Research article

Treatment with Green Tea Prevents Intracerebroventricular Streptozotocin Induced Cognitive Impairment and Oxidative Stress in Mice

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Abstract

Green tea polyphenols have demonstrated significant antioxidant, anti-carcinogenic, anti-mutagenic and antidiabetic in numerous human, animal and in vitro studies. Hence present study was design to evaluate the influence of green tea in streptozotocin induced oxidative stress in mice.
Morris water maze, Elevated plus maze and passive avoidance apparatus was used for the evaluation of learning and memory. Brain thiobarbituric acid reactive substance was also estimated.
Intracerebroventricular administration of streptozotocin reduces the learning and memory and increase the concentration of thiobarbituric acid reactive substance in mice. Green tea significantly improves the learning and memory and reverses the increase thiobarbituric acid reactive substance concentration in mice.
The result of present study indicates that green tea improve the learning and memory. It also reduces the streptozotocin induced oxidative stress.

Key Word: Hippocampus, degenerative disease, green tea

Introduction

Hippocampus has been recognized to be an important integration center for learning and memory and it is particularly sensitive and responsive to change in insulin and glucose concentration [1]. Insulin brain receptor and degenerative disease have exhibited certain relationship [2]. It has been shown that centrally available insulin plays an important role in stages of cognitive dysfunction like Alzheimer's disease (AD) [3]. It is reported that insulin should be administered in brain degenerative disease to enhance memory in such patients [4]. Intracerebroventricular (ICV) streptozotocin (STZ) treated rats has been described as an appropriate model for sporadic Alzheimer's disease (SAD) in human as both are characterized by progressive deterioration of memory, cerebral glucose and energy metabolism [5]. Reduced cerebral glucose metabolism decreases the cortical acetylcholine release which may have a causal role in loss of cognitive function in the sporadic form of AD [6]. Again Streptozotocin (ICV injection) administration cause abnormalities in metabolic pathways being under control of the insulin receptor signaling has already been linked to increased oxidative stress [7]. Oxidative stress and free radicals have been implicated on the prime candidates mediating the behavioral impairment and memory deficits in

age related neurodegenerative disorders such as AD and Parkinson's disease [8]. Thus antioxidants have been purposed reversing the neuronal and behavioral abnormalities.

Green tea, a popular beverage, is now being recognized for its herbal remedy and its medicinal properties have been widely explored [9]. The tea plant, *Cammelia sinesis*, is a member of Theaceae family, black and green tea are produced from its leaves [10]. The polyphenols found in the tea are commonly known as ''flavanols'' or ''catechins''. The main catechins in green tea are epicatechin, epicatechin-3-gallate, epigallocatechin, and epigallocatechin-3-gallate (EGCG), with latter being highest in concentration [11]. Green tea polyphenols have demonstrated significant antioxidant, anti-carcinogenic, anti-mutagenic properties and antidiabetic in numerous human, animal and in vitro studies [12].

Therefore, the present study was designed to evaluate the effect of green tea on the learning and memory and marked effect on streptozotocin (ICV injection) induced learning and memory.

Materials and Methods

Extraction of drug
The leaf was identified by the Forest Research Institute, Dehradun. After authentication, the powdered dry leaf was extracted with water using Soxhlet apparatus.

Animals
All the experiments were carried out using Albino mice of either sex produced from IVRI, Bareilly, U.P. India. The animals were housed, 12 hr. light and 12 hr. dark cycle in the departmental animal house with free access to water and standard diet.

All experiments were performed as per the norms of the ethical committee and the studies were approved and clearance obtained by the 'Institutional Review Board'.

Drug treatment
In the present study the mice were divided in to various group for employing memory models and estimation of Thiobarbituric Acid Reactive Substance (TBARS). Control group animals were given plain drinking water *ad libitum* whereas treated group animals were given 0.5% green tea extract in the drinking water for two months [13]. Insulin (Cadila) was used as the standard drug and was administered 2IU/kg i.p.[14]. Streptozotocin (Sigma, USA) (3 mg/kg, intracerebroventricular) was administered single dose under the anesthesia. All experiments were conducted at the same time of the day to minimize circadian influence.

Morris water maze
Morris water maze [15] was employed to evaluate learning and memory. It consisted of a circular water tank (diameter 150 cm. and height 45 cm.) and was filled with water up to 30 cm. (at 25^0 C). The tank was divided into four equal quadrants with the help of two threads, fixed at right angle to each other on the rim of the pool. A platform (10 cm^2) of 29 cm. height was located in the center of one of these four quadrants. The position of the platform and clues were kept constant throughout the training session. In the present study, the target quadrant was Q_4. Each animal was subjected to four consecutive trials on each day with an interval of 5 min, during which they were allowed to remain on the platform for 20 sec. In case the animal was unable to locate the hidden platform with in 120 sec. It was gently guided by hand to the platform and was allowed to remain there for 20 sec. Escape latency time (ELT) to locate the hidden platform in water maze was noted as an index of acquisition. Rats were subjected to acquisition trial for four consecutive days. On the 5th day, the platform was removed and time spent by animal in each quadrant was noted. The time spent by the animal in target quadrant and (Q_4) in search of missing platform was noted as an index of retrieval.

Acquisition trial
Each mouse was subjected to four trials on each day (after 16 day of drug treatment). A rest interval of 5 min was allowed in between each trial. Four trials per day were repeated for four consecutive days. Starting position on each day to conduct four-acquisition trial was changed as

described below and Q_4 was maintained as target quadrant in all acquisition trials.

Day 1	Q_1	Q_2	Q_3	Q_4
Day II	Q_2	Q_3	Q_4	Q_1
Day III	Q_3	Q_4	Q_1	Q_2
Day IV	Q_4	Q_1	Q_2	Q_3

Mean escape latency time (ELT) calculated each day during acquisition trial was used as an index of acquisition.

Retrieval trial

On day 5th, the platform was removed. Each mouse was placed in water maze and allowed to explore the maze for 120 sec. Each rat was subjected to four such trials and each trial was started from different quadrant. Mean time spent in target quadrant i.e. Q_4 in search of missing platform provided an index of retrieval. Care was taken that relative location of water maze with respect to other subject in laboratory serving as visual clues were not disturbed during the total duration of the study.

Elevated plus maze

Plus maze [16] consisted of two open (50 x 10 cm) and two enclosed (50x10x40 cm) arms, connected by a central platform (5 x 5cm). The apparatus was elevated to a height of 25 cm above the floor. A fine line was drawn in the middle of the floor of each enclosed arm. On the day first (i. e. 16th day of drug treatment) each mice was placed at the end of an open arm, facing away from the central platform. Transfer latency time (in seconds) was recorded first day (training session). The mouse was allowed to explore the maze for 2 min and returned to home case. Retention of this learn task (memory) was examined 24 hr after the first day trial (i.e.16th day, 24 after last dose).

Passive avoidance test

Passive avoidance [16] test was performed by the method of the apparatus consisted of two compartments, an illuminated compartment (27 cm X 30 cm X 21 cm) and a dark compartment (10 cm X 30 cm X 21 cm). Shock was delivered via a small dark compartment consisted of a grid floor. These compartments were separated by a guillotine door. Control and treated mouse were placed in an illuminated compartment. After 10 s the door was raised and latency period to enter (LTE) the dark compartment was noted. Upon entry, the door was closed and a foot shock was immediately administered (100 V for 2 s). The rat was placed again in the illuminated chamber 24 h after the acquisition trial and response (LTE) was noted up to 300 s during the retention trial. The difference between LTE in the acquisition and retention trial was computed and considered as a measure of memory.

Intracerebroventricular (ICV) administration of streptozotocin (STZ)

ICV injection of STZ was performed as described previously [17]. Briefly, adult mouse were anesthetized with thiopentone (Neon Laboratories, India, 45 mg/kg, i.p.). The head was placed in position in the stereotaxic apparatus and a midline sagittal incision was made in the scalp. Following coordinates were used for ICV injection: 0.8 mm posterior to bregma, 1.5 mm lateral to saggital suture and 3.6 mm ventral from the surface of the brain. STZ (Sigma, USA) was dissolved in citrate buffer (pH 4.4) just prior to injection. The STZ group was injected bilaterally with Intracerebroventricular STZ (3mg/kg) in single dose. The concentration of STZ in citrate buffer was adjusted so as to deliver 10 μl of the solution. Mouse in sham operated group was given ICV injection of the same volume of citrate buffer in STZ injected mice. After ICV injection, povidone–iodine solution was applied and the cut skin was sutured after second injection followed by daily application of antiseptic powder (Neosporin). Post operatively, the rats were fed with milk by oral gavage and normal pellet diet for 4 days, followed by normal pellet diet alone.

Estimation of thiobarbituric acid reactive substance (TBARS)

Animals were sacrificed by cervical dislocation and the brain was removed. The brain was homogenized in 5 ml of 30 mM Tris-HCl + 2.5 mM $CaCl_2$ buffer (pH 7.6 at 5^0C). Homogenate was centrifuged at 750g to separate cellular

debris. The supernatant was accurately divided into two parts. Both portions were centrifuged at 8200g to obtain the mitochondrial fraction. One fraction was utilized for determination of TBARS [18] and the other portion was employed for protein estimation [19].

For the estimation of TBARS in both mitochondrial pellet and supernatant, each fraction was suspended in 4ml of distilled water. To each, 1 ml of TBA reagent (mixture of equal volume of 0.67% TBA aqueous solution and glacial acetic acid) was added. Reaction mixture was heated for 60 minutes at 95^0C on a water bath. After cooling with tap water, 5 ml of n-butanol was added. Solution was shaken and centrifuged at 750g for 15 minutes. Butanol layer was pippeted out for spectrophotometric measurement at 532 nm (Shimadzu, UV1601, Japan). Absorbance was read against blank prepared identically without addition of mitochondrial fraction. A standard curve for MDA using 1,1,3,3- tetramethoxypropane was plotted. The extent of lipid peroxidation was expressed as nanomoles of TBARS formed per mg of protein.

For the estimation of total protein in both mitochondrial and supernatant fractions, each fraction was suspended in distilled water. 5 ml of Lowry's reagent (freshly prepared mixture of 1% w/v copper sulphate, 2% w/v sodium potassium tartrate and and 2% w/v sodium carbonate in 0.1 N NaOH in the ratio of 1:1:98 respectively) was added in both portions and mixed thoroughly. Mixture was allowed to stand for 15 minutes at room temperature and then 0.5 ml of 1:1 v/v diluted Folin-Ciacalteu reagent was added. Contents were vortexed and incubated at 37^0C for 30 minutes. Optical density was read spectrophotometrically (Shimadzu, UV1601, Japan) at 750 nm against suitably prepared blank. A standard curve using 25-200 mg of BSA was plotted. The amount of total protein was expressed in mg.

Quantitative estimation of serum glucose

Serum glucose was estimated spectrophotometrically at 505 nm by glucose oxidase/peroxidase method [20] using a commercially available kit (Span Diagnostic Ltd, Surat, INDIA).

1500μL working glucose reagent was added to 20μL of serum, 20μL of standard solution of glucose (100mg/dL) and 20μL of purified water to prepare test, standard and blank sample respectively. All test tubes were incubated at room temperature for 30 min. To each test tube 1500μL of purified water was added. The absorbance of test and standard was measured against blank 505 nm spectrophotometrically (Shimadzu, UV1601, Japan). Concentration of glucose (mg/dl) = O.D. test / O.D. std. x 100

Statistical analysis

All results were expressed as mean ± SEM. Data was analyzed by using one way ANOVA followed by Tukey's test and Bonferroni test. $p < 0.05$ was considered to be statically significant.

Results

Effect on Escape Latency Time (ELT) and Time Spent in Target Quadrant (Using Morris Water Maze)

In STZ treated mice ELT increases significantly ($p < 0.001$) during acquisition trials conducted on day 1 to day 4 when compair with control group (fig-1) and markedly reduced time spent in target quadrant (Q_4) in search of missing platform during retrieval trial. (fig-2).
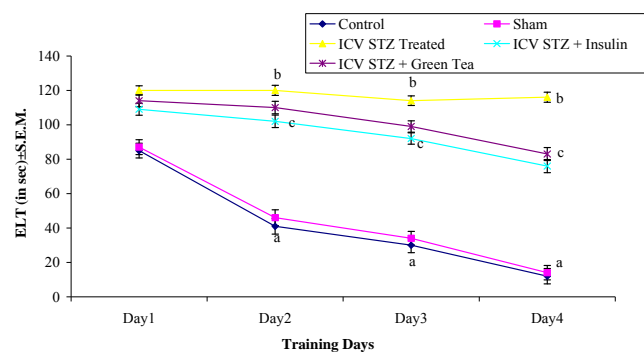
Fig-1. Effect of green tea on ELT (acquisition trails conducted on day 1 to day 4) using morris water maze.

Green tea and insulin reduces significantly ($p < 0.001$) ELT in STZ treated mice during acquisition trails conducted on day 1 to day 4

(fig-1) and significantly prevented STZ induced decrease in time spent in target quadrant (Q_4) in search of missing platform during retrieval trial conducted on day 5 (fig-2).
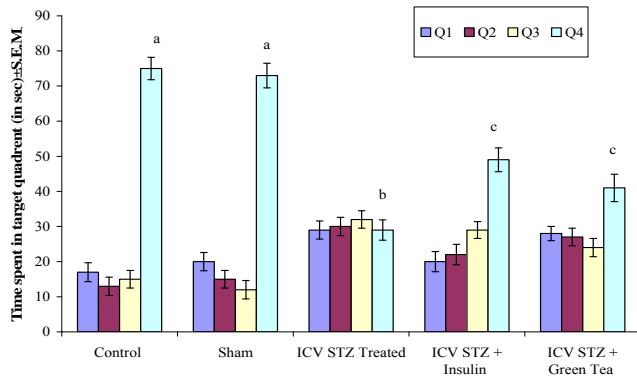

Fig-2. Effect of green tea on retrieval trails (conducted on day 5) using morris water maze.

Effect on Transfer Latency (TL) (Using Elevated Plus Maze)

Transfer Latency is the time (in sec.) taken by the animal to move from the open arm into one of the covered arms with all its four lags. Significant reduction in TL value retention indicated improvement of memory. STZ intracerbroventricular injection significantly ($p<0.001$) increse the TL in the mice. Insulin treateament show improvement ($p<0.05$) in STZ induced memory impairement. Green tea also reverse significantly ($p<0.05$) reduce TL in STZ treated mice(fig-3).
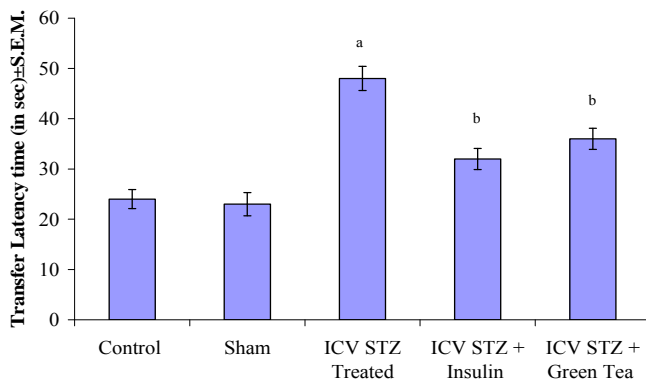

Fig-3. Effect of green tea on TL time using elevated plus maze.

Effect on Step-down Latency (SDL) (Using Passive Avoidance Paradigm)

Step-down latency is the time (in sec.) taken by the mouse to step down from the wooden platform to grid floor. SDL is reflected the long term memory of animals. Significatly increase in SDL value indicated improvement of memory. STZ intracerbroventricular injection remarkably reduce SDL in mice. Insulin treateament show improvement ($p<0.05$) in STZ induced memory impairement. Green tea also reverse significantly ($p<0.05$) reduce SDL in STZ treated mice(fig-4).
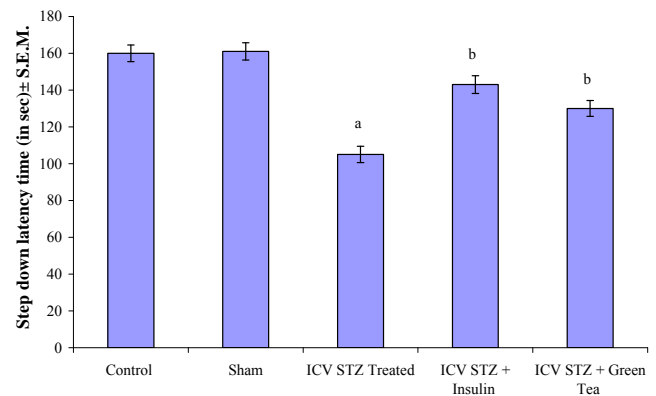

Fig-4. Effect of green tea on step down latency time on passive avoidance paradigm.

Effect on thiobarbituric acid reactive substances (TBARS)

STZ intracerbroventricular injection significantly ($p<0.001$) increase TBARS concentration in brain mitochondria and supernatant fraction. Administration of green tea extract in mice significantly ($p<0.05$) reduce TBARS concentration in brain mitochondria and supernatant fractions (Fig-5).
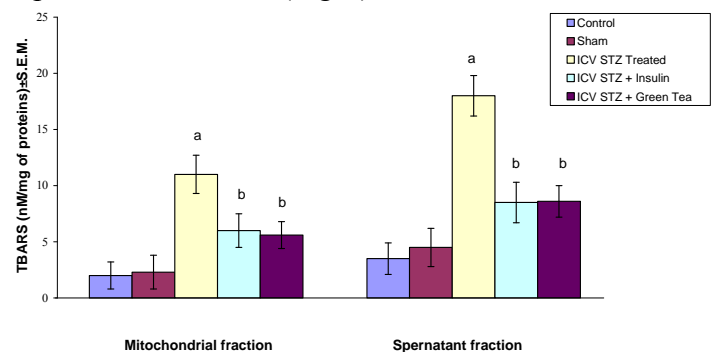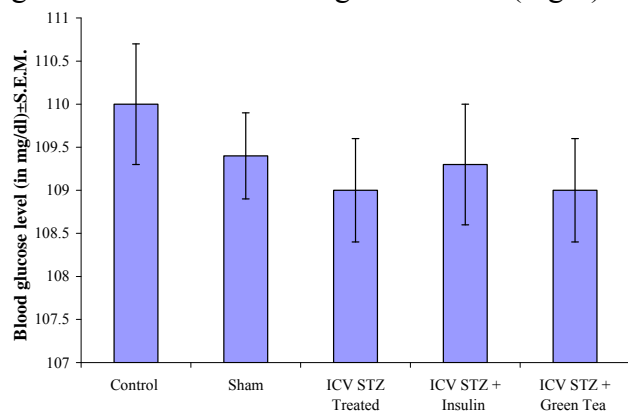

Fig-5. Effect of green tea on thibarbituric acid reactive substaces (TBARS).

Effect on blood glucose level

Insulin and green tea does not have any significant effect on blood glucose level (Fig-6).

Fig-6. Effect of green tea on Blood glucose level.

Discussion and conclusion

Intracerebroventricular STZ models in rodents has been described as an appropriate animal model for sporadic Alzheimer type dementia [21] as both are characterized by a progressive deteriortation of memory, cerebral glucose and energy metabolism as well as presence of oxidative stress [22].

Earlier studies have shown that green tea extract contains many of polyphenolic antioxidants such as catechins, epicatechin and epigallocatechin gallate. Epicatechin, one of its polyphenolic constituent, has been found to enhance learning and memory ability in mice using passive avoidance paradigm when injected intracisternally [23]. Studies show that green tea extract and (-)-epigallocatechin-3-gallate (EGCG) possess potent neuroprotective activity in cell culture as well as in the mice model of Parkinson's disease. Consistently, EGCG markedly increased protein kinase C in the membrane and the cytosolic fractions of mice hippocampus, the learning site of brain [24]. Further, EGCG shows neuroprotective effect against neuronal damage following global ischemia in the gerbils [11]. Also, green tea extract pretreatment has been found to promote recovery from the ischemia reperfusion-induced inhibition of active avoidance [24]. The oxidative damage to the rat synapse in the cerebral cortex and hippocampus during aging may contribute to the deficit of cognitive functions supporting the view that oxidative stress is a causal factor in brain senescence [25]. It is seen that in the central nervous tissue the activity of superoxide dismutase, glutathione peroxidase and lipid peroxidation products decreases while the activity of glutathione reductase and catalase increases after drinking green tea, thus indicating the beneficial effects of green tea extract [26].

In the present study we observed that green tea significantly improve the learning and memory in STZ induced memory impairment. Green tea also reduces the TBARS concentration in brain mitochondria and supernatant fraction which is increase by the ICV administration of STZ. It is reported that reduce blood sugar also impairment of memory [27] long term treatment of insulin and green tea at the given dose not a have any significant affect on blood glucose level.

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