

Neurochemical and behavioral effects of green tea (*Camellia sinensis*) as observed in animals exposed to restraint stress

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Abstract: Clinical studies on psychiatric patients suggest that life events stress precipitates depression. The possible involvement of 5-Hydroxy tryptamine (5-HT; Serotonin) in depression and other behavioral deficits is also suggested by clinical studies. As a natural stimulant, green tea (*Camellia Sinensis*) diminishes stress, worry and anxiety, allowing the brain to focus and concentrate better. Previously we have reported that beneficial effects of green tea might be associated with altered levels of 5-HT, which in turn may help in coping with stress. Present study therefore deals with monitoring the behavior and neurochemical profile of single restrained stress in animals previously administered (for 5 weeks) with green tea. Activities in light dark activity box were monitored 1hr post restraint stress. Cumulative food intake values were monitored 24hr post restraint stress. 24hr after restrained stress, rats were decapitated to collect plasma and brain samples. Brain samples were kept stored at -70°C until neurochemical analysis by HPLC-EC. Findings illustrate that although food intake was decreased in both green tea- as well as water treated rats, stress-induced anxiogenic effects were attenuated in green tea treated rats. Tone of 5-HT was also normalized in restrained animals. Results suggest beneficial effects of green tea in coping the stressful conditions/stimuli are related to altered 5-HT metabolism.

Key words: *Camellia sinensis*, serotonin, stress, light dark box activity.

INTRODUCTION

Green tea (*Camellia sinensis*) is a popular beverage in East Asia, and also used as a herbal remedy in Europe and North America. Green tea is considered to be anti-inflammatory, antioxidative, antimutagenic, and anticarcinogenic (Benelli *et al.*, 2002; Weisburger and Chung, 2002) and can prevent cardiac disorders. Epidemiologically, it has been suggested that green tea consumption prevents type II diabetes. The amelioration of insulin resistance by green tea is associated with the increased expression level of glucose transporter IV in a fructose-fed rat (Wu *et al.*, 2005). Green tea extract contains polyphenols, theanine and caffeine (Zheng *et al.*, 2004). The extract also includes pyrroloquinoline quinone, a newly discovered vitamin which prevents cognitive deficit caused by oxidative stress (Ohwada *et al.*, 2008). Green tea contains pigallocatechin gallate and epigallocatechin which improve glucose tolerance and reduces weight gain of rats fed on normal and high-fat diet (Snoussi *et al.*, 2014). Green tea extracts also inhibit intestinal glucose uptake by inhibiting the sodium-dependent glucose transporter of rabbit intestinal epithelial cells (Kobayashi *et al.*, 2000), and to reduce serum glucose level in alloxan-induced diabetic rats (Maruyama *et al.*, 2009).

Green tea extracts are more stable than pure epigallocatechin gallate, the major constituents of green

tea, because of the presence of other antioxidant constituents in the extract (Kaszkın *et al.*, 2004). In general, herbal medicines are complex mixtures of different compounds that often act in a synergistic fashion and exert their full beneficial effect as total extracts.

The calming effect of green tea may seem contradictory to the stimulatory property of tea's caffeine content but it could be explained by the action of L-theanine. This amino acid actually acts antagonistically against the stimulatory effects of caffeine on the nervous system (Kakuda *et al.*, 2000). Research on human volunteers has demonstrated that L-theanine creates a sense of relaxation in approximately 30-40 minutes after ingestion via at least two different mechanisms. First, this amino acid directly stimulates the production of alpha brain waves, creating a state of deep relaxation and mental alertness similar to what is achieved through meditation. Second, L-theanine is involved in the formation of the inhibitory neurotransmitter, gamma amino butyric acid (GABA). GABA influences the levels of two other neurotransmitters, dopamine and serotonin, producing the key relaxation effect (Mason, 2001). L-theanine has a significant effect on the release or reduction of neurotransmitters like dopamine and serotonin, resulting in improved memory and learning ability.

Clinical studies on psychiatric patients suggest that a life event stresses precipitate depression (O'Dougherty *et al.*, 2012). The possible involvement of 5-HT in depression and other behavioral deficits is also suggested by clinical

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studies (Mahar *et al.*, 2014). The present study was designed to monitor the effects of green tea on behavior and neurochemistry of rat brain serotonin, pre and post stress. Results may be beneficial in treating stress and related disorders.

MATERIALS AND METHODS

Animals

Study was carried out on locally bred male Albino Wistar rats (150-250g) purchased from HEJ Research Institute of Chemistry. Rats were kept individually in specially designed cages in a quiet room with free access to water and cubes of standard rat food for at least 1 week before starting the experiment so that rats adopt the environment.

Experimental protocol

Twenty four rats were randomly assigned to four groups each containing six animals: (i) Unrestrained-water- (ii) Unrestrained-green tea-, (iii) Restraint-water- and (iv) Restraint green tea administered rats. Rats had free access to tap water and green tea extract (1g/L) respectively for a period of five weeks. After five weeks, animals of restraint-water- and restraint green tea administered groups were exposed to 2hr restraint stress. 1hr post restraint stress, elevated plus maze and light dark box activities of animals were monitored for assessing the anxiolytic effects of green tea. Animals were decapitated 24hr post termination of restraint stress and brain samples were collected and stored at -70 °C until neurochemical analysis by HPLC-EC.

Food intakes and body weights

Cumulative food intakes (g) were determined by taking the difference of food given on day 1, between 8:00 and 9:00h and food left next day and every day (between 8:00 and 9:00h). Body weights were also monitored at the same time and change in body weights were calculated (body weight on monitoring day / body weight on preceding day) × 100 as reported previously (Haleem *et al.*, 2013; Ikram and Haleem, 2011).

Immobilization procedure

The animals were immobilized as described before (Haleem and Ikram, 2013). Wire grids of 10 in. × 9 in. fitted with a Perspex plate of 9 x 6.5" were used. Immobilization was effected by pressing the forelegs of the rat through the gaps in the metal grid and taping them together with zinc oxide plaster. Hind limbs were also taped and the head of the animal rested on the Perspex plate. At the end of the 2h immobilization period the animals were released and returned to their home cages.

Light dark box activity

Light dark box activity was performed as described before (Ikram and Haleem, 2010). Specifically designed two Perspex boxes of equal dimensions (26x26x26 cm) were used to monitor the activity. One box is transparent and

other is black walled. There is also an entry between them. To determine light and dark box activity, animal was taken out from home cage and was placed for the first time in the light box. Time spent in the light and dark boxes was monitored for 5 minutes.

Elevated plus maze activity

The elevated plus-maze is a cross shaped maze that has two open arms and two close arms enclosed by sides, but with an open roof. The entire maze is elevated 50cm above the floor. Rodent's unconditioned aversion to light and open spaces contribute to its effectiveness as a test for anxiety. Test involves placement of animal in the center of maze and observing the number of entries and time spent in open/fear inducing arm.

Brain dissection

After decapitation, skull plates were cut and membrane covering the brain was removed with the help of fine forceps. Using spatula, brain was taken out and washed with ice-cold saline. The collected brains were immediately stored at -70°C for neurochemical estimations using High performance liquid chromatography with electrochemical detection (HPLC-EC) (Ikram *et al.*, 2011; Ikram *et al.*, 2014).

HPLC-EC determination of 5-HT & 5-HIAA

HPLC-EC determination was carried out as described before (Mirza *et al.*, 2013). A 5µ Shim-pack ODS separation column of 4.0mm internal diameter and 150mm length was used. Separation was achieved by a mobile phase containing methanol (14%), octyl sodium sulfate (0.023%) and EDTA (0.0035%) in 0.1M phosphate buffer of PH 2.9 at an operating potential of 2000-3000 psi on Shimadzu HPLC pump. Electrochemical detection was achieved on Shimadzu LEC 6A detector at an operating potential of +0.8V (Ikram *et al.*, 2012).

Plasma corticosterone estimation

Blood was collected immediately after decapitation in the heparinized centrifuged tubes. Blood samples were promptly centrifuged at 4 °C, and separated plasma was used for corticosterone determination. The extraction procedure for the plasma corticosterone was essentially the same as described by Peterson and Pierce (1960). Method used to estimate the plasma corticosterone concentration via fluorimetric assay was the same as described by Mattingly (1962). In glass stoppered tubes 200µl of plasma was extracted with 1.5ml dichloromethane followed by vigorous shaking and centrifugation. The upper layer was aspirated and 1 ml of aliquots of dichloromethane layer was transferred to a glass tube and shaken with 1 ml of a sulfuric acid-ethanol reagent (7: 3 v/v). The solvent was removed by aspiration and the acid-alcohol reagent transferred to a small cuvette for fluorescence determination in a fluorimeter at 470 nm

excitation and 570 nm emission wavelengths. In order to prepare stock standard of corticosterone 10mg of corticosterone was dissolved in 10ml ethanol. 0.1ml of this solution was diluted to 100ml with distilled water (100 µg/dl) to prepare working standards. These solutions remain stable for months at 40°C.

STATISTICAL ANALYSIS

Results are represented as means $\pm$ S.D. Statistical analysis was performed by two-way ANOVA (SPSS ver 17.0). Post hoc comparison of groups was performed by Newman-Keuls test. Values of $p < 0.05$ were considered as significant.

RESULTS

Fig. 1 shows the effect of 2hr restrained stress on food intake. Two-way ANOVA showed significant effect of stress ($F=9.94$; $df=1,20$; $p<0.01$), green tea ($F= 631.92$ $df= 1,20$; $p<0.01$) as well as interaction between the two ($F= 4.09$; $df= 1,20$; $p<0.05$) on food intake. Post hoc analysis by Newman keuls test showed that stress significantly decreased ($p<0.01$) food intake in both water and green tea treated rats as compared to respective unstressed controls.

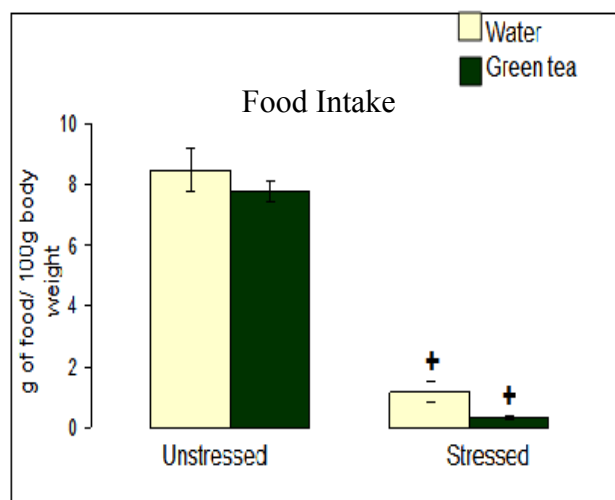


Fig 1: Effects of green tea in rats exposed to restrained stress, on food intake values. Values are means $\pm$ SD ($n=6$). Significant differences by Newman-Keuls: + $p<0.01$ from respective unstressed controls, following two-way ANOVA.

Fig. 2 shows effects of 2hr restrained stress on light dark box activity. Data on number of entries in light compartment (fig 2a) as analyzed by two way ANOVA showed that the effects of stress ($F= 0.457$; $df=1,8$) were non-significant. While effects of green tea ($F= 6.56$; $df=1,20$; $p<0.05$) as well as interaction between stress and green tea ($F= 2.19$; $df= 1, 20$; $p<0.05$) were significant. Post hoc analysis by Newman keuls test showed that stress decreased the number of entries in light

compartment in water treated animals ($p<0.01$) as compared to their respective unstressed controls. Fig. 2b shows the effect of 2hr restrained stress on time spent in light compartment of the light dark activity box. Two way ANOVA showed significant effects of stress ($F=207.67$; $df= 1,20$; $p<0.01$), green tea ($F=153.83$; $df=1,20$; $p<0.01$) as well as interaction between the two ($F=4.57$ $df= 1,8$ $P<0.05$). Post hoc analysis by Newman keuls test showed that green tea increased ($p<0.01$) time spent in light compartment as compared to water treated controls. Restrained water treated animals exhibited a decrease in the time spent in light compartment as compared to similarly treated unrestrained animals.

Light Dark Box Activity

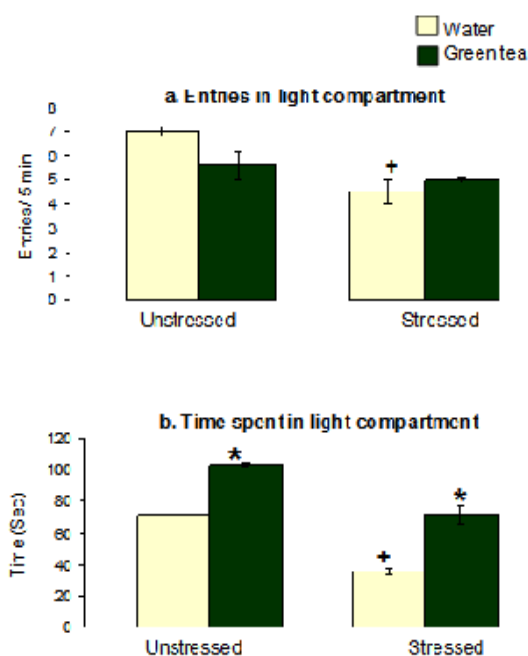


Fig 2: Effects of green tea in rats exposed to restrained stress, on light dark box activity. Values are means $\pm$ SD ($n=6$). Significant differences by Newman-Keuls: + $p<0.01$ from respective unstressed controls; * $p<0.01$ from respective water treated controls following two-way ANOVA.

Fig. 3a shows the effect of 2hr restrained stress on plasma corticosterone level. Two-way ANOVA showed insignificant effect of stress ($F=5.18$ $df= 1,8$), significant effect of green tea intake ($F 42.07$ $df 1,8$; $p<0.01$) and insignificant interaction between stress and green tea ($F 0.11$ $df 1,8$) on plasma corticosterone level. Post hoc analysis Newman keuls test showed that stress significantly increase ($P<0.01$) the plasma corticosterone level in both water and green tea treated rats. Fig. 3b shows the effect of 2hr restrained stress on plasma glucose level. Two-way ANOVA showed insignificant effect of stress ($F=4.71$ $df= 1,8$), insignificant effect of green tea intake ($F= 0.16$ $df= 1,8$) and significant

interaction between stress and green tea ($F=8.67$, $df= 1,8$ $p<0.05$) on plasma glucose level. Post hoc analysis by Newman keuls test showed decreased level of plasma glucose in green tea treated animals as compare to water treated controls.

Light Dark Box Activity

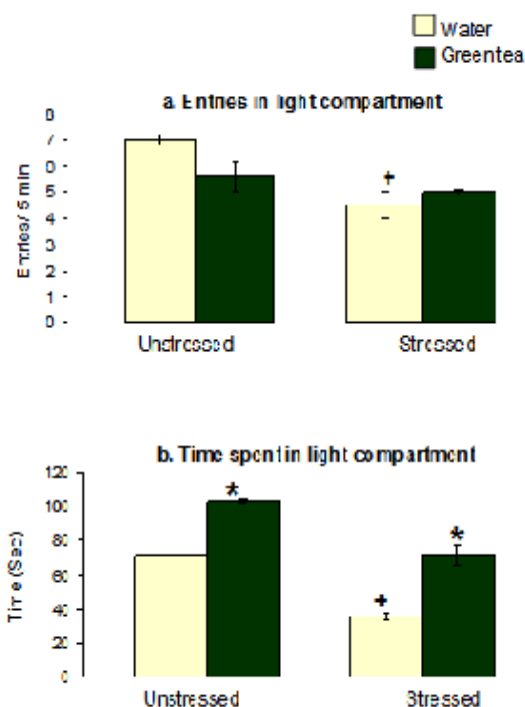


Fig 3: Effects of green tea in rats exposed to restrained stress, on plasma glucose and corticosterone levels. Values are means $\pm$ SD ($n= 6$). Significant differences by Newman-Keuls: + $p<0.01$ from respective unstressed controls; * $p<0.01$ from respective water treated controls following two-way ANOVA.

Fig. 4a show the effect of 2 hrs restraint stress on concentration of brain 5HT. 2-way ANOVA showed insignificant effect of stress ($F=4.46$ $df= 1,8$), significant effect of green tea intake ($F= 113.71$ $df= 1,8$ $P<0.01$) and significant interaction between stress and green tea ($F= 99.53$ $df= 1,8$ $P<0.01$) on brain 5HT level. Post hoc analysis by Newman keuls test showed that green tea significantly decrease the brain 5HT levels as compare to water treated ones. Stress increased the levels of 5HT in brain in both water and green tea treated rats as compare to similarly treated unrestraint while in green tea treated animals, stress significantly increased brain 5HT level as compare to similarly treated unrestraint animals. That stress significantly increase ($P<0.05$) brain 5HT level in green tea treated rats. Fig. 4b shows the effect of 2 hr restrained stress on concentration of brain 5HIAA. 2-way ANOVA showed significant effect of stress ($F=13.44$ $df= 1,8$ $P<0.01$), green tea ($F=14.92$ $df= 1,8$ $P<0.01$) and

interaction between the to ($F=30.69$ $df= 1,8$ $P<0.01$) on brain 5HIAA level. Post hoc analysis Newman keuls test showed that stress significantly increased a ($P<0.01$) brain 5HIAA level in water treated rats, while green tea treated restraint animals show decreased levels of 5HIAA as compare to water treated restraint animals.

Light Dark Box Activity

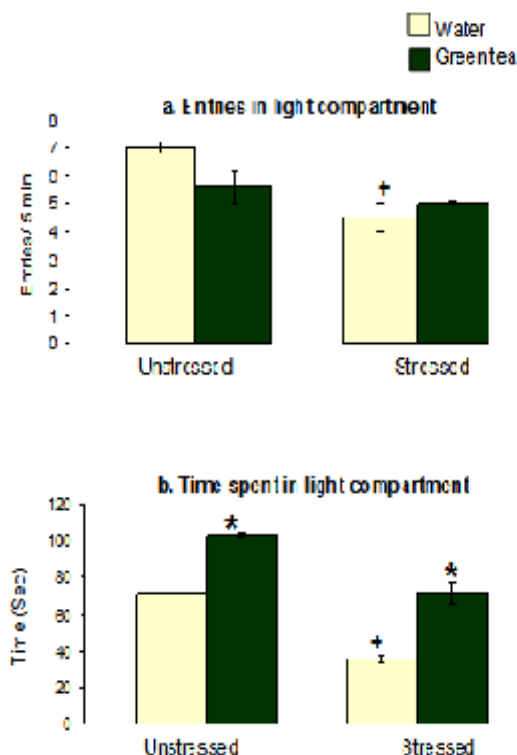


Fig 4: Effects of green tea in rats exposed to restrained stress, on 5-HT and 5-HIAA levels. Values are means $\pm$ SD ($n= 6$). Significant differences by Newman-Keuls: + $p<0.01$ from respective unstressed controls; * $p<0.01$ from respective water treated controls following two-way ANOVA.

DISCUSSION

Present results show a decrease in the food intake by green tea in restraint animals. Sayama *et al* (2000) also have reported suppression of food intake in mice by feeding them 4% green tea diet. Green tea reduces body weight by increasing energy expenditure and fat oxidation (Auvichayapat *et al.*, 2008). Green tea treated restrained animals spent more time in light compartment as compared to the water treated controls, thereby exhibiting anxiolytic effects of green tea extract. Others have reported that green tea polyphenol epigallocatechin gallate/ derivatives could be an interesting alternative therapeutic approach to treat anxiety. Have reported that epigallocatechin gallate can induce anxiolytic activity

which could result from an interaction with GABA_A receptors (Vignes *et al.*, 2006). L-theanine can also produce anxiolytic-like effects in the elevated plus maze and the marble burying assay in naïve mice at doses that did not significantly affect motor behavior (Wise *et al.*, 2012).

Water treated restrained animals showed increased levels of plasma corticosterone as compared to green tea treated stressed ones. Previous research showed that in response to stress, the brain activates several neuropeptide-secreting systems. This eventually leads to the release of adrenal corticosteroid hormones (de Kloet *et al.*, 2005). In the present study we found that 2hr restrained stress significantly increase plasma corticosterone level in both water and green tea treated rats, as reported that corticosterone (in rodents) are important mediators of stress system (McEwen, 2007). However, the stress induced raise in corticosterone was higher in water treated rats as compared to green tea treated ones. This shows that green tea tends to decrease the level of stress-induced raise in plasma corticosterone and therefore can help in coping with anxiety.

5-HT is a peripherally and centrally occurring transmitter, which is involved in regulation of anxiety-related behavior. 5-HT plays an important role in the development and the persistence of anxiety disorders (Filaferro *et al.*, 2014). A consistent observation from preclinical studies is that activation of the hypothalamic-pituitary-adrenal (HPA) axis raises levels of extracellular fluid (ECF) serotonin (5-HT) in various forebrain regions, including hippocampus a critical neural locus for antidepressant medications (Goel *et al.*, 2014; Hestermann *et al.*, 2014; Rentesi *et al.*, 2010). In the present study we found that together with plasma corticosterone level, stress also increase the level of brain 5HT in both water and green tea treated rats. This is supported by the results of Linthorst and colleagues (2002) who interpret an extremely increased 5-HT release in some animals as an anxiety / panic-stimulated release. However, water treated rats showed higher levels of 5HT in brain as compared to green tea treated ones which shows that green tea tends to normalize the stress induced raise in brain 5HT. Stress also increased brain 5HIAA in water- and green tea treated rats.

CONCLUSION

In conclusion, present results suggest that 5-HT plays an important role in the beneficial effects of green tea resulting in coping with stress-induced behavioral deficits. Present results show that green tea significantly attenuated anxiogenic effects as evaluated in a light dark activity box. Green tea decreased plasma levels of glucose in unrestrained but not restrained animals. Synergistic attenuation of elevated plasma corticosterone in restraint animals, along with a decreased 5-HT metabolism,

suggests that anxiolytic effects of green tea are mediated via 5-HT-HPA interaction.

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