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Protective effect of tea against copper (Cu) toxicity in erythrocytes

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Abstract: Oxidative damage has been implicated in the reduced survival of erythrocytes during copper toxicity. Tea is particularly rich in flavonoids including catechins and theaflavins that are known to possess a well-established protective effect against oxidative damages. This study was designed to compare the efficacies of green tea (GT) and black tea (BT) towards prevention of copper-induced reduced survival of erythrocytes in infant rats. In BT, total catechin concentration was significantly lower than in GT. GT catechins were oxidized and epimerized during the manufacture of BT to form theaflavins. Tea samples were administered to the infant rats exposed to copper. Animals received teas during the last 10 days of copper exposure that was continued for 20 days. Results showed that both GT and BT samples restrained copper-induced morphological alterations of erythrocytes to a similar extent. Potency of both tea samples were at par in inhibiting the oxidation of membrane lipids and preserving antioxidant reserve in erythrocytes during copper exposure. Feeding with GT and BT demonstrated comparable efficacy in resisting untimely hemolysis in copper-exposed animals. In conclusion, these findings emphasize on the equal contribution of GT and BT in preventing copper-induced reduced survival of erythrocytes.

Keywords: *Green tea; Black tea; Copper, Cu; Erythrocytes; Reduced survival; Rat*

Introduction

The beneficial effect of the consumption of tea (*Camellia sinensis*) has emerged out to be a subject of growing interest throughout the world. Salutary health effects of tea depend largely on its antioxidant potential and free-radical scavenging properties, which are associated with its constituent flavonoids like catechins, flavonols and theaflavins.[1–3] Although there are various kinds of teas, essentially all are produced from the same species of plant "*Camellia sinensis*".[4] Green tea (GT) is favoured in East Asian countries such as Japan and China, whereas black tea (BT) is more popular in Western countries and India. Until recently, GT, the non-fermented tea was considered to be more effective than the highly fermented BT as an antioxidant. This was thought to be due to conversion of monomeric catechins in GT to dimeric theaflavins in BT by enzymatic oxidation (fermentation) during the manufacturing process. Explanation for this was attributed to the decrease of catechin content, which constitutes about 80–90% of total flavonoids in GT to 20–50% in BT, in consequence of fermentation process.[5,6] Further studies indicated that theaflavins counterbalance the lack of catechins in BT and the conversion of catechins to theaflavins during fermentation process does not alter their free-radical scavenging property.[7] It has been demonstrated that both GT and BT are equally potent in imparting beneficial effects such as inhibition of low-density lipoprotein (LDL) oxidation, improvement of endothelial function and prevention of cardiovascular diseases.[6–8]

Copper (Cu) is essential for mammalian nutrition in trace amounts. It functions as catalyst for several metalloenzymes associated with biochemical and physiological processes.[9] However, exposure to high levels of copper over a prolonged period can result in chronic copper toxicosis. This situation is characterized by gradual hepatic accumulation of copper consequent to disruption in copper trafficking system.[10,11] The toxicity remains sub-clinical till copper is stored in the liver. Eventually it is released in massive amounts causing sudden increase in blood copper concentration, followed by intra-vascular hemolysis.[12] Young children are more susceptible to copper poisoning than their adult counterparts due to their immature biliary excretory system. This leads to the development of copper-induced cirrhosis in young children known as, "Indian childhood cirrhosis".[13,14] Copper toxicity is closely associated with oxidative

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stress which develops in consequence of the release of unbound ionic copper through redox cycling, promoting the generation of reactive oxygen species (ROS).[11, 15–17] D-penicillamine, a slow acting chelator, is currently used as first line drug in the treatment of copper toxicosis. Although short-term use of this drug does not cause any major side-effect, long-term use results in adverse effects including pancytopenia, gastrointestinal disturbances, proteinurea, etc.[18,19] Therapeutic approaches with both enzymatic and non-enzymatic antioxidants failed to show appreciable response due to various complications and contradictory results.[20,21] Worldwide use of tea in ameliorating chronic diseases and a recent report on the potency of tea against copper-mediated LDL oxidation[7] prompted us to study its role in the management of copper-induced hemolytic outcome. Present paper depicts comparative effects of GT and BT in combating redox imbalance, which eventually prevents untimely death of erythrocytes during chronic exposure to cupper in rats.

Materials and Methods

Chemicals

Radioactive sodium chromate (labeled with ^{51}Cr; sp act 94.2 Ci/g) was procured from Board of Radiation and Isotope Technology (Mumbai, India). Alanine aminotransferase (ALT) measurement kits were obtained from TECO Diagnostics (Anaheim, CA, USA). Blood glucose, urea, creatinine measurement kits were obtained from Cayman Chemicals (Michigan, USA). 2′,7′-dichlorofluorescein diacetate (DCFDA) was obtained from Invitrogen Molecular Probes (Eugene, OR). All other fine chemicals (unless mentioned) were purchased from Sigma-Aldrich Corporation.

Analysis of GT and BT Component by High-Performance Liquid Chromatographic (HPLC)

GT and BT (Tocklai Vegetative clone TV 25 *Camellia sinensis*, var. *assamica*) aqueous infusions were used as the source of catechins and theaflavins for HPLC analysis. Each infusion was prepared by adding 25 ml boiling milli-Q™ water on 1 g tea and brewing for 5 min. After brewing, aqueous infusions were centrifuged at 8,000 × *g* for 10 min. The supernatant were filtered through 0.45-μm membrane filter (Pall Gelman Laboratory, South Wagner road, Ann arbor, USA) before analysis on HPLC. HPLC analysis was done by the modified method of Yao et al.,[22] using gradient elution system. The analyses were performed using an HPLC system – HPLC Waters 600 Separation Module (quaternary solvent delivery pumps, in-line degasser), with a Photo Diodes Array (PDA) detector (Model Waters 2998) and the results were processed by Empower Waters Software (Waters Corporation, Singapore). Separations were performed on an Xterra RP C18 5-μm (250 mm × 4.6 mm i.d.) column. The mobile phase consisted of acetonitrile (B) and 0.2% acetic acid in water (A) and each solvent were filtered through a 0.45-μm pore size hydrophilic polyethersulfone filter (Pall Gelman Laboratory, South Wagner road, Ann arbor, USA). The composition of mobile phase was initially set at 8% of acetonitrile (B) and 92% of 0.2% acetic acid in water (A). Solvent B then gradually increased to 31% at 25 min, to 40% at 35 min, to 100% at 37 min and hold for 3 min. The Photodiode Array Detector (PAD) was set at 200–600 nm wavelength and the chromatogram was detected at 274 nm. The separations were performed at room temperature with a 1 ml min^{-1} flow rate and the injection volume was 20 μl. Authentic standards of gallic acid, catechin (C), epicatechin (EC), epigallocatechin (EGC), epigallocatechin gallate (EGCG), gallocatechin gallate (GCG), epicatechin gallate (ECG), theaflavin (TF), theaflavin 3 monogallate (TF3MG), theaflavin 3′ monogallate (TF3′MG) and theaflavin 3,3′ monogallate (TF33′MG) were used to identify peaks and calculate the concentration of components in tea samples. Each peak was confirmed by comparing the retention times and absorption spectra of unknowns to those of standard compounds. Total thearubigin and theaflavin content were measured spectrophotometrically by the method described by Roberts and Smith.[23]

Analysis of Volatile Flavour Compounds by GC-MS from GT and BT Samples

Volatile flavour compounds were extracted from tea samples by dynamic headspace sampling techniques.[24] For each extraction, 20 g of tea samples were kept into 250 ml of round bottom flask attached with two open arm of 8-mm diameter. One arm was connected with a charcoal filter, through which environment air can enters into the flask. Another arm was connected with an 8-mm diameter column containing an adsorbent bed. In this system, external air was pulled over the tea sample through the adsorbent bed that was connected to a vacuum pump, at a particular flow rate. The air containing the volatiles compounds were trapped by the adsorbent bed. The extraction was carried out at 50°C for 2 hr. Trapped volatiles were eluted from the adsorbing matrix into glass vial with 200 μl of HPLC grade dichloromethane containing

1 µl of ethyl hexanoate as an internal standard.

The both GT and BT extracts were analysed on a Shimadzu QP2010 GC-MS system. A ZB-5 column (30 m × 0.25 mm i.d., film thickness 0.25 µm) was used for the separation of volatile compounds with helium as a carrier gas and the results were processed by GCMS solution (ver. 2.6 software). The injection volume was 2 µl with split ratio 2:1. The injector temperature was set at 260°C. The column oven temperature of GC initially was set at 50°C for 2 min, then increase to 60°C at a rate of 2°C min^{-1} and hold for 2 min, again raised to 210°C at 3°C min^{-1} and hold for 2 min, finally to 270°C at 10°C min^{-1} and hold for 7 min. Column flow rate was 1 ml min^{-1}. The conditions for the operation of mass spectrometer were set as follows: ion source temperature, 200°C; interface temperature, 280°C; electron energy, 70 eV; scanning range of *m/z*, 40–600 a.m.u. The compounds were identified by comparing mass spectrum of the component to that of mass spectral library from NIST 05 (National Institute of Standards and Technology, Gaithersburg, USA) and Wiley 8.0 (Wiley, New York, USA). The area (micro Volt unit) was calculated from TIC (Total ion chromatogram) automated computerized integrator.

Preparation of GT and BT Solids

Decaffeinated GT and BT solids were prepared in our laboratory by brewing either 25 g of GT or BT in 1 L boiling water. The tea was filtered (Whatman No. 1 filter), followed by evaporation of the clear liquid using Rotavapor R-114 and the freeze-dry system lyophilizer. Caffeine was extracted from the tea brew using an equal volume of chloroform. The water phase was evaporated as described above for the production of decaffeinated BT and GT solids.[25] Decaffeinated teas were prepared every other day and stored in the refrigerator.

Animals

Female Sprauge–Dawley 25 day old infant rats (45–48 gm) were bred and housed in the animal house of Indian Institute of Chemical Biology, Kolkata. All animal studies were performed following the mandates approved by Animal Ethics Committee (Committee for the purpose of control and supervisions of experiments of animals, Govt. of India). They were housed in a room on a 12-hr light/dark cycle with a free access to food and water *ad libitum*. Rat pups were kept in cages with their littermates. Animals were grouped according to drug treatment with 6 animals in each group.

Animal Treatment

Copper sulphate ($CuSO_4$) was dissolved in 0.9% NaCl and administered everyday by feeding ryles tubes in doses ranging from 5 to 15 mg kg^{-1} body weight day^{-1} for 30 days for assessing their mean survival time against copper exposure. For all other experiments, animals were given $CuSO_4$ at a dose of 10 mg kg^{-1} for 20 days. Selection of this dose for the development of copper toxicity was based on an integrated overview of the physiologic and pathologic responses associated with range of copper intake. This is also in well accordance with the daily intake of greater than 5 mg kg^{-1} body weight day^{-1} as suggested by Aggett,[26] for development of copper toxicity. The treatment groups were fed different doses of decaffeinated GT and BT (50–100 mg kg^{-1} body wt day^{-1}) after 10 days of $CuSO_4$ exposure and continued till the last day of $CuSO_4$ exposure in the respective groups. Rats in the control group received equivalent level of 0.9% NaCl during the same days. Animals were sacrificed 24 hr after the last dose of exposure.

Biochemical Analysis

The activity of serum ALT was analysed by using a Merck Clinical Chemistry Autoanalyzer using commercial test reagents (Teco Diagnostics, California, USA).

Haemoglobin (Hb) was measured using hemoglobinometer. Blood glucose, urea, creatinine were measured according to method provided by commercially available kits (Cayman Chemicals, Michigan, USA).

Isolation of Erythrocytes and Preparation of Red Blood Cell Membrane

Blood was collected from the experimental animals in heparinized tubes. Plasma and buffy coat were separated from erythrocytes by centrifugation at 900 × *g* for 10 min. The packed erythrocytes were then washed three times at 900 × *g* for 5 min with isotonic PBS, pH 7.4. Packed erythrocytes were hemolyzed in hypotonic phosphate buffer (5 mM), pH 7.4. It was centrifuged at 27,000 × *g* for 30 min at 0–4°C. This washing process was repeated until the red blood cell (RBC) membrane obtained in the pellet was almost free of haemoglobin.[27] Protein concentration was determined using bovine serum albumin (BSA) as the standard.[28]

Measurement of Hemolysis

Haemoglobin (Hb) leakage into the extracellular fluid or hemolysis was determined by measuring the extent of hemolysis of a 50% erythrocyte suspension in nor-

mal saline (0.9% NaCl), pH 7.4, in comparison to that of the same in distilled water. The extent of hemolysis was determined spectrophotometrically at 540 nm after 30 min incubation at room temperature[29] and expressed as percentage of the total Hb.

Estimation of Copper

In order to determine copper levels in erythrocytes and plasma, we followed a modified method of Odabasi *et al.*[30] Blood samples were drawn from infant rat using Na_2-EDTA as anti-coagulant. Samples were centrifuged for 10 min at 1,000 × *g* at 4°C. Plasma and erythrocytes were then separated. The erythrocytes were washed with 0.9% NaCl three times and hemolysed with cold distilled water. Plasma and erythrocyte lysate were stored at –70°C until the analysis. To determine total urinary copper excretion for 10 days, urine was collected throughout 24 hr for the said period and centrifuged at 2,300 × *g* for 10 min, and 1.0 ml portions of the supernatant was wet-digested with 1.0 ml of mixed acid ($HClO_4$: HNO_3 = 1 : 5, *v/v*). Fecal matters were collected for 24 hr and was dried at 80°C for 24 hr, and approximately 0.2 g portions were wet-digested with 2.0 ml of the mixed acid. The acid-digested samples were diluted to 5 ml with double-distilled water.[31,32] Plasma, erythrocyte, urine and fecal Cu^{2+} levels were measured by atomic absorption spectrophotometry (flame atomization procedure).

Study of Cellular Morphology

Packed erythrocytes were fixed in 2% glutaraldehyde dissolved in phosphate buffer saline (PBS) at pH 7.4 in a polyethylene tube. The erythrocytes were gently inverted and allowed to settle for 2 hr. Samples were then pipetted to collagen-coated cover slips in a moist petri dish. Cells were then dehydrated through ascending ethanol solutions, processed through critical point, drying in liquid carbon dioxide and coated with gold-palladium. Coated samples were examined in Jeol Scanning Electron Microscope (Model JSM 5200, Tokyo, Japan) at 20 kV. Total 200–1200 cells were counted for each sample. Erythrocyte shapes were compared between various groups.[33]

Measurement of Lipid Peroxidation

Fresh erythrocytes were centrifuged and suspended in PBS. The erythrocyte membrane was prepared by the method of Dodge *et al.*[27] Concentration of thiobarbituric acid reactive substance (TBARS) was measured by the spectrophotometric method based on the reaction of lipid peroxides with thiobarbituric acid (TBA). Measurements were done at 532 nm, using malonaldehyde bis (Bimethyl Acetal) as standard.[34] Quantitation was based upon the molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} and the TBARS content was expressed in nmole per mg protein.

Measurement of Intracellular ROS

The intracellular ROS content of erythrocytes was measured immediately after isolation by incubating with fluorescent probes dihydroethidium (DHE) and DCFDA, followed by the measurement of the oxidation of DHE to ethidium by superoxide anion ($O_2^{\bullet-}$) and DCFDA to dichlorofluorescein (DCF) by hydrogen peroxide (H_2O_2), respectively.[35, 36] Briefly, erythrocytes (5% hematocrit) were incubated in PBS, pH 7.4, in the presence of 10 µM DCFDA and 5 µM DHE for 30 min at 37°C. Cells were then quickly washed twice with PBS and subjected to flow cytometry (FACS Calibur from BD Biosciences, Mountain View, CA, USA). Samples were analysed with an excitation wavelength of 488 nm and emission wavelengths of 585 nm for $O_2^{\bullet-}$ and 525 nm for H_2O_2, respectively.

Catalase Activity Determination

Catalase activity was measured in supernatant after centrifugation of erythrocyte lysate at 27,000 × *g* for 30 min at 0–4°C Catalase activity was estimated through the breakdown of H_2O_2, followed spectrophotometrically at 240 nm.[37]

Determination of Redox Potential of Erythrocytes

Redox potential in RBC was determined from the ratios of pyridine nucleotides. Redox potential was assayed from NADH/ [NAD+ +NADH] and NADPH/ [NADP+ +NADPH] ratios.[33]

Assessment of Reduced Glutathione (GSH) and Oxidized Glutathione (GSSG)

Blood samples were collected in heparin-containing vacutainers and were cooled immediately. Plasma was separated within 1 hr of blood collection by centrifugation (800 × *g*) at 4°C. GSH present in plasma was measured according to the method of Tietze.[38] Briefly, isolated plasma was first deproteinized with 35% metaphosphoric acid. Acid supernatant was then neutralized with 0.3 M Na_2HPO_4. Total GSH of plasma was determined, by adding 0.6 mM DTNB, 0.5 U of glutathione reductase

(GR) and 0.2 mM NADPH with supernatant. The formation of GSH–DTNB conjugate was then measured at 412 nm. Plasma GSSG level was determined enzymatically.[39] Then, 0.5 ml of 0.25 M N-ethyl maleimide (NEM) was added to alkylate reduced GSH in order to prevent the oxidation of this tripeptide. Further, 2 ml of ice-cold 30% (*w/v*) trichloroacetic acid (TCA) was then added as precipitating agent, followed by centrifugation at 1000 × *g* for 15 min. Clear supernatant was extracted thrice with ice-cold ether to remove TCA and NEM. Excess ether was removed with a stream of nitrogen gas. GSSG was assayed by following its reduction to GSH catalyzed by using GR. Estimation was done from the oxidation of NADPH at 340 nm. Concentration of GSH was determined by subtracting GSSG from total glutathione value in GSH equivalents.

Measurement of Life Span of Red Blood Cells

Red blood cell survival was measured from the half-life of erythrocyte over time according to standard method.[40, 41] Briefly, [^{51}Cr]-labeled sodium chromate, (specific activity 1.27 × 10^3) was injected into rat *via* the cavernous vein at the dose of 20 μCi kg^{-1}. Blood was collected from rat, and volumes of erythrocytes were calculated from blood volume. Erythrocytes were washed in 20 mM HEPES-Tris (pH 7.4) and then lysed by adding distilled water. The radioactivity of lysates was measured using a gamma-counter (K2700B/ECIL). Radioactivity present in the red cells was calculated. The count obtained on the first day of injection was taken as 100% value. The radioactivity present in each sample on any subsequent day is related to this initial 100% value as percent of initial activity. The day at which 50% radioactivity disappeared was termed as $t^{1/2}$.

Statistical Analysis

All data were given as mean ± SD. Differences between two groups were compared by unpaired Student's *t*-test. For multigroup comparisons, analysis of variance was determined by ANOVA followed by Student–Newman–Keuls test. Differences between the means with a *P* value of less than 5% ($P < 0.05$) were considered to be statistically significant. The statistical analysis was done by using GraphPad Instant Software (Graph-Pad, La Jolla, CA, USA).

Results

Polyphenols in GT and BT

The concentrations of catechins and theaflavins in both GT and BT preparations were measured using HPLC. As shown in Table 1, contents of catechin monomers, particularly EGCG and EGC were present in much higher amount in GT in comparison to their respective counterparts in BT. The overall catechin concentration in GT accounted for 8.01 g/100 g dry leaves that decreased to only 1.07 g/100 g dry leaves in BT. Meanwhile, four kinds of theaflavin monomers in BT were not found in

Table 1: Concentration of Main Components of Tea Infusions Analysed in HPLC

Compounds	GT (mg g^{-1} dry weight tea)	BT (mg g^{-1} dry weight tea)
Gallic acid	2.59 ± 0.16	2.21 ± 0.15
C	1.72 ± 0.15	ND
EC	9.02 ± 0.48	4.26 ± 0.14
EGC	30.29 ± 1.49	ND
ECG	8.23 ± 0.34	2.18 ± 0.18
GCG	2.44 ± 0.15	1.62 ± 0.14
EGCG	30.09 ± 1.12	2.65 ± 0.14
TF	ND	0.25 ± 0.02
TF3MG	ND	0.56 ± 0.03
TF3′MG	ND	0.19 ± 0.01
TF33′MG	ND	0.49 ± 0.03

Note: Concentration is represented at mg g^{-1} dry weight tea. All data are expressed as mean ± SD. Results are representative of three independent experiments. C= catechin, EC= (–) epicatechin, EGC= (–) epigallocatechin, ECG= (–) epicatechin gallate, GCG= (–) gallocatechin gallate, EGCG= (–) epigallocatechin gallate, TF= theaflavin, TF3MG= theaflavin-3-monogallate, TF3′ MG= theaflavin-3′-monogallate and TF33′ DG= theaflavin-3,3′-digallate. ND = not detectable.

Table 2: Identification of Volatile Flavour Compounds in GT and BT

Compounds	Green tea (GT)	Black tea (BT)
Myrcene	0.09	ND
Ocimene	0.01	ND
Limonene	0.09	ND
5-Methyl-5,6-dihydro-2(^{1}H)-pyridione	ND	0.11
α-Pinene	0.04	ND
Linalool oxide I (trans, furanoid)	0.03	0.11
Linalool oxide II (cis, furanoid)	0.1	0.18
Linalool	0.28	0.36
1-Ethyl-2,5-pyrrolidinedione	0.05	0.3
^{4}H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	ND	0.14
Linalool oxide III (trans, pyranoid)	ND	0.03
Linalool oxide IV (cis, pyranoid)	ND	0.18
Camphor	0.06	ND
1-Methoxyadamantane	0.2	0.13
Methyl salicylate	0.09	0.23
Decanal	0.02	0.04
Phenylethanol acetate	ND	0.21
Geraniol	ND	0.06
β-Cyclocitral	0.02	ND
2,6-Octadien-1-ol-2,7-dimethyl	0.05	ND
4,8-Dimethyl-1,7-nonadien-4-ol	ND	0.12
Indole	0.08	0.21
Naphthalene	0.12	0.24
α-Ionone	0.03	0.06
Geranylacetone	0.05	0.08
β-Ionone	0.11	0.23
Farnesene	0.03	0.05
Benzofuranone	0.05	0.12
Nerolidol	0.08	0.08

Note: Values are represented as ratio of peak area to that of internal standard. ND = not detectable.

GT. Total theaflavin content in BT was 0.15 g/100 g dry leaves. TF3MG was most abundant (0.06%) followed by TF33′DG (0.05%), TF (0.03%) and TF3′MG (0.02%). Oligomeric catechin thearubigin content in BT was 9.17% (data not shown). Volatile flavour compounds extracted from both GT and BT were analysed on a GC-MS system. Peak areas of compound identified in the mass spectrum obtained from GT and BT are shown in Table 2.

Effect of GT and BT on Copper-induced Hemolysis

A model for chronic copper toxicity in infant rats under *in vivo* conditions was set up by exposing the animals to increasing concentrations of copper (5–15 mg kg^{-1} body wt) and monitoring their survival at different time periods. As evident from Figure 1(A), exposure to copper beyond 20 days affected the mean survival of all the

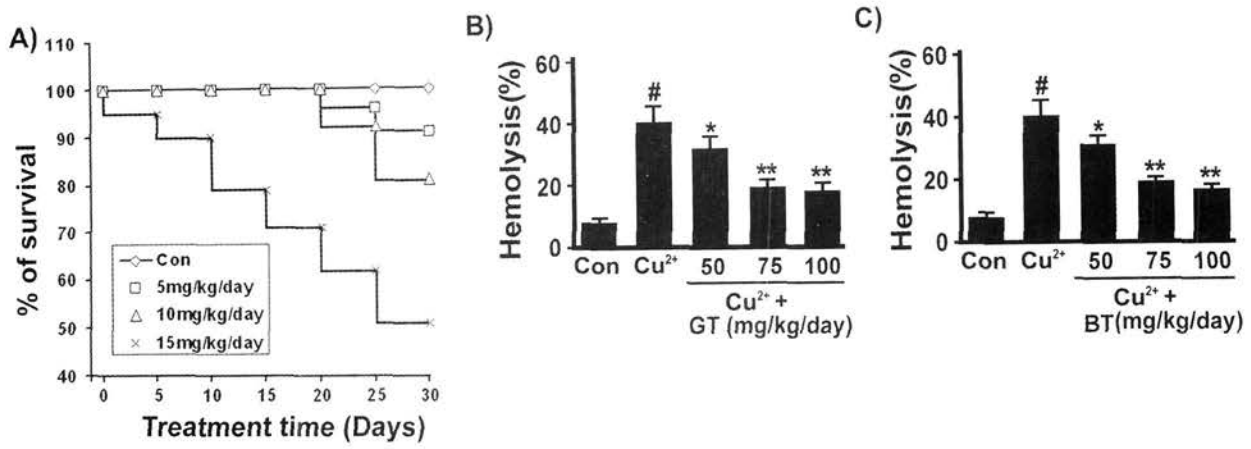

Figure. 1. (A) Survival curve of rats exposed to increasing doses of copper for a period of 30 days. Results are representative of three independent experiments with five animals in each group. Preventive effect of GT **(B)** and BT **(C)** against hemolysis during 20 days of copper exposure. Results are mean ± SD of three independent experiments with five animals in each group. # $P < 0.01$ vs. respective controls. *P <0.05, ** $P < 0.01$ vs. respective copper-exposed levels without GT or BT. Tea was given to the animals during the last 10 days of copper exposure.

animals. Rats receiving lower doses (5 mg kg^{-1} and 10 mg kg^{-1}) showed 100% survival up to 20 days, whereas animals exposed to copper at 15 mg kg^{-1} could not tolerate the treatment showing 30% drop in their survival after similar period of exposure. This made us select 10 mg kg^{-1} day^{-1} as the exposure level for a period of 20 days towards the development of copper toxicity in our further experiments.

Copper toxicity is associated with oxidative stress which eventually leads to a hemolytic outcome. Here, we tried to find out whether consumption of tea during the last 10 days of exposure period could combat copper-induced hemolysis of erythrocytes. Both GT and BT showed significant prevention in dose-dependent manner, as evident from Figures 1(B) and 1(C), respectively. Treatment with doses above 75mg kg^{-1} day^{-1} failed to exhibit any further prevention, and this made us select this as the optimal dose for subsequent experiments.

Effect of GT and BT on Body Weight and Food Intake of Copper-fed Rats

As shown in Table 3, copper-fed infant rats had a reduced rate of weight gain during the period of exposure in comparison to the control animals of the same age group. Weight gains in the GT- and BT-treated animals were close to that observed in the control rats. This was supported by the food intake of the rats per day. Copper exposed animals took lesser food than control ones, which improved, to the level of normal intake in response to feeding tea.

Toxicity Study in Copper-exposed Rats With and Without Treatment with Tea Extracts

Serum concentration of urea and creatinine as well as the activity of liver marker enzyme ALT increased; whereas hemoglobin and blood glucose levels were decreased in copper exposed rats as compared to control ones. Feeding with GT and BT restrained copper-induced alterations in these parameters, and the values in the treated animals were close to that observed in the control animals. However, neither GT nor BT had any toxic effect on control animals (Table 4).

Copper Deposition and Excretion in Rats

Copper feeding for 20 days led to increased accumulation of copper to a significant extent from the control levels in both erythrocytes (Fig. 2A) and plasma (Fig. 2B). Preparations of tea were capable of inhibiting this

Table 3: Body Weight and Food Intake by Infant Rat

Groups	Body weight (g)	Food intake (g day^{-1})
Con	128.2 ± 20.1	2.36 ± 0.5
Cu^{2+}	94.6 ± 11.8#	1.58 ± 0.3#
Cu^{2+} + GT	119.8 ± 15.1**	2.18 ± 0.4**
Cu^{2+} + BT	122.7 ± 17.2**	2.24 ± 0.5**

Note: All data are expressed as mean ± SD. Results are representative of five independent experiments with six animals in each group. Unpaired *t*-test was applied to determine the statistical significances. #P<0.001 vs. control group. **P <0.01 vs. Cu^{2+} exposed group.

Tabe 4: Toxicity Studies in Control and Cu^{2+} Exposed Infant Rats Before and After GT and BT Treatments

Parameter	Con	Con + GT	Con + BT	Cu^{2+}	Cu^{2+} + GT	Cu^{2+} + BT
Hb (g%)	13.9 ± 2.1	13.6 ± 2.1^{NS}	13.8 ± 2.0^{NS}	11.1± 0.9$^{\#}$	13.4 ± 1.5*	13.5 ± 1.7*
Blood Glucose (mg dl^{-1})	76.2 ± 3.6	75.4 ± 3.5^{NS}	76.0 ± 3.4^{NS}	60.4 ± 4.8$^{\#}$	74.1 ± 3.7*	75.3 ± 3.9*
Urea (mg dl^{-1})	24.6 ± 2.8	25.9 ± 3.2^{NS}	25.4 ± 3.3^{NS}	51.9 ± 8.7$^{\#*}$	27.0 ± 4.1**	26.6 ± 3.5**
Creatinine (mg dl^{-1})	0.39 ± 0.02	0.44 ± 0.03^{NS}	0.43 ± 0.03^{NS}	1.12 ± 0.3$^{\#*}$	0.51 ± 0.1**	0.48 ± 0.1**
ALT (U L^{-1})	28.6 ± 4.2	32.5 ± 5.1^{NS}	31.3 ± 4.1^{NS}	140.5 ± 24.6$^{\#*}$	40.1 ± 5.3**	38.7 ± 5.5**

Note: All data are expressed as mean ± SD. Results are representative of five independent experiments with six animals in each group. Unpaired *t*-test was applied to determine the statistical significances. NS = non significant, #P<0.05, #*P<0.01 vs. control group. *P<0.05, **P<0.01 vs. Cu^{2+}-exposed group.

enhanced copper accumulation, and the extent of inhibition was more or less similar with GT and BT.

Urinary copper excretion got elevated to some extent during copper exposure, which increased further to a much-elevated level following GT or BT treatment (Fig. 2C). Copper exposure was associated with significant increase in its excretion *via* fecal route. However, feeding with GT or BT did not show much enhancement over this level, as evident from Figure 2(D).

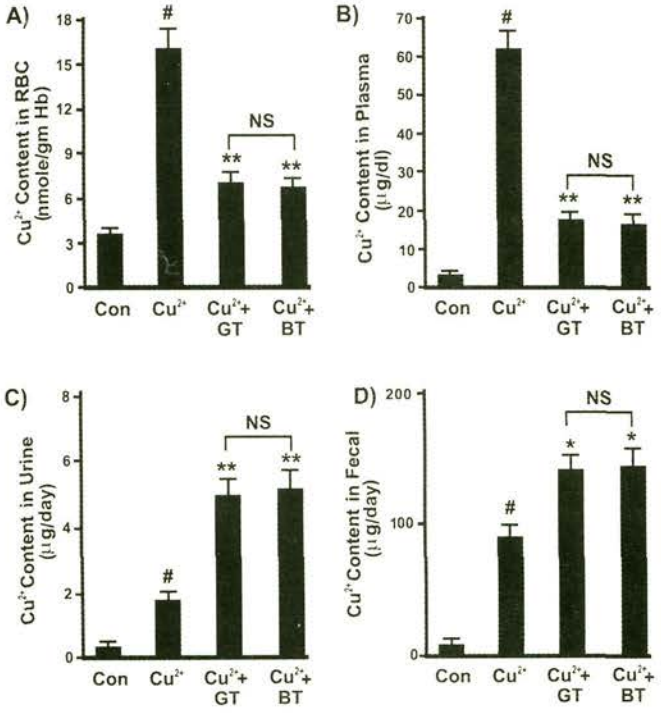

Figure 2. Effect of tea samples on copper levels in erythrocyte, plasma, urine and feces after 20 days of exposure in rats. Deposition of copper in response to 10 day treatment with GT or BT in erythrocytes **(A)** and plasma **(B)**. Effect of GT or BT on the excretion of copper *via* urinary **(C)** and fecal routes **(D)**. Results are mean ± SD of three independent experiments with five animals in each group. # P < 0.01 vs. respective controls. ** P <0.01 vs. respective copper-exposed levels without GT or BT. NS = non-significant vs. Cu^{2+} + GT.

Bioavailability of Catechin and Theaflavin within Erythrocyte

Our next attempt was to determine the bioavailability of tea ingredients within erythrocytes after consumption of tea extracts. Figure 3 depicts total concentrations of catechins and theaflavins observed within RBC after consumption of GT and BT. The results showed that catechin content was three times higher in GT-fed rats as compared to that observed in BT-fed animals. Unlike GT feeding, BT feeding resulted in the presence of theaflavin in erythrocytes (as shown in Fig. 3).

Treatment with Tea Prevents Copper-induced Morphological Alteration of Erythrocytes

Scanning electron micrographs revealed morphological alterations in erythrocytes from copper-exposed rats.

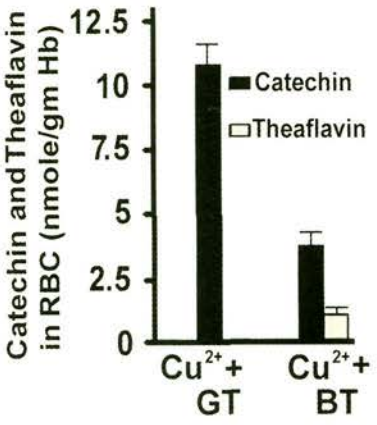

Figure 3. Total concentrations of catechins and theaflavins within erythrocytes after consumption of GT or BT for 10 days in copper-exposed rats. Results are mean ± SD of values obtained from HPLC analysis in three independent experiments with five animals in each group.

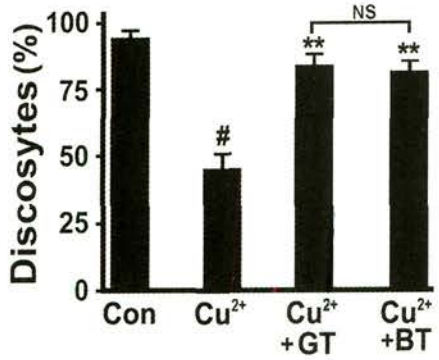

Figure 4. Copper-induced morphologic alteration of erythrocytes with and without feeding of GT or BT. Percentage of discocytes was calculated from the distribution of erythrocytes, as observed under scanning electron microscope. Values are mean ± SD of three separate experiments with five animals in each group. # P <0.01 vs. control group. ** P <0.01 vs. copper-exposed group, not fed with GT or BT. NS = non-significant vs. Cu^{2+} + GT.

Only discocytes were found in the unexposed control samples. Copper exposure led to shape transformation of discocytic cells resulting in a decrease in the percentage of discocytes by approx. 55% from the control level. Feeding with GT or BT helped to maintain normal biconcave disc configurations of the erythrocytes to a great extent, as revealed from the discocyte levels in tea-fed copper exposed rats (Fig. 4).

Effect of GT and BT on Copper-induced Lipid Peroxidation and ROS Generation in Erythrocytes

Considering the involvement of copper in the induction of oxidative stress, our next experiment was to find out the extent of oxidative damage in RBC in presence and absence of GT or BT treatment in copper-exposed rats. Figure 5A shows significant increase in TBARS level in the exposed animals, indicating enhanced peroxidation of RBC membrane lipids after copper exposure. Both of the tea preparations were significantly capable of restricting the formation of TBARS almost to the control level. This result was further supported by the protective effects of GT and BT towards copper-induced ROS generation in erythrocytes.

This was measured using the cell permeable oxidation sensitive dyes DCFDA and DHE in a flow cytometer. These dyes are oxidized by ROS into fluorescent products, DCF and ethidium, respectively. In the dot plot analysis (Fig. 5B), cells located in the upper-left quadrant exhibited only DCF fluorescence lower-right quadrant corresponded to ethidium fluorescence, while those in the upper right quadrant exhibited both DCF and ethidium fluorescence. About 3.2% of the cells from the control group exhibited fluorescence, indicating a basal level of total ROS production. Copper exposure increased

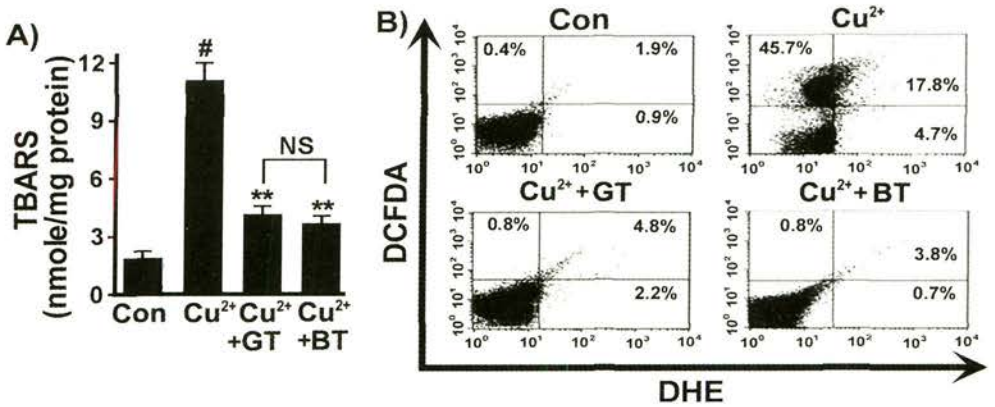

Figure 5. Effect of tea samples on copper-mediated oxidative stress and ROS generation in erythrocytes. **(A)** Inhibitory effect of GT or BT on lipid peroxidation of erythrocyte membrane from copper-exposed rats. Values are mean ± SD of three separate experiments with five animals in each group. # P <0.01 vs. control group. ** P <0.01 copper-exposed group, not fed with GT or BT. NS = non-significant vs. Cu^{2+} + GT. **(B)** Presence of H_2O_2 and $O_2^{\bullet-}$ was determined flow cytrometrically from dot plot analysis showing oxidation of DCFDA and DHE, respectively. The vertical axis represents labeling with DCFDA and horizontal axis with DHE. Accordingly, distributions of cells in different quadrants of the dot plot are as follows: lower left = control cell population with no fluorescence; upper left = DCFDA bound cells; lower right = DHE bound cells; upper right = both DCFDA and DHE bound cells. Results are representative of three independent experiments with five animals in each group.

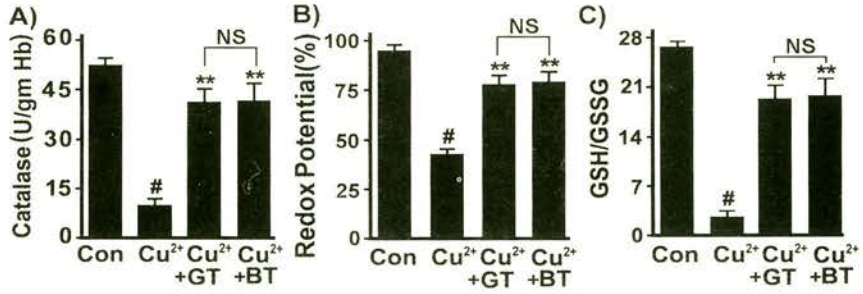

Figure 6. Antioxidant status in erythrocytes of copper-exposed rats in response to administration of GT or BT. **(A)** Redox potential, **(B)** GSH / GSSG ratio, **(C)** catalase activity in erythrocytes of different groups of animals. Values are mean ± SD of three separate experiments with five animals in each group. $^{\#}P < 0.01$ vs. respective controls. $^{**}P < 0.01$ vs. respective copper-exposed levels without GT or BT. NS = non-significant vs. Cu^{2+} + GT.

the percentage to 68.2%, as evident from Figure 5(B). Maximum increase was observed in the fluorescence of DCF indicating oxidation of DCFDA by H_2O_2. Extent of increase in ethidium fluorescent cells was much less, indicating lesser increase in $O_2^{\bullet -}$ levels compared to H_2O_2 in these cells. Treatment with GT or BT attenuated total ROS generation and decreased the levels to 7.8% and 5.3%, respectively.

Antioxidant Status in the Erythrocytes of Copper-exposed Rats in Response to Feeding With GT and BT

Preventive role of tea on copper-induced oxidative stress in erythrocytes was further supported by the effect of GT and BT on the status of the cellular antioxidant system. This was assessed by measuring redox potential, GSH/GSSG ratio and catalase activity in RBC. As evident from Figure 6(A), there was a reduced level of redox potential after copper exposure, and feeding with GT or BT was capable of increasing it significantly towards the control level. Figure 6B indicated oxidation of GSH to GSSG after copper exposure. Copper-catalyzed oxidation of GSH was markedly reduced in GT- or BT-treated erythrocytes, as evident from GSG/GSSG ratio within erythrocytes. Copper exposure led to down-regulation of catalase activity in RBC, whereas treatment with GT or BT successfully checked the decline (Fig. 6C).

Effect of GT and BT on Copper-induced Reduced Survival of Erythrocytes

Figure 7 demonstrates inhibitory role of GT and BT on the copper-induced destruction of erythrocytes. Measurement of RBC life span following decay of Cr^{51} over time provided is evident for the increased survivability of erythrocytes in response to treatment with both tea preparations. Reduced $t^{1/2}$ level (representing half-life of erythrocytes) in the exposed animals increased significantly in response to treatment with GT and BT (inset of Fig. 7), suggesting it to be an effective measure against enhanced erythrocyte destruction during copper exposure.

Discussion

Tea is one of the most widely consumed beverages in the world and the present study was undertaken to compare the effects of GT and BT on reduced survival of erythrocytes during copper toxicity. Although copper is considered as one of the essential micronutrient trace elements, high intake of this metal may lead to accumulation of "free" copper in the body. This form of copper is not tightly bound to proteins or other molecules and can participate in Fenton-type reactions, generating toxic hydroxyl radical that can damage cellular components.[11] Role of antioxidants in combating such aberrant conditions are well documented.[42] Most of the beneficial health effects of tea are attributed to their antioxidant and free-radical scavenging properties which are essentially due to its constituent flavonoids, a group of polyphenols.[43] Chromatographic analysis of the flavonoids presented in this study corroborates with the previous reports indicating catechins as the major components of GT, whereas BT was found to be rich in theaflavins and thearubigin that are produced from catechins through oxidation and epimerization during fermentation process.[44, 45] Treatment for 10 days with the selected dose of tea samples helped to restrict copper-induced changes in liver function, kidney function and blood toxicity parameters in the exposed rats. Moreover, the treatment had no major changes in these parameters in control animals.

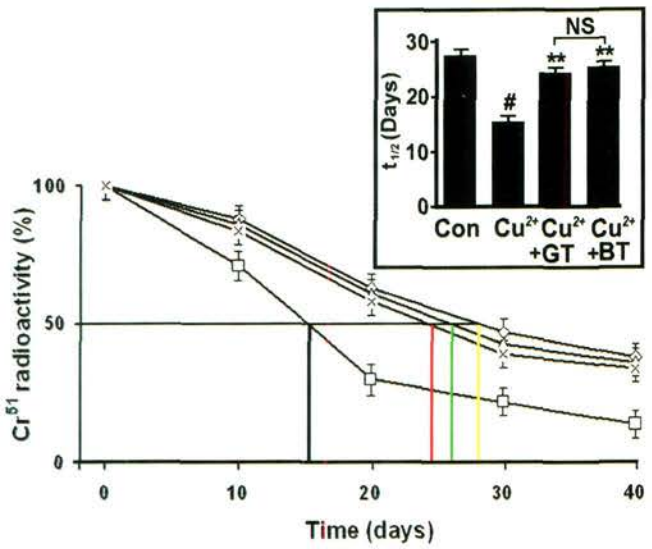

Figure 7. Restrain on copper-mediated reduced survival of erythrocytes by feeding tea. Life span of erythrocytes was assessed from the decay of Cr^{51} radioactivity over time. $t^{1/2}$ representing mean half-life of erythrocytes is indicated as follows: yellow line (control), black line (copper exposed), red line (copper + GT) and green line (copper + BT). $t^{1/2}$ levels in different groups of animals are shown in the inset. Values are mean ± SD of three separate experiments with five animals in each group. $^{\#}P < 0.01$ vs. control group. $^{**}P < 0.01$ copper-exposed group, not fed with GT or BT. NS = non-significant vs. Cu^{2+} + GT.

Determination of deposition and excretion of copper were necessary to assess the status of copper load in the exposed animals. Plasma is the easiest and most commonly used matrix for evaluating the presence of essential trace elements , but it is prone to be influenced in a variety of circumstances.[46] Considering this, we further confirmed the copper load by measuring the level in erythrocytes, which gives a stable picture[47] and are the target cells in the present study. Both GT and BT were highly efficient in hindering sharp rise in plasma copper level after 20 days of exposure. Efficacy of the tea samples in combating copper overload in erythrocytes followed a similar trend as observed in plasma, supporting protective role of GT and BT against development of copper toxicosis. Enhancement of copper excretion *via* urinary and fecal routes in the exposed rats may be explained by the animals' inherent adaptive measure against copper overloading. Further promotion of the excretion process in response to feeding with GT and BT supported the beneficial role of tea in preventing copper toxicity. Among the two excretory routes, renal excretion was more prominent during GT and BT treatment.

The potential health effects of GT and BT depend not only on the amount consumed but also on the bioavailability of their concerned components within target tissues. Since this study deals with copper-induced hemolytic outcome, it was necessary to evaluate total catechin and theaflavin bioavailability within RBC under our experimental conditions. Results obtained from GT- and BT-fed animals were in well accordance with the chromatographic analysis of the flavonoids in respective tea products.

Ability of the erythrocytes to change shape during their flow in circulation may be linked up with cellular deformability.[48] Toxicity to erythrocytes is associated with loss of cellular deformability that develops in consequence of altered cell shape.[49] Copper exposure led to profound changes in the structure of erythrocytes, as evident from the loss of normal discoid shape of the RBC. Since cellular deformability is one of the major parameters responsible for determining the lifespan of erythrocytes,[50] decreased deformability is likely to account for the increased red cell destruction in copper-exposed animals. Both GT and BT were equally effective in protecting the cells from copper-induced morphological alteration.

Oxidative damage of erythrocytes has been reported to be a possible mechanism for premature hemolysis during several pathological conditions.[51] Inhibitory role of GT and BT towards the reduced survival of erythrocytes consequent to metal catalyzed oxidative threat during copper toxicosis is likely to take place through the antioxidant role of catechins and theaflavins that contributes to the pathoprevention by virtue of their ability to donate electrons or hydrogen from hydroxyl groups to free radicals.[52] Although, as expected, the catechin content was much lower in BT, the benefits of drinking this fermented tea preparation in terms of impeding untimely hemolysis was identical to GT.

Previous reports suggest that the antioxidative effects of GT and BT may differ depending on the polyphenol composition.[6, 43] However, data available are not uniform with regard to the antioxidative capacity of theaflavins as compared with catechins. According to Lee *et al.*,[53] antioxidative capacity per serving of GT is much higher than that of BT in commercial tea products. On the other hand, Yoshida *et al.*[54] have shown that theaflavin digallate exhibited higher antioxidative capacity towards LDL oxidation in macrophages in comparison to EGCG. They have further postulated that the better efficacy of theaflavin which are essentially polymers of catechins might be linked up with the greater number of hydroxyl

(OH) groups in it.[54] This may be related to the degree of hydroxylation of the compound that was suggested to be positively correlated with its antioxidant efficiency.[55] In contrast to these reports, Yoshino *et al.*[56] showed that GT and BT infusions had similar antioxidant activities in rat liver homogenates. Later on, this notion was supported by the reports indicating equal efficacy of both types of tea in ameliorating endothelial function[6] and providing cardio protection.[8] Present study clearly suggests that drinking BT has benefits equal to those of drinking GT in preventing reduced survival of erythrocytes during copper toxicity.

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