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Effects of (+)-Catechin Supplementation on the Some Biochemical Parameters in Sera and Erythrocytes of Ovariectomized Wistar Rats Induced by the Carcinogen Potassium Bromate ($KBrO_3$)

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Abstract: The aim of this research was to examine the effects of (+)-catechin on the levels of Malondialdehyde (MDA), homocysteine, vitamin C, cholesterol, GSH, GSSG and lipophilic vitamins in serum and erythrocytes of old and ovariectomized rats after exposure to the carcinogen potassium bromate. Female Wistar rats (n = 30) were randomly divided into 3 groups. The 1st group was used as a control, the 2nd group was treated with $KBrO_3$ and 3rd group received C + $KBrO_3$. Rats in the $KBrO_3$ and C + $KBrO_3$ groups were injected intraperitoneally with a single dose of $KBrO_3$ (80 mg kg^{-1}) in physiological saline. After 2 days, those in the C + $KBrO_3$ group were intraperitoneally injected with catechin (30 mg kg^{-1}) 4 times $week^{-1}$. Physiological saline was injected into the control group rats. The results indicate that the serum cholesterol level in the $KBrO_3$ group was higher than in the other 2 groups ($p<0.01$). MDA level was lower in the C + $KBrO_3$ group than the others ($p<0.001$). The amount of α-tocopherol increased in the $KBrO_3$ treated groups ($p<0.05$, $p<0.01$). Vitamins C and D_2 levels were higher in the $KBrO_3$ and C + $KBrO_3$ groups than the control group ($p<0.001$). In erythrocytes, the cholesterol level decreased in the $KBrO_3$ group and the vitamin D_3 level decreased in the C + $KBrO_3$ group ($p<0.005$). MDA level in the $KBrO_3$ group was higher than the control and C + $KBrO_3$ group ($p<0.05$). GSH, GSSG and α-tocopherol levels were lower in erythrocytes from the $KBrO_3$ and C + $KBrO_3$ groups than the control group ($p<0.001$). The GSH/GSSG ratio was high in the $KBrO_3$ group; there was no difference between the control and C + $KBrO_3$ groups. In conclusion, it can be said that administration of catechin decreased MDA in the serum and erythrocytes of old and ovariectomized Wistar rats and prevented the decrease of erythrocyte cholesterol.

Key words: (+)-Catechin, serum, erythrocytes, α-tocopherol, Malondialdehyde (MDA), glutathione, ovariectomized Wistar rats, potassium bromate

INTRODUCTION

Flavonoids are polyphenolic antioxidants naturally present in vegetables, fruits and beverages such as tea and wine. They have been shown to have potential for protecting against chronic diseases and atherosclerosis. They can act as free radical scavengers and transient metal ion chelators. Catechin has pharmacological effects such as anticarcinogenic, antioxidant, dental and antimicrobial activities and is active in preventing cardiovascular disease (Kang *et al*., 1999; Demeule *et al*., 2000). The pure catechins to be more powerful antioxidants than vitamins C, E and β-carotene in an *in vitro* lipoprotein oxidation model (Joe *et al*., 1998).

Catechins have also been shown to protect neurons, enhance resistance of red blood cells to oxidative stress, inhibit ultra-violet radiation-induced oxidative stress in skin and reduce the blood cholesterol level by protecting low density lipoproteins from oxidation (Chu *et al*., 2004). However, catechins have been reported to enhance colon carcinogenesis in rats, suggesting that they may have carcinogenic as well as anticarcinogenic effects (Furukawa *et al*., 2003). These compounds show antithrombic activity, which might be due to an antiplatelet effect rather than anticoagulation (Son *et al*., 2004). Catechins, have been considered potential chemotherapeutic agents since they inhibit carcinogenesis in several animal models (Hirose *et al*., 2001). The protective activity of catechins is generally attributed to the free radical-scavenging (Kang *et al*., 1998).

Potassium Bromate ($KBrO_3$) is an oxidizing agent that has been reported to be carcinogenic in rats, causing renal tumours (Chipman *et al*., 1998; Cadenas and Barja, 1999).

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It increases the level of 8-oxodeoxyguanosine (8-OH-deoxyguanosine, 8-oxodG) in the kidney DNA of treated rats. In female rats, ovariectomy artificially induces a marked reduction in endogenous estrogen concentrations subsequently causing a negative bone remodeling balance that augments bone loss and increases the incidence of osteopenia (Wronski *et al*., 1988). Estrogens influence the growth and functioning of female and male reproductive tissues, maintain the skeletal and central nervous system, provide cardioprotective effects in the cardiovascular system and protect against colon cancer and aging skin (Ososki and Kennelly, 2003). Plant-derived phytoestrogens are compounds structurally or functionally mimic mammalian estrogens and therefore, are considered to play an important role in the prevention of cancers, heart disease, menopausal symptoms and osteoporosis (Setchell *et al*., 1998; Adlercreutz, 2002; Kronenberg and Berman, 2002). The aim of this study was to examine the effects of (+)-catechin on the levels of MDA, homocysteine, vitamin C, cholesterol, GSH, GSSG and lipophilic vitamins in serum and erythrocytes from old and ovariectomized female Wistar rats exposed to potassium bromate ($KBrO_3$).

MATERIALS AND METHODS

Animals: A total 32 old ovarectomized female Wistar rats were used in this study. The animals were housed in cages where they had *ad libitum* rat chow and water in an air-conditioned room with a 12 h light/dark cycle and were randomly divided into 3 groups. The 1st group was used as a control, the 2nd group received potassium bromate ($KbrO_3$) and 3rd group received C + $KBrO_3$. Rats in the $KBrO_3$ and C + $KBrO_3$ groups were injected intraperitoneally with a single dose of potassium bromate (80 mg kg^{-1}) in physiological saline. Two days later, the rats in C + $KBrO_3$ group were intraperitoneally injected with (+)-catechin (30 mg kg^{-1}) 4 times $week^{-1}$. Physiological saline was injected into the control group rats. These treatments were continued for 5 weeks, then each rat was decapitated and blood samples were collected and stored in -85°C prior to biochemical analysis.

Sample preparation for biochemical analyses: Blood samples were collected into tubes. Blood was centrifuged at 2, 500×g at 4°C for 10 min and serum was separated. The erythrocyte pellet was washed 3 times with 0.9% NaCl and centrifuged for 5 min at 2.500×g after each wash. The pellet and serum samples were prepared freshly for each biochemical analysis.

Determination of erythrocyte glutathione: GSH and GSSG were separated and analyzed by fully automatic High Performance Liquid Chromatography (HPLC). The HPLC equipment consisted of a pump (LC-10 ADVP), a UV-visible detector (SPD-10AVP), a column oven (CTO-10ASVP), an autosampler (SIL-10ADVP), a degasser unit (DGU-14A) and a computer system with class VP software (Shimadzu, Kyoto, Japan). A discovery RP-Amide C16 column (150×4.6 mm, 5 µm; Sigma, USA) was used and the mobile phase was 50 mM $NaClO_4$ + 0.1% H_3PO_4 (Serada *et al*., 1997) at a flow rate of 1 mL min^{-1}. Samples were detected at 215 nm with the column oven set at 40°C. Briefly, a 300 mg erythrocyte pellet was homogenized in 2 mL, 5 mM EDTA, 50 mM $NaClO_4$, 0.1% H_3PO_4 buffer. Proteins were precipitated by adding 500 µL of 10% metaphosphoric acid (Yoshida, 1996). The samples were vortexed for 20 sec and centrifuged at 8000×g for 10 min at 4°C. The supernatants were transferred to autosampler vials for HPLC. Quantification was carried out by external standardization using Class VP software. The results were expressed as µg g^{-1} wet cell pellet.

Determination of lipid-soluble vitamins in serum and erythrocytes: Serum samples (200 µL) or 300 mg erythrocyte pellets were homogenized in 3 mL acetonitrile/methanol (3.1, v v^{-1}) and the samples were vortexed for 30 sec and centrifuged at 6000×g for 10 min at 4°C. Supernatants were transferred to autosampler vials for HPLC. Acetonitrile/methanol (75.25, v v^{-1}) was used as the mobile phase at a flow-rate of 1 mL min^{-1}. The temperature of the analytical column was kept at 40°C. A Supelcosil™ LC 18 DB column (250×4.6 mm, 5 µm; Sigma, USA) was used. Retinol (vitamin A) and retinol acetate were detected at 320 nm and vitamins D_2, D_3 and K_1, α-tocopherol, α-tocopherol acetate and α-tocopherol at 215 nm. Individual vitamins were identified by frequent comparison with authentic external standard mixtures analyzed under the same conditions. Quantification was carried out by external standardization using Class VP software. The results were expressed as µg g^{-1} wet cell pellet for erythrocytes and µg dL^{-1} for serum.

Cholesterol in serum and erythrocytes: Serum samples (200 µL) or 300 mg erythrocyte pellets were extracted in 3 mL acetonitrile/isopropyl alcohol (70:30, v v^{-1}) and the mixtures were vortexed for 30 sec and centrifuged at 6000×g for 10 min at 4°C. Supernatants were transferred to autosampler vials for HPLC. Acetonitrile-isopropanol (70:30 v v^{-1}) was used as mobile phase at a flow rate of 1 mL min^{-1} (Bragagnolo and Amaya, 2003). A Supelcosil LC 18™ DB column (250×4.6 mm, 5 µm) was used. Samples were detected at 202 nm and the column oven was set at

40°C (Katsanidis and Addis, 1999). Quantification was carried out by external standardization using class VP software. The results were expressed as μmol g^{-1} wet cell pellet for erythrocytes and μmol dL^{-1} for serum samples.

Vitamin C and MDA: Serum samples (200 μL) or 300 mg erythrocyte pellets were homogenized in 2.5 mL of 5 mM 1-hexane sulphonic acid sodium salt + 0.1 H_3PO_4 buffer. Proteins were precipitated by adding 0.5 mL of metaphosphoric acid (5% w v^{-1}) and the samples were centrifuged at 10,000 g for 5 min. The supernatants were transferred to autosampler vials for HPLC. A Discovery RPAmide16 (150×4.6 mm, 5 μm, Sigma, USA) column was used. The mobile phase was 5 mM 1-hexane sulphonic acid sodium salt + 0.1 H_3PO_4 buffer with acetonitrile (90 + 10%) at a flow rate of 1 mL min^{-1}. Samples were detected at 244 nm and the column was maintained at 37°C. Quantification was carried out by external standardization using class VP software. The results were expressed as nmol for MDA and μg for VC.

RESULTS

The serum cholesterol level was increased in the $KBrO_3$ group ($p<0.01$) but there was no difference between the control and C + $KBrO_3$ groups ($p>0.05$). MDA was decreased in the C + $KBrO_3$ group but there was no difference between the control and $KBrO_3$ groups ($p>0.05$). The α-tocopherol levels in the $KBrO_3$ and C + $KBrO_3$ groups were higher than the control ($p<0.05$, $p<0.01$, respectively). The α-tocopherol acetate, δ-tocopherol, retinol and vitamin D_3 levels did not differ among the groups ($p>0.05$). However, the vitamin C and D_2 levels in the $KBrO_3$ and C + $KBrO_3$ groups were higher than in the control group ($p<0.001$) (Table 1).

In the erythrocytes; GSH and GSSG were lower in the $KBrO_3$ and C + $KBrO_3$ groups than the control group ($p<0.05$). However, the GSH/GSSG ratio was high in the $KBrO_3$ group; there were no differences between the control and C + $KBrO_3$ groups Cholesterol did not differ between the control and C + $KBrO_3$ groups but was lower in the $KBrO_3$ group ($p<0.05$). MDA were increased in the $KBrO_3$ group ($p<0.05$), but there was no difference between the control and C + $KBrO_3$ groups ($p>0.05$) The vitamin D_3 level was lower in the C + $KBrO_3$ group than the $KBrO_3$ and control groups ($p<0.05$). α-tocopherol acetate and α-tocopherol were lower than control levels in the $KbrO_3$ and C + $KBrO_3$ groups ($p<0.001$). Vitamin K_1 was the same in the control and C + $KBrO_3$ groups and decreased in the $KBrO_3$ group ($p<0.01$) (Table 2).

Table 1: Biochemical parameters in the serum samples

Biochemical parameters	Control	$KBrO_3$	C+ $KbrO_3$
Retinol (μg dL^{-1})	37.55±2.49[a]	40.04±1.62[a]	37.91±1.57[a]
δ-tocopherol (μg dL^{-1})	25.82±2.01[a]	29.80±1.94[a]	29.24±0.64[a]
Vitamin D2 (μg dL^{-1})	17.32±0.59	31.66±2.24[d]	29.24±0.65[d]
Vitamin D3 (μg dL^{-1})	47.66±1.76[a]	55.33±0.82[a]	57.41±2.73[a]
α-tocopherol (μmol dL^{-1})	2.21±0.04	2.54±0.07[b]	2.58±0.06[c]
α-tocopherol acetate (μg dL^{-1})	53.44±2.92[a]	45.62±4.18[a]	47.66±1.62[a]
Cholesterol (μmol dL^{-1})	42.21±1.42[a]	54.65±3.16[c]	45.76±2.64[a]
MDA (nmol dL^{-1})	850.50±41.76[a]	969.29±29.12[b]	530.12±28.91[d]
Vitamin C (μg dL^{-1})	15.86±1.39	38.62±3.08[d]	34.00±3.45[d]

Table 2: Biochemical parameters in the erythrocytes

Biochemical parameters	Control	$KBrO_3$	C+ $KbrO_3$
Vitamin D3 (μg g^{-1})	0.30±0.02[a]	0.29±0.03[a]	0.19±0.01[b]
α-tocopherol (μg g^{-1})	2.97±0.14	2.18±0.13[c]	1.81±0.06[d]
α-tocopherol acetate (μg g^{-1})	2.09±0.11	0.56±0.01[d]	0.40±0.01[d]
Vitamin K1 (μg g^{-1})	0.64±0.01[a]	0.23±0.001[d]	0.53±0.001[a]
Cholesterol (μmol g^{-1})	4.86±0.13[a]	4.09±0.08[b]	4.61±0.09[a]
MDA (nmol g^{-1})	60.04±1.57[a]	85.04±3.14[b]	66.08±1.72[a]
GSH (nmol g^{-1})	520.21±30.45	330.11±35.56[d]	120.13±10.14[d]
GSSG (nmol g^{-1})	80.07±5.11	20.01±2.18[d]	13.16±1.37[d]
GSH/GSSG	3.11	8.36	3.99

Values are the mean±SEM; ANOVA: one-way ANOVA p-value; a: $p>0.05$, b: $p<0.05$, c: $p<0.01$, d: $p<0.001$ using the post hoc Fischer's LSD test

DISCUSSION

Lipid peroxidation products are involved in some of the pathophysiological effects associated with oxidative stress in cells and tissues (Ostrowska *et al*., 2004; Komatsu and Hiramatsu, 2000). Aldehydes produced by lipid peroxidation are rather long-lived and can therefore, diffuse from the site of their origin to attack intracellular and extracellular targets (Esterbauer *et al*., 1991). They disrupt important structural and protective functions associated with biomembranes in various pathological events *in vivo* (Barclay, 1993).

We found that the MDA level in the serum and erythrocytes of $KBrO_3$ group were than the control and C + $KBrO_3$ groups. It has been reported that catechins suppress the formation of lipid peroxidation products in tissues and subcellular fractions (Ostrowska *et al*., 2004). Glutathione and MDA levels have been commonly used to assess antioxidant defenses and lipid peroxidation. MDA has also been extensively used to evaluate the extent of tissue and cell damage through lipid peroxidation (Mejia and Mares, 2002). Many transition metalions are also able to break down lipid hydroperoxides to trigger free radical-mediated lipid peroxidation (Aust *et al*., 1985). Therefore, the oxidative breakdown of the membrane polyunsaturated fatty acids is accompanied by the formation of secondary products. These compounds are highly reactive and may act as secondary toxic messengers of the primary free radical event (Mitchell and Petersen, 1997). In consequence, the probability of cell injury may increase (Tuma *et al*., 1987). Aldehydes generated during lipid peroxidation also form glutathione

conjugates, leading to a significant decrease in cellular glutathione concentration, which may partly account for the cytotoxicity of oxidative stress (Kinter and Roberts, 1996).

Lipid peroxidation is a degradative process causing functional abnormalities and pathological changes that lead to and exacerbate myriad chronic diseases (Willcox *et al*., 2003). Catechins may decrease the concentration of lipid free radicals and terminate the initiation and propagation of lipid peroxidation (Ostrowska *et al*., 2004). Because catechin derivatives are water-soluble antioxidants, they could reduce the mobility of the free radicals into the lipid bilayer as well.

In our results, the α-tocopherol level was higher in the C + $KBrO_3$ group than the control group. Catechins prevent the loss of the lipophilic antioxidant α-tocopherol by repairing tocopheryl radicals and by protecting the hydrophilic antioxidant ascorbate, which also repairs this radical (Ostrowska *et al*., 2004). Α-tocopherol is a major antioxidant in the plasma or serum for protection of low-density lipoproteins (Zhu *et al*., 1999). α-tocopherol scavenges active oxygen radicals in biological systems and protects unsaturated lipid from peroxidation, producing the α-tocopheroxyl radical (Mukai *et al*., 2005). Catechins may counteract a decrease in α-tocopherol by acting as antioxidants *in vivo* (Nanjo *et al*., 1993).

The present study has shown that the serum cholesterol level in the $KBrO_3$ group was higher than in the control and C + $KBrO_3$ groups. Oxidized cholesterol has various deleterious actions such as cytotoxicity, carcinogenicity, atherogenicity, inhibition of DNA synthesis, suppression of immune function and modulation of lipid metabolism both *in vitro* and *in vivo*. It has been reported that catechin inhibits cholesterol oxidation in lipoproteins through its radical scavenging action (Loest *et al*., 2001).

A correlation has been reported between the antiproliferative potency of flavonoids and their ability to inhibit the cellular accumulation of vitamin C: thus, quercetin and genistein are strongly antiproliferative and affect ascorbic acid accumulation. The inhibition is dose-dependent and can be observed after as little as 10 min incubation. Flavonoids with little antiprolifeative effect, naringenin and catechin, have little effect on ascorbic acid accumulation. Therefore, the antiproliferative properties of these molecules could be linked to their capacity to affect ascorbate accumulation (Osada *et al*., 2001). In our study, catechin partly inhibited the accumulation of vitamin C in serum.

Glutathione (GSH) level was low in both the $KBrO_3$ and C + $KBrO_3$ groups. GSH plays a key role in protecting cells against electrophiles and free radicals and it is a substrate or a cofactor for a number of protective enzymes such as GSHpx, the GSH S-transferases and glyoxalase (Kuo *et al*., 1997). In addition, GSH has several vital roles including antioxidation, maintenance of the intracellular redox state and modulation of the immune response and detoxification of xenobiotics (Navarro *et al*., 1997). With respect to cancer, glutathione metabolism can play both protective and pathogenic roles. It is crucial in the removal and detoxification of carcinogens and alterations in this pathway can have a profound effect on cell survival. However, by conferring resistance to a number of chemotherapeutic drugs, elevated levels of glutathione protect tumor cells in bone marrow, breast, colon, larynx and lung (Kuo *et al*., 1997). Reduced GSH can directly scavenge radicals and peroxides via mixed disulfide formation, or upon oxidization to GSSG. Various oxygen radical stresses deplete GSH and form GSSG in the short term (Kuo *et al*., 1997). In particular, a decreased mitochondrial GSH pool has been reported in rats and this may play an important role in pathogenesis (Balendiran *et al*., 2004). Catechins and gallic acid are poor inhibitors of GSH reductase, possibly owing to the absence of key structural features from the molecules. Inhibition of GSH-reductase by plant polyphenols and the consequent low level of reduced GSH may impair the detoxification of anticancer drugs by GSH conjugation and sensitize drug-resistant cells to chemotherapeutic agents (Lu, 1999). GSH is depleted by $KBrO_3$, suggesting an interaction (Chipman *et al*., 1998). Our results show that the decrease in erythrocyte GSH in the $KBrO_3$ treated groups was not prevented by catechin.

CONCLUSION

In conclusion, MDA levels in the sera and erythrocytes of ovariectomized Wistar rats were decreased by catechin administration. In addition, decrease of erythrocyte cholesterol was prevented by catechin treatment. However, the erythrocyte GSH levels were low in both the catechin and $KBrO_3$ treated groups.

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