Evaluation of the in-vitro protective effect of plant extract (astaxanthin) on chromosomal breakage in Fanconi anemia cell culture
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Received 12 November 2012
Accepted 11 January 2013

Middle East Journal of Medical Genetics 2013, 2:45–49

Background
Fanconi anemia (FA) is an inherited bone marrow failure syndrome associated with congenital abnormalities and hypersensitivity to DNA cross-linking agents. The high frequency of chromosomal breaks in FA lymphocytes has been related to the increased oxidative damage shown by these cells. Reactive oxygen species (ROS) are derived from the metabolism of molecular oxygen. It is now assumed that ROS are involved both in the initiation and in the progression of cancer. Antioxidants have the ability to transform ROS into stable and harmless compounds. Astaxanthin is a natural source of antioxidant; its effect might protect cells from oxidative damage. Its antioxidant activity is far higher than that of vitamin E.

Aim of work
This study was designed to compare between the antioxidant effect of both astaxanthin and vitamin E as measured by their ability to reduce the frequency of induced chromosomal breakage in patients with FA.

Participants and methods
The current study included 15 patients with FA, nine females and six males, ranging in age from 4 to 21 years. The diagnosis of FA was confirmed by induction of chromosomal breakage by diepoxybutane. Astaxanthin and vitamin E were added at the start of the peripheral blood lymphocyte cultures to provide the possibility to improve the pro-oxidant state of the cells; then caffeine was added during the last 6 h of culture to induce chromosomal breakage.

Results and conclusion
The level of breakage was markedly reduced using astaxanthin and vitamin E; however, there was no significant difference between the effects of both substances. Astaxanthin was found in a wide diversity of natural sources; also, it is 10 times more potent than vitamin A and much safer than vitamin E. Our study is the first to investigate the effect of astaxanthin on chromosomal breakage in vitro. We conclude that the administration could be beneficial for patients with FA to improve their hematopoietic state.

Keywords: antioxidant, astaxanthin, fanconi anemia, reduction of chromosomal breakage, vitamin E

Introduction
Fanconi anemia (FA) is an inherited bone marrow failure syndrome associated with congenital abnormalities, hypersensitivity to DNA cross-linking agents, progressive bone marrow failure leading to death or the need for stem cell transplantation, recurrent infection, and a predisposition to acute myeloid leukemia and specific solid tumors (Shimamura and Alter, 2010).

The relationships of oxidative stress with the FA phenotype rely on a group of evidence that includes excessive formation of DNA oxidative damage (both in vivo and in vitro); cellular protection by hypoxia, antioxidants, and antioxidant enzymes; impaired expression and/or activity of antioxidant enzymes; the redox-dependent action mechanisms of mitomycin C and diepoxybutane; and excessive reactive oxygen species (ROS) formation (in vivo or in vitro) (Shukla et al., 2012).

ROS are derived from the metabolism of molecular oxygen. It can damage DNA, leading to mutations. Indeed, these species can act at several steps in multistage carcinogenesis. It is now assumed that ROS are involved both in the initiation and in the progression of cancer (Behe and Segal, 2007).

Antioxidants have the ability to transform ROS into stable and harmless compounds or by scavenging both ROS and RNS by a redox-based mechanism (Santoconoa...
et al., 2006). Low levels of antioxidants, or inhibition of the antioxidant enzymes, cause oxidative stress and may damage or kill cells (Muller and Williams, 2009). Among these antioxidants is vitamin E, which is found naturally in some foods, and added to others, also available as a dietary supplement (Traber and Packer, 1995).

Astaxanthin is also an antioxidant, which might protect cells from oxidative damage. Astaxanthin is a red carotenoid pigment, which is present in certain marine animals and plants such as fish, shrimps, and algae. It serves important metabolic functions in animals, including conversion of vitamin A (Bendich, 1989), enhancement of immune response (Park et al., 2010), and protection against diseases such as cancer (Goto et al., 2001). Astaxanthin has been shown to be one of the most effective antioxidants against lipid peroxidation and oxidative stress in in-vitro and in-vivo systems (Barros et al., 2001). Its antioxidant activity is far higher than that of vitamin E (Hayakawa et al., 2008).

**Participants and methods**
This study included 15 patients with FA, nine females and six males, ranging in age from 4 to 21 years.

All patients were subjected to the following:

(1) Complete blood count (CBC).
(2) Four sets of peripheral blood lymphocytes culture for 72 h were prepared:
   (a) In the first culture, induction of chromosomal breakage by the diepoxybutane (DEB) using 0.1 μg/ml was performed to confirm the diagnosis of FA according to Kutler et al., 2003 and Auerbach et al., 1989. The culture was exposed to DEB for 48 h.
   (b) Induction of chromosomal breakage by caffeine was performed using 2.2 mmol/l (0.02gm/5 ml) added to the other three cultures 6 h before harvest according to Pincheira et al. (2001).
   (c) DL-α-Tocopherol (vitamin E) was added at the start of culture for the correction of chromosomal breakage induced by caffeine using a 100 μmol/l final concentration according to Pincheira et al. (2001).
   (d) Astaxanthin was added at the start of another culture for the correction of chromosomal breakage induced by caffeine using 5 μmol/l according to Wojcik et al. (2008).

**Results**

**Hematological findings**
The hematological findings of the patients are presented in Table 1.

**Cytogenetics results**
The cytogenetic study was carried out on 15 FA patients. The study included induction of chromosomal breakage by DEB and caffeine and correction of the caffeine-induced breakage by astaxanthin and α-tocopherol (Table 2).

**Diepoxybutane test**
All patients were subjected to the DEB test to confirm the diagnosis of FA. The levels of breakage induced by DEB ranged from 3.1 to 11.6 breaks/cell (br/cell) and the average chromosomal breakage was 7.2 br/cell (Fig. 1).

**Caffeine test**
Induction of breakage by caffeine showed high levels but not as much as DEB. The range of breakage was 0.72–1.85 br/cell and the average was 1.2 br/cell (Fig. 2).

**α-Tocopherol test**
α-Tocopherol was used to test its efficacy in the reduction of the chromosomal breakage induced by caffeine. The levels of corrected breakage ranged from 0.35 to 1.15 br/cell and the mean was 0.74 br/cell (Fig. 3).

<table>
<thead>
<tr>
<th>Case number</th>
<th>Age (years)</th>
<th>Hb (g/dl)</th>
<th>RBC</th>
<th>WBC</th>
<th>Platelets</th>
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<tr>
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<td>1.1</td>
<td>4500</td>
<td>14 000</td>
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<tr>
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<td>6</td>
<td>1.6</td>
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<td>19 000</td>
</tr>
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<td>19</td>
<td>9</td>
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<td>20</td>
<td>11.6</td>
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</tr>
<tr>
<td>6</td>
<td>21</td>
<td>13.2</td>
<td>5.2</td>
<td>4000</td>
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</tr>
<tr>
<td>7</td>
<td>10</td>
<td>9.5</td>
<td>2.99</td>
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<td>8</td>
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<td>11</td>
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<td>7</td>
<td>8</td>
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<td>3600</td>
<td>80 000</td>
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<tr>
<td>Range</td>
<td>4–21</td>
<td>4.4–13.2</td>
<td>1.1–4.44</td>
<td>1700–6000</td>
<td>19 000–425 000</td>
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<tr>
<td>Mean ± SD</td>
<td>12.6±5.4</td>
<td>8.7±2.4</td>
<td>3.07±1.1</td>
<td>3726±1485</td>
<td>96 333±101 175</td>
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<tr>
<td>Normal values</td>
<td>–</td>
<td>11 g/dl</td>
<td>4–6×10^12/μl</td>
<td>4000–11 000/μl</td>
<td>150 000–450 000/μl</td>
</tr>
</tbody>
</table>

Hb, hemoglobin; RBC, red blood cell; WBC, white blood cell.
Astaxanthin was used to test its efficacy in the reduction of the induced chromosomal breakage. The levels of corrected breakage ranged from 0.32 to 1.50 br/cell and the average was 0.785 br/cell (Fig. 4).

**Statistical analysis**

The *t*-test was used to detect the significance of using α-tocopherol and astaxanthin for correction of the induced chromosomal breakage; the results showed a highly significant difference between the chromosomal breakage from cultures with caffeine alone and culture with caffeine and α-tocopherol added (*P*<0.0001). The level of breakage was markedly reduced with the use of this vitamin. Also, the results showed a highly significant difference between the chromosomal breakage from culture with caffeine alone and culture with caffeine astaxanthin added (*P*<0.0001); the level of breakage was markedly reduced with the use of astaxanthin.

However, there was no significant difference between the protective effect of α-tocopherol and astaxanthin (*P*>0.5).

There was a highly significant difference between the breakage induced by caffeine and DEB (*P*<0.0001), with a markedly increased chromosomal breakage level using DEB. Only a few cells have shown the characteristic chromosomal breakage with the use of caffeine.

The Pearson correlation test was used to determine the correlation between the sensitivity to DEB and the hematological findings. There was an inverse correlation between the hematological findings and the chromosomal breakage; this means that the increased chromosomal breakage values are associated with decreased hemoglobin (Hb) level, red blood cell (RBC), white blood cell (WBC), and platelet count (*r* values, −0.12, −0.037, −0.24, −0.42, respectively (*P*<0.05; Figs 5 and 6).

<table>
<thead>
<tr>
<th>Table 2 Cytogenetics results of the patients</th>
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<tr>
<td><strong>Diepoxybutane (br/cell)</strong></td>
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<td>14</td>
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<td>15</td>
</tr>
</tbody>
</table>

Mean ± SD 7.2 ± 2.86 1.2 ± 0.33 0.74 ± 0.22 0.78 ± 0.31

Range 3.2–11.6 0.8–1.85 0.35–1.15 0.32–1.3

**Figure 1**
Chromosomal breakage induced by diepoxybutane with characteristic isochromatid exchange; the red arrows indicate isochromatid exchange and the green arrows indicate simple breaks.

**Figure 2**
Chromosomal breakage induced by caffeine with characteristic isochromatid exchange; red arrows indicate isochromatid exchange and the green arrows indicate simple breaks.
Discussion

FA is an example of disorders that have chromosomal aberrations and susceptibility to cancer in common (Alter et al., 2003). FA patients have shown an abnormal reaction to oxidative stress that may exert an effect on the disease pathology. Thus, understanding the relationship between oxidative stress and disease progression in FA is very crucial, and the aim of the current study was to evaluate the role of antioxidants in improving the course of the disease as evidenced by reduction of induced chromosomal breakage. Astaxanthin and α-tocopherol are antioxidants; their antioxidant effects are evaluated by studying the correction of the caffeine-induced chromosomal breakage.

The current study included 15 patients with FA, nine females and six males; their ages ranged from 4 to 21 years. The CBC results of the patients showed that three patients (No. 5, 6, 8) of 15 patients had a normal CBC profile. Their ages ranged from 20 to 21 years and the average CBC values were as follows: the Hb level was 11.9 g/dl, the RBCs count was 4.54 x 10^6/µl, the WBC count was 4966/µl, and the platelets count was 112 660. The ages of these patients and their CBC profile might indicate the stability of their clinical condition probably because of the improvement in medical care. From 1981 to 1990, the median age of death was only 19 years; by 2000, the median age had reached 30 years (Alan and Andrea, 2010).

The cytogenetic results were promising in terms of the antioxidant effect of both vitamin E and astaxanthin, thus suggesting that astaxanthin and vitamin E were highly effective in protection against oxidative damage expressed as chromosomal breakage. However, there was no significant difference between the protective effect of α-tocopherol and astaxanthin ($P>0.5$). The level of breakage was markedly reduced with the use of vitamin E, which is in agreement with the previous observations of Pincheira et al. (2001).

Although the biological action of astaxanthin has been reported for both in-vitro and in-vivo studies, this is the first in-vitro study to show the effect of astaxanthin as a potent antioxidant in the reduction of chromosomal breakage in FA. Astaxanthin was added to the blood culture of patients with FA and the results indicated a highly significant difference
between the chromosomal breakage from culture with caffeine alone and culture with caffeine and astaxanthin added ($P<0.0001$). The level of breakage was markedly reduced with the use of astaxanthin. This finding suggested that astaxanthin is a powerful antioxidant with a potential to correct the induced chromosomal breakage.

On comparing astaxanthin and vitamin E, there is a wide diversity in natural sources of astaxanthin than vitamin E. Some researches did not find any adverse effects of consuming vitamin E in food (Verhagen et al., 2006). However, high doses of α-tocopherol supplements can cause hemorrhage and interrupt blood coagulation in animals, and in-vitro data suggested that high doses inhibit platelet aggregation and may lead to development of cancer (Sesso et al., 2008; Klein et al., 2011). In contrast, natural astaxanthin from Haematococcus microalgae has never been associated with any toxicity as reported in the literature, and numerous animal and human studies tend to support its safety (Maher, 2000). Many human safety studies in addition to the human clinical trials and the animal trials have never reported any adverse effects for astaxanthin (Capelli and Cysewski, 2007). Thus, astaxanthin could be very beneficial to FA patients. Also, it is worth mentioning that astaxanthin is available in many forms such as oil astaxanthin extract, powdered extract, and in a water-soluble form (Capelli and Cysewski, 2007).

Conclusion
From the present work, it is evident that astaxanthin is a potent antioxidant with the ability to reduce induced chromosomal breakage (in vitro) in patients with FA. Astaxanthin, a bioactive natural carotenoid, could be an effective adjuvant therapy for patients with FA to improve their pro-oxidant status, thus leading to improvement of the hematological condition; also, by improving the chromosomal stability, it might protect FA patients from development of cancer.

Acknowledgements
Conflicts of interest
There are no conflicts of interest.

References