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

S. Safaverdi[1], F. Roshani[1], M. Lamehi Rashti[2], Sh. Golkhoo[3], Z.M. Hassan[4*], L. Langroudi[4]

[1]Department of physics, Alzahra University, Tehran, Iran
[2]Institute for Research in Fundamental Sciences, Tehran, Iran
[3]Research Institute of women, Alzahra University, Tehran, Iran
[4]Department of Immunology, Tarbiat Modares University, Tehran, Iran

Background: Trace elements play an important role in a number of biological processes. Astaxanthin (ASX), a carotoid pigment found in certain marine plant and animals, has shown anti cancer and anti free radical properties. This work intended to understand the effect of Astaxanthin in breast cancer (invasive ductal carcinoma, IDC) by using micro-pixe method. For this aim the concentration of trace elements were compared in healthy, cancerous and cancer treated with astaxanthin in the breast and liver tissues of breast cancer bearing mice, using proton induced X-ray emission (PIXE). ***Materials and Methods:*** Proton induced X-ray emission (PIXE) was used in a study intending to compare the concentration of trace elements in breast and liver tissues of mice bearing tumor, three groups of mice: healthy, cancerous, and cancerous treated by astaxanthin, were considered. Astaxanthin was supplied from Research Institute of women, Alzahra University. ***Results:*** Comparing the untreated tumor tissue, treatment with Astaxanthin significantly decreased the amount Fe, P, S, and Ca elements level in tumor tissue of the breast cancer. It is also found that the concentrations of those elements in liver of the untreated mice and the liver of treated mice with astaxanthin were fairly equal. Astaxanthin significantly decrease the accumulation of elements in the site of tumor, and caused the breast cancer cell membrane to lose their desire to collect the elements from healthy tissues. ***Conclusion:*** The micro -pixe technique could calculate elemental concentrations in tissues. Changes in metallic elements may affect microenvironment and cell functions, which might led lead to cell degeneration or death, the results shows that astaxanthin reduces vital element concentration in tumor site, thus it could be used as an anti tumor agent. **Iran. J. Radiat. Res., 2009; 7 (1): 33-39**

Keywords: *Breast cancerous tissues, proton-induced X-ray emission, astaxanthin, trace elements.*

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 [(2-5)]. 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 ß carotene, cantaxantine and zeaxantine (3, 3'-dihydroxyl-ß-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-

Corresponding author:
Dr. Z.M. Hassan,
Department of Immunology, Tarbiat Modares University, Tehran, Iran.
E-mail: *hassan_zm@modares.ac.ir*

ing oxidant-stimulated cell division the human body maintains an array of endogenous 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.

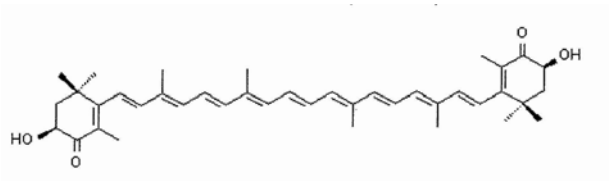

Figure 1. structure of astaxanthin.

Astaxanthin, the king of alpha-carotene as derived from Phaffia rhodozyma, is one the most potent antioxidants 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 applications include determining the element and its concentration in biological samples [14-18].

MATERIALS AND METHODS

Astaxanthin ($C_{40}H_{52}O_4$) 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 neurodegenerative 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 astaxanthin 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

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 intratumoraly, 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-direction. 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 K_α and K_β 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.

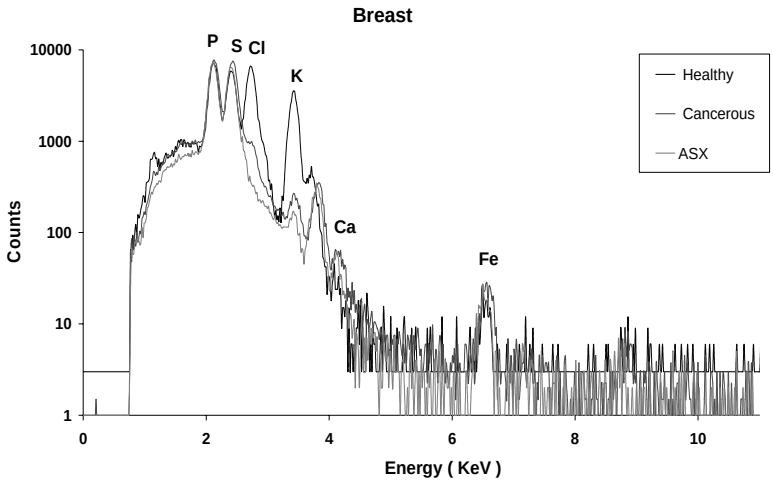

Figure 2. the X-ray spectra recorded from the breast compared healthy, cancerous and injected cancerous with astaxanthin.

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/cm^2) 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 1. Comparison of elemental concentrations (ng/cm^2) for healthy, cancerous and treated cancer with astaxanthin breast tissues.

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

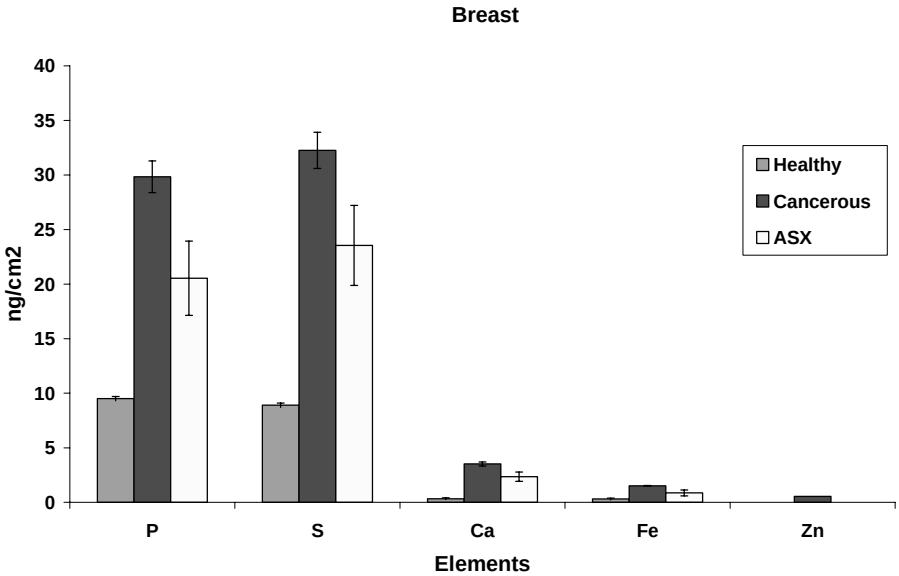

Figure 3. The bar chart is shown for comparison of elemental concentrations (ng/cm^2) for healthy, cancerous and treated cancerous with astaxanthin breast tissues.

The concentrations of the element (ng/cm^2) 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.

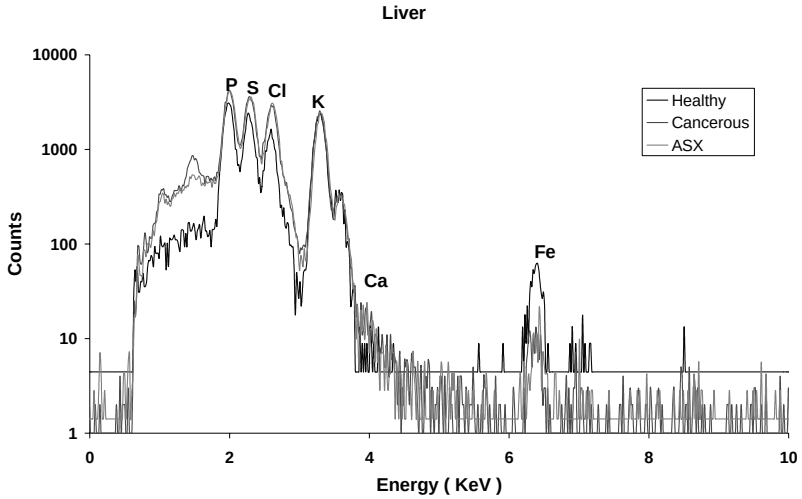

Figure 4. The X-ray spectra recorded from the liver compared healthy liver tissues, liver of the mice with breast cancer and the liver of mice with breast cancer that was injected with astaxanthin.

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

Elements	Healthy	Cancerous tissue	Cancerous tissue treated with Astaxanthin (ASX)
P	0.59±0.02	0.72±0.01	0.71 ± 0.01
S	0.49±0.04	0.73±0.01	0.72 ± 0.01
Ca	0.023±0.01	0.026±0.005	0.021 ± 0.006
Fe	0.13±0.03	0.021±0.005	0.024 ± 0.006

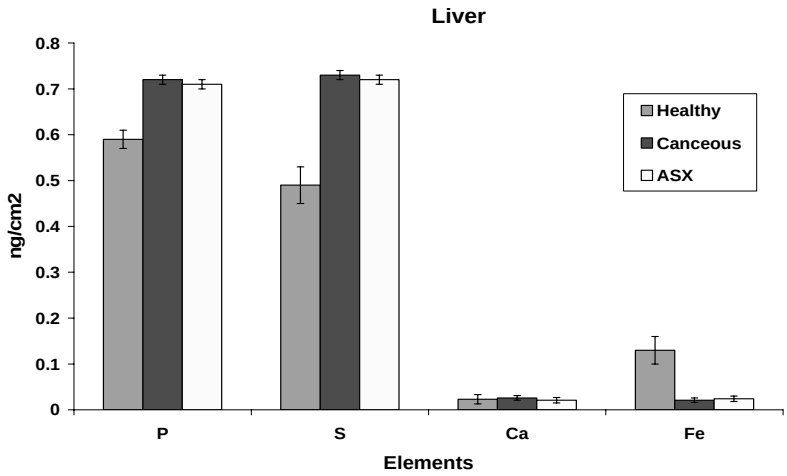

Figure 5. The bar chart is shown for comparison of elemental concentrations (ng/cm^2) for healthy liver tissues, liver of mice with breast cancer and the liver of mice with breast cancer that was treated with astaxanthin.

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 [15].

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.

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