Micro-pixe analysis in invasive ductal carcinoma tissues after treatment of astaxanthin

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INTRODUCTION

The understanding of breast disease has prompted active area of research to focus on the role of trace element concentrations in breast disease. For over 30 years, an active area of research has focused on the role of trace element concentrations in breast disease, to understand the disease process (1). Trace elements play an important role in all biological systems. They take part in all metabolic processes, being components of different enzymes, catalyzing chemical interaction in living cells (2–3). Trace element deficiency or excess has been found in patients with certain diseases, including cancer.

Astaxanthin, a carotenoids pigment which belongs to a larger class of phytochemicals known as terpenes is found in certain marine animals and plants, such as fish, shrimps, algae and fungi (6), and it has been reported to be used widely as a food supplement in poultry and aquaculture. It is also, classified as a xanthophyll, which means “yellow leaves”. Possessing a special structure as shown in figure 1, astaxanthin has a powerful singlet oxygen radical and peroxyl radical scavenger property which is shown to be more than β-carotene, canthaxantin and zeaxantine (3, 3′-dihydroxy-β-carotene). This anti oxidizing activity protects cells from the many different oxidizing agents present (7). Antioxidant compounds can decrease mutagenesis, and thus carcinogenesis, both by decreasing oxidative damage to DNA, and by decreas-
ing oxidant-stimulated cell division the human body maintains an array of endoge-
inous antioxidants such as catalase and superoxide dismutase; however, exogenous
dietary antioxidants such as ascorbic acid (vitamin C), α-tocopherol (vitamin E) and
carotenoids play important roles in reducing oxidative damage as well, and their serum
levels have the potential to be manipulated.

Figure 1. structure of astaxanthin.

Astaxanthin, the king of alpha-
carotene as derived from Phaffia
rhodozyma, is one the most potent antioxi-
dants in nature. Studies suggest that
Astaxanthin can deliver 1000 times the antioxidant power of vitamin E (8).

Studies performed by the international
Agency for Research on Cancer have
proposed that some trace elements or their compounds are involved in carcinogenic
processes. Those elements include: Be, Cr,
Co, Ni, As, Cd, Sb, Pb, Hg, and Pt. The
carcinogenic effect of Mn, Fe, Cu, Zn, Se,
and Sr has not been proved yet (9-12).

In this work, Proton induced X-ray
emission (PIXE) was used in a study
intending to quantify the levels of the elements in healthy, breast cancerous and
treated cancerous with astaxanthin breast
samples. The analytical results revealed a
tendency of higher concentrations of
elements in cancerous tissues. In this study
several trace elements showed a reduced
concentration in astaxanthin treated
cancerous tissues in comparison to
cancerous control tissues.

PIXE was also used to study the
concentration of trace elements in liver and
in untreated and treated mice bearing
tumor. Higher concentration of trace
elements was detected in the breast cancer
mice in comparison with treated ones. The
analytical results revealed the same
tendency in accumulation of the elements in
treated mice.

Particle induced X-ray emission is a
powerful elemental analytical technique
used routinely by bio-physicists, biologists,
archaeologists and art conservators to
answer questions about the identification
and quantization of trace elements.
Bombardment with ions of sufficient energy
(usually MeV protons) produced by an ion
accelerator, will cause inner shell ionization
of atoms in a specimen (13). Other applica-
tions include determining the element and
its concentration in biological samples (14-18).

MATERIALS AND METHODS

Astaxanthin (C₄₀H₅₂O₄) compound was
purified by the biomedical laboratory in
Research institution of women, University
of Alzahra (19). Research showed that due to
astaxanthin’s potent antioxidant activity, it
might be beneficial in cardiovascular,
immune, inflammatory and neurodegenera-
tive diseases. The presence of oxygen-
containing functional groups on these rings
classifies astaxanthin among the
xanthophylls. This structure was useful in
energy transfer and dissipation and gave
carotenoids their characteristic colors (20).

Existing data on the potential for astaxan-
thin to directly prevent cancer is limited to
in vitro cell culture studies and in vivo
studies with rodent models (21, 22). Eight-ten
weeks old inbred Balb/c mice Pasture
Institute, Tehran, Iran they were given
sterilized water and autoclaved standard
mouse chow ad labium throughout the
study. Tumor cells: Spontaneous mouse
mammary tumor (SMMT) spontaneously
developed in female Balb/c mice which were
then transplanted subcutaneously to 9
healthy female mice. SMMT is an invasive
ductal carcinoma. Animals were housed
three to a cage maintained in a 12-h
light–dark cycle (light on 6 a.m.–6 p.m.) and
at an ambient temperature of 22 °C and
relative humidity of 65%. Animal-use
protocol of this experiment was approved by
Micro-pixe analysis of IDC treated with astaxanthin

the Animal Use and Care Committee of the Tarbiat Modares University.

Three groups including three female mice were considered. The first group was healthy untreated mice, the second group was breast cancerous mice 12 days post transplantation, and third group contained mice which 12 days post transplantation received a single dose of aqueous solution 25 mg of Astaxanthin compound intratumorally, who were sacrificed on day 24.

The mice were scarified under slight carbon dioxide anesthesia. The breast and liver tissues were immediately isolated and frozen by liquid nitrogen. Making use of the quick-freezing element of a cryo-microtome (Leica Company Model CM1850) lateral sections were cut at a thickness of 50 µm in -15 °C stable temperatures. After cutting, the sections were mounted on the target holder for micro PIXE analysis. The sections were dried for twenty four hours at 4 °C and 100 Pa.

Left beam line was set at an angle of 45 °C, which was a powerful tool for simultaneous multi-element analysis of biological samples. The microbeam opened the possibility to obtain one overall X-ray spectrum of the sample. The protons were accelerated with the Van De Graff (High Voltage Engineering Company in Burlington, Massachusetts), to energy of 2.0 MeV. The proton beam from Van De Graff is projected with the object slit of the microprobe and projected on the target by quadruples lenses in x- and y-directions. The achieved beam spot was 10 × 10 µm. The target was moving computer control in both x and y directions. Analyzing an area of 2.5 × 2.5 of sample surface, X-ray spectrum was collected by an energy dispersive solid state Si (Li) detector at an angle of 135 °C. The multichannel analyzer opened for a predefined time interval and subsequently the data were read out and written to memory. The beam current was 10-30 pA. Each sample takes approximately 3 hours to perform the experiment. The data were analyzed using GUPIX, a program that fits the element Kα and Kβ peaks under consideration, taking account of line overlaps and subtracts the background. Each sample analysis was repeated three times.

RESULTS AND DISCUSSION

Breast tissues

In order to assess the concentration of Fe, S, Ca, P and Zn in the tumor tissues, 6 tumor bearing mice, untreated and treated with astaxanthin, were sacrificed and the micro-PIXE analysis, on the breast tissues samples, was performed and spectrum was obtained. The experimental results are presented in figure 2 which shows the comparison among these three groups. The spectrum was normalized by phosphorous. Figure 2 shows a typical X-ray spectrum.

![Figure 2](image_url)

Figure 2, the X-ray spectra recorded from the breast compared healthy, cancerous and injected cancerous with astaxanthin.
The data were analyzed by GUPIX, an interactive software package which was used to analyze and convert raw spectral data into elemental concentrations. The concentrations of the element (ng/cm²) were collected and are shown in table 1. The concentrations of P, S, Ca, and Fe have found to be different in healthy, cancerous and treated cancerous tissues. An increase in elemental concentrations in breast cancerous tissues was observed, as compared with breast healthy tissues. It was also observed that the concentration of Zn had not only increased, but also it is only detected in breast cancerous tissues. The results also revealed a tendency of lower elemental concentrations in the astaxanthin treated cancerous tissues in comparison with cancerous ones. The decrease was pronounced for P, S, Ca, Fe, and Zn was so decreased to the level which was not detected. For observing comparison, the bar chart is shown schematically in figure 3.

**Liver tissues**

This technique has also been used in other studies, which aimed to investigate the differences in the concentrations of trace elements in liver samples of the same mice that their breast was analyzed. Figure 4 shows the comparison of the obtained spectra from micro PIXE analysis among healthy liver tissues, liver of the mice with breast cancer and the liver of mice with breast cancer that was treated with astaxanthin.

<table>
<thead>
<tr>
<th>Elements</th>
<th>Healthy</th>
<th>Cancerous tissue</th>
<th>Cancerous tissue treated with astaxanthin (ASX)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>9.50±0.2</td>
<td>24.84±3.40</td>
<td>20.54 ± 1.45</td>
</tr>
<tr>
<td>S</td>
<td>8.90±0.2</td>
<td>32.26±3.67</td>
<td>23.55 ± 1.66</td>
</tr>
<tr>
<td>Ca</td>
<td>0.33±0.08</td>
<td>3.52±0.43</td>
<td>2.35 ± 0.18</td>
</tr>
<tr>
<td>Fe</td>
<td>0.31±0.08</td>
<td>1.25±0.27</td>
<td>0.86 ± 0.0</td>
</tr>
<tr>
<td>Zn</td>
<td>_</td>
<td>0.5 ± 0.18</td>
<td>_</td>
</tr>
</tbody>
</table>

Table 1. Comparison of elemental concentrations (ng/cm²) for healthy, cancerous and treated cancer with astaxanthin breast tissues.

**Figure 3.** The bar chart is shown for comparison of elemental concentrations (ng/cm²) for healthy, cancerous and treated cancerous with astaxanthin breast tissues.
The concentrations of the element (ng/cm²) in liver samples are shown in table 2. The results indicated an increase in concentration of P, S, Ca, and Fe in the liver of the mice with breast cancer in comparison to healthy ones. It was also found that the concentrations of these elements in liver of the mice with breast cancer and the liver of mice with breast cancer which was treated with astaxanthin to be fairly equal. The bar chart is also shown schematically in figure 5.

**Table 2.** Comparison of elemental concentrations (ng/cm²) for healthy liver tissues, liver of mice with breast cancer and the liver of mice with breast cancer that was treated with astaxanthin.

<table>
<thead>
<tr>
<th>Elements</th>
<th>Healthy</th>
<th>Cancerous tissue</th>
<th>Cancerous tissue treated with Astaxanthin (ASX)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>0.59±0.02</td>
<td>0.72±0.01</td>
<td>0.71 ± 0.01</td>
</tr>
<tr>
<td>S</td>
<td>0.49±0.04</td>
<td>0.73±0.01</td>
<td>0.72 ± 0.01</td>
</tr>
<tr>
<td>Ca</td>
<td>0.023±0.01</td>
<td>0.026±0.005</td>
<td>0.021 ± 0.006</td>
</tr>
<tr>
<td>Fe</td>
<td>0.13±0.03</td>
<td>0.021±0.005</td>
<td>0.024 ± 0.006</td>
</tr>
</tbody>
</table>

**Figure 4.** The X-ray spectra recorded from the liver compared healthy liver tissues, liver of the mice with breast cancer and the liver of mice with breast cancer that was injected with astaxanthin.

**Figure 5.** The bar chart is shown for comparison of elemental concentrations (ng/cm²) for healthy liver tissues, liver of mice with breast cancer and the liver of mice with breast cancer that was treated with astaxanthin.
CONCLUSION

The use of analytical techniques for medical physics applications is receiving an increasing amount of attention both by analytical scientists as well as medical doctors (23). This study was focused on three parameters: the first is to set out a suitable technique to measure the elements. We aimed to use the micro-PIXE as a suitable technique for determining elemental concentration at a cellular level over sections of breast cancers. Fisher and Fisher (24) used atomic absorption spectrophotometer to measure the concentration of the elements. Homogenizing the samples is the pre stage for measuring the elements by absorption spectrophotometer; this can lead to a loss of elements during sample preparation. The result presented here, shows that the technique has promised and could obtain elemental concentrations in tissues. Due to the fact that excessive accumulation or an imbalance of metallic elements may disturb the cell functions, and may result in degeneration or cell death, the main difficulty with the process was the preparation of the suitable samples for analyzing. The best way to do this is to freeze the samples immediately and cut with cryo-microtome.

The second goal was to measure the P, Ca, Fe and S in the tumor and liver tissue. The results showed that the concentration of the elements in the breast cancer is highly increased in the tumor tissue while it had a slight increase in the liver tissues comparing with control healthy animal. The results of the present research were in agreement with the previous study of Urszula Majewska (25) which showed an increase in the consumption of trace elements in the breast cancer tissue. The third goal was to evaluate the Astaxanthin compound on the accumulation of elements. The result showed that Astaxanthin significantly decreased the accumulation of elements in the tumor site and caused the breast cancer cell membrane lose their desire to collect the elements from healthy tissues; therefore, the concentration of the elements after injecting had decreased in comparison with the cancerous group and the concentration of the elements in the liver tissues had not changed obviously. The predominant element of interest for this study was iron; however, the peaks from other elements were presented to demonstrate the capabilities of the technique. It could be appreciated that the peaks obtained for Fe were often less clear and that is due to the severely low concentrations of the elements in tissues (of the order of a few ppm).

It is evident from the spectrums, that several discrete peaks are seen sitting on a continuous distribution which has a maximum at low energies. The decrease in the continuum at the lowest energies is due to absorption of X-rays in the window in front of the Si(Li) detector. The background at low energies prevents easy and precise analysis of the light elements in the spectrum. The main processes which contribute to the background are: Incident projectile bremsstrahlung and Secondary electron bremsstrahlung (13).

Using a suitable sample preparation technique, it would be possible to preserve the structure and the content as close as possible to that of the living tissue and by improving scan times, it should be possible to detect and quantify more trace elements in order to better understand the role of elements and their changes affecting tissue microenvironment and cellular behavior in cancer, hence the effects of anticancer compounds for therapy.

REFERENCES
