

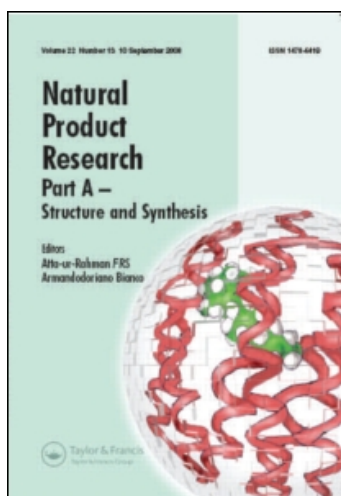
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Protective role of tea catechins on erythrocytes subjected to oxidative stress during human aging

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Protective role of tea catechins on erythrocytes subjected to oxidative stress during human aging

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Antioxidant effect of tea catechins has been shown in many epidemiological studies. In the present study we report the protective mechanism of tea catechins (EGCG, ECG, EGC, EC) on various oxidative stress parameters, which are elevated during aging in humans. We hereby report the *in vitro* effect of tea catechins on erythrocyte malondialdehyde (MDA), reduced glutathione (GSH), and on membrane sulphhydryl (–SH) group in humans. Results show an age-dependent increase in erythrocyte MDA level and a decrease in GSH and membrane –SH group concentration. We report that tea catechins show significant protection to erythrocyte against oxidative stress induced by *tert*-butyl hydroperoxide (*t*-BHP). The effect was more pronounced in older age group compared to lower age group. The findings suggest a possible role of tea catechins as anti-aging compounds.

Keywords: tea catechins; aging; erythrocytes; oxidative stress

1. Introduction

Tea (*Camellia sinensis*) is one of the most popular beverages worldwide. Tea contains polyphenolic compounds collectively known as catechins belonging to the flavanoid family. Several catechins have been identified in green tea extract (Zeeb, Nelson, Albert, & Dalluge, 2000), but epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechingallate (ECG), epicatechin (EC) have been extensively investigated. Epidemiological studies and clinical observations have shown chemopreventive properties of tea catechins (Shanafelt et al., 2006; Thangazham et al., 2007). Catechins are known to possess antioxidant (Cao, Sofic, & Prior 1997; Tjiburg, Mattern, Folts, Weisgerber, & Katan, 1997; van Acker et al., 1996), anticancer (Yang et al., 2006), hypoglycemic (Rizvi, Zaid, Anis, & Mishra, 2005), cardiovascular and neurodegenerative disorders, and they scavenge reactive oxygen species (ROS) and reactive nitrogen species (RNS). Catechins are believed to react with biomolecules either directly or after cellular metabolism but the exact mechanism underlying these processes remains speculative. An increase in the production of ROS and compromised antioxidant status may cause oxidative stress, which may contribute to aging and other age-related diseases (Fusco, Colloca, Lo Monaco, & Cesari, 2007).

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Aging is the accumulation process of diverse detrimental changes in the cells and tissues with advancing age, resulting in an increase in the risks of disease and death (Harman, 2006). There are many theories which attempt to explain the process of aging. The oxidative stress hypothesis offers the best mechanistic elucidation of the aging process and other age-related phenomenon (Beckman & Ames, 1998; Junqueira et al., 2004). Aerobic cells produce ROS as a byproduct of their metabolic processes. ROS cause oxidative damage to macromolecules under conditions when the antioxidant defence of the body is overwhelmed. A certain amount of oxidative damage takes place even under normal conditions, however, the rate of this damage increases during the aging process as the efficiency of antioxidative and repair mechanisms decrease (Gil et al., 2006).

Many *in vitro* studies have demonstrated that several parameters of blood are negatively affected by increased oxidative stress. In fact, changes in membrane fluidity and inactivation of membrane bound receptors and enzymes (Halliwell & Gutteridge, 1986), ionic parameters (Maridonneau, Barquet, & Garay, 1983), an increase in lipid peroxidation, oxidation of glutathione (GSH) and protein sulphhydryl (-SH) groups (Tsantes, Bonaovas, Travlou, & Sitara, 2006) have been described following the application of oxidative stress. Recently we have reported a significant age-dependent decline in plasma antioxidant capacity, measured in terms of FRAP values (Rizvi, Jha, & Maurya, 2006). Since antioxidant capacity of the plasma is related to dietary intake of antioxidants (Cao, Booth, Sadowski, & Prior, 1998), it is important to study the use of tea catechins in the prevention of oxidative stress and aging.

The present study was undertaken to evaluate the anti-aging effect of tea catechins (EGCG, EGC, ECG, EC) on markers of oxidative stress in erythrocytes from different age subjects. We report a protective effect of tea catechins on oxidation-induced increase malondialdehyde (MDA) content and intracellular reduced GSH and membrane -SH on human erythrocytes subjected to increased oxidative stress.

2. Materials and methods

The study was carried out on 80 normal healthy subjects of both sexes between the ages of 18 and 85 years. The criteria for selection of subjects were the same as described earlier (Rizvi et al., 2006; Rizvi & Maurya, 2007). Briefly, the subjects were divided into three groups, namely young, middle and old. The subjects were screened for diabetes mellitus, asthma, tuberculosis or any other major illness. None of the subjects were smokers or were taking any medication. All subjects gave their informed consent for the use of their blood samples for the study. The protocol of study was in conformity with the guidelines of the Institutional Ethical Committee.

Human venous blood from different healthy volunteers was obtained by venipuncture in heparin. The blood was centrifuged at $1800 \times g$ for 10 min at 4°C. After removal of plasma, buffy coat and upper 15% of the packed red blood cells (RBCs), the RBCs were washed twice with cold PBS (0.9% NaCl, 10 mM Na₂HPO₄, pH 7.4). Erythrocyte ghosts from leucocyte-free RBCs were prepared by osmotic shock procedure (Marchesi & Palade, 1967).

2.1. Determination of MDA content

Erythrocyte MDA was measured according to the method of Esterbauer and Cheeseman (1990). Packed erythrocytes (0.2 mL) were suspended in 3 mL Krebs-Ringer phosphate

(KRP) buffer, pH 7.4. The lysate (1 mL) was added to 1 mL of 10% trichloroacetic acid (TCA) and mixture was centrifuged for 5 min at $1000 \times g$. The supernatant (1 mL) was added to 1 mL of 0.67% thiobarbituric acid (TBA) in 0.05 mol L^{-1} NaOH and boiled for 20 min at temperature $>90^\circ\text{C}$, cooled and the absorbance read at 532 nm (OD1) and 600 nm (OD2). The net optical density (OD) was calculated after subtracting absorbance at OD2 from that at OD1. The concentration of MDA in erythrocytes was determined from a standard plot. Concentration of MDA is expressed as nmol mL^{-1} of packed erythrocytes.

2.2. Determination of erythrocyte GSH and membrane –SH group content

Erythrocyte GSH was measured following the method of Beutler (1984) and membrane bound –SH groups were estimated according to the method of Kitajima, Yamaguchi, and Kinoto (1990). Both the methods were based on the ability of the –SH group to reduce 5,5'-dithiobis, 2-nitrobenzoic acid (DTNB) and form a yellow coloured anionic product whose OD is measured at 412 nm. Concentration of GSH is expressed in milligram per millilitre packed RBCs and was determined from standard plot. The concentration of –SH group is expressed as nmol mg^{-1} protein. The protein content of the erythrocyte ghosts was estimated according to the method of Lowry, Rosenbrough, Farr, and Randall (1951), using bovine serum albumin as the standard. Absorbance was measured at 690 nm.

2.3. Experiments with tea catechins and induction of oxidative stress

The effect of tea catechins (EGCG, ECG, EGC, EC) on erythrocyte GSH status and MDA content was investigated as follows. Blood was washed two to three times with KRP containing 5 mmol L^{-1} glucose (KRP-G), pH 7.4. Erythrocytes were then suspended in 4 volume of KRP-G. The *in vitro* effect of tea catechins was evaluated by incubating erythrocytes in the presence of $10^{-5} \text{ mol L}^{-1}$ (final concentration) of each catechin (EGCG, ECG, EGC, EC) separately for 60 min at 37°C . The erythrocytes were again washed two to three times with KRP, pH 7.4 and finally, packed erythrocytes were used for the assay. In parallel control experiments, blood was incubated without catechins. For –SH group estimation, erythrocyte ghosts (0.4–0.6 mg protein) were incubated with indicated final concentrations of catechins in 3 mL of 0.1 mol L^{-1} phosphate buffer for 1 h at 37°C before estimation of –SH groups.

Oxidative stress was induced *in vitro* by incubating washed erythrocytes/erythrocyte ghosts with *t*-BHP ($10^{-5} \text{ mol L}^{-1}$ final concentration). The concentration of *t*-BHP used in the present study to induce oxidative stress of erythrocyte was in the range of concentration used in other published reports (Di Simplicio et al., 1998).

Statistical analysis was performed using the software PRISM 4.

3. Results and discussion

Subjecting erythrocytes to increased oxidative stress by incubating them with *t*-BHP caused an increase in MDA content above basal level in all the age groups in human erythrocytes (Figure 1). Erythrocytes from the old age group show greater susceptibility to oxidation; incubation with *t*-BHP caused a 42% increase in MDA while middle age have 28% and young age have 25% increase in MDA content. The presence of tea catechins in

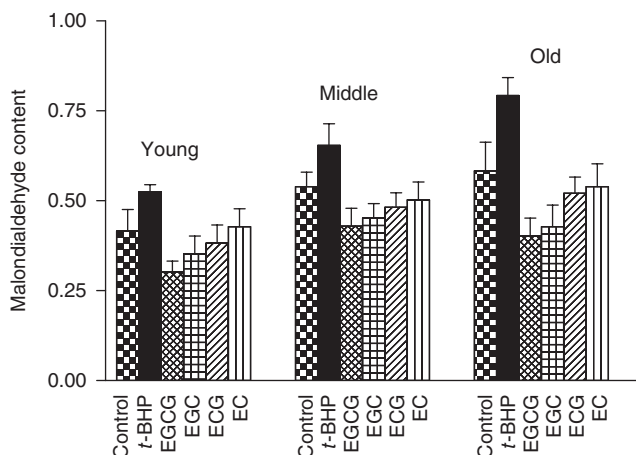


Figure 1. Effect of EGCG, EGC, ECG, EC (10^{-5} molL⁻¹ final concentration) on *tert*-butyl hydroperoxide (*t*-BHP) (10^{-5} molL⁻¹) induced changes in malondialdehyde (MDA) content in different age groups in human erythrocytes. Concentration of MDA is expressed as nmol mL⁻¹ of packed erythrocytes. Values represent mean \pm SD.

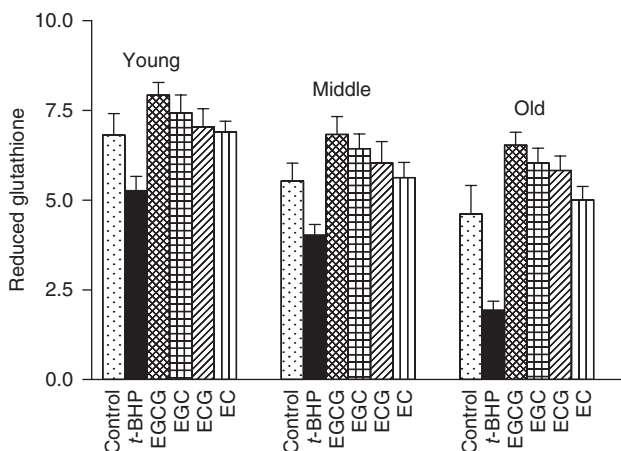


Figure 2. Effect of EGCG, EGC, ECG, EC (10^{-5} molL⁻¹ final concentration) on *tert*-butyl hydroperoxide (*t*-BHP) (10^{-5} molL⁻¹) induced changes in reduced glutathione (GSH) content in different age groups in human erythrocytes. Concentration of GSH is expressed in milligram per millilitre packed erythrocytes. Values represent mean \pm SD.

the incubation medium protected the erythrocyte from *t*-BHP-induced oxidative stress, as evident by decrease in MDA level in all age groups but effect is more pronounced in old age groups. A significant ($p < 0.05$) protective effect of tea catechins was observed at concentration of 10^{-5} M and the order of effectiveness of individual catechins was EGCG > EGC > ECG > EC.

Intracellular GSH level decreases as a function of human age. In the old age group, decrease in GSH content is 51% while in the middle age group it is 20% and in the young age group it is 16%, suggestive of more oxidative stress in higher age groups (Figure 2). The induction of oxidative stress following incubation with *t*-BHP resulted in decrease in

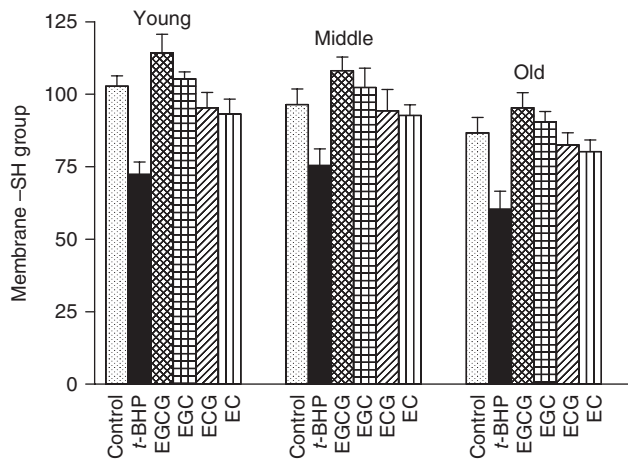


Figure 3. Effect of EGCG, EGC, ECG, EC (10^{-5} mol L⁻¹ final concentration) on *tert*-butyl hydroperoxide (*t*-BHP) (10^{-5} mol L⁻¹) induced changes in membrane -SH group content in different age groups in human erythrocytes. The concentration of -SH group is expressed as nmol mg⁻¹ protein. Values represent mean \pm SD.

GSH content in all three age groups but the decrease is more pronounced in the old age group showing more oxidative stress in higher age group erythrocytes. Tea catechin demonstrated a protective effect against *t*-BHP induced GSH oxidation, this effect being more pronounced in old age erythrocyte than young, signifying the inherent lower level of antioxidant defence in old age. The relative efficiency of different catechins was in the order of EGCG > EGC > ECG > EC.

Figure 3 shows that membrane -SH group content was significantly decreased as a function of human age. Incubation with *t*-BHP caused a decline in the -SH group content in all age groups but this decline was more pronounced in the old age group (28%) compared with the middle age group (22%) and the young age group (22%), suggestive of an increased oxidative stress in higher age groups. The presence of tea catechins protected -SH group oxidation against *t*-BHP induced oxidative stress. Although the pattern of the antioxidant effect of tea catechin on membrane -SH group content was similar to the effect of the catechin on MDA and GSH. EGCG and ECG display more significant protection of -SH group as compared to EGC and EC.

Under physiological conditions, ROS are eliminated by enzymatic and nonenzymatic antioxidant defence systems. Knowledge of the absorption and metabolism of tea catechins is important before any conclusions may be drawn regarding their potential to exert biological activity *in vivo*, as suggested by *in vitro* studies. A report (Isbrucker, Edwards, Wolz, & Davidovich, 2006) has demonstrated that dietary administration of EGCG (up to 500 mg kg⁻¹) to rats and dogs for several weeks was not found to be toxic. Almost 50% of orally administered radiolabelled catechin has been shown to be excreted as metabolites in urine (Das, 1971). Plasma total catechin concentrations are reported to range from 0.63 to 1.8 μ mol L⁻¹ after ingestion of a single large dose of green tea. This plasma concentration is achieved after 1.5–2.6 h; levels returned to baseline values after 24 h (Leenen, Rodenberg, Tijburg, & Wiseman, 2000; van het Hof, Kivitis, Weststrate, & Tijburg, 1998). Thus, it is clear that plasma concentrations of catechins are achievable in the micromolar range.

Free radical-induced formation of lipid peroxides is thought to play an important role in the aging process and other oxidative stress-related diseases (Rizvi & Maurya, 2007). As a result of lipid peroxidation, a number of unstable intermediary metabolites, namely MDA and 4-HNE, are produced from cellular molecules and damage various biomolecules leading to the aging process. Dietary flavanols, especially catechins, are known to protect oxidation of biomolecules against oxidant species by preventing lipid peroxidation (Guo, Zhao, Li, Shen, & Xin, 1996; Steffen, Schewe, & Sies, 2005). GSH metabolism was also affected in the presence of tea catechins. GSH has many biological functions, including maintenance of membrane protein –SH groups in the reduced form, the oxidation of which can cause altered cellular structure and function. It has been reported that there is age-dependent decline in GSH (Gil et al., 2006; Rizvi & Maurya, 2007). The human erythrocyte is an easily accessible cell type that is rich in –SH functions: the importance of the erythrocyte –SH group in overall cellular redox balance has been emphasised. Membrane oxidative damage has a considerable effect on membrane mechanical properties. Membrane –SH group oxidative damage may be an important molecular mechanism, inducing changes in the membrane microelasticity or whole cell deformability under conditions of physiological and pathological oxidative stress.

The normal human body has a very complex and efficient antioxidant system consisting of a number of interrelated antioxidant compounds and enzymes. Mechanism(s) that are thought to be involved in the increased oxidative stress as a function of human age include not only oxygen free radicals generation but also changes in the tissue/plasma content and the activity of the antioxidant defence system. These results may have implications in designing strategies for the use of tea catechins in age-related diseases. We assume that a high intake of catechin-rich diet by higher age groups may provide some protection against the development of age-related diseases and slow down the aging process.

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