GREEN TEA ATTENUATES MEMORY IMPAIRMENT INDUCED BY BILATERAL COMMON CAROTID ARTERY OCCLUSION IN MICE

Arun Kumar[1]*, Vijay Juyal[1]

[1]Department of Pharmaceutical Sciences, Bhimtal campus, Kumaun University, Bhimtal-263136, Nainaital, Uttarkhand, India.
*Corresponding author contact id: arun_pharma1@rediffmail.com

Summary

Green tea polyphenols have demonstrated significant antioxidant, anti-carcinogenic, anti-mutagenic and antidiabetic activity in numerous human, animal and in vitro studies. Hence present study was designed to evaluate the influence of green tea in ischemia induced memory loss 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. Brain ischemia reduces the learning and memory performance and also increase the concentration of thiobarbituric acid reactive substance in mice. Green tea significantly improved the learning and memory and reversed the increased 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 ischemia induced oxidative stress.

Key Word: Ischemia, memory, green tea

Introduction

One of the most persistent consequences of brain ischemia in humans is amnesia. Memory loss has been observed in patients with a history of transient global cerebral ischemia induced by cardiac arrest or coronary artery occlusion (1,2). Several tests have been developed to study cognitive functions in rodents. These tests differ for the cognitive domains that they explore. Studies on cognitive functions in rats subjected to chronic cerebral ischemia have so far preferentially explored

learning and reference memory using the Water Maze test and the Radial Arm Maze test. These tests include a training phase and are frequently associated with reward or punishment. The results of these studies are almost consistent with the finding of an alteration of learning and reference or visuospatial memory at different time intervals from the induction of chronic ischemia. Cognitive deficits have been found to be associated with damage in the CA1 region of the hippocampus in studies using the bilateral common carotid artery occlusion model (3,5). Cholinergic and glutamatergic neurons in hippocampus are crucially involved in learning and memory. Dysfunction in any one or both of these systems leads to severe form of dementia (6,7). Cerebral ischemia induces enhanced free radical [$NO^{\bullet}$, $ONOO^{-}$, $^{\bullet-}O2$, 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 (8,9). PARP is a multifaceted enzyme involved in various cytotoxic mechanisms like inflammation, mitochondrial dysfunction, necrosis and apoptosis. It is noteworthy that all these cell death machineries are well recognized in global cerebral ischemia-induced delayed neuronal death (10,11). This suggests that inflammation and oxidents plays a detrimental role in ischemic neuronal death, which deserves in-depth investigation. Antioxidants (12), ubiquitin-proteasome system and proteasome inhibitors (13), growth factors (14), glutamate receptor blockers (15), estrogens (16) and inflammation (17) are the various novel agents that have been found experimentally effective for containing cerebral ischemia/reperfusion induced cellular pathology.

Green tea, a popular beverage, is now being recognized for its herbal remedy and its medicinal properties have been widely explored (18). The tea plant, *Cammelia sinesis*, is a member of Theaceae family, black and green tea are produced from its leaves (19). 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 (20). Green tea polyphenols have demonstrated significant antioxidant, anti-inflammatory, anti-carcinogenic properties and antidiabetic in numerous human, animal and in vitro studies (21).

Material and methods

Extraction of drug

The leaves were identified by the Forest Research Institute, Dehradun, India (Accession no. 157030). 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'.

Global cerebral ischemia and reperfusion

On day 15, the mice were anesthetized with ketamine(110 mg/kg) and xylazine (15 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 incisionwas sutured (22). 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 Common carotid artery occlusion surgery was 75%. The mice were housed in their cages until the memory test.

Morris water maze

Morris water maze (23) 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 5^{th}, 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 (24) 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. 16^{th} 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.16^{th} day, 24 after last dose).

Passive avoidance test

Passive avoidance (24) 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.

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 (25) and the other portion was employed for protein estimation (26).

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.

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 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).

Pre treatment with green tea and Vitamine-E reduced significantly ($p<0.001$) ELT in ischemia reperfusion injured mice during acquisition trails 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).

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 also reverse significantly ($p<0.05$) reduce TL in Ischemia reperfusion injured mice(fig-3).

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 also reversed significantly ($p<0.05$) reduce SDL in ischemia reperfusion injured mice(fig-4).

Effect on thiobarbituric acid reactive substances (TBARS)

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

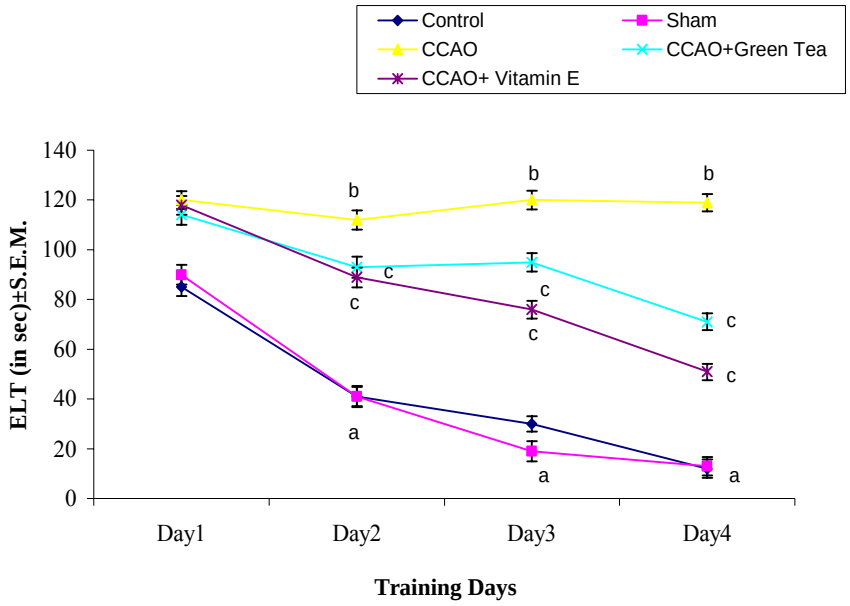

Fig. 1. Effect of green tea on ELT (acquisition trails conducted on day 1 to day 4) using morris water maze. Control represents administration of normal saline (10 ml kg^{-1} i.p.) 30 min before acquisition in mice. Sham represents, that only surgical procedure was followed before acquisition trials. CCAO represents, both common carotid artery was occluded before acquisition trials. CCAO + green tea represents, green tea (0.05%) was administered for 60 days then both common carotid artery was occluded before acquisition trials. Vitamin-E (200 mg/kg) was used as standard drug. Each group (n = 10) represents mean ± S.E.M. a = $p < 0.001$ Vs ELT on first day of same groups. b = $p < 0.001$ Vs ELT on control group for the same day. c = $p < 0.05$ Vs ELT on indomethacin and green tea treated group for the same day.

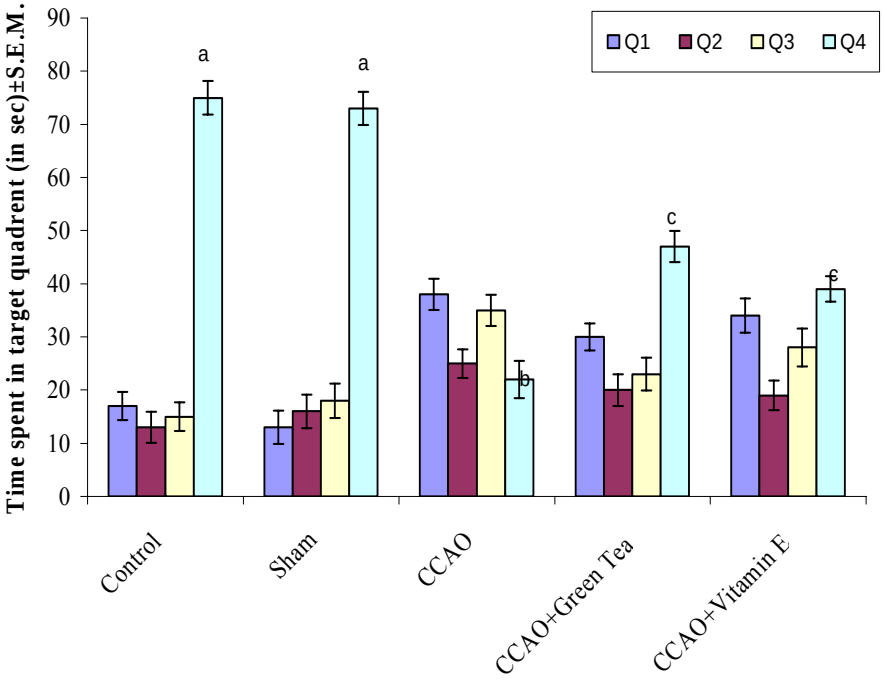

Fig-2. Effect of green tea on retrieval trails (conducted on day 5) using morris water maze. Control represents, administration of normal saline (10 ml kg^{-1} i.p.) 30 min before retrieval trials in mice. Sham represents, that only surgical procedure was followed before retrieval trials. CCAO represents, both common carotid artery was occluded before retrieval trials. CCAO + green tea represents, green tea (0.05%) was administered for 60 days then both common carotid artery was occluded before retrieval trials. Vitamin-E (200 mg/kg) was used as standard drug. Each group (n = 10) represents mean ± S.E.M. = $p< 0.001$ 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 = $p< 0.05$ Vs time spent in target quadrant i.e. Q4 in CCAO group.

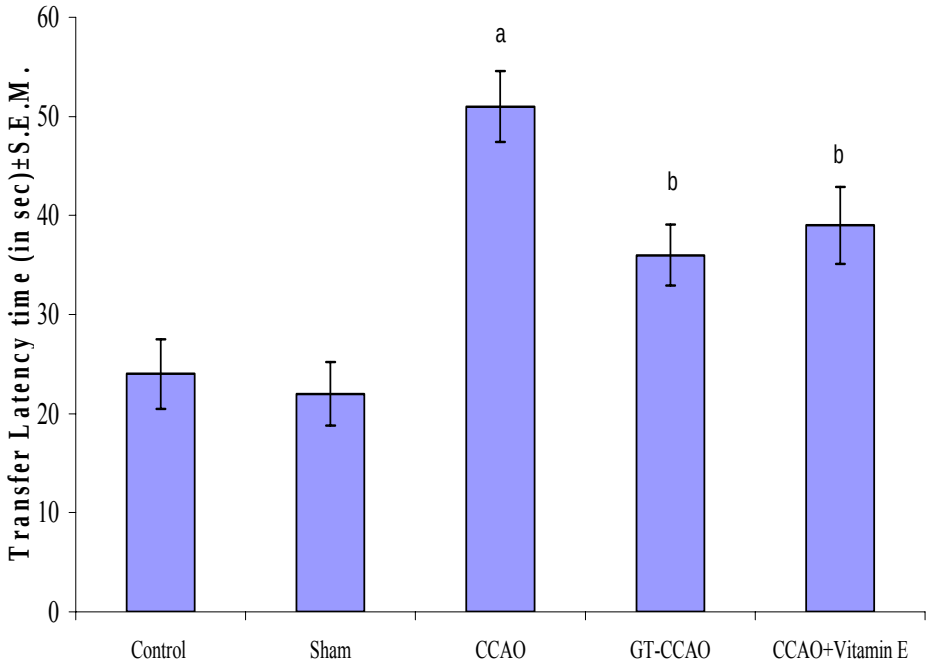

Fig-3. Effect of green tea on TL time using elevated plus maze. Control represents, administration of normal saline (10 ml kg^{-1} i.p.) 30 min before trails in mice. Sham represents, that only surgical procedure was followed before trials. CCAO represents, both common carotid artery was occluded before trails. CCAO + green tea represents, green tea (0.05%) was administered for 60 days then both common carotid artery was occluded before trials. Vitamin-E (200 mg/kg) was used as standard drug. Each group (n = 10) represents mean ± S.E.M. a = $p< 0.001$ Vs control group. b = $p< 0.05$ Vs CCAO group.

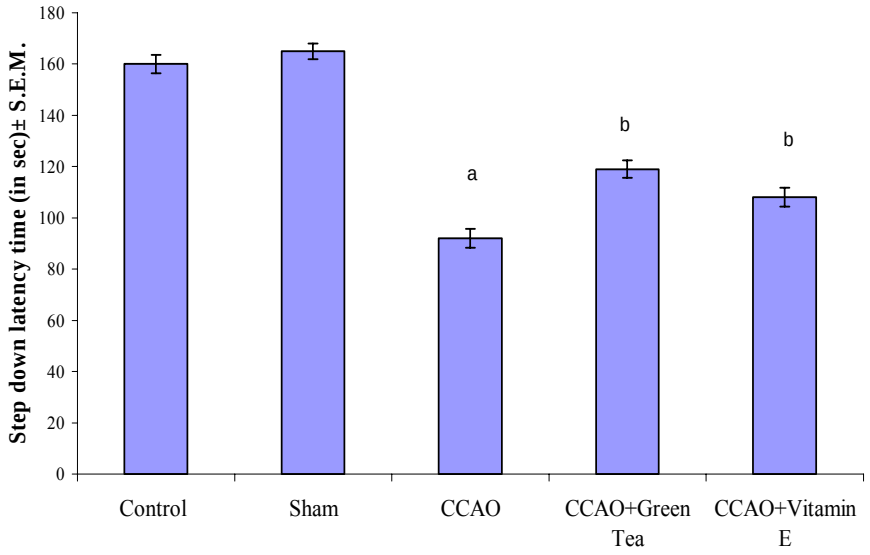

Fig-4. Effect of green tea on step down latency time on passive avoidance paradigm. Control represents, administration of normal saline (10 ml kg^{-1} i.p.) 30 min before trails in mice. Sham represents, that only surgical procedure was followed before trails. CCAO represents, both common carotid artery was occluded before trials. CCAO + green tea represents, green tea (0.05%) was administered for 60 days then both common carotid artery was occluded before trials. Vitamin-E (200 mg/kg) was used as standard drug. Each group (n = 6) represents mean ± S.E.M. a = $p < 0.001$ Vs control group. b = $p < 0.05$ Vs STZ treated group.

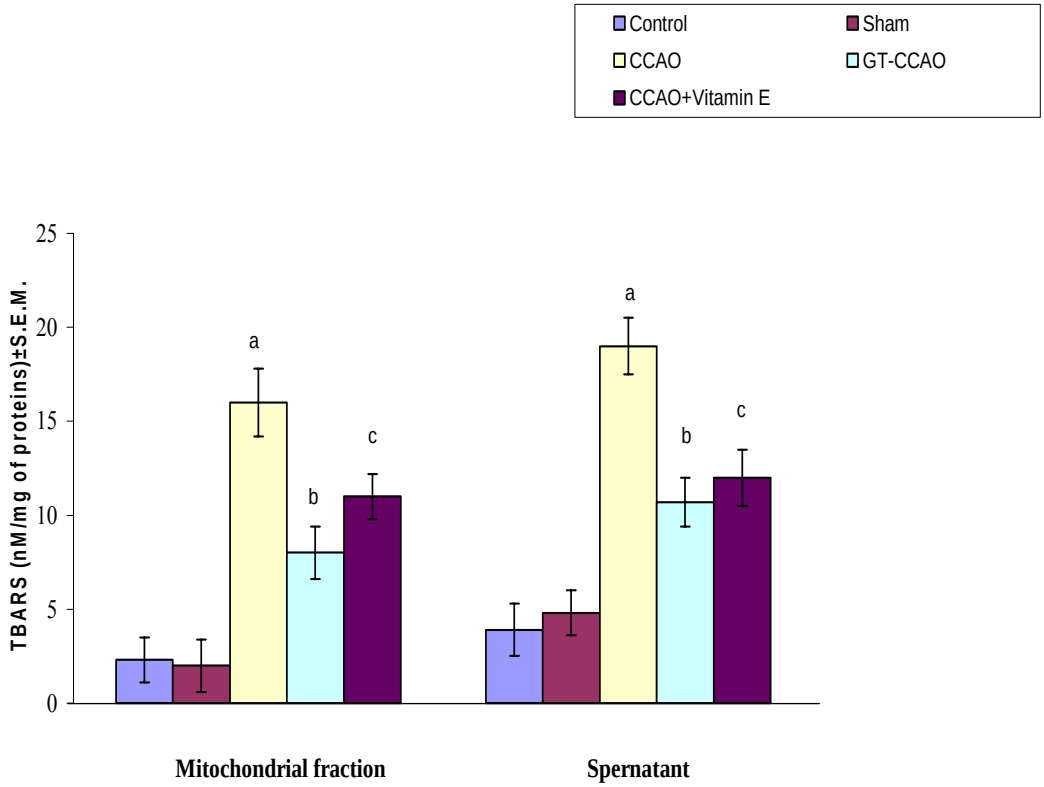

Fig-5. Effect of green tea on thibarbituric acid reactive substaces (TBARS). Control represents, administration of normal saline (10 ml kg^{-1} i.p.) in mice. Sham represents, that only surgical procedure was followed. CCAO represents, both common carotid artery was occluded. CCAO + green tea represents, green tea (0.05%) was administered for 60 days then both common carotid artery was occluded. Vitamin-E (200 mg/kg) was used as standard drug. Each group (n =10) represents mean ± S.E.M. a = $p < 0.001$ Vs control group. b = $p < 0.05$ Vs ICV STZ treated group.

Discussion

Global cerebral ischemia has been widely used in experimental studies. Temporarily occluding the bilateral common carotid arteries is sufficient to induce brain injury in the striatum, cerebral cortex and hippocampus, which have been shown to be involved in mood, learning, and memory processes (27). Cerebral ischemia has been reported to impair short-term memory and spatial memory because hippocampal neurons are particularly vulnerable to the deleterious effects of ischemia and reperfusion (28,29). Therefore, in the present investigation we employed elevated plus-maze test to assess short-term memory (30) and morris water maze to assess spatial memory (31). Ischemia reperfusion injury causes a robust increase in typical markers of inflammation and oxidative stress in the rat hippocampus (32). Reactive oxygen species (ROS) have been indicated as one of the earliest and most important components of tissue injury after reperfusion of the ischemic organ (33). The brain is very susceptible to the damage caused by oxidative stress, due to its rapid oxidative metabolic activity, high polyunsaturated fatty acid content, relatively low antioxidant capacity, and inadequate neuronal cell repair activity (34). The effect of antioxidants and antioxidant rich extract from natural product such as vitamin E, vitamin C, coca, apple jush, melatonin on the cognitive defect has been widely investigated. Thus it has been believed that antioxidants improve cognitive impairement through protection of neurones against oxidative stress (35). 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 intapenitorially (36).

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 TBARS concentration in brain mitochondria and supernatant fraction which is increased by the ischemia reperfusion injury in brain.

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