

METODOLOGICAL ISSUES IN PROTEIN AND LIPIDIC EXPRESSIONS IN BRAIN TISSUE EXPOSED TO Co⁶⁰ BASED ON DESI/MALDI-MS

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ABSTRACT

The present paper attempts to present some issues in the methodology of identifying lipid and protein changes in brain tissue induced by radiation. The goal was to address the analysis of the methodology and to investigate the feasibility of the generation of lipid/protein profiles of irradiated brain tissue, in order to identify radioinduced changes. Lipids and proteins are biomolecules with diverse structures and functionalities that participate in important intracellular processes. Changes in the lipid and the tissue protein profiles may indicate a cellular response to an external stimulus as well as the emergence of neoplasms or neurodegenerative diseases such as Alzheimer's. DESI-MS is a convenient method for identifying lipids and their spatial distribution in tissue beyond analytical quantification. DESI-MS allows the creation of an image of several low lipid m/z classes. MALDI-MS has already been a method used in the study of macromolecules as structural, membrane, hormone, neuromediator and immunological peptides. Through a full-scan matrix scan, with a m/z spectrum between 500-1000 for lipids and with a mass spectrum of 1000-15000 Da for proteins, the molecular profile can be analyzed. Generated pixel shape 2D chemical image. The produced image allows to associate the tissue distribution of the lipids and proteins with their chemical profile identified, allowing the verification of the changes radioinduced. Radiation triggers intense oxidative stress by increasing reactive oxygen species (ROS) and free radicals, causing DNA damage with consequent alterations in proteomics and cellular lipid explaining such changes in the lipid and protein expressions. The cellular morphophysiological changes are responsible for both the clonogenic inhibition and the induction of the apoptotic process. The images' production was directly dependent on the rigorous execution of the methodological procedures. Innumerable interferences could impair the image's generations and protein and lipid analysis. The main interferences in the present MS methodology were addressed and discussed. Following a well-established methodology, DESI can generate lipid spectra and MALDI protein spectra in brain tissues. Near future, it will be possible to detect a change in the distribution and types of lipids and proteins between the white and gray matter regions.

1. INTRODUCTION

The World Health Organization's International Agency for Research on Cancer (IARC/WHO) has shown that cancer is a major cause of morbidity and mortality worldwide, with about 14 million new cases and 8.2 million deaths directly related to neoplasias since 2012 [1]. It is estimated that the number of annual cases will increase approximately 57.14%, reaching 22 million new cases in the next two decades [1]. Neoplastic malignant lesions are characterized by intense disordered cell proliferation associated with mechanism failures in cell repair, apoptosis's and clonogenic control, which may lead to clinical physiological abnormal alterations, affecting the organs, tissues and cells which hold the ability of infiltrations and metastases [2].

The association of an early diagnosis and the prescription of efficient treatments, including surgery, chemotherapy and radiation therapy (RT) becomes essential. Thus, if the harmful deterministic effects of ionizing radiation (IR) provide the neoplastic clonogenic control with acceptable deleterious effects in the adjacent healthy tissues, the RT holds a good indication and is supported [3]. It is known that IR causes non selective damage to healthy and neoplastic tissues and the intensity of its effects are manifested according to the inherent cell radiosensitivity, tissue kinetics and the way of the cellular organization of the tissues [4]. However, even with the adoption of strict protocols to minimize deleterious effects in organs of risk, ensuring adequate kinetics of repair in healthy tissues, effects induced by IR, even in low doses, have already been identified through changes mainly in the protein metabolite and intracellular lipids [11,12,14].

The metabolomics allows the analytical study of biomolecules as well as their final products and the cellular processes involved in their synthesis. Lipidomic analysis allows dynamically identification of the changes involved in lipid expression induced by tissue's irradiation [4]. The most abundant lipids are glycerophospholipids (PLs) present in biological membranes and involved in signaling, regulation, proliferation and apoptosis [5,6]. The use of analytical techniques such as Mass Spectrometry (MS) allows the determination of elemental compositions, molecular structures and quantitative and qualitative analyzes of complex molecular mixtures. Through DESI (Desorption Electro Spray Ionization) and MALDI (Matrix Assisted Laser Desorption Ionization) two ambient ionization techniques, organic tissues can be subjected to the analysis of their structural components such as lipids (phospholipids) [7] and proteins, respectively. The characterization of phospholipid is of extreme importance due to the diversity of cellular functions besides the possibility of being used as a biomarker in neoplasms [7]. The use of the DESI-MS system in the full scan mode in both healthy and tumor tissues showed a great variation in the content of phospholipids between the tissues, allowing also decision making for surgical margins enlargement based on molecular information from the quantitative analysis and qualitative analysis of lipids in the organs affected by neoplasias, decreasing the chance of relapses and new surgeries to remove lesions [15]. MALDI-MS is being used to identify phospholipids, toxins from microorganisms, identification of microorganism species and proteins in various tissues as transmembrane proteins [16,17,18,19]. DESI-MS presents numerous applications besides the obtaining of the lipid profiles of normal and neoplastic tissues such as identification of metabolites in microbiology, falsification of money and study of cerebral neurotransmitters [20,21,22].

Recently, numerous evidences of alterations in the metabolome were verified mainly by the lipid alterations in the tissues. It seems evident that RT can induce changes in the human metabolome. To improve this knowledge, the present paper addresses some methodological issues in brain protein and lipid's expressions, due to Co⁶⁰ exposition, based on DESI/MALDI-MS, lighting on the visibility of quantifying such molecular changes induced by IR.

2. MATERIALS AND METHODS

2.1 Group selection

An amount of 28 healthy Wistar rats was selected, with an age of 11 weeks and body weight between 300 ± 15 g. The animals were divided in two groups: one control (n = 4), denominated GC, and another one submitted to total body irradiation (TBI), called GIR (n = 24) subdivided into 06 sub-groups containing 04 animals. The animals were submitted to the same photo period of 12h day/night with free access to food and water. Animals of the same lineage, family, with the same weight and age, were used to linearize the experiment, provided by the laboratory of the Federal University of Minas Gerais, Central Bioterium. All experiments were previously submitted and approved to CEUA-UFMG, protocol 339/2014, following standardization for animal care and euthanasia.

2.1 Irradiation Protocol

The animals of the GIR group (n=24) had their total body irradiated through the exposition to a Co-60 source, all together placed in 6 boxes, with a 5 Gy absorbed dose, in the Laboratory of Irradiation Gamma-LIG of the Center of Development of the Nuclear Technology - CDTN. They were irradiated following a distinct time kinetics. The following data were set: date of calibration of the Co-60 source on Oct.14th, 2013; activity at the date of calibration of 43749,21 Ci; activity at the animal irradiation date of 26940,00 Ci; distance from the chimney of 1,6 m; current dose rate of 87.91 Gy.h⁻¹; applied dose of 5 Gy; exposure time of (hr:mm:ss) 0:03:25.

2.2 Euthanasia and organ preservation

The animals were fasted without deprivation of water in the boxes. Each one was submitted individually to the anesthetic procedure following CEUA protocol. The calculation of the anesthetic solutions of 5% Ketamine and 2% Xylazine at the doses of 80mg of ketamine + 15 mg of xylazine, both/kg of animal to be administered parenterally (intraperitoneal) was carried out, maintaining the V/V ketamine/Xylazine (2:1) ratio. After the administration of the anesthetics, tests were carried out to investigate the sedation and the consequent guillotining. The organs were removed, weighed, frozen in CO₂ ice rapidly and stored at -80°C.

2.3 Cryostat

The organs were transported on dry ice to avoid denaturation and losses of the tissue components. The cuts were performed on the Leica CM1850 cryostat. Sagittal sections were preserved preserving a cerebral hemisphere. The chosen hemisphere was sectioned at 12 μm thick and placed on the common foils for analysis by DESI. For MALDI, the cuts were with the same thickness; however, they were deposited in special blades that conduct electricity, being necessary the aid of a multimeter to identify the correct surface of analysis. After the cuts, the slides were stored in the ultra-freezer at $-80\text{ }^{\circ}\text{C}$.

2.4 DESI

Immediately prior to analysis, plates with slide samples were transferred to a vacuum desiccator until thawed. It was run for 15-20 min with care not to dehydrate excessively so as not to reduce the effectiveness of the DESI. The best solvents were acetonitrile (ACN):(DMF) N,N-dimethylformaldehyde (1:1) and EtOH:DMF (1:1) since they do not cause morphological changes. The infusion pump was adjusted with a flow rate of $0.7\text{ }\mu\text{L}\cdot\text{min}^{-1}$. The nitrogen nebulizer gas was turned on at 160 psi pressure. The high voltage source was connected to the ion source and a voltage of 5 kV applied. The mass spectra were acquired in the 200-1000 mass range., with an incidence angle of $\sim 49^{\circ}$ between the source and the sample surface. The slide was laid with 150 μm for rat brain and 200 μm for human brain and the slide scanning started.

2.4 MALDI

The matrix was dissolved in 60% can and 40% H_2O with 0.2% Trifluoroacetic acid (TFA). The matrix was sublimated and the slides were placed in the adapter. In order to optimize the analyzes in the Maldi-TOF/TOF instrument (UltraFlexxtreme),the slides were scanned to determine the scanning area limits.Laser shots (500) were applied (resolution of 150 μm) and the mass spectra acquired in a 1000-15000 mass range. The analysis was performed using the FlexImaging 3.0 software.

3. RESULTS

The protocols for matrix formation required for ionization in MALDI should be reviewed and adjusted according to the type of sample. As illustration, the first MALDI processing is presented in Fig. 1. It is a partial analysis of the images of a brain slice. The analysis was not completed due to the following reasons: i) long analysis time; ii) the needs for calibration; and iii) interruption of the cleaning process in order to guarantee the reception of the signal at an adequate intensity. Preliminary analyzes already showed characteristic spots of proteins in the spectra that need proteomic protocols to be identified (FIG. 2).

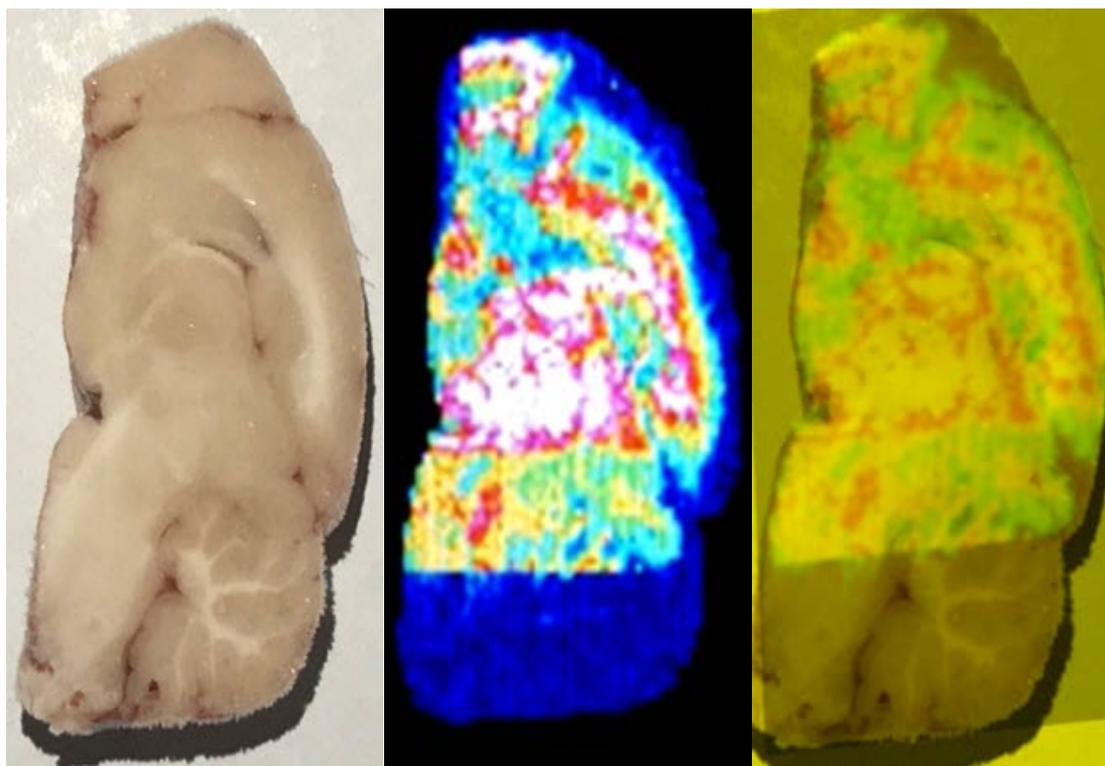


FIG.1: Image of a sagittal brain section from an irradiated animal, recorded on a MALDI/TOF instrument and superposition of the high metabolic areas with sagittal brain section.

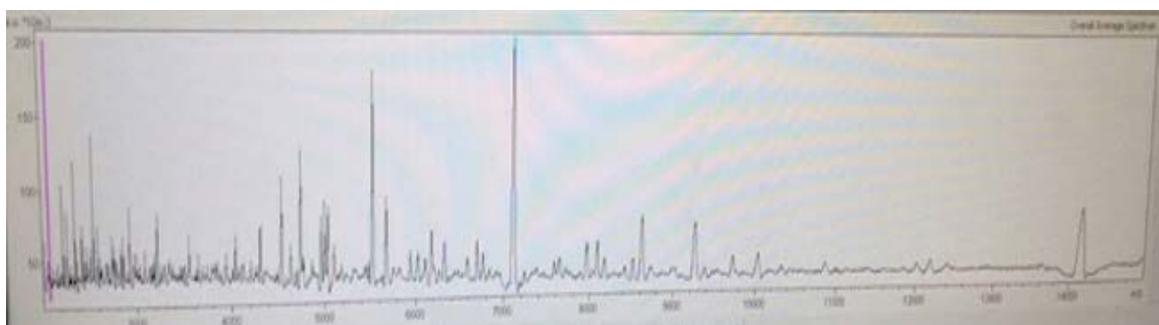


FIG. 2: Typical protein spectrum taken in a small area in a sagittal brain section from an irradiated animal, took in a MALDI/TOF instrument.

The images's production was directly dependent on the rigorous execution of the methodological procedures. Innumerable interferences could impair the image's generations and protein and lipid analysis.

The main interferences in our MS methodology can be addressed, as follows:

I. Sterile material should be used in the organ's extraction, without being washed directly with detergent. Organs and instruments should be always watered with deionized water (Milli-Q water) avoiding direct contact with the hands in order to avoid contamination with fatty acids of the skin. The organs should be removed and frozen in the shortest possible time in order to preserve the morphology and prevent enzymatic degradation. The brain must be

withdrawn with extremely careful by removing the temporal bone part and the meninges. Appropriate instruments must be used to avoid fractures in the brain tissue, impairing the generation of full cutting sections.

II. During the preparation of the foils, the direct contact with the skin should be avoided so that no contamination of fatty acids with the sample occurs. An ideal cutting temperature for each type of organ's tissue must be checked. A negative temperature range from 15-25 °C should be applied to the brain, heart and lungs; 10-15°C to gonads; 25-50 °C to kidney and liver (Leica CM1850 technical reference). The Tissue-Tek OCT should not be overused to avoid contaminating the tissues and making the samples unfeasible. The cuts should be adjusted to 12µm thickness and the glass plate was not used to capture the cut as it creates micro grooves that damage the cut.

III. Immediately before carrying out the analyzes, the slides should be transferred to a vacuum desiccator for 15-20 min until thawed. It is necessary no excessive dehydration so as not to reduce the effectiveness of the DESI. The defrost period can be used to start the machine.

IV. The following variables should be optimized, such as solvent type (DMF:ACN as 1:1), solvent volume, solvent flow rates, gas pressure rate, nebulizing gas voltage, distance and angles of the probe and the MS input capillary. The probe should be at a distance of 3 mm from the sample surface and 5 mm input from the MS inlet capillary. The probe should be at an angle of 55° to the surface of the sample and ~15° to the sample surface for optimal transfer of ions. The speed of the infusion pump should be adjusted to obtain the optimum pixel for optimization and image.

V. The solvent with DMF (DMF:EtOH or DMF:ACN) provided an increase in ion signal strength with a well defined spray area being ideal for imaging applications. Besides DMF is histologically compatible with medical procedures allowing H & E histochemistry after DESI /MS.

VI. A heating element that surrounds the MS inlet capillary may improve the sensitivity by desolvation of the charged analyt micro droplets, produced during ionization. A rope warmer wrapped around the transfer capillary assembly should be maintained at 100 °C.

VII. It should be brought in mind that two factors influence the quality of the MALDI spectra: the matrix and the technique used for matrix deposition. The choice of the stratigraphic matrix is related to the analysis of a certain type of protein (intact, crosslinked, soluble, cleaved by proteolysis). A mixture of two matrices is required to guarantee the good data acquisition with better resolution (protein peaks More evident). We should also mention an important factor that should be considered in MALDI that is the matrix deposition. Such matrix must be made with thin layers without contaminants. The cleaning of the blade compartment must be carried out carefully. A high-resolution optical image should be generated to determine scanning locations for analysis and to verify alignment and positioning with the sample.

4. DISCUSSION

The IR provides interactions to the organic molecules that present sufficient energy capable of provoking electronic excitation and ionization, causing in the breakdown of chemical bonds in the biological molecules of the tissues [8]. Gamma rays ($R\gamma$) are emitted radiations of radioactive nuclides, through nuclear disintegrations, or of positron annihilation processes in matter [9]. Gamma rays are low LET (Linear Energy Transfer) radiations usually employed in RT that produce ionization and consequently free radicals damaging biological tissues. Dose and dose rate are essential in the analysis of energy absorbed in RT patients. The clinical response to a deterministic effect is directly related to the frequency and dose rate absorbed when exceeding a dose threshold relative to the sensitivity of the tissue exposed to its effect [10].

From literature, data from brain tissue of guinea pigs suggest a cellular response to oxidative stress. Polyunsaturated fatty acids and cell membrane structural lipids are widely susceptible to gamma irradiation damage causing alteration of permeability, cell signaling, and apoptosis [12,13,14].

In cells, membrane integrity is imperative to ensure signaling of the protein and lipid metabolome. However, IR induces the oxidation of lipids and proteins by Reactive Oxygen Species (ROS).

Lipid peroxidation is a cytotoxic event defined by successive biochemical events triggered by free radicals determining the extensive oxidation of unsaturated fatty acids and membranes. The by-products formed generate structural and functional alterations in the cell membrane causing alteration in the ionic and molecular substrate flow, triggering several processes [11]. Metabolism and serum phospholipid levels (PLs) were altered following exposure to ionizing radiation [12].

5. CONCLUSIONS

DESI can generate lipid spectra and MALDI protein spectra in brain tissues. Near future, it will be possible to detect a change in the distribution and types of lipids and proteins between the white and gray matter regions.

Thus, lipid-induced changes, especially in phospholipids, and in cortical and spinal proteins deserve greater detail and deepening in the attempt to elucidate the metabolic pathways that suggest such expressions. With the possibility of identifying other lipids and proteins and the metabolic pathways involved, we can contribute in the future to the use of metabolic markers of cellular injury and to contribute to the review of certain radiotherapeutic protocols.

The experiments are still being adjusted to confer reliability on the large volume of data generated. The high efficacy and qualitative and quantitative precision will allow a careful analysis of the possible alterations found in irradiated tissues. To validate the experimental data will be used statistical methods allowing the fingerprint of lipids and proteins obtained by DESI and MALDI respectively, are duly compared and discussed with absolute safety and reliability.

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