Kromatografska izolacija izvanstaničnih vezikula iz cerebrospinalne tekućine bolesnika s teškom ozljedom mozga

Krapić, Mia

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University Graduate Programme
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Mentor: Assist. Prof. Kristina Grabušić

SVEUČILIŠTE U RIJECI
ODJEL ZA BIOTEHNOLOGIJU
Sveučilišni diplomski studij
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The master's thesis defence was held on the 27 September 2018, before the committee consisting of:

1. *Assist. Prof*. Kristina Grabušić, PhD
2. *Assist. Prof*. Mladenka Malenica Staver, PhD
3. *Assoc. Prof.* Miranda Mladinić Pejatović, PhD

The thesis has 45 pages, 14 figures and 38 literary citations.

Summary

Severe traumatic brain injury (TBI) is intracranial damage caused by external force and is the leading cause of death and disability among young people. The damage to brain tissue involves a complex and mostly long-lasting cascade of pathological processes such as inflammation, excitotoxicity and apoptosis, which are targeted for management during the critical care. Although a full recovery after severe TBI is possible, the mechanisms of neuroregeneration and thus targeted measures to support neuro-recovery are unknown. However, intercellular communication between neurons and glia cells is cellular activity vital for the renewal of old synapses and establishment of new ones.

Newly described potential mediators of intercellular communication in the brain are extracellular vesicles (EVs). EVs are nanosized particles enveloped with phospholipid bilayer and capable of transporting proteins, lipids and nucleic acids between cells. The previous study discovered that cerebrospinal fluid (CSF) of patients with severe TBI shows dynamic changes in both physical properties of EVs and levels of neuroregeneration-associated proteins. This EV dynamics indicates possible neuro-recovery response of the brain. The goal of this research was to enable analysis of EV-proteome by isolating total EVs from clinical CSFs after severe TBI.

An in-house size-exclusion chromatography (SEC) based on sepharose CL-6B was developed in this work and used it to separate total EVs from CSF pools of 6 control patients and 6 severe TBI patients. After collection, SEC fractions were characterised by 3 independent methods: immunoblot analysis for CD9 protein (i) and acetylcholinesterase (AChE) assay (ii) as well-established EV protein markers and Bradford assay (iii) to determine the total protein concentration. These quantifications of SEC fractions resulted in partially overlapping curves with AChE and CD9 peaks detected in earlier fractions than

total protein peak. The results indicate a successful separation of EVs from soluble proteins enabling the proteome analysis by mass spectrometry.

Key words: Size-exclusion chromatography, severe traumatic brain injury, extracellular vesicles, neuroregeneration, cerebrospinal fluid

Sažetak

Teška traumatska ozljeda mozga je intrakranijalno oštećenje uzrokovano vanjskom silom te je vodeći uzrok smrtnosti i invaliditeta u mladih ljudi. Ozljeda moždanog tkiva uključuje složenu i dugotrajnu kaskadu patoloških procesa poput upale, ekscitotoksičnosti i apoptoze, a liječenje bolesnika u kritičnom stanju je usmjereno na ublažavanje tih negativnih procesa. Iako je potpuni oporavak nakon ozljede moguć, mehanizmi neuroregeneracije kao i potencijalne mjere koje bi je poticale još uvijek nisu otkrivene. Međutim, oporavak starih sinapsi i stvaranje novih ovisi o međustaničnoj komunikaciji između neurona i glija stanica.

Novi potencijalni posrednici međustanične komunikacije u mozgu su izvanstanične vezikule (IV). IV su nanočestice obavijene fosfolipidnom membranom i mogu prenositi lipide, proteine i nukleinske kiseline između stanica. U prethodnom istraživanju pokazalo se da u cerebrospinalnoj tekućini pacijenata s teškom traumatskom ozljedom mozga dolazi do dinamičnih promjena fizikalnih svojstava IV-a i razina proteina uključenih u neuroregeneraciju. Te promjene ukazuju na moguće procese neuroregeneracije. Cilj ovog istraživanja bio je izolirati ukupne IV iz kliničkih uzoraka cerebrospinalne tekućine nakon teške traumatske ozljede mozga i time omogućiti analizu njihova proteoma.

Kromatografska metoda na bazi sefaroze CL-6B razvijena je tijekom ovog rada i primijenjena za razdvajanje ukupnih IV-a iz cerebrospinalne tekućine 6 kontrolnih i 6 bolesnika s teškom traumatskom ozljedom mozga. Sakupljene kromatografske frakcije karakterizirane su pomoću 3 metode: imunoblot analizom za CD9 protein (i), esejem za acetilkolinesteraznu aktivnost (AChE) (ii), koji su poznati proteinski markeri IV-a te Bradford esejem (iii) kojim se odredila koncentracija ukupnih proteina. Kvantifikacija frakcija rezultirala je krivuljama koje su se djelomično preklapale. Vrhovi krivulja AChE i CD9

detektirani su u ranijim frakcijama u odnosu na ukupne proteine. Rezultati ukazuju na uspješno razdvajanje IV-a od solubilnih proteina čime je omogućena njihova daljnja proteomska analiza masenom spektrometrijom.

Ključne riječi: gel-filtracija, teška traumatska ozljeda mozga, izvanstanične vezikule, neuroregeneracija, cerebrospinalna tekućina

Content

1. INTRODUCTION

1.1. Severe traumatic brain injury

Severe traumatic brain injury (TBI) is the most serious form of brain damage caused by mechanical force and usually results in disabling or fatal consequences (1,2). The symptoms and complications can be various due to the brain complexity, type of injury and localisation (1). It is a heterogenous injury with permanent or temporary consequences which involve cognitive/motor dysfunctions, amnesia, seizures, loss of consciousness or coma. Patients with severe TBI are treated at the intensive care unit with invasive procedures and surgeries to improve their critical condition and prevent further neurological damage (1-3).

1.1.1. Definition, diagnosis and incidence of severe TBI

The severity of the clinical picture classifies TBI patients into 3 categories of injury: mild, moderate and severe (Table 1) (1). Diagnosis of TBI is made by the evaluation of a clinical picture with Glasgow coma scale (GCS) score, followed by confirmation with computed tomography or magnetic resonance imaging (Figure 1) (1,2). GCS is used to determine the severity of a brain injury by allocating points based on verbal response, motor response and the ability of eye opening (1-3).

Table 1. Classification of traumatic brain injury with Glasgow coma scale (GCS) score

Type of response	Point range	TBI	Sum of points
verbal response	1-5	Mild	13-15
motor response	1-6	Moderate	9-12
eye opening	1-4	Severe	3-8

Based on the GCS score, patients are diagnosed with an injury type which determines the course of their treatment. The main problem are patients with mild and moderate TBI because these injury types do not necessarily manifest with any visible symptoms or they have mild consequences such as headaches, confusion and impaired motor or cognitive functions (3). Even though the symptoms are mild, they can be the reflection of ongoing pathological processes which can lead to more severe cognitive deficits, epilepsy and neurodegenerative diseases (1). Therefore, it is necessary to properly evaluate the extent of the injury and to monitor patients in the hospital and after they have been discharged. On the other hand, individuals with severe TBI are clinically the most difficult cases with immediate onset of serious complications which require urgent medical assistance at the intensive care unit (2). Usually, they are experiencing loss of consciousness or coma, seizures, brain contusions and hematomas (Figure 1) (1,3).

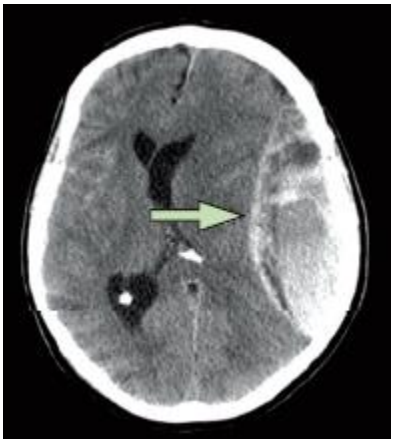

Figure 1. Computed tomography of a life-threatening severe TBI
(Maas et al. Lancet Neurology, 2017)

Severe TBI is a major public health issue because it is the leading cause of deaths linked with injuries. However, difficulties in severe TBI categorisation and diagnosis are causing inconsistencies in the epidemiological data (1).

Epidemiology of severe TBI varies between countries whereby low-income countries have higher death and injury rates (1). Approximately 10% of more than 50 million TBI cases per year worldwide is severe (1,2). Severe TBI has a high mortality and disability rate especially among young people, mostly due to traffic accidents. The incidence is increasing in the elderly population which is prone to fall accidents (1). Individuals who survive severe TBI have permanent neurological damage resulting in long term consequences such as cognitive dysfunctions in 43% of patients and seizures in 40-50% (4).

1.1.2. Pathophysiology of severe TBI

Pathophysiology of severe TBI is a very complex cascade of damaging events which lead to the loss of brain functions (Figure 2). Primary injury occurs as a direct result of external force which causes bleeding, tissue damage and necrosis. Secondary injury quickly follows as a response to the primary physical damage (1).

Secondary injury begins with disruption of extracellular neurotransmitter levels which has excitotoxic effect on neurons. Consequently, the over-stimulation of neurons increases membrane permeability and intercellular ion levels which leads to an excessive activation of proteases and free radical formation (5-7). Free radicals and membrane damage can have a negative influence on antioxidant system and electron transport chain. Furthermore, mitochondrial dysfunction causes a decrease of energy production and unfavourable anaerobic conditions. These events lead to injury/death of neurons, axons and glia cells within hours after injury (6,8).

Microglia are immune cells which act as a primary response to pathogens or injury in the central nervous system (CNS) (9). At the injury site they increase in number and activate an immune response by producing chemokines,

cytokines and prostaglandins and consequently stimulating the infiltration of other inflammatory cells (5,6,9). Days after injury, inflammatory cells or mitochondrial dysfunction can trigger programmed cell death also known as apoptosis (7,8). At the same time, demyelination occurs because of the lack of myelin producing cells (6). These events can further disrupt the blood-brain barrier and cerebrovascular autoregulation which can cause increased intracranial pressure (ICP), oedema development, ischaemia or decreased cerebral perfusion pressure (7). Neurodegeneration initiated by secondary injury can persist years after the trauma, causing the long-term repercussions on a patient's health (1).

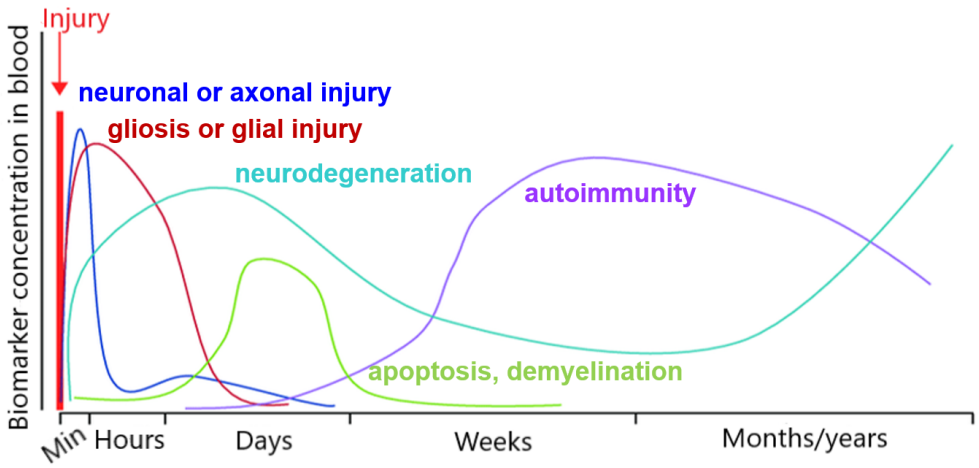

Figure 2. Pathophysiology of severe traumatic brain injury (TBI) is a complex cascade of damaging processes which can last for months or years after injury. (modified according to Maas et al. Lancet Neurology, 2017)

1.1.3. Treatment and recovery of patients with severe TBI

Shortly after the severe TBI clinicians are forced to quickly make important decisions regarding surgical procedures, medication and rehabilitation. Quality clinical assessment and diagnosis are crucial for the survival, treatment and

recovery of patients (1). In a severely injured brain physiology and circulation of the cerebrospinal fluid (CSF) is often impaired and the brain starts to swell which leads to an increased ICP (1,2). The primary goal of treatment is to stabilise the patient's breathing and blood pressure with intubation and ventilation to prevent the increase of ICP. Often, individuals require sedation to prevent movements and subsequent ICP elevation. The brain trauma can sometimes induce seizures which have to be managed with prophylactic anticonvulsants to reduce the risk of further damage (2,8).

Depending on the patient's condition, the increased ICP can be treated with different medication, hyperventilation or more aggressive surgical procedures such as ventriculostomy and decompressive craniectomy (1,2). Ventriculostomy involves the insertion of a catheter in the ventricles to drain the excess CSF. Decompressive craniectomy is a procedure based on the removal of a piece of skull to manage the brain swelling. These procedures are risky, and they can have direct impact on the outcome of a patient's recovery (1,2,8).

Critical patients frequently undergo surgeries to stabilise fractions, lesions or hematomas in the brain or other injured body parts. Patients sometimes have to fight the accompanying infections which can cause sepsis, pneumonia, organ dysfunctions and therefore aggravate the existing condition (8).

The choice of treatment in the acute phase of severe TBI can certainly influence the progression of neuroregeneration processes (1). An open question remains which interventions can be done in this early stage to improve neurorecovery.

Recovery after severe brain trauma is different on a case by case basis and it depends on the type of injury, treatment and complications. However, for majority of people and their families it is a long lasting and difficult process (1,8). The outcome is evaluated 3 months after the injury with Glasgow Outcome Scale (GOS) which describes the level of recovery to determine

further rehabilitation. Death and vegetative state are the most difficult cases, followed by severe, moderate and low disability (1). Early rehabilitation and constant monitoring are necessary to help patients with movement, cognitive and emotional problems which disrupt the quality of their lives. Besides physical disability, frequent consequences of severe TBI are headaches, anxiety, depression, fatigue and trouble with sleeping (1,3). Long term consequences such as brain atrophy or neurodegenerative disorders can affect brain morphology years after injury (1,10).

1.2. Extracellular vesicles

Extracellular vesicles (EVs) are small, heterogenic spherical particles enclosed by a phospholipid bilayer with the diameter ranging between approximately 100-1000 nm (11). We can find them in blood, saliva, urine and CSF because they are involved in many important cellular processes including cargo transport, coagulation, cell survival, immunological response and intercellular communication (12). Although intensive research in the past 30 years has broadened our knowledge on the subject it has led to inconsistencies in nomenclature and categorization. It is certain that 3 different subpopulations of EVs exist, but researchers have not yet agreed on the nomenclature and classification because of their diversity and technical difficulties present in their isolation (12).

1.2.1. Types and characteristics of extracellular vesicles

EVs are most commonly divided into three main subpopulations: exosomes, microvesicles and apoptotic bodies based on their size, density, origin and morphology (Figure 3) (11,13).

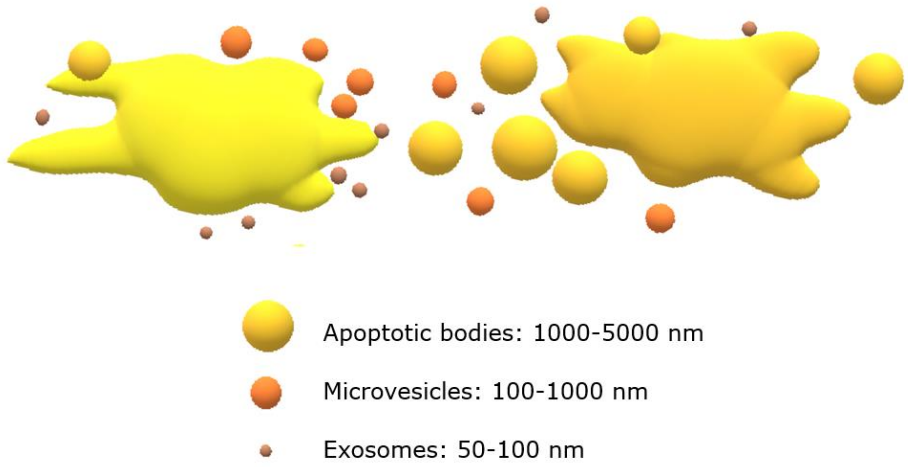

Figure 3. Cells actively secrete extracellular vesicles (EVs) which are mediators of intercellular communication in physiologic and pathologic conditions.

Exosomes are 50-100 nm large vesicles secreted from late endosomes (multivesicular bodies) with exocytosis, predominantly released by tumour and immune cells (13). The formation of EVs such as exosomes starts when the plasma membrane is budding inwards. After an early endosome is formed, it transforms into the late endosome which contains intraluminal vesicles filled with cargo. The late endosome can either fuse with a lysosome for content degradation or with a plasma membrane which enables secretion of EVs into the extracellular space (Figure 4) (11,12,14). They contain proteins involved in membrane transport, late endosome formation and antigen presentation. Exosomes can also transport different lipids, messenger ribonucleic acids (mRNA) and micro ribonucleic acids (miRNA) (11). On the other hand, microvesicles are EVs with the diameter raging between approximately 100-1000 nm and their secretion is a regulated process (13). The increase of intercellular calcium levels induces cytoskeleton and membrane reorganisation and subsequent budding of the plasma membrane (11). They are most commonly secreted in low amounts from platelets and erythrocytes, but in higher quantities from tumour cells. Apoptotic bodies are EVs with a 1000-5000 nm diameter released by apoptotic cells (13). They contain

cytoskeleton, DNA fragments and other parts of dying cells and therefore are phagocytised to prevent immunologic reaction (11).

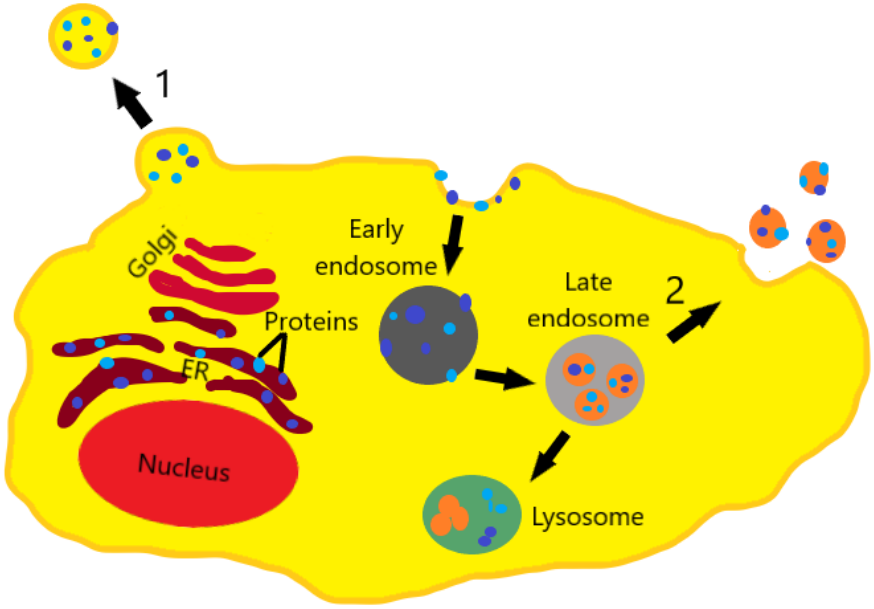

Figure 4. Two different pathways of extracellular vesicle (EV) formation in living cells. EVs can be produced by direct budding of the plasma membrane – 1, or by fusion of the late endosome containing intraluminal vesicles with the membrane - 2. ER – endoplasmic reticulum

The content of EVs depends on the pathway of their formation, type of native cell and processes occurring inside them. It includes lipids, nucleic acids and especially proteins that are being intensely investigated because of their potential application in the immuno-specific methods of isolation (11,15,16). Some proteins present on the surface or in the core are common for most EV subgroups and therefore are used as biomarkers for their detection and characterization. Some of these biomarkers are tetraspanins, flotillins, Rab family, Arf family, annexins and acetylcholinesterase (AChE) (Figure 5) (11,15,16).

Tetraspanins such as CD9, CD63, CD81 and CD82 are abundant proteins consisting of four domains passing through the phospholipid bilayer. As transmembrane proteins, they are reliable biomarkers of EVs frequently used

in their detection. Tetraspanins are important for cell adhesion, fusion, organization and signalling (13).

AChE is an enzyme also recognized as an EV biomarker that is present in three different forms as a membrane-associated and non-membrane-associated protein. Although we can find it in high amounts in the CNS, it is also present in other tissues and organs. The activity of this enzyme can easily be measured and quantified, enabling the detection of EVs (16,17).

Rab protein family are guanosine triphosphate (GTP) binding proteins which have a role in fusion of late endosome with plasma membrane and in sorting of cargo into EVs (11,18). Flotillins are located at the inner part of a membrane enriched with lipid rafts. They are involved in membrane fusion and EV transport (11,19). Annexin protein family can bind to phospholipid membrane and they are involved in calcium level regulation, membrane fusion and trafficking (11,20). Arf proteins are GTP binding proteins important for vesicle formation and budding (11).

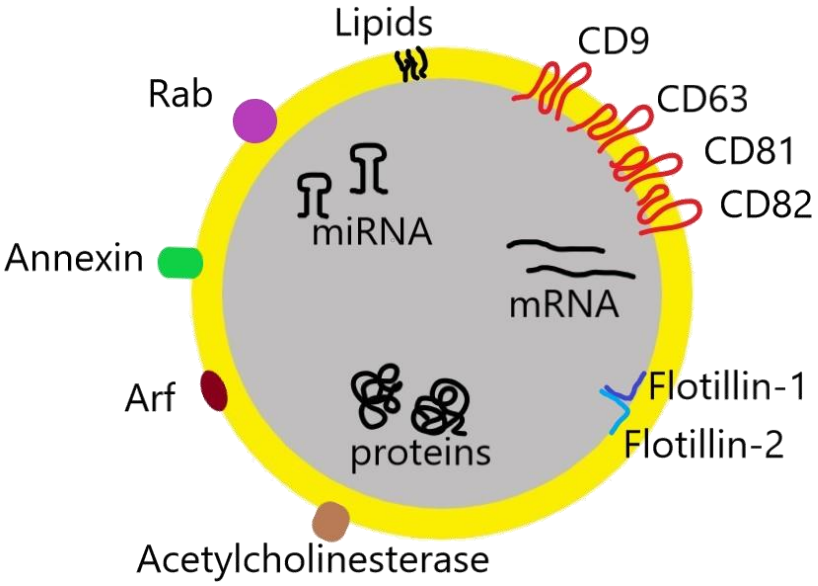

Figure 5. The composition of an extracellular vesicle (EV). EV can transport proteins and nucleic acids in their core and protein biomarkers and lipids on their surface.

1.2.2. Extracellular vesicles as mediators of intercellular communication in CNS

Until recently it was thought that cells communicate only through direct contact or by releasing soluble molecules. Scientists have discovered new mediators of intercellular communication, EVs which have previously been considered as cellular garbage (11). In the brain, EVs are enabling much more complex neuron-neuron communication than soluble molecules (21). EVs can transport different biological molecules such as proteins, lipids, mRNA and miRNA selectively to distant cells without degradation. After fusing with the recipient cell their content can affect signalling transduction and consequently EVs can mediate both physiological and pathological processes (21). They can reflect the ongoing conditions present in cells they originate from and therefore EVs are being investigated as biomarkers of different diseases (18,22).

1.2.3. Extracellular vesicles in severe TBI

After the severe TBI it is necessary to repair the damage by restoring synapses, secreting growth factors, inducing remyelination and stem cell proliferation. However, it is not certain if these neuroregenerative processes appear (4,23).

There are only several studies on human clinical samples which are primarily focused on discovering the EVs involvement in TBI pathophysiology mostly in blood samples. Nekludov et al. investigated the quantity of EVs from blood samples of severe TBI and control patients (24). Higher levels of EVs from endothelial cells, thrombocytes and leucocytes were present in severe TBI samples when compared to control. On the other hand, research on the CSF is more limited because of the small volumes of collected samples. Existing

research analysed inflammatory molecules and coagulation enzymatic processes in patients with severe TBI days after injury. EVs from severe TBI patients showed increased procoagulant activity and higher levels of inflammasome proteins (25,26). Other research was focused on EV protein and genetic composition analysis in order to find injury biomarkers (27,28).

The systemic acute response was investigated in a study on animal models of brain injury. They showed that circulating EVs increase in number and that deterioration of response depends on EV cell origin (29). However, positive impact of EVs was observed in animal models which secreted microglial vesicles containing increased levels of miR-124-3p. This molecule reduces inflammation and stimulates neurone growth (30).

Therefore, scientists are searching for more reliable and precise biomarkers of neural plasticity necessary for the prediction of a patient's recovery and treatment. It was considered only recently that some EVs could have a role in neuroregeneration processes, but until recently none of the existing studies has investigated these processes after severe TBI (4,23).

Kuharić et al. investigated the role of EVs in neuroregeneration during the first 7 days after severe TBI by using the CSF samples (31). Protein analysis detected changes in levels of Arf6, Rab7a and Flotillin-1 which are EV markers involved in neuron formation and regeneration. Nanoparticle tracking analysis (NTA) showed induced larger EVs 4 days after brain injury indicating their possible role in neuroregeneration processes.

1.3. Methods for the isolation of extracellular vesicles

Various existing methods for vesicle isolation are based on different physical, chemical and biological principles. The protocols are not standardized between research laboratories and consequently, the results differ (11). EV isolation methods have technical obstacles which can result in loss of samples, impaired structure and diminished quality of research. Furthermore, EVs are difficult to

isolate and detect due to their diversity, different sizes and low concentrations in body fluids (22). Frequently used methods used for their isolation are: ultracentrifugation, polymer precipitation, immuno-affinity purification and size-exclusion chromatography.

1.3.1. Ultracentrifugation

Ultracentrifugation method is most commonly applied for the isolation of EVs (16,32). It requires an expensive ultracentrifuge device which can be used for different types of ultracentrifugation. Differential ultracentrifugation involves multiple centrifugation steps at different speeds and duration. After each centrifugation step, the pellet is resuspended in buffer and supernatant thrown away. Cells, larger biomolecules and cellular debris are being pelleted at lower speeds, while smaller EVs at high speeds (32,33). This method cannot be used for the isolation of vesicles completely based on their size because the density of EVs plays an important role in their precipitation (16).

On the other hand, EV isolation with density gradient ultracentrifugation is based on their size, density and mass. The medium has higher density at the top of the tube which enables separation of EVs into layers. The main limitation of this type of ultracentrifugation is limited sample volume which can be loaded on the medium (15,16).

The quality of EV isolation depends on the protocol, rotor type and clinical sample properties. However, the high velocities (approximately 100.000 x g) can damage the structure of EVs or cause unwanted aggregation (16,33). Furthermore, the pellet could be contaminated with other particles or a portion of vesicles could remain unprecipitated and thrown away as the supernatant (Figure 6) (33). These losses of EV result in low yields which can affect subsequent analyses.

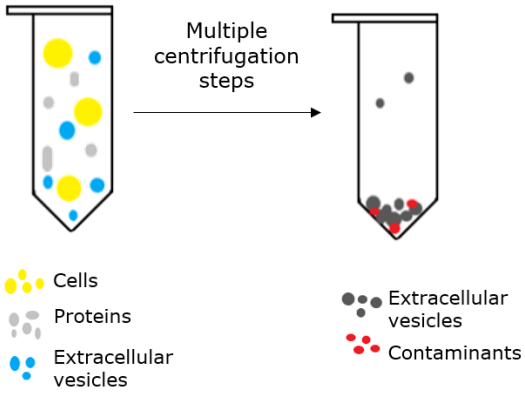

Figure 6. The principle of extracellular vesicles isolation with ultracentrifugation. After multiple centrifugation steps of a biological sample the pellet is containing contaminants and vesicles with changed functionality.

1.3.2. Polymer precipitation

Polymer precipitation with the commercial kit is a simple method which enables isolation of EVs within a specific size range regardless of their composition (Figure 7) (16). The protocol usually involves the overnight incubation of a sample with a solution containing polymer at 4-5°C. It is followed by low-speed centrifugation step for the precipitation of EVs (15,16). The excess polymer is thrown away as a supernatant and the pellet is resuspended in buffer to release EVs. Polymers such as polyethylene glycol form a network around EVs resulting in high vesicle yields. The main restriction of polymer precipitation is the contamination of EVs with the polymer and other compounds pelleted in the sample (15,16,33).

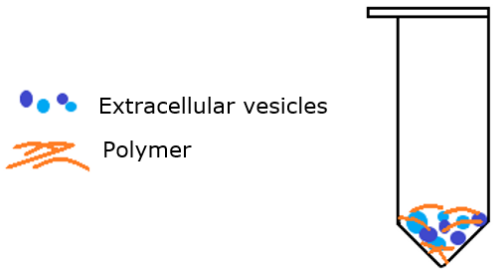

Figure 7. The principle of extracellular vesicles isolation with polymer precipitation. The polymer forms a network between extracellular vesicles which enables the isolation of different subpopulations.

1.3.3. Immuno-affinity purification

The principle of the immuno-affinity purification methods is based on the use of magnetic beads coated with streptavidin which can bind the biotinylated antibody of interest (Figure 8) (16). Antibodies for specific surface EV markers such as tetraspanins (CD9, CD81, CD63) enable their selective isolation, but only for the specific type of EVs which contain these markers (16,32). Antigens of interest are not exclusively present on EVs which diminishes the purity of isolated EVs. Consequently, immuno-affinity purification can be used for the isolation of one EV subpopulation resulting in low concentration for further analysis. It is also questionable whether purified EVs keep their original functionality and properties (15,16,32,33).

Another immune-affinity purification-based method is ELISA (The enzyme-linked immunosorbent assay). In this method wells are coated with antibody specific for EV marker. The primary antibody binds to EV antigen and labelled secondary antibody enables detection. This method also provides the isolation of a specific EV subtype which makes it useful in EV biomarker research (32).

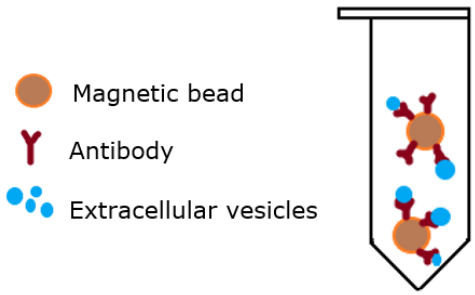

Figure 8. The principle of extracellular vesicle isolation with immuno-affinity purification. Magnetic beads coated with streptavidin bind to the biotinylated antibody which is specific for the extracellular vesicle protein marker.

1.3.4. Size-exclusion chromatography

Size-exclusion chromatography (SEC) or gel-filtration enables high-purity separation of particles based on their size (15). It is often combined with centrifugation and filtration for removing cellular debris and larger molecules (16,33). SEC column consists of a stationary porous phase such as sepharose while the mobile phase is continuously added to maintain the sample separation under the influence of gravitational force. The buffer is used as eluent because it preserves the biological activity of sample. Smaller molecules are entering pores in the sepharose and therefore are eluted in the later fractions. Larger particles like EVs are eluted in the earlier fractions because they are passing along the sepharose (Figure 9) (15). Column length, width and the compactness of stationary phase directly influence the quality of sample separation. SEC can be used for the isolation of multiple EV populations in the sample, leaving their structure intact (15). This is a cost-effective, simple and quick method for the separation of EVs from soluble proteins which are then suitable for further downstream analyses.

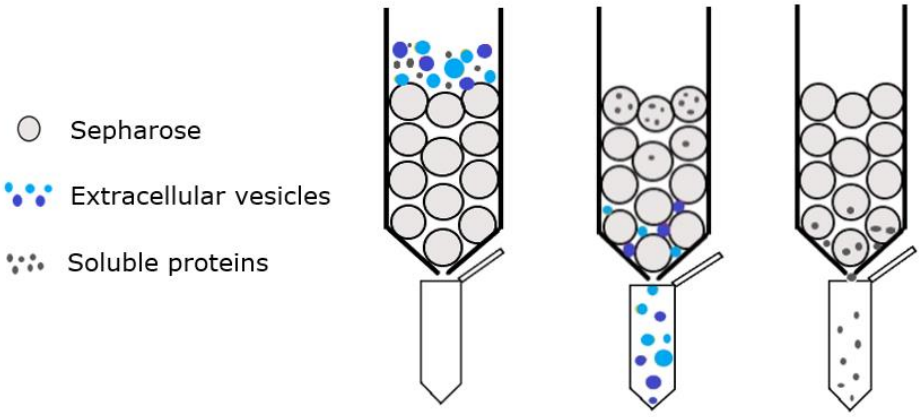

Figure 9. The principle of size-exclusion chromatography (SEC). Smaller particles such as soluble proteins are entering pores on the sepharose and are eluted in later fractions as oppose to larger extracellular vesicles.

2. OBJECTIVES

The main objective of this research was to isolate total EVs from clinical samples of CSF in the way that the quality and quantity of isolated EVs is suitable for downstream analyses including EV visualisation by electron microscopy and proteome characterisation by mass spectrometry.

The specific goals of this scientific work were:

1. To develop size-exclusion chromatography suitable for isolation of total EVs from clinical samples of cerebrospinal fluid

2. To apply size-exclusion chromatography to isolate total EVs from 4 CSF pools: control, severe TBI day 1, day 2-3, day 4-7.

3. To assess the quality of separation of EVs and soluble proteins from CSF by:

    - Determining the protein concentrations by Bradford assay
    - Measuring acetylcholinesterase activity as EV marker
    - Detecting levels of transmembrane CD9 protein by immunoblot

3. MATERIALS AND METHODS

3.1. Chemicals

2-mercaptoethanol (C_2H_6OS), *Sigma Aldrich*, St. Louis, USA
5,5'-dithio-bis(2-nitrobenzoic acid), *Sigma Aldrich*, St. Louis, USA
30 % acrylamide/bisacrylamide solution (29:1), *Alfa Aesar*, Karlsruhe, Germany
Acetic acid (CH_3COOH), *Kemika*, Zagreb, Croatia
Acetylthiocholine chloride, *Sigma Aldrich*, St. Louis, USA
Ammonium persulfate (APS), *Sigma Aldrich*, St. Louis, USA
Bovine serum albumin (BSA), *Affymetrix*, Santa Clara, USA
Bradford reagent, *Bio-Rad*, Hercules, USA
Bromophenol blue, *Sigma Aldrich*, St. Louis, USA
Calcium chloride ($CaCl_2$), *Kemika*, Zagreb, Croatia
Dulbecco's Modified Eagle Medium (DMEM), *Lonza*, Basel, Switzerland
ECL Prime, *GE Healthcare*, Chicago, Illinois, USA
Fetal calf serum, *Gibco*, Gaithersburg, USA
Glycerol, *Sigma Aldrich*, St. Louis, USA
Glycin ($C_2H_5NO_2$), *Carl Roth*, Karlsruhe, Germany
Hydrochloric acid (HCl), *Kemika*, Zagreb, Croatia
L-glutamine, *Gibco*, Gaithersburg, USA
Magnesium chloride hexahydrate ($MgCl_2$ x $6H_2O$), *Kemika*, Zagreb, Croatia
Methanol (CH_3OH), *Kemika*, Zagreb, Croatia
Nonfat dry milk, *Santa Cruz*, Dallas, USA
NP-40, *Roche*, Basel, Switzerland
Penicillin-streptomycin, *Gibco*, Gaithersburg, USA
Ponceau S stain, *Sigma Aldrich*, St. Louis, USA
Potassium chloride (KCl), *Kemika*, Zagreb, Croatia
Potassium dihydrogen phosphate (KH_2PO_4), *Kemika*, Zagreb, Croatia

Protease inhibitor, *Roche*, Basel, Switzerland

Protein ladder, *Thermo Scientific*, Rockford, USA

Pyruvate, *Pan biotech*, Aidenbach, Germany

Sepharose CL-6B, *GE healthcare*, Little Chalfont, United Kingdom

Sodium chloride (NaCl), *Kemika*, Zagreb, Croatia

Sodium deoxycholate, *Sigma Aldrich*, St. Louis, USA

Sodium dihydrogen phosphate-2-hydrate (NaH_2PO_4 x $2H_2O$), *Kemika*, Zagreb, Croatia

Sodium dodecyl sulfate (SDS), *Sigma Aldrich*, St. Louis, USA

Tetramethylethylendiamine (TEMED) ($C_6H_{16}N_2$), *Sigma Aldrich*, St. Louis, USA

Tris Base, *Sigma Aldrich*, St. Louis, USA

Tween 20, *Sigma Aldrich*, St. Louis, USA

3.2. Patients, collection and processing of cerebrospinal fluid

Research included 6 control patients and 6 patients with isolated severe TBI treated at the Clinical Hospital Centre Rijeka, Rijeka, Croatia. The study was approved by the ethical review board of the Clinical Hospital Centre Rijeka and by the ethical committee of the Faculty of Medicine Rijeka, Rijeka, Croatia. Control patients gave an informed consent to participate in the research and for severe TBI patients an informed consent was obtained by a family member. Severe TBI patients had isolated head trauma with a GCS score ≤8 and their CSFs were collected with a ventriculostomy catheter for the ICP management. TBI samples were collected at the end of day 1, day 3 and day 7 after injury. Control CSFs were remains after lumbar puncture for diagnostic purposes from patients with excluded CNS pathology. Samples were processed after collection using centrifugation at 500 x g for 10 minutes followed by another centrifugation at 2000 x g for 30 minutes. Filtration trough an 0.2 μm filter was conducted on supernatants. Samples were stored at -80°C until separation of EVs with SEC.

3.3. Separation of extracellular vesicles by size-exclusion chromatography

Individual CSF samples of 6 TBI patients were combined in equal volumes by the time points of collection resulting in 2 ml of the following 3 CSF-pools: day 1 (d1), day 2-3 (d2-3), day 4-7 (d4-7). The fourth pool consisted of 6 control patients whose CSFs were also collected in equal volumes resulting in the final 2 ml. In 2 ml of each pool was added 200 μl of Dulbecco's Modified Eagle Medium (DMEM) for the visualization and easier tracking of samples through the column (Figure 10). An in-house developed SEC column was 60 cm high (FALCON serological pipette of 50 ml) and filled with 50 ml of sepharose CL-6B which was left to sediment during the night. The column top was extended with plastic tube containing a net which served as filter. The bottom of the

column was filled with nylon wool to prevent sepharose from leaking. Phosphate buffered saline (PBS, pH 7.4, 140 mM NaCl, 2.7 mM KCl, 6.5 mM NaH_2PO_4 x $2H_2O$, 1.5 M KH_2PO_4, 0.7 mM $CaCl_2$, 0.7 mM $MgCl_2$ x $6H_2O$) was used as the mobile phase and fraction collection started after the first 10 ml of PBS passed through. During each SEC 80 fractions per 0.7 ml were collected and stored at 4°C for further analyses.

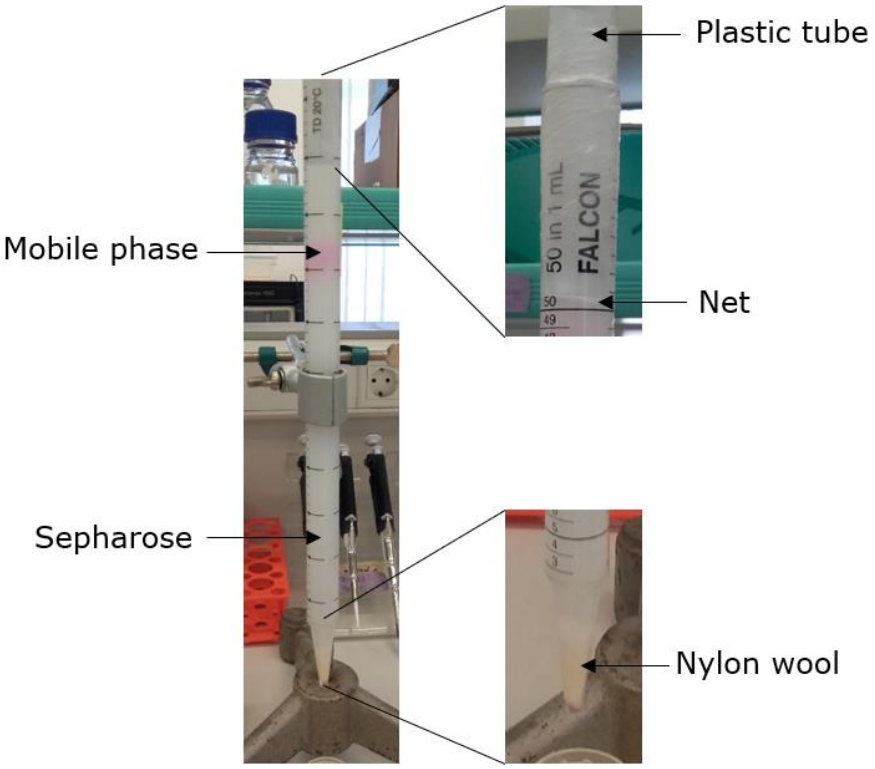

Figure 10. Visualization of a CSF sample traveling through the in-house developed size-exclusion chromatography column filled with sepharose CL-6B. The top of the column contains plastic tube with net and nylon wool is placed at the bottom.

3.4. Human neuroblastoma cell line SH-SY5Y

Cell cultures were cultivated in plastic flasks with the surface of 25 cm^2 in complete 10% DMEM for cell culture (DMEM, FCS 10 % (w/v), 2 mM L-glutamine, penicillin-streptomycin 1000 iU/ml, 0,11 mg/ml pyruvate) at 37°C and in humid conditions with 5% CO_2.

3.5. Western blotting

Cell lysates from cell line SH-SY5Y were prepared with the trypsinisation of cells in 50 ml tubes, centrifugation at 1500 x g for 10 minutes, rinsing with 2 ml of PBS and centrifugation for 8 minutes at 1500 rpm in new tubes. RIPA buffer (25 mM Tris, HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) with protease inhibitor were mixed with sample, incubated at 4°C for 20 minutes and centrifuged 20 minutes at 16100 x g. Supernatant was transferred in new 1.5 ml tubes and mixed with 5 x Laemmli buffer (1 M Tris-Cl pH 6.8, 50 % glycerol (v/v), 10 % SDS (w/v), 0.05 % bromophenol blue (w/v)), 2-mercaptoethanol and incubated at 95°C for 10 minutes.

After the second ultracentrifugation equal volumes of CSF samples and RIPA buffer were mixed with the protease inhibitor and incubated at 4°C for 20 minutes. Centrifugation at 16100 x g was followed by mixing of the supernatant with 5 x Laemmli buffer, 2-mercaptoethanol and incubation at 95°C for 10 minutes. SH-SY5Y cell lysate was loaded in 10 µl volume and CSF samples were loaded in 50 µl volume on the gel together with protein ladder. Stacking gel consisted of 5% acrylamide (5% Bisacrylamide (v/v), 130 mM Tris (pH 6.8) 0.1 % SDS (v/v), 0.1 % APS (v/v), 0.1 % TEMED (v/v)) and separating gel was 12% sodium dodecyl sulfate-polyacrylamide gel (12% Bisacrylamide (v/v), 380 mM Tris-Cl (pH 8.8), 0.1% SDS (v/v), 0.1% APS (v/v), 0.04% TEMED (v/v)). The electrophoresis (SDS-PAGE) was run first at 90V for approximately 30 minutes and then at 130V for additional 2 hours using the running buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS (w/v)). After electrophoreses, proteins were transferred to polyvinylidene fluoride (PVDF) membrane (Merck Millipore, Burlington, Massachusetts, USA) which was previously activated in methanol for 15 seconds, rinsed in water for 2 minutes and equilibrated in the transfer buffer (25 mM Tris base, 192 mM glycin, 20% methanol (v/v)) for 20 minutes at room temperature. After

assembling the transfer cell, the transfer was set for 90 minutes at 60V. The membrane was stained with the Ponceau S stain (Ponceau S 0.5% (w/v) in 1% (v/v) acetic acid) and rinsed with water. The next step was blocking with 5% nonfat dry milk in TBS (20 mM Tris base, 150 mM NaCl) and incubation over night with 1:1000 CD9 rabbit antibodies (Cell Signaling, Leiden, The Netherlands) in 5% bovine serum albumin (BSA), 1 x TBS and 0.1% Tween 20 at 4°C. Primary antibody was washed with three times in 1 x TBS and 0.05% Tween 20. Membrane was incubated for 1 hour with secondary 1:2000 anti-rabbit antibodies (Cell Signaling, Leiden, The Netherlands) in 5% nonfat dry milk, 1% TBS and 0.1% Tween 20. CD9 visualization was obtained with ECL Prime (GE Healthcare, Chicago, Illinois, USA) and chemiluminescence was measured using the Uvitec Alliance 4.7 imaging system.

3.6. Bradford assay

The standard curve for determining the total protein concentration was made by the measurement of the absorbances (620 nm wavelength) of BSA protein dilutions (2, 4, 6, 8, 10 µg/ml) on a microplate reader (Tecan Trading, Männedorf, Switzerland). Bradford reagent diluted with water was applied on a 96-well plate (200 µl per well). Negative control (PBS 20 µl) and samples (20 µl per well) were measured in duplicate. Optical density was measured at 620 nm on microplate reader (Tecan Trading, Männedorf, Switzerland).

3.7. Acetylcholinesterase activity measurement

Acetylcholinesterase activity assay was used for the absorbance measurement on a 96-well plate according to protocol (34,35). Samples (40 µl per well), positive control (40 µl of DMEM) and negative control (40 µl of PBS) were measured in duplicate and in each well 160 µl of master mix (125 mM 100 x acetylthiocholine chloride 2 µl, 5 mM 50 x 5,5’-dithio-bis(2-nitrobenzoic acid)

4 µl, PBS 154 µl) was added. Optical density was measured at 405 nm on a microplate reader (Tecan Trading, Männedorf, Switzerland) after 1 hour of incubation.

3.8. Slot blot

Each fraction from number 3 to 45 at the volume of 50 µl was mixed with 12.5 µl of 5 x Laemmli buffer without glycerol (1 M Tris-Cl pH 6.8, 10 % SDS (w/v), 0.05 % bromophenol blue (w/v)) and incubated at 95°C for 10 minutes. As a negative control PBS was used. Nitrocellulose membrane (0.45 µm, Bio-Rad, Hercules, USA) was soaked in water for 2 minutes and then in 1 x transfer buffer until samples were loaded on a on a slot blot system (Hoefer, Holliston, USA). After samples have entered slots under the influence of pump producing vacuum, each slot was rinsed with 1 ml of 1 x PBS three times. With the pump still on, membranes were lifted off and incubated over night with 1:1000 CD9 rabbit antibodies (Cell Signalling, Leiden, The Netherlands) in 5% BSA, 1 x TBS and 0.1% Tween 20 at 4°C. Membranes were washed three times with 1 x TBS and 0.05% Tween 20 and incubated for 1 hour with secondary 1:2000 anti-rabbit antibody conjugated with horseradish peroxidase (Cell Signalling, Leiden, The Netherlands) in 5% nonfat dry milk, 1% TBS and 0.1% Tween 20. The chemiluminescent signals were produced by ECL Prime (GE Healthcare, Chicago, Illinois, USA) and visualised using Uvitec Alliance 4.7 imaging system.

3.9. Data analysis

Data from AChE activity measurement and Bradford assay were processed using Microsoft Office Excel software. Quantification of slot blot signals was conducted by ImageJ software. Graphs of AChE activity, protein concentration and CD9 protein levels were made using OriginLab software and non-linear curve fitting analysis.

4. RESULTS

4.1. Patients with severe TBI and control patients

The study included 6 patients with isolated severe TBI defined as GCS score ≤8. All patients were male with the age ranging from 24 to 56 and a median of 45 years. Four patients experienced a motor vehicle accident, 1 a bicycle accident and 1 obtained brain injury by falling from height (Table 2).

Table 2. Patients with isolated severe traumatic brain injury.

Patient	Age	Gender	Type of injury	GCS	GOS	Intracranial pathology
1	39	M	Fall from height	6	4	EDH, SDH, traumatic SAH, focal brain injury
2	33	M	Motor vehicle accident	7	3	Traumatic SAH, traumatic thalamic hemorrhage, DAI
3	53	M	Motor vehicle accident	4	2	Traumatic SAH, bilateral frontal lobe contusions, DAI
4	51	M	Motor vehicle accident	4	3	Traumatic SAH, mesencephalon contusion, DAI
5	56	M	Bicycle accident	8	2	SDH, traumatic SAH, multiple cerebral contusion sites
6	24	M	Motor vehicle accident	3	3	EDH, traumatic SAH, DAI

GCS – Glasgow coma scale, GOS – Glasgow outcome scale
EDH - epidural hematoma, SDH - subdural hematoma, SAH - subarachnoid haemorrhage, DAI - diffuse axonal injury

All analysed patients had traumatic subarachnoid haemorrhage and 4 of them had diffuse axonal injury. The injury caused epidural hematoma in 2 patients and subdural hematoma in 2 patients. Additionally, 1 patient obtained focal brain injury, 1 traumatic thalamic haemorrhage, 1 bilateral frontal lobe

contusions, 1 mesencephalon contusion and 1 multiple cerebral contusion sites.

Favourable outcome in recovery estimated by the GOS score 3 months after discharge was found in 1 patient with the GOS score of 4 corresponding to moderate disability. Three analysed patients had severe disability with the GOS score 3 and 2 patients were in a persistent vegetative state (GOS score 2).

The control group consisted of 5 male and 1 female patient who were excluded from having CNS pathology after the lumbar puncture procedure. Patients age range was from 19 to 84, with a median of 53 years (Table 3).

Table 3. Demographic characteristics of control patients.

Patient	Age	Gender
1	81	M
2	34	M
3	19	M
4	47	F
5	59	M
6	84	M

4.2. CD9 EV protein biomarker is present in severe TBI and control CSF pools

In the previous research the transmembrane CD9 protein was detected at high amounts in the individual severe TBI-CSF samples (31). Because of the high levels that were found in the samples and its transmembrane nature, CD9 is a reliable protein marker for detection of EVs. To test if CD9 is ubiquitously present in CSF pools prepared for SEC separation the western blotting method was used. CD9 was detected in cell lysate of SH-SY5Y cell line which was a positive control and in all CSF pools. The weakest signal of CD9 was detected in the control CSF where the CD9 protein level was visible at the longer

exposition of 5 minutes. In the severe TBI pools the CD9 protein was readily detected whereby the strongest signal was present in the d1 sample and the weakest in the d4-7 sample. Total protein levels were detected by Ponceau staining and were consistent with the CD9 levels detected by immunoblot (Figure 11).

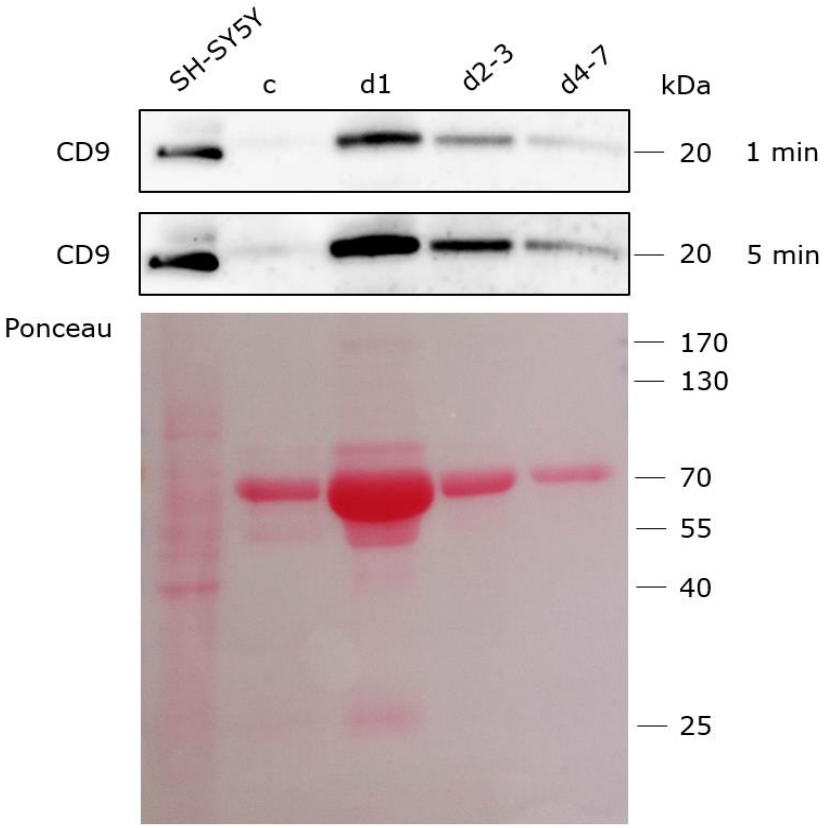

Figure 11. Extracellular vesicle marker CD9 is detected in the control (c), severe TBI d1 (day 1), d2-3 (day 2-3) and d4-7 (day 4-7) CSF pools. The presence of CD9 protein was investigated in CSF cell lysates with western blot method. Cell lysate of SH-SY5Y cell line was used as a positive control. kDa - kilodalton

4.3. Distribution of total proteins concentration in size-exclusion chromatography fractions

After the brain trauma, a large amount of different proteins and molecules are released into the CSF. These soluble proteins interfere with EV detection and analysis. The previous research detected the presence of enlarged EV during

acute phase after severe TBI (31). To investigate the composition of these vesicles, each CSF pool was separated independently with SEC.

Collected fractions from SEC of 4 CSF pools were first characterised by determining the total protein concentration by Bradford assay performed on a 96-well plate. To calculate the unknown concentrations, a standard curve was formed for BSA protein dilutions with known concentrations (Figure 12). The highest protein concentrations were detected in severe TBI day 1 and the lowest in day 4-7 compared to control whereby the continuous protein concentration values above 10 µg/ml (marked grey) were present in fraction range 33-57 in the control pool, 25-52 in d1 pool, 30-48 in d2-3 pool and 35-48 in d4-7 pool (Table 4).

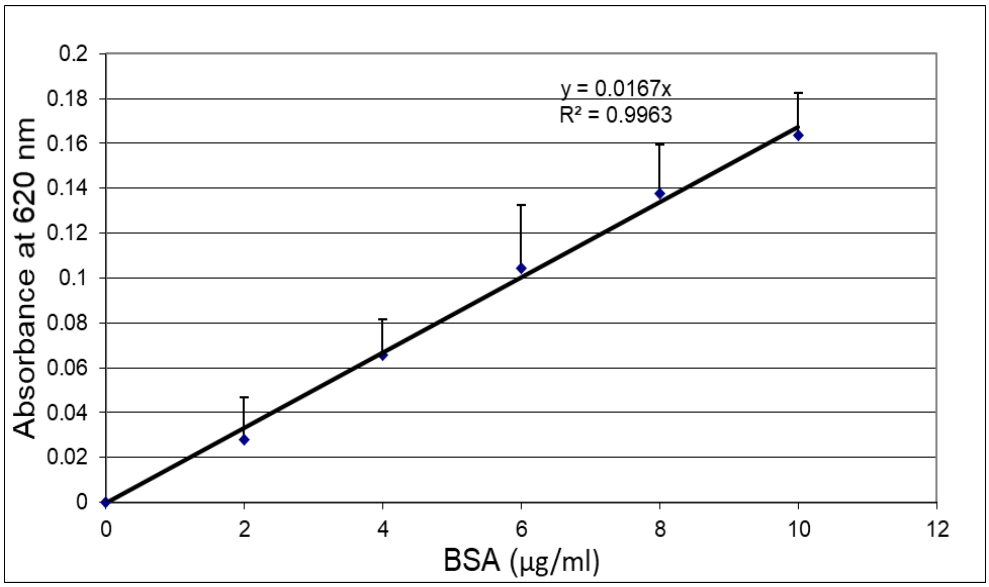

Figure 12. Standard curve for the calculation of total protein concentration in size-exclusion chromatography fractions was determined on a 96-well plate to enable simultaneous characterisation of collected fractions. Absorbance at 620 nm wavelength was measured for known concentrations of bovine serum albumin (BSA). Linear equation (marked y) was used to determine total protein concentration in each fraction. R^2- correlation coefficient

Table 4. Total protein concentration (µg/ml) in fractions of 4 CSF pools determined by Bradford assay.

Fraction number	Control	day 1	day2-3	day4-7	Fraction number	Control	day 1	day2-3	day4-7
1	0	9	0	0	**41**	207	441	182	182
2	0	0	0	0	**42**	210	278	168	168
3	0	4	0	0	**43**	223	435	144	144
4	0	9	0	0	**44**	213	321	116	116
5	0	0	0	0	**45**	215	333	88	88
6	0	0	0	0	**46**	174	289	61	61
7	0	7	4	0	**47**	172	209	33	33
8	0	0	0	0	**48**	141	98	34	34
9	0	6	0	0	**49**	117	57	7	7
10	0	9	0	0	**50**	89	44	4	4
11	0	2	0	0	**51**	67	17	1	1
12	0	0	0	0	**52**	47	13	0	0
13	0	0	2	0	**53**	36	0	0	0
14	0	0	0	0	**54**	24	0	0	0
15	0	0	0	0	**55**	20	0	0	0
16	0	2	0	0	**56**	12	4	1	1
17	0	4	0	0	**57**	11	0	1	0
18	0	0	13	0	**58**	7	0	1	0
19	0	19	7	0	**59**	8	0	0	0
20	0	9	0	0	**60**	2	0	0	0
21	0	20	0	0	**61**	15	0	0	0
22	0	33	0	0	**62**	3	0	0	0
23	0	46	0	0	**63**	0	0	0	0
24	0	0	0	0	**64**	0	0	0	0
25	0	51	6	0	**65**	0	0	0	0
26	0	40	0	0	**66**	5	0	0	0
27	0	62	0	0	**67**	0	0	0	0
28	0	85	0	0	**68**	0	0	0	0
29	0	107	0	0	**69**	0	0	1	0
30	3	58	10	0	**70**	0	0	3	0
31	0	164	17	0	**71**	0	0	0	0
32	6	169	19	0	**72**	0	0	0	0
33	12	269	27	0	**73**	2	0	1	1
34	28	346	49	0	**74**	0	0	4	4
35	35	394	70	70	**75**	0	0	1	1
36	66	262	101	101	**76**	0	0	5	0
37	92	430	151	151	**77**	2	0	0	0
38	101	396	179	179	**78**	0	0	0	0
39	151	480	187	187	**79**	0	0	0	0
40	186	493	188	188	**80**	0	0	0	0

4.4. Detection of EVs with acetylcholinesterase measurement

After determining total protein levels, the activity of AChE as EV marker was measured in 96-well plate to detect EV. The continuous AChE activity values above 0.01 (marked grey) were detected in fraction range 26-53 in control, 21-47 in severe TBI d1 pool, 25-44 d2-3 pool, while d4-7 showed increased activity in fractions 33-44 (Table 5).

4.5. Detection of EV protein marker CD9 in fractions after size-exclusion chromatography

After the detection of total protein levels and AChE activity, the slot-blot system to simultaneously detect CD9 protein levels in fractions after size-exclusion chromatography was used. Fraction range 3-45 was loaded on a membrane in a slot blot system for each performed SEC. This fraction range was analysed because the larger particles such as EVs are expected to exit the SEC column before the smaller particles like soluble proteins detected by the Bradford assay. The control CSF had positive CD9 signal in fractions 30-45, while the strongest signal was present in fractions 33-43. The severe TBI d1 showed presence of CD9 in fraction range 12-45 with strongest signal present in fraction range 12-14 and 27-42. In d2-3 and d4-7 CD9 was detected in fractions 28-45 and 32-43 with strongest signal present in fractions 32-42 and 33-40 (Figure 13).

Table 5. Detection of AChE activity in size-exclusion chromatography fractions of 4 CSF pools by measuring substrate dependent change in absorbance at 405 nm.

Fraction number	Control	day 1	day2-3	day4-7	Fraction number	Control	day 1	day2-3	day4-7
1	0	0	0	0	**41**	0.168	0.225	0.057	0.057
2	0	0	0.002	0	**42**	0.144	0.178	0.032	0.032
3	0.001	0	0	0	**43**	0.122	0.119	0.021	0.021
4	0.003	0	0	0	**44**	0.098	0.085	0.010	0.010
5	0.001	0	0	0	**45**	0.081	0.070	0.002	0.002
6	0	0	0	0	**46**	0.065	0.039	0	0
7	0.002	0	0	0	**47**	0.050	0.022	0.003	0.003
8	0.002	0	0	0	**48**	0.038	0.009	0	0
9	0.001	0	0	0	**49**	0.027	0.009	0	0
10	0	0.010	0	0	**50**	0.019	0.006	0	0
11	0.004	0.008	0.001	0	**51**	0.011	0.071	0.015	0.015
12	0.004	0.014	0.004	0	**52**	0.010	0.014	0.001	0
13	0.002	0.017	0.004	0	**53**	0.010	0.002	0.003	0
14	0.003	0.004	0	0	**54**	0.008	0.002	0.001	0
15	0.002	0.001	0	0	**55**	0.003	0.014	0.003	0
16	0.002	0.001	0	0	**56**	0.004	0.025	0	0
17	0	0.001	0	0	**57**	0.010	0.024	0.007	0.007
18	0	0.003	0.001	0	**58**	0.005	0.021	0.009	0.009
19	0.003	0	0	0	**59**	0.005	0.011	0.009	0
20	0.003	0.007	0.006	0	**60**	0.005	0.008	0.005	0
21	0.002	0.020	0	0	**61**	0.005	0.012	0.006	0
22	0.001	0.056	0	0	**62**	0.006	0.016	0.004	0
23	0.005	0.122	0.007	0	**63**	0.012	0.009	0.005	0
24	0.006	0.237	0.009	0	**64**	0.012	0.013	0.001	0
25	0.008	0.361	0.016	0	**65**	0.012	0.010	0.003	0
26	0.013	0.515	0.025	0	**66**	0.008	0.006	0.002	0
27	0.020	0.694	0.039	0	**67**	0.010	0.009	0	0
28	0.033	0.855	0.073	0	**68**	0.010	0.005	0	0
29	0.057	0.959	0.089	0	**69**	0.006	0.010	0.001	0
30	0.098	1.024	0.118	0	**70**	0.005	0.007	0.009	0
31	0.140	1.068	0.133	0	**71**	0.006	0.007	0.001	0
32	0.192	1.061	0.164	0	**72**	0.005	0.006	0	0
33	0.219	0.997	0.154	0.154	**73**	0.002	0.005	0	0
34	0.250	0.900	0.147	0.147	**74**	0.002	0.010	0	0
35	0.273	0.766	0.149	0.149	**75**	0.002	0.006	0.001	0
36	0.276	0.662	0.129	0.129	**76**	0.002	0.005	0.001	0
37	0.237	0.577	0.094	0.094	**77**	0	0.003	0.001	0
38	0.245	0.475	0.088	0.088	**78**	0	0.009	0	0
39	0.221	0.381	0.061	0.061	**79**	0.001	0.011	0	0
40	0.209	0.299	0.040	0.040	**80**	0.002	0.012	0	0

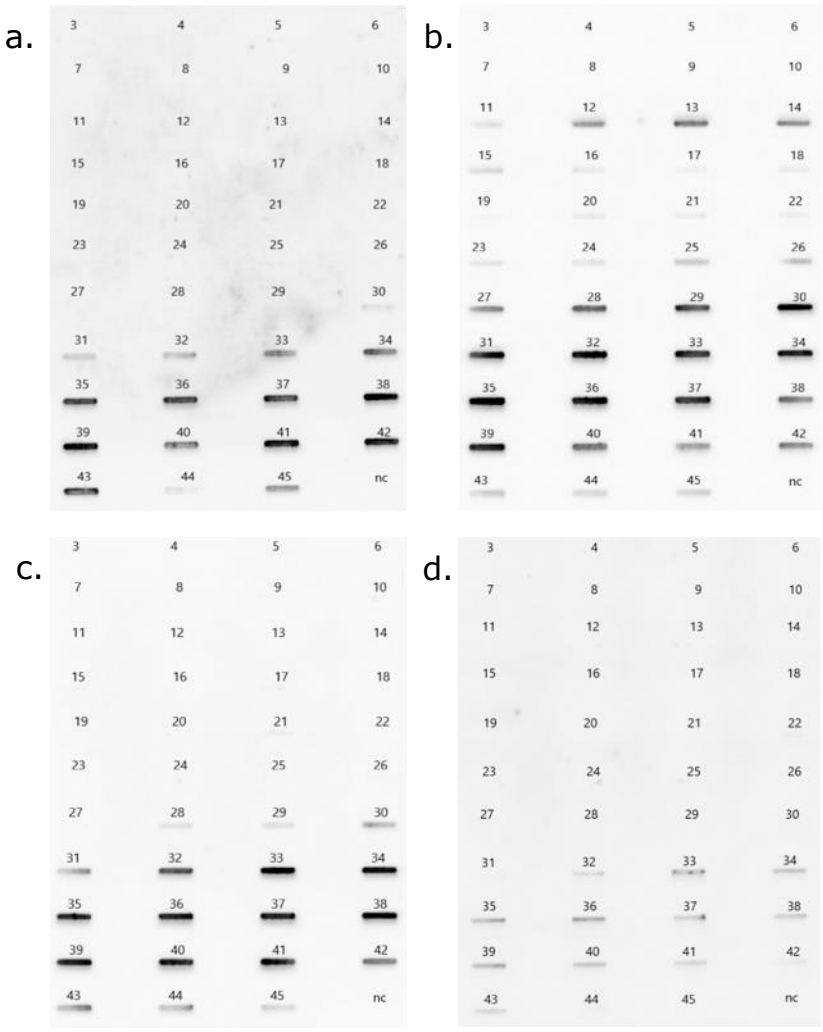

Figure 13. Extracellular vesicle biomarker CD9 is detected by immunoblot method in fractions after the separation of CSF samples with size-exclusion chromatography. Fractions 3-45 for control (a.) severe TBI day 1 (b.) day 2-3 (c.) and day 4-7 (d.) were loaded on the membrane in a slot blot system. Membranes were incubated with antibody against CD9 protein and signal was detected by chemiluminescence. Phosphate buffered saline was used as a negative control - marked nc.

4.6. Applied size-exclusion chromatography resulted in separation of EVs from soluble proteins

To assess the quality of EV isolation by SEC, all three characterizations of fractions, including AChE activity measurement, Bradford assay and CD9 immunoblot quantification, were depicted in a single graph for each SEC

(Figure 14). Generally, all four separations showed equal distribution of measured values resulting in normal distribution of AChE activity, CD9 and protein level curves. AChE peaks in control, severe TBI day 1 and day 2-3 samples were detected in fraction range 25-40, followed by CD9 peaks in fraction range 30-45 and protein peaks in fraction range 35-55 (Figure 14 a-c). Severe TBI day 4-7 showed an overlap of AChE and protein peaks in fraction range 37-50, but CD9 protein was also detected in earlier fraction range 30-40 (Figure 14 d). Unlike other samples, severe TBI day 1 showed additional increase of CD9 protein levels in fraction range 10-15 (Figure 14 b).

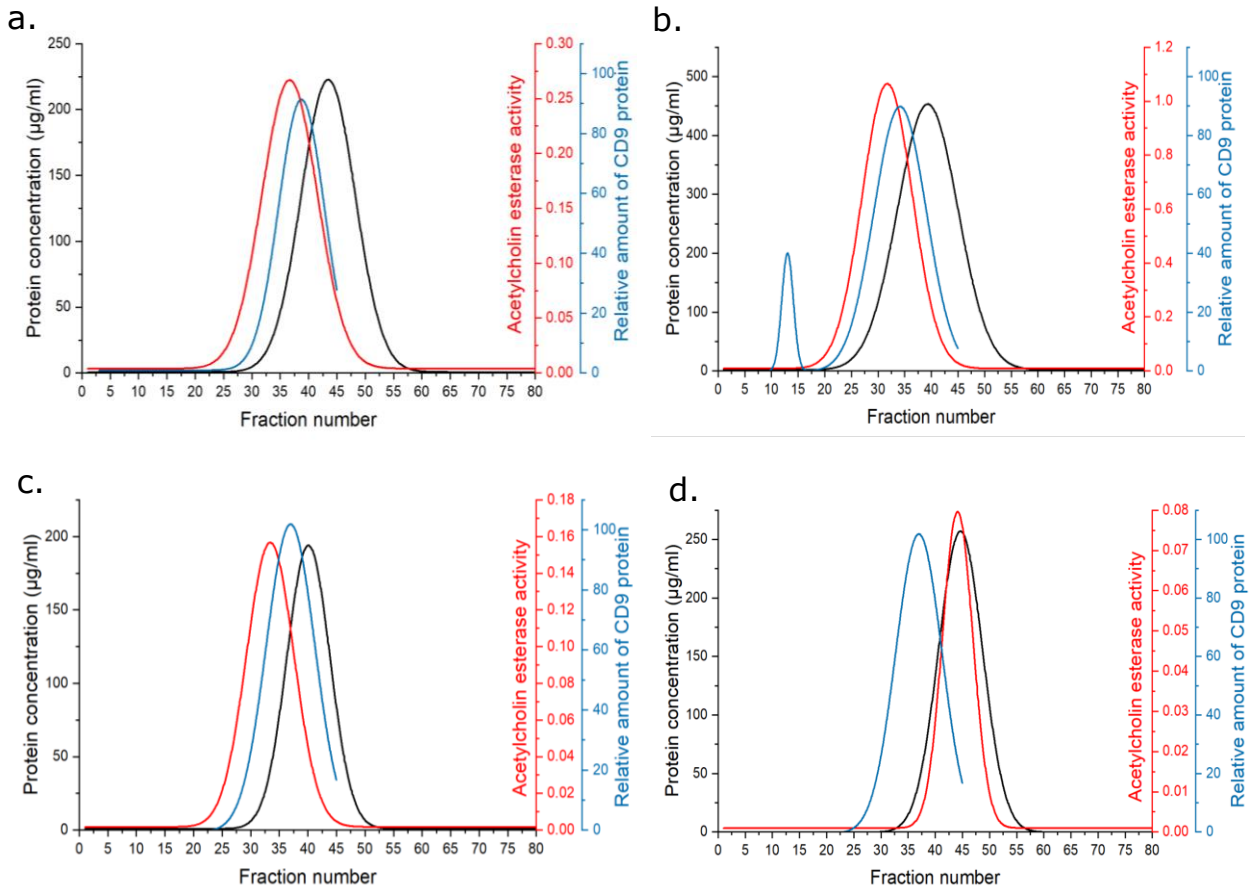

Figure 14. Quality of the extracellular vesicles (EVs) separation from soluble proteins by size-exclusion chromatography. CD9 (blue curve) and acetylcholinesterase (red curve) as EV protein biomarkers were detected in earlier fractions than soluble proteins (black curve) in control (a.), severe TBI day 1 (b.), day 2-3 (c.) and day 4-7 (d.) samples.

5. DISCUSSION

EVs are mediators of intercellular communication that potentially have a role in neuro repair processes, but the mechanisms of these processes are unknown (23). The main problem in EV research is non-standardised protocols and the methods used for their isolation (11). Furthermore, none of the existing studies investigated the quality of their separation. To enable downstream characterisation of EVs after severe TBI, a non-commercial SEC method for the isolation of total EVs from CSF was developed. This research provides the protocol for EV separation from soluble proteins present in CSF samples. Isolated EVs can undergo further molecular and functional analyses to examine their involvement in the neuro-recovery.

Commonly used methods for the isolation of EVs such as ultracentrifugation, immunoaffinity purification and precipitation with polymer often result in damaged and contaminated EVs which is lowering their yields and changing biological properties (15). Also, the complexity of biological samples containing a high amount of proteins, protein aggregates and lipoproteins additionally reduces the efficiency of EV isolation (36). On the other hand, SEC enables the isolation of total EVs without co-contamination and disruption of their structure or biological activity (16). However, the quality of SEC separation depends on the column length and type of stationary phase. The existing studies used a short SEC column (approximately 6 cm) and sepharose CL-2B as a stationary phase (37). In this research the SEC method was developed in-house because it is not commercially available. To improve the separation a longer column (approximately 60 cm) was filled with a denser sepharose CL-6B.

To examine if CD9 EV biomarker is present in CSF pools, the western blotting method was used. The CD9 protein was reported to be present in many EV subgroups and its presence was confirmed in the individual CSF samples (15,31). Results showed that CD9 was present in the control and severe TBI

pools indicating that it can be used for further characterization of EVs (Figure 11). Ponceau staining of the western blot membrane confirmed the successful transfer of proteins. A strong signal of 70 kDa protein was present in all CSF samples, which could represent the serum albumin accumulated during the post-trauma bleeding. The highest protein levels were detected in the day 1 pool and the lowest in day 4-7. However, small volumes of CSF pools analysed on the gel do not necessarily reflect ongoing conditions in the CNS.

Quantification of EV and total proteins showed that in control, severe TBI – d1 and d2-3 EV biomarkers are present in earlier fractions while soluble proteins are detected in later (Figure 14 a-c). Surprisingly, d4-7 AChE peak is shifted towards the protein concentration peak with almost complete overlapping (Figure 14 d). This could indicate that AChE has changed its activity at this point or that the majority of detected proteins are present on the EVs surface. The main deficiency of AChE activity measurement is inability to differentiate which forms of an enzyme are present/detected in each sample. Also, AChE exists as a soluble or membrane-bound protein, meaning that detected enzymes are not exclusively present on the EVs (17). On the other hand, CD9 as a transmembrane protein is a more reliable marker of EVs due to its high quantity on the EVs membrane (11). It was detected in the earlier fractions of all 4 samples which indicates their separation from soluble proteins. Same as AChE, CD9 can be present in different biological membranes and not only on EVs. Both AChE and CD9 are proteins present in different tissues and therefore are not CNS-specific markers. Ideally, fractions should be characterised with biomarkers expressed only on EVs secreted from CNS.

As expected it was expected after Ponceau staining, day 1 after severe TBI shows high protein concentrations in the CSF because of the tissue damage, blood-brain barrier (BBB) disruption and excretion of pro-inflammatory molecules (Figure 14 b). Other pools have similar levels of protein concentration compared to the control (Figure 14 c-d). However, it is

important to note that CSF obtained by lumbar puncture is not ideal to be used for control because it is physiologically different when compared to ventriculostomy CSF (5,38). Furthermore, in the SEC column larger particles and EVs are travelling faster and therefore it is expected to detect increased levels of CD9 protein in early fractions which would represent larger vesicles. This signal was present only in d1 pool in fraction range 10-15 probably because few damaged EVs could stick together and form a larger EV (Figures 13b and 14b). This signal could be detected only on day 1 because of the tissue pieces and cellular debris cleaned up in the following days.

Only a few studies are characterising EVs in cerebrospinal fluid of people with traumatic brain injury (27,28). The main reason is limited access to clinical samples due to small volumes collected with invasive surgical procedures. Consequently, research is often conducted on a small number of patients with clinically different injuries which should be studied separately. Unavoidable limitation of this study is that measurements on CSF pools represent average events occurring in the brains of 6 patients which differ in age, sex, clinical history and type of injury. Unlike most studies, this one included only patients who had isolated severe TBI with a GCS score 3-8.

Although SEC does not result in complete separation of EVs, we can say that collected fractions were enriched with them. Modifications of column properties such as its length or the type of stationary phase could improve the separation of EVs, but this level of separation from soluble proteins is sufficient enough for next steps: electron microscopy for the visualization of EVs and mass spectrometry analysis of EVs protein content. Mass spectrometry would enable identification of CNS-specific markers which are suitable for immuno-isolation of EVs. The resolution at mass spectrometry depends on the purity of the biological sample. In this research abundant soluble proteins were separated from EVs, which is why a higher accuracy of mass spectrometry analysis is expected.

6. CONCLUSION

Results indicate that non-commercial SEC method developed in this study can be used for the separation of EVs from soluble proteins in clinical CSF. The quality of EV separation in this research was confirmed by the EVs presence in earlier and soluble proteins in later SEC fractions. Furthermore, measurement of AChE activity and EV protein markers like CD9 are suitable to assess quality of EVs isolation. The unanswered question remains why is AChE activity peak shifted towards the protein concentration on day 4-7 after injury.

The main restriction of this research is that results reflect average conditions occurring in the CSF of a small number of patients. Also, AChE activity measurement and Bradford assay are not completely sensitive methods. Therefore, improvement of this approach by modifying the SEC column or changing the EV detection methods can be suggested.

The future steps would be visualisation of EVs with electron microscopy and EV proteome analysis with mass spectrometry.

7. REFERENCES

1. Maas AIR, Menon DK, Adelson PD, Andelic N, Bell MJ, Belli A *et al.* Traumatic brain injury: integrated approaches to improve prevention, clinical care, and research. *Lancet Neurol* 2017; 16: 987–1048.

2. Dinsmore J. Traumatic brain injury: an evidence-based review of management. *Contin Educ Anaesth Crit Care Pain* 2013; 13: 189–195.

3. Parikh S, Koch M, Narayan RK. Traumatic brain injury. *Int Anesthesiol Clin* 2007; 45: 119–135.

4. Park K, Biederer T. Neuronal adhesion and synapse organization in recovery after brain injury. *Future Neurol* 2013; 8: 555–567.

5. Ottens AK, Kobeissy FH, Golden EC, Zhang Z, Haskins WE, Chen S-S *et al.* Neuroproteomics in neurotrauma. *Mass Spectrom Rev* 2006; 25: 380–408.

6. Werner C, Engelhard K. Pathophysiology of traumatic brain injury. *Br J Anaesth* 2007; 99: 4–9.

7. Bramlett HM, Dietrich WD. Long-Term Consequences of Traumatic Brain Injury: Current Status of Potential Mechanisms of Injury and Neurological Outcomes. *J Neurotrauma* 2015; 32: 1834–1848.

8. Maas AI, Stocchetti N, Bullock R. Moderate and severe traumatic brain injury in adults. *Lancet Neurol* 2008; 7: 728–741.

9. Filiano AJ, Gadani SP, Kipnis J. Interactions of innate and adaptive immunity in brain development and function. *Brain Res* 2015; 1617: 18–27.

10. Bergeson AG, Lundin R, Parkinson RB, Tate DF, Victoroff J, Hopkins RO *et al.* Clinical Rating of Cortical Atrophy and Cognitive Correlates Following Traumatic Brain Injury. *Clin Neuropsychol* 2004; 18: 509–520.

11. Kalra H, Drummen G, Mathivanan S. Focus on Extracellular Vesicles: Introducing the Next Small Big Thing. *Int J Mol Sci* 2016; 17: 170.

12. van der Pol E, Boing AN, Harrison P, Sturk A, Nieuwland R. Classification, Functions, and Clinical Relevance of Extracellular Vesicles. *Pharmacol Rev* 2012; 64: 676–705.

13. György B, Szabó TG, Pásztói M, Pál Z, Misják P, Aradi B *et al.* Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. *Cell Mol Life Sci* 2011; 68: 2667–2688.

14. Yuana Y, Sturk A, Nieuwland R. Extracellular vesicles in physiological and pathological conditions. *Blood Rev* 2013; 27: 31–39.

15. Li P, Kaslan M, Lee SH, Yao J, Gao Z. Progress in Exosome Isolation Techniques. *Theranostics* 2017; 7: 789–804.

16. Peterson MF, Otoc N, Sethi JK, Gupta A, Antes TJ. Integrated systems for exosome investigation. *Methods* 2015; 87: 31–45.

17. Hicks D, John D, Makova NZ, Henderson Z, Nalivaeva NN, Turner AJ. Membrane targeting, shedding and protein interactions of brain acetylcholinesterase: Brain acetylcholinesterase. *J Neurochem* 2011; 116: 742–746.

18. Basso M, Bonetto V. Extracellular Vesicles and a Novel Form of Communication in the Brain. *Front Neurosci* 2016; 10.

19. Meister M, Tikkanen R. Endocytic Trafficking of Membrane-Bound Cargo: A Flotillin Point of View. *Membranes* 2014; 4: 356–371.

20. Lizarbe M, Barrasa J, Olmo N, Gavilanes F, Turnay J. Annexin-Phospholipid Interactions. Functional Implications. *Int J Mol Sci* 2013; 14: 2652–2683.

21. Chivet M, Hemming F, Pernet-Gallay K, Fraboulet S, Sadoul R. Emerging Role of Neuronal Exosomes in the Central Nervous System. *Front Physiol* 2012; 3.

22. Xu R, Greening DW, Zhu H-J, Takahashi N, Simpson RJ. Extracellular vesicle isolation and characterization: toward clinical application. *J Clin Invest* 2016; 126: 1152–1162.

23. Werner JK, Stevens RD. Traumatic brain injury: recent advances in plasticity and regeneration. *Curr Opin Neurol* 2015; 28: 565–573.

24. Nekludov M, Mobarrez F, Gryth D, Bellander B-M, Wallen H. Formation of Microparticles in the Injured Brain of Patients with Severe Isolated Traumatic Brain Injury. *J Neurotrauma* 2014; 31: 1927–1933.

25. Morel N, Morel O, Petit L, Hugel B, Cochard J-F, Freyssinet J-M *et al.* Generation of Procoagulant Microparticles in Cerebrospinal Fluid and

Peripheral Blood After Traumatic Brain Injury: *J Trauma Inj Infect Crit Care* 2008; 64: 698–704.

26. de Rivero Vaccari JP, Brand F, Adamczak S, Lee SW, Perez-Barcena J, Wang MY *et al.* Exosome-mediated inflammasome signaling after central nervous system injury. *J Neurochem* 2016; 136: 39–48.

27. Manek R, Moghieb A, Yang Z, Kumar D, Kobessiy F, Sarkis GA *et al.* Protein Biomarkers and Neuroproteomics Characterization of Microvesicles/Exosomes from Human Cerebrospinal Fluid Following Traumatic Brain Injury. *Mol Neurobiol* 2017. 55: 6112–6128

28. Patz S, Trattnig C, Grünbacher G, Ebner B, Gülly C, Novak A *et al.* More than Cell Dust: Microparticles Isolated from Cerebrospinal Fluid of Brain Injured Patients Are Messengers Carrying mRNAs, miRNAs, and Proteins. *J Neurotrauma* 2013; 30: 1232–1242.

29. Hazelton I, Yates A, Dale A, Roodselaar J, Akbar N, Ruitenberg MJ *et al.* Exacerbation of Acute Traumatic Brain Injury by Circulating Extracellular Vesicles. *J Neurotrauma* 2018; 35: 639–651.

30. Huang S, Ge X, Yu J, Han Z, Yin Z, Li Y *et al.* Increased miR-124-3p in microglial exosomes following traumatic brain injury inhibits neuronal inflammation and contributes to neurite outgrowth *via* their transfer into neurons. *FASEB J* 2018; 32: 512–528.

31. Kuharić J, Grabušić K, Tokmadžić VS, Štifter S, Tulić K, Shevchuk O *et al.* Severe Traumatic Brain Injury Induces Early Changes in the Physical Properties and Protein Composition of Intracranial Extracellular Vesicles. *J Neurotrauma* 2018. (ahead of print)

32. Momen-Heravi F, Balaj L, Alian S, Mantel P-Y, Halleck AE, Trachtenberg AJ *et al.* Current methods for the isolation of extracellular vesicles. *Biol Chem* 2013; 394: 1253-62

33. Witwer KW, Buzás EI, Bemis LT, Bora A, Lässer C, Lötvall J *et al.* Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. *J Extracell Vesicles* 2013; 2: 20360.

34. Khan S, Jutzy JMS, Aspe JR, McGregor DW, Neidigh JW, Wall NR. Survivin is released from cancer cells via exosomes. *Apoptosis* 2011; 16: 1–12.

35. Savina A, Vidal M, Colombo MI. The exosome pathway in K562 cells is regulated by Rab11. *J Cell Sci* 2002; 115: 2505-15

36. Böing AN, van der Pol E, Grootemaat AE, Coumans FAW, Sturk A, Nieuwland R. Single-step isolation of extracellular vesicles by size-exclusion chromatography. *J Extracell Vesicles* 2014; 3: 23430.

37. Welton JL, Webber JP, Botos L-A, Jones M, Clayton A. Ready-made chromatography columns for extracellular vesicle isolation from plasma. *J Extracell Vesicles* 2015; 4: 27269.

38. Sommer JB, Gaul C, Heckmann J, Neundörfer B, Erbguth FJ. Does Lumbar Cerebrospinal Fluid Reflect Ventricular Cerebrospinal Fluid? A Prospective Study in Patients with External Ventricular Drainage. *Eur Neurol* 2002; 47: 224–232.

Acronyms and abbreviations

AChE	Acetylcholinesterase
APS	Ammonium persulfate
BBB	Blood-brain barrier
BSA	Bovine serum albumin
DMEM	Dulbecco's Modified Eagle Medium
CNS	Central nervous system
CSF	Cerebrospinal fluid
EV	Extracellular vesicle
ELISA	The enzyme-linked immunosorbent assay
FCS	Fetal calf serum
GCS	Glasgow coma scale
GOS	Glasgow outcome scale
GTP	Guanosine triphosphate
ICP	Intracranial pressure
kDa	Kilodalton
miRNA	Micro ribonucleic acid
mRNA	Messenger ribonucleic acid
NTA	Nanoparticle tracking analysis
PBS	Phosphate buffered saline
PVDF	Polyvinylidene fluoride
RIPA	Radioimmunoprecipitation assay buffer
SDS	Sodium dodecyl sulfate
PAGE	Polyacrylamide gel electrophoresis
SEC	Size-exclusion chromatography
TBI	Traumatic brain injury
TBS	Tris buffered saline
TEMED	Tetramethylethylendiamine

8. CURRICULUM VITAE

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WORK EXPERIENCE

August 2017–September 2018 — Master's thesis „Size-exclusion chromatography-based isolation of extracellular vesicles from cerebrospinal fluid of patients with severe traumatic brain injury"

- Faculty of Medicine, Rijeka (Croatia) – Department of physiology, immunology and pathophysiology
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March 2018 — Teaching assistant in General chemistry

Department of biotechnology, Rijeka (Croatia)

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2017-2018 — Student job as a sales promoter of food supplements

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2014–2017 — Student jobs

June 2016 — Internship

Jadran galenski laboratorij JGL d.d., Rijeka (Croatia)

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EDUCATION AND TRAINING

2016–2018 — Graduate university program "Biotechnology in Medicine"

Department of biotechnology, Rijeka (Croatia)

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2013–2016 **Undergraduate university program "Biotechnology and Drug Research"**

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2009–2013 **High school - gymnasium**

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PERSONAL SKILLS

Mother tongue(s): Croatian

Foreign language(s)

	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken interaction	Spoken production	
English	C1	C1	C1	C1	C1
Italian	B2	B2	B1	B1	B1

Levels: A1 and A2: Basic user - B1 and B2: Independent user - C1 and C2: Proficient user
Common European Framework of Reference for Languages

Communication skills: Good communication skills acquired through different student jobs and public presentations. Working as a sales promoter and in customer service has helped me acquire experience needed to solve problems both independently and as a part of a team.

Digital skills

SELF-ASSESSMENT				
Information processing	Communication	Content creation	Safety	Problem solving
Proficient user	Proficient user	Proficient user	Proficient user	Proficient user

Digital skills - Self-assessment grid

ADDITIONAL INFORMATION

Memberships: Fifth generation of Start-up incubator Rijeka

Projects: Member of the student start-up project SmartFood

Conferences and congresses

- The first regional conference of digital medicine: The future is now,

Zagreb, Croatia 2016 (passive participant)

- Student congress of neuroscience Rijeka, Rijeka, Croatia 2018 (active participant - oral presentation)

  Abstract title: Chromatography-based isolation of intracranial extracellular vesicles from patients with severe traumatic brain injury

  Authors: Mia Krapić, Mladenka Malenica Staver, Janja Kuharić, Natalija Kučić, Vlatka Sotošek Tokmadžić, Pero Lučin, Kristina Grabušić

- The International Student Congress (ISC) of the Medical University of Graz, Graz, Austria 2018 (active participant - poster presentation)

  Abstract title: Size-exclusion chromatography-based isolation of extracellular vesicles from cerebrospinal fluid of patients with severe traumatic brain injury

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Honours and awards

- First place award in the National student Start-up competition, Zagreb, 2016
- Finished the Undergraduate university program "with great honor" - magna cum laude
- Approved financial resources for the project "Characterisation of EV in patients with TBI" by the Student council of the University of Rijeka, 2017
- Approved financial resources for the active participation at the congress by the Student council of the University of Rijeka and University of Rijeka Foundation, 2018
- Third place award for poster presentation at the International Student Congress (ISC) of the Medical University of Graz, 2018

Courses

American summer school: pathophysiology of public health issues and diseases, Rijeka, 2017