

Effects of Chronic Lithium Chloride and L-Arginine Treatment on Prevention of Streptozotocin Induced Cognitive Deficits by Ellagic Acid

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ABSTRACT

Administration of streptozotocin (STZ) through intracerebroventricular (ICV) route manifests AD symptoms in rats. STZ deregulates the control over GSK-3 and eNOS activities through disruption of phosphoinositides mediated signaling. We attempted to elucidate the functions of GSK-3 and eNOS in memory enhancing activity of ellagic acid (EGA) in STZ (ICV) triggered AD type dementia. A 3 mg/kg dose of STZ was injected gently in lateral cerebral ventricles of rats on day 1 and 3. The rats were given EGA (35 mg/kg *b.w.*) through oral route for four weeks daily. LiCl (GSK-3 inhibitor) and L-Arginine (NO precursor) were administered for four weeks to explore the modulation of GSK-3 and eNOS respectively by EGA in STZ (ICV) injected rats. MWM and EPM paradigms were utilized for evaluation of memory of rats. The rats were sacrificed on day 28 to determine markers of oxidative stress (TBARS, GSH, SOD, CAT), nitrite, AChE, LDH, TNF- α and eNOS in brain. STZ (ICV) initiated cognitive deficits through enhancement of brain oxidative stress, nitrite, TNF- α , AChE, LDH activity and reduction in eNOS levels. EGA attenuated the rise in oxidative stress, inflammation and LDH activity in STZ (ICV) treated rats. Decrease in nitrite content, AChE activity and resurrection of eNOS activity by EGA averted STZ (ICV) induced memory dysfunction in rats. Chronic inhibition of GSK-3 by LiCl (100 mg/kg, *i.p.*) enhanced these effects of EGA in STZ (ICV) injected rats which thereby exhibited marked cognitive improvement. L-Arginine group manifested inflation in brain oxidative stress, TNF- α content, AChE and LDH activities. L-Arginine (200 mg/kg, *i.p.*) surged the nitrite content even though eNOS expression was diminished in brain of EGA and STZ (ICV) administered rats resulting in profound loss of memory. It can be concluded that GSK-3 and eNOS are involved in memory enhancing activity of EGA in STZ (ICV) injected rats.

Keywords: Ellagic acid; GSK-3; endothelial NOS; Memory; Streptozotocin.

INTRODUCTION

Ellagic acid (EGA) constitutes natural polyphenol in nuts, pomegranates, berries and beverages¹. EGA enriched dietary supplements (e.g. PomActiv™) enjoy wide popularity owing

to its potent antioxidative, anti-inflammatory, vasorelaxing, hypolipidemic, antidiabetic and antitumor properties². A clinical study revealed upper GIT as major site of absorption of EGA with extensive *in vivo* protein binding and intestinal transformation³. Approximately 90% of orally administered EGA is



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detected unchanged in blood plasma, urine and faeces, however, a small fraction (10%) of EGA is metabolized to urolithins by gut microbiota in rodents. The broad chemoprotective properties of EGA are validated by its antiapoptotic, cardioprotective, genoprotective, hepatoprotective, neuroprotective, nephroprotective and ulceroprotective activities against a plethora of toxins such as arsenic trioxide, mercuric chloride, 6-hydroxidopamine, cisplatin and acetic acid⁴. The neuroprotective activity of EGA has been evaluated in animal studies against A β ₂₅₋₃₅, colchicine, scopolamine and diazepam signifying the memory enhancing potential of EGA⁵.

Earlier we demonstrated that EGA prevented cognitive deficits in rats treated with intracerebroventricular streptozotocin (STZ-ICV)¹. STZ is a neurotoxic hexose linked *N*-methyl-*N*-nitrosourea analog which precipitates Alzheimer's disease (AD) like dementia in rodents. Centrally administered STZ triggers microglia induced inflammation, redox imbalance, and depletion of cholinergic transmission and ATP/ADP ratio in brain⁶. Disruption of insulin signaling and downstream phosphoinositide-3-kinase (PI3K) pathway is key feature of STZ-induced memory dysfunction^{7,8}. Phosphoinositides mediated signaling regulates miscellaneous cell functions including cell survival, brain development, neuronal plasticity, glucose metabolism, transcription, cellular transport and neurodegeneration through downstream targets glycogen synthase kinase (GSK-3) and endothelial nitric oxide synthase (eNOS). GSK-3 has basal serine-threonine kinase activity, ubiquitously expressed in brain and is implicated in formation of neurofibrillary tangles (NFTs), A β plaques, cholinergic hypoactivity and cognitive deficits⁹. Involvement of GSK-3 in familial and sporadic forms of AD has prompted its pharmacological targeting (e.g. Tideglusib, LY2090314, Enzastaurin, lithium chloride) with substantial amelioration in AD manifestations¹⁰. Nitric oxide is a retrograde messenger involved in synaptogenesis, neurogenesis and memory functions^{11, 12}. Neuronal and endothelial isoforms of NOS fulfill the physiological NO demands with mutual cooperation rendering eNOS activity pivotal during traumatic or neurotoxic brain insult and is substantiated by a study that revealed intracranial inhibition of eNOS in chicks instigated AD like symptoms¹³.

ICV injection of STZ compromises the PI3K-Akt mediated control over GSK-3 and eNOS activities in brain^{7,8}. Aberrant overactivation of GSK-3, fading of eNOS expression and loss of cognitive abilities in response to STZ administration in brain of rats is reported by many studies^{8,9}. In a previous study chronic EGA administration prevented the loss of memory in STZ (ICV) treated rats⁵. *In vitro* study has disclosed prevention of lipid peroxidation and endothelial apoptosis by EGA through modulation of eNOS activity¹⁴. However, the molecular mechanism of neuroprotective activity of EGA is still eluded. We propose that suppression of GSK-3 overactivity and resurrection of eNOS activity in brain of rats is involved in prevention of memory loss by EGA in STZ (ICV) model.

MATERIALS AND METHODS

Drugs

A suspension of ellagic acid (Himedia Labs, Mumbai, India) was prepared in 0.1% gum acacia maintaining the temperature 45 \pm 5 $^{\circ}$ C and pH 6-8¹. Streptozotocin (SRL Pvt Ltd., Mumbai, India) was dissolved in 5% DMSO in aCSF vehicle (0.147 M NaCl, 0.0029 M KCl, 0.0016 M MgCl₂, 0.0017 M CaCl₂, 0.0022 M dextrose in 10 ml water for injection, pH 7.3)¹. L-Arginine and lithium chloride (Himedia Labs, Mumbai) were dissolved in normal saline. The drug solutions were freshly prepared right before administration.

Experimental animals

The experimental design was approved by Institutional Animal Ethics Committee of the institute. Wistar rats (180-220 g, either sex) were acquired from Central Animal Facility (CAF), AIIMS, New Delhi and were reared at CAF of the institute as per guidelines of CPCSEA, Ministry of Forests and Environment, Government of India. Prior to surgery three rats per cage (44 \times 29 \times 16 cm³) were harbored (temperature, 21-25 $^{\circ}$ C; humidity, 30-50%; 12:12 light-dark cycle) and nurtured with customary rodent diet (pellets from Ashirwad Industries, Mohali, India) and water *ad lib*. Post-surgery each rat was placed individually in separate cage (30 \times 23 \times 14 cm³) for 7 days allowing access to food and water gratis.

Stereotaxic surgery of rat brain

The body of anesthetized rat (chloral

hydrate, 350 mg/kg, *i.p.*) was placed on warm pad with head positioned in the stereotaxic frame (INCO, Ambala, India). The scalp was incised (mid-sagittal), skin was retracted and the skull was uncovered. A hole was drilled through the parietal bone to access a lateral cerebral ventricle (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)^{1, 15}. On day one STZ (3 mg/kg *b.w.*) was injected in randomly chosen lateral cerebral ventricle over 10 min duration (1 μ l/min)⁹. After injection the Hamilton[®] microneedle was not displaced for 5 min to facilitate diffusivity of drug in CSF. Post drug treatment the hole was repaired with dental cement, skin was sutured and Neosporin[®] was applied *pro re nata* to prevent contamination. STZ administration was repeated in remaining lateral ventricle once after 48 h. Sham rats received same volume (10 μ l) of ICV-vehicle only on day 1 and 3. Sepsis was avoided by injecting cephalosporin sodium (Ranbaxy) (30 mg/kg, *i.p.*) once postoperatively. Postsurgical hypothermia was prevented by keeping the rats warm ($37 \pm 0.5^\circ\text{C}$).

Experimental design

The animals were acclimatized two weeks before experiments. AD type dementia was induced in rats by injecting STZ (ICV) on day 1 and day 3 one hour after EGA administration¹. EGA (35 mg/kg) was administered through oral route for four weeks daily¹. L-Arginine (NO precursor; 200 mg/kg, *i.p.*)^{16, 17} and lithium chloride (GSK-3 inhibitor; 100 mg/kg, *i.p.*)¹⁸ were administered to EGA and STZ (ICV) treated rats for 28 consecutive days. The rats were subjected to Morris water maze (MWM) and elevated plus maze (EPM) tests from day 22 onwards for evaluation of learning and memory functions. After behavioral tests animals were partially anesthetized using diethyl ether and sacrificed by decapitation for biochemical estimations in whole rat brain (Fig. 1).

The rats were indiscriminately disseminated to five different groups having six animals in each group: (i) Sham group (Sham-treated) rats were administered ICV-vehicle (10 μ l); (ii) STZ (ICV) group (STZ-ICV) rats were given streptozotocin (3 mg/kg in 10 μ l vehicle) alone; (iii) EGA+STZ group (EGA+STZ) was administered EGA (35 mg/kg, *p.o.*) for 4 weeks daily and STZ (ICV) (3 mg/kg, 10 μ l); (iv) LiCl group (EGA+STZ+LiCl) received EGA (35 mg/kg, *p.o.*), STZ (ICV) (3 mg/kg, 10 μ l) and LiCl

(100 mg/kg, *i.p.*); (v) L-Arginine group (EGA+STZ+L-Arginine) received EGA (35 mg/kg, *p.o.*), STZ (ICV) (3 mg/kg, 10 μ l) and L-Arginine (200 mg/kg, *i.p.*).

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 min¹.

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 trial¹⁹. A black colored metallic (iron) circular tank (radius 100 cm, height 60 cm) was filled to a depth of 30 cm with water (temperature $25 \pm 1^\circ\text{C}$) and two threads were fixed at right angle to each other on the rim of the pool to divide the tank into four equal quadrants. A clockwise nomenclature was assigned to quadrants viz. North (N), East (E), South (S) and West (W)¹. 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 cm², search area: target ratio of 314:1) was submerged 2 cm below surface of water in the centre of target quadrant (W) of this tank¹. 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 thigmotaxis²⁰ and mean time spent in all four quadrants was noted¹. 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 study¹.

Elevated plus maze test

EPM consisted of a wooden apparatus having a square central platform (area 100 cm²) 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 trial¹.

Whole brain tissue preparation

The whole brain was dissected out and rinsed with ice-cold sterile normal saline (0.9 g/L sodium chloride). 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 15×10³ 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, nitrite, LDH and TNF- α .

Determination 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 was measured by method provided by Ohkawa *et al.*²¹ Ellman method was used for GSH estimation²². SOD activity was determined by method of Winterbourn *et al.*²³ CAT activity is assessed following the method of Claiborne²⁴.

Measurement of nitrite content in rat brain

Briefly, a mixture of 0.5 ml of Greiss reagent (equal volumes of 1% sulphanilamide in 3 M HCl and 0.1% N-1-Naphthyl ethylene diamine dihydrochloride in water) and 0.1 ml supernatant was incubated at room temperature in the dark for 10 min, and the absorbance was measured at $\lambda_{\text{max}} = 548 \text{ nm}$ (Shimadzu UV-1700, Pharmaspec)²⁵. Total nitrite content ($\mu\text{M}/\text{mg}$ of protein) was determined from standard curve of sodium nitrite (10-100 μM).

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 μM acetylthiocholine iodide hydrolysed/min/mg protein ($\epsilon = 1.36 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$, $\lambda_{\text{max}} = 412 \text{ nm}$)²⁶.

Determination of LDH activity in rat brain

The total reaction mixture (3 ml) contained 1 ml of 200 mM Tris-HCl buffer (pH 7.4), 0.15 ml of 100 mM KCl, 0.15 ml of 50 mM sodium pyruvate, 0.20 ml of 2.4 mM NADH and supernatant²⁷. A decrease in extinction ($6220 \text{ M}^{-1}\text{cm}^{-1}$) at $\lambda_{\text{max}} = 340 \text{ nm}$ for 2 min at 25°C was measured and result was expressed in 'n' μM NADH oxidized/min/mg protein.

Determination of brain TNF- α and eNOS by ELISA

The rat brain TNF- α (Krishgen, Mumbai) and eNOS (KinesisDX, California) levels were determined by double antibody sandwich ELISA as per instructions provided in kits. The supernatant obtained by centrifuging the brain homogenate at 2500 rpm for 20 min 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 $\lambda_{\text{max}} = 450 \text{ nm}$ in ELISA microplate reader (iMARK, BIORAD) within 15 minutes of stopping reaction. Concentration of eNOS (ng/ml) and TNF- α (pg/ml) in unknown sample was calculated from standard curve.

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 $\lambda_{\max}=650$ nm with a standard curve (0.25-2.50 mg/ml of bovine serum albumin)²⁸.

Histopathology of rat brain

The rats (n=1) were injected with chloral hydrate (400 mg/kg, *i.p.*) and given transcatheter 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 μ m 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 $\times 45$ 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 \pm SEM and statistical significance was achieved at $p < 0.05$.

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).

LiCl and L-Arginine modulate the impact of EGA on memory of STZ (ICV) treated rats in MWM

The different groups showed no significant difference in mean escape latency (MEL) on day 22, but major differences appeared on day 23 of acquisition trials in MWM. STZ alone group had significantly ($p < 0.001$) higher MEL with respect to sham-treated group and thereby exhibited decrease in spatial learning. EGA+STZ group manifested improvement in learning evident by declined ($p <$

0.001) MEL with respect to STZ (ICV) alone group. LiCl group exhibited decline of MEL (day 23, $p < 0.001$; day 24, $p < 0.01$; day 25 and 26, $p < 0.05$) in comparison to EGA+STZ group (Fig. 3A). L-Arginine group displayed considerable rise of MEL (day 23 and 24, $p < 0.01$; day 25, $p < 0.05$; day 26, $p > 0.05$) in comparison to EGA+STZ group.

On day 27 a reduction ($p < 0.001$) in percentage of time spent in target quadrant [TSTQ (%)] during probe trial in STZ (ICV) alone group denoted loss of spatial memory with respect to sham-treated group. EGA+STZ group showed significant ($p < 0.001$) rise in TSTQ (%) in comparison to STZ (ICV) alone group. LiCl group displayed increase of TSTQ (%) ($p < 0.01$) when compared to EGA+STZ group. L-Arginine group had low ($p < 0.05$) TSTQ (%) with respect to EGA+STZ group (Fig. 3B). These results revealed rise in memory functions of rats in response to chronic LiCl treatment while L-Arginine treatment enhanced the STZ (ICV) triggered cognitive deficits in EGA treated rats.

LiCl and L-Arginine modulate the impact of EGA on memory of STZ (ICV) injected rats in EPM task

During acquisition trial on day 27 of EPM task none of the groups showed significant difference in mean transfer latency (TL). During retrieval trial on day 28 STZ (ICV) group exhibited an increase ($p < 0.001$) in mean TL disclosed loss of memory in comparison to sham-treated group. EGA+STZ group showed decline ($p < 0.001$) of TL in comparison to STZ (ICV) group. LiCl group exhibited improvement in cognitive abilities evident by substantial decrease ($p < 0.01$) of TL with respect to EGA+STZ group. L-Arginine group displayed enhanced ($p < 0.01$) TL when compared with EGA+STZ group (Fig. 4).

LiCl and L-Arginine modulate the effects of EGA on brain oxidative stress of STZ (ICV) administered rats

Centrally administered STZ raised the brain TBARS content, and diminished the GSH level, SOD and CAT activity ($p < 0.001$) in comparison to sham-treated rats. EGA treatment prevented the STZ triggered surge in brain TBARS content, and decline of GSH level, SOD and CAT activity ($p < 0.001$). LiCl group showed attenuation of brain TBARS level ($p < 0.01$), and rise of GSH content ($p < 0.05$), SOD ($p < 0.01$) and CAT activity ($p < 0.05$).

with respect to EGA+STZ group. L-Arginine group manifested higher brain TBARS content ($p < 0.01$), and lower GSH level ($p < 0.05$), SOD ($p < 0.001$) and CAT activity ($p < 0.05$) with respect to EGA+STZ group (Table 1).

LiCl and L-Arginine modulate the effects of EGA on brain nitrite level of STZ (ICV) injected rats

Total nitrite content in brain estimates the extent of NO transmission. STZ (ICV) group showed significant ($p < 0.001$) elevation in the brain nitrite content in comparison to sham-treated rats. EGA+STZ group had lower ($p < 0.001$) brain

nitrite content as compared to STZ (ICV) group. LiCl or L-Arginine treatments enhanced ($p < 0.05$ and $p < 0.001$ respectively) the brain nitrite content in separate groups of EGA and STZ treated rats (Table 1). However, L-Arginine group showed exorbitant rise ($p < 0.001$) in brain nitrite content when compared with LiCl group.

LiCl and L-Arginine modulate the effects of EGA on brain AChE activity of STZ (ICV) injected rats

STZ (ICV) administration increased the brain AChE activity ($p < 0.001$) with respect to sham-treated group. EGA+STZ group displayed decrease

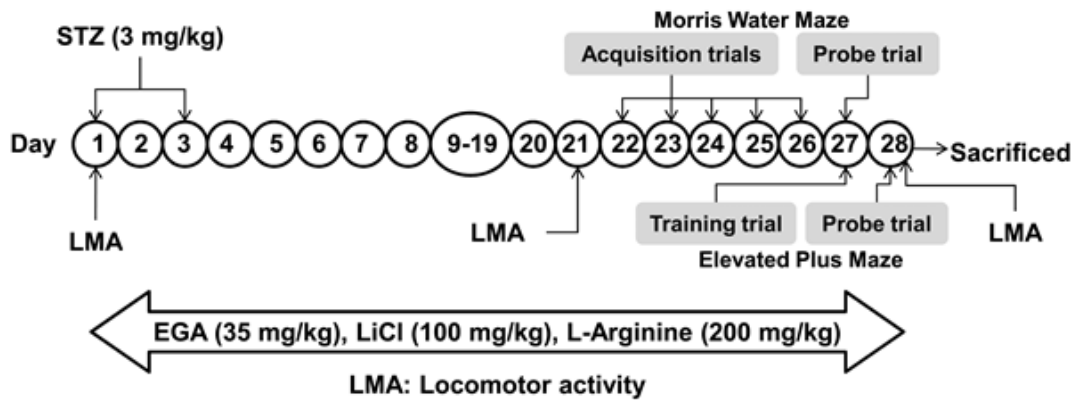
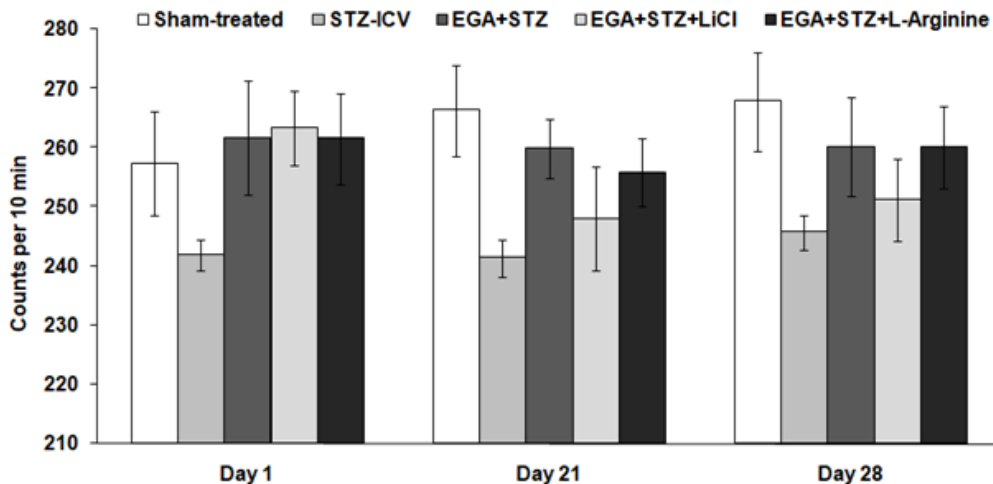


Fig. 1: Drug treatment schedule and experimental protocol



Two-way ANOVA followed by Bonferroni *post-hoc* test was applied. Values are denoted as mean±SEM (n=6). (STZ: Streptozotocin; EGA: Ellagic acid; LiCl: Lithium chloride).

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

($p < 0.001$) in the AChE activity when compared with STZ (ICV) group. LiCl and L-Arginine groups showed significant attenuation of AChE activity ($p < 0.001$ and $p < 0.05$ respectively) with respect to EGA+STZ group. These results showed that facilitation of NO biosynthesis enhanced the AChE inhibitory activity of EGA and cholinergic activity in brain of rats (Table 2).

LiCl and L-Arginine modulate the effects of EGA on brain LDH activity of STZ (ICV) injected rats

Central administration of STZ compromised the neuronal coherence evident by enhanced ($p < 0.001$) brain LDH activity in comparison to sham-treated group. A decrease ($p < 0.001$) in brain LDH activity was noted in EGA+STZ group in comparison to STZ (ICV) group which manifested resurrection of neuron membrane integrity. LiCl group showed decline ($p < 0.05$) in LDH activity with respect to EGA+STZ group. L-Arginine treatment enhanced (p

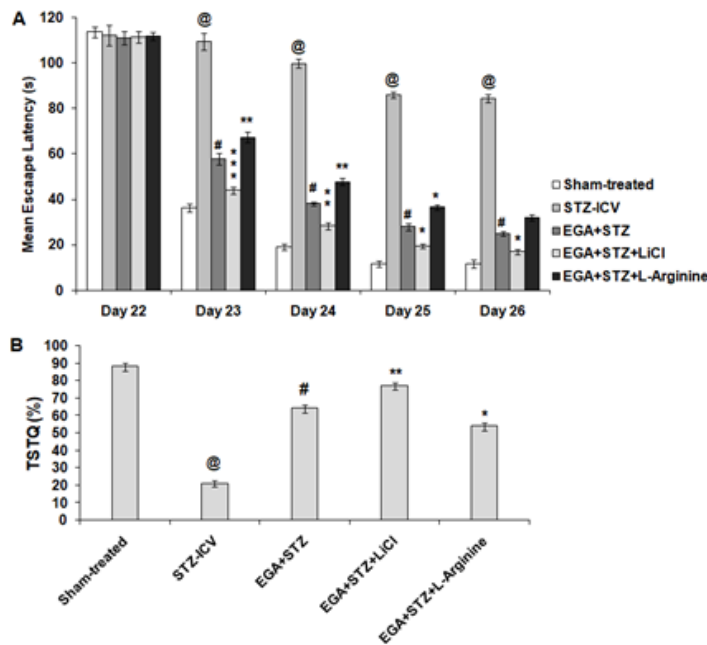
< 0.01) the STZ (ICV) induced surge in LDH activity in EGA treated rats (Table 2).

LiCl and L-Arginine modulate the effects of EGA on brain TNF- α content of STZ (ICV) injected rats

STZ (ICV) treatment enhanced ($p < 0.001$) the brain TNF- α content as compared to sham-treated group. Chronic administration of EGA attenuated ($p < 0.001$) the brain TNF- α levels in comparison to rats that received STZ (ICV) alone. Administration of LiCl reduced ($p < 0.01$) the TNF- α content in brain of EGA and STZ (ICV) treated rats. L-Arginine treatment increased ($p < 0.05$) the STZ (ICV) induced surge in brain TNF- α content in EGA treated rats when compared to rats administered with EGA and STZ only (Table 2).

LiCl and L-Arginine modulate the effects of EGA on brain eNOS level of STZ (ICV) injected rats

STZ (ICV) group displayed lower ($p < 0.001$)



Comparison of (A) mean escape latency during five days acquisition trials (Analyzed by two-way ANOVA followed by Bonferroni *post-hoc* test), (B) percentage of time spent in target quadrant [TSTQ (%)] during probe trial (Analyzed by one-way ANOVA followed by Tukey's *post-hoc* test). Values are denoted as mean±SEM (n=6). Significance at @ $p < 0.001$ vs. sham-treated group; # $p < 0.001$ vs. STZ-ICV; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. EGA+STZ group. (STZ: Streptozotocin; EGA: Ellagic acid; LiCl: Lithium chloride).

Fig. 3: Effect of LiCl and L-Arginine on learning and memory of EGA and STZ (ICV) injected rats in MWM

brain eNOS levels with respect to sham-treated rats. Chronic EGA treatment revitalized ($p < 0.001$) the eNOS content in brain of STZ (ICV) injected rats with respect to STZ (ICV) alone group. LiCl group exhibited fall ($p > 0.05$) in brain eNOS level with respect to EGA+STZ. Chronic administration of L-Arginine downregulated ($p < 0.001$) the eNOS expression in brain of EGA and STZ (ICV) treated rats (Table 2). L-Arginine group portrayed decrease ($p < 0.05$) in brain eNOS content with respect to LiCl group.

Histology of rat brain

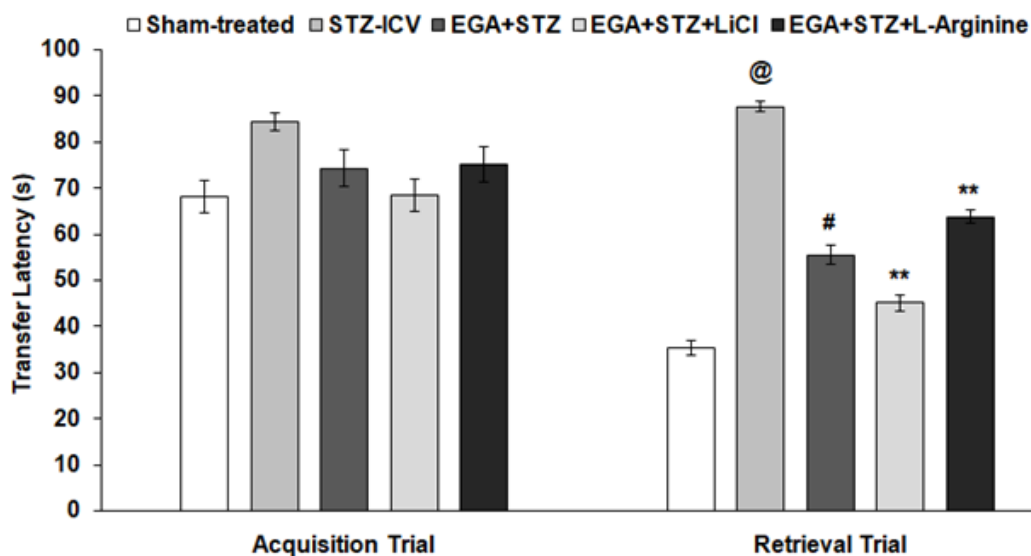
STZ (ICV) administration catapulted neurodegeneration in cortical region of rat brain. EGA treatment for 28 days arrested the noxious effects of STZ (ICV) in rat brain. LiCl potentiated the neuroprotective activity of EGA in STZ (ICV) treated rats. L-Arginine group portrayed enhanced neurodegeneration in comparison to EGA+STZ group (Fig. 5).

(ICV) model of AD was evaluated in the current study. Administration of STZ in brain manifests symptoms akin to age associated progressive neurodegenerative disorders such as AD⁶. The association of central cholinergic activity with memory has witnessed several pharmacological, electrophysiological and biochemical evidences. The cholinergic projection neurons of the basal forebrain and upper brainstem innervate brain regions (cortex, hippocampus) involved in memory²⁹. In present study STZ (ICV) injection compromised the cholinergic function evident by enhanced AChE activity in brain of rats. Chronic EGA administration attenuated the brain AChE activity in STZ (ICV) treated rats. LiCl (GSK-3 inhibitor) and L-Arginine (NO precursor) treatments enhanced the brain cholinergic function through inhibition of AChE activity in EGA and STZ (ICV) treated rats. Previous studies demonstrated improvement in brain cholinergic function through increase in central NO transmission and inhibition of GSK-3 activity by L-Arginine or LiCl^{30, 31}.

DISCUSSION

The functions of GSK-3 and eNOS in modulation of memory abilities by EGA in STZ

Commensurate to previous findings, administration of STZ in lateral cerebral ventricles of rats expedited the biogenesis of brain free radicals and diminished the antioxidant guard in the current



Mean transfer latency of different groups during retrieval trial in EPM is compared. Analyzed by one-way ANOVA followed by Tukey's *post-hoc* test. Values are denoted as mean \pm SEM (n=6). Significance at @ $p < 0.001$ vs. sham-treated group; # $p < 0.001$ vs. STZ-ICV; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. EGA+STZ group. (STZ: Streptozotocin; EGA: Ellagic acid; LiCl: Lithium chloride).

Fig. 4: Effect of LiCl and L-Arginine on memory of EGA and STZ (ICV) injected rats in EPM

study. TBARS is a widely acclaimed biomarker of lipid peroxidation conspicuously enhanced by STZ (ICV) treatment. GSH, SOD and CAT constitute endogenous brain antioxidants diminished in STZ (ICV) treated rats. Oxidative stress is an early event which hastens generation of reactive oxygen species, compromise antioxidative status and provides impetus to various age associated derangements in brain manifesting profound neurodegeneration and AD like cognitive deficits⁶. Oral administration of EGA attenuated rise of brain

TBARS and decline of GSH, SOD and CAT activity in STZ (ICV) treated rats. Uninhibited GSK-3 overactivity is known to hasten lipid peroxidation, protein oxidation and PARP activity³². In the present study control of GSK-3 activity by chronic administration of LiCl (non-selective GSK-3 inhibitor) lowered the brain TBARS levels and enhanced the GSH, SOD, CAT activities in EGA and STZ (ICV) treated rats. However, L-Arginine (NO precursor) treatment spurred the STZ (ICV) induced oxidative stress in EGA administered rats.

Table 1: Effect of EGA and its modulation by LiCl and L-Arginine on brain thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and total nitrite in STZ (ICV) treated rats.

Animal group	TBARS (nM/mg protein)	GSH (μ M GSH/mg protein)	SOD (μ M NBT reduced/min/mg protein)	CAT (μ M H ₂ O ₂ decomposed/min/mg protein)	Nitrite (μ M/mg protein)
Sham-treated	1.312±0.199	0.34±0.009	0.028±0.005	16.24±0.424	76.83±2.416
STZ-ICV	10.94±0.344 [@]	0.013±0.0033 [@]	0.177±0.005 [@]	1.859±0.27 [@]	174.3±2.013 [@]
EGA+STZ	4.544±0.369 [#]	0.229±0.011 [#]	0.079±0.0047 [#]	11.7±0.504 [#]	120.5±3.374 [#]
EGA+STZ+LiCl	2.764±0.197 ^{**}	0.286±0.014 [*]	0.051±0.0048 ^{**}	13.96±0.615 [*]	133.8±2.93 [*]
EGA+STZ+L-Arginine	6.266±0.404 ^{**}	0.173±0.015 [*]	0.122±0.002 ^{***}	9.279±0.518 [*]	152.9±2.944 ^{***} ^{\$\$\$}

Values are denoted as mean±SEM (n=5). Significance at [@]p < 0.001 vs. sham-treated group; [#]p < 0.001 vs. STZ-ICV; ^{*}p < 0.05, ^{**}p < 0.01, ^{***}p < 0.001 vs. EGA+STZ; ^{\$\$\$}p < 0.001 vs. EGA+STZ+LiCl group. (STZ: Streptozotocin; EGA: Ellagic acid; LiCl: Lithium chloride).

Table 2: Effect of EGA and its modulation by LiCl and L-Arginine on brain acetylcholinesterase (AChE), lactate dehydrogenase (LDH) activity, TNF- α and eNOS levels in STZ (ICV) treated rats

Animal group	AChE (μ M acetylthiocholine iodide hydrolysed /min/mg protein)	LDH (μ M NADH oxidized/min/mg protein)	TNF- α (pg/ml)	eNOS (ng/ml)
Sham-treated	0.0354±0.0017	0.0529± 0.0104	29.29±3.118	23.23±1.175
STZ-ICV	0.1463±0.0054 [@]	0.3419±0.0103 [@]	89.66±1.411 [@]	1.415±0.4389 [@]
EGA+STZ	0.0875±0.004690 [#]	0.1489±0.0052 [#]	51.6±1.416 [#]	15.08±0.858 [#]
EGA+STZ+LiCl	0.0553±0.004 ^{***}	0.105±0.00612 [*]	40.37±1.795 ^{**}	13.11±0.687
EGA+STZ+L-Arginine	0.0679±0.0042 [*]	0.194±0.00884 ^{**}	61.66±1.72 [*]	9.323±0.891 ^{***} [§]

Values are denoted as mean±SEM (n=5). Significance at [@]p < 0.001 vs. sham-treated group; [#]p < 0.001 vs. STZ-ICV; ^{*}p < 0.05, ^{**}p < 0.01, ^{***}p < 0.001 vs. EGA+STZ; [§]p < 0.05 vs. EGA+STZ+LiCl group. (STZ: Streptozotocin; EGA: Ellagic acid; LiCl: Lithium chloride).

Amyloid α aggregates and several neurotoxins (e.g. STZ, aluminum chloride, colchicine) trigger release of pro-inflammatory cytokines (e.g. IL-1, IL-6, TNF- α) by reactive microglia and astrocytes⁶. Chronic elevation of TNF- α level in brain, CSF and plasma is associated with AD like neurodegenerative changes³³. Oral administration of EGA lowered the STZ (ICV) triggered increase of TNF- α in brain of rats. Inhibition of GSK-3 by LiCl facilitated the anti-inflammatory activity of EGA in STZ (ICV) injected rats. Chronic administration of L-Arginine abolished the decrease of brain TNF- α level by EGA in STZ (ICV) treated rats. Furthermore, STZ (ICV) treated rats showed high brain LDH activity which signified loss of cell viability. Lipid peroxidation and chronic inflammation infringe the plasma membrane and breach neuronal integrity that incites LDH activity³⁴. EGA averted STZ (ICV) induced neurodegeneration evident by abrogation of brain LDH activity. LiCl enhanced whereas L-Arginine declined the neuroprotective activity of EGA in STZ (ICV) injected rats. Furthermore, the histology of rat brain substantiated the present results.

The aforementioned biochemical results well supported the MWM and EPM findings. STZ

(ICV) triggered brain oxidative stress, inflammation and cholinergic deficit reduced the spatial memory of rats. Chronic treatment with EGA abrogated the oxidative stress, inflammation, LDH, AChE activity and thereby prevented the loss of memory in STZ (ICV) treated rats. Administration of LiCl (GSK-3 inhibitor) enhanced the memory restorative activity of EGA in STZ (ICV) treated rats. However, contrary to our hypothesis facilitation of NO transmission by chronic L-Arginine (NO precursor) treatment decreased the memory of EGA and STZ (ICV) administered rats. Elevation in oxidative stress, inflammation and fall of antioxidative defense culminated in loss of memory of L-Arginine group rats. Furthermore, exploration of total brain nitrite content and eNOS levels by ELISA in brain of rats substantiated these results.

NO is ascribed a vital second messenger biosynthesized by constitutively expressed eNOS and is associated with fabrication of long-term memories. The eNOS mediated NO biosynthesis in cortex and hippocampus is associated with long term potentiation and synaptic plasticity¹². Nevertheless, excessive nitrite levels in brain leads to neurodegeneration through peroxynitrite

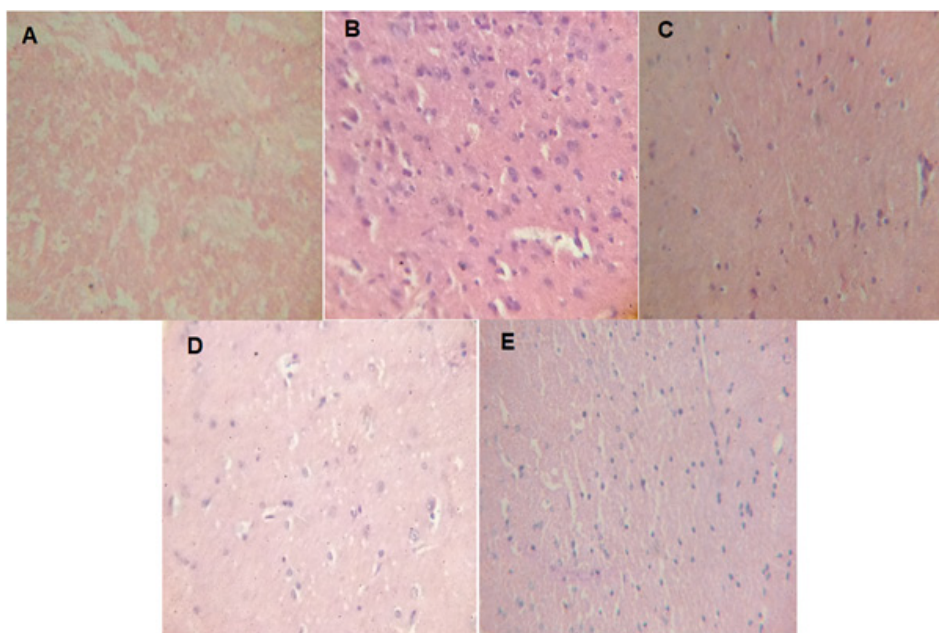


Fig. 5: Histology of rat brain cortex (H&E, 45x). (A) Sham-treated group, (B) STZ (ICV) group, (C) EGA+STZ group, (D) LiCl group (EGA+STZ+LiCl), (E) L-Arginine group (EGA+STZ+L-Arginine). (STZ: Streptozotocin; EGA: Ellagic acid; LiCl: Lithium chloride)

mediated protein oxidation, lipid peroxidation, oxidative modification of DNA and mitochondria³⁵. STZ (ICV) causes excessive NO production through pathological iNOS activation that results in neurodegeneration and loss of memory. In the present study central administration of STZ heightened the NO (nitrite) levels in brain although decreased the expression and activity of eNOS. EGA revived the eNOS content and halted the exorbitant rise of brain nitrite levels in STZ (ICV) treated rats. LiCl and L-Arginine treatments increased the NO production in brain of EGA and STZ (ICV) treated rats. However, LiCl group showed no significant difference in brain eNOS content in comparison to EGA+STZ group. L-Arginine group exhibited profound downfall in eNOS expression (not activity of eNOS) in comparison to LiCl group and EGA+STZ group. NO regulated eNOS expression has been demonstrated in previous studies³⁶. These results imply that increase in nitrite content by L-Arginine downregulated the eNOS expression in brain of rats that resulted in loss of cognitive functions.

CONCLUSION

Suppression of GSK-3 activity by LiCl potentiated the antioxidative, anti-inflammatory,

anti-AChE and cytoprotective activity of EGA in STZ (ICV) treated rats. This implies that EGA attenuated the STZ (ICV) incited aberrant overactivity of GSK-3 in brain of rats and revived their diminishing memory functions. L-Arginine treatment enhanced STZ (ICV) initiated brain oxidative stress, inflammation, cytotoxicity, nitrite content and AChE activity in EGA administered rats. Furthermore, ELISA results showed that L-Arginine aided the STZ (ICV) induced decrease of brain eNOS expression (not activity of eNOS) and thereby reduced the memory of EGA treated rats. These results highlighted that EGA prevented loss of memory in STZ (ICV) rats through suppression of GSK-3 activity and upregulation of eNOS in brain.

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