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NATURAL DRUGS

THE EFFECT OF GREEN, BLACK AND WHITE TEA ON THE LEVEL OF α AND γ TOCOPHEROLS IN FREE RADICAL-INDUCED OXIDATIVE DAMAGE OF HUMAN RED BLOOD CELLS

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Abstract: The present study was undertaken to investigate the effect of aqueous tea extracts on lipid peroxidation and α and γ tocopherols concentration in the oxidative damage of human red blood cells (RBC). RBC was taken as the model for study of the oxidative damage was induced by cumene hydroperoxide (cumOOH). The antioxidative property of leaf green tea, leaf and granulate of black tea and white tea at levels 1, 2, 4 g/150 mL of water were evaluated. The correlation was observed between reducing power of tea extract and formation of malondialdehyde – MDA (an indicator of lipid peroxidation) in oxidative damage of RBC. All tea extracts at level of 4 g/150 mL of water significantly decreased concentration of MDA. The extract of green tea in comparison to black and white tea extracts at the same levels seems to be a better protective agent against oxidative stress. The antioxidant synergism between components extracted from leaves of green tea and endogenous α tocopherol in the oxidative damage of red blood cells was observed. The consumption of α tocopherol in oxidative damage of RBC was the lowest after treatment with the highest dose of green tea extract. All tea extracts did not protect against decrease of γ tocopherol in human erythrocytes treated with cumOOH.

Keywords: green tea, black tea, white tea; reducing power; erythrocytes; oxidative damage, vitamin E; antioxidant synergism

Tea is the most popular beverage widely used in large areas of the world. Many different types of tea: green, black and white are produced and sold in many countries. In Poland, people, at average, consume the black tea at least twice a day. Less popular is drinking green tea. A number of studies suggest that the consumption of green and black tea beverage may bring positive health effects (1- 4). These effects depend on tea flavonoids which possess strong antioxidant properties. These compounds are absorbed in the digestive tract and give rise of concentration of major flavonoids in plasma (2, 5). Tea flawonoids protect the macromolecules such as DNA and lipids from damage caused by free radical induced oxidative stress (6-9).

Lipid peroxidation and DNA damage are associated with a variety of chronic health problems, such as cancer, ageing, atherosclerosis. The mechanisms which exert protection of lipids against peroxidation are very complicated and require more research.

Polyunsaturated fatty acids-rich erythrocytes membranes are particularly susceptible to oxidative stress. Many compounds (e.g. vitamin E) can protect the erythrocytes membranes against lipid peroxidation. For this reason erythrocyte membranes (in whole red blood cells or ghost of erythrocytes) have been used as a model for oxidative stress and antioxidant studies.

A lot of data confirm that green and black tea polyphenols protect red blood cell membranes against oxidative damage in vitro (10-14). It has been confirmed that tea polyphenols can protect erythrocyte membranes against lipid peroxidation and lysis induced by water-soluble compounds [hydrogen peroxide or 2,2”-azobis(2-amidino-propane hydrochloride)] which can be used as free radical source (11, 13). Tea polyphenols also protect erythrocytes against lipid peroxidation induced by organic hydroperoxides membrane soluble initiators such as *tert*-butylhydroperoxide (14).

The present study was aimed to evaluate the antioxidant properties of green, black and white tea. The protective power of different concentration of tea extracts on the formation of MDA and consumption of tocopherols in oxidative damage of red blood cells was determined

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EXPERIMENTAL

Chemicals

α and γ tocopherol were purchased from Sigma-Aldrich Chemie (Germany), racemic tocol from Matreya (Pleasant Gap. PA USA). *n*-hexane and 2-propanol were HPLC grade and were purchased from J.T. Baker (Holland). All other chemicals were supplied by Sigma-Aldrich Chemie (Germany).

Preparation of tea extracts

The tea extracts were prepared using 0.75 g, 1.5 g or 3 g of commercially available leaf green tea Silver Moon (Bio-Active Poland), black tea Iguana black, tea granulate Lipton (Unilever Poland), leaf black tea Yunan (POSTIT Poland) and white tea Pandino (Bio-Active Poland.). The leaves of teas were soaked in 150 mL of boiling water for 5 min. The proportions of tea and water were selected to represent a typical quantity consumed by tea drinkers in Poland. After brewing and cooling to 20°C the antioxidant capacity of fresh tea extracts was determined.

Antioxidant activity of tea extracts

The reducing power

The reducing power of tea extracts were assessed using an assay based upon the reduction of ferric chloride (15). Tea extract (20 μL of each extract corresponding to 100; 200 or 400 μg of tea) was added to 5 mL of mixture of phosphate buffer (0.05 M, pH 6.6) and potassium ferricyanide (1% w/v water solution). The samples were incubated at 50°C for 20 min. Then 2.5 mL of trichloroacetic acid (TCA) (10% w/v water solution) was added to each sample and after centrifugation the supernatant (2.5 mL) was added to 0.5 mL of $FeCl_3$ (0.1% water solution). Then the absorbance was measured (Spectronic Genesys 2PC) at 700 nm. An increase in the absorbance of the reaction mixture compared with sample without tea extract indicated an increase of reducing power of the studied teas. Data presented are arithmetic means of 3 independent measurements.

Effect of tea extracts on *in vitro* oxidative damage of human red blood cells

Preparation of human blood cells: blood samples from healthy people were collected into tubes containing sodium citrate solution**.** Packed red blood cells were isolated by centrifugation at 1500×g for 10 min at 4°C. The plasma and buffy coat were removed and the red cells were washed three times with 0.9% saline.

Oxidative treatment of erythrocytes: a 10% suspension of erythrocytes in PBS was prepared and the Hb concentration was measured by Drabkin method. The suspension was divided into 2 mL samples each. All samples were incubated with 66.7 mM/L cumene hydroperoxide (25 mL of solution in PBS and ethanol; 1:1) in the presence or absence of water tea extracts (20 μL) at 37°C for 60 min.

TBARS assays: following the incubation, samples with cumene hydroperoxide and with cumene hydroperoxide plus tea extract, were taken for determination of MDA concentration. The concentration of malondialdehyde was measured in reaction with thiobarbituric acid, forming with MDA thiobarbituric acid reactive substances (TBARS) according to Stock method (16). The results were expressed in mM of MDA/g Hb as mean ± SD of three measurement of each brand of tea, each at 3 doses. MDA was not detected in any sample of intact red cells investigated prior to the addition of cumOOH.

Determination of α and γ tocopherol in erythrocytes: series of suspensions of erythrocytes were incubated with cumene hydroperoxide (as described above) in the presence or absence of each tea extract at 37°C for 5 and 30 min. Since all of the study tea extracts in a dose 400 μg significantly protected the RBC against oxidation induced by cumOOH, such a dose was chosen to study the effect of tea extracts on the level of α and γ tocopherols. The concentrations of these antioxidants were determined in washed red blood cells before addition of cumOOH, in red blood cells after incubation with cumOOH and after incubation with cumOOH plus tea extracts (20 μL).

Following the incubation, packed red blood cells were isolated by centrifugation at 1500×g for 10 min at 4°C. In erythrocytes, α and γ tocopherols levels were determined by HPLC method described earlier (17). Briefly, the packed erythrocytes (0.1 mL) were pipetted into glass tubes containg BHT in ethanol (0.025%, w/v, 2 mL), ascorbic acid (15%, w/v, 0.2 ml), pyrogallol (25% w/v, 0.2 mL) and 0.1 mL internal standard (racemic tocol). The samples were heated at 60°C for 30 min after addition of 10% KOH. The tubes were then cooled to a room temperature. Water and BHT in hexane (0.025%, 2 mL) were added, and tocopherols were extracted into the hexane by vortex mixing for 1 min. The hexane phase was isolated and evaporated under a stream of nitrogen at room temperature. The residue was redissolved in the mobile phase and injected on the chromatographic column. HPLC was performed with Shimadzu spectrofluorimetric detector. The tocopherols were separated on Separon SGX NH_2 analytical column, by isocratic elution with 97:3

(v/v) n-hexane and 2-propanol. Fluorimetric detection was performed at the wavelengths λ_{ex} = 298 nm, λ_{em} = 325 nm. Experiments with each brand of tea and with each of dose were carried out in triplicates. The results were expressed in μg/g of Hb.

Statistical method

Data are presented as means ± SD. Comparisons between study samples were done using the paired t-test. Statistics were performed using the STATISTICA programme (ver. 6.0, Statsoft, Poland).

RESULTS

The ability to reduce of Fe^{3+} by the extracts of green (Silver Monn), black (Iguana, Lipton, Yunan) and white (Pandino) tea corresponding to100, 200 or 400 g of tea in 20 μL of water is shown in Figure 1. The reducing power was dose dependent. A dose of 100 g of all types of tea showed nearly the same reducing power. The maximal reducing power was found in Silver Moon green tea at level 400 μg/20 μL of water.

In order to study the antioxidant effects of the various tea brand extracts, the model of oxidative stress induced by cumene hydroperoxide in red blood cells was chosen. Incubation of a suspension of normal whole erythrocytes with cumOOH for 60 min resulted in significant increase of malondialdehyde concentration. The concentration of MDA was 0.042 ± 0.007 mM/g Hb. The effect of tea extracts at levels 100, 200 and 400 μg/20 μL on formation of thiobarbituric acid reactive substances is shown in Figure 2.

TBARS formation was significantly decreased when the extracts of green tea Silver Moon at the levels of 200 or 400 μg/20 μL were added to 2 mL of suspension of red cells with cumOOH. The extracts of black teas Iguana and Lipton at the levels of 200 or 400 μg/20 μL also significantly decreased the lipid peroxidation in oxidative treatment of red blood cells. Black tea Yunan showed significant antioxidant capacity only at the highest level (400 μg/20 μL). The white tea Pandino significantly reduced TBARS formation at the levels of 200 and 400 μg/20 μL. All brands of teas studied at level 100 μg/20 μL did not protect red blood cells against oxidative damage by cumOOH. Decreased TBARS formation was dose dependent only for green tea Silver Moon and black tea Iguana and Lipton.

As it is shown in Table1, a correlation between the ability of green, black and white tea to reduce Fe^{3+} at 3 levels (100, 200, 400 μg/20 μL) and to decrease the concentration of MDA in protected against oxidative damage red blood cells was observed.

The reducing power of teas measured as the ability to reduce of Fe^{3+} strongly correlated with the antioxidative effect of teas expressed as formation of MDA in oxidative damage of erythrocytes.

Changes in the level of the antioxidants, α and γ tocopherols after incubation of red blood cells with cumOOH or cumOOH and tea extracts (400 μg of tea in 20 μl water) are presented in Figures 3 and 4.

The addition of oxidant to the RBC suspension after incubation for 5 or 30 min resulted in a loss of α tocopherol in the membrane (60% and 72%, respectively) and in a loss of γ tocopherol (43 and 47%, respectively) compared to control. In the incubation mixture of the RBC and cumOOH, in the present of green tea Silver Moon extracts, the loss of α tocopherol was significantly inhibited after 30 min of incubation as compared with incubation with cumOOH only. The extracts from other teas did not significantly influence the change of the level of α tocopherol in oxidatively damaged red blood cells.

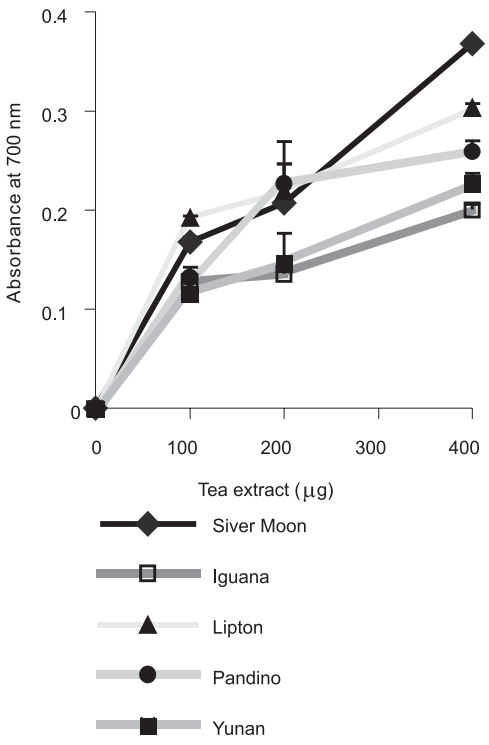

Figure1. Increase of samples' absorbance (measured as described under "Experimental") after reduction of ferric chloride by 20 μL extracts of teas corresponding to 100, 200 and 400 μg of tea. Data are expressed as mean ± SD (n = 3).

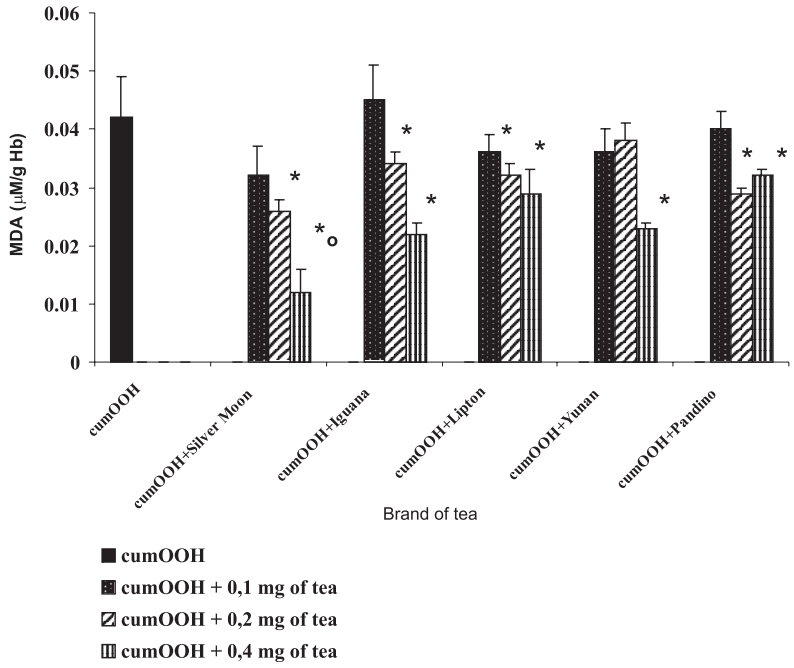

Figure 2. Effect of 20 μL of extracts of teas corresponding to 100, 200 and 400 μg of a given kind of tea on the formation of MDA during peroxidation of red blood cells. Normal RBC were incubated with cumOOH or with cumOOH plus tea extract for 1 h at 37°C. Mean ± SD from triplicates are given

* Significance of differences between means: tea extracts + cumOOH and cumOOH only; $p < 0.05$

o Significance of differences between means: 0.4 mg of Silver Moon and others teas in the same dose; $p < 0.05$

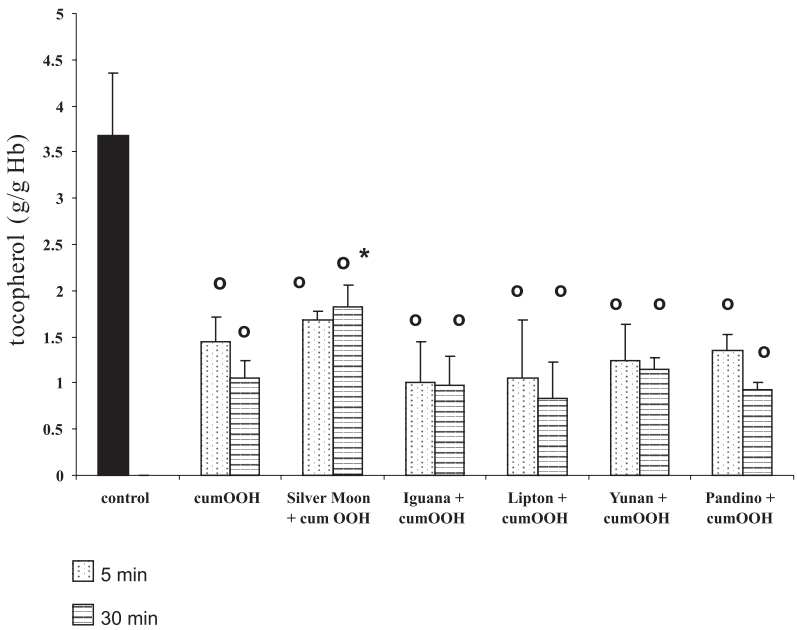

Figure 3. Effect of tea extracts corresponding to 400 μg of tea on the concentration of α tocopherol in red blood cells damaged by cumOOH during incubation at 37°C for 5 or 30 min. Control represents level of α tocopherol in intact red blood cells. Data are expressed as mean ± SD (n = 3).

o Significantly different from control value ; $p < 0.05$

* The value significantly different from a tocopherol concentration in RBC treated with cumOOH and others teas during 30 min; $p < 0.05$

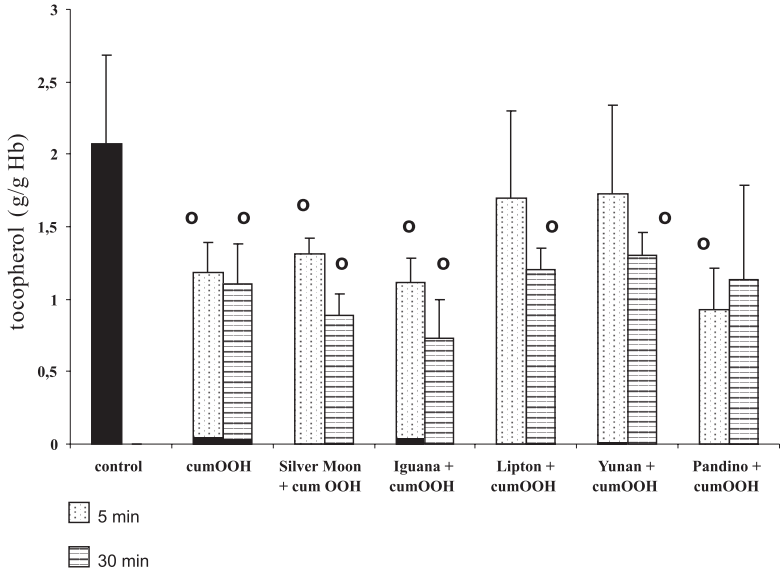

Figure 4. Effect of tea extracts (400 µg tea/20 µL of water) on the concentration of γ tocopherol in red blood cells damaged by cumOOH during incubation at 37°C for 5 and 30 min. Control represents level of γ tocopherol in intact red blood cells. Data are expressed as mean ± SD (n = 3).
° Significantly different from control value ; $p < 0.05$

The decay of γ tocopherol was not significantly inhibited by all tea extracts.

DISCUSSION AND CONCLUSION

It may be suspected that tea polyphenols which can be determined in plasma and urine in human subjects can protect the biological membranes against lipid peroxidation (16). In the present study the antioxidant potential of green (Silver Moon), black (Iguana, Lipton, Yunan) and white tea (Pandino) available in Poland were assessed. The tea varieties on the Polish market are probably different from those in other countries. The teas investigated in the present study had various antioxidant capacities, depending on the brand and doses of the tea used. The results obtained after using the reaction with Fe^{3+}, indicate that the green, black and white tea, manufactured in various conditions have similar antioxidant potential at low doses, corresponding to half tea spoons of tea in a cup of water (approximately 150 mL). The greatest reducing power for the highest concentration of the studied tea extracts, corresponding to full 2 tea spoons on cup of water was observed in Silver Moon tea relative to the other teas.

To study the effect of antioxidant activity of teas on biological system, suspensions of human whole erythrocytes were exposed to an oxidant cumene hydroperoxide in the presence or absence of variety of brand and concentration of tea extracts. Cumene hydroperoxide was chosen as an initiator of oxidative process in red blood cells. This organic hydroperoxide is soluble in membranes and in used concentration it is not a significant factor for RBC lysis (18). The extent of lipid peroxidation and the antioxidative effect of tea in cell membranes was assessed by measuring TBARS formation and changes in the level of α and γ tocopherols in erythrocytes. TBARS assay is not the specific method for assessing the extent of lipid peroxidation but it is sufficient when we compare the oxidative process in lipids in various samples.

In the present study the data received from TBARS assay showed that rather higher concentration of teas in water infusion, corresponding to two tea spoons in a cup of water, could significantly decrease the TBARS formation in oxidative dam-

Table 1. Correlation between the reducing power of tea extract and formation of MDA in oxidative damage of RBC after addition of tea extracts.

Tea	Silver Moon	Iguana	Lipton	Yunan	Pandino
Correlation coefficient	-0.9844	-0.9217	-0.8128	-0.9434	-0.9442

aged red blood cells. The low doses of green, black and white tea which were equivalent to a half and an one tea spoon in a cup of water do not protect against oxidative damage of membrane lipids. These data agreed with the data representing the reducing power of teas.

In RBC obtained from healthy subjects, significant oxidative damage is prevented by a very efficient antioxidant system, consisting of a number of antioxidant compounds and enzymes. In this system vitamin E plays a very important role. In the present study addition of cumOOH, to the RBC suspension during incubation resulted in partial consumption of α and γ tocopherols presented in erythrocyte membranes. Under described conditions of the experiment, only green tea extract could prevent the consumption of α tocopherol when RBC were incubated with cumOOH during 30 min. These data could confirm an opinion that there is a synergistic antioxidative effect of the green tea polyphenols with α tocopherol, suggested by some authors, who observed the antioxidant effects of green tea polyphenols in human low-density lipoprotein or in some other compartments (8, 13, 19). The synergistic interaction between tea extract and α tocopherol was observed despite the fact that polyphenols are water-soluble, while vitamin E is lipophilic. In the present study, addition of the tea extract to the suspension of RBC caused the regeneration of α tocopherol during the first 5 min of incubation and the regeneration was prolongated to 30 min. of incubation. The extracts from black and white tea do not protect α tocopherol against consumption during oxidative reactions in RBC. These data may indicate that only polyphenols from green tea and other coexisting in tissue endogenous antioxidants could effectively protect the biological membrane against loss of α tocopherol. In the body, γ tocopherol as an antioxidant plays less effective role than α tocopherol. In the present study no effect of green, black and white tea extracts on γ tocopherol regeneration reactions was observed.

In conclusion, the presented results indicate that green tea had the highest antioxidant activity and only this tea may effectively protect α tocopherol in RBC membrane against cumOOH induced oxidation compared to black and white tea. The white tea demonstrates similar properties as the black tea. Further studies are required to investigate whether a large variation of flavonols might be responsible for the higher antioxidative effect of green teas compared to black and white teas (20).

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