Elemental mapping by synchrotron radiation X-Ray microfluorescence in cellular spheroid of prostate tumor cells

R. G. Leitão[1]; C. A. N. Santos[2]; A. Palumbo Jr.[3]; P. A. V. R. Souza[3], G. R. Pereira[4], M. J. Anjos[1, 5], L. E. Nasciutti[3]; R. T. Lopes[1]

[1] *Nuclear Instrumentation Laboratory, Federal University of Rio de Janeiro, 21941-972, Rio de Janeiro-RJ, Brazil*

[2] *Biotechnology Laboratory, National Institute of Metrology, Standardization and Technology, 25250-020, Duque de Caxias-RJ, Brazil*

[3] *Biomedical Science Institute, Federal University of Rio de Janeiro, 21941-590, Rio de Janeiro-RJ, Brazil.*

[4] *Non-Destructive Testing, Corrosion and Welding Laboratory, Federal University of Rio de Janeiro, 21941-942, Rio de Janeiro-RJ, Brazil*

[5.] *Physics Institute, Stated University of Rio de Janeiro,20550-900, Rio de Janeiro-RJ, Brazil*

roberta@lin.ufrj.br

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Prostate cancer is the sixth most common type of cancer and the third most common in males in Western industrialized countries. Cellular spheroid serves as excellent physiologic tumor models as they mimic avascular tumors and micrometastases. Trace elements play a significant role in biological processes. They are capable of affecting human health by competing with essential elements for available binding sites and by the activation or inhibition of reactions between metabolic enzymes. It is well known that zinc levels in the peripheral zone of dorsal and lateral lobes of the prostate are almost 10 times higher than in other soft tissues. Prostate tumor cells were isolated of the prostate tissue samples that were collected from patients submitted to surgery. The measurements were performed in XRF beam line at the Synchrotron Light National Laboratory (LNLS) in Campinas, Brazil. The results showed that all elements were heterogeneously distributed in different areas of the spheroids analyzed. P, S and Cl showed similar elemental distribution in all the samples analyzed while K, Ca, Fe, and Cu showed different elemental distribution. In all spheroids analyzed, Zn presented more intense distributions in the central region of the spheroid. The relationship between the function of Zn in the secretory epithelial cells and the carcinogenic process suggests that more studies on elemental mapping in spheroids are necessary.

Keywords: X-Ray Fluorescence; Elemental distribution; Prostate tumor cells.

1. INTRODCTION

During the last decades, element analysis in different fields such as medicine, biology, and environmental science has been well established [1]. Trace elements play a significant role in biological processes. They are capable of affecting human health by competing with essential elements for available binding sites and by the activation or inhibition of reactions between metabolic enzymes [2]. Both excess and deficiency of trace elements have been associated with many diseases including cancer. Even though extensive work has been carried out to find an association between trace elements and cancer, and to understand the mechanisms involved in carcinogenesis, no definite conclusions have been drawn so far [3].

Micro X-Ray Fluorescence (μXRF) is a non-destructive technique very often used in biological investigations to obtain information regarding the distribution of elements in tissues and cell samples. μXRF investigates sample regions with a spatial resolution in a micrometer scale. It allows the determination of not only the concentration of elements but also their distribution [4].

The formation of three-dimensional cell microspheres such as spheroids, embryoid bodies, and neurospheres has been highlighted as a useful culture technique [5]. Spheroids are sphere shaped cell colonies formed by self-assembly that allow various growth and functional studies of diverse tissues. Spheroids serve as excellent physiologic tumor models as they mimic avascular tumors and micrometastases and are known to provide more reliable and meaningful therapeutic readouts. These advantages of tumor spheroids have been widely recognized [6].

Diseases of the prostate gland such as Prostate cancer (PCa) is the most frequently health problems in men past middle age and the most prevalent type of cancer in men. Several factors such as the late and doubtful diagnostics of PCa, high incidence of PCa and the increase of the mortality rate in the world due to prostate cancer have made the prostatic gland the object of study of many researchers with the objective of developing methods of prevention, new diagnostic techniques and treatments.

In this context, the goal of this study was to analyze the elemental distributions in spheroids derived from tissues with PCa using Synchrotron Radiation micro X-Ray Fluorescence (μSRXRF). Therefore, it helps to elucidate the mechanisms and effects of trace elements on prostate functions.

2. MATERIALS AND METHODS

2.1 Population Characteristics

This study was conducted after approval from the Internal Review Board of the Clementino Fraga Filho Teaching Hospital at the Federal University of Rio de Janeiro, Brazil. The prostate samples were collected from 3 patients submitted to surgery at the Andarai Hospital in Rio de Janeiro city – Brazil. The age-range of the patients was 40 – 85 years old.

2.2 Cell Culture and spheroids

The specimens were maintained under sterile conditions in flask-contained "Dulbecco's Modified Eagle's Medium – DMEM, Sigma Chemical Co." culture medium supplemented with 5% fetal bovine serum (FBS) plus antibiotic/antimycotic mixture Penicillin 100 U/mL, Streptomycin 100 μg/mL and Fungizone 25 μg/mL (Gibco). To isolate prostate cells, specimens of malign tissue were diced into approximately 1 mm^3 pieces using forceps and scissors with 0.1% collagenase enzymatic solution (Worthington Biochemicals, Lakewood, NY, USA) prepared in culture medium DMEM without se-rum and incubated at 37°C and 5% CO_2 atmosphere for 1 hour. At the end of the dissociation, the cell homogenate was centrifuged; the cell pellet was resuspended in 1 mL of culture medium supplemented with 10% FBS. The cells were seeded in 25 mm^3 flasks and left to allow attachment in a defined medium composed of DMEM supplemented with 10% FBS and placed in a tissue culture incubator at 37°C under humidified air containing 5% CO_2. The cells were fed 3 times a week and at confluence, they were detached with 0.25% trypsin/EDTA (Sigma). After one or two weeks, the same procedure was realized and after establishment of prostate cell primary cultures (epithelial and stromal cells); the cells were detached and replated.

Prostate spheroid cultures were made from primary prostate cells. Cells were seeded at 2×10^4 cells/well on a 96-well tissue culture plate (round bottom) (STARSTEDT, USA) on a base of 1 % agarose. The 3D cultures were performed in DMEM culture medium supplemented with 10% FBS. After 72 hours, the spheroids already had a spherical shape and were daily monitored by microscopy. Every 5 days, approximately 90% of the total culture medium volume was replaced by fresh medium. In all the studies, the prostatospheres were cultured for at least 7 days prior to their use. After 10 days of incubation, the spheroids were fixed with 4.0% paraformaldehyde for 30 min. Following three washes with phosphate buffer solution (PBS) 0.1 M, the spheroids were placed in PBS solution, pH 7.4 at 4°C until analysis. Before μXRF analysis, the spheroids were repeatedly washed in MILLI-Q water. Finally, with a micropipette (100-1000μL) they were taken and deposited on ultralene film (4 μm) fixed in a sample holder and dried in air at room temperature. Figure 1 shows spheroids PCa 1, PCa 2 and PCa 3.

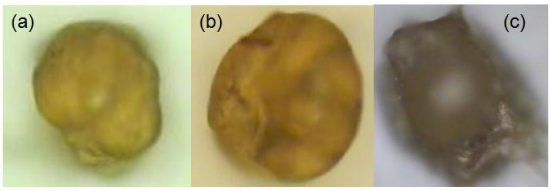

Figure 1: Photo of spheroids: (a) PCa 1, (b) PCa 2, (c) PCa 3.

2.3 Experimental Organization

The measurements were carried out using X-ray microfluorescence with synchrotron radiation (μSRXRF) technique in the XRF beam line at the Synchrotron Light National Laboratory, in Campinas, São Paulo, using a polychromatic beam with maximum energy of 20 keV for excitation and a Si(Li) detector with a resolution of 165 eV at 5.9 keV. The experiment was performed in standard geometry (45° x 45°), exciting with a white beam and using a 20 μm diameter optical capillary collimation. The X-ray fluorescence spectra obtained were evaluated by the QXAS software distributed by the International Atomic Energy Agency [7]. The two-dimensional maps were obtained after normalization of the intensities of characteristic X-ray lines to the value of the ionization chamber which measure the photon flux of the incident beam in the sample. The counting live time for each pixel was of 20 s/step and the step size was of 30 μm/step in both directions. A schematic drawing of the experimental setup for an X-ray microfluorescence is shown in Figure 2. The elemental mapping was obtained using PyMCA software developed at the ESRF synchrotron [8].

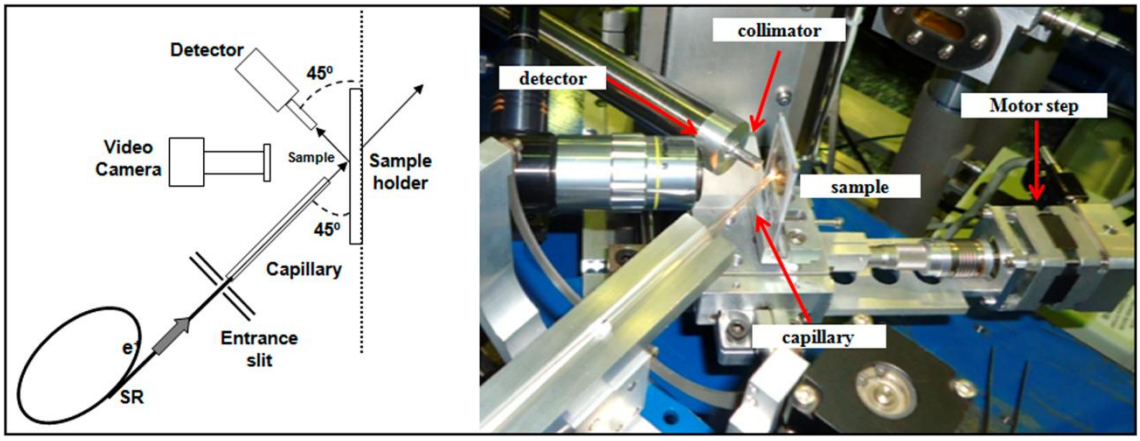

Figure 2: The experimental arrangement for X-ray microfluorescence measurements.

3. RESULTS AND DISCUSSION

Figure 3 shows a typical X Ray fluorescence spectrum of a sample of PCa spheroids using the μSRXRF technique. It was possible to detect the following elements: P, S, Cl, K, Ca, Fe, Cu and Zn. The X-Ray fluorescence mapping of PCa spheroids is shown in Figures 4-7 for the elements P, S, Cl, K, Ca, Fe, Cu and Zn, respectively.

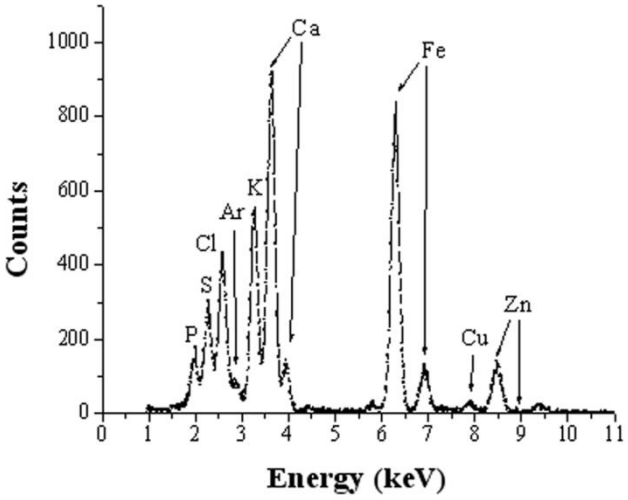

Figure 3: X-ray fluorescence spectra of a PCa spheroid sample.

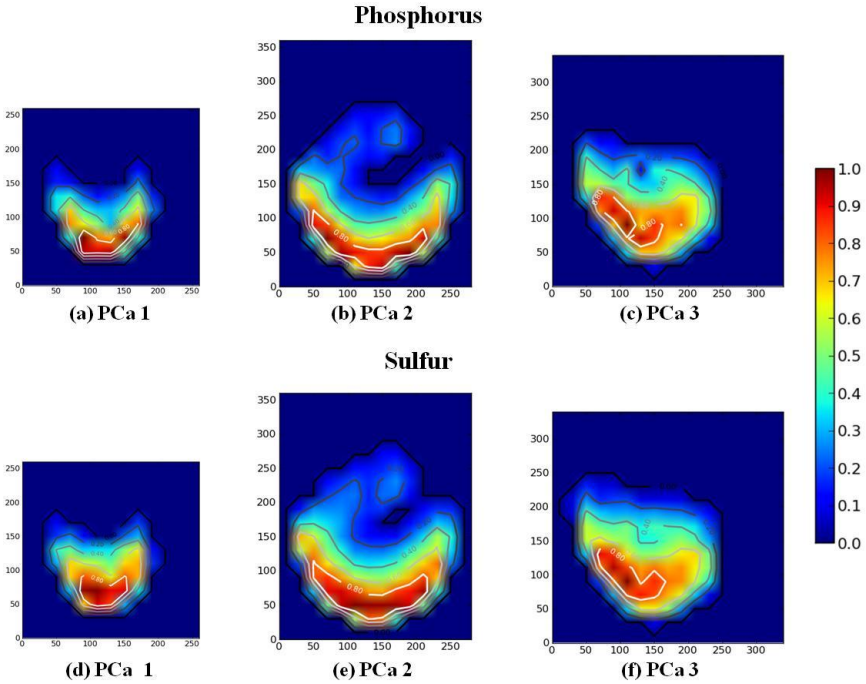

Figure 4: Image of phosphorus mapping the spheroids of: (a) PCa 1, (b) PCa 2, (c) PCa 3 and Image of sulfur mapping the spheroids of: (d) PCa 1, (e) PCa 2, (f) PCa 3.

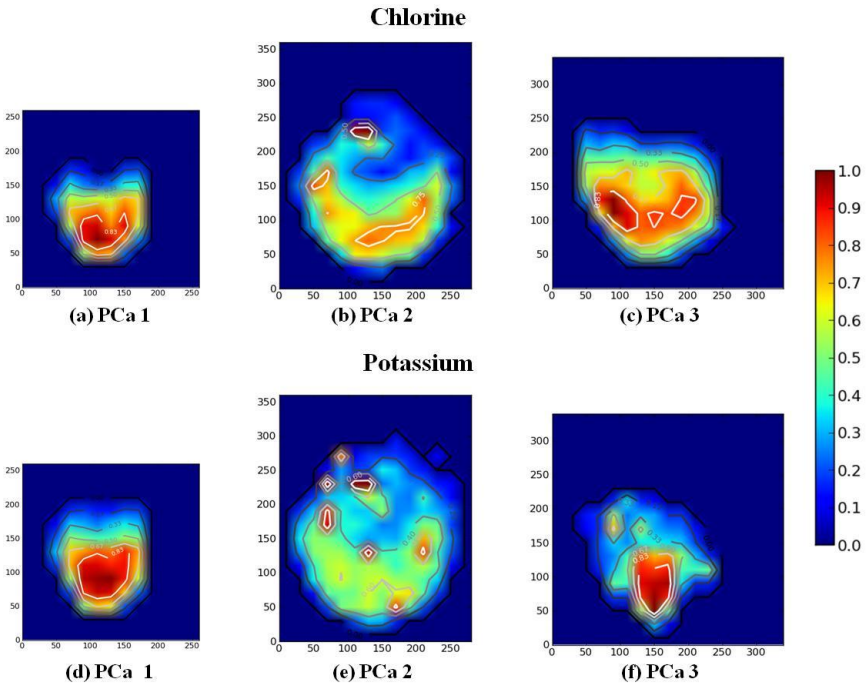

Figure 5: Image of chlorine mapping the spheroids of: (a) PCa 1, (b) PCa 2, (c) PCa 3 and Image of potassium mapping the spheroids of: (d) PCa 1, (e) PCa 2, (f) PCa 3.

Figure 4 and figure 5 (a, b, c) show mappings of P, S and Cl, the typical circular shape is not observed because of the absorption effect caused by low energy K-lines of these elements, the higher intensities are observed on the wrap of spheroids. In addition can be observed also that those elements presented similar elemental mappings more intense on the wrap of spheroids.

Figure 5 (d, e, f) shows that K elemental distribution is concentrated in a particular region of spheroids, and that spheroids PCa 1 and PCa 2 present the same distribution pattern for this element.

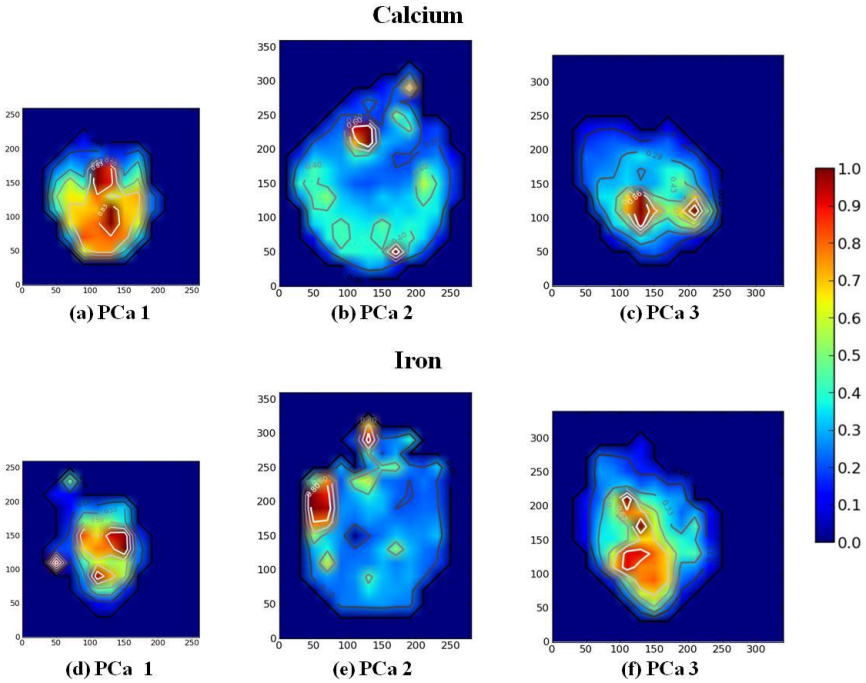

Figure 6: Image of calcium mapping the spheroids of: (a) PCa 1, (b) PCa 2, (c) PCa 3 and Image of iron mapping the spheroids of: (d) PCa 1, (e) PCa 2, (f) PCa 3.

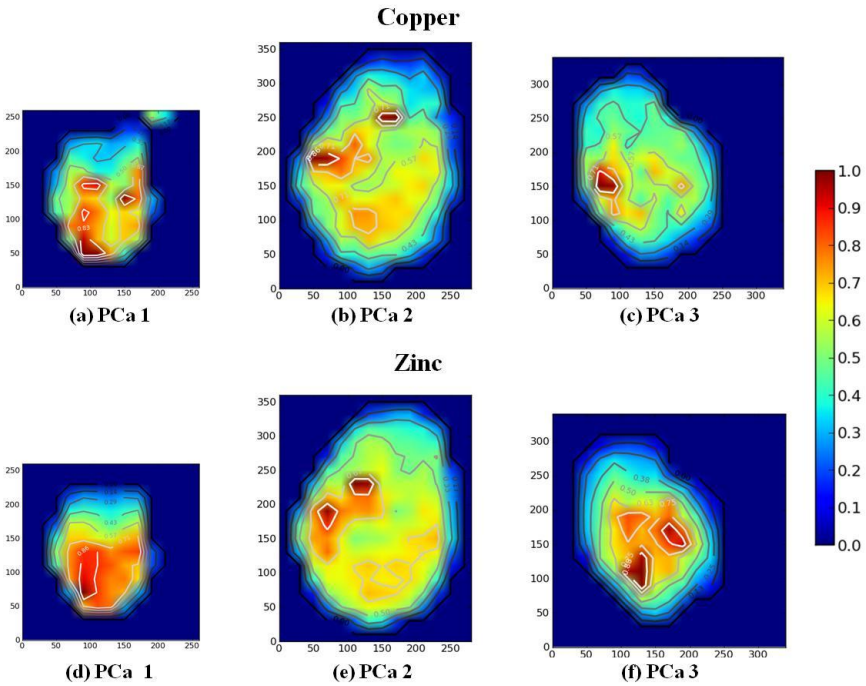

Figure 7: Image of copper mapping the spheroids of: (a) PCa 1, (b) PCa 2, (c) PCa 3 and Image of zinc mapping the spheroids of: (d) PCa 1, (e) PCa 2, (f) PCa 3.

The analysis of images for Ca and Fe (Figures 6) indicates very small intense regions for all spheroids analyzed. In addiction Figure 6 (d, e, f) shows that all spheroids analyzed presented an non-uniform Fe distribution. It can be observed that for PCa spheroids there is a small area that is more intense. PCa 1 and PCa 3 spheroids presented more intense elemental mapping of Fe in the central area.

The analysis of Figure 7 (a, b, c) shows that copper is distributed heterogeneously along the full extent of spheroids in low concentrations. Spheroids presented small regions (four in PCa 1, two in PCa 2, one in PCa 3) with high intensity of Cu.

Analyzing Figure 7 (d, e, f), it can be seen that Zn mapping presents a heterogeneous distribution in the entire spheroid. In addition, it is possible to see more intense Zn distribution in the central region of the PCa 1 and PCa 3 spheroids. The comparison of Cu and Zn mapping in Figure 7, shows that they have a similar distribution in PCa 1 and PCa 2 spheroids.

It is well known that zinc levels in the peripheral zone of dorsal and lateral lobes of the prostate are almost 10 times higher than in other soft tissues [10]. The normal secretory epithelial cells are highly specialized cells that synthesize, accumulate and secrete enormously high levels of citrate into the prostatic fluid; which is a major function of the prostate gland [11]. The highest levels of zinc are found in the mitochondria and prevent citrate oxidation by Krebs cycle. The decrease in citrate oxidation represents 65 % of the ATP efficiency [10, 12].

In prostate cancer, the malignant cells undergo a metabolic transformation from citrate-producing to citrateoxidizing cells. This occurs due to the loss of the ability of the malignant cells to accumulate zinc. The absence of high mitochondrial zinc levels prevents the inhibition of m-aconitase activity. Citrate is then oxidized and the typical complete oxidation of glucose restores efficiently the ATP production. Detailed descriptions of the relationships between citrate metabolism and zinc in prostate can be found in the literature [12, 13].

This study indicates that the distribution of zinc is more intense in the central region of the PCa spheroids, suggesting that there is an accumulation of epithelial cells in this region. Thus, a histomorphological study is necessary to evaluate how different cell types are distributed in spheroids and to evaluate the formation of different tissues in spheroid.

4. CONCLUSION

Mapping by μSRXRF proved to be a powerful technique to study the distribution of 8 elements: P, S, Cl, K, Ca, Fe, Cu and Zn in spheroid samples. All the elements were heterogeneously distributed in different areas of the spheroids analyzed. P, S and Cl showed similar elemental distribution. Elemental mapping of K showed a particular more intense region. Ca and Fe showed very small more intense distribution regions. Prostate spheroids presented small regions more intense of Cu. Zinc presented more intense distributions in the central region of the two PCa spheroid samples. The relationship between the function of zinc in the secretory epithelial cells and the carcinogenic process suggests that more studies on elemental mapping in spheroids are necessary.

5. ACKNOWLEDGEMENTS

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