Evaluation of the level of DNA damage and repair in human lymphocytes cultured in the presence of Beta- Carotene using comet assay (single cell gel electrophoresis)

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Abstract:

Background: Reactive oxygen species (free radicals) can cause various damage to different parts of the body, including the blood. Oxidative DNA damage can be measured in lymphocytes by various techniques which is a useful way to evaluate the degree of oxidative stress. The Comet assay is important technique for assess the damage or repair of DNA in human cultured lymphocytes.

Aim of the study: To assess the levels of DNA damage and to measure the proportion of the DNA cellular repair in human lymphocytes cultured in vitro conditions, the impact of the presence of Beta-Carotene by measuring the Comet tail moment.

Subjects and Methods: The study included 50 individuals aged between 20-50 years, during the period from12 October 2014 to 19 November 2015 and from healthy individuals, non-smokers, and non-conception of any type of vitamins before 1-2 weeks of sampling. Ten milliliters of hole blood sample taken to hepernized container, 10 samples (5 males, 5 females) to study the toxicity of different concentrations of Beta-Carotene (100, 10000) µg/ml on cultured lymphocyte by trazoleom assay; then take another samples (40) to assess the level of DNA damage in cultured lymphocytes by Comet assay in presence of the two different concentrations of Beta-Carotene (100, 10000) µg/ml, (20 individuals every concentration).

Results: There were a damage occur in DNA of the cultured lymphocytes by the effect of the presence of hydrogen peroxide, and there was repair occur by the presence of Beta-Carotene at the concentrations (100 and 10000) µg/ml, and also there were a significant change in the average of tail moment (in Comet assay) as an indicator of a positive effect for Beta-Carotene to protect DNA of the cultured lymphocyte cells.

Conclusions: This study demonstrated the protective effects of in vitro applications of Beta Carotene in different concentrations (100, 10000) µg/ml on DNA damage induced by H2O2 in lymphocyte cultures of healthy individuals, via Comet assay (tail moment), which showed that the most effective concentration of Beta-Carotene as antioxidant was in the concentration of 10000 µg.

Keywords: Beta Carotene, lymphocytes, comet assay.

Introduction:

To protect cells from active oxygen species, organisms have developed enzyme-dependent (superoxide dismutase, catalase, and glutathione peroxidase), and enzyme–independent (vitamins, uric acid, and glutathione) antioxidant defenses[1].

Antioxidant functions are associated with lowered DNA damage, and lipid peroxidation, or inhibited malignant transformation[2]. An imbalance between pro – and antioxidants in the intracellular microenvironment can produce oxidative stress[3]. Oxidative stress is considered to play a critical role in aging and the development of various diseases, including cancer and other degenerative diseases[1].

The comet assay is a simple and sensitive tool for measuring strand breaks of DNA in single cells. Different types of cells are embedded in a thin layer of agarose on a microscope slide and lysed with salt solution**.** The presence of breaks in DNA causes a relaxation in the super coiled loops of DNA in the nucleoid. When an electrical charge is passed through the gel, the relaxed areas of the DNA loops are pulled to the anode, forming a comet a tail, and the DNA in the nucleoid are the comet head. Comets are visualized by fluorescent microscopy, by using ethedium bromide as staining dye and the amount of DNA in the tail, relative to the head, is proportional to an amount of strand breaks, tail moment is defined as product of the tail length and the fraction of total DNA in the tail [4]**.**

Lymphocytes cells can be isolated from hole blood and incubated in vitro with an anti-oxidant, like Beta Carotene agent of interest prior to the comet assay, and the resulting DNA damage, or not can be measured. The effect of DNA after treated cells have been investigated, and assess the possible genoprotective or genotoxic effects. This application as a nutraceutical researches. The micro culture tetrazolium assay (MTT) was originally developed by Mosmann [5]. This method can be used to measure cytotoxicity, proliferation or activation for cultured lymphocytes in (100, 10000) µg/ml of Beta Carotene. The results can be read on a multiwall scanning spectrophotometer (ELISA reader) and show a high degree of precision, no washing steps are used in the assay[6].

Materials and methods:

Human subjects, cell culture, and treatments: Fifty individuals apparently healthy, age-compatible, nonsmoking volunteers (31 females and 19 males) were recruited, excluding anyone consuming a diet with supplements or taking prescribed medication. The study was approved by the local ethics committee. Venous blood samples were collected and lymphocyte cultures were set up after lymphocyte isolation with ficoll centrifugation. The

culture medium used was composed of RPMI1640 (CAPRICORN Scientific, Germen), containing10% (FCS) fetal calf serum (Sigma), Penicillin G solution (final concentration 0.1 mg/ml), L-Glutamine (BDH), and Streptomycin solution (final concentration 0.1 mg/ml). The lymphocyte suspension was utilized for cell culture according to the procedure described by Potter[7].The mixture of lymphocytes culture media was incubated in the Sterile incubator (Gallen kamp size one, model 1H-150, England), for 5 minite after added two concentrations (100, 10000) µg/ml of Beta Carotene (groups 1, 2), 5% H2O2 alone or as a mixture; for used for comet assay(20 individuals, every group), another (10 individuals,5mal,5femal)for MTT assay.

Preparation of Beta Carotene Solutions (Santa Cruz Biotechnology,Inc.,sc-202485): To prepare stock solution of Beta Carotene, that has the concentration of (10000 µg/ml), weighted 0.1 gm of the powder and put it in sterilized tube contained of (10 ml of DMSO solution added to it 2.5 µl Twen 80 solution supported from BDH / England for in vitro diagnostics), this solutions for accelerate solubility of Beta Carotene, from stock solution of Beta Carotene (10000 µg/ml) and by dilution method were prepared the solution of the concentration 100 µg/ml by added RPMI media for preparation of group 2.

Alkaline comet assay (alkaline single-cell gel electrophoresis)

Alkaline comet assay (SCGE) was performed in order to detect the level of genotoxicity in untreated(control) and treated lymphocyte cultured in presents of different concentrations (100, 10000) µg/ml of Beta Carotene. In brief, lymphocytes were resuspended in 0.5 mL of phosphate buffered saline (PBS), and 5 µL of cell suspension was mixed with 35 µL of 1% (w/v) low-melting-point agarose (LMPA; Sigma-Aldrich) and added to slides coated with 0.5% (w/v) normal-melting-point agarose (NMPA; Sigma-Aldrich). Cover slips were added and slides were incubated on ice packs until solidification of the agarose. Cover slips were then removed and 40 µL of 1% (w/v) LMPA was added to the slides. Slides were incubated in a lysis solution (2.5 M NaCl, 100 mM EDTA disodium salt, 10 mM Tris; pH 10) at 4°C in the dark for 2h. Slides were incubated in electrophoresis buffer (300 mM NaOH, 1 mM EDTA disodium salt; pH>13) in the dark for 20 min and electrophoresis was performed at 24 V (300 mA) for 30 min. After neutralization (0.4 M Tris; pH 7.5), slides were stained with 10mg/mL of ethedium bromide and observed under a fluorescence microscope (Olympus-Japan). A computerized image analysis system (Comet Assay IV, Perceptive Instruments, UK) was employed. Tail moment was used as the measure of DNA damage. A minimum of 4 SCGE slides were prepared for each treatment and, in total, 50 nuclei were analyzed per treatment [8],[9].

MTT assay: The cell viability percentage and inhibition percentage were evaluated by using methyl thiazolyl tetrazolium bromide (MTT, Sigma, USA) assay [5]**.**

In order to evaluate the variables in this study, using analysis of variance (ANOVA)in complete design. Differences between means have analyzed by least significant difference (LSD) at ($P \leq 0.05$) and expressed as mean±standard error of the mean(SEM), and t-test has been used to test the significant differences.

Results:

1- The Comet tail moment values for cultured lymphocytes treated with the 100 µg /ml concentration of Beta Carotene (group 1):

The results in table1 were showed the mean ±SEM tail moment values for the cultured lymphocytes treated with the100 µg/ml concentration of Beta Carotene, 5% H2O2and the control (group1). There was highly significant elevation in the mean tail moment value in the cultured lymphocytes treated with hydrogen peroxide, compared with this treated with Beta Carotene, and also with control, each one alone (LSD=1.407, $P \leq 0.05$). Also there were highly significant elevation in the mean tail moment value in the cultured lymphocytes treated with hydrogen peroxide, and these treated with the All (BC, H2O2).

2-The Comet tail moment values for cultured lymphocytes treated with the 10000 µg /ml concentration of Beta Carotene, (group 2):

The results were in table 2, presented that the mean±SEM of tail moment values for the cultured lymphocytes treated with the 10000µg/ml concentration of Beta Carotene,5% H2O2.There were highly significant elevation in the mean tail moment value in the cultured lymphocytes treated with hydrogen peroxide, compared with this treated with Beta Carotene, and also with control (LSD=4.295, $P \leq 0.05$). Also there were highly significant elevation in the mean tail moment value in the cultured lymphocytes treated with hydrogen peroxide, and these treated with the All (BC,H2O2). Figure1 showed a photographs of single cell gel electrophoresis (SCGE) stained with ethidium bromide, in group2 treatments.

3-The results MTT assay:

There were no toxic effect of Beta Carotene on cultured humane peripheral lymphocytes in both concentrations (100, 10000) µg/ml, in which the calculations are from these formulas: **Viability%**=[mean OD of test/mean OD of control)] x100, and **Inhibition%=(**1–mean OD of test/mean OD of control**)**×100, as shown in table 3.

Table 1: The Comet tail moment (µm) value of the cultured lymphocytes with different treatments, (group1).

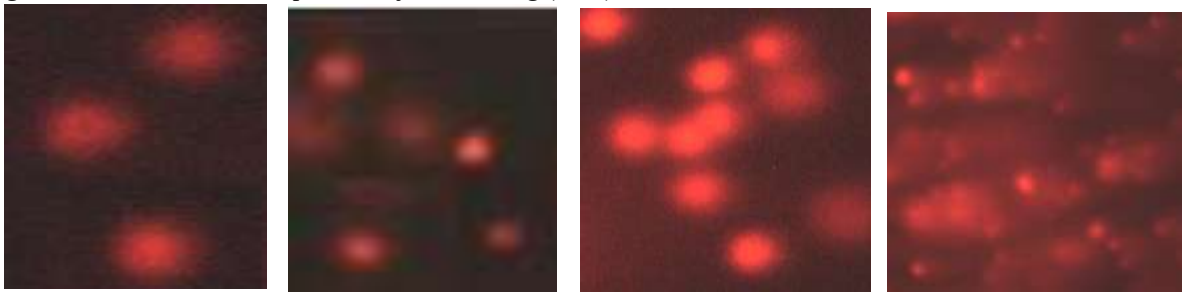
Factors	Treatments	Concentration 100 µg / ml (20 Samples)			
		Mean	±	SEM	**Sig.**
Tail Moment	Beta Carotene	1.547	±	0.234	bc
	H2O2	15.299	±	0.581	a
	ALL(BC,H2O2)	2.047	±	0.288	b
	Control	1.538	±	0.101	bc
P ≤ 0.05		**LSD =1.407**			

*Means in column, followed by similar letters (Sig.) are not significantly different; but the not similar letters are significant different; at 0.05 probability level, using (LSD) test

Table 2: The Comet tail moment value of the cultured lymphocytes with different treatments (group2).

Factors	Treatments	Concentration 10000 µg / ml (20 Samples)			
		Mean	±	SEM	**Sig.**
Tail Moment	Beta Carotene	0.309	±	0.060	c
	H2O2	22.997	±	0.846	a
	ALL(BC,H2O2)	11.881	±	0.617	b
	Control	1.913	±	0.159	c
P ≤ 0.05		**LSD =4.295**			

*Means in column, followed by similar letters (Sig.) are not significantly different; but the not similar letters are significant different; at 0.05 probability level, using (LSD) test.



a b c d

Figure 1: Photographs of single cell gel electrophoresis (SCGE) stained with ethidium bromide a,b,c,d, in group2, a-Cultured lymphocytes in All (BetaCarotene,H2O2), b-Cultured lymphocytes in Beta Carotene, c-Cultured lymphocytes in control, d-Cultured lymphocytes in H2O2,x400(Florescent Microscope).

Table 3: The values of interactions between viability percentage and inhibition percentage in different concentrations of Beta Carotene in MTT assay, (10 samples)

Factors	**Concentrations, Viability%, and Inhibition%**	**Beta Carotene**			
		Mean	**±**	**SEM**	**Sig.**
Interactions	**100 Viability%**	**124.000**	**±**	**0.115**	**d**
	100 Inhibition%	**-24.000**	**±**	**0.173**	**g**
	10000 Viability%	**150.000**	**±**	**0.231**	**a**
	10000 Inhibition5	**-50.000**	**±**	**0.577**	**g**
P ≤ 0.05		**LSD =1.161**			

* The letters express significant, and when they are seem express non-significant between these values.

Discussion:

Beta Carotene is one of the major antioxidants of fresh fruits and vegetables. The antioxidative properties of Beta Carotene have been implicated in the molecular basis for preventing several diseases, primarily owing to the decreased the oxidative stress in disease initiation and progression[10]. It has been demonstrated that Beta Carotene can suppress in vivo oxidative stress dependent lipid peroxidation[11].Many health claims have been made for natural compounds derived from vegetables, fruits and plants. This interest has increased the number of studies aiming to identify and characterize the biological effects of the active natural compounds [12]. Carotenoids, for instance, absorb excess energy from other molecules through a non-radioactive energy transfer mechanism; this is possible due to the presence of conjugated double bonds in their structures [13], and this characteristic may be responsible for the antioxidant activity related to carotenoids [14],especially by the ability to quench singlet oxygen molecules[15].The products of normal oxidative metabolism, potentially dangerous oxidants (free radicals) can damage cells and tissues

in a number of ways: by damaging biomolecules and cell components, by triggering the activation of specific signaling pathways, by creating toxic products, by altering gene expression and enzyme activity, and by disrupting normal repair mechanisms. Antioxidants prevent free-radical-induced tissue damage by preventing the formation of radicals, scavenging them, or promoting their decomposition. Normal diets including antioxidants and micronutrients help cells to decrease the deleterious effects of oxidative stress. Due to their high antioxidant content, fruit and vegetable-rich diets are inversely related to the risk of diseases related to oxidative damage [(16)]. The most important polyphenolic components of plants, flavonoids may stabilize free radicals by complexing with them [(17)]. The evaluation of lymphocyte nuclei with the comet assay demonstrated that hydrogen peroxide(H2O2) treatment caused significantly higher DNA damage in comparison to untreated controls, and after 5 mints of treatment with the mixture of (hydrogen peroxide and Beta Carotene), hydrogen peroxide H2O2-induced DNA damage significantly decreased,(tables1,2 above). Previous studies demonstrated that Beta Carotene protected peripheral blood lymphocytes against H2O2-induced oxidative DNA damage in vivo[(18)]. In other studies, it was also shown that using Beta Carotene to protects various cells, including isolated human lymphocytes, protected the cells against oxidative stress-inducing agents such as γ-radiation that use pathways similar to that of H2O2 [(19),(20)]. In the present study, there were induced oxidative damage in vitro lymphocyte cultures of healthy individuals through elevated free radicals levels by hydrogen peroxide, and we demonstrated the protective effects of Beta Carotene against DNA damage using the comet assay, results of the comet assay showed that the protective effects of Beta Carotene were different with different concentrations, between (100, 10000) µg/ml, furthermore, that the higher concentration (10000 µg/ml) were the higher protective lymphocytes against H2O2-induced oxidative damage (lower tail moment). When Beta Carotene was applied in combination with H2O2-treated cells, Beta Carotene interact with singlet O2, either via a physical quenching mechanism, in which the excited energy from singlet O2 is transferred to the carotenoid, and in which the carotenoid is destroyed in the process by the addition of O2 to its double-bond system [(21)]. Beta Carotene also reduce the levels of the highly oxidizing free radicals such asO2•—, RO•, and HO•[(22)].

Conclusion: This study proved the beneficial antioxidant capacity of beta-carotene in reducing the degree of the oxidative stress as manifested by the high levels of oxidative DNA damage measured via comet assay (tail moment), which showed that the most effective concentration of Beta-Carotene as antioxidant was in the concentration of 10000 µg/ml, as well as for that when were a combination of Beta-Carotene and hydrogen peroxide the most effective concentration was also in 10000 µg / ml.

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