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The First Pyrolysis Protocol Based on Experimental Measurements in the Atomic Level Structured Cancer Studies

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Abstract

Background: *Oxygen balance is critical for cell life and is regulated by an intricate oxygen-sensing process in the body. The same mechanism can also be used by cancer cells to survive, to grow and to disseminate what is key for cancer patients' life. We took highly advanced interdisciplinary approach Isotope Ratio Mass Spectrometry to experimentally search for methodological issues fundamental for oxygen evaluation on an atomic level of cancer tissue biology – by stable isotope ratio assessment.*

Objectives: *The aim of presented studies was to assess the reference mass of the sample for oxygen estimation in future cancer tissue studies.*

Material and Methods*: Experimental O-isotope determination in animal tissues made by IRMS (Thermo Finnigan MAT 253) following pyrolysis at 1350°C and chromatographic separation (70°C) of* H_2 *and CO in a He gas stream.*

Results: *Oxygen signals were identified in all the examined experimental animal tissue samples. The obtained oxygen isotope ratio values appeared 12,7 ± 0,5 ‰ and 11,8 ± 1,0/2 ‰ for vacuum line and the vacuum dryer experimental parts, appropriately.*

Conclusions: *Homogeneity of the tissue sample is critical for oxygen isotope ratio measurements in cancer. It must be taken in consideration when the level of homogenization of the sample allows to achieve the results versatile for cancer tissue studies with prospective clinical impact. Presented studies are the call for overcoming interdisciplinary barriers to intensify and develop isotope ratio cancer studies which give hope to understand more cancer disease and save cancer patients' lives.*

Key words: *biomarkers, isotopes, pyrolysis, structure analysis, neoplasms*

Introduction

Normal human cells need oxygen to survive and act properly. However, the amount must be right since oxygen balance is critical for cell life and because of that fact is regulated by an intricate oxygen-sensing process in the body, the discovery of which earned the 2019 Nobel Prize in Medicine. The same mechanism can also be used by cancer cells for their own survival. Hypoxic cancer cells acquire abilities key for cancer patients' health and life – the ability to metastasize and resist chemotherapy and radiation treatment. Although, it is not completely explained how hypoxic tumors gain these abilities, however it was proved that activating Hypoxia Inducible Factor (HIF) turns on genes and proteins that can degrade extracellular matrix, enabling cancer cells to disseminate. There is proved the relation of hypoxia and angiogenesis, and neovascularization in many cancer types. In oxygen deprivation states HIF expression is impaired and induces activity of the other proangiogenic factors as Stromal cell derived factor-1 (SDF-1), which recruits in turn bone marrow derived progenitor cells CD133(+), from circulatory system to the hypoxic areas which starts angio – and vasculogenesis. Epithelial to mesenchymal transition caused by HIF action makes cancer cells to be more mobile and more resistant to therapy. Hypoxia in tumors is also a major factor in their resistance to immunotherapy agents. Moreover, cancer cells can also adapt to low-oxygen environments by turning on an alternative way for generating energy – one that doesn't require oxygen [1–5].

The complex relationship between oxygen and cancer has been a subject of ongoing research. The lack of practical results of these efforts so far justifies a search for wider understanding of the problem with the use of modern methods which may alight pathways of cancer growth and metastasizing in oxygen deprivation states.

Interdisciplinary studies on cancer, highly developing today, had brought such a possibility. Awarded the four Nobel Prizes Mass Spectrometry had opened a new window on cancer studies by structured cancer tissue studies on an atomic level – the stable isotope ratio assessment.

Stable isotopes are non-radioactive atoms of the same chemical element, which differ only in their number of neutrons [6]. The studies with the use of stable isotopes are known from decades and since 1990s their popularity in metabolic studies started to increase [7].

Isotope ratio assessment by Isotope ratio Mass Spectrometry (IRMS) relies on the fact that biochemical processes cause change of isotopic profile of elements of reacting molecules due to different rates of isotopic species what is named an isotopic fractionation. The measurement of the ratio of a heavier stable isotope to a lighter one is very precisely expressed as a delta value which is 'per mil' (‰) deviation from a standard.

Stable isotopes are used for the dynamic assessment of in vivo metabolism and they may be particularly important for cancer studies and the validation of new treatment modalities. In 2015 it was successfully completed the first protocol of stable isotope ratio assessment in tumor tissues based on original research and the same year the first histoclinical studies were performed and for the first time revealed proves for prospective clinical impact of isotope ratio measurements in cancer [8, 9].

Highly developing stable isotope cancer studies during next few years had brought many intrigued findings which alight cancer biology at the atomic level as well as potential clinical implications of stable isotopes estimations, and the results of isotopic studies were published by the most demanded scientific journals e.g. Nature group, however none of them concentrated on oxygen [10, 11].

At this still early stage of isotope ratio assessments in cancer tissue it is fundamental to establish universal methodology of the studies as well as patterns of measurements and references values of samples.

The aim of presented studies was to assess the reference mass of tissue sample for oxygen estimation in future cancer studies. The standard mass was understood as the mass of the sample selected in such a way that it was possible to obtain the appropriate peak intensity (mV) of CO (the values within the linear range of the used devices).

Material and Methods

Experimental part

Material: animal tissue (meat RFN type (EU), class 1 – pork loin of the protein content in the fresh meat 16.8% (67.2% dry matter) and fat content which did not exceeded 30%, and connective tissue content which did not exceeded 20%).

Preparation procedures

Animal tissues were cut into pieces of an average size of 0.3 cm x 0.3 cm x 0.3 cm and frozen – 70°C till the time of experiment and thawed prior to drying. Drying: The pieces of animal tissue were placed on the sides of bottles 5 centimetres high and weighed. Drying was carried out using two methods: by means of a vacuum line and in a vacuum dryer. Drying in a vacuum oven took place at the temperature of 30°C for 24 hours; drying in a vacuum line lasted 5 days (120 h). Drying in a vacuum line took place in a desiccator with the use of a drying agent – phosphorus pentoxide (P_2O_5). The vacuum in the system was 0.001 mm Hg. After the drying process, the samples were weighed and on this basis the percentage of dry mass was obtained.

Homogenization

Homogenization followed drying, the pieces of animal tissue were homogenized by a vibrating grinder that had two balls made of agate. Grinding the pieces of animal tissue by hand, first with a scalpel, and then with a mortar made of percelite, was used. The samples obtained in this way – for the vacuum line and the vacuum dryer, which were determined respectively "L" and "S", were sent for isotope ratio assessment with the use of pyrolysis.

Isotope ratio measurements

Samples were first weighed (180 ± 20 µg) into Ag-foil capsules. The capsules were then folded and loaded into plastic sample trays. Samples were then loaded rapidly onto the automated carousel of a ThermoFinnigan elemental analyzer (Bremen, Germany), which was evacuated, purged with helium, and opened to the reactor. Before beginning the analysis, I monitored the background voltage on masses 28, 29, 30 until stable. O-isotope determination was made by IRMS (Thermo Finnigan MAT 253) following pyrolysis at 1350°C and chromatographic separation (70°C) of H_2 and CO in a He gas stream. Measured values were calibrated to repeat analyses of a gelatin (certified reference material) provided by Elemental Microanalysis company and caffeine (measured by different research group and published) standards and are reported to VSMOW on the VSMOW-SLAP scale. Analytical precision, based on repeated analysis was 0,5 ‰ (δ18OVSMOW;1σ n = 5).

One needs to pay attention to the fact, that N-containing compounds could yield less accurate results, despite quantitative conversion of the standard oxygen into CO. Analysts believe that the problems is partially caused by interfering gases (third peak on the chromatogram) produced by a secondary decomposition of N – and C-containing polymers formed during the decomposition of the analyte [12–14].

Results

Oxygen signals were identified in all the examined experimental animal tissue samples.

Obtained results of oxygen isotope ratio assessment regarding the conditions of performed experiments are presented in Table 1, identification of the method and summary of the procedure and results are presented in Table 2 and Figure 1.

Table 1. Characteristic of results of oxygen isotope ratio measurements in experimental animal tissue studies

	Samples	Animal tissue mass (g)	Animal tissue mass after drying (g)	% of dry animal tissue mass	% of dry animal tissue mass (mean value)
Vacuum dryer	1	0,03188	0,00898	28,16813	30,21254
	2	0,03344	0,00975	29,1567	
	3	0,02434	0,00742	30,4848	
	4	0,03068	0,00894	29,1395	
	9	0,41910	0,14297	34,11358	
Vacuum line	5	0,06190	0,01709	27,609047	28,93006
	6	0,07765	0,02282	29,38828	
	7	0,06820	0,01986	29,12023	
	8	0,06594	0,01952	29,60267	

Identification of used method and the summary of oxygen isotope ratio measurement procedure and results plus EA-Py-CF-IRMS chromatogram of gelatin are presented in Table 2 and Figure 1.

Table 2. Identification of used method δ18OVSMOW/EA-Py-CF-IRMS

"L" analysis			
Analysis	Parameter	Result±s	Unit
1.	δ 18OVSMOW	12,7 ± 0,5	‰
"S" analysis			
Analysis	Parameter	Result±s	Unit
2.	δ 18OVSMOW	11,8 ± 1,0/2	‰

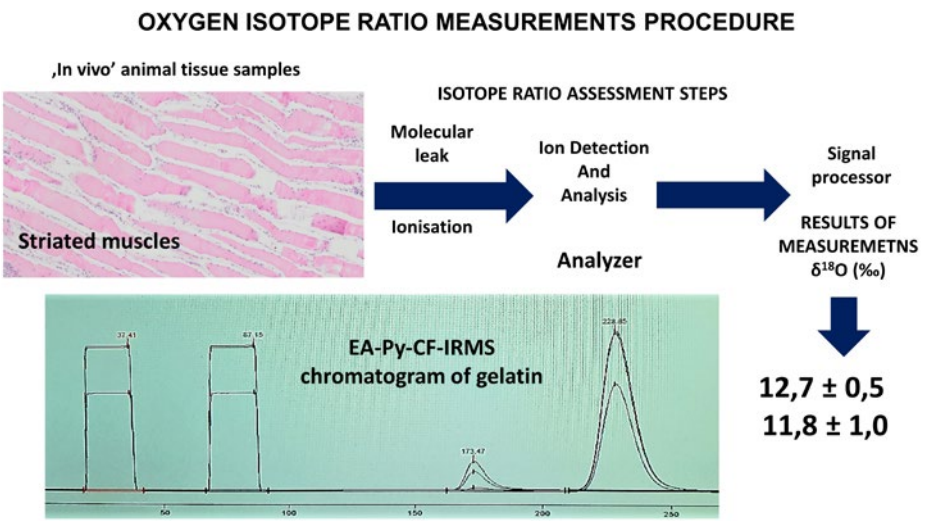

Figure 1. Oxygen isotope ratio measurement procedure and results plus EA-Py-CF-IRMS chromatogram of gelatin

Discussion

The stable-isotopic composition of the body tissues depends on the isotopic composition of food sources and on shifts due to isotopic fractionation during metabolism. It already have been demonstrated that cardiovascular diseases, smoking, anaemia, liver diseases, obesity and pregnancy affect the isotopic composition of human tissues [15–21].

The research in literature strongly suggests a relationship between stable isotope biochemistry, and human pathology, including cancer [10, 11, 22–25].

Nitrogen and carbon highly predominate as the subject of isotopic studies on cancer regarding their part in cell life sustention and proliferation [10, 11, 24]. A little is known about the other elements e.g. copper and zinc in cancer, the studies on oxygen fractionation in cancer had not been found in the literature according to the authors knowledge [23, 25, 26].

Isotopic studies on oxygen reflects in the literature environmental studies on water resources and oxygen fractionation is analyzed with hydrogen as one of the two elements of water chemical structure. Growing risk of climatic

changes makes this area of studies highly developing. Isotope ratios of tap water have previously been studied as a potential tool to link public supply waters with water source characteristics at local to continental scales, providing information on the footprint of and potential risks associated with the water sources used. The tap water isotope signatures identified here could be widely applied to characterize water supplies and associated sustainability challenges in different regions worldwide [27, 28]. Stable isotopes in water (δ 2 H and δ 18 O) are important indicators of hydrological and ecological patterns and they are incorporated into geological and biological systems in a predictable manner. Physical processes result in spatial variation of δ 2 H, δ 18 O in water across the landscape (so-called "isoscapes") and provide the basis for hydrological, ecological, archaeological and forensic studies [29].

The relation of oxygen and cancer biology is complex and not completely known. In cancer patients tumor hypoxia leads to a poor prognosis due to the potential of increased aggressiveness, metastatic potential and resistance to chemo – and radiation therapy [30, 31]. Although HIF-1α transcription does not require oxygen, in normoxia, HIF-1α is rapidly degraded [32].

HIFs also stimulate cancer stem cells (CSCs) [33]. Furthermore, one mechanism of therapy resistance can be attributed to the special capacities of CSCs.

Regarding the part of oxygen in cancer cell survival, growth, dissemination and resistance to universal therapies and taking into consideration promising results of already performed IRMS studies on the other elements it seems to be reasonable to choose oxygen fractionation as the current aim of evaluation which may benefit in better understanding of cancer biology and triggers for metastasizing.

In presented studies the oxygen signals were successfully identified in all the examined samples, however a large standard deviation can indicate that samples were not enough homogenous. Regarding the obtained results homogeneity of the sample reveals as the condition of expected precision in oxygen studies and the evaluation of stable isotopes of oxygen seems to be much more challenging than previously performed with the use of continuous low isotope ratio mass spectrometry (CF-IRMS) estimations of nitrogen and carbon.

Finally, some general methodical issues may be underline. To improve the precision of the results, the intensity of the peak that comes from the sample should be close to the intensity of the standard gas. The mass of the sample should be selected to obtain the appropriate peak intensities (mV) of CO (at appropriate dilution), so that they fall within the linear range of the introducing device (elemental analyzer) and the mass spectrometer. To improve the precision of the results, the intensity of the sample peak should be close to that of the reference gas.

Conclusions

Homogeneity of the tissue sample is critical for oxygen isotope ratio measurements in cancer. The use of a vibrating grinder for homogenization did not give satisfactory results. It must be taken in consideration when the level of homogenization of the sample allows to achieve the results versatile for cancer tissue studies with prospective clinical impact. Presented studies are the call for overcoming interdisciplinary barriers to intensify and develop isotope ratio cancer studies which give hope to understand more cancer disease and save cancer patients' lives.

The authors declare no conflict of interest.

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