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EFFECT OF GREEN TEA EXTRACT AND VITAMIN C ON OXIDANT OR ANTIOXIDANT STATUS OF RHEUMATOID ARTHRITIS RAT MODEL

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ABSTRACT

Elevated free radical generation in inflamed joints and impaired antioxidant system has been implicated in rheumatoid arthritis (RA). Green tea extracts (GTE) have been shown to reduce inflammation in inflammatory arthritis murine model. This study investigates possible mechanisms by which vitamin C and GTE protect joints in RA rat model. This study included forty adult male rats that were divided into four groups (10 rats each); control group, collagen II induced RA group (CII), CII treated with vitamin C (CII + Vit C) and CII treated with GTE (CII + GTE) in physiology laboratory, Assiut University, Egypt. After 45 days of treatment, plasma levels of lipid peroxides (LPO), nitric oxide (NO), ceruloplasmin (CP), superoxide dismutase (SOD), uric acid (UA) and glutathione (GSH) were detected using colorimetric methods, PGE_2 using ELISA and copper (Cu) and zinc (Zn) using spectrometer. In CII group, levels of LPO, NO, PGE_2, UA, CP, Cu were higher while SOD, GSH, Zn were lower than controls. In groups treated with vitamin C and GTE, levels of SOD, GSH were increased while levels of LPO, NO, PGE_2, Cu, CP were decreased compared with CII group. Levels of UA were decreased and Zn increased in GTE treated group compared with CII group. GTE treated group showed higher Zn and low Cu levels compared with vitamin C treated group. This study suggests proper GTE and vitamin C intake may effectively normalize the impaired oxidant/ antioxidant system and delaying complication of RA.

KEY WORDS

Rheumatoid arthritis, Green tea extract, Vitamin C, Oxidant, Antioxidant.

INTRODUCTION

Rheumatoid arthritis (RA) is a polyarticular autoimmune disease affecting about 1% of the adult population (1). The process of disease progression is characterized by hyperplasia of synoviocytes, mainly of synovial fibroblasts, resulting in bone and joint destruction (2). The murine model of collagen-induced arthritis (CII) has been used extensively to improve our understanding of autoimmune-mediated arthritis and to identify potential new therapeutic agents to treat RA (3). This disease model is an Ag-induced arthritis that is cartilage restricted.

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Several lines of evidence suggest that oxidative stress, associated with the generation of free radicals, and insufficiency of antioxidant defense systems can be resulted in pro-oxidant/antioxidant imbalance and joint damage in RA (4). Pro-inflammatory cytokines such as interleukin-1beta (IL-1β) and tumor necrosis factor alpha (TNF-α) are involved in the formation of toxic peroxynitrite by increasing the activity of nitric oxide synthase (NOS) enzyme. Nitric oxide (NO) is potent inflammatory mediator because of its strong reactivity with oxygen, superoxide and iron-containing compounds (4). Prostaglandins are well known as proinflammatory mediators, and inhibition of cyclooxygenase (COX) has long been used in the management of joint inflammation. Levels of prostaglandin E_2 (PGE_2) are increased in various states of inflammation (5).

The anti-inflammatory effects of copper (Cu) and zinc (Zn) have been documented in animals (6) and humans (7). Cu and Zn are constituents of the superoxide-dismutase (SOD)

enzyme, which performs intracellular antioxidant functions (8). Zn is indispensable in many steps of inflammatory reactions as prostaglandin biosynthesis, stimulation of lymphocytes and immune response and scavenging of toxic free oxygen radicals. Zn is likewise an important element in collagen tissue formation and bone metabolism (7). It is reported that 30 to 50% increases in serum Cu level during an acute phase reactions response triggered by release of IL-1 and ceruloplasmin (CP). The latter is a potent antioxidant extracellular enzyme increases during acute phase reactions in order to scavenge toxic free oxygen radicals (7).

The identification of common dietary substances capable of affording protection or modulating the onset and severity of arthritis may have important health implications. Extensive studies in several animal models in the past 2 decades have verified the antioxidant, anti-inflammatory, and antioncogenic properties of a polyphenolic mixture derived from green tea (*Camellia sinensis*). The most abundant of the polyphenolic compounds in green tea is epigallocatechin gallate (EGCG), with other catechins such as epicatechin (EC), epigallocatechin (EGC) and epicatechin gallate (ECG) also present (9). Haqqi et al demonstrated that oral administration of a polyphenolic fraction from green tea can ameliorate inflammation in a murine model of inflammatory arthritis (10). In studies using human chondrocytes derived from osteoarthritis cartilage, it had showed that EGCG is effective inhibitor of production of as nitric oxide (NO) and PGE_2 by transcriptional and translational regulation (11). Mohamadin and his colleagues found the supplementation of green tea extract (GTE) attenuates cyclosporine A-induced oxidative stress in rats (12). Undoubtedly, vitamin C was shown to be the most effective antioxidant in preventing isoprostane formation. Ascorbic acid has also been shown to be an effective antioxidant with other biomarkers of lipid peroxidation, such as, cholesterol ester hydroperoxides (13).

Various forms of antioxidant therapy have demonstrated promising results in experimental RA models (14). Accordingly, the present study was aimed to evaluate the physiological effect of dietary intake of vitamin C and GTE on indices of oxidative stress of rat model of RA. The plasma levels of oxidative stress markers as lipid peroxides (LPO), NO, glutathione (GSH), SOD, CP, uric acid (UA), Cu and Zn were determined. Moreover, the effect of vitamin C and GTE on plasma levels of PGE_2 were also investigated.

MATERIALS AND METHODS

Chemicals: L-Ascorbic acid, thiobarbituric acid, reduced glutathione, naphthylenediamine dihydrochloride, sulphanilamide, sodium nitrite, sodium azide, 5,5-dithio bis (2-nitrobenzoic acid), epinephrine and p-phenylene diamine dihydrochloride, complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) were fine grade and obtained from Sigma-Aldrich (St. Louis, MO, USA).

Animal treatment: Forty healthy adult male albino rats *(Rattus norvegicus)* with average body weight (150–170 gms) were utilized for this study. They were obtained from animal house in Faculty of Medicine, Assiut University, Assiut, Egypt. The Ethical Committee of Assiut University approved all animal procedures. The animals were conditioned at room temperature at natural photoperiod for 1 week before the start of experiment. A commercial balanced diet and tap water ad libitum were provided. The duration of experiments was 45 days. The animals were divided into 4 groups (10 rats each) as following; 1. Control group served as negative Control; 2. Collagen II induced arthritis group (CII) served as positive control. Bovine collagen type II (Chondrex) was dissolved in 0.01N acetic acid and emulsified in an equal volume of CFA containing 1mg/ml heat-killed *Mycobacterium tuberculosis* (Sigma-Aldrich). Arthritis was induced by the initial immunization with 100µg/100µl emulsion by an intradermal injection in the base of the tail. Twenty-one days later after initial immunization, the rats received a boost intradermal injection (base of tail) of 100µg/100µl of bovine CII emulsified in IFA (15). Rates were examined, beginning 3 wk after primary immunization, for signs of developing arthritis; 3. CII + vitamin C – treated (CII + Vit. C) group. Animals were injected by collagen II as group CII, and received vitamin C daily for 45 days via oral rout (50 mg/kg/day) beginning with the first day of adjuvant injection (16); 4. CII + GTE – treated (CII + GTE) group. Animals were injected with CII as group CII to induced arthritis, and received GTE orally for 45 days beginning with first day of adjuvant injection. The GTE was made as previously described (17) by soaking 15g of instant green tea powder in 1L of boiling distilled water for 5 minutes. The solution was filtered to make 1.5% GTE. This solution was provided to rats as their sole source of drinking water.

The animals of different groups were sacrificed under light anesthesia 1 day after the end of the treatment. The blood samples from all groups were collected from the orbital vein in heparinized tubes at end of experiment (45 days) and were centrifuged at 5000 rpm for 10min for plasma separation. The plasma sample was divided into aliquots and kept at -20°C until biochemical analyses.

Biochemical analysis: The plasma level of LPO was

measured as thiobarbituric acid reactivity (TBARS). The product of the reaction between malondialdehyde and thiobarbituric acid was measured (18). Nitric oxide plasma level was determined as total nitrite after deproteinzation with zinc sulphate ($ZnSO_4$) (30%), nitrate reduction with cadmium (activated by 2 % HCL) and color developed by reaction with Griess reagent (1% sulfanilamide/ 0.1% naphthyelthylene diamine diHCL, w/v in 2.5% H_3PO_4) was recorded at 550nm against reagent blank using sodium nitrite as standard (19). GSH levels were determined chemically (20). SOD activity was determined according to its ability to inhibit the autooxidation of epinephrine at alkaline medium (21). CP activity was determined using a para-phenylenediamine dihydrochloride method (22). Uric acid level was determined by enzymatic colorimetric method (23). PGE_2 plasma level was detected using ELISA kit (Cat NO. KGE004, R&D system GmbH, Germany). The minimum detectable plasma level of PGE_2 was 27.5 pg/ml. Plasma samples were diluted with deionized water (0.5:4.5 v/v), Cu and Zn levels were then measured with an atomic absorption/flame emission spectrophotometer (Schimadzu Seisakusho LTD, model AA-630-02, Japan), using an air-acetylene flame and hollow cathode lamps. Standards were obtained from Buck Scientific, NY, USA. The lamp current (mA), the wave length (nm) and standard concentration (lot number) were for Cu {10 mA, 324.7 nm, 996 µg/ml Cu in 2% HNO_3 (lot # 9805K)}, for Zn {10 mA, 213.9 nm, 999 µg/ml Zn in 2% HNO_3 (lot # 9711V)}. The concentration of trace elements (Zn and Cu) was expressed as µg/mL.

Statistical analysis: The results are expressed as mean ± standard error (SE). Differences between groups were assessed by one-way ANOVA using the SPSS version 10 software packages for windows. The level of significance was accepted with p <0.05. Correlation was made between variant using Pearson correlation.

RESULTS

Table 1 shows the comparison of the effect of green tea extract and vitamin C on the plasma levels of LPO and NO among different studied groups. Levels of LPO and NO were significantly elevated in CII (P<0.000 for both) while LPO only was significantly elevated in CII + Vit C treated group (P<0.05) compared to controls. The levels of LPO and NO were reduced in both vitamin C (P<0.05, P<0.000) and GTE treated groups (P<0.01, P<0.000) in comparison with CII group.

Table 2 showed that in CII group, plasma levels of GSH, SOD and Zn were significantly lower (P<0.000 for all) while, UA, Cu and CP were significantly higher (P<0.000 for all) than negative control group. In both vitamin C and GTE treated groups, the levels of GSH, SOD and Zn (P<0.000, P<0.05, P<0.000 and P<0.001, P<0.05, P<0.000) were significantly increased but Cu and CP levels were significantly decreased (P<0.05, P<0.000 and P<0.000, P<0.000) in comparison with CII group. In both Vit C and GTE treated groups, levels of SOD (P<0.001 for both) and Zn (P<0.000, P<0.01) were significantly lower while levels of UA (P<0.01 and P<0.05) and Cu (P<0.000 and P<0.01) were higher than negative controls. In Vit C treated group, CP was higher (P<0.05) than untreated controls. In GTE treated group, plasma levels of Zn increased (P< 0.000) while Cu decreased (P<0.05) compared to Vit C treated group.

Figure 1 shows that plasma levels of PGE_2 were significantly higher (P<0.000) in CII treated group (683.000±43.190 pg/ml) than control group (192.500± 12.200 pg/ml). The plasma levels of PGE_2 were significantly reduced in vitamin C (329.400 ± 74.380 pg/ml) and GTE (305.400± 57.210 pg/ml) treated

Table 1: Comparison of the effect of vitamin C and green tea extract (GTE) on the plasma levels of lipid peroxide and nitric oxide among different studied groups

Parameters	Controls (n=10)	CII-treated Group (n=10)	CII + vitamin C-treated group (n=10)	CII + GTE-treated group (n=10)
Lipid peroxide (nmol/ml)	3.218 ± 0.441	8.000 ± 1.193	5.626 ± 0.526	5.182 ± 0.485
Significance		P<0.001	P<0.05 ^{1}P<0.05	P>0.05 ^{1}P<0.01 ^{2}P>0.05
Nitric oxide (ng/ml)	3.619 ± 0.215	8.520 ± 1.248	4.629 ± 0.091	5.275 ± 0.151
Significance		P<0.001	P>0.05 ^{1}P<0.001	P>0.05 ^{1}P<0.001 ^{2}P>0.05

Data are expressed as mean ± SE. CII group: collagen II -induced RA group; CII + Vit. C group: CII group treated with vitamin C; and CII + GTE: CII group treated with green tea extract. P significance versus controls, ^{1}P versus CII treated group, ^{2}P versus vitamin C- treated group.

Table 2: Comparison of the effect of vitamin C and green tea extract (GTE) on the studied oxidant/ antioxidants among different groups

Parameters	Controls (n=10)	CII-treated Group (n=10)	CII + vitamin C-treated group (n=10)	CII + GTE-treated group (n=10)
Glutathione (nmol/ml) Significance	4.265 ± 0.249	2.749 ± 0.306 P<0.001	4.330 ± 0.366 P>0.05 ^{1}P<0.001	4.242 ± 0.184 P>0.05 ^{1}P<0.001 ^{2}P>0.05
Superoxide dismutase (U/ml) Significance	344.700 ± 41.220	109.800 ± 18.720 P<0.001	197.800 ± 14.380 P<0.001 ^{1}P<0.05	195.900 ± 30.450 P<0.001 ^{1}P<0.05 ^{2}P>0.05
Uric acid (mg/dl) Significance	4.319 ± 0.139	8.422 ± 0.853 P<0.001	6.948 ± 0.755 P<0.01 ^{1}P>0.05	6.417 ± 0.600 P<0.05 ^{1}P<0.05 ^{2}P>0.05
Copper (µg/ml) Significance	2.374 ± 0.098	3.645 ± 0.166 P<0.001	3.268 ± 0.064 P<0.001 ^{1}P<0.05	2.892 ± 0.083 P<0.01 ^{1}P<0.001 ^{2}P<0.05
Ceruloplasmin (mg/dl) Significance	95.860 ± 11.320	225.500 ± 13.090 P<0.001	166.700 ± 13.070 P<0.05 ^{1}P<0.001	103.600 ± 11.960 P>0.05 ^{1}P<0.001 ^{2}P>0.05
Zinc (µg/ml) Significance	3.662 ± 0.982	0.616 ± 0.245 P<0.001	0.859 ± 0.346 P<0.001 ^{1}P>0.05	4.802 ± 1.463 P<0.01 ^{1}P<0.001 ^{2}P<0.001

Data are expressed as mean ± SE. CII group: collagen II -induced RA group; CII + Vit. C group: CII group treated with vitamin C; and CII + GTE: CII group treated with green tea extract. P significance versus controls, ^{1}P versus CII treated group, ^{2}P versus vitamin C- treated group.

groups compared to CII-treated group (P<0.001 for both). No significance difference was found between Vit C and GTE treated groups or between them and controls.

In CII group, a positive correlation was found between NO and both LPO (r= 0.717, P<0.05) and PGE_2 (r= 0.694, P<0.05). Meanwhile, in CII + Vit C group, a negative correlation was found between NO and glutathione (r= -0.778, P<0.01).

DISCUSSION

Rheumatoid arthritis is an autoimmune disease that causes chronic inflammation of the joints and tissue around them with infiltration of macrophages and activated T cells. The pathogenesis of this disease is linked predominantly with the formation of free radicals at the site of inflammation and decreased antioxidant levels, which may worsen the symptoms of disease (4). In the current work, RA rat (CII) group showed significant increased in plasma levels of LPO, NO, PGE_2, uric acid, copper and ceruloplasmin but significant decreased in levels of glutathione, superoxide dismutase and zinc compared with control group. Fermor and his colleagues suggested that many factors such as inflammation and mechanical loading in RA can lead to increased production of inflammatory mediators such as NO and PGE_2 (24). Similarly, previous studies (14) reported increased in plasma levels of LPO and NO but decrease in SOD activities in RA. IL-1beta has been shown to induce production of NO by synovial cells and chondrocytes, which leads to increased vasodilation, permeability and cartilage resorption in arthritic joints (25), inhibits proteoglycan synthesis, increases susceptibility to other oxidants/oxidative stress, modulates activity of metalloproteinases and induces apoptosis in human chondrocytes (26). The above data proposed antioxidant therapy strategies for the prevention and treatment of RA.

In this study, elevated levels of PEG_2 in RA group compared to control. Also, a positive correlation between NO and PGE_2 was found in RA. Similarly, other studies reported increased in PEG_2 levels in RA (24). PGE_2 enhances the susceptibility of human chondrocytes to NO-induced apoptosis and potentiate the effects of other mediators, such as bradykinin

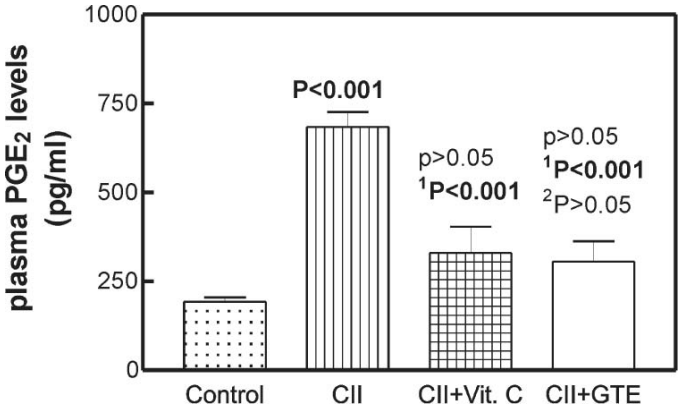

Figure 1: Effect of vitamin C (Vit C) and green tea extracts (GTE) on the plasma levels of prostaglandin E_2 ($PGE_{2)}$ among different studied groups. P significance versus controls, ^{1}P versus CII treated group, ^{2}P versus vitamin C- treated group.

and IL-1beta, which induce vasopermeability (26). The decreased antioxidant status of RA patients has been explained either by excessive need for antioxidants due to the inflammatory processes itself or decreased antioxidant nutrient intake (27).

Green tea is one of the most commonly consumed beverages in the world with no reported side effects. Catechins of green tea are hypothesized to help protect against RA by contributing, along with antioxidant vitamins (i.e., vitamins C and E) and enzymes [i.e., superoxide dismutase (SOD) and catalase], to the total antioxidant defense system (28). In this study, administration of Vit C and green tea extract, leads to reduction of plasma levels of LPO, NO, CU, CP, PGE_2 and elevation of glutathione and SOD compared to RA. The levels of NO, GSH, PEG_2 in both Vit C and GTE returned back to normal. Meanwhile, in GTE treated group levels of LPO and CP and in Vit C treated group, levels of UA returned back to normal. In this respect, Rennie et al, found that vitamin C supplementation increase levels of antioxidants and decrease LPO along with improved symptoms of RA (29). The reduction of NO and PGE_2 by GTE in RA were shown in some studies (11). Previous studies reported that intake of GTE increases the activity of SOD in serum and the expression of catalase in the aorta, enzymes implicated in cellular protection against reactive oxygen species (28). This action is combined with direct action on oxygen species by a decrease in plasma levels of nitric oxide (26). These results suggest that catechins could have a direct (antioxidant) or indirect (increase of activity or expression) effect. Thabrew and his coworkers indicated increases in serum SOD activity in RA treated with antioxidant herbal preparations resulted either from transcriptional activation of these enzymes or removal of oxidative stress (30).

Studies conducted using EGCG found that in human chondrocytes derived from osteoarthritis (OA) cartilage, EGCG inhibited the transcription nuclear factor kappaB (NF-kB) in conjunction with IL-1b-inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX2), resulting in reduction of NO and PGE_2 in vitro (11). It has also been shown that EGCG selectively inhibits the IL-1b-induced phosphorylation of c-Jun-N-terminal kinase (JNK) p46 isoform resulting in lower levels of phospho-c-Jun and DNA-binding activity of activation protein-1 (AP-1), a transcription factor implicated in the inflammatory response, in human OA chondrocytes (31). This is important since JNK kinase is a prime culprit in inflammatory and degenerative diseases (32). The significant decrease of PGE_2 in rheumatoid rats treated with GTE may be due to inhibition of COX2 activity (33).

In this study, treatment with GTE reduced cupper and raised zinc levels than Vit C treated group. Previous study reported that EGCG possesses antioxidant activity higher than chain-breaking antioxidants such as vitamin E and vitamin C (34).

The levels of CP and Cu were significantly elevated in RA rats than control group which reduced markedly after treatment with Vit C and GTE. Cu was significantly reduced in GTE compare to Vit C treated groups. Similarly, many investigators found the plasma levels of CP and Cu were significantly elevated in RA (35). Ninety percent of the serum copper is incorporated to the ceruloplasmin, which is one of the main extracellular antioxidants (36). Therefore, the increase of serum copper may be due to increase of ceruloplasmin, whose role in adjuvant arthritis is to neutralize free oxygen radicals, mainly anion superoxide in an attempt to stop the process of turning chronic (37). Increased levels of CP observed in the present study may be related to its scavenging action of superoxide radicals that are generated during the inflammatory process of RA.

Zinc is metal antioxidant that has a stabilizing effect on membranes possibly by displacing bound transition metal ions and thereby preventing peroxidation of membrane lipids (38). The levels of Zn in the current study were significantly lower in RA rats. Moreover, in groups treated with GTE, levels of Zn elevated compared to RA and Vit C treated group but levels did not reached normal. Previously, Tuncer et al found plasma zinc levels are decreased significantly in RA which may be caused by elevated IL-1 (8). With acute inflammation, Zn may move into the liver and the reduced plasma concentration may not be indicative of overall deficiency (39, 40). During inflammatory processes, zinc increase seems to occur in the liver and in the injured tissue, such as synovial fluid of arthritic

patients. As zinc is a cofactor for the protein and nucleic acid synthesis, it is conceivable that a portion of the accumulated zinc in the liver is involved in the increased synthesis of acute phase proteins. The interleukin-1, produced by stimulated macrophages, increases metallothionein-mediated hepatic uptake (39). The increased metallothionein synthesis is necessary for the protein synthesis of the acute phase. In RA, decreased absorption and hepatic accumulation were described, after a zinc tolerance test (41).

GSH plays an important role in the protection of cells and tissue structures. Its role includes detoxication of xenobiotics, free radicals, peroxides and regulation of immune function. In this study GSH level was significantly decreased in RA. In groups treated with Vit C and GTE, levels of GSH elevated to reach normal levels. Low levels of GSH in RA had been reported (42). In addition, Kraus and his coworkers found that Zn-deficient rats have lowered GSH concentrations (43). This finding may explain the reduction of plasma level of GSH in RA in our study. Moreover, Miesel and Zuber suggested that the participation of xanthin oxidase in the depletion of serum GSH in RA (44).

Uric acid is considered as one of non enzymatic antioxidants, but increased production of uric acid means increased free radical production due to activation of the xanthine oxidase enzyme system (45). The levels of uric acid were significantly elevated in our RA rats which were significantly reduced in group treated with GTE compared to RA group. Smolenska and his colleagues found high levels of uric acid in RA (46). Forrest et al suggested that hyperuricemia may enhance some aspects of rheumatoid inflammation and modulate rheumatoid autoimmunity (47). Reaction of uric acid with free radicals, such as hydroxyl radical results in allantoin production suggesting that uric acid acts as a free radical scavenger and thus is converted to allantoin. Increased allantoin levels suggest the possible involvement of free radicals in rheumatoid arthritis (48).

In conclusion, our study suggests proper antioxidant intake management may reduce free radical generation and improve antioxidant status in RA. GTE, and vitamin C may effectively normalize in different degrees the impaired the oxidant/ antioxidant system and may be useful in delaying the complication of RA. Moreover, they display considerable potency in anti-inflammatory action and have prominent effects on RA by decreasing PGE_2 level in RA rat model. With the global availability of green tea, its low cost and proven lack of toxicity, green tea catechins or compounds derived from them could one day be useful as a conventional medicine or as effective adjunct therapies for the treatment of RA. Thus, there is experimental evidence to support further studies to investigate the anti-oxidative effects of green tea at the molecular level.

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