EVALUATING THE DEGREE OF OXIDATIVE DNA DAMAGE & APOPTOSIS IN HUMAN LYMPHOCYTES CULTURED IN THE PRESENCE OF BETA–CAROTENE USING COMET ASSAY, AND FAS L (CD95)

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ABSTRACT

Background: Reactive oxygen species can cause various damage to different parts of the body, including the blood. It can damage the proteins, lipids as well as the DNA components of the blood. Oxidative DNA damage can be measured in lymphocytes by various techniques which is a useful way to assess the overall degree of oxidative stress. This study measures the levels of DNA damage and assess the proportion of the DNA cellular repair in human lymphocytes cultured in vitro conditions, the impact of the presence of beta-carotene using comet assay (single cell gel electrophoresis) ,and to evaluate the apoptosis in these cells if it may occur. Subjects and Methods: The study included 50 individuals aged between 20-50 years, during the period from October 2014 to November 2015. All participants were healthy, non-smokers with no family history of any disease. Also, they were not taking any type of vitamins or dietary supplements. Ten milliliters of total blood sample were collected in hepernized containers. Random blood samples of ten participants (5 males, 5 females) were used to study the effects of different concentrations of beta-carotene (100 and 10000) µg /ml on cultured lymphocytes by trazoleom assay. Samples from the remaining 40 participants were used to assess the levels of DNA damage in cultured lymphocytes using single cell gel electrophoresis in presence of the two different concentrations of beta-carotene (100 and 10000) µg /ml, and also to measure Fas L (CD95). Results: treating the lymphocytic cells
with hydrogen peroxide caused a considerable damage to the DNA, however, lymphocytic cells treated with beta-carotene (at concentrations of 100 and 10000 Ug/ml) showed less significant DNA damage. This was also associated with a significant change in the average tail lengths (in comet assay), indicating the positive effects of beta-carotene on the lymphocytes. Fas L (DC95) was not detected among the healthy lymphocytes, which was not associated with lymphocytic stimulation in response to beta-carotene. Conclusions: 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. A high concentration (10000ug/ml) of beta-carotene proved to be highly Beneficial to the lymphocytes compared to a lower concentrations.

KEYWORDS: Beta-carotene; Lymphocytes; Comet assay; Oxidative DNA damage.

INTRODUCTION
The cultured human lymphocytes are frequently used as a standard model for individual eukaryotic cells that can be vitally evaluated cytologically by phase contrast and differential contrast microscopy. They can be assessed both cytochemically and biochemically for various markers which affect the nuclear DNA including various forms of DNA damage and repair. To protect cells from active oxygen species, organisms have developed enzyme – dependent antioxidants like (superoxide dismutase, catalase, and glutathione peroxidase), and enzyme–independen antioxidants like (vitamins, uric acid, and glutathione). Antioxidant functions are associated with a lowered DNA damage, and lipid peroxidation, or inhibited malignant transformation. Beta-carotene is a natural pigment which is synthesized by plants and is responsible for the bright colors of various fruits and vegetables. It was found that beta-carotene possess an anti-oxidant properties and it can participate in prevention of DNA damage inflicted by oxygen free radicals. An imbalance between pro – and antioxidants in the intracellular microenvironment can produce oxidative stress.

Oxidative stress is considered to play a critical role in aging and the development of various diseases, including cancer and other degenerative diseases. Fas ligand (CD95) is a type-II transmembrane protein that belongs to the tumor necrosis factor (TNF) family. Its binding with its receptor induces apoptosis. Fas ligand/receptor interactions play an important role in the regulation of the immune system and the progression of cancer. 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 visualize by fluorescent microscopy, by using ethedium bromide as a staining dye and the amount of the DNA in the tail, relative to the head, is proportional to an amount of strand breaks.[5] Lymphocytes cells can be isolated from the whole 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. The micro culture tetrazolium assay (MTT) was originally developed by Mosmann[6], this method can be used to measure cytotoxicity, proliferation or activation for cultured lymphocytes in (100 and 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.[7]

MATERIALS AND METHODS

Human subjects, cell culture, and treatments

Fifty healthy non-smoking individuals, age-compatible (31 female and 19 male) were recruited, excluding anyone consuming a diet with supplements or taking prescribed medication. The study was approved by the Human Ethics Committee at College of Medicine, Al-Nahrain University. Venous blood samples were collected and lymphocytic cultures were set up after lymphocyte isolation with ficoll centrifugation. The culture medium used was composed of RPMI1640 (CAPRICORN Scientific, Gemen), containing 10% (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.[8] The mixture of lymphocytes culture media was incubated in the sterile incubator (Gallen kamp size one, model 1H- 150, England), for 5 minutes after adding two concentrations (100, 10000) µg/ml of beta-carotene (groups1,2), 5% H2O2 alone or as a mixture; for used for comet assay (40 individuals), (10 individuals) for MTT assay.

Preparation of Beta-carotene Solutions (Santa Cruz Biotechnology ,Inc.,sc-202485)

For prepare stock solution of beta-carotene, that has the concentration of (10000 µg/ml), weight 0.1 gm of the powder and put it in a sterilized tube containing (10 ml of DMSO
solution added to it 2.5 μl Twen 80 solution supported from BDH/ England for in vitro diagnostics). This is to accelerate solubility of beta-carotene, from a stock solution of (10000µg/ml), then by dilution we prepared the solution of the concentration 100µg/ml.

**Alkaline comet assay (alkaline single-cell gel electrophoresis)**

Alkaline single-cell gel electrophoresis (SCGE) was performed in order to detect the levels of genotoxicity in treated and untreated 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 length (µm) 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.[9,10]

**Cell viability assay**

The cell viability was evaluated by using methyl thiazolyl tetrazolium bromide (MTT, Sigma, USA) assay.[6]

**Immunocytochemistry (ICC) Assay for determination Fas ligand of lymphocytes**

Immunocytochemistry is a technique used to detect the presence of proteins in cell suspension and cultured cells by the use of a specific antibody, which binds to it, then allowing visualization and examination using light microscope. It is a simple tool for the determination of cellular contents of individual cells. Samples can be analyzed include blood smears, swabs, cultured cells, and cell suspensions.[11]
In this study, we used anti-antibody for Fas ligands (CD95) estimation (Santa cruz biotechnology, INC, Sc-2050).

**Statistical Analysis:** Data were analyzed using the statistical package of SPSS version 18. Mean and standard error or means were measured for the continuous variables. Analysis of variance (ANOVA) and Least Significant Deference (LSD) between means at level of significance (0.05). All hypothesis testing two tailed considering a significant P value at or below 0.05. Regression and Correlation (r) was calculated to check the relationship between variables and t-test has been used to test the significant differences.

**RESULTS**

The Comet tail length values for cultured lymphocytes treated with the 100 µg/ml concentration of beta-carotene and 5%H2O2 (group 1)

Results of this group are shown in table (1). There was a highly significant elevation in levels of the mean comet tail length in cultured lymphocytes in the beta-carotene and 5%H2O2 subgroups with the remaining subgroups of this cohort, (P value <0.005).

<table>
<thead>
<tr>
<th>Factors</th>
<th>Treatments</th>
<th>Concentration 100 µg / ml (40 Samples)</th>
<th>Mean± SEM</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tail Length (µm)</td>
<td>BetaCarotene</td>
<td>7.500±0.526*</td>
<td>b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hydrogen Peroxide</td>
<td>106.96±1.146*</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ALL(BC,H2O2)</td>
<td>75.32±0.426</td>
<td>c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>7.6±0.197*</td>
<td>b</td>
<td></td>
</tr>
</tbody>
</table>

* Significant difference between the “All” subgroup and the other subgroups in this cohort.

The Comet tail length values for cultured lymphocytes treated with the 10000 µg /ml concentration of beta-carotene (group 2)

Results of this group are shown in table (2). There was a highly significant elevation in levels of the mean comet tail length in cultured lymphocytes in the beta-carotene and 5%H2O2 subgroups with the remaining subgroups of this cohort, (P value <0.005).

Also there was a highly significant elevation in the mean tail length levels in the cultured lymphocytes treated with hydrogen peroxide compared to the All (BC,H2O2) subgroup.
Table (2): Comet tail length (µm) in the cultured lymphocytes with different treatments modalities, treated with 10000ug/ml beta-carotene (group2). P value = or less than 0.005.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Treatments</th>
<th>Concentration 10000 µg / ml (40 Samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tail Length</td>
<td>Beta Carotene</td>
<td>3.22±0.190*</td>
</tr>
<tr>
<td>(µm)</td>
<td>Hydrogen Peroxide</td>
<td>120.75±1.495*</td>
</tr>
<tr>
<td></td>
<td>ALL (BC, H2O2)</td>
<td>9.67±3.646</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>7.84±0.281*</td>
</tr>
<tr>
<td>LSD P ≤ 0.05</td>
<td></td>
<td>4.295</td>
</tr>
</tbody>
</table>

* Significant difference between the “All” subgroup and the other subgroups in this cohort.

In addition, there was a significant negative correlation between the mean tail length of the cultured lymphocytes treated with beta-carotene compared to the mean tail length levels in the control cultured lymphocytes. Photographs of cultured lymphocytes in different treatments were showed in figure 1.

![Photographs of cultured lymphocytes in different treatments](image)

**Figure 1:** Photographs of single cell gel electrophoresis (SCGE) stained with ethidium bromide (A, B, C, and D) in group2. A- Cultured lymphocytes in group 2; All (Beta-Carotene, H2O2 subgroup). B- Cultured lymphocytes in group 2 (Beta-Carotene subgroup). C- Cultured lymphocytes in group 2 (control subgroup). D- Cultured lymphocytes in group 2 (H2O2 subgroup), at x400 (Fluorescent Microscope).
2-Results of Fas L (CD 95)
In this study there were no detectable levels of Fas L in cultured peripheral blood lymphocytes (PBLs) in the healthy individuals, age (20-50 years) in control and in the treated with beta-carotene (in the two concentrations 100, 10000 µg/ml) for 5 minutes. The estimation of Fas L by anti-Fas L antibody by immune-cytochemistry assay suggested that there is no expression of Fas L in these cells, and there are no stimulation for the healthy peripheral blood lymphocytes by beta-carotene in the two concentrations (100, 10000 µg/ml), as in vitro treatment.

DISCUSSION
Beta-carotene is one of the major antioxidants present in 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 levels of oxidative stress in disease initiation and progression. It has been demonstrated that beta-carotene can suppresses the in vivo oxidative stress dependent lipid peroxidation. Many health claims have been made for the 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. 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, and this characteristic may be responsible for the antioxidant activity related to carotenoids, especially by the ability to quench singlet oxygen molecules. The products of normal oxidative metabolism are potentially dangerous oxidants (free radicals) which are capable of damaging the 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 contents, fruit and vegetable-rich diets are inversely related to the risk of diseases related to oxidative damage. 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, (tables1, 2 above) after 5 minutes of treatment with hydrogen peroxide and beta-carotene, hydrogen peroxide-induced DNA.
damage significantly decreased. Previous studies demonstrated that beta-carotene protected the peripheral blood lymphocytes against H2O2-induced oxidative DNA damage in vivo (19). In other studies, it was also shown that using beta-carotene to protects the 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. (20, 21) In the present study, there were induced oxidative damage in vitro lymphocyte cultures of healthy individuals through elevated free radicals levels, 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, and 10000 µg/ml). Furthermore, the higher concentration (10000 µg/ml) were the higher protective lymphocytes against H2O2-induced oxidative damage, when beta-carotene was applied in combination with H2O2 -treated cells. Beta-carotene interact with singlet O2 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. (22) Beta-carotene also reduces the highly oxidizing free radicals such as O2•, RO•, and HO•. (23) The most important polyphenolic components of plants, flavonoids may stabilize free radicals by complexing with them. (24)

Peripheral neutral killer cell (NK) from healthy persons expressed Fas L only on activation and the sera from healthy persons did not contain a detectable levels of Fas L (soluble Fas L). (25) Then in our study there were no detectable levels of Fas L in the cultured peripheral blood lymphocytes of the healthy individuals when treated with beta-carotene in different concentrations (100, 10000 µg/ml) for 5 min, the estimation of Fas L by anti-Fas L antibody by immune-cytochemistry assay, suggested that no expression of Fas L in these cells, and there was no stimulation for the healthy peripheral blood lymphocytes by beta-carotene in different concentrations (100, 10000 µg/ml), as in vitro treatment, in which they differs with in vivo treatments as in other experiments, and also the difference in the environments.

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. A high concentration (10000ug/ml) of beta-carotene proved to be highly Beneficial to the lymphocytes compared to a lower concentrations.
REFERENCES


