Black Tea Supplementation Improves Antioxidant Status in Rats Subjected to Oxidative Stress

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The protective effect of black tea extract (BTE) against $HgCl_2$-induced oxidative damage in Wistar rats was investigated. Rats were injected with $HgCl_2$ (5 mg/kg body weight in 0.9% NaCl) to induce oxidative stress. The aqueous BTE (2.5%) was prepared from CTC (curl, tear, crush) grade tea. BTE was fed to control and $HgCl_2$-treated rats by gavage at a dose of 1 ml/(100 g body weight d). Biomarkers of oxidative stress, such as the erythrocyte plasma membrane redox system (PMRS) activity, lipid peroxidation, and advanced oxidation protein products (AOPP), increased by 166, 31, and 373%, respectively, in response to $HgCl_2$ treatment, while intracellular glutathione and plasma antioxidant potential, *i.e.* ferric reducing ability (FRAP) decreased by 75 and 22%, respectively. BTE protected the rats against $HgCl_2$-induced oxidative damage and raised the antioxidant potential in control rats. Due to its strong antioxidant effect *in vivo*, black tea intake may provide a significant health-promoting effect to humans.

Key words: Oxidative Stress, Black Tea, Antioxidants

Introduction

Tea [*Camellia sinensis* (L.) Kuntze] is one of the most popular beverages consumed worldwide and is a rich source of polyphenolic compounds. Green tea contains considerable amounts of catechins such as epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), and epigallocatechin gallate (EGCG). In contrast the major components of black tea are theaflavins (mixture of theaflavin-3-gallate, theaflavin-3'-gallate, theaflavin-3,3'-digallate) and thearubigins, which are oxidation products of quinones and flavonols that are formed when black tea is manufactured from freshly plucked green leaves (Bonnely *et al.*, 2003). The worldwide consumption of black tea is 80% compared to 18% for green tea (Fu *et al.*, 2011). Green tea has antioxidant, antimutagenic, anticarcinogenic, and free radical scavenging activities (Boehm *et al.*, 2009; Thakur *et al.*, 2012; Maurya and Rizvi, 2009) due to its high content of polyphenols.

Earlier, we reported on the antiaging and antidiabetic role of tea catechins, which can be attributed to the strong antioxidant activity of these compounds (Maurya and Rizvi, 2009; Rizvi and Zaid, 2005). The multimeric polyphenols of black tea, theaflavins and thearubigins, which are generated during the fermentation of tea leaves, possess even stronger antioxidant activity than their precursor catechins (Leung *et al.*, 2001). These components of black tea are also known to possess antipyretic, anti-inflammatory, and antimicrobial properties, and they have been shown to provide protection against peroxidation of lipids (Wang *et al.*, 2011; Augustyniak *et al.*, 2011). In contrast to green tea, the biological health effects of black tea have been less investigated.

During cellular respiration aerobic organisms continuously generate free radicals which have been implicated to cause damage to cellular molecules (Lykkesfeldt, 2007). Although all aerobic organisms are endowed with inherent antioxidant defence mechanisms which can be augmented by dietary supplementation, several human disease conditions are known to precipitate formation of free radicals causing a state of oxidative stress. Diabetes, atherosclerosis, cancer, and aging are known to be associated with oxidative stress (Halliwell and Gutteridge, 2007). Dietary supplementation of antioxidants has been considered a feasible approach to mitigate oxidative stress in human health and disease, however, the results have been contradictory (Lotito and Frei, 2006). Although free radicals are perceived to be

responsible for oxidative damage to important biomolecules, a certain amount of these species perform important physiological functions and are thus indispensable.

In the present study we have used a rat model of oxidative stress. Male Wistar rats were treated with $HgCl_2$ to generate experimental oxidative stress (Augusti *et al*., 2008). The *in vivo* antioxidant effect of black tea extract (BTE) has been studied on the level of markers of oxidative stress (plasma membrane redox system, antioxidant potential of plasma, erythrocyte lipid peroxidation, protein oxidation, and intracellular glutathione) in oxidatively stressed rats. In earlier *in vitro* studies, the protective role of black tea against the oxidative damage of human red blood cells was determined (Halder and Bhaduri, 1998), however, there is still no report on the *in vivo* effect of black tea on important markers of oxidative stress. The aim of the present study was to examine the efficacy of BTE as an antioxidant supplementation in a rat model of oxidative stress.

Material and Methods

Chemicals

Reduced glutathione [2,4,6-tri-2-pyridyl-*s*-triazine (TPTZ)], 4,7-diphenyl-1,10 phenanthroline disulfonic acid disodium salt (DPI), and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma Aldrich (Bangalore, India). All other chemicals were of highest purity available from Merck (Mumbai, India) and HIMEDIA Labs (Mumbai, India). Premium quality CTC (curl, tear, crush) black tea (Brooke Bond Red Label, India) was purchased at the local market.

Preparation of black tea extract (BTE)

Preparation of an aqueous BTE followed the method described by Wei *et al.* (1999). Briefly, 1.25 g of black tea leaves were added to 25 ml of boiling water in a beaker, and the mixture was steeped for 15 min. The infusion was cooled to room temperature and then filtered. The tea leaves were extracted a second time with 25 ml of boiling water and filtered; finally both filtrates were combined to obtain a 2.5% (w/v) aqueous BTE. The resulting clear solution was similar to tea brews consumed by human. This BTE was fed to rats by gavage at a dose of 1 ml/[100 g body weight (BW) d] at a temperature of 37 °C (Das *et al*., 2006).

Animal model and study protocol

The experiment was carried out with 28 male Wistar rats [(4 ± 0.5) months old] with body weights of (150 ± 15) g. They were housed in a temperature-controlled facility at (25 ± 5) °C with a 12-h/12-h light/dark cycle for at least 1 week, were fed with a normal laboratory diet of nutrient-rich pellets containing total energy as fat, protein, and carbohydrates, and had free access to drinking water. After the stabilization period of one week, the rats were randomly divided into four groups, containing seven animals each (n = 7). Group I rats (control) received no treatment/supplementation. Group II rats were injected intraperitoneally $HgCl_2$ at a dose of 5 mg/kg BW in 0.9% NaCl to induce oxidative stress (Augusti *et al*., 2008). Group III rats were administered BTE via gavage (oral route) at a dose of 1 ml/(100 g BW d) 10 d before $HgCl_2$ injection, and BTE administration continued daily up to 30 d after $HgCl_2$ treatment; the BTE dose selected was according to Wei *et al*. (1999). Group IV rats were given only BTE for 30 consecutive days. The animals of the first group were simultaneously administered water until the 30th day.

Collection of blood and isolation of red blood cells (RBCs) and plasma

After the treatment periods, rats were sacrificed under light anaesthesia. Blood samples were collected by cardiac puncture into anticoagulant syringes (rinsed with 10 units/ml heparin), and RBCs were pelleted by centrifugation at 800 x *g* for 10 min at 4 °C. After removal of plasma (immediately frozen at –80 °C until use for biochemical assays), buffy coat, and the upper 15% of packed red blood cells (PRBCs), the RBCs were washed twice with cold phosphate-buffered saline (PBS) (0.9% NaCl and 10 mM Na_2HPO_4, pH 7.4) and then used for experiments. All protocols for experiments had been approved by the Animal Care and Ethics Committee of University of Allahabad.

Measurement of erythrocyte plasma membrane redox system (PMRS) activity

The activity of the erythrocyte PMRS was estimated by following the reduction of ferricyanide

according to Avron and Shavit (1963) modified by Rizvi *et al*. (2006). Briefly, PRBCs (0.2 ml) were suspended in PBS containing 5 mM glucose and 1 mM freshly prepared potassium ferricyanide to a final volume of 2.0 ml. The suspension was incubated for 30 min at 37 °C and then centrifuged at 800 x *g* at 4 °C. The supernatant was assayed for its ferrocyanide content using DPI by measuring the absorption at 535 nm (ε = 20,500 $M^{-1} cm^{-1}$). The results are expressed in μmol ferrocyanide/(ml PRBCs 30 min).

Measurement of antioxidant activity by ferric reducing ability of plasma (FRAP) assay

The total antioxidant potential of the plasma samples was determined using a modification of the FRAP assay of Benzie and Strain (1996). FRAP reagent was prepared by mixing 0.3 M acetate buffer, pH 3.6, 20 mM ferric chloride, and 10 mM TPTZ in 40 mM HCl in the ratio 10:1:1 (v/v/v). Three ml of FRAP reagent were thoroughly mixed with 100 μl of plasma. The absorbance was read at 593 nm at 30-s intervals for 4 min. Aqueous solutions of known Fe^{2+}concentration in the range of 100 to 1000 μmol/l were used for calibration. The FRAP values [μmol Fe(II)/l] of the plasma were calculated using the regression equation.

Determination of erythrocyte malondialdehyde (MDA) concentration

The erythrocyte MDA concentration was measured according to the method of Esterbauer and Cheeseman (1990), with slight modifications. Packed erythrocytes (0.2 ml) were suspended in 3 ml PBS containing 0.5 mM glucose, pH 7.4. The lysate (0.2 ml) was added to 1 ml of 10% trichloroacetic acid (TCA) and 2 ml of 0.67% thiobarbituric acid (TBA), heated for 20 min at 90–100 °C, and cooled. The mixture was centrifuged at 1,000 x *g* for 5 min, and the absorbance of the supernatant was read at 532 nm. The concentration of MDA in erythrocytes was calculated using the extinction coefficient (ε = 31,500 M/cm) and is expressed as nmol/ml of packed erythrocytes.

Determination of erythrocyte reduced glutathione (GSH) concentration

Erythrocyte GSH concentration was measured following the method of Beutler (1984) using Ellman's reagent DTNB. The concentration of GSH is expressed in μmol/ml PRBCs.

Assay of advanced oxidation protein products (AOPP)

Determination of AOPP levels was performed by modification of the method of Witko-Sarsat *et al.* (1996). Two ml of plasma were diluted 1:5 in PBS, 0.1 ml of 1.16 M KI was added, followed 2 min later by 0.2 ml glacial acetic acid. The absorbance of the reaction mixture was immediately read at 340 nm against a blank containing 2 ml of PBS, 0.1 ml of KI, and 0.2 ml of acetic acid. AOPP concentrations are expressed as μmol/l of chloramine-T equivalents.

Statistical analysis

All data are presented as means ± SEM, and statistical analyses were conducted using the software PRISM version 4. Differences among treatments were determined using a *t*-test.

Results and Discussion

Acute exposure of rats to $HgCl_2$ caused a continuous reduction in their body weight over the experimental period of 30 days (Fig. 1), in agreement with the previous report of Klein *et al*. (1972). Weight loss is known to be a basic aspect of mercury toxicity and has been attributed to reduced food intake. Mercury is one of the strongest toxicants present in the environment and plays an active role in intracellular formation of free radicals (Hussain *et al*., 1999). Sublethal doses of $HgCl_2$ have been shown to induce severe oxidative stress (Kim and Sharma, 2005) leading to liver injury (Farina *et al*., 2004).

The ferric reducing ability of plasma (FRAP) assay, developed by Benzie and Strain (1996), is a reliable method to measure the plasma antioxidant potential. $HgCl_2$-treated rats had significantly ($P < 0.01$) lower FRAP values than the control rats (Fig. 2), which is indicative of the oxidative stress condition in these rats. Control rats supplemented with BTE alone exhibited an enhanced FRAP value signifying the antioxidant property of BTE. In rats treated with both $HgCl_2$ and BTE the FRAP value did not differ significantly from that of the control rats. Thus, BTE supplementation provided antioxidant defence in rats from oxidative stress generated after $HgCl_2$ treatment.

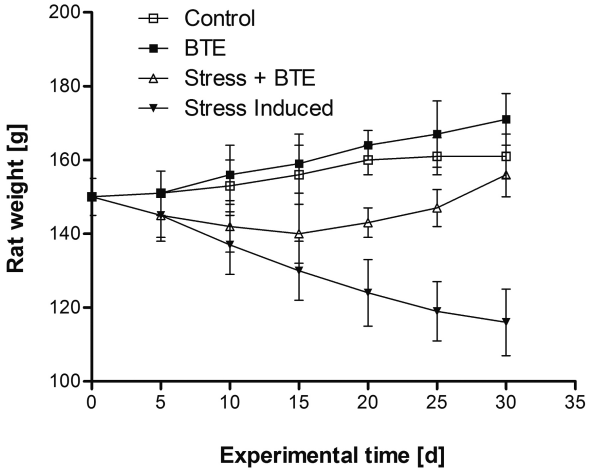

Fig. 1. Body weight of rats subjected to $HgCl_2$ treatment (stress) and black tea extract (BTE) supplementation during the experimental period of 30 days. Each value represents the mean ± SEM of seven rats in each group.

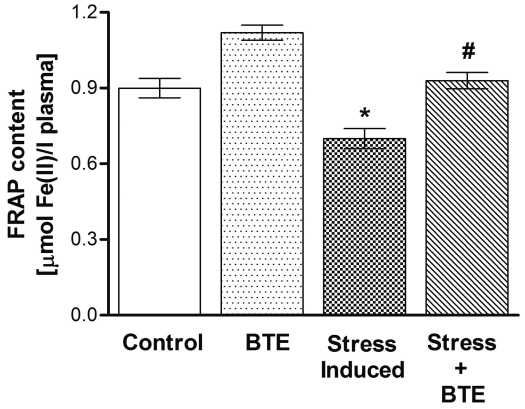

Fig. 2. Antioxidant potential (as FRAP values) of rats administered a single dose of $HgCl_2$ (stress) and/or a daily dose of black tea extract (BTE). *Significantly different ($P < 0.01$) from control (untreated). #Significantly different ($P < 0.01$) from $HgCl_2$.

BTE has been earlier shown to increase the blood serum enzymatic antioxidant capacity, such as superoxide dismutase and catalase, after free radical induction by ethanol, and *in vivo* and *in vitro* studies reported the antioxidant and free radical scavenging activity of BTE (Wang *et al.*, 2011; Luczaj and Skrzydlewska, 2005).

Black tea and green tea extracts are known to retain their antioxidative efficacy when supplemented to humans (Henning *et al.*, 2004). Consumption of tea increases the antioxidant status of a person between 3.5–76% (Yashin *et al.*, 2012). Moreover, a number of studies revealed a significant increase in plasma antioxidant capacity in humans approximately 1 h after consumption of moderate amounts of black tea, *i.e.* 1–6 cups per day (Gupta *et al.*, 2002). Regarding bioavailability, the maximum plasma concentration of theaflavin was 1 ng/ml in volunteers after oral intake of 700 mg theaflavin (equivalent to 30 cups of black tea) (Lee *et al.*, 2004).

Most eukaryotic cells, including erythrocytes, have a plasma membrane redox system (PMRS) which is involved in the regulation of cellular physiological processes and performs several functions, including hormonal signal transduction and protection of cells from oxidative stress. The PMRS transfers electrons from intracellular substrates to extracellular electron acceptors (Hyun *et al.*, 2006a). The PMRS is activated to maintain a balanced NAD^+/NADH ratio which is essential for normal energy metabolism, homeostasis, and for protection against oxidative stress (Rizvi *et al.*, 2006). Here we present evidence that the erythrocyte PMRS activity is increased significantly ($P < 0.001$) in rats subjected to $HgCl_2$ treatment (Fig. 3). The importance of the red blood cell PMRS during conditions resulting in oxidative stress has recently been highlighted (Rizvi *et al.*, 2011). This is the primary defence system to protect cells from exogenous oxidants (Hyun *et al.*, 2006a, b).The PMRS appears to play a protective role during mitochondrial dysfunction to provide cells with a survival mechanism during stress conditions, and during caloric restriction by lowering oxidative stress (Pandey and Rizvi, 2011).

The PMRS activity increases during aging and type 2 diabetes concomitant with a decreased plasma antioxidant potential (Rizvi *et al.*, 2006; Rizvi and Srivastava, 2010). An elevated PMRS activity is thought to act as a protective mechanism against oxidative stress (Rizvi *et al.*, 2006, 2009), thus the increased activity of the erythrocyte PMRS in $HgCl_2$-induced rats is indicative of oxidative stress (Fig. 3). Rats consuming only BTE displayed a significantly ($P < 0.001$) lower activity of the erythrocyte PMRS, demonstrating the improvement of the plasma antioxidant potential after BTE supplementation. To the best of our knowledge, this is the first report of an *in vivo* effect of BTE supplementation on the red blood cell PMRS activity. The level of PMRS reached nearly the basal value in $HgCl_2$-treated and with

BTE supplemented rats, signifying the improvement of antioxidant defence by BTE.

Like other biological membranes, the RBC cell membrane is also prone to lipid peroxidation under oxidative stress that involves cleavage of polyunsaturated fatty acids at their double bonds leading to the formation of malondialdehyde (MDA). MDA can react with the free amino groups of proteins, phospholipids, and nucleic acids leading to structural modifications which can induce dysfunction of the immune system (Kingsley *et al*., 2009). An increased level of MDA in erythrocytes is found in many disease conditions that are accompanied by oxidative stress (Pandey and Rizvi, 2010). MDA may react with haemoglobin via a redox reaction to form superoxide, hydrogen peroxide, free radical intermediates, and oxidized products of haemoglobin (D'Agnillo and Alayash, 2001). An increased membrane lipid peroxidation has been reported in diabetes, atherosclerosis, liver disease, and inflammation (Lykkesfeldt, 2007). In our *in vivo* experimental system, induction of oxidative stress by $HgCl_2$ caused a significant ($P < 0.01$) increase in the MDA level above the basal value (Fig. 4), which conforms with a comparable previous *in vitro* study (Durak *et al*., 2010). Administration of BTE significantly ($P < 0.01$) protected the erythrocytes from $HgCl_2$-induced oxidative stress, as evidenced by a decrease in the MDA level in BTE-supplemented rats (Fig. 4). Treatment with BTE alone led to a significant ($P < 0.01$) decrease in the lipid peroxidation level compared to the control, thus substantiating the antioxidant effect of black tea compounds.

Proteins are especially vulnerable to oxidative stress; the attack of reactive oxygen species on proteins results in amino acid modifications, which can serve as an early marker for oxidative protein damage and are used in the quantification of oxidative protein damage (Levine *et al*., 1990). Oxidants, mainly hypochlorous acid and chloramines, produced by myeloperoxidase in activated neutrophiles, form dityrosine-crosslinked protein products known as AOPPs and are considered reliable markers for estimating the degree of protein oxidative modification (Witko-Sarsat *et al*., 1996). Accumulation of oxidized protein products is associated with a number of diseases, including amyotrophic lateral sclerosis, Alzheimer's disease, respiratory distress syndrome, muscular dystrophy, and rheumatoid arthritis (Berlett and Stadtman, 1997). The level of oxidized protein is determined by the rates of both protein oxidation and oxidized protein degradation (Kayali *et al*., 2007).

We observed a significant ($P < 0.001$) increase in the AOPP concentration in the plasma of

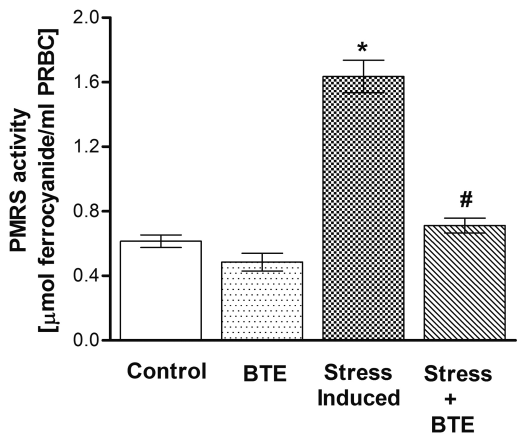

Fig. 3. Plasma membrane redox system (PMRS) activity of rats administered a single dose of $HgCl_2$ (stress) and/or a daily dose of black tea extract (BTE). *Significantly different ($P < 0.001$) from control (untreated). #Significantly different ($P < 0.001$) from $HgCl_2$.

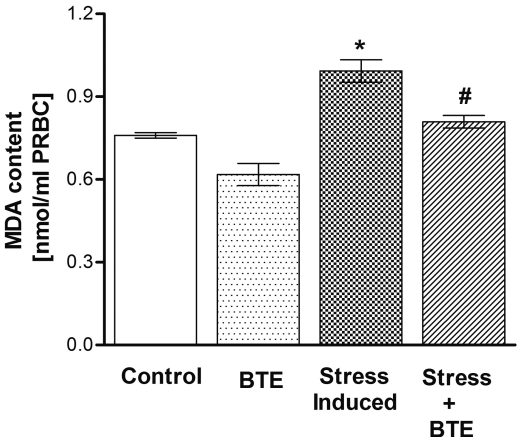

Fig. 4. Erythrocyte malondialdehyde (MDA) concentration of rats administered a single dose of $HgCl_2$ (stress) and/or a daily dose of black tea extract (BTE). *Significantly different ($P < 0.01$) from control (untreated). #Significantly different ($P < 0.01$) from $HgCl_2$. BTE alone-treated groups showed a significant ($P < 0.01$) decrease in MDA level compared to the control.

$HgCl_2$-treated rats, and BTE supplementation significantly ($P < 0.001$) protected against plasma protein oxidation (Fig. 5). We hypothesize that BTE may provide protection against oxidation-induced damage also to membrane proteins under conditions that challenge the body's redox status. Protection from protein oxidation by BTE is another indicator of the strong antioxidant effect of BTE. Oxidative damage may lead to a loss of specific protein functions.

Reduced glutathione (GSH) is the major intracellular nonprotein thiol compound which plays a major role in the protection of cell and tissue structures from oxidative injury (Zhu *et al*., 2006). Induction of oxidative stress in rats by $HgCl_2$ caused significant ($P < 0.001$) depletion of erythrocyte intracellular GSH (Fig. 6), similar to observations by Rossignol and Frye (2011). The decrease in intracellular GSH could be due to both oxidative stress and binding of mercury to glutathione and subsequent elimination of intracellular glutathione (Miura *et al*., 1995). BTE supplementation moderately, but significantly, counteracted the loss/decrease of GSH induced by $HgCl_2$ (Fig. 6). BTE treatment alone also caused an increment in the erythrocyte GSH level, further underlining the antioxidant potential of BTE. Oxidative stress resistance of many cells is associated with high intracellular levels of GSH. Glutathione deficiency has been reported to be related to many diseases such as Kwashiorkor, Alzheimer's disease, Parkinson disease, liver disease, cystic fibrosis, sickle cell anemia, AIDS, cancer, CHD, stroke, and diabetes (Wu *et al.*, 2004).

Conclusion

This *in vivo* study demonstrates the protective effect of BTE against $HgCl_2$-induced oxidative damage in rats and supports the effect of BTE on the antioxidant defence system of cells as suggested by the modulation of the PMRS activity. The results signify that black tea intake may contribute towards a significant health-promoting effect in humans especially in conditions which challenge the antioxidant defence of the body. While BTE polyphenols appear to be responsible for this protective effect, one should note that some of the health benefits of black tea may be attributed to a mixture of polyphenols in tea rather than individual monomeric compounds (Widlansky *et al*., 2005). In view of the high consumption of black tea in several parts of the world and the promising results obtained with BTE *in vivo*, further studies are required to investigate the bioavailability and metabolism of black tea polyphenols in humans.

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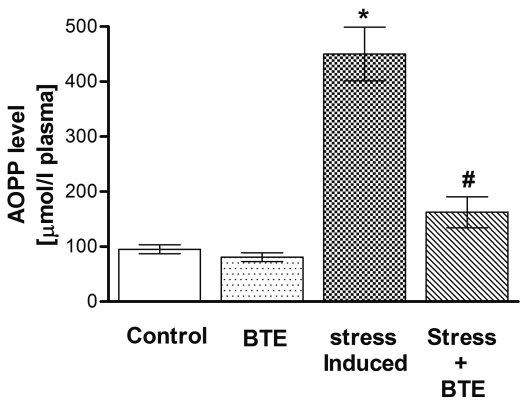

Fig. 5. Advanced oxidation protein products (AOPP) level of rats administered a single dose of $HgCl_2$ (stress) and/or a daily dose of black tea extract (BTE). *Significantly different ($P < 0.001$) from control (untreated). #Significantly different ($P < 0.001$) from $HgCl_2$.

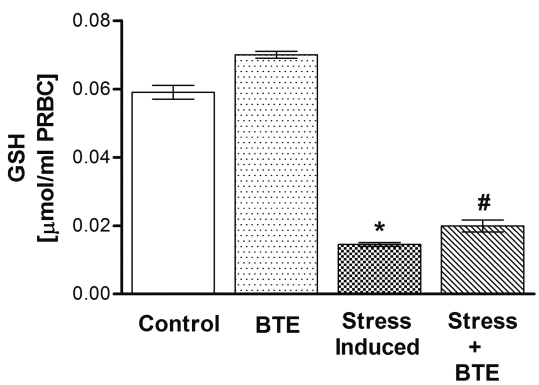

Fig. 6. Erythrocyte reduced glutathione (GSH) concentration of rats administered a single dose of $HgCl_2$ (stress) and/or a daily dose of black tea extract (BTE). *Significantly different ($P < 0.001$) from control (untreated). #Significantly different ($P < 0.01$) from $HgCl_2$.

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