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CONTRIBUȚII PERSONALE LA IMPLEMENTAREA ȘI DEZVOLTAREA MICROSCOPIEI CONFOCALE ÎN CADRUL UNIVERSITĂȚII DE MEDICINĂ ȘI FARMACIE ”VICTOR BABEȘ” DIN TIMIȘOARA

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ABSTRACT

The first description of an optical device that allowed microscopic evaluation of biological materials in a confocal system belonged to Marvin Minsky. After a quiet period, the interest for the confocal system resurged in the 1980s, following the wide-spread use of immunofluorescence. The first prototypes of confocal microscopes were developed by Amos and White, along with Carlsson. The first axial image set showing enhanced nuclear detail belonged to Brakenhoff and colleagues.

Confocal microscopy is commonly used in biology, medicine and material studies. The biological samples used are variable, from cell cultures to whole organs. The viability of the samples is determined by the processing techniques involved: living cells for *in vitro* and *in vivo* studies, tissue fragments or whole organs maintained in special media, for example in organ baths, cells and tissues fixed in various fixative agents, respectively. None the less important is the processing of tissues on ice, which in special circumstances may keep the tissue viable.

The fluorescent markers employed in confocal microscopy can be either endogenous (synthesized by target cells or intrinsic autofluorescence) or exogenous. The exogenous markers commonly employed are fluorescently labelled antibodies, substances that are intrinsically fluorescent or are metabolized to a fluorescent compound.

The types of datasets usually acquired in confocal microscopy include 2D, 3D images and their corresponding time-lapse counterparts (4D). These images can be further analysed to yield objective data, like morphometric measurements (size, diameter, areas, volumes, object density, distances between objects, and so on). Furthermore, fluorescence intensities can be measured which can reveal statistically relevant differences that the naked eye cannot distinguish.

In the clinical setting, confocal microscopy is used in two approaches: *ex vivo* and *in vivo*. Each of them can further employ two distinct imaging techiques. The first one evaluates the light reflected by the tissues, after illumination with a specifically determined wavelength for the tissue in question (reflectance confocal microscopy). The second technique uses fluorophores or contrasting agents which can be stimulated by light with a specific wavelength.

The surgically removed tissues can be evaluated in confocal microscopy in their native state, fixation not being required. Toluidine blue and methylene blue can be used as markers for fluorescent labelling, while acridine orange can be used to highlight nuclei, due to its affinity for DNA.

For *in vivo* confocal imaging, the most commonly employed technique is the endoscopic approach. One endoscopic system integrates the confocal system in the intrinsic optical system, leaving the working channel free to be used by the biopsy forceps, for example. The second types can be considered as an add-on to any endoscope, as it utilizes the work channel to insert the confocal system.

The source of energy in the cells is represented by oxidative degradation of substrates in the mitochondria. The deleterious effects of uncontrolled oxidative reactions are countered by an ensemble of compounds and antioxidant systems.

Oxidative stress was defined as the imbalance between free radicals production and the antioxidant capabilities in the cells. Dean Jones proposed a different definition of oxidative stress, after analysing the two redox systems of GSH/GSSG and CyS/CySS. According to Jones, oxidative stress is rather an imbalance in the control and signalling of redox processes.

In order to quantify oxidative stress, several techniques are described. Most of the studies describing oxidative stress quantify either oxidative activity (i.e. reactive oxygen species generation), either the antioxidant capacity. Both approaches are rarely seen in the published literature.

AIM OF THE THESIS

The aim of this thesis was to develop new research directions by implementing and further development of confocal microscopy techniques in the University of Medicine and Pharmacy "Victor Babes" Timisoara. Furthermore, I also aimed to improve the infrastructure of the confocal microscopy lab.

The main objectives were as follows:

1. Implementation of immunofluorescence techniques using frozen tissues;
2. Implementation of ROS monitoring techniques, by using DCF on frozen tissues and DHE *in vivo* or on frozen samples;
3. Implementation of an experimental model for *in vivo* dynamic ROS monitoring in rats with chronic kidney disease induced by unilateral urinary obstruction;
4. Implementation of image analysis techniques and algorithms on confocal microscopy acquired datasets;
5. Designing and building of a new portable cryoembedding device.

STUDY NO.1 THE ROLE OF MONOAMINEOXIDASES IN CARDIOVASCULAR OXIDATIVE STRESS IN EXPERIMENTAL DIABETES MELLITUS

The aim of this study was to characterize the immunohistochemical expression of both MAO-A and MAO-B isoforms in aortic rings and myocardium of diabetic Wistar rats.

Diabetes mellitus was induced by intraperitoneal injection of streptozotocin (50mg/kg). The control group received citrate buffer solution (0.01ml/L, pH=4.5). After a 2 month period of diabetes mellitus, myocardium tissue fragments were OCT embedded and frozen. 6um thick sections were incubated with primary anti-MAO-A (Abcam ab126751, 1:50), anti-MAO-B (Abcam ab125010, 1:50) and Texas Red – labelled secondary antibodies (SantaCruz SC2780, 1:200). Nuclei were counterstained with DAPI (SantaCruz sc3598). The slides were coverslipped using an aqueous mounting medium for fluorescence (Vectashield, VectorLabs). The slides were then examined using

an Olympus Fluoview FV1000 confocal microscope. Fluorescence intensity was measured in linear regions of interest and expressed as Arbitrary Units (AU).

The MAO-A expression was similar in both control and diabetes mellitus rats (641.21 AU and 628,37AU, respectively). The MAO-B was overexpressed in diabetic rats as compared to control group (728.79 AU vs. 321.9AU).

In this study we showed for the first time that MAO-B isoform is overexpressed in the cardiomyocytes of rats with experimentally induced diabetes mellitus. Confocal microscopy enabled us to produce objective data for quantification of MAO expression.

STUDY NO.2 CHARACTERIZATION OF H202 LEVELS IN THE OXIDATIVE STRESS OF THE PATIENTS WITH CARDIAC DISEASE

The aim of this study was to quantify the H2O2 levels in human myocardial cells harvested from patients with coronary heart disease and associated diabetes mellitus, using confocal microscopy techniques.

This study was performed on 30 patients admitted into the Institute for Cardiovascular Diseases Timisoara and scheduled for open heart surgery. The patients were assigned to three study groups:

1. control group, which included patients with valvular lesions, without prior coronary disease (CTRL);
2. coronary heart disease patient group, without associated diabetes mellitus (CHD);
3. coronary heart disease patient group, with associated diabetes mellitus. (CHD-DM).

During surgery, after cardiopulmonary bypass was instated, small myocardium fragments were harvested from the right atrial appendage. The tissue fragments designated for confocal microscopy evaluation were embedded in OCT and then frozen. 8um thick sections were incubated in the dark for 30 minutes with 2’7’-dichlorofluorescein diacetate (DCF)(Sigma-Aldrich, D6882). The sections were immediately examined on an Olympus Fluoview FV1000 confocal microscope, using a 40x objective, NA=0.9. Spectral deconvolution was performed in order to limit the contribution of autofluorescence to the DCF staining. An automated image analysis protocol was constructed in Icy, which included the following steps:

- identification of TIFF files in the designated folder;
- opening of each image;
- applying the detection parameters to form ROIs;
- overlaying the ROIs on the original image to designate the areas in which to quantify the signal;
- extracting the ROI statistics and exporting them into xls files, having similar naming as the original file;
- the superimposed ROI on the original image were saved as a new TIFF file, with the suffix _processed.

Following data evaluation, we noticed that even though there was apparent difference in DCF intensity, it did not reach statistical significance (ANOVA p=0.4142). The average values were: 341.6AU (SEM=24.95) for CTRL, 405,8AU (SEM=39.94) for CHD and 400,1AU (SEM=13.31) for CHD-DM. Confocal microscopy added value to our experiments by bringing morphological characterization of DCF distribution and by allowing fine tuning of detection settings in order to limit autofluorescent signal.

STUDY NO.3 ROS SPECIES COMPARTIMENTALIZATION IN THE KIDNEYS OF RATS WITH EXPERIMENTAL DIABETES MELLITUS

Through this study we aimed to characterize ROS generation in the different compartments of the renal parenchyma in chronic kidney disease, by inducing diabetes mellitus in rats.

Experimental diabetes mellitus was induced in 12 Wistar rats, by injecting a single dose of streptozotocin intraperitoneally. Representative renal cortex fragments were harvested 2 months after diabetes mellitus was instated and frozen in OCT. 20 um thick cryosections were incubated with DHE for 30 minutes, in the dark, at room temperature. The slides were immediately examined on an Olympus FluoView FV1000 confocal microscope (20x UPLSAPO objective, NA=0.75, ex/em 405/100nm bandpass filter at 500nm, 4us/pixel sampling speed). An automated image processing protocol was constructed in Icy to quantify DHE fluorescence intensity, as follows<

- identification of TIFF images in designated folder
- nuclei segmentation using HK means plugin
- ROI statistics computing for each individual detected nucleus
- Export of results in an xls file

At the end of the experiment, the rats were evenly distributed in 2 groups, according to their weight: G1 with weights up to 400g and G2 with weights over 400g. The average values for DHE fluorescence intensity were over 900AU in 2/6 rats in G1 and 6/6 in G2. ANOVA analysis for compartmental variances in individual rats yielded significant values in 2/6 G1 rats (p=0.01 and p=0.04) and 2/6 G2 rats (both p<0.0001). Tukey multiple comparison test showed significant differences between glomerular and vascular, glomerular and tubule-interstitial in 2/6 G2 rats. 4/6 G1 rats showed higher glomerular than vascular and conversely, 4/6 G2 rats showed higher vascular than glomerular values, all the while 5/6 G2 rats had higher vascular levels than tubule-interstitial. The ANOVA analysis of the groups showed significant influence for the weight factor (p=0.006). We defined a new parameter, weight adjusted ROS – wROS, which is calculated by dividing the fluorescence intensity to the rats weight. For both glomerular and vascular compartments, the wROS values were higher in G2 vs. G1 rats.

This study allowed us to notice a relationship between the rat's weight and detected ROS levels in confocal microscopy. The glomerular and vascular compartments showed prominent changes in relationship to the weight factor.

The image processing algorithm employed in this study provided consistency in quantification and increased speed in evaluating a large dataset.

STUDY NO.4 THE ROLE OF MAO IN INCREASING ROS LEVELS IN BLOOD VESSELS OF RATS WITH EXPERIMENTAL DIABETES MELLITUS

The aim of this study was to characterize the ROS production at vascular level in an experimental model of diabetes mellitus in Wistar rats, in relationship to the activity or inhibition of MAO.

Experimental diabetes mellitus was induced by injecting 50mg/kg streptozotocin in Wistar rats. In order to study the MAO contribution to H_2O_2 production, the following experimental incubations were used:

1. control;
2. diabetes mellitus;
3. control + MAO inhibitors (Clorgilin and Deprenil, 10uM);
4. control + H_2O_2;
5. diabetes mellitus + MAO inhibitors (Clorgilin and Deprenil, 10uM);
6. control + MAO 0.1U;
7. control + MAO 10 U;
8. control + MAO 1 U.

After incubation, the samples were OCT embedded and frozen. 20um thick sections were incubated with DHE at room temperature for 30 minutes. Then they were examined under an Olympus FluoView FV1000 confocal microscope. We used 405nm and 488nm excitation lasers, detecting the emission with a 100nm barrier filter at 500nm. For each sample 3 representative images we acquired, using 2 channel setting, DAPI (for autofluorescent component of the elastic lamina) and DHE. The images were then processed in an automated protocol developed in Icy, using the following operations:

- Channel extraction for individual processing
- Threshold based segmentation for each channel
- Boolean XOR operation for removing the DAPI channel autofluorescent component from the DHE channel
- ROI statistics computing and exporting of the results in an .xls file
- Generation of a new image containing the original with superimposed final ROI, as a control image for checking proper image segmentation.

We noticed an increase in ROS levels in the setting of diabetes mellitus, incubation with H_2O_2 and MAO, while the inhibitors reduces the detected DHE fluorescence.

Our study has confirmed the utility of confocal microscopy for indirect characterization of MAO activity at vascular level in a model of experimental diabetes mellitus in rats.

STUDY NO.5 *IN VIVO* CHARACTERIZATION OF ROS LEVELS IN THE KIDNEY OF THE RAT WITH CHRONIC KIDNEY DISEASE

The aim of this study was to implement a viable working model for *in vivo* confocal microscopy quantification of ROS in the kidneys of rats with unilateral urinary obstruction.

We performed a randomized controlled study on 36 adult male Wistar rats, which were assigned to 6 experimental groups: 3 control groups and 3 unilateral urinary obstruction groups. For both experimental settings, the 3 groups were evaluated at 2, 6 and 10 days after ligation.

The *in vivo* examination of the left ligated kidney was performed on an Olympus Fluoview FV1000 (ex 405 and 488nm, em – 100nm barrier filter at 555nm, 20x UPLSAPO objective, NA=0.75). We used an aluminium plate with a central hole acting as a viewing window. We performed two analysis of the datasets: quantification of fluorescence integrated intensity (IIF), for global quantification of DHE, and nuclear fluorescence intensity measurements.

The following analysis groups were designated:

1. Analysis groups based un excitation stimulus and intervention type (GA):
    a. control, excitation 405nm – C405
    b. control, excitation 488nm – C488
    c. ligated, excitation 405nm – L405
    d. ligated, excitation 488nm – L488
2. Analysis groups based on day of examination and intervention type (GE):
    a. control, examined on day 2 – C2
    b. control, examined on day 6 – C6
    c. control, examined on day 10 – C10
    d. ligated, examined on day 2 – L2
    e. ligated, examined on day 6 – L6
    f. ligated, examined on day 10 – L10

IIF quantification results

In all analysis groups we detected the highest ROS levels on day 6, as compared to days 2 and 10. We compared the individual average values for GE groups, we notice higher levels for L405 vs C405, while in the 488 excitation, C488 presented higher values in comparison to C405. The unpaired t-Test showed us the following results:

Excitation wavelength	**Compared groups**	**Significance levels**
405	C2 vs C6	p=0.0013
	L2 vs L6	p=0.0128
488	C2 vs C6	p<0.0001
	L2 vs L6	p=0.017
	C6 vs C10	0.005
	C6 vs L6	0.0071

We wanted to quantify the sole contribution of the ligation effect to the ROS generation, therefore we calculated the absolute difference between the average values of the ligated groups and control ones. This showed us an increase on the 10th day from the 6th day in the 405nm excitation assay.

Nuclear intensity measurements

The highest fluorescence intensity values were recorded on the 6th day for both L405 and L488, followed by a decrease on 10th day. ANOVA analysis showed very significant value within the groups ($p<0.0001$), while Tukey's multiple comparison test showed significant differences between days 2 and 6, 6 and 10, for both excitation assays (405 and 488nm).

For the control groups, the trend was similar, with highest values on day 6, which decreased on day 10, for both 405 and 488nm assays. The ANOVA analysis showed very significant differences between the groups ($p<0.0001$), while Tukey's multiple comparison test showed significant differences between days 2 and 6, 2 and 10, respectively, irrelevant of the excitation stimulus.

The comparison of the control and ligated groups over time showed similar trends. Significant differences were noted only on the 10th day, for both 405 nm ($p=0.0052$) and 488nm ($p=0.0021$) excitation assays. Higher values were recorded for ligated groups vs. control in both assays on the 10th day.

The ROS levels detected over time and linked to the ligation factor would indicate that the oxidative stress elicited by unilateral urinary obstruction has an initial plateau period in the early days after intervention, followed by an increase on the 10th day.

This study set the conditions for a more complex and truer evaluation of ROS generation by using *in vivo* confocal microscopy evaluation. The experimental model described here can be further used for interventional studies on modulation of ROS generation using various pharmacological agents.

STUDY NO.6 DEVELOPMENT OF A PORTABLE CRYOEMBEDDING DEVICE

The aim of this study was to develop a device that is portable, lightweight, simple in construction, and allows remote cryoembedding followed by transporting the frozen blocks to the processing site.

We constructed a prototype of the device presented in the patent no. RO130705(A3). We used an aluminium bar, 20x5x3.7cm, in which we bored conical cavities, 2.5cm at the base, 1cm deep. The insulating casing was constructed of two superimposed layers, an inner shell 4cm thick polyurethane and a 2cm polystyrene outer shell. The insulating layer creates a vat in which the aluminium bar is placed. Above the bar, up to the upper edge of the vat, there is a 3.5cm open space that represents the work space in which the freezing is performed. Above the ensemble vat – bar we placed an insulating lid, 28x13x6cm, composed of two layers of polyurethane, 3.5 and 2.5cm thick. The inner layer has the adequate size to be inserted in the work space. In this lid, corresponding to the conical shaped holes in the bar, we bore 4 holes 2 cm in diameter. Thus, after the lid is placed on top, there is enough space left to

accommodate 4 typical cryostat freezing chucks. The entire ensemble of vat – bar – lid was then placed in a commercially available 16 inch toolbox (Stanley). On the side of the toolbox we bore a hole that went all the way into the aluminium bar, so that we could insert the stem of a bimetallic thermometer (Durac, temperature range -100^{o}C to 40^{o}C, 1% accuracy). Total weight of the device is 2.2kg.

We monitored the device after it was cooled in a ULT freezer down to -80 ^{o}C and placed in an air conditioned room at 23^{o}C. We noticed that the device warmed up at a rate of 1oC every 5 minutes, reaching -30^{o}C in 215 minutes.

We tested the autonomy of the device by performing a mock freezing experiment. The data we recorded are:

- -76^{o}C starting temperature,
- -66^{o}C after 45 minutes,
- Freezing 4 OCT blocks warmed it up to -44^{o}C
- Another 45 minutes later, the final temperature was -38^{o}C.

We analysed frozen kidney samples that we stained with DHE and noticed a better preservation of nuclear morphology and better cytoplasmic staining, as compared to the images acquired in study no. 3. The images acquired showed better tissue morphology preservation, better resembling the images obtained *in vivo* in study no. 5.

We designed a portable device for cryoembedding in remote sites, that is cheap and easy to manufacture, that can be operated by anyone with minimum training required. This device can be employed in clinical and research practices, its main advantage being the ability of freezing tissues in remote locations where cryoembedding infrastructure is lacking.

PERSONAL CONTRIBUTIONS

1. Development of custom designed protocols for image analysis using datasets acquired in confocal microscopy
2. Documentation, for the first time, of the overexpression of MAO-B isoform in experimentally induced diabetes mellitus in rats
3. Documentation of the compartimentalization of ROS generation in the kidneys of diabetic rats
4. Implementation of an experimental model for dynamic monitoring of ROS using *in vivo* confocal microscopy
5. Development and construction of a novel portable device for cryoembedding of tissue fragments.