streptozotocin. Data are presented as mean± SEM analyzed by one way ANOVA post hoc Newman Keuls multiple comparison test, Control vs STZ - *p<0.05, **P<0.01, ***P<0.001; STZ vs STZ+R- $P<0.05, $$$P<0.01,$$$$P<0.001.
Fig 4 Effect of rivastigmine on proteasome activity: Bar diagram represents the proteasome activity of trypsin & chymotrypsin enzymes in both cortex & hippocampus regions of rat brain. Abbreviations: Con – control; R – rivastigmine, STZ- streptozotocin. (n=6) Data are presented as mean± SEM analyzed by one way ANOVA post hoc Newman Keuls multiple comparison test, Control vs STZ - **P<0.01, ***P<0.001; STZ vs STZ+R- $$$$P<0.001.
Fig 5 Effect of rivastigmine on neuronal degeneration and apoptosis (A) Fluorescent images showing the degeneration of neurons stained by Flurojade-C (n=6) (B) Blots showing the level of apoptosis marker protein cleaved caspase-3 & bar diagram represents the quantification of cleaved caspase-3 abundance with respect to loading control β-Actin in both cortex & hippocampus regions of rat brain. (n=6) (C) Bright field images showing the neuronal morphology in rat brain and bar diagram represents the quantification of degenerated neurons. (n=6) Scale-50µm. Abbreviations: Con – control; R - Rivastigmine, STZ- streptozotocin. Data are presented as mean± SEM analyzed by one way ANOVA post hoc Newman Keuls multiple comparison test, Control vs STZ - ***P<0.001; STZ vs STZ+R- $$$$P<0.001.
Fig. 6 Effect of rivastigmine on astrocytes activation and neurons by immunofluorescence (A) Images showing the activated astrocytes by GFAP (green) (B) DAPI (blue) (C) NeuN (red) (D) Merged images. The images were captured at 10X to visualize the specific regions and certain area (square box) was zoomed further and captured at 40X magnification. Bar diagram showing the quantification of GFAP and NeuN immunoreactivity in both cortex and HP region analyzed by Leica Qwin software. SEM (n=6) Scale-100µm. Abbreviations: Con – control; R - Rivastigmine, STZ- streptozotocin. Data are presented as mean ± analyzed by one way ANOVA post hoc Newman Keuls multiple comparison test, Control vs STZ - ***P<0.001; STZ vs STZ+R - $$$P<0.001.
Fig – 7 Image illustrating the neuroprotective effect of Rivastigmine against the Alzheimer’s related pathological markers, oxidative stress, nitrosative stress, endoplasmic reticulum stress and neuronal apoptosis observed during Alzheimer’s disease Pathology indicating its antioxidative and antiapoptotic activity along with its interference in protein degradation machinery.