NEUROPROTECTIVE EFFECT OF GREEN TEA ON ISCHEMIA AND REPERFUSION-INDUCED CEREBRAL INJURY

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Summary

The present study is designed to investigate the effect of extract of green tea on ischemia and reperfusion-induced cerebral injury. Global cerebral ischemia was induced by bilateral carotid artery occlusion for 60 min. followed by reperfusion for 7 days. Pretreatment with green tea extract markedly reduced cerebral infarct size and attenuated impairment in memory. The protective effect of green tea extract also decrease in mitochondrial thiobarbituric acid reactive substance (TBARS) and serum nitrite level.

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Keywords: Green tea, Cerebral injury, learning and memory

Introduction

Cerebral cerebral ischemia (GCI) is one of the major leading causes of morbidity and mortality worldwide. Preclinical and clinical studies have shown memory impairments, cognitive deficits, and brain damages in GCI subjects (1) It is believed that the ischemia-induced brain damage is associated with cognitive and memory dysfunction (2). Therefore, cerebral ischemia has been widely used as an invivo model to test neuroprotective effects of new drugs or to explore new neural mechanisms of existing drugs used for brain illnesses (3). Cognitive deficits have been found to be associated with a damage in the CA1 region of the hippocampus in studies using the bilateral common carotid artery occlusion model (4). Interestingly, only subtle histological damage have been found in gerbils submitted to the narrowing of both common carotid arteries (5) and in rats submitted to the creation of an arteriovenous fistula (6). Cerebral ischemia induces enhanced free radical [$NO^{\bullet}$, $ONOO^{-}$, $^{\bullet-}O_2$, and $^{\bullet}OH$] formation, which leads to DNA strand break thereby activating poly-(ADP-ribose) polymerase (PARP), an enzyme involved in maintaining genomic DNA integrity (7,8,9). Furthermore GCI increase the brain inflammation by activation of release of inflammatory substances (10).

Green tea, a popular beverage, is now being recognized for its herbal remedy and its medicinal properties have been widely explored (11). The tea plant, *Cammelia sinesis*, is a member of Theaceae family, black and green tea are produced from its leaves (12). 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 (13). Green tea polyphenols have demonstrated significant antioxidant, anti-inflammatory, anti-carcinogenic properties and antidiabetic in numerous human, animal and in vitro studies (14).

In the present study, we examined the possible effects of green tea on the ischemia induced behavioural consequences of global cerebral ischemia (GCI) by investigating the effects of a two-months pre-administration of green tea (0.025%, 0.05% and 0.1%, in drinking water) on behavioural and pathological changes induced by GCI. The behavioural changes were tested by using Morris water maze, elevated plus maze and passive avoidance test. These results may help reveal the mechanisms of green tea, and shed new light on the treatment of Alzheimer’s disease.

Material and methods

Extraction of drug

The leaf of green tea was identified by the Forest Research Institute, Dehradun (Accession no. 157030). After authentication, the powdered dry leaf was extracted with water using Soxhlet apparatus. It was also certified by Wockhardt, Sonal, H.P, India, that green tea extract contain 57% polyphenols, 28% EGCG and 8% caffeine (Reference No.-WOC/10/ANAL/213).

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

Animals were pretreated with green tea (0.025%, 0.05%, 0.1%, for two months in drinking water) before common carotid artery occlusion (CCAO) and again for five consecutive days during acquisition trials and retrieval trial. Vitamin-E (200 mg/kg, p.o.) was administered daily, after CCAO, for seven days and again for five consecutive days during acquisition trials and retrieval trial.

Global cerebral ischemia and reperfusion

The mice were anesthetized with chloralhydrate (400 mg/kg). Common carotid artery occlusion was induced by isolation of the bilateral common carotid arteries through a ventral midline incision in the neck, followed by occlusion of the arteries using cotton thread for 60 min. At the end of the occlusion, the cotton thread was removed and the arteries visually inspected for reflow, then the midline incision was sutured (15). Sham operated animals underwent the same procedure, but without arterial occlusion. All mice were maintained at normothermia using a warm water circulating blanket until they were able to regulate their own temperature 2 h post-surgery. The survival rate of the mice after CCAO surgery was 75%. The mice were housed in their cages. Acquisition trials were conducted seven days after CCAO.

Morris water maze

Morris water maze (16) 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 5^{th} 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 (17) 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

This was another sensitive-model employed in the present study for testing learning and memory (17). This apparatus comprised of a box (27X27X27 cm) having three walls of wood and one wall of plexiglass, featuring a grid floor (3-mm stainless steel rods set 8-mm apart) with a wooden platform (10X7X1.7 cm) in the centre of the grid floor. Electric shock (20 V AC) was delivered to the grid floor. The box was illuminated with a 15-W bulb during the experimental period. Training was carried out in two similar sessions. Each mouse was gently placed on the wooden platform set in the centre of the grid floor. When the mouse stepped down and placed all its paws on the grid floor, shocks were delivered for 15 s and step-down-latency (SDL) was recorded. SDL was defined as the time taken by the mouse to step down from the wooden platform to the grid floor with all its paws on the grid floor. Animals showing SDL in the range of 2 – 15 sec. during the first test were used for the second session and the retention test. The second session was carried out 90 min after the first test. When the animals stepped down before 60 s, electric shocks were delivered for 15 sec. During the second test, animals were removed from shock-free-zone if they did not step down for a period of 60 sec. Retention was tested after 24 h in a similar manner, except that the electric shocks were not applied to the grid floor. Each mouse was again placed on the platform, and the SDL was recorded, with an upper cut-off time of 300 sec.

Estimation of thiobarbituric acid reactive substance (TBARS):

Animals were sacrificed by cervical dislocation under light anaesthesia then 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 pipetted 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 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.

Estimation of serum nitrite concentration

At the end of 7 days of reperfusion after the global cerebral ischemia, serum nitrite concentration was estimated using method of Sastry *et al.* (20). Blood sample was taken from retro-orbital sinus of mice and then serum was separated by centrifugation at 4000 rpm for 15 minutes. 400 µl of carbonate buffer (pH 9.0) was added to 100 µl of serum or standards sample followed by addition of small amount (~0.15 g) of copper cadmium alloy. The tubes were incubated at room temperature for 1 h to reduce nitrate to nitrite. The reaction was stopped by adding 100 µl of 0.35 M sodium hydroxide. Following this, 400 µl of zinc sulfate solution (120 mM) was added to deproteinate the serum samples. The samples were allowed to stand for 10 min and then centrifuged at 4000 g for 10 min. Greiss reagent (250 µl of 1.0%

sulfanilamide prepared in 3 N HCl and 250 µl of 0.1% N-naphthylethylenediamine prepared in water) was added to aliquots (500 µl) of clear supernatant and serum nitrite was measured spectrophotometrically (Shimadzu, UV1601, Japan) at 545 nm. The standard curve of sodium nitrite was plotted to calculate concentration of serum nitrite.

Estimation of cerebral infract size

At the end of 7 days of reperfusion after the global cerebral ischemia, animals were sacrificed by cervical dislocation and the brain was removed. The brain was kept overnight in freezer. Frozen brain was sliced into uniform coronal sections of about 1mm thickness and stained with 1% 2,3,5- triphenyl tetrazolium chloride (TTC) in phosphate buffer (0.1M, pH 7.4) and incubated for 30 minutes at 37^0C (21). The sections were refrigerated in 4% formaldehyde in phosphate buffer for 30 minutes and the image of each sectioned was taken. TTC is converted to red formazone pigment by NAD and dehydrogenase and thereof stained the viable cells deep red. The infracted cells have lost the enzyme and cofactor and thus remained unstained dull yellow. The brain slices were placed over glass plate. A transparent plastic grid with 100 squares in 1 cm^2 was placed over it. Average area of each brain slice was calculated by counting the number of square on either side. Similarly, numbers of squares falling over non-stained dull yellow area were also counted. Infracted area was expressed as a percentage of total brain volume. Whole brain slices were weighed. Infracted dull yellow part was dissected out and weighed. Infracted size was expressed as percentage of total wet weight of brain.

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 statistically significant.

Results

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

In ischemia reperfusion injured mice, ELT increased significantly ($p<0.001$) during acquisition trials conducted on day 1 to day 4 when compare 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).

In sham group there is no sinnificance difference in ELT and in time spent in target quadrant (Q_4) in search of missing platform during retrieval trial conducted on day 5, when compared with the control group (fig-1,2).

Pre treatment with Vitamine-E reduced significantly ($p<0.001$) ELT in ischemia reperfusion injured mice during acquisition trials conducted on day 1 to day 4 (fig-1) and significantly prevented ischemia reperfusion injury induced decrease in time spent in target quadrant (Q_4) in search of missing platform during retrieval trial conducted on day 5 (fig-2).

Pre treatment with green tea (0.025%, 0.05%, 0.1%, in drinking water) reduced significantly ELT in ischemia reperfusion injured mice during acquisition trials conducted on day 1 to day 4 (fig-3) and significantly prevented ischemia reperfusion injury induced decrease in time spent in target quadrant (Q_4) in search of missing platform during retrieval trial conducted on day 5 (fig-4)

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. Significat increase in SDL value indicated improvement of memory. Ischemia reperfusion injury remarkably reduced SDL in mice. Vitamine-E treatement show improvement ($p<0.05$) in ischemia reperfusion injury induced memory impairement. Green tea (0.05%, 0.1%, in drinking water) also reversed significantly ($p<0.05$, $p<0.001$, respectively) reduce SDL in ischemia reperfusion injured mice (fig-5). Whereas pretreatment with green tea 0.025% did not show any significant effect in ischemia reperfusion injured mice

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. Ischemia reperfusion injury significantly ($p<0.001$) incresed the TL in the mice. Vitamine-E treatement show improvement ($p<0.05$) in ischemia reperfusion injury induced memory impairement. Green tea (0.025%, 0.05%, 0.1%, in drinking water) also reverse significantly ($p<0.05$) reduce TL in ischemia reperfusion injured mice (fig-6).

Effect on thiobarbituric acid reactive substances (TBARS)

Ischemia reperfusion significant ($p<0.001$) increase in TBARS concentration of mice brain mitochondria and supernatant fraction. Pretreatment of green tea extract in mice significantly ($p<0.001$) reduced TBARS concentration in brain mitochondria and supernatant fractions (Fig-7).

Effect on serum nitrite concentration

Ischemia reperfusion significant ($p<0.001$) increase in serum nitrite concentration in mice. Pretreatment with green tea extract in mice significantly ($p<0.05$) reduced serum nitrite concentration in mice (Fig-8).

Effect on cerebral infract size

Ischemia reperfusion significant ($p<0.001$) increase in infract size of mice brain. Administration of vitamin-E in mice significantly ($p<0.001$) reduced infract size in mice brain. Pre treatment with green tea extract (0.05%, 0.1%, in drinking water) in mice significantly ($p<0.05$) reduced infract size in mice brain. Green tea extract 0.025% did not show significant effect on infract size on mice brain (Fig-9).

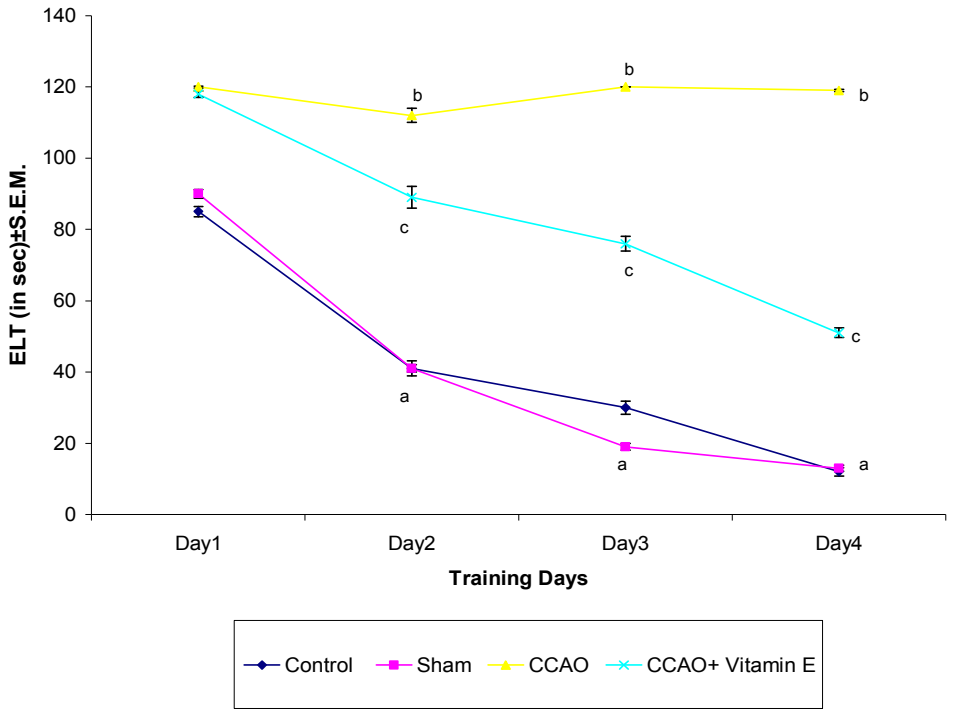

Figure -1. Effect of vitamin-E on ELT (acquisition trails conducted on day 1 to day 4) using morris water maze. Control represents normal water was given for drinking to mice, before conducting acquisition trails. CCAO represents common carotid artery occlusion was done in mice, before conducting acquisition trials. Sham represents only surgical procedure was performed. Each group (n = 6) represents mean ± S.E.M. a = $p < 0.001$ Vs ELT on first day of same groups. b = $p < 0.001$ Vs ELT of control group for the same day. c = $p < 0.001$ Vs ELT of CCAO group for the same day.

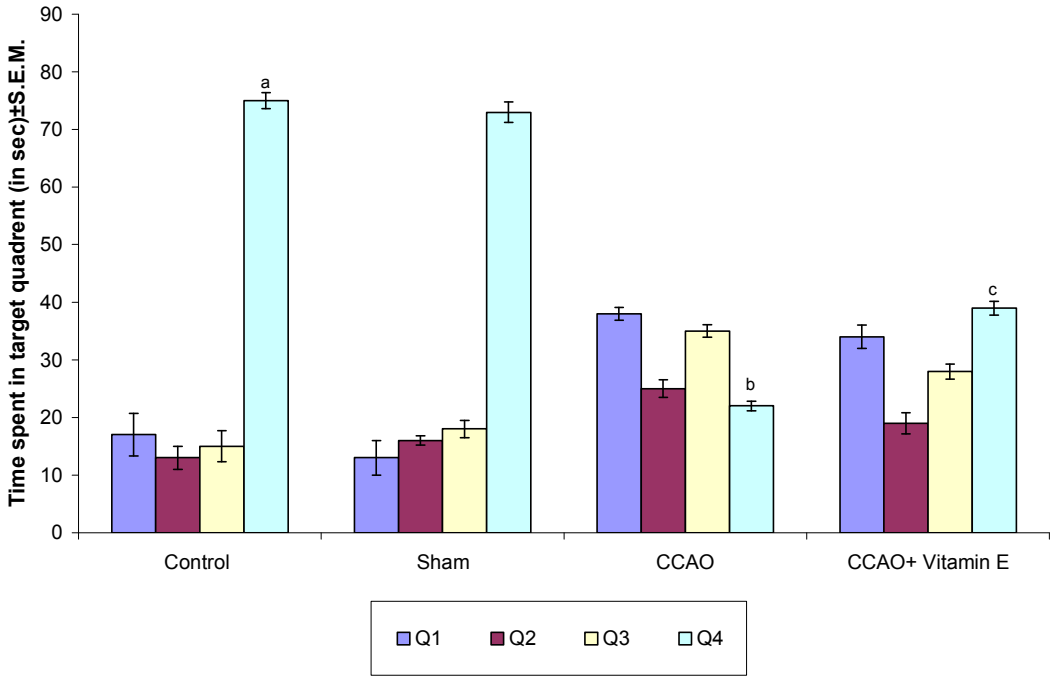

Figure -2. Effect of vitamin-E on retrieval trials (conducted on day 5) using morris water maze, in CCAO mice. Control represents normal water was given for drinking to mice, before conducting acquisition trials. CCAO represents common carotid artery occlusion was done in mice, before conducting acquisition trials. Sham represents only surgical procedure was performed. Each group (n = 6) represents mean ± S.E.M. a = $p< 0.001$ Vs time spent in other quadrants in control group. b = $p< 0.001$ Vs time spent in target quadrant i.e. Q4 in control group. c = $p< 0.001$ Vs time spent in target quadrant i.e. Q4 in CCAO group.

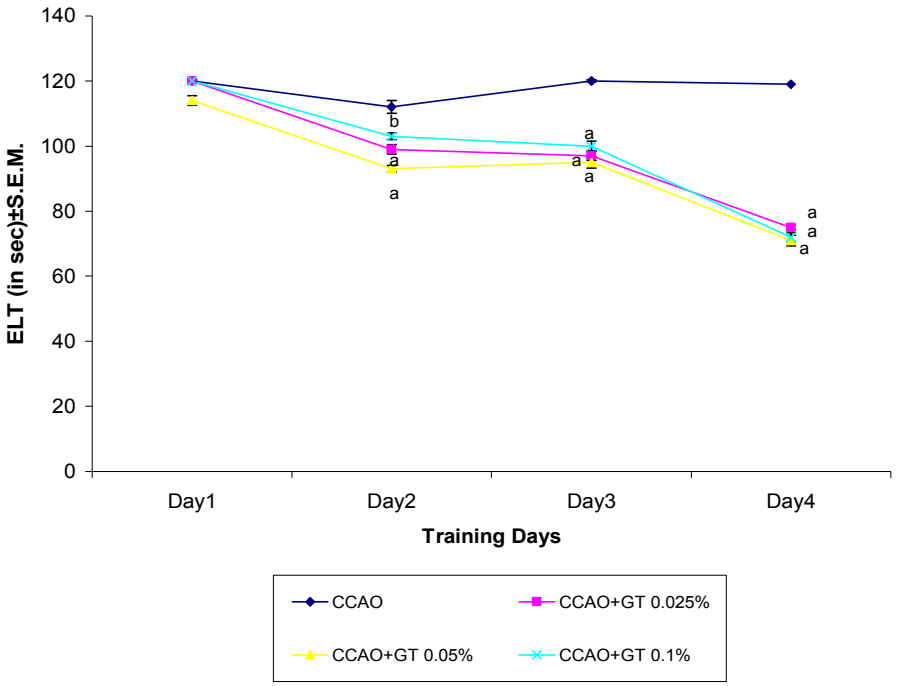

Figure -3. Effect of green tea on ELT (acquisition trials conducted on day 1 to day 4) using morris water maze. CCAO represents common carotid artery occlusion was done in mice, before conducting acquisition trials. CCAO+GT (0..025%, 0.05%, 0.1%) represents mice were pretreated with green tea than CCAO done, before conducting acquisition trials. Each group (n = 6) represents mean ± S.E.M. a = $p < 0.001$ Vs ELT of CCAO group for the same day. b = $p < 0.05$ Vs ELT of CCAO group for the same day.

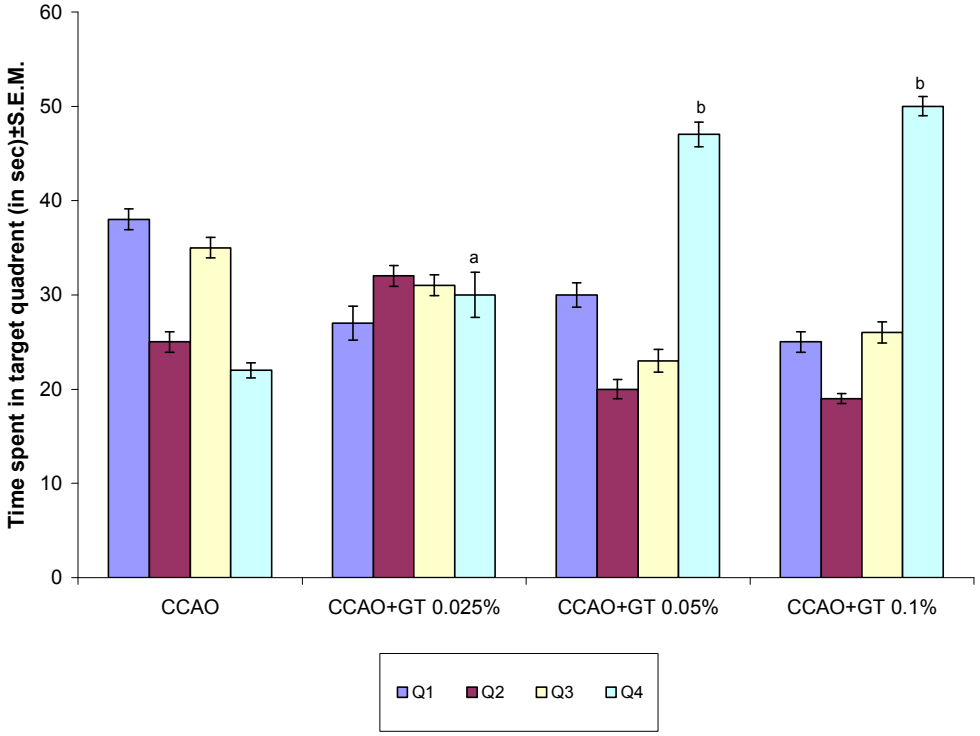

Figure -4. Effect of green tea on retrieval trials (conducted on day 5) using morris water maze, in CCAO mice. CCAO represents common carotid artery occlusion was done in mice, before conducting retrieval trial. CCAO+GT (0.025%, 0.05%, 0.1%) represents mice were pretreated with green tea than CCAO done, before conducting retrieval trial. Each group (n = 6) represents mean ± S.E.M. a = $p< 0.001$ Vs time spent in target quadrant i.e. Q4 in CCAO group. b = $p< 0.05$ Vs time spent in target quadrant i.e. Q4 in control group.

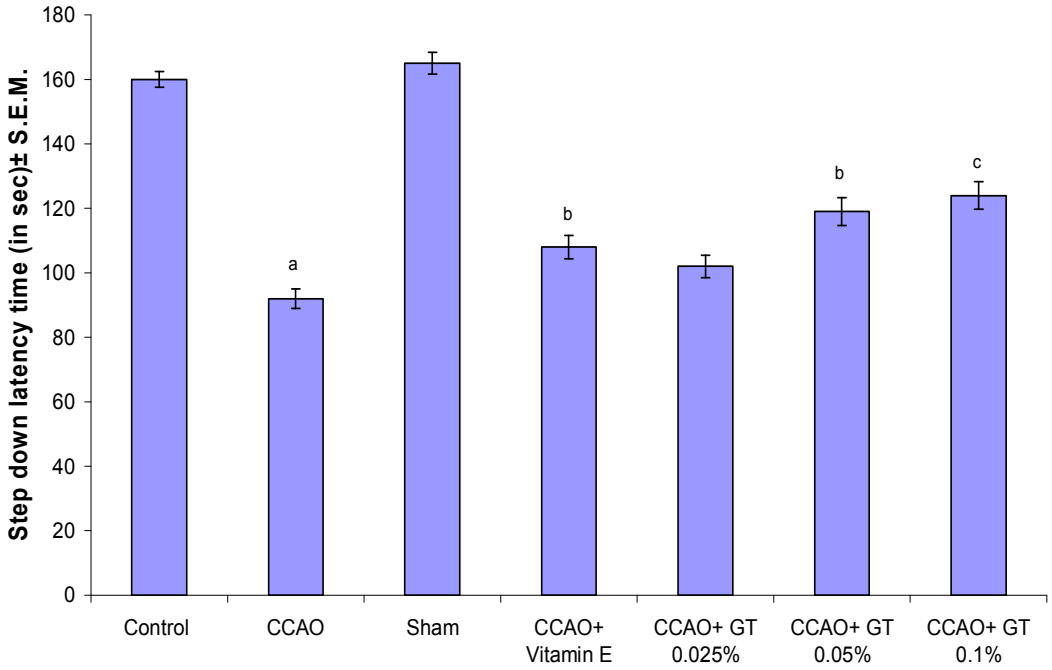

Figure -5. Effect of green tea on step down latency time on passive avoidance paradigm, in CCAO mice. Control represents normal water was given for drinking to mice, before conducting acquisition trial. Vitamin-E (200 mg/kg, p.o.) was used standard drug. Each group (n = 6) represents mean ± S.E.M. a = $p< 0.001$ Vs control group. b = $p< 0.05$ Vs CCAO treated group. c = $p< 0.001$ Vs CCAO treated group.

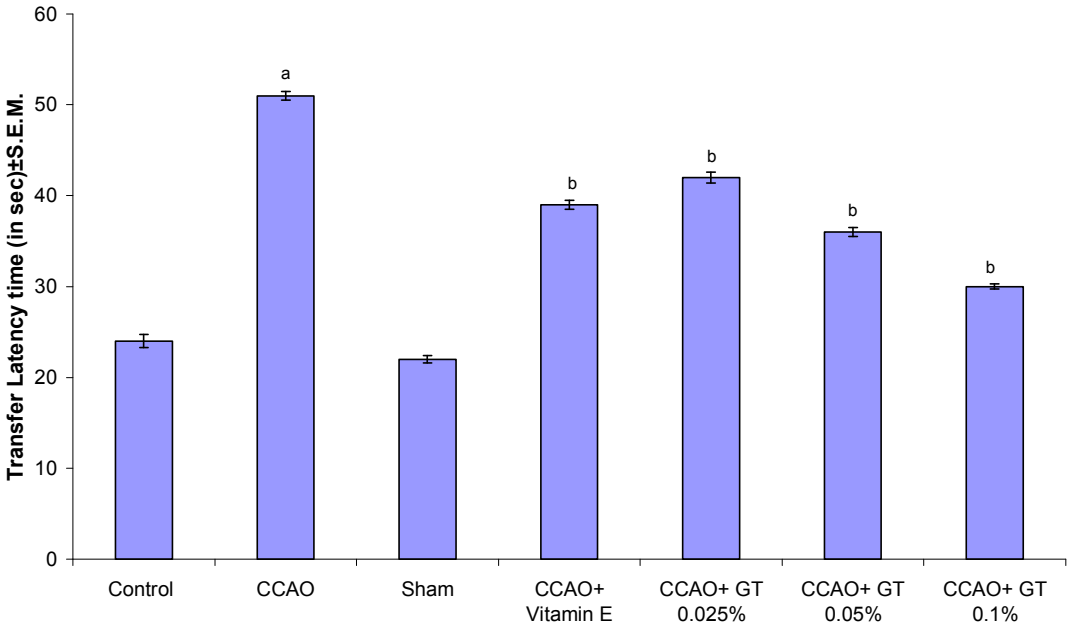

Figure -6. Effect of green tea on TL time using elevated plus maze, in CCAO mice. Control represents normal water was given for drinking to mice, before conducting acquisition trail. Vitamin-E (200 mg/kg, p.o.) was used standard drug. Each group (n = 6) represents mean ± S.E.M. a = $p< 0.001$ Vs control group. b = $p< 0.001$ Vs CCAO group.

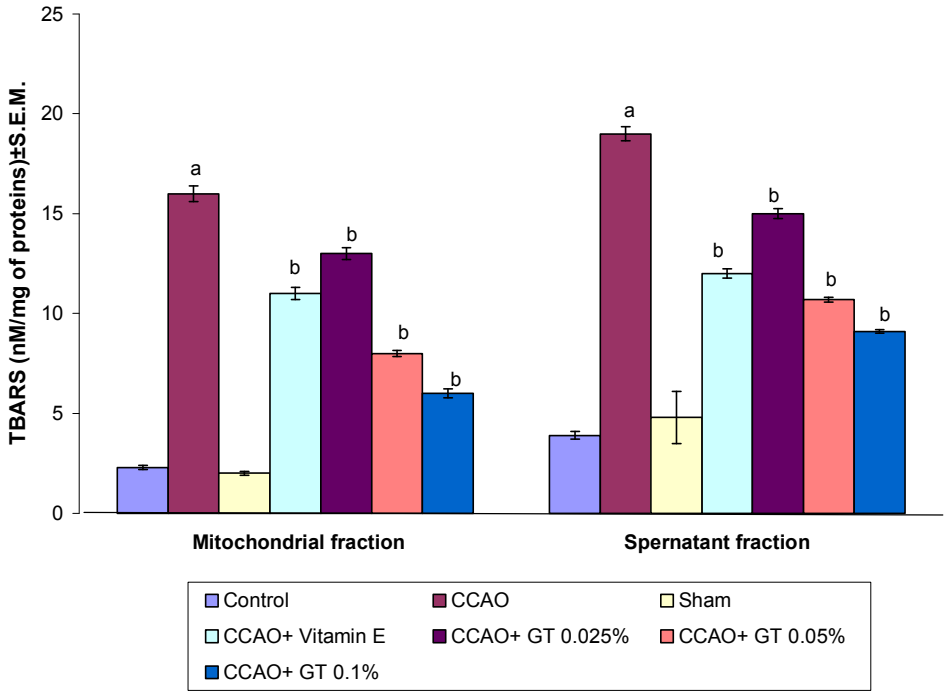

Figure -7. Effect of green tea on thibarbituric acid reactive substances (TBARS) on CCAO mice brain. Control represents normal water was given for drinking to mice. Sham represents that only surgical procedure was followed. CCAO represents both common carotid artery was occluded. CCAO + GT represents, green tea (0.025%, 0.05%, 0.1%) was administered for two months then both common carotid artery was occluded. Vitamin-E (200 mg/kg, p.o.) was used as standard drug. Each group (n =6) represents mean ± S.E.M. a = $p < 0.001$ Vs control group. b = $p < 0.001$ Vs CCAO group.

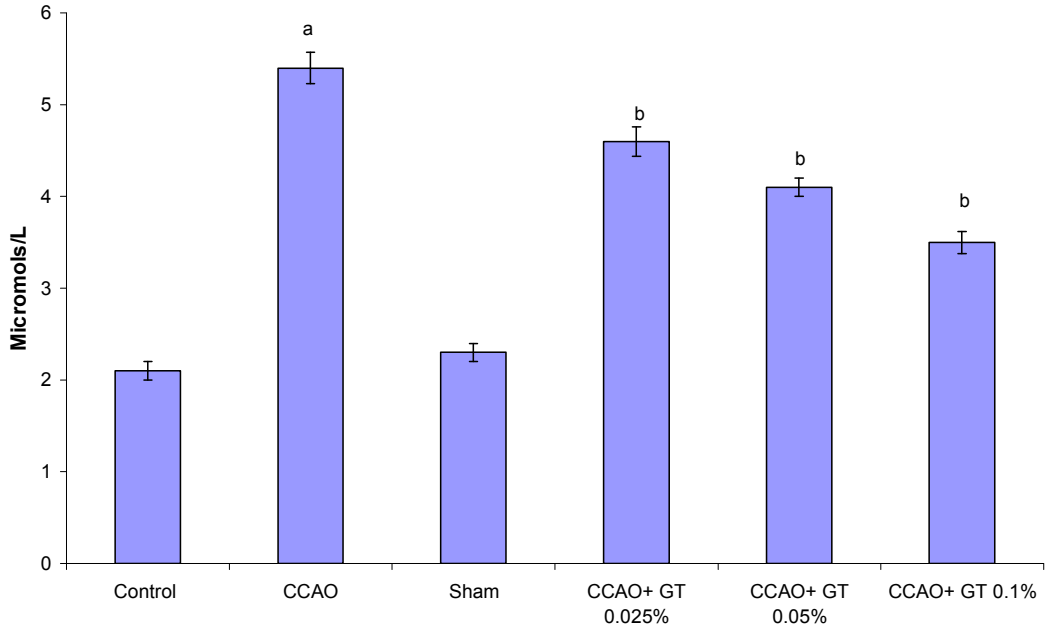

Figure -8. Effect of green tea on serum nitrite concentration in mice. Control represents normal water was given for drinking to mice. Sham represents, that only surgical procedure was followed. CCAO represents, both common carotid artery was occluded. CCAO + GT a represents, green tea (0.025%, 0.05%, 0.1%) was administered for two months then both common carotid artery was occluded. Each group (n =10) represents mean ± S.E.M. a = $p < 0.001$ Vs control group. b = $p < 0.05$ Vs CCAO group.

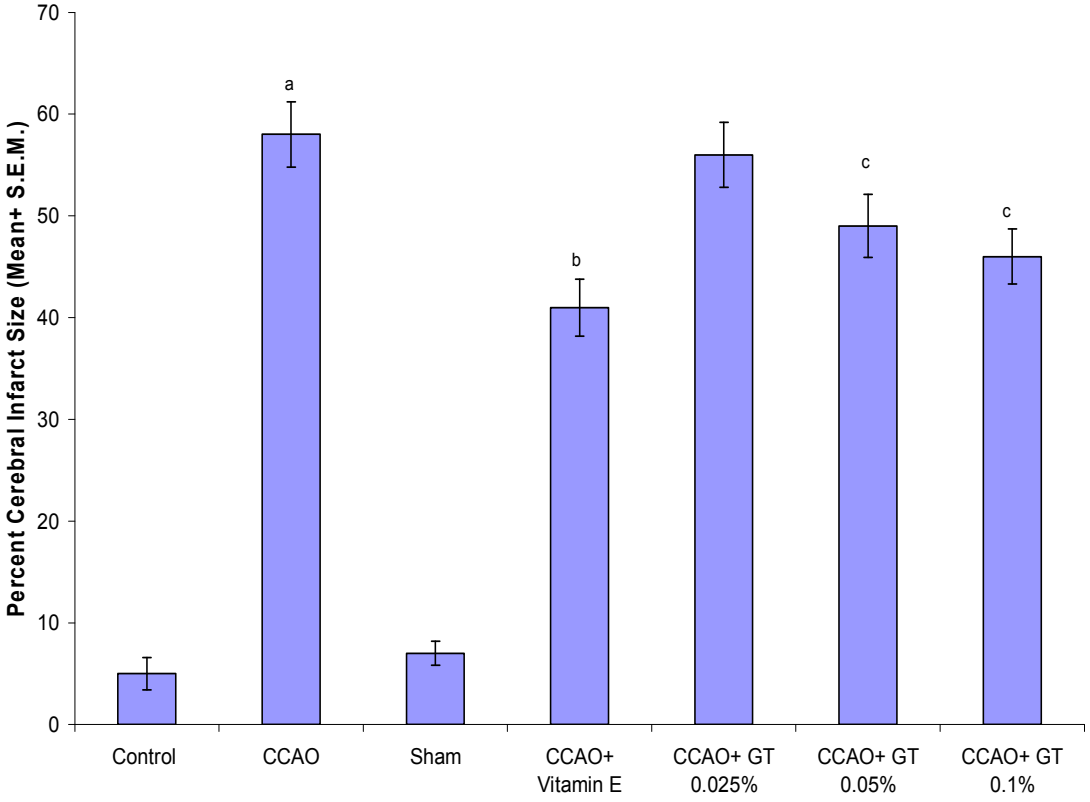

Figure -9. Effect of green tea and vitamin-E on reperfusion induced cerebral infarct size measured by volume method in mice. Sham represents that mice were subjected to surgical procedures without cerebral ischemia and reperfusion. Each group (n =10) represents mean ± S.E.M. a=p< 0.001 Vs control group. b=p< 0.001 Vs CCAO group. c= p<0.05 Vs CCAO group.

Discussion

The hippocampus is a brain region that demonstrates selective vulnerability to ischemic damage. It is also a structure directly involved in learning and memory processes. Cognition deficiency is classically described after induced global ischemia either in man or in animals (22). During the ischemic period and during early

reperfusion a massive release of excitatory amino acids, an intracellular overload with calcium and an increase in free radicals are the hallmarks of a phase called excitotoxicity (23). Ischemia/reperfusion is well known to activate membrane phospholipase A2 and promote the liberation of free fatty acids, mainly arachidonic acid, from which a series of eicosanoids are produced, including prostaglandins, leukotrienes, and thromboxanes. These and other bioactive eicosanoids are potent vasoconstrictors and increase vascular permeability, thus contributing to edema formation and neuronal injury after ischemia (24)

Green tea polyphenols prevent oxidative damage to proteins by restoring protein carbonyl levels and antioxidant enzyme status (25). Antioxidative enzymes are activated by green tea catechin intake, and the antioxidative potency increases with continued ingestion of green tea (26). Green tea polyphenols have been reported to have potent radical scavenging abilities (27) and can chelate metals to prevent metal-catalyzed free radical formation (2). Green tea polyphenols can inhibit ROS accumulation by inhibiting xanthine oxidase, which catabolizes purines to produce uric acid and ROS (28). Increased ROS production can lead to oxidative damage of lipids and DNA and, ultimately, apoptotic cell death and cognitive impairment. Green tea polyphenols can protect DNA from •OH radical-induced strand breaks and base damage through fast chemical repair of DNA radicals (29). Furthermore pre-treatment with green tea improve GCI induced memory impairment (30). Green tea phytochemicals also inhibit COX-2 and NOS expression and produces anti-inflammatory action (31,32). In the present study we observed that green tea significantly improved the learning and memory in ischemia reperfusion induced memory impairment. Green tea also reduced the brain TBARS and serum NO concentration which was increased by the ischemia reperfusion injury in mice.

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