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Glutathione, Vitamin D and Antioxidant Status in the Blood of Patients with Colorectal Cancer: A Pilot Study

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Abstract: A case-control study was conducted to evaluate some parameters of antioxidant and nutritional status in patients with colorectal cancer (CRC) and healthy controls. The present study was based on 25 patients with diagnosed CRC, ranging in age from 35 to 76 years with the mean age of 51.3±11.3 years. There were healthy volunteers (age-and sex-matched) serving as the control subjects. We measured reduced glutathione (GSH, a marker of antioxidant response) in erythrocytes along with the examination of plasma antioxidants (vitamin E, A and beta-carotene) and vitamin D. The results showed that the levels of vitamin D and GSH in the blood of the patients with CRC were significantly lower than the control. However, there was no significant difference in the level of vitamin A and beta-carotene in the blood of the patients with CRC as compared to control group. Interestingly, our results showed a significant increase in the blood level of Vitamin E in patients with CRC as compared to control. Our results indicate significant role of oxidative-induced injury in the CRC carcinogenesis. The decreased concentrations of vitamin D and GSH in CRC cases as compared to controls could be used as predictors for having CRC. However, no association between vitamin A and beta-carotene and CRC was detected. Higher plasma concentration of vitamin E was noticed in CRC cases comparing to controls.

Key words: Glutathione, vitamins D, E and A, beta-carotene, colorectal cancer

INTRODUCTION

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells which can lead to death. Cancer is caused by both external and internal factors, which act together, or separately to initiate or promote the development of cancer (American Cancer Society, 2012). In Jordan, cancer is considered the second cause of death and a major cause of morbidity and mortality. Colorectal cancer (CRC) ranked as the second cause for all new cancer cases among Jordanians; first among men and second among women according to the National Cancer Registry (2009).

There are a lot of nutritional and pathological factors, including low intake of fruits and vegetables (Van Duijnhoven and Bueno-De-Mesquita, 2009), high saturated fat consumption (Oba and Shimizu, 2006) and reactive oxygen species (ROS) involved in the process of CRC initiation and progression (Zalewski, 2004). It is known that ROS has been implicated in the pathogenesis of certain diseases, including cancer (Tsai *et al*., 2003). To control the overproduction of ROS, the cells protect themselves against oxidative damage by antioxidant mechanisms that help to lower ROS concentrations in the body. Different antioxidant systems including nonenzymatic antioxidants such as GSH, vitamin A, C, beta-carotene and E and various antioxidant enzymes will defend against ROS attacks (Ozgonul *et al*., 2009).

Oxidative stress due to damage by ROS is known to influence the response of patients to therapy and high levels of GSH cause drug resistance in the tumor tissue (Obrador *et al*., 2001; Carretero *et al*., 1999; Lai *et al*., 1991; Calvert *et al*., 1998). The measurements of erythrocytes GSH levels may be used as indicators of intracellular GSH levels. In addition, vitamin D was found to exert an anti-cancer activity by inhibiting angiogenesis and regulating cellular proliferation and differentiation (Yin *et al*., 2009).

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In the present study, the following parameters were assessed in the erythrocytes and plasma to elucidate the oxidant and antioxidant status in patients with newly diagnosed CRC before therapy. Erythrocyte GSH levels were estimated as an index of antioxidant status as well as plasma level of antioxidant vitamins such as vitamin A, E and beta-carotene.

MATERIALS AND METHODS

Subjects: Twenty five patients, newly diagnosed with CRC (prior to any treatment), were recruited from 5 large Jordanian hospitals that have oncology departments, from January 2011-December 2011. The study protocol was approved by the ethics committees of each hospital. Control group (38 participants) was selected from the community (hospital personnel, outpatients, visitors and accompanying persons) free of cancer and matched to patients with CRC in age, sex, occupation and marital status. All participants were free of diabetes mellitus, liver disease and rheumatoid arthritis. The sample size was 63 subjects (35 males and 28 females). Medical history of participants was collected. Written informed consent was obtained from all participants before their interview and blood withdrawal.

Anthropometric measurements: Body weight was measured to the nearest 0.1 kg, with minimal clothing and without shoes, using a calibrated portable scale. Height was measured to the nearest 1.0 cm with participants in the full standing position without shoes using a calibrated portable measuring rod (Lee and Nieman, 2010). Body mass index (BMI) was calculated as the ratio of weight in kilograms to the square of height in meters (Lee and Nieman, 2010).

Biochemical analysis: Biochemical analyses were carried out for 25 CRC patients (newly diagnosed and without any previous cancer therapy treatment) and 38 matched. Before recruitment, they were asked about consuming vitamin supplements and if one of the cases or controls taking any kind of vitamin supplementation he/she was excluded. A 10 mL fasting venous blood sample was taken for the analyses of plasma vitamins (A, E and D), beta-carotene as well as GSH level in erythrocytes.

Vitamin E, A, beta-carotene and vitamin D measurements: Plasma antioxidants (Vitamin E, A and beta-carotene) and vitamin D were measured. Blood samples were obtained by venous arm puncture in heparinized tubes and the plasma was separated by centrifugation at 2500 g for 15 min.

Vitamin E and A analyses: The analyses of vitamin A and E were performed on a reversed-phase High Performance Liquid Chromatography (HPLC) method. The analysis was carried out on Varian prostar. Separation of compounds was performed on C18 column (150 mm long, 4.6 mm internal diameter, 0.45 µm particle diameter) by using isocratic elution.

Sample preparation: Fifty microliters each of retinyl acetate and alpha-tocopheryl acetate working standards were added to 100 µL of plasma, then mixed vigorously on vortex-mixer for 10 s. One hundred microliters of hexane were added to the solution and mixed for 45 s on vortex. The solution was centrifuged at 800 g for 5 min, 75 µL of the hexane layer was transferred to test tube. The hexane was evaporated under a stream of argon. Finally, the lipid residue was dissolved in 25 µL of diethyl ether, mixed gently and 75 µL of methanol were added.

Method of analysis: One hundred microliters of the prepared solution was injected into the HPLC. Vitamins were eluted isocratically at a flow rate of 2.5 mL/min using methanol as the mobile phase. Ultraviolet absorbance was monitored at 280 nm wavelength. The retention time of vitamin A and E was 2.4 min and 6.2 min, respectively (Catignani *et al*., 1983). Repeated measures were done.

Beta-carotene analysis: Reversed-phase HPLC method was conducted to estimate plasma level of beta-carotene in controls and cases. The analysis was carried out on Varian prostar. Separation of beta-carotene was performed on C18 column (150 mm long, 4.6 mm internal diameter, 0.45 µm particle diameter) by using isocratic elution.

Sample preparation: To prepare the sample for beta-carotene analysis, 200 µL of ethyl alcohol were added to 200 µL of plasma and then mixed on vortex-mixer for 1 min. Two mL of hexane were added to the solution and shacked for 5 min. The solution was centrifuged at 4000 g for 2 min, hexane layer was transferred to test tube. The last 2 steps were repeated 3 times then the layers were mixed. The hexane was evaporated under a stream of argon. Finally, the residue were dissolved in 200 µL of solution of 50:50 methylene chloride and methanol and mixed gently.

Method of analysis: Beta-carotene was eluted isocratically at a flow rate of 1.7 mL/min using the mobile phase. The mobile phase consisted of 70% acetonitrile, 10% methanol and 20% methylene chloride. Ultraviolet absorbance was monitored at 464 nm wavelength. The injected volume of samples and standards was 100 µL and retention time of beta-carotene was 6.5 min (Rock and Swendseid, 1992). Each sample was analyzed in duplicate.

Vitamin D analysis: Vitamin D was analyzed using ELISA technique, 25-Hydroxy Vitamin D EIA. Mean and acceptable ranges were 33 (26-40) nmol/L and LOT number was 14211.

GSH status analysis: Plasma was separated by centrifugation at 2500 g for 15 min at 4°C. After separation of plasma, the buffy coat was removed and the packed cells washed twice with physiologic saline. A known volume of erythrocytes was hemolyzed with hypotonic phosphate buffer, pH 7.4.

Measurement of GSH levels in RBCs was based on that originally described by Anderson (1985). Estimation is based on the development of yellow color when 5, 5'dithio 2-nitrobenzoic acid (DTNB) is added to compounds containing sulphydryl groups. Fifty µL of each sample was mixed with 100 mL distilled water, 700 mL of 0.298 mM nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) dissolved in sodium phosphate buffer at pH 7.5 and 100 mL of 6 mM dithiobisnitrobenzoic acid (DTNB; Ellman's reagent). The assay was started by the addition of 50 mL GSH reductase (GR; 266 U/mL) and the rate of 5-thio-2-nitrobenzoate (TNB) formation was monitored at 412 nm, every 10 s for 2.5 min using a spectrophotometer at the Hashemite University.

Statistical analysis: Statistical analysis was performed with SPSS IBM-20 software. The significance level was set at $p<0.05$. Data were presented as mean±SD, mean±SEM and percentages. T-test was used to detect the significance of differences between patients and controls

RESULTS

Demographic characteristics of the study subjects are shown in Table 1. CRC and control groups had comparable baseline characteristics including age, gender, occupation, marital and smoking status. The mean±SEM of plasma level of beta-carotene, vitamin A, E and D is indicated in the Table 2. There was a statistically significant difference ($p<0.05$) in the level of vitamin E and D in CRC group (cases) compared to controls. Cases group showed a higher blood level of vitamin E compared to the controls, while controls showed higher vitamin D level than cases. On the other hand, the levels of plasma vitamin A and beta-carotene did not show any significant change between cases and controls group. As shown in Fig. 1, GSH concentration decreased of 1.7-fold in the cases group and this change was significant ($p<0.001$).

DISCUSSION

Our study shows that plasma level of vitamin E was significantly higher in CRC cases as compared to controls. This finding is contrary to many studies (Kabat

Table 1: Selected socio-demographic characteristics of controls and CRC cases participated in the study

Characteristic	Controls n = 38	Cases n = 25	p-value
Age (mean±SD)	53.9±10.88	51.3±11.3	0.56
Height (mean±SD)	1.68±9.2	1.67±8.0	0.498
Weight (mean±SD)	82.3±15.0	73.8±14.8	0.828
BMI (mean±SD)	28.6±7.4	26.3±4.8	0.587
Gender (%)			
Males	52.8	68.8	0.221
Females	47.2	31.2	
Marital status (%)			
Married	91.7	80.4	0.322
Single	0	6.3	
Divorced and widow	8.3	13.3	
Education (%)			
Illiterate	2.8	20	0.274
High school and below	41.6	46.6	
Above high school	55.5	33.4	
Tobacco use (%)			
Yes	14	12.5	0.754
No	86	87.5	
Health problems* (%)			
Yes	52.8	53.3	0.608
No	47.2	46.7	
Employed (%)			
Yes	44.4	47.8	0.505
No	55.6	52.2	
Family history for CRC (%)			
Yes	19.4	26.7	0.411
No	80.6	73.3	

BMI: Body mass index, Significance is at $p\leq0.05$

*Diseases other than diabetes mellitus, liver disease and rheumatoid arthritis

Table 2: Plasma level of vitamin A, E and D and beta-carotene (Mean±SEM) according to the occurrence of colorectal cancer

Parameter	Controls n = 38	Cases n = 25	p-value
Vitamin A (µg/L)	331.7±15.3	303.6±32.9	0.392
beta-carotene (µg/L)	86.1±2.3	87.6±4.1	0.729
Vitamin E (mg/L)	6.7±0.5	8.1±0.6	0.001
Vitamin D (nmol/L)	51.1±5.1	41.5±2.6	0.003

Significance is at $p<0.05$

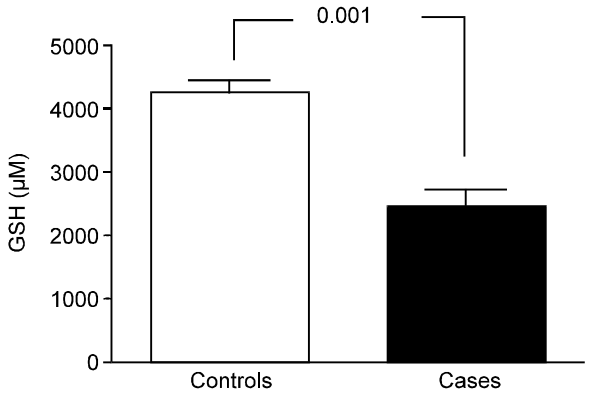

Fig. 1: Antioxidant GSH in CRC cases vs. controls. GSH content was determined on erythrocytes of CRC patients in comparison to controls group. Data are expressed±SEM ($p<0.05$)

et al., 2012; Skrzydlewska *et al*., 2005; Erhardt *et al*., 2003; Comstock and Bush, 1992; Nomura *et al*., 1985). Most of the studies found that patients with CRC had a lower level of vitamin E than controls although it was insignificant. Nomura *et al*. (1985) revealed that no protective effect of vitamin E against specific cancer (including CRC) was detected among their cases (285 patients) as compared to controls (302) (Nomura *et al*., 1985). In addition, Comstock *et al*. (1991) showed that while higher serum vitamin E levels had a protective effect on lung cancer, none of other sites showed any significant association (Comstock and Bush, 1992). It has been proposed that the antioxidant role of vitamin E can be attributed to its ability in quenching highly reactive lipid peroxide intermediate by giving hydrogen molecule and this prevents abstraction of hydrogen molecule from polyunsaturated fatty acid (Comstock and Bush, 1992). Also, the lower serum level of vitamin E among cancer patients could be due to impaired absorption or transport across the gastrointestinal tract (Bhagat *et al*., 2011). In this case, the explanation compared with our data may reside in the observation that vitamin E was not measured in plasma, but in the serum of the examinees (Kabat *et al*., 2012; Skrzydlewska *et al*., 2005; Erhardt *et al*., 2003; Comstock and Bush, 1992; Nomura *et al*., 1985). Another explanation is a possible defect of vitamin E utilization as an antioxidant to protect against CRC.

Regarding plasma levels of vitamins A and beta-carotene, no significant differences ($p = 0.392$ and 0.729, respectively) was detected between the two groups. Those findings are in agreement with many studies (Kabat *et al*., 2012; Erhardt *et al*., 2003; Nomura *et al*., 1985). Kabat *et al*. (2012) reported that serum antioxidants [alpha-carotene, beta-carotene, beta-cryptoxanthin, lutein+zeaxanthin, lycopene, alpha-tocopherol and gamma-tocopherol] measured at baseline generally showed no association with risk of colorectal cancer, although serum beta-carotene at baseline showed a non-significant inverse association with colon cancer alone (Kabat *et al*., 2012). Erhardt *et al*. (2003) revealed that the median plasma beta-carotene concentration also tended to be lower in the adenoma group (-25.5%), but the difference was not significant (Erhardt *et al*., 2003). In the stepwise multiple logistic regression, neither plasma beta-carotene concentrations nor plasma alpha-tocopherol concentrations were related to adenoma prevalence. The only type of cancer which beta-carotene can inhibit is lung cancer; an inverse significant association was reported in many studies (Fritz *et al*., 2011; Min and Min, 2014).

Plasma levels of vitamin D showed a significant ($p = 0.003$) lower concentration in CRC cases as compared to controls. Similar results documented by Giovannucci *et al*. (2010); they reported an inverse association between vitamin D status and CRC patients (Giovannucci, 2010). Several studies revealed that the anti-tumoural action of vitamin D in CRC relies on different mechanisms at the cellular level. Those mechanisms include inhibition of cell proliferation, sensitization to apoptosis, induction of epithelial differentiation and cell detoxification metabolism and inhibition of angiogenesis (Pereira *et al*., 2012). The combined effect of these mechanisms, in a cell-type-and cell-context-dependent manner, may determine the anti-tumoural action of vitamin D (Stubbins *et al*., 2012).

GSH is the major intracellular antioxidant that is likely playing an important role in protection against cancer development. GSH also is responsible for the detoxification of many carcinogens through Phase II conjugation; the maintenance of immune function by regulating mitogenic response and lymphocytic proliferation and gene expression and cellular differentiation, proliferation and apoptosis (Upadhya *et al*., 2004). In the present study, GSH is significantly ($p = 0.001$) decreased in patients with CRC as compared to controls. The decrease in the GSH levels may be due to the increased turnover of GSH for preventing oxidative damage in these patients (Ortega *et al*., 2011). Grubben *et al*. (2006) illustrated that low glutathione levels were correlated with high clinical risk for development of colon cancer (Grubben *et al*., 2006). This reduced level of GSH could be explained by different mechanisms. One of them is that in growing tumors, cysteine, whose concentration in blood is low, may become rate-limiting for GSH synthesis and cell growth; normally cystine is reduced to cysteine and used preferentially for protein GSH synthesis (Grubben *et al*., 2006).

Our results have proved that there was a profound disruption in the non-enzymatic level of antioxidants, such as vitamin E and GSH as well as vitamin D in CRC patients. The present results and the results of previous studies show that CRC is associated with oxidative stress.

Study limitations: The main limitations of our study are; number of cases is not representative and participants were selected conveniently.

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