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Manish kumar and Nitin Bansal
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Original Article

Caffeic Acid Phenethylester Averts Streptozotocin-Induced Memory Deficits: Role of GSK-3 and NF-kappa B

Manish Kumar [1, 2], Nitin Bansal [2, *]

[1] PhD Research Scholar, IKG Punjab Technical University, Kapurthala (Punjab) 144603, India
[2] Department of Pharmacology, ASBASJSM College of Pharmacy, Bela (Ropar) 140111, India

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ABSTRACT

Objective: Earlier we reported attenuation of memory deficits by caffeic acid phenethylester (CAPE) in STZ (ICV) injected rats. Intracerebroventricular (ICV) administration of streptozotocin (STZ) in rodents instigates Alzheimer's type cognitive decline through augmentation of GSK-3 associated pathogenesis. This study delineates the role of GSK-3 in prevention of cognitive deficits by CAPE using STZ (ICV) model of AD like dementia in rats. **Methods:** CAPE (6 mg/kg, *i.p.*) was administered for four weeks daily and STZ (3 mg/kg) was slowly injected in lateral cerebral ventricles of rats twice at a gap of 48 h. Lithium chloride (100 mg/kg, *i.p.*) was administered for four weeks daily to explore the role of GSK-3 in CAPE and STZ (ICV) injected rats. The Morris water maze and elevated plus maze paradigms were used for memory assessments. Markers of oxidative stress (TBARS, GSH, SOD, CAT) and inflammation (TNF-α) including NF-kappaB, and AChE activity were quantified in brain of rats. **Results:** STZ (ICV) injection deteriorated the spatial memory of rats through elevation of the brain oxidative stress, AChE activity, TNF-α, NF-kappaB levels. CAPE enhanced the memory functions through attenuation of rise in oxidative stress, AChE activity, TNF-α and NF-kappaB levels in STZ (ICV) treated rats. Chronic administration of LiCl (GSK-3 inhibitor) further potentiated the decline of TBARS, AChE activity, TNF-α, NF-kappaB levels and elevation of GSH, SOD and CAT activities by CAPE in brain of STZ (ICV) injected rats. **Conclusion:** These results suggested that CAPE prevents STZ (ICV) triggered memory dysfunctions in rats through attenuation of GSK-3 activity.

Keywords: Caffeic acid phenethylester; Glycogen synthase kinase-3; Oxidative stress; NF-kappaB; Inflammation; Streptozotocin.

Corresponding author *
Prof (Dr) Nitin Bansal
Head, Department of Pharmacology
ASBASJSM College of Pharmacy, Bela (Ropar)
Punjab 140111
Email ID: nitindsp@rediffmail.com

1. INTRODUCTION

Age associated dementia is a debilitating brain disorder inflicting considerable morbidity and mortality in elderly people. Alzheimer's disease (AD) is the prime determinant of dementia culminating progressive neurodegeneration

(limbic system, neocortical and basal forebrain regions) and profound loss of cognitive abilities in advanced stages.[1] Deposition of amyloidβ (Aβ) plaques, neurofibrillary tangles (NFTs) and cholinergic deficit are neuropathological features manifested at various stages of AD. NFTs are argyrophilic fibrillar aggregates of highly phosphorylated and misfolded microtubule-associated tau protein that spontaneously forms paired-helical filaments (each 10 nm) inside the neuroplasm. The extent of tau hyperphosphorylation directly correlates with retrograde degeneration of neurons, synaptic dysfunction and cognitive decline in AD patients.[2] The pattern of intraneuronal NFTs and neuropil threads formation in specific brain regions (entorhinal, perirhinal cortex, hippocampus, association cortex) is summarized by six stages of Braak and Braak and included in 1997 NIA-Reagan clinical diagnostic criteria of AD.[3]

Tau is synthesized in perikaryon, transported anterograde in axons and stabilize the microtubular assembly by interacting with tubulin protein. Amongst a number of kinases (e.g. cyclin-dependent protein kinase-5, cAMP-dependent protein kinase) glycogen synthase kinase-3 (GSK-3) is a major tau kinase whose age associated rise in expression precipitates taupathy commensurate with AD like spatiotemporal pattern of NFTs and decline in memory.[4] Recession in antioxidant shield, enhanced generation of free radicals and pro-inflammatory cytokines in brain constitute primary neuropathological events in progression of AD hastened by GSK-3 overactivity. A number of evidences indicate rise in expression of amyloid precursor protein (APP), β-secretase-1, NF-kappaB activity and cholinergic deficit through unregulated GSK-3 activity. GSK-3 intensifies the downregulation of pro-survival transcription factors and upregulation of apoptotic factors leading to cell death under stress conditions such as oxidative stress and insult by diverse neurotoxins such as streptozotocin. Suppression of GSK-3 activity (e.g. AZD-1080, AR-A014418, lithium chloride, SB216763, SB415286, TDZD-8, tideglusib) correlates with reduction in oxidative stress, inflammation and NFTs in brain.[5]

Caffeic acid phenethylester (CAPE) is a natural bioactive polyphenolic ester present in many plants and propolis of honeybee hives. The catechol ring of CAPE (2-phenylethyl (2*E*)-3-(3,4-dihydroxyphenyl) acrylate) is key to the highly desirable bioactivities evaluated in cancer, diabetes, inflammatory and many neurodegenerative disorders. The restrain over eicosanoids pathogenesis, pro-inflammatory cytokine release (e,g. interleukins, tumour necrosis factor) and facilitation of Nrf2 signaling sufficiently indicate the potent anti-inflammatory and antioxidative properties of CAPE.[6] Furthermore, reduction in brain APP formation, β-secretase-1 activity, insulin resistance and neuroapoptosis amply substantiates the benefits of CAPE in AD pathology.[7] Earlier in animal studies we observed that CAPE averted streptozotocin (STZ) triggered dementia akin to AD.[8] Streptozotocin is a neurotoxic nitrosourea derivative that disrupts the control over basal GSK-3 activity in brain.[9] Furthermore, intracerebroventricle (ICV) administration of STZ upregulates the expression of GSK-3 in brain of rats and thereby hastens redox imbalance, inflammation and profound loss of cognitive functions.[10] In this study we examined the role of GSK-3 in modulation of memory functions by CAPE using STZ (ICV) model of AD.

2. MATERIALS AND METHODS

Experimental animals and drug treatment

The research design of this study was approved by Institutional Animal Ethics Committee of the institute. Adult Wistar rats (180-220 g, either sex) were procured from Central Animal Facility (CAF), AIIMS, New Delhi and were kept at CAF of the institute following the guidelines of CPCSEA, Ministry of Forests and Environment, Government of India. The rats were harbored under typical laboratory environment (temperature, 21-25°C; humidity, 30-50%; 12:12 light-dark cycle) in group of three per cage (44×29×16 cm^3) and nourished with customary rodent pellet diet (Ashirwad Industries, Mohali, India) and water *ad lib*. The rats were acclimatized to laboratory conditions two weeks prior to experimentation and randomly divided into four different groups having six animals in each group. Sham-treated group received 5% DMSO-aCSF solution (10 µl) on day 1 and 3; STZ (ICV) group rats were given streptozotocin (3 mg/kg in 10 µl vehicle) alone twice at gap of 48 h; CAPE+STZ group (CAPE+STZ) was administered CAPE (6 mg/kg, *i.p.*) for four weeks daily and STZ (ICV) (3 mg/kg); LiCl group (CAPE+STZ+LiCl) received CAPE (6 mg/kg, *i.p.*), STZ (ICV) (3 mg/kg) and lithium chloride (100 mg/kg, *i.p.*). CAPE (SRL, Mumbai) was dissolved in sterile DMSO diluted with isotonic saline (1:5) and was administered (dose 6 mg/kg) through intraperitoneal route for four weeks daily.[8] Slow ICV injection of STZ (3 mg/kg *b.w.*)[10, 11] was given on day 1 and 3 one hour after CAPE administration to simulate AD like dementia in rats. Lithium chloride (Himedia, Mumbai) is a non-selective GSK-3 inhibitor, dissolved in normal saline and administered (dose 100 mg/kg, *i.p.*)[11] to CAPE and STZ (ICV) treated rats for four weeks daily. Morris water maze (MWM) and elevated plus maze (EPM) tests were conducted day 22 onwards to assess the memory functions. After behavioral tests animals were partially anesthetized using diethyl ether and sacrificed by decapitation for biochemical estimations in rat whole brain (Fig 1).

The ICV administration of STZ (SRL, Mumbai) was accomplished through stereotaxic surgery of rat brain. Barring sham group all other groups received STZ in lateral cerebral ventricle on day 1. A 3 mg/kg dose of STZ was freshly prepared using 5% dimethylsulfoxide (DMSO) in aCSF (0.147 M NaCl, 0.0029 M KCl, 0.0016 M $MgCl_2$, 0.0017 M $CaCl_2$, 0.0022 M dextrose, pH 7.3)[8] vehicle. The rats were anesthetized using chloral hydrate (350 mg/kg,

i.p.)[8] and depth of anesthesia was determined by lack of pedal pain in tail/toe pinch-response method. The body was placed on a warm pad (37±0.5°C) and head was positioned in the frame of stereotaxic apparatus (INCO, Ambala, India). The partially shaved scalp was wiped with 70% ethyl alcohol swab before incision. A middle sagittal incision was made in the scalp, skin was retracted and skull was uncovered. A burr hole was drilled through the parietal bone (stereotaxic coordinates: antero-posterior from bregma = -0.8 mm, mediolateral from mid-sagittal suture = ±1.5 mm, dorso-ventral from the skull = ±3.6 mm)[12] to access a randomly selected lateral cerebral ventricle. STZ (3 mg/kg in 10 µl vehicle) was slowly injected over 10 min duration (1 µl/min) and after injection the microneedle (28 gauge) was not displaced for 5 min to prevent pressure backflow (reflux) of drug solution out of ventricle along the injection track and thereby enhance diffusivity of drug in CSF. Sham rats received same volume of 5% DMSO-aCSF vehicle only. ICV treatment was repeated in remaining lateral ventricle once after 48 h. After drug administration the holes were repaired with dental cement and skin was sutured. Neosporin® was applied *pro re nata* to prevent contamination. To avoid sepsis Reflin® (cephazolin sodium, Ranbaxy) was injected (30 mg/kg, *i.p.*) once postoperatively. Postsurgical hypothermia was prevented by keeping the rats warm. Post-surgery each rat was placed individually in separate cage (30×23×14 cm^3) for 7 days allowing access to food and water gratis.

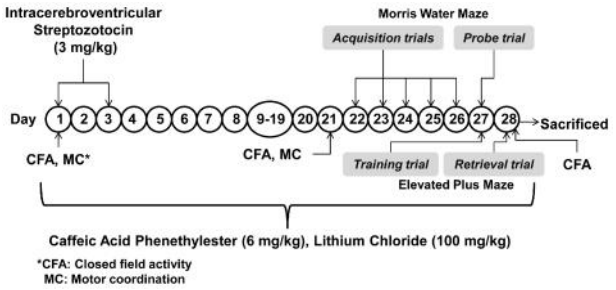

Fig 1: Experimental design

Closed field activity

The mean ambulatory score of different groups was determined on day 1, 21 and 28 using actophotometer (INCO, Ambala, India) for a period of 10 min in a dark room and expressed as counts per 10 min.[8]

Rotarod test

Rotarod (Rolex Scientific Instruments, Ambala, India) apparatus was used to assess motor impairment in rats on day 1 and 21. For habituation the rats were placed on constantly rotating rod (10 rpm) for 3 min. To determine motor coordination each animal was placed on rod whose rotations gradually increased from 6 to 40 rpm over 5 min and latency to fall off (s) from rotating rod was noted.

Morris water maze test

Spatial navigation memory of rodents was assessed by using MWM in place-condition paradigm which involves learning using allocentric and egocentric cues to escape on a submerged platform placed in a fixed location with start position of each animal randomized with each trial.[13] A black colored metallic (iron) circular tank (radius 100 cm, height 60 cm) was filled to a depth of 30 cm with water (temperature 25±1°C) and two threads were fixed at right angle to each other on the rim of the pool to divided the tank into four equal quadrants. A clockwise nomenclature was assigned to quadrants *viz.* North (N), East (E), South (S) and West (W).[8] The standard procedure was adopted having three phases. On day 21 each rat was familiarized with MWM by allowing maze exploration for 120 s without platform. During acquisition phase a black painted metal (iron) platform (area 11 cm^2, search area: target ratio of 314:1) was submerged 2 cm below surface of water in the centre of target quadrant (W) of this tank.[8] The platform was camouflaged by making the water opaque. In place-condition paradigm submerged platform was kept in a fixed location (W) throughout the training session with start position of each animal randomized with each trial. The rat was smoothly released in the water with head facing the wall of tank and allowed 120 s to locate submerged platform. The start location of each animal was randomized *viz.* from N to W on day 22, E to N on day 23, S to E on day 24, W to S on day 25, N to W on day 26 for each trial. Each rat received four training trials consecutively per day with inter-trial gap of 30 s on the platform. The animal unable to locate the hidden platform within 120 s was guided gently onto platform. The time taken by each rat to locate the hidden platform denotes mean escape latency (MEL). After 24 h of the last acquisition trial retention of memory of each rat was determined in probe trial. The platform was removed, each rat was placed 180° from original platform position to navigate the tank for 60 s to eliminate thigmotaxis[14] and mean time spent in all four quadrants was noted. The reference memory is denoted by percentage of mean time spent by the animal in target quadrant [TSTQ (%)] searching for the camouflaged platform. The distal visual cues and position of experimenter remained same during whole study.[8]

Elevated plus maze test

EPM consisted of a wooden apparatus having a square central platform (area 100 cm^2) connected to two open arms (50 cm×10 cm) and two laterally covered arms with open roof (50 cm×40 cm×10 cm), placed 60 cm above the floor. The duration of entry of rat from the open arm into one of the covered arms (transfer latency) with its entire four paws denoted memory of the animal. On day 27 each rat was placed at the distal-most end of an open arm with head opposite to the central platform and allowed to explore the maze for 90 s. The animal which failed to passage into a closed arm within 90 s was gently guided in one of the covered arms. The rat was further given exploration time of 20 s and then returned to its home cage. The reference

memory of this EPM learning was evaluated 24 h after the last trial.[8]

Whole brain tissue preparation

The whole brain was dissected out and rinsed with ice-cold sterile normal saline (0.9 g/L sodium chloride) to eliminate the extraneous tissue residues and blood. Brain tissue homogenate (10% w/v) was prepared in 50 mM sodium potassium phosphate buffer (1% v/v Triton X-100, pH 7.4) at 4°C, centrifuged at 15×10^3 rpm for twenty minutes (4°C) in high speed refrigerated centrifuge (CPR-30 Remi Compufuge, India), and supernatant was separated from sediment for estimation of AChE, GSH, SOD, CAT.

Determination of markers of oxidative stress in rat brain

The markers of oxidative stress *viz.* thiobarbituric acid reactive substance (TBARS), glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) were measured. TBARS (nanomole/mg protein) was measured by method provided by ohkawa et al [15]. Ellman method was used for GSH estimation (micromole GSH/ mg protein). [16] SOD activity activity (micromole NBT reduced/min/mg/protein) was determined by method of winterbourn et al. [17] CAT activity (micromole H2O2 decomposed/min/mg protein) is assessed following the method of Claiborne.[18]

Estimation of AChE activity in brain of rats

Briefly, the reaction mixture consisted of 0.05 ml of the supernatant, 3 ml of phosphate buffer (100 mM, pH 8), 0.1 ml of 10 mM 5,5'-dithiobis-(2-nitrobenzoic acid), 0.1 ml of acetylthiocholine iodide (1585 mM). Acetylcholinesterase (AChE) activity is expressed as micromole (µM) acetylthiocholine iodide hydrolysed/min/mg protein ($\varepsilon=1.36\times10^4\ M^{-1}cm^{-1}$, λ_{max}=412 nm).[19]

Determination of tumor necrosis factor-α (TNF-α) and nuclear factor-kappa B (NF-kappaB) in rat brain

The rat brain TNF-α (Krishgen, Mumbai) and NF-kappaB (KinesisDX, California) levels were determined by double antibody sandwich ELISA as per instructions provided in kits. The brain homogenate was centrifuged at 2500 rpm for 20 min and supernatant was used for ELISA procedures. The sample was added to rat monoclonal antibody pre-coated wells (96 wells), treated with secondary antibodies labeled with biotin followed by Streptavidin-Horseradish Peroxidase and incubated at 37°C for 1 h after covering the plate. Afterwards, treatment with chromogenic solution A and B or TMB substrate produced bluish color, stop solution was added to stop the reaction and absorbance was noted at λ_{max}=450 nm in ELISA microplate reader (iMARK, BIORAD) within 15 minutes of stopping reaction. Concentration of NF-kappaB and TNF-α in unknown sample was calculated from standard curve. The brain NF-kappaB is expressed as 'n' ng/ml and TNF-α level as 'n' pg/ml.

Estimation of total protein in rat brain

The reaction mixture consisted of 0.25 ml of supernatant, 0.75 ml phosphate buffer, 5 ml of Lowry's reagent and 0.5 ml of Folin-Ciocalteu reagent (1 N). After incubation the protein content was determined spectrophotometrically at λ_{max}=650 nm with a standard curve (0.25-2.50 mg/ml of bovine serum albumin).[20]

Histopathology of rat brain

The rats (n=1) were injected with chloral hydrate (400 mg/kg, *i.p.*) and given transcardial perfusion with 10% neutral buffered formalin solution (10% NBF) by using gravity fed perfusion setup. The head was decapitated; whole brain was dissected out and fixed in 10:1 ratio of fixative (10% NBF) to tissue for 6 days at 4°C. Afterwards, the brain was stored in 70% ethanol at 4°C until sectioning. 5 µm sections were trimmed out by microtome and treated with haematoxylin-eosin (H&E) stain. Slides were then cover-slipped with permanent mounting medium (synthetic resin DPX) and examined in light microscopy at ×45 magnifications.

Statistical analysis

One-way ANOVA followed by Tukey's *post-hoc* test and two-way ANOVA followed by Bonferroni *post-hoc* test were utilized to interpret inter-group variation using software GraphPad Prism5 (GraphPad Software Inc., USA). All the values are denoted as mean±SEM and statistical significance was achieved at $p<0.05$.

3. RESULTS

Effect on ambulatory activity of rats

The different groups exhibited no significant difference between mean locomotor activity on day 1, 21 and 28 (Fig 2).

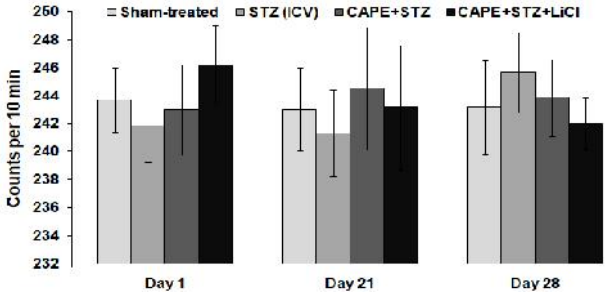

Fig 2: Mean locomotor activity exhibits no significant variation in actophotometer.

Two-way ANOVA followed by Bonferroni *post-hoc* test was applied. Values are denoted as mean±SEM (n=6).

Effect on motor coordination of rats

In the present study, the mean fall off latency (s) from the rotating rod did not differed significantly among rats of all the groups as determined on day 1 and 21 (Fig 3).

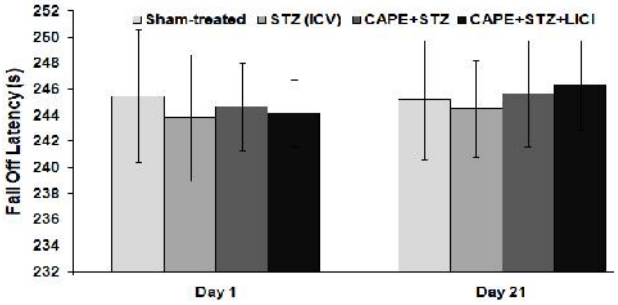

Fig 3: Different groups have no significant variation in latency to fall-off (s) determined utilizing rotarod apparatus.

One-way ANOVA followed by Tukey's *post-hoc* test was applied. Values are denoted as mean±SEM (n=6).

Chronic administration of lithium chloride enhances the attenuation of decline in memory by CAPE in STZ (ICV) injected rats in MWM

The significant variation in MEL amongst different groups emerged out on day 23 of acquisition trials. ICV administration of STZ alone deteriorated the spatial learning of rats evident by enhancement in MEL ($p<0.05$) of rats with respect to sham-treated rats. Improvement in learning abilities of STZ (ICV) injected rats by chronic administration of CAPE (6 mg/kg) was portrayed by lowering of ($p<0.05$) MEL when compared to rats of STZ (ICV) alone group. Suppression of brain GSK-3 activity by chronic administration of LiCl potentiated the lowering of MEL ($p<0.05$) by CAPE in STZ (ICV) injected rats with respect to rats administered with CAPE and STZ (ICV) only (Fig 4A).

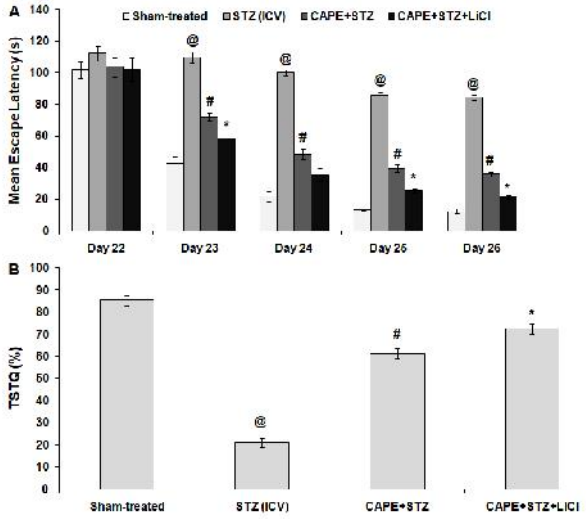

Fig 4: Chronic inhibition of GSK-3 by lithium chloride improves the memory enhancing activity of CAPE in STZ (ICV) model of AD in MWM.

Comparison of (A) mean escape latency (s) during five days acquisition trials by utilizing two-way ANOVA followed by Bonferroni *post-hoc* test; (B) percentage of time spent in target quadrant [TSTQ (%)] during probe trial by using one-way ANOVA followed by Tukey's *post-hoc* test. Values are denoted as mean±SEM (n=6). Significance at $^{@}$ $p<0.05$ vs. sham-treated group; $^{\#}p<0.05$ vs. STZ (ICV) group; $^{*}p<0.05$ vs. CAPE+STZ group. (STZ: Streptozotocin; CAPE: Caffeic acid phenethylester; LiCl: Lithium chloride).

All the animals were subjected to retrieval trial on day 27 to evaluate the reference memory. STZ (ICV) group exhibited profound memory loss denoted by decrease ($p<0.05$) in TSTQ (%) with respect to sham-treated group. Treatment with CAPE increased the memory of STZ (ICV) injected rats showed by significant ($p<0.05$) enhancement in TSTQ (%) when compared to rats that received STZ (ICV) alone. LiCl group exhibited elevation ($p<0.05$) in TSTQ (%) in comparison to CAPE+STZ group (Fig 4B). These results suggest that chronic inhibition of brain GSK-3 activity by LiCl improved the learning and memory of CAPE and STZ (ICV) treated rats.

Chronic administration of lithium chloride facilitates CAPE induced attenuation of memory loss in STZ (ICV) injected rats in EPM

During training trial in EPM the mean transfer latency (TL) of different groups exhibited no significant variation. However, significant differences in TLs of different groups were noted during retrieval trial. Degradation of cognitive abilities of STZ (ICV) group was exhibited by elevation of mean TL ($p<0.05$) in comparison to sham-treated group. Reduction in TL ($p<0.05$) of CAPE+STZ group with respect to STZ (ICV) alone group portrayed resurrection of STZ (ICV) induced memory dysfunctions in rats chronically administered with CAPE. Long-term administration of LiCl facilitated the decrease in TL ($p<0.05$) by CAPE in STZ (ICV) injected rats in comparison to rats administered with CAPE and STZ (ICV) only (Fig 5).

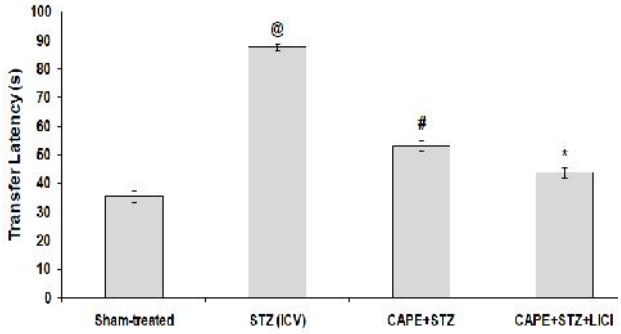

Fig 5: Chronic administration of lithium chloride (GSK-3 inhibitor) improves the memory enhancing activity of CAPE in STZ (ICV) model of AD in EPM.

Mean transfer latency (s) of different groups during retrieval trial in EPM is compared by employing one-way ANOVA followed by Tukey's *post-hoc* test. Values are denoted as mean±SEM (n=6). Significance at $^{@}p<0.05$ vs. sham-treated group; $^{\#}p<0.05$ vs. STZ (ICV) group; $^{*}p<0.05$ vs. CAPE+STZ group. (STZ: Streptozotocin; CAPE: Caffeic acid phenethylester; LiCl: Lithium chloride).

Chronic administration of lithium chloride enhances the mitigation of oxidative stress by CAPE in brain of STZ (ICV) injected rats

TBARS is a widely acknowledged indicator of lipid peroxidation product malondialdehyde. Quantification of GSH, SOD, CAT activities determine the endogenous antioxidant defense. These biochemical parameters are well established biomarkers of oxidative stress. ICV administered STZ enhanced the brain TBARS content, and diminished the GSH level, SOD and CAT activity ($p<0.05$) in comparison to sham-treatment. Long-term administration of CAPE prevented the STZ (ICV) triggered elevation in brain TBARS content, and decline of GSH level, SOD and CAT activity ($p<0.05$) with respect to STZ (ICV) alone treatment.

LiCl group exhibited reduction of brain TBARS level ($p<0.05$), and rise of GSH content ($p<0.05$), SOD ($p<0.05$) and CAT activity ($p<0.05$) with respect to CAPE+STZ group (Fig 6).

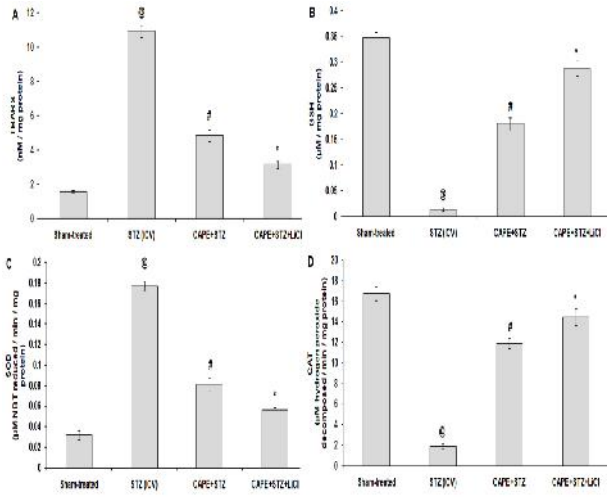

Fig 6: Chronic inhibition of GSK-3 by lithium chloride improves the decline of oxidative stress by CAPE in brain of STZ (ICV) injected rats.
Statistical analysis of brain (A) thiobarbituric acid reactive substances (TBARS) content, (B) reduced glutathione (GSH) level, (C) superoxide dismutase (SOD) activity and (D) catalase (CAT) activity in different groups (One-way ANOVA followed by Tukey's *post-hoc* test was applied). Values are expressed as mean±SEM (n=5). Significance at $^{@}p<0.05$ vs. sham-treated group; $^{\#}p<0.05$ vs. STZ (ICV) group; $^{*}p<0.05$ vs. CAPE+STZ group. (STZ: Streptozotocin; CAPE: Caffeic acid phenethylester; LiCl: Lithium chloride).

Chronic administration of lithium chloride enhances the suppression of AChE activity by CAPE in brain of STZ (ICV) injected rats

Pathophysiological increase in AChE activity compromises cholinergic activity in the brain and is associated with cognitive deficits. STZ (ICV) group showed increase in the brain AChE activity ($p<0.05$) with respect to sham-treated group. CAPE+STZ group exhibited decline ($p<0.05$) in the AChE activity when compared with STZ (ICV) group. LiCl group showed significant decrease of AChE activity ($p<0.05$) with respect to CAPE+STZ group (Fig 7). These results exhibited improvement in cholinergic facilitation by CAPE through chronic suppression of GSK-3 activity by LiCl in brain of STZ (ICV) injected rats.

Chronic administration of lithium chloride potentiates the repression of TNF-α level by CAPE in brain of STZ (ICV) injected rats

STZ (ICV) treatment enhanced ($p<0.05$) the brain TNF-α content as compared to sham-treatment. Chronic administration of CAPE attenuated ($p<0.05$) the TNF-α levels in brain of STZ (ICV) injected rats in comparison to rats that received STZ (ICV) alone. Administration of LiCl further reduced ($p<0.05$) the TNF-α content in brain of CAPE and STZ (ICV) treated rats when compared to rats administered with CAPE and STZ (ICV) only (Fig 7).

Chronic administration of lithium chloride reduced the NF-kappaB content in brain of CAPE and STZ (ICV) injected rats

STZ (ICV) treatment increased ($p<0.05$) the brain NF-kappaB content as compared to sham-treatment. Chronic administration of CAPE attenuated ($p<0.05$) the STZ (ICV) triggered rise of brain NF-kappaB levels in comparison to rats that received STZ (ICV) alone. Administration of LiCl reduced ($p<0.05$) the NF-kappaB content in brain of CAPE and STZ (ICV) treated rats when compared to rats administered with CAPE and STZ (ICV) only (Fig 7).

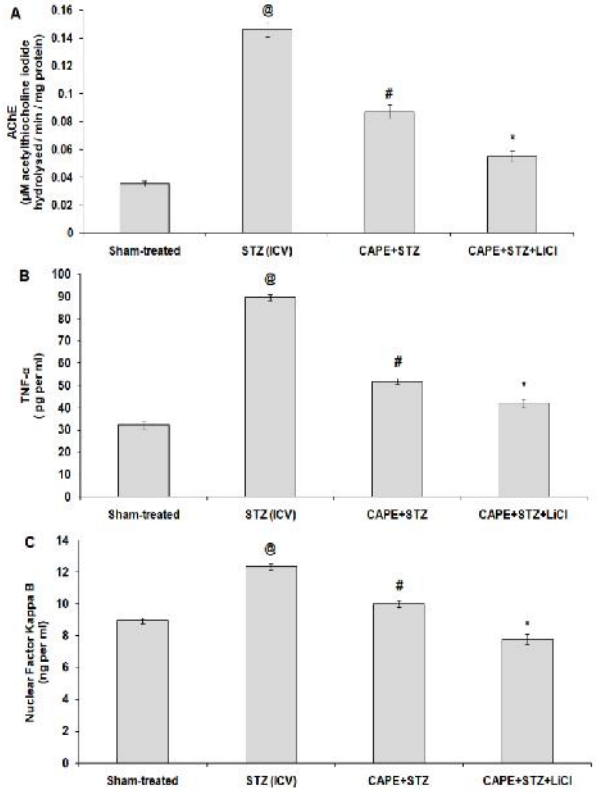

Fig 7: Attenuation of brain acetylcholinesterase (AChE) activity, TNF-α and NF-kappaB levels by CAPE in STZ (ICV) injected rats and its modulation by chronic LiCl administration.
Statistical analysis of brain (A) AChE activity, (B) TNF-α content and (C) NF-kappaB levels in different groups (One-way ANOVA followed by Tukey's *post-hoc* test was applied). Values are expressed as mean±SEM (n=5). Significance at $^{@}p<0.05$ vs. sham-treated group; $^{\#}p<0.05$ vs. STZ (ICV) group; $^{*}p<0.05$ vs. CAPE+STZ group. (STZ: Streptozotocin; CAPE: Caffeic acid phenethylester; LiCl: Lithium chloride).

Histopathology of rat brain cortex

The cortical region of rat brain exhibited gross neurodegeneration in response to STZ (ICV) injection when

compared to brain of sham-treated rats. Treatment with CAPE for four weeks daily arrested the STZ (ICV) accrued neurodegenerative changes in rat brain cortex in comparison to brain of rats that received STZ (ICV) only. Chronic inhibition of GSK-3 by LiCl potentiated the neuroprotective activity of CAPE in STZ (ICV) treated rats. LiCl group showed less neurodegeneration in comparison to CAPE+STZ group (Fig 8).

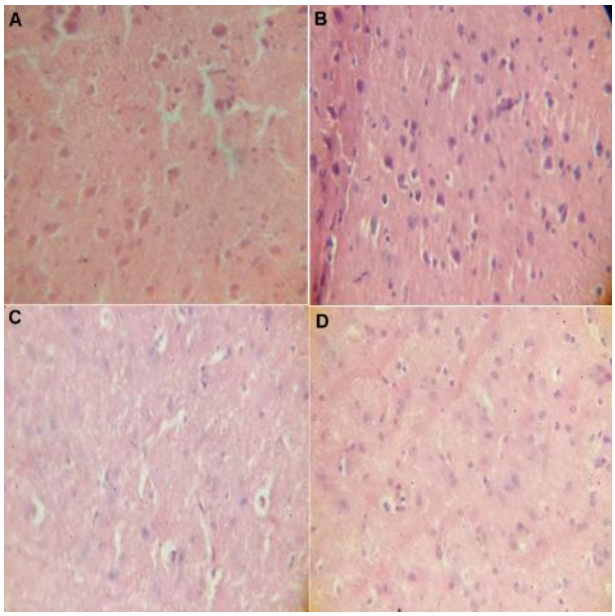

Fig 8: Histology of rat brain cortex (H&E, 45x). (A) Sham-treated group, (B) STZ (ICV) group, (C) CAPE+STZ group, (D) LiCl group (CAPE+STZ+LiCl).
(STZ: Streptozotocin; CAPE: Caffeic acid phenethylester; LiCl: Lithium chloride).

4. DISCUSSION

In previous study we demonstrated therapeutic activity of CAPE in STZ (ICV) induced dementia.[8] Centrally administered STZ (dose range 1-3 mg/kg) compromises the brain insulin signaling through down-regulation of phosphoinositide-3-kinase-Akt pathway and thereby elevates expression and activity of GSK-3 in brain of rodents that subsequently manifest AD traits over a period of 2-3 weeks.[10] Furthermore, STZ hastens brain oxidative stress, deposition of neuritic/senile plaques, formation of paired-helical filaments, genotoxicity and hyperstimulation of glial cells culminating profound cognitive deficits in rodents.[9] GSK-3 is ubiquitously expressed constitutively active kinase present in brain negatively modulated by phosphoinositide-3-kinase (PI3-kinase) and Wnt signaling. The aberrant overactivity and age related increase in expression of GSK-3 isoforms has been associated with several neurodegenerative disorders such as AD. GSK-3 hyperactivation in brain conjures tau hyperphosphorylation, free radical biogenesis and inflammation that are hallmark features of AD.[21] Several studies substantiate mitigation of oxidative stress, inflammatory cascade and amelioration of AD symptoms through suppression of GSK-3 activity by natural products. In this study we aimed to elucidate the role of GSK-3 in modulation of brain functions by CAPE using STZ (ICV) model of AD.

In the present study with respect to sham-treatment ICV administration of STZ alone increased the TBARS content and decreased the GSH level, SOD and CAT activities in the brain of rats. These results exhibited conspicuous rise in lipid peroxidation and diminution of antioxidant status in brain of rats in response to STZ (ICV) administration. Brain is vulnerable to oxidative stress owing to its low antioxidant reservoir, high oxygen and glucose utilization. Age associated downfall of endogenous antioxidants (e.g. reduced GSH, SOD, CAT) in brain ensue unhindered progression of AD pathology through free radicals mediated lipid peroxidation that generates numerous neurotoxic secondary products such as malondialdehyde, 4-hydroxy-2-nonenal, acrolein and isoprostanes. Lipid peroxidation is a consistent feature in early stages of AD pathology that initiates neurodegenerative cascade and is indicated by measurement of brain TBARS content.[22] Chronic treatment with CAPE (6 mg/kg) halted the STZ (ICV) induced rise in TBARS and decline of GSH, SOD and CAT activities, thereby resurrected the endogenous antioxidative guard in brain of rats. Suppression of GSK-3 activity in STZ (ICV) injected rats through chronic treatment with LiCl (100 mg/kg) further decreased the oxidative stress and strengthened the endogenous antioxidants activities in brain of CAPE treated rats. Many reports indicate surge in free radicals mediated toxicity by GSK-3 activity through inhibition of Nrf2/ARE pathway and depletion of GSH levels.[23] Decline in oxidative stress and restoration of antioxidant defense by inhibition of GSK-3 overactivity has been demonstrated in rodents.[21]

Several studies have revealed involvement of acetylcholine (ACh) and muscarinic receptors in learning and memory processes. Downfall in ACh levels through degenerative changes in cholinergic neurons, decrease in cholineacetyltransferase (ChAt) or increase in AChE activities are key features of AD affected brain explored during several clinical and pre-clinical studies.[24] Commensurate with previous findings, STZ (ICV) injection enhanced the AChE activity in brain of rats. Treatment with CAPE suppressed the STZ (ICV) triggered brain AChE overactivity. Inhibition of GSK-3 by LiCl further decreased the AChE activity in brain of CAPE and STZ (ICV) injected rats. Previous reports have also validated the aberrant cholinergic activity imparted by hyper-expression and activity of GSK-3 and amelioration by GSK-3 inhibitors such as LiCl.[25]

Chronic release of miniscule amounts of inflammatory chemokines through reactive microglia and astrocytes has been detected in early stages of AD. However, with progression of Aβ plaque deposition and NFTs formation the surge in pro-inflammatory cytokines (TNF-α) precipitate

profound neurodegenerative changes in brain.[26] Previous studies have demonstrated reduction of inflammation and improvement in AD like symptoms in rodents administered with anti-inflammatory drugs (COX inhibitors e.g. ibuprofen, rofecoxib and TNF-α inhibitors e.g. infliximab, adalimumab) or some natural products (e.g. Andean compound, curcumin, *Ginkgo biloba*, resveratrol).[25] TNF-α is a marker of inflammation found invariably associated with Aβ plaques and NFTs in brain, CSF and plasma of AD inflicted patients. The NF-kappaB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a transcription factor that regulates the transcription of immune related genes such as enzymes (e.g. COX-2, LOX, iNOS), adhesion molecules (ICAM-1, VCAM-1), pro-inflammatory cytokines and chemokines (e.g. IL-1, Il-6, TNF-α), and factors that affect neuronal survival. Constitutively active NF-kappaB is abundantly found in whole brain (including cortex and hippocampus) and is associated with synaptic plasticity, long-term potentiation, prenatal brain development and protects the brain against injuries. However, activation of NF-kappaB in response to brain insult by free radicals, radiation, bacterial lipopolysaccharides and several neurotoxins such as STZ leads to exacerbation of release of inflammatory chemokines.[27] Several studies determined that GSK-3 precipitates degenerative changes in brain by enhancing the release of inflammatory cytokines through NF-kappaB pathway.[28] In the present study we determined TNF-α and NF-kappaB levels in whole brain homogenate of rats by ELISA. STZ (ICV) injection incited profound elevation of inflammatory cascade in brain of rats. The TNF-α and NF-kappaB levels were enhanced in response to STZ (ICV) treatment in brain of rats. Treatment with CAPE reduced the TNF-α and NF-kappaB levels in brain of STZ (ICV) injected rats. Chronic inhibition of GSK-3 activity by LiCl exaggerated the reduction of TNF-α and NF-kappaB levels by CAPE in brain of STZ (ICV) injected rats. These results indicated that rise in NF-kappaB activity through GSK-3 was abrogated by CAPE in STZ (ICV) injected rats.

The ambulatory activity and motor coordination of rats were not significantly altered by various drug treatments implying no effect of these activities on behavioral results in MWM and EPM tests. STZ (ICV) administration heightened the MEL during hidden platform trial and reduced the TSTQ (%) during probe trial in MWM test, and increased the TL in EPM test corroborating reduction of spatial memory of rats. A decrease of MEL and increase of TSTQ (%) in MWM test, and decline of TL in EPM test substantiated the improvement of cognitive abilities in STZ (ICV) injected rats by chronic administration of CAPE (6 mg/kg). Inhibition of GSK-3 activity by LiCl further improved the memory of CAPE and STZ (ICV) treated rats evident by decrease in MEL during acquisition trials and increase of TSTQ (%) during probe trial in MWM, and decrease in TL during retrieval trial in EPM test.

5. CONCLUSION

Chronic administration of CAPE arrested the surge in brain oxidative stress, inflammation, cholinergic deficit and decline of memory in STZ (ICV) injected rats. Furthermore, suppression of GSK-3 activity in STZ (ICV) treated rats potentiated the improvement of memory functions by CAPE. From above discussion it may be summarized that in the presently employed STZ (ICV) model of AD CAPE prevented memory loss through suppression of GSK-3 and NF-kappaB activities.

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