Sveučilište u Rijeci
University of Rijeka

MEDICINSKI FAKULTET - FACULTY OF MEDICINE

MEDRI

HRZZ
Croatian Science Foundation

Workshop

"Traumatic Brain Injury
- insights through extracellular vesicles"

01 June 2023
Lecture hall "Vijećnica", Faculty of Medicine, Rijeka

The workshop is being organised in frame of the HRZZ-funded project "Identification of circulating biomarkers for neuro-recovery of brain-injured patients".

Organisers: Lara Valenčić Seršić, Janja Tarčuković, Kristina Grabušić.

PROGRAMME

PART I: 10:00 - 13:00

Pero Lučin: Extracellular nanoparticle content within preparations of beta-herpes virions

Olga Shevchuk: Mass spectrometry-based proteomics for unravelling immune response in pyelonephritis

Lada Kalagac Fabris: A review on the current approach to the multimodal monitoring in traumatic brain injury

Vlatka Sotošek: Neuroimmunomodulation in patients with severe traumatic brain injury

13:00 - 14:00 LUNCH

PART II: 14:00 -17:00

Mario Kurtjak: Sizes and shapes of cerebrospinal extracellular vesicles

Mladenka Malenica: Nano-palpation of extracellular vesicles

Karmen Wechtersbach: Imaging of extracellular vesicles from human cerebrospinal fluid by transmission electron microscopy (TEM)

Kristina Pilipović: Investigating the effects of chemically functionalized single-walled carbon nanotubes in the in vitro model of traumatic brain injury

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ABSTRACTS

Mass spectrometry- based proteomics for unravelling immune response in pyelonephritis

Olga Shevchuk, Department of Immunodynamics, Institute for Experimental Immunology and Imaging, University Hospital Essen, Essen, Germany

Mass spectrometry (MS) based proteomics has increasingly been considered an indispensable technology for biological and biomedical research. The unbiased, 'discovery' proteomics analysis (e.g., 'shotgun' proteomics) can now provide genome-scale coverage and quantification of both proteins and posttranslational modifications. However, verification of these discovery findings requires higher specificity, higher precision, and accuracy in quantification and higher sample throughput. In our study, we combined mass spectrometry and a molecular biology-based approach to unravel the molecular and cellular mechanism underlying pyelonephritis (PN). For this, biopsies from nephrectomized patients with histopathological PN were analyzed by label-free liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based proteomics and compared with healthy regions of renal cell carcinoma (RCC) resections as a control. The proteomic profile of the kidney disclosed upregulation of biological processes involved in the immune and defense response, including significant elevation of proteins with antimicrobial function. Upregulation of selected AMPs was validated in urine through enzyme-linked immunosorbent assay (ELISA) and complemented

by assessing the clinical parameters of the additional PN cohort. Additionally, targeted proteomics as an alternative for antibody-based verifications as well as for large-scale clinical studies will be discussed.

A review on the current approach to the multimodal monitoring in traumatic brain injury

Lada Kalagac Fabris, Maša Biberić, Siniša Zrna, General Hospital Pula, Pula, Croatia

Traumatic brain injury is one of the greatest challenges for resuscitation and intensive care specialists as it remains the leading cause of death and disability worldwide. With the standardized application of the Glasgow Coma Scale, it is now possible to distinguish moderate from severe brain injury in a timely manner and to choose the correct therapeutic approach early.

Clinical outcomes are determined not only by the severity of the initial injury but also by inflammatory, biochemical, and excitotoxic responses that lead to secondary brain injury. Sophisticated neuromonitoring systems should correctly read the entire series of changes that occur at the level of brain flow, intracranial pressure, and cellular metabolism, in order to optimize the therapeutic approach and the outcome of the treatment itself. Continuous monitoring of intracranial pressure (ICP) and cerebral perfusion pressure (CPP) has become the cornerstone in neuromonitoring, as it reflects the predisposition to further cerebral injury and herniation. Monitoring of jugular venous oxygen saturation ($SjvO_2$) is another conventional practice that serves as an extended approach for estimation of the balance between global cerebral oxygen delivery (DO_2) and utilization and reflects cerebral oxygen deficit. Other recently developed devices include intracerebral microdialysis probes that, in addition to providing information on CPP, can be also used for online analysis of extracellular/interstitial biochemical changes in glucose, lactate, pyruvate, glycerol, and glutamate. Biochemical changes have been considered early markers of cerebral ischemia, diffusion hypoxia, and loss of cellular structural integrity before low CPP is detected.

Although the combined use of different types of neuromonitoring has been shown to be good for understanding secondary brain injury in intensive care units, there are some recent randomized clinical trials that have shown that all these devices have not shown a significant improvement in outcome in severely injured patients. Furthermore, there is uncertainty about which physiologic variables are most clinically relevant, how and when they should be monitored, and whether monitoring is cost-effective and affects outcome. Highly complex in their application, such advanced devices will continue to develop as they offer extended monitoring capabilities and provide valuable information about pathophysiological events in the injured brain, which cannot be obtained from morphological images such as computed tomography (CT) and magnetic resonance imaging (MR).

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Neuroimmunomodulation in patients with severe traumatic brain injury

Vlatka Sotošek, Department of Anesthesiology, Reanimatology, Emergency and Intensive Medicine, Faculty of Medicine, University of Rijeka, Rijeka, Croatia

Severe traumatic brain injury is a potentiality devastating condition which leads to a high morbidity and mortality rate. It is a major cause of disability and permanent neurological damage in patients younger than 45 years in industrialized countries and it is a silently growing epidemic. Much of mortality is related to severity of primary neurological impairment and secondary brain injury caused by hypoxia and hypotension. Despite significant improvement of intensive care treatment and neurosurgical procedures in patients with severe traumatic brain injury, majority of these patients are highly susceptible to infections. Severe traumatic brain injury is a neuro-inflammatory condition which is characterized by the release of proinflammatory cytokines, such as interleukin (IL)-1, IL-6, tumor necrosis factor (TNF) alpha, IL-18, IL-21, chemokines, cytotoxic proteases, and reactive oxygen species that activate endothelium adhesion molecules and increase the permeability of blood–brain barrier allowing extravasation of leukocytes. Activated leukocytes and soluble mediators induce disturbance of a normally balanced relationship between central nervous and immune systems. Commonly reported abnormalities in immune system after the brain injury are reduced number and activity of T lymphocytes, NK and NKT cells, diminished mitogen-induced cytokine production and proliferation *in vitro*, impaired phagocytic activity of granulocytes and monocytes and depressed delayed-type hypersensitivity skin test. Additionally, transient non-reactivity of monocytes after endotoxin stimulation, down-regulation of antigen presentation ability and enhanced anti-inflammatory cytokine production were observed both in animals and humans following severe traumatic brain injury. The result is down-regulation of immune system that is not just a simple deletion of different cell type involved in immune response, but rather involves complex immunoregulatory reaction. Knowing more about the immunological changes upon brain injury could led to optimize therapy in order to find new approaches for successful management of patients after severe brain injury.

Sizes and shapes of cerebrospinal extracellular vesicles

Mario Kurtjak[1], Vera Tomas[2], David Fabijan[1], Hrvoje Križan[3], Sami Kereïche[4], Metka Lenassi[5], Vedrana Krušić Alić[6], Janja Tarčuković[2,7], Maša Biberić[8], Siniša Zrna[8], Kristina Grabušić[6], Robert Tempest[9], Stefano Prato[10], Ivan Štajduhar[11], Mladenka Malenica[3]

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Extracellular vesicles (EVs) appear in a variety of shapes and sizes, and their morphology is believed to be related to their cells of origin, biogenesis, and physiological and pathological state. However, it is still unknown what exactly is affected. Is it the average size, the most common size, a change in size diversity or range, or perhaps a change in the distribution of the subpopulations with different shapes, morphology, and internal structure? And how to accurately determine these values and compare different samples extracted from a body fluid?

In our attempt to most unambiguously evaluate the near-native morphology of EVs from human cerebrospinal fluid (CSF), we compared nanoparticle tracking analysis, tunable resistive pulse sensing, dynamic light scattering, nano-flow cytometry, atomic force microscopy (AFM) in liquid and cryogenic transmission electron microscopy (cryo-TEM) on the same sample. We were the first to combine AFM in liquid for surface morphology investigation and cryo-TEM for internal structure differentiation of EVs from CSF. EVs were isolated by size exclusion chromatography from a pool of CSF from patients with traumatic brain injury and identified by immunoblot on CD9 and CD81 antibodies. The statistical analysis and interpretation of the results, the advantages, and limitations of each method, as well as certain issues we encountered that could lead to misinterpretation, will be presented. Moreover, we identified five different shapes of EVs by AFM and developed a computer program to examine individual 3D structures of EVs in an AFM image and sort them by their shape. Thus, we could obtain the frequency and distributions of subpopulations differing in surface morphology. After manual categorization, we also developed machine learning models for future automatic subpopulation classification and attribution of biomechanical properties, which will be tested for diagnostics in patients with brain-related conditions.

Nano-palpation of extracellular vesicles

Hrvoje Križan[1], Mario Kurtjak[2], Ana Svetić[3], Pietro Parisse[3], Loredana Casalis[4], Simone dal Zilio[3], Marco Lazzarino[3], Ana Blagaić[5], Marin Tota[1], Marko Loparić[6], Ivana Vinković Vrček,[7] **Mladenka Malenica[1]**

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Extracellular vesicles (EVs) can carry bioactive molecules and participate in important events, such as intercellular communications in normal or pathological biological processes, signaling and transport of therapeutic agents. EVs can cross the blood-brain barrier, avoid degradation, and evade the immune system, thus enabling local and long-distance communication. They can also modulate the biomechanical properties of target cells and play a crucial role in metastatic spreading. Unfortunately, these mechanisms are still poorly understood, but they are definitely related to the biomechanical properties of EVs, like stiffness, softness, elasticity, and motility. These properties affect the internalization and externalization of EVs by a cell, their adhesion to the surface, life in circulation, tissue targeting, and release of biomolecules. Currently, the International Society of Extracellular Vesicles recommends atomic force microscopy (AFM) as one of the main high-resolution imaging techniques for assessing the heterogeneity and morphological properties of EVs. AFM as a basic scanning probe microscopy exploits the interaction between the tip and the sample surface during raster scanning to contour surface features. It has been increasingly recognized as a valuable method for imaging nano-sized samples in near-native condition in liquid. It provides 3D topographic images of EVs, from which their size distribution can be extracted and native morphology observed. We were the first to show these characteristics for EVs from human cerebrospinal fluid. For nanomechanical mapping, a simple indentation of EVs with the AFM tip is performed and the force-distance curve recorded. These curves are observed and interpreted through indentation models. The data about biomechanical properties from the multiple force measurements can be further processed into force volume maps within a defined 3D volume of the sample to map interactions (i.e., chemical, electrical, or physical) between the probe and the sample. Our future goal is to correlate force measurements with specific features in AFM topography images and immuno-based interactions to unveil the roles of structure and function of EVs.

Imaging of extracellular vesicles from cerebrospinal fluid by transmission electron microscopy (TEM)

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Extracellular vesicles (EVs) are of general interest in clinical applications as therapeutic vehicles and as biomarkers for a variety of diseases. The size of many EVs is below the resolution of even the most enhanced optical microscopes and nanometer resolution of transmission electron microscope (TEM) makes it an essential tool in the study of their morphology and size distribution. TEM is the most common type of electron microscopies for EV imaging where it is mainly used for monitoring the quality and purity of EV-containing samples because it can better than other methods discriminate single EVs from similarly sized non-EV particles. Several properties of EVs complicate TEM investigation and decrease the reliability of the obtained results. Electron microscopes are conventionally designed for observation of dry solid samples under high vacuum, whereas EVs are in the form of buffer suspensions after their isolation from the biofluids. For these reasons, preparation protocol should be well selected and well optimized to keep their structural and topological characteristics as close as possible to their native form.

Our aim was to optimise two protocols for TEM visualization of EVs from human cerebrospinal fluid. EVs from cerebrospinal fluid were isolated with size exclusion chromatography which enabled separation of EVs from free proteins. The first protocol involved fixation of EVs, deposition of a drop of fixed sample on a copper grid, removal of excess liquid by blotting and negative staining with uranyl acetate. The second protocol was performed for imaging of ultrathin sections of EVs embedded in Epon resin. EVs were first concentrated with ultracentrifugation to make a pellet, then fixed, embedded in specimen-processing gel, dehydrated, embedded in Epon resin, polymerized, and viewed in a TEM.

EVs were defined by TEM on the basis of their round shape, electron dense lipid bilayer and size of 60-300 nm. Our results show that TEM techniques are a powerful tool for the investigation of the ultrastructure of EVs.

Investigating the effects of chemically functionalized single-walled carbon nanotubes in the *in vitro* model of traumatic brain injury

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Traumatic brain injury (TBI), one of the leading causes of mortality in young adults, still lacks effective treatment. Tissue engineering has emerged as a potential therapeutic approach, with carbon nanotubes (CNTs) being a potential candidate due to their conductivity, biocompatibility, size, strength, and flexibility. It has previously been found that, when applied

to the culture medium, single-walled CNTs (SWCNTs) modulate morpho-functional properties of astrocytes. Therefore, our research group aimed to investigate whether chemically functionalized SWCNTs can influence astrocyte survival and function after TBI *in vitro*.

For *in vitro* TBI, we used the rapid stretch-induced injury of primary mouse astrocytes. Cell survival rates and the effects of SWCNTs application on the severely injured astrocytes were evaluated. We monitored the changes in the expression of the astrocyte marker glial fibrillary acidic protein (GFAP), and excitatory amino acid transporters (EAATs) as well as the alterations in the secretory function of these cells, including the changes in the exosome release from the cultured astrocytes.

Results of our studies indicate that SWCNTs increase the survival of injured astrocytes with changes in the expression of certain astrocyte-specific proteins as well as alterations of the secretion of cytokines and chemokines.

Our findings point to a potential protective effect of SWCNTs following *in vitro* TBI, however, further studies are needed to investigate their safety, efficacy, and long-term effects *in vivo*. This research was fully supported by the Croatian Science Foundation grant UIP-2017-05-9517 to KP.