Universidade do Minho
Escola de Medicina

Helena Isabel Alvites Cavaleiro

Combinatory use of Gellan Gum and Mesenchymal Stem Cells to Promote Traumatic Brain Injury Regeneration

outubro de 2023

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Trabalho realizado sob orientação de

Doutor António José Braga Osório Gomes Salgado
e da
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Outubro de 2023

DIREITOS DE AUTOR E CONDIÇÕES DE UTILIZAÇÃO DO TRABALHO POR TERCEIROS

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I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

RESUMO

Estratégia combinatória com hidrogéis de goma gelana e células mesenquimatosas promover regeneração em casos de Traumatismo Cranicoencefálico

O Traumatismo Cranioencefálico (TCE) é caracterizado pela perda da função normal do cérebro devido a um impacto mecânico na cabeça. Esse impacto mecânico pode ser único, ou repetido, mais leve ou mais severo podendo originar diferentes tipos de lesões e sintomas. O TCE grave é uma das principais causas de morte e incapacidade em todo o mundo e uma grande preocupação socioeconômica e de saúde pública. Ao longo dos anos, a abordagem clínica para casos de TCE melhorou, no entanto não há ainda terapia disponível para prevenir ou mitigar a progressão da lesão nem os sintomas crónicos que caracterizam a doença. Há fortes evidências de que a terapia com células estaminais mesenquimatosas (MSCs) atenua défices funcionais e danos anatômicos em modelos animais de TCE. O principal objetivo desta tese foi estudar, in vitro e in vivo, o potencial terapêutico de MSCs humanas colhidas dos tecidos perivasculares do cordão umbilical (HUCPVCs). Em primeiro lugar, desenvolvemos um modelo in vitro de TCE em fatias organotípicas do córtex. Nosso modelo recém-implementado reproduziu as principais características da fisiopatologia do TCE e provou seu valor como uma plataforma para avaliar o papel protetor de MSCs. Especificamente, os fatores secretados pelos HUCPVCs foram capazes de proteger da morte celular e recuperar a regulação negativa de fatores de crescimento neuronal, induzida por trauma. In vivo, usamos uma matriz protetora de goma gelana para transplantar HUCPVCs diretamente na cavidade da lesão. HUCPVCs melhoraram a capacidade sensório-motora e cognitiva dos animais tratados e levaram à redução de marcadores astrocíticos e microgliais no córtex. Além disso, a análise proteômica revelou que HUCPVCs encapsuladas na goma gelana secretaram mais fatores de crescimento neuronal, como BDNF e GDNF, bem como S100B e VEGF-A, reforçando a vantagem em usar esta estratégia. Concluindo, o nosso estudo trouxe contribuições importantes para a investigação de TCE, pois implementamos com sucesso um novo modelo in vitro onde tanto, lesões primárias como secundárias são representadas. Ainda, a nossa proposta de uma abordagem combinatória para tratar o TCE diretamente na zona da lesão, utilizando HUCPVCs em matrizes de goma gelan diretamente no local da lesão, mostrou resultados promissores e permitirá, no futuro projetar novas abordagens clinicas para o tratamento de TCE.

Palavras-chave: TCE, modelos in vitro, Células mesenquimatosas, Neuromodulação, Neuroproteção

ABSTRACT

Combinatory use of gellan gum hydrogels and Mesenchymal Stem Cells to Promote Traumatic Brain Injury Regeneration

Traumatic Brain Injury (TBI) is characterized by the loss of normal brain function due to an external mechanical impact on the head. This mechanical impact may be single or repeated, which may together with trauma severity originate different types of lesions and disease progression. Severe TBI is a leading cause of death and disability worldwide, and a major public health and socioeconomic concern. Throughout the years clinical management of TBI has improved, however, currently, there is no available therapy to prevent or mitigate injury progression and chronic outcome. There is strong evidence that Mesenchymal Stem Cells (MSCs) therapy attenuates functional deficits and anatomical damage in animal models of TBI.

The main goal of this thesis was to address, in vitro and in vivo, the therapeutic potential of human MSCs harvested from the umbilical cord perivascular tissues (HUCPVCs). Firstly, we developed an in vitro model of TBI using organotypic slices from cortex. Our newly implemented model reproduced both first and secondary injury mechanisms and the main hallmarks of TBI pathophysiology. Additionally, our new model proved value as a platform to screen MSCs protection. Specifically, the HUCPVCs secreted factors were able to protect from cell death, and to recover the downregulation of neuronal growth factors, induced by trauma. In vivo, we used a protective scaffold to transplant directly into the lesion cavity the HUCPVCs. HUCPVCs led to sensorimotor and cognitive recovery together with reduction of astrocytic and glial markers at the cortex of treated animals. Moreover, proteomic analysis, revealed that HUCPVCs engrafted inside the gellan gum scaffold, secreted more neuronal growth factors such as BDNF and GDNF, as well as S100B and VEGF-A, reinforcing the advantage of using it. Overall our study made important contributions to TBI research as we successfully implemented a new in vitro model to study TBI where both first and secondary injuries are represented. Additionally, we design a novel combinatorial approach to treat TBI directly at the lesion site using gellan gum scaffolds and HUCPVCs with promising results in supporting disease recovery.

Keywords: TBI, in vitro models, Mesenchymal stem cells, neuromodulation, neuroprotection

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LIST OF ABBREVIATIONS

#

2D – two dimensional

3D – three dimensional

A

ACR-AC - American College of Radiology Appropriateness Criteria

ACTH - Adrenocorticotropic Hormone

AD – Alzheimer's Disease

AEDs – Antiepileptic drugs

ALS – Amyotrophic Lateral Sclerosis

ALS – Amyotrophic lateral sclerosis

APOE - Apolipoprotein E

ASCs – Adipose Stem Cells

B

BBB – Blood Brain Barrier

BDNF – Brain Derived Neurotrophic factor

BM – Bone Marrow

C

CCI – Controlled Cortical Impact

CDC – Centers for Disease Control and Prevention

CHI – Closed Head Injury

CNS – Central Nervous System

CSF – cerebrospinal fluid

CT – Computerized tomography

CTE – Chronic traumatic encephalopathy

D

DAI – Diffuse Axonal Injury

DAMPS - Damage Associated Molecular Patterns

DCs – Dendritic Cells

E

ELISA - Enzyme-Linked Immunosorbent Assay

ER – Emergency room

F

FDA – U.S Food and Drug Administration

FGF – fibroblastic growth Factor

FPI - Fluid Percussion Injury

G

G – gravitational force units

GC – Glasgow Coma

GCS – Glasgow Coma Scale

GFAP – Glial Fibrillary Acidic Protein

GLAST - GLutamate ASpartate Transporte

GLT-1 - Glial Glutamate Transporter -1

GSH-GG - Glutathione Modified Gellan Gum

H

HA – Hyaluronic Acid

HGF – Hepatocyte Growth Factor

HPL – Human Platelet Lysate

HUCPVCs – Human Umbilical Cord Perivascular Cells

I

Iba-1 – Ionized calcium-binding adaptor molecule

ICAM1 - intercellular adhesion molecule 1

ICP – Intracranial Pressure

IL – Interleukin

IPSCs – Induced Pluripotent Stem Cells

L

LA – Linear Acceleration

LDH – Lactate Dehydrogenase

M

MAG – Myelin Associated Glycopeptide

MAS - Modified Ashworth Scale

MBP – Myelin Basic Protein

MiRNAs - MicroRNAs

MMPs – metalloproteinases

MOG - Myelin Oligodendrocytes Glycoprotein

MRI – Magnetic Resonance Imaging

MSCs – Mesenchymal Stem Cells

N

NF – Neurofilament

NF-H – Neurofilament heavy

NF-L – Neurofilament light

NF-M – Neurofilmanet middle

NGF – Nerve Growth Factor

NLRs – NOD –like Receptors

NSCs – Neural Stem Cells

NSE - Neuron specific enolase

NSS – Neurological Severity Score

O

OGD – Oxigen Glucose Deprivation

P

PBBI - Penetrating ballistic-like brain injuries

PD – Parkinson's Disease

pNF-H – phosphrilated Neurofilament heavy

PTA – Post traumatic Amnesia

PTE – Post Traumatic Epilepsy

R

RA – Rotational Acceleration

S

SA – Sodium Alginate

SDF-1 – Stromal Cell Derived Factor -1

SOD1 – Superoxide Dismutase 1

T

TAI – Transmissible Axonal Injury

TBI – Traumatic Brain Injury

TGG – thiolated Gellan Gum

TLRs – Toll-like Receptors

TNF – Tumor Necrosis Factor

U

UC – Umbilical Cord

UCH-L1 - Ubiquitin C-terminal hydrolase-L1

V

VEGF – Vascular Endothelial Growth Factor

W

WD – Weight Drop

WDI – weight Drop Injury

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THESIS AIMS AND OUTLINE

The main aim of this thesis was to develop a therapeutic strategy for Traumatic Brain Injury (TBI), by combining Mesenchymal Stem Cells (MSCs) with a GRGDS-modified gellan gum hydrogels (GG-GRGDS). Due to in vitro models' great importance, and contradictorily, to the lack of reliable TBI in vitro models in the literature, part of this thesis was focused on establishing a new model of TBI, replicating, in vitro, the Controlled Cortical Impact (CCI), widely used to study TBI in rodents. Our model consisted of biomechanical damaging organotypic slices from cortex using an impactor device from Leica. To test the new in vitro model as a platform to screen therapies, we evaluated the secretome of MSCs from perivascular tissues (HUCPVCs) as TBI therapeutic agent. In vitro, HUCPVCs proved promising in reverting cell death and glial activation induced by the mechanical impact on organotypic brain slices. Therefore, these cells were tested in an in vivo model of TBI as proof of concept. Before transplanting into TBI brains, cells were placed inside gellan gum (GG) matrices modified with the fibronectin mimetic – GRGDS (GG-GRGDS), to confer cells-protection and restrain them at the injury site. GG is a porous matrix, allowing the cells inside to release the secreted factors into the extracellular space.

Hereupon, this thesis is divided into four chapters:

Chapter I: includes a general introduction to the main topics addressed in this study. An overview is provided for TBI epidemiological data, as well as the main pathophysiologic components. Moreover, we describe the existing experimental models of TBI, as well as the current therapeutic approaches and the ones being developed or explored. Additionally, the main therapeutic strategies using MSCS are mentioned, with special emphasis being given to combinatorial approaches of MSCs and hydrogel matrices.

Portions of sections 1, 2, and 4 were adapted from the author's previous publications: Luís Rocha, Helena Cavaleiro et al. Advanced Functional Materials 2020.Francesca Pischiutta, Helena Cavaleiro et al. Regenerative Medicine 2021; Francesca Pischiutta, Helena Cavaleiro et al Experimental Neurology 2022; Luís A. Rocha

Chapter II: describes the implementation and characterization of a new in vitro model to study TBI.

This chapter shows that CCI in vitro replicates basic features of TBI in vivo, and deeply affects neurons and glia. This new model will be fundamental to carrying out a rapid screening of therapeutic candidates,

allowing better selection before in vivo testing. Additionally, in the present work, the successful optimization of this model allowed to test and evaluate the therapeutic effect of HUCPVCs in reverting CCI in vitro induced damage.

This chapter is based on the publication: Cavaleiro and Pischiutta et al, Frontiers in Cell. Neurosc. 2023

Chapter III: presents one of the main objectives of the thesis, which is to evaluate the therapeutic potential of MSCs combined with GG-GRGDS hydrogel. Following TBI at the left parietotemporal cortex, mice were followed for 13 weeks. Animals treated with HUCPVCs and hydrogel, significantly improved locomotor and cognitive functions. 13 weeks post trauma HUCPVCs treatment reduced microglial activation and it was observed a tendency towards an increased number of NeuN positive cells and decreased astrocytic activation

Manuscript in preparation: Cavaleiro and Pischiutta, et al

Chapter IV: is a general discussion of the results reported during this thesis, as well as future experiments that would reinforce the findings reported in this document.

CHAPTER I

Introduction

CHAPTER I. Introduction

This chapter is partially based on the following publications:

- Luís A. Rocha, Deolinda Silva, Sandra Barata-Antunes, **Helena Cavaleiro**, Eduardo D. Gomes, Nuno A. Silva, and António J. Salgado. (2020). Cell and Tissue Instructive Materials for Central Nervous System Repair. *Advanced Functional Materials.* https://doi.org/10.1002/adfm.201909083.

- Francesca Pischiutta, Enrico Caruso, Alessandra Lugo, **Helena Cavaleiro**, Nino Stocchetti, Giuseppe Citerio, António Salgado, Silvano Gallus and Elisa R. Zanier. (2021). Systematic review and meta-analysis of preclinical studies testing mesenchymal stromal cells for traumatic brain injury. *Regenerative Medicine*. https://doi.org/10.1038/s41536-021-00182-8.

- Francesca Pischiutta, Enrico Caruso, **Helena Cavaleiro**, António Salgado, David J.Loane and Elisa R. Zanier. (2022). Mesenchymal stromal cell secretome for traumatic brain injury: Focus on immunomodulatory action. E*xperimental Neurology*. https://doi.org/10.1016/j.expneurol.2022.114199

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1. Traumatic Brain injury

1.1. Causes, demographics and economic burden

The most common causes of TBI of any severity are falls and road traffic accidents, which together account for >50% of all cases (Peeters et al. 2015; Roozenbeek, Maas, e Menon 2013). The remaining cases of TBI are due to assault, sport-related injuries, violence, and blast injuries. Blast injuries are specifically related to military services and are characterized as the less prevalent cause of TBI (Wilk et al. 2010) (**Figure 1**). Specifically, with the aging population of high-income countries, the prevalence of elderly with TBI is increasing, mainly due to falls in a domestic environment. In developing countries, traffic-incident-related injuries mainly contribute to an increase of TBI prevalence (GBD 2019 Ageing Collaborators 2022). TBI may be classified regarding injury severity, from mild to severe. A person with a mild TBI or concussion may experience short-term symptoms and recover safely at home, following a medical check-up, within a couple of weeks or months. On the other hand, someone with a moderate or severe TBI may experience long-term effects from the injury, requiring ongoing and specialized care (Bramlett e Dietrich 2015).

Estimates from 2016, indicate that 55 (fifty-five) millions of individuals with TBI were living with serious disabilities, and that TBI related disability is increasing by 8,5% comparing records from 1990 to 2016 (GBD 2016 Traumatic Brain Injury and Spinal Cord Injury Collaborators 2019). Globally, approximately 69 million people suffer TBI per year. From those, about 13 million are moderate to severe cases (Dewan et al. 2018). The latest findings from the global burden of diseases indicate higher TBI incidence rates across Central Europe, Eastern Europe, and Central Asia, comparing to other global regions. A closer look reveals Syria emerged with alarmingly high rates with around 1322 cases per every 100 000 people, between 1990 and 2016 while Slovenia (1092 per 100 000) and Czech Republic (1022 per 100 000) had the next highest incidence rates (GBD 2019 Ageing Collaborators 2022).

Although TBI has been extensively reported in different populations, it is important to have in mind that the real incidence and impact are difficult to determine with a high level of accuracy, mainly due to TBI heterogeneity, regarding causes, severities, and outcome. Additionally, not all individuals seek much needed medical assistance following a TBI (National Academies of Sciences et al. 2022). Moreover, individuals' likelihood of seeking medical attention could be affected by the proximity and accessibility of healthcare resources. This is evident as regions with inadequate medical services report significantly fewer cases of TBI («Global Civil Registration and Vital Statistics

Scaling Up Investment Plan 2015-2024» s.d.). Adding to its high prevalence, TBI is a major cause of death with more than 56,000 patients TBI-related death in the United States (Taylor et al. 2017).

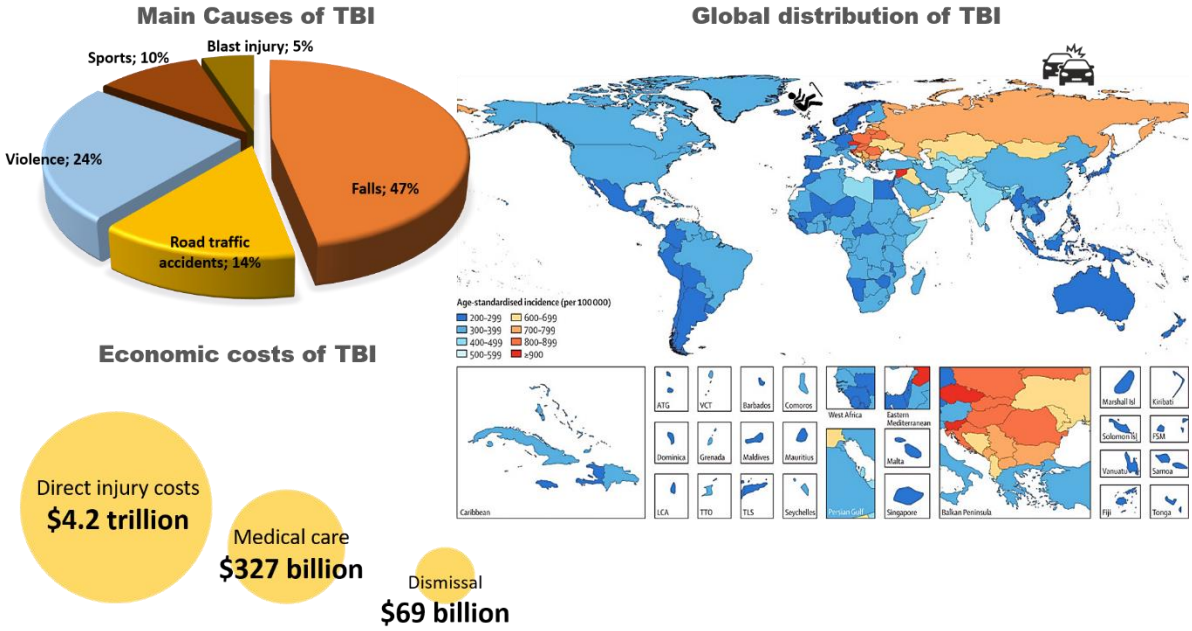


Figure 1. Causes, global distribution and economic costs TBI-related. TBI is a major cause of death and disability world-wide. TBI causes are accidents that can happen during our daily tasks, and are highly heterogeneous. The most common case of TBI are falls, followed by violence and road traffic accidents. Sports and blast injuries, both affect specific niches of the society, such us, close-contact sports and military respectively. TBI injuries come along with serious disabilities, that bring a strong economic impact to the patient, the health care system and society. The map above, show age-standardized incidence of TBI per 100 000 population by location for both sexes for 2016. Central Europe, eastern Europe, and Central Asia, showed higher incidence rates of TBI comparing with other world regions. Syria had the highest incidence rate of any country, followed by Slovenia, and the Czech Republic. *Figure adapted from GBD 2016 (GBD 2016 Traumatic Brain Injury and Spinal Cord Injury Collaborators 2019).*

The economic costs associated with injury, in general, are high, including injury direct costs, medical care, economic difficulties due to work loss, and costs related with reduced quality-of-life (**BOX 1**). In detail, in 2019, CDC estimated that the values spent in case of injury were divided in the following manner: $4.2 trillion spent directly into injury-related costs, $327 billion in medical care, $69 billion due to job capacities loss and, consequently, work loss (Peterson et al. 2021). Additionally, there is a strong correlation between TBI severity and economic waste, with the severest cases showing a high economic burden (Miller, DePadilla, e Xu 2021). In what regards to Portugal, a study performed in 2010 revealed that a total 77 549 people had a TBI per year at that

time and that the mean costs normalized on the total prevalence of the country were 6 519 € per person (Gustavsson et al. 2011).

BOX 1 I Economic costs associated with brain injury
Doctors and hospitals
Nursing home bills (in case of rehabilitation)
Lost wages and salaries
Speech and occupational therapist bills
Adaptive equipment, including crutches, wheelchairs or other devices
Costs of home modifications for handicap accessibility
Prescription drug costs
Costs of home modifications for handicap accessibility

1.2. Diagnosis

In the case of TBI the medical procedure to follow in the emergency room (ER) will depend on trauma severity, on the symptoms exhibited by the patient, and on the hospital's protocols. The regular protocol at the ER usually involves: an initial assessment, in attempt to stabilize the individual's vital signs, ensuring mostly a normal airway function, adequate breathing and blood flow. Any life-threatening conditions or injuries will be addressed immediately. The healthcare providers will conduct a detailed physical examination, including neurological assessment, to evaluate the extent and severity of the TBI. An important aspect to monitor in a TBI patient is the intracranial pressure (ICP) inside the skull and the brain (Bernard 2023). Raised ICP reduces cerebral perfusion risking ischemia and, when severe and sustained, brain herniation. To reduce ICP and other conditions such as innate inflammatory response, as well as to reduce the cerebral metabolic rate, hypothermia or decompressed cranectomy are the most used clinical procedures. Additionally, surgery or medication may be administered to manage pain, reduce brain swelling and remove blood clots, control seizures or to prevent general infection (Rakhit et al. 2021).

Described below are explored the major diagnosis techniques used in the ER and later on the disease progression, to access TBI severity and evolution through the time.

1.2.1 Diagnosis techniques

Worldwide, TBI severity is clinically classified using the Glasgow Coma (GC) Scale. A drawback of GC Scale is that it is often biased by prehospital sedation and tracheal intubation. Therefore, it is a crude tool which may not adequately capture the gravity of TBI. Additionally, to the GC Scale, TBI characterization and diagnosis are comprised of different techniques that have evolved and improved through time in order to increase diagnosis accuracy. The main approaches and technologies used nowadays are (1) biomechanical studies quantifying direct or indirect forces to the brain, (2) the use of advanced neuroimaging for the identification of underlying functional disturbances and structural injury, and (3) employment of blood/Cerebral Spinal Fluid (CSF) biomarkers.

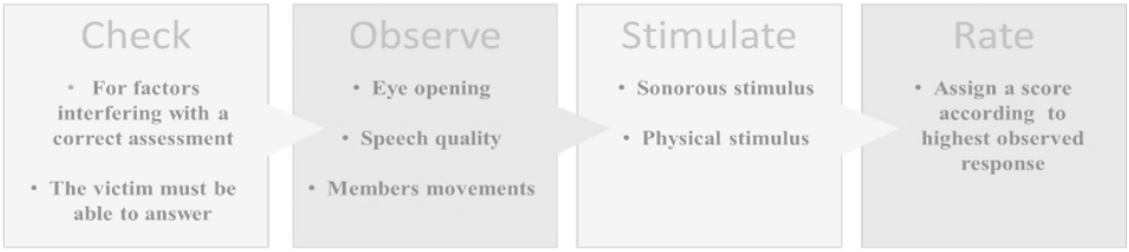
1.2.1.1. Glasgow Coma Scale

Since the GC Scale was introduced in 1974 (Teasdale e Jennett 1974) it has become the most common method to describe a patient's level of consciousness globally. Prior to this, ill-defined terms such as comatose, obtunded, and decerebrate posturing were used to compare different series of head injuries. Such classification was hard and made individual patient reports often inaccurate.

The GC Scale (**Figure 2**) provided a simple measure of conscious level, showing low inter-observer variability amongst the clinical staff evaluating the patient (Braakman et al. 1978). The three aspects of consciousness evaluated are arousal, awareness, and activity, and the GC Scale uses an explanation in words to describe these components as Eye Opening, Verbal Response, and Motor Response, respectively. The GC Score is the numerical equivalent of the GC Scale. Broadly, a GC Score of 13–15 defines mild TBI, the lowest level of severity. The patient is fine and replying to all the tested stimuli. GC Score of 9–12 is given to moderate TBI and, broadly, classifies a patient with a decreased level of consciousness. GC Score of 3–8 means that the patient is in coma and has low or none responsiveness (Barlow 2012) There is an approximately linear relationship between decreased sum scores and increasing mortality in patients with TBI (Reith et al. 2016). While it is true that the GC scale and GC score have been lauded for their ease of use, it is also crucial to recognize that there are scenarios where they may not effectively depict an individual patient situation in a clinical context. For instance:

1. The patient health state frequently compromises the measurement of some individual components specified on the scale. For example, if the eyes are closed due to severe swelling the assessment of eye opening will be compromised. Also, if the patient has severe lower facial injuries, or is intubated, the verbal response cannot be assessed.
2. Many clinicians will admit to doubt which value to attribute to each patient and to guess or estimate it when using the GC score, trying to include the patient within the range. This is a dubious clinical practice and one reason why some studies suggest poor inter-observer variability (Reith et al. 2016; Marion e Carlier 1994).
3. In some cases, the use of the GC scale, per se, would be preferable, since the patient condition would clearly be described using words instead of numbers. A GC score may lead to loss of crucial information, since the same numerical classification may represent different clinical situations. For instance, if someone is assigned a "GC Score 10", this might mean that the patient obeys commands, opens eyes to speech and has no verbal response; or it might mean that the patient may demonstrate localized reactions towards physical discomfort or pain, with reflexive eye opening and improper speech.
4. GC Score has limitations regarding long-term prognostic. This is because multiple pathologies with different clinical outcomes may lead to a low score as a first evaluation, and progress into serious cases (Jennett et al. 1976; Sternbach 2000).

GLASGOW COMA SCALE



Eye-opening

Criterion	Rating	Score
Open before stimulus	Spontaneous	4
After spoken or shouted request	To sound	3
After finger tip stimulus	To pressure	2
No opening at any time, no interfering factor	None	1
Closed by local factor	Non testable	NT

Verbal response

Criterion	Rating	Score
Correctly gives name, place and date	Oriented	5
Not oriented but coherent communication	Confused	4
Intelligible single words	Words	3
Only moan/groans	None	1
Factor interfering with communication	Non testable	NT

Best motor response

Criterion	Rating	Score
Obey 2-part request	Obeys command	6
Brings hand above clavicle to stimulus on head neck	Localizing	5
Bends arm at elbow rapidly but features not predominantly abnormal	Normal flexion	4
Bends arm at elbow, features clearly predominantly abnormal	Abnormal flexion	3
Extends arm at elbow	Extension	2
No movement in arms/legs, no interfering factor	None	1
Paralyzed or other limiting factor	Non testable	NT

Figure 2. General procedure to evaluate a TBI patient using Glasgow Coma Scale. The main criteria, tests and attributed scores to evaluate a TBI at ER are depicted. The table is adapted from: Glasgow Coma Scale

1.2.1.2. Biomechanical studies

Kinematics studies the movements of systems and objects while excluding the underlying forces that drive their motion. Current research is seeking to identify the kinematics signature of concussion. Traditional force measurements include linear acceleration (LA), measured as gravitational force units (g), and rotational acceleration (RA) measured in units of radians per second squared (rad/s^2) (Rowson et al. 2016).

Revolutionary monitoring equipment, using an array of accelerometers connected to the athlete helmet can now measure, in vivo, the forces involved in sport-related TBI (Duma et al. 2005). While helmeted devices have always been common in football they are becoming increasingly prevalent in other sports like boxing, snow sports (like skiing, snowboarding or ice hockey), and soccer (O'Connor et al. 2017). Despite their increasing popularity, these systems remain costly and inaccurate due to poor coupling with the head. Helmet-detected acceleration can be up to ten times greater than real head acceleration leading to overestimated values of the forces involved in TBI (Rowson et al. 2012; Manoogian et al. 2006).

The aforementioned limitations suggest a dire need for alternative devices with better skull coupling. In light of this concern, popular non-helmeted sensors include mouth guard sensors, base-of-skull or ear canal sensors (L. C. Wu et al. 2016). The most commonly used research systems are the X-Patch, an adhesive patch worn behind the ear, and the X-Guard, a device embedded in a custom mouthguard. Both of these systems use a triaxial accelerometer that track head acceleration forces (A. S. McIntosh et al. 2019; Dsouza et al. 2022; «X2 Biosystems Introduces Their Next Generation X-Patch Pro Head Impact Monitor» 2016). However, background noise associated with skin contact or mouse motion significantly interferes with these sensing mechanisms.

Importantly, none of the devices mentioned previously have undergone peer review, with their scientific validity being questionable (O'Connor et al. 2017; Dsouza et al. 2022). An additional approach to TBI surveillance includes quantitative and qualitative video analyses. This analysis monitors the number of head impacts, injury mechanisms, and clinical signs of TBI. In summary,

these are relatively new technologies and their clinical utility requires further investigation to support their validity.

1.2.1.3. Neuroimaging

As described above the GC Score alone is insufficient to have an accurate prediction of TBI outcome. Gladly, neuroimaging techniques are available and play a critical role evaluating the extent of the primary injuries, allowing triaging of patients and determining if surgery will be needed (L. G. F. Smith et al. 2019). The imaging method is chosen based on the victim symptoms and physical examination by a doctor. Nevertheless, due to the disease's high variability it can be challenging to have a definitive consensus on which technique to follow. Current neuroimaging recommendations for patients with TBI are listed in the American College of Radiology Appropriateness Criteria (ACR-AC), which are evidence-based guidelines to assist physicians and other providers in selecting the most appropriate imaging technique or treatment for a specific clinical condition (Subramaniam et al. 2019). These guidelines are reviewed annually by experts to enhance quality of care and contribute to the most efficacious use of radiology. In general, non-contrast computerized tomography (CT) is the most broadly accepted investigation to detect potential surgical brain injuries in people with suspected moderate to severe TBI. It is a diagnosis technique widely available in hospitals and health centers that shows a rapid acquisition time (L. G. F. Smith et al. 2019). CT creates a two-dimensional image of the area aimed to analyze, demonstrating a great capacity to evaluate skull fractures and to detect subdural or epidural hematomas. Still, it lacks sensitivity for detecting deep cerebral lesions, underestimating signs of parenchymal contusions, detection of diffuse axonal injury (DAI), and showing limitations in detecting signs of intracranial hypertension (Smith et al. 2019). Magnetic resonance imaging (MRI) has a significantly higher sensitivity compared to CT (96,4% vs 63,4% respectively) (YUH et al. 2013; Mittl et al. 1994; Orrison et al. 1994). It may be used after the initial assessment and treatment as it detects subtle changes in the brain that the CT scan might miss. Despite advantageous, due to the exposure to high frequencies MRI is only used in well justified cases. Besides that, other limitations associated with MRI are the reduced availability of the equipment, longer imaging times, sensitivity to patient motion, and incompatibility with certain medical and life-support devices (e.g, pacemakers, nerve stimulators, ferromagnetic intracranial aneurysm clips, most routinely used ventilators).

Mild TBI changes are the most difficult to detect using imaging techniques since the brain-induced changes are highly subtle. Luckily, significant advances have been made in the last decade to image milder TBI, and advanced imaging techniques, such as diffusion tensor imaging, magnetic resonance spectroscopy, perfusion weighted imaging, or functional MRI, can reveal abnormalities that are not detected using conventional imaging methods. For example, diffusion tensor imaging can image white matter tracts; more sensitive tests like fluid-attenuated inversion recovery can detect small areas of damage; and susceptibility-weighted imaging is sensitive to identify bleeding. One must be mindful that negative results on imaging studies, including advanced imaging techniques, may not accurately predict disease progression. Even though some patients will display normal findings on CT and MRI, for instance, they may present highly variable neurological symptoms. In these cases, the patient should be followed with caution in order to better track new symptoms and clinical behavior alterations.

1.2.1.4. Blood and Cerebral Spinal Fluid Biomarkers

Predicting prognosis of TBI patients in early stages has crucial importance. However, monitoring the degree of primary brain injury and continuing secondary damage is still not fully achieved with GC Score or the above-mentioned imaging techniques.

The mechanical impact on the brain damages resident cells and impacts the structural cellular proteins that are released into the surrounding bio-fluids. Additionally, the first mechanical impact activates intracellular calpains and caspases, which contribute to myelin breakdown. It is widely recognized that brain cellular proteins released into cerebral spinal fluid (CSF), plasma and blood after a TBI correlate with brain damage – these fluid-detected proteins are called biomarkers. Measure of bio-fluids biomarkers is a practical screening tool, which allows prognosis at early stages, identifying patients at high risk and allowing a better redirection of therapeutic and rehabilitation strategies (McDonald, O'Brien, e Shultz 2022). Importantly, TBI is not a single acute event but a progressive neurodegenerative process made of multiple interconnected cascades and cells. Biomarkers allow to track these mechanisms over time and thus to follow the ongoing neurodegenerative process. Biomarkers mirror several different mechanisms, such as axonal injury, dendritic injury, neuronal cell body injury, demyelination, and synaptic injury (**Figure 3**).

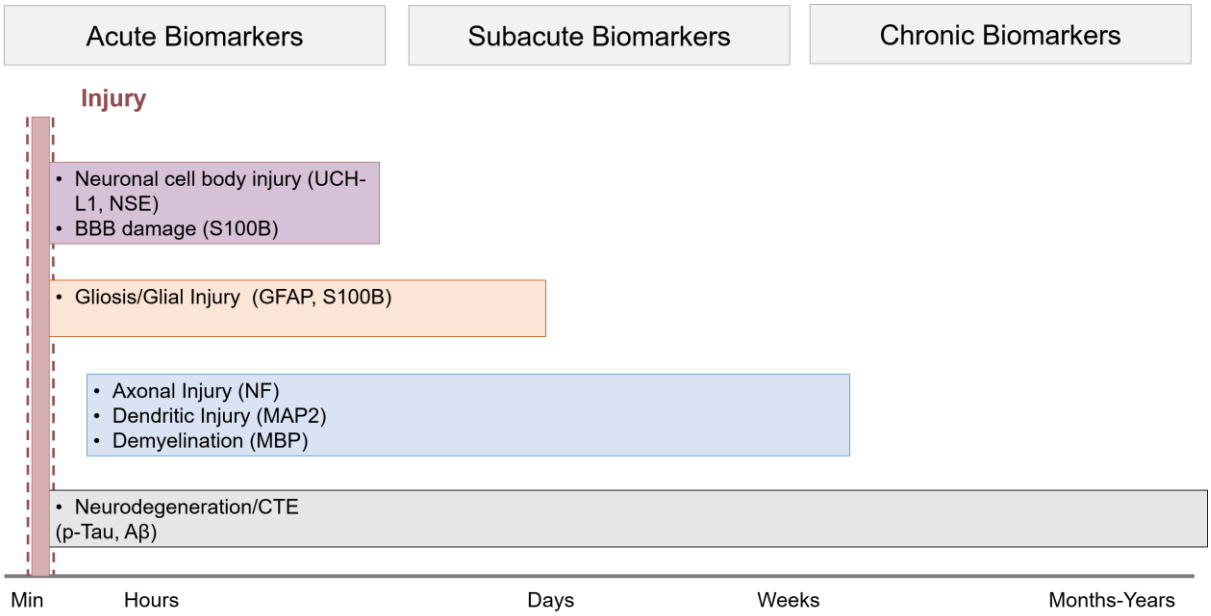


Figure 3. Bood and Cerebral Spinal Fluid Biomarkers evaluated throughout the course of TBI progress. Cell death and degeneration during TBI course is a continuous process. Biomarkers are an important new technique which allows to classify injury severity, follow disease progression and adapt medical procedures, depending on the patient need. UCHL-1, NSE and S100B are acute biomarkers released to the external bio fluids between minutes to hours after TBI. GFAP, is released in subacute faze, and indicates astrocytic death and severe injuries. NF, MAP2, MBP, p-Tau, and Aβ, are indicators of strong neuronal death and demyelination, being chronic biomarkers of TBI. Those are released in chronic time points, in severe cases of neuronal degeneration and cognitive deficits.

Several studies have shown elevated biomarker levels in the blood or CSF after experimental TBI (Manivannan et al. 2018). Additionally, biomarkers can be accessed in animal models, which opens the possibility of better understanding the mechanisms involved and allows the development of new diagnostic and therapeutic approaches.

According to Wang et al. (K. K. Wang et al. 2018, 2), the ideal and reliable fluid biomarker of TBI should meet the following criteria:

1) be readily measured in accessible biofluids and allow multiple measurements over time.

2) be available in the acute phase after trauma and show significantly higher values in TBI patients than in healthy people.

3) correlate with injury severity and associate with other clinical diagnostic approaches like GCS (Guzel et al. 2008), CT, and MRI.

4) ideally, a fluid biomarker should be translational in nature in at least two animal models of TBI currently being used in basic research.

Described below are the most used and emergent biomarkers to track TBI evolution.

Neuronal cell body injury markers

Neuron specific enolase (NSE) and Ubiquitin C-terminal hydrolase-L1 (UCH-L1) are currently used biomarkers to evaluate acute neuronal cell body injury. NSE is an enzyme found almost exclusively in the cytoplasm of neurons but also in neuroendocrine cells (Guzel et al. 2008). NSE is also abundantly expressed in red blood cells, which impelled researchers to use a hemolysis correction when quantifying it in blood samples (Verfaillie e Delanghe 2010). UCH-L1 is a protein that mainly resides in the neuronal cell body cytoplasm, authorized in 2018 by the FDA to be measured in a blood test for mild traumatic brain injury (Commissioner 2020). Both NSE (Stein et al. 2011; Haque et al. 2016) and UCH-L1 (Papa et al. 2010; Brophy et al. 2011) elevations in blood have been documented in severe and mild TBI, including non-penetrating injuries.

Axonal injury and demyelination markers

Neurofilament (NF) proteins are found exclusively in neurons, and their main function is to provide structural support for the axon and to regulate axon diameter. NF are axonal cytoskeletal proteins composed of an assembly of three chains (Neurofilament light – NF-L; Neurofilament middle - NF-M; Neurofilament heavy – NF-H). NF chains may be released into surrounding fluids due to transmissible axonal injury (TAI), serving as delayed axonal injury biomarkers (K. K. Wang et al. 2018). Phosphorylation of NF-H is specific to axons, forming the basis of interest in the use of phosphorylated NF-H (pNF-H) as a blood biomarker of axonal injury (Anderson et al. 2008). NF protein release into biofluids is a delayed process with respect to the initial insult (days following insults). Thus, it could reflect ongoing axonal degeneration and might be linked to cognitive decline and development in chronic TBI subjects. All the NF chains were already corroborated as biomarkers in both human and animal models. For instance, pNF-H correlated to mechanical impact responses, being elevated in serum following a weight drop closed head injury model (M. Liu et al. 2015). Additionally, CSF levels of pNF-H were as well high following bouts among amateur boxers (Neselius et al. 2013), and after TBI in children, compared to non-injured individuals (Zurek, Bartlová, e Fedora 2011; Žurek e Fedora 2012). Martínez-Morillo and colleagues showed that NF-M protein concentrations were increased in CSF and serum samples from patients with severe TBI (Shahim et al. 2020). In addition, NF-L serum levels appear elevated in American football players over the course of a season (Oliver et al. 2019).

Besides NFL two other promising candidates as TBI biomarkers are the Microtubule associated protein (MAP-2) (Mondello et al. 2012)·(Gottschalk e Hefti 2022) and the Brain Derived

Neurotrophic Factor (BDNF) (Korley et al. 2016). For instance, early measurements show increased levels of MAP-2 at ventricular CSF, and these results enhanced prognostic predictions of mortality at 6 months (Mondello et al. 2012; Svingos et al. 2022). Regarding BDNF, when measured at the injury day, it provides a 6-month prognostic information and accurately predicts TBI recovery (D. Simon et al. 2016). Interestingly, it was found that BDNF has a higher prognostic value among mTBI subjects than moderate/severe TBI subjects. Nevertheless, further evaluation is needed to determine how useful MAP-2 and BDNF are as TBI biomarkers.

Myelin basic protein (MBP) is a subacute injury demyelination marker. White matter axons are surrounded by a myelin sheath produced by oligodendrocytes, in the CNS and Schwann cells peripherally. MBP is the main structural component of the multi-layered myelin sheath covering nerve fibers. MBP guarantees the correct structure of myelin (Saab e Nave 2017) and when degraded leads to axon deterioration together with the surrounding myelin sheath (demyelination)(Ottens et al. 2008; M. C. Liu et al. 2006). MBP release into the surrounding biofluids after TBI was observed in both humans (Sarkis et al. 2022, 202; Su et al. 2012) and animal models (M. Liu et al. 2015). Despite the previous observations, attempts at utilizing MBP as a blood-based biomarker to measure injury severity and outcome have generated variable outcomes. Peripheral nerve myelin expressing this protein limits the biomarker specificity in diagnosing or predicting outcomes in severe TBI cases damaging such nerves. Further investigations into revealing transcripts in bone marrow and immune system surrounding this protein highlights additional complexities (Zelenika, Grima, e Pessac 1993).

Astroglial biomarkers

Astrocytes represent 30-40% of CNS cells, being essential for the brain blood barrier (BBB) maintenance as well as normal synaptic function (Sofroniew e Vinters 2010). Glial fibrillary acidic protein (GFAP) is the main intermediate filament, and its role is to guaranty astrocytes integrity and resilience (Eng et al. 1971). After TBI, astrocytes undergo physical damage and GFAP is released into the CSF and blood. GFAP is classified as an acute TBI marker, as its levels increase as soon as 1h post injury (Papa et al. 2016) comparing to healthy patients. To add, GFAP levels remain elevated until 72h post injury, expressing an elevation peak at 20h post injury (Papa et al. 2016). Moreover, studies have shown a strong correlation between variations in GFAP levels and both CT pathological changes and patient outcomes. The consensus among various studies strongly

indicate that considering all measured biomarkers for distinguishing between abnormal and normal head CT scans resulting from TBI, GFAP is the most effective (Papa et al. 2014).

S100B is a calcium-binding protein mainly produced by astrocytes(Gerlach et al. 2006). Also, S100B protein is not brain specific and is expressed in melanocytes, chondrocytes, adipocytes and skeletal muscle (Chargé e Rudnicki 2004; Sorci et al. 2013). Thus, extracranial contributions like bone fractures may influence S100B measurements and the interpretation of the results in a clinical context (Müller et al. 2020). S100B is one of the most powerful acute biomarkers to predict patient's outcome. Levels of S100B within 12-36h post TBI in neurointensive care unit strongly correlate with GCS at 6 months, and serum levels superior to 0.7 ng/ml correlate 100% with mortality (Kellermann et al. 2016). Overall, S100B has high potential as a diagnostic biomarker, although there's certain limitations that need to be taken into consideration, as for example the lack of specificity and sensitivity. Thus, it may be necessary to use S100B in combination with other markers, diagnostic tools or clinical approaches. The markers S100B, GFAP, and UCH-L1 can be used in combination to distinguish between favorable and unfavorable outcomes and survival and death in patients with severe TBI (Lee et al. 2015). Multiple studies have confirmed the strong predictive value of these markers for unfavorable outcomes in severe TBI patients (Thelin, Nelson, e Bellander 2017; Vos et al. 2010).

Non-protein emerging biomarkers

Besides the protein biomarkers mentioned previously, emerging non-protein biomarkers are pointed out as useful trackers for TBI evolution. Studies have shown dysregulation after TBI of these non-protein markers and their utility in diagnosis and prognosis, as well as their potential to determine mechanistic insights.

MicroRNAs (miRNAs) are small non-coding RNAs that play a critical role in gene expression regulation. Recent research uncovered a promising set of miRNAs in the context of TBI, including miR-151–5p, miR-195, miR-20a, miR328, miR-362–3p, miR30d, miR-451, miR-486, miR-505 and miR-92a. Those were detected in acute CSF and/or serum samples and allowed to differentiate varying degrees of TBI ranging from mild to severe (Bhomia et al. 2016). Furthermore, the same group of molecules also showed the ability to distinguish between mild TBI patients versus healthy volunteers or orthopedic injury controls. Additionally, findings revealed that two specific microRNAs (mir142–3p and mir423–3p) could reliably indicate if mild TBI patients were at risk for post-concussion syndrome (Mitra et al. 2017).

Exosomes and Microvesicles are also pointed as TBI biomarkers. These small particles are encapsulated in a lipid bilayer and released, in both health and injury situations, into the surrounding biofluids. When released under disease conditions, these microscopic bodies contain specific miRNA or protein biomarkers that could potentially serve as diagnostic tools (Moyron et al. 2017). For instance, research displayed elevated levels for several protein biomarkers, such as synaptophysin, UCH-L1 and GFAP, within exosomes or microvesicles in CSF from TBI patients (Manek et al. 2018). Circulating exosomes containing Tau has great potential to diagnose chronic TBI and the potential to develop chronic traumatic encephalopathy (CTE) (Stern et al. 2016). Furthermore, circulating nucleic acids are a field of study as potential biomarkers. Specifically, total plasma-cell free DNA is pointed as a mortality rate predictor among individuals with severe TBI (Rodrigues Filho et al. 2014; Macher et al. 2012; Hazeldine et al. 2021). Despite the previous, limitations arise when considering the fact that this form of testing does not specifically target brain related elements alone. As such, its effectiveness as a definite diagnostic tool for identifying TBIs remains uncertain.

Techniques for TBI Biomarker Measurement

When it comes to detecting biomarkers linked to TBI, the most widely used techniques in different human fluids include Enzyme-Linked Immunosorbent Assay (ELISA) – both regular and multiplex ELISA. Additionally, other techniques, such as mass spectrometry (MS) are pointed as a valuable alternative. The approach is selected based on parameters such as the particular biomarker to be studied, sample composition, and sensitivity level required.

In regular ELISA, the biomarker of interest (antigen) reacts with two types of antibodies: the capturing antibody and the detection antibody. The detection antibody produces a color change that indicates the presence of an antigen. Comparison of the generated color with a calibration curve enables quantifying the analyte of interest. In comparison, multiplex ELISA has the advantage to react simultaneously with more than one antigen in a single sample. Usually, the measurement is performed with markers that reflect distinct and unique axes of TBI pathology. CNS disorders research has long relied on TBI biomarkers related panels, which provide complementary and more comprehensive data, while improving the patient characterization and risk stratification with TBI cases. Nevertheless, it can be difficult to optimize the analysis conditions for several antigens–antibody complexes in multiplexing, especially if their concentration or biochemical characteristics are substantially different.

MS assesses proteins in an antibody-independent manner. It separates ionized particles such as atoms, molecules, and clusters by using differences in the ratios of their charges to their respective masses (mass/charge; m/z) and can be used to determine the molecular weight of the particles(Y. J. Kim, Lee, e Hur 2022). MS measures are highly sensitive, making it an ideal diagnostic and prognostic tool. The majority of investigators using MS techniques for biomarker discovery have applied them to experimental models of TBI, most often in rodents (Roux et al. 2016; Zheng et al. 2017). However, some investigators have applied mass spectroscopy-focused approaches for biomarker discovery in patients with TBI (Creech et al. 2022; Shultz et al. 2022). These human studies have not been restricted only to blood — tissue and urine have also been informative sources of the post-TBI proteome, highlighting the use of MS as a tool in TBI.

1.3. Acute and chronic Symptoms and pharmacological treatments

As mentioned before, TBI may be classified regarding the forces involved in the accident and the injury severity. Symptoms may be different based on the injury extent and the brain area affected, and lead to short- or long-term health complications (Eme 2017). For instance, mild TBI may only result in a brief loss of consciousness or confusion, while moderate to severe TBI may lead to an extended loss of consciousness, coma, or even death. Importantly, when mild TBI happens repetitively it may lead to serious complications overtime that, in some cases, are comparable or surpass those induced by severe TBI (Fehily e Fitzgerald 2017). Additionally, specific differences regarding the victim's age (Moro et al. 2022; Krukowski et al. 2018; Islam et al. 2021), individual genome, and medical and social history will influence recovery and outcome after trauma.

For instance, TBI host genomics may modulate the diverse mechanisms involved in injury response, affecting repair and neurodegeneration. Recent works show the relevance of specific genes that influence the victim's response and recovery after trauma (Kurowski et al. 2017; Kals et al. 2022). Apolipoprotein E (APOE) gene is the most extensively studied in the TBI context. APOE is involved in the brain lipid metabolism and plays a crucial role in neuronal repair and inflammation (Parhizkar e Holtzman 2022; Y. Wu et al. 2019). Variations in this gene have been associated with an increased risk of developing CTE after repeated head injuries (Atherton et al. 2022) and with serum tau levels following mild TBI (Lippa et al. 2022). Additionally, there is a diverse pool of other genetic polymorphisms within neuroinflammatory mediators associated with TBI. Some examples

are tumor necrosis factor α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), transforming growth factor-β (TGF-β), microtubule-associated protein tau (MAPT), angiotensin converting enzyme (ACE) and brain-derived growth factor (BDNF) gene polymorphisms (Kals et al. 2022).

TBI symptoms are vast and may be classified into somatic, cognitive, or affective symptoms **(BOX 2)**. Additionally, they may be distinguished from acute or chronic. Briefly, immediately after trauma the somatic symptoms most often reported include headache, dizziness/nausea, spasticity, fatigue or lethargy, loss of balance, altered vision, and sleep pattern alterations (Renga 2021). At a short term, headache is the most frequently reported somatic symptom, and may be found in both acute or chronic time points, depending on the resolution time. Dizziness is another common symptom that generally resolves within 2 months but may continue in patients with moderate or severe TBI. Cognitive dysfunction includes confusion, disorientation, distractibility, mental slowness, impaired memory (post-traumatic amnesia), and post-traumatic seizures (Pavlovic et al. 2019). Affective symptoms following TBI comprise personality changes, depression, anxiety, impulsivity, irritability, emotional lability, and apathy. Studies show that TBI victims are at higher risk of suicide and suicidal attempts compared with others (Howlett, Nelson, e Stein 2022). Altogether and through disease progression, the first initial injury may evolve and give rise to serious chronic complications. Below are exposed the most common TBI associated disabilities.

BOX 2 I Clinical outcomes post TBI

Cognitive

Memory deficits

Attention and concentration difficulty

Difficulties with speech

Executive dysfunction

Fine motor difficulties

Psychologic

Depression

Anxiety

Irritability and personality changes

Fatigue

Somatosensory and vestibulocochlear dysfunction

Headaches

Nausea and vomiting

Light and sound sensitivity

Hyperalgesia

Tinnitus

Visual symptoms and oculomotor dysfunction

Light sensitivity

Blurry vision

Convergence difficulty

Double vision

1.3.1. Post-Traumatic Amnesia

Seventy percent of TBI hospitalized patients experience post-traumatic amnesia (PTA). PTA is a transient period of time following TBI during which the individual experiences loss of memory of events that occurred immediately before and after the injury. During PTA, the victim may be disoriented, confused, and have trouble processing new information (T. D. Parker et al. 2022). The length of PTA correlates with TBI severity, and it can last from minutes to days, weeks, or even months. Specifically, PTA duration has been associated with skull fractures, intracranial hemorrhage, and increased intracranial pressure, which lead to neurological impairments, associated with longer-term functional deficits (Ponsford, Spitz, e McKenzie 2016).

1.3.2. Spasticity

Spasticity is one of the most significant challenges of moderate/severe TBI. Mechanistically it occurs due to damage of upper motor neurons leading to a loss or disruption of descending inhibitory signals resulting in altered activity of the stretch reflex (Bose, Hou, e Thompson 2015). Spasticity is a motor disorder characterized by increased muscle tone and involuntary muscle contractions (Lance 1980). It is often described as muscle overactivity and may occur sporadically or continuously. Patients often experience impaired functional mobility, pain, spasms, postural issues, and difficulty with self-care activities. Healthcare professionals evaluate muscle tone using the Modified Ashworth Scale (MAS), which assigns a rating from 1-5 based on resistance in a range of motion. Grading spasticity through MAS can provide valuable insight into treatment efficacy and help detect any alterations in symptoms (Harb e Kishner 2023).

Treatment:

Treatment for spasticity typically involves a combination of therapies, including physical therapy, medication, and in some cases, surgery. By rule, the first approach is non-pharmacological such as stretching, splinting, rotatory movements, and electrical stimulation (Synnot et al. 2017). When pharmacological therapy is needed, it is crucial to consider that many of the commonly used medications for spasticity act centrally and may impact neurorecovery (Davidoff 1985; E. Chang et al. 2013). The current pharmacological treatments for spasticity include oral medications like baclofen, that may also be delivered intrathecally, tizanidine, diazepam, and dantrolene sodium (Synnot et al. 2017). Furthermore, botulinum toxin (Botox) injections are used to specifically target

spastic muscles to avoid generalized weakness (Gracies et al. 2015). Although promising, injection therapy can be time-consuming and technically challenging.

1.3.3. Post traumatic seizures

Seizures are classified by the international League Against Epilepsy as a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity of the brain (Falco-Walter 2020). Post-traumatic epilepsy (PTE) is one of the most devastating long term consequences of TBI (Fordington e Manford 2020). In an epilepsy population, approximately 20% of prevalent cases may be due to previous TBI (Falco-Walter 2020; Guekht et al. 2010). Post Traumatic Seizures may be specific to one brain area, named focal seizures, or they can be generalized, affecting the entire brain (Andrade-Machado, Benjumea Cuartas, e Muhammad 2021). They can follow shortly after injury, in months or even years later. Importantly, more than 80% of patients who develop seizure activity TBI related will present them in the first 2 years after injury, and after that time, the chance to develop a seizure decreases.

Treatment:

Since TBI patients are at high risk to develop epilepsy, it is hard not to consider medication currently used to prevent seizures. Despite this, research on using epileptic drugs to prevent TBI seizures is poorly conclusive. The most widely prescribed epileptic drugs are phenytoin, levetiracetam, and valproate (Wat et al. 2019). Specifically, it was found that while treatment options like phenytoin (Temkin et al. 1990) or carbamazepine can lower early post-traumatic seizure incidence rates, it does not affect later seizures or mortality rates following TBI (Thompson et al. 2015). Valproate and levetiracetam underwent comparisons against phenytoin, but no benefit was found, and it was concluded that levetiracetam has no advantages compared to phenytoin (Y. Yang et al. 2016). Interestingly enough a survey conducted among clinicians revealed that the majority do not use prophylactic anti-epileptic drugs. Out of those who use anti-epileptic drugs, roughly 38% only prescribed them for a month. Medications most commonly utilized in acute units for status epilepticus administration can also be applied when treating patients with developing seizures (Mee et al. 2019).

1.3.4. Post-traumatic Neuroendocrine Disorders

The pituitary gland is sensitive to the lack of blood supply and acceleration and deceleration injuries. Therefore, TBI may disrupt the gland's normal secretion, leading to hormonal imbalances including hypopituitarism (Kałas et al. 2023; Kgosidialwa e Agha 2019). These neuroendocrine abnormalities are rare in mTBI, but, in severest cases, the prevalence is 50% to 80%. Hypopituitarism will further exacerbate cognitive and affective symptoms. This condition can manifest with a variety of symptoms depending on which hormones are affected. The most described feelings are fatigue, weakness, decreased libido, menstrual irregularities, and weight gain (Gray et al. s.d.).

Addressing hormonal imbalance and attempting to control hormone production may assist both cognitive and physical recovery. However, in the acute phase post-trauma, accurately measuring hormone impairment may be trick and poorly reliable. Despite this, it is essential to check Adrenocorticotropic Hormone (ACTH)/cortisol deficiency since it is potentially life-threatening. An acute phase cortisol level below 300 nmol/l is suggestive of adrenal insufficiency and treatment should be considered (Cooper e Stewart 2003; Bensalah et al. 2018). In chronic phases, 3 months or later post-injury it is essential to check for thyroid function, gonadotrophins and sex steroid levels. For the growth hormone, for instance, the best time point for assessment is 6 to 12-months post-injury.

1.3.5. Neurodegenerative diseases

Parkinson's and Alzheimer's disease

As mentioned previously, cognitive impairments such as attention and memory deficits, slow cognition, and distraction are commonly reported after TBI (Dikmen et al. 2009). Long-term follow-up by advanced neuroimaging and post-mortem analysis reveals that specific brain areas, like for instance the hippocampus, are strongly affected by TBI (Bigler e Maxwell 2011). Studies show that TBI, namely the most severe cases, confer a long-term risk for cognitive impairment, increasing the prevalence of neurodegenerative conditions such as Alzheimer's disease (AD) and Parkinson's disease (PD) (Brett et al. 2022; D. Wu et al. 2021). Importantly, the chance of developing neurodegenerative diseases after TBI can vary depending on various factors, like trauma severity and the number of injuries acquired, the individuals age, and other risk factors. Regarding age, the risk of developing TBI-associated dementia is higher when the individual gets the trauma at an older age. Probably this is due to a potentiation of the combined effects of ageing (which naturally

affects brain structures promoting dementia) and the effects of brain injury (Mendez 2017)·(Johnson e Stewart 2015; Susman et al. 2002). PD is the most prevalent movement disorder for which TBI is a major non-genetic risk factor. Specifically, a study published in 2014 shows that TBI victims have a 56% higher chance to develop PD than those without a TBI history (Gardner et al. 2014). Research highlights that parkinsonism has frequently emerged as a result of repeated mild TBI due to contact sports. PD affects the entire basal ganglia and is classically characterized by dopamine dysregulation in the nigrostriatal circuit, neuronal mitochondrial dysfunction, and loss of dopaminergic projections to the striatum from the Substantia Nigra pars compacta (SNpc), culminating in progressive death of dopaminergic neurons (DNs) (Tysnes e Storstein 2017; D. K. Simon, Tanner, e Brundin 2020). Additionally, PD is characterized by Lewy bodies and Lewy neurite formation due to misfolded and α-syn aggregates (Mehra, Sahay, e Maji 2019). Normally, α-syn is present within synapses and neuronal nucleus. Excessive phosphorylation at serine129 leads α–syn to aggregate (Eliezer et al. 2001). Interestingly, aggregates do not form indiscriminately but with apparent selectivity toward specific neuron types characterized by lacking extensive myelin and, therefore more, vulnerable. Given the link between PD pathology and traumatic brain injury, it would be worthwhile to investigate whether diffuse axonal injuries connected with mild or repetitive TBI increase the vulnerability of specific neuron types towards PD pathology, in an attempt to counteract this tendency (Braak et al. 2003). While not all individuals who experience TBI will develop Alzheimer's disease, the chances of developing it are increased about 1.5 times after a TBI (Y et al. 2017). Despite this, the relationship between the two conditions is complex and not fully understood. AD is characterized by memory quality decline, such as having issues in remembering newly learned information; cognitive decline, such as lack of cohesive speech, problem-solving and decision-making capacity, attention and concentration; disorientation, especially in new environments, not being able to identify family members; or behavioral and mood changes.

TBI induces several cellular changes in the brain associated with AD, which is believed to potentiate the disease development after injury. As mentioned previously, TBI will lead, in chronic time points, to amyloid plaque formation that clumps together and disrupt cell communication, neurofibrillary tangles, neuronal loss, inflammation, and oxidative stress. In summary, the gradation and malfunction of the brain cells, caused by the initial mechanical impact and consequent secondary injury, ultimately lead to cognitive impairment and memory deterioration found in AD patients (Trejo-Lopez, Yachnis, e Prokop 2022). Nevertheless, research is ongoing to unravel intricate

patterns of disease evolution and which are the specific mechanisms of action between TBI and AD.

Chronic Traumatic encephalopathy

CTE is a neurodegenerative brain disease, usually developed after exposure to repetitive head trauma. Sporadically, CTE may be due to a single moderate or severe TBI, which for reasons that remain to be clarified, progresses gradually to dementia. CTE was challenging to discover since the symptoms may not appear until years or decades after the traumatic events. The first reports described it as the ''punch drunk'' syndrome or dementia pugilistic among boxers until the current nomination (CTE) was reached. It is mostly reported in individuals with a history of repetitive brain trauma such as athletes in a range of contact, collision or combat sports, but also in martial arts, soccer, ice hockey, and others (VanItallie 2019) . Non-sport-related CTE are combat-related and recognized in young active military personnel and retired veterans (Lakis et al. 2013). The disease hallmark is the buildup of hyperphosphorylated tau (p-tau) protein in the brain. Other pathological proteins, such as Amyloid β and α–syn are found. Gross anatomic changes include generalized cerebral atrophy involving the frontal and temporal lobes. Others, such as thalamus and hypothalamus, also experience atrophy. Additionally, there is an expansion of the lateral and third ventricles and thinning of the corpus callosum (McKee et al. 2015). Symptomatically, CTE is similar to AD regarding cognition deterioration, although CTE manifests primarily with behavioral and personality changes such as depression and suicide, poor impulse control, and social disinhibition. There is a progressive scale of symptoms evolution with regards CTE. First symptoms are usually light and tend to escalate trough time (D. H. Smith et al. 2019). Early-stage CTE is frequently asymptomatic. The few symptoms such as headache, attentional impairment, inability to focus, depression, and irritability, are short-duration and not definitive. Short-term memory deficits, aggressive behavior, mood swings, and organization and planning issues characterize the next stage. As CTE progress, cognitive impairment and memory loss become evident. In the later stages of CTE, dementia is the major sign (D. H. Smith et al. 2019).

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by the loss of upper and lower motor neurons, which manifests clinically as progressive skeletal muscle weakness (Feldman et al. 2022). The majority of ALS cases are characterized by cytosolic

inclusions containing TDP-43, p62, and FUS, except for specific ALS subtypes such as familial ALS with superoxide dismutase 1 (*SOD1*) mutations, which appear to have unique features (Farrawell et al. 2015). Ubiquitin-positive inclusions seem to be a universal feature of all sporadic and familial ALS (Tran e Lee 2022). Although some studies found an association between TBI and ALS, existing epidemiological data have debated whether TBI sufficiently correlates with ALS. Therefore, ALS remains less linked to TBI compared to severe cognitive diseases such as CTE and PD (McKee et al. 2010; Thomsen et al. 2015; Armon e Nelson 2012). Of the aforementioned cognitive diseases, ALS is the least associated with TBI. The hypothesis is that TBI-induced ALS is a subset of susceptible patients based on a history of repetitive mild TBI and genetic predisposition.

1.4. Pathophysiology of TBI

1.4.1. Lesion Types

There are numerous definitions of TBI (**Figure 4**), including lesion classification, which is essential for acute management, treatment, prognosis, and neurorehabilitation requirements.

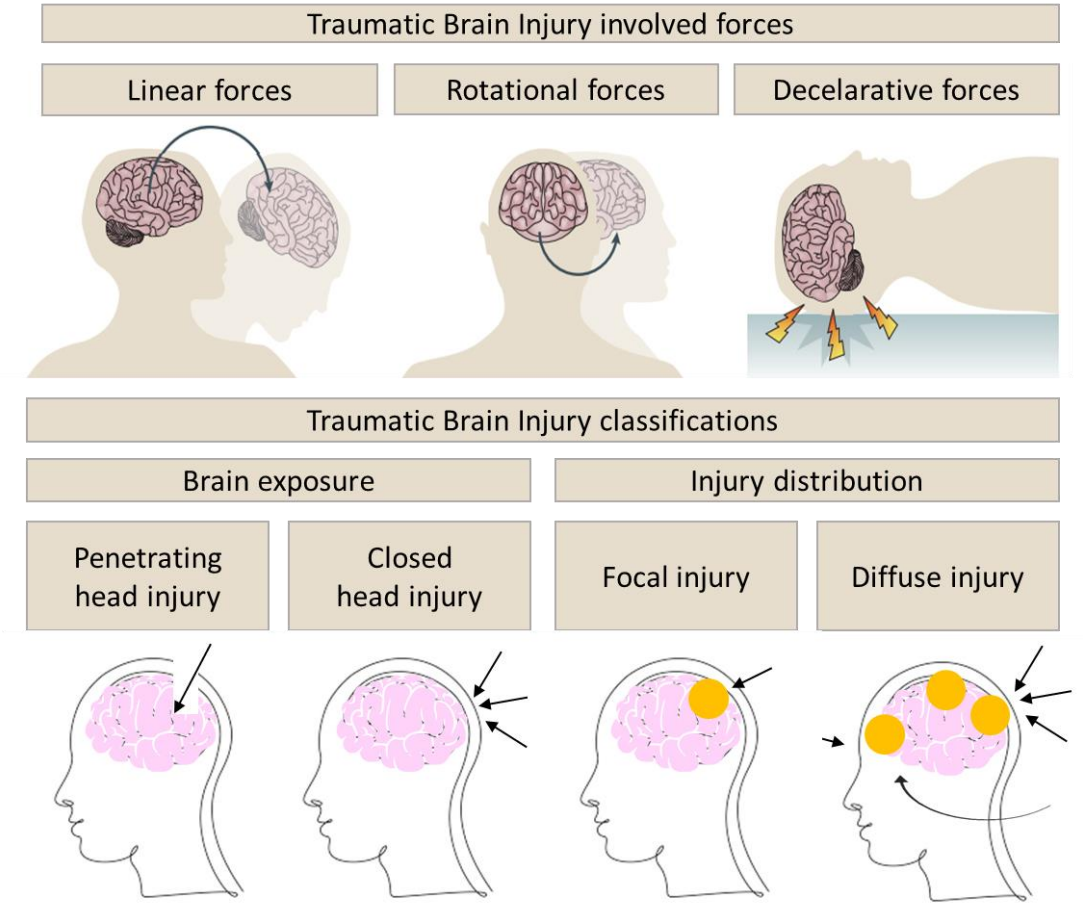


Figure 4: Main forces involved during TBI and different types of injuries and lesions depending on brain exposure to the mechanical impact and injury distribution on the brain tissue.

TBI pathogenic processes and consequent lesion type depend on the forces involved during the accident and how those are transmitted through the brain tissue. Specifically, besides blunt impact, the main forces involved during head trauma are acceleration and deceleration. These may be 1) linear, if obligate the head to move in the anterior-posterior direction; 2) rotational, provoking a rotative movement of the brain inside the skull, for instance as a result of a punch in a box fight; and 3) decelerative, which damage the brain at the moment in which the head decelerates, such as when the head hits the ground after a fall (Dixon 2017). Importantly, TBI is a complex condition that arises multiple set of forces, and the outcome will rarely result from a single force at a time.

The forces involved in the traumatic event will generate intracranial pressure gradients, which result in shearing and strain forces culminating in axonal injury. Depending on the damage location, TBI may be focal or diffuse (**Figure 4**). Focal brain injury is produced by collision forces to the skull that cause brain tissue compression. It is broadly characterized by subdural or epidural hematomas, intraparenchymal hematomas, and contusions (Karibe et al. 2014). In focal brain injuries, the axonal injury and cell damage are mostly restrained to the site of the impact (Mesfin et al. 2023). On the other side, in diffuse brain injury, there is widely distributed damage to axons, diffuse vascular injury, hypoxic-ischemic injury and brain swelling. Diffuse brain injury results from rapid acceleration–deceleration of the head, as seen, for example, in high-speed car accidents (Chelly et al. 2011; Rungruangsak e Poriswanish 2021; Moe et al. 2019). During the accident, the entire brain is exposed to trauma forces in a heterogeneous manner. Consequently, shear, tensile ,and compressive forces are applied in the brain with different intensities and distributions (Chavko et al. 2011).

TBI may be additionally classified as closed, in case of a non-penetrating injury; or open, when the dura is exposed.

1.4.2 Cellular mechanisms

TBI pathophysiology involves a complex cascade of events that occur both at the time of the initial injury and in the subsequent hours to days and months after trauma. The primary injury is a consequence of the first mechanical impact on the tissue, which leads to disruption of cellular integrity and immediate damage.

After the first insult to the brain, a set of mechanisms and constitute the secondary injury will develop over time and intensify the first mechanical damage. Protective mechanisms are activated in an attempt by the organism to counteract tissue injury. Despite the previous, inflammatory and detrimental processes will prevail intensifying the first initial damage and promoting pathological outcomes (Huber-Lang, Lambris, e Ward 2018; Johnson et al. 2013; Begum et al. 2020; Corps, Roth, e McGavern 2015). Local and peripheral secondary mechanisms are active for minutes to months after the first injury and include excitotoxicity and cell death, cerebral edema, ischemia and hypoxia, inflammatory response, BBB disruption, oxidative stress, neuroplasticity and repair (Ladak, Enam, e Ibrahim 2019).

Excitotocicity, cell death and neuroplasticity

After the initial injury, there is a massive increase in interstitial glutamate concentration which overstimulates neurons and consequently increases cell damage (Dorsett et al. 2017). After impact, glutamate increases massively in the extracellular space, as it is released by damaged cells, including astrocytes and microglia (Dorsett et al. 2017; van Landeghem et al. 2001; Yi et al. 2007). Later, glutamate levels keep rising due to BBB leakage, and there is a reduced glutamate reuptake by astrocytes, which show downregulation of glutamate transporters such as Glutamate Aspartate Transporter (GLAST) and Glial Glutamate Transporter -1 (GLT-1) (Pajarillo et al. 2019; Rao et al. 1998). The altered glutamate concentration at the extracellular space will lead to a consequent calcium influx into neurons and astrocytes. Therefore, there is an increased glutamate efflux as a result of Na^+ /K^+-ATPase inhibition and subsequent collapse of the ions gradients. Consequently, the immense intracellular ionic accumulations lead to cerebral edema, which can lead to swelling and further increase of intracranial pressure, further compromising blood flow and oxygen supply to the brain and causing additional damage (Papazian et al. 2018). Of significant importance is that, following TBI, the brain initiates neuroplasticity and repair processes aimed at adapting to the lesion and promoting healing to the damaged tissues. These processes involve molecular, synaptic, and cellular changes (Sophie Su, Veeravagu, e Grant 2016). During the acute phase of TBI, there is a pronounced expression of genes that promote neuroplasticity. For instance, neurotrophins such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) have been described (Grundy et al. 2000). These neuronal factors may provide protection from a secondary injury, although this protective effect is transient and reflective of excessive neuroexcitation during primary injury.

Inflammatory response

TBI elicits a marked local and systemic inflammatory response which plays a crucial role in secondary injury progression (Corps, Roth, e McGavern 2015; E. J. Needham et al. 2019; Drieu et al. 2022). Previous works suggested that neuroinflammation had only an adverse effect, but today it is known that inflammatory mechanisms play also a beneficial role (Yuan e Wu 2022; Loane e Kumar 2016, 201) (**Figure 5**). Therefore, therapies aiming to modulate inflammation instead of shutting it down completely, will be preferable as potential TBI therapies.

Inflammation in TBI is a set of complex multifactorial events, including pro- and anti-inflammatory cytokines, chemokines, adhesion molecules, complement factors, and reactive oxygen and

nitrogen species. Both local and systemic inflammation have specific timings and interact in complex ways, overlapping and potentiating the initial inflammatory condition **(Figure 5).**

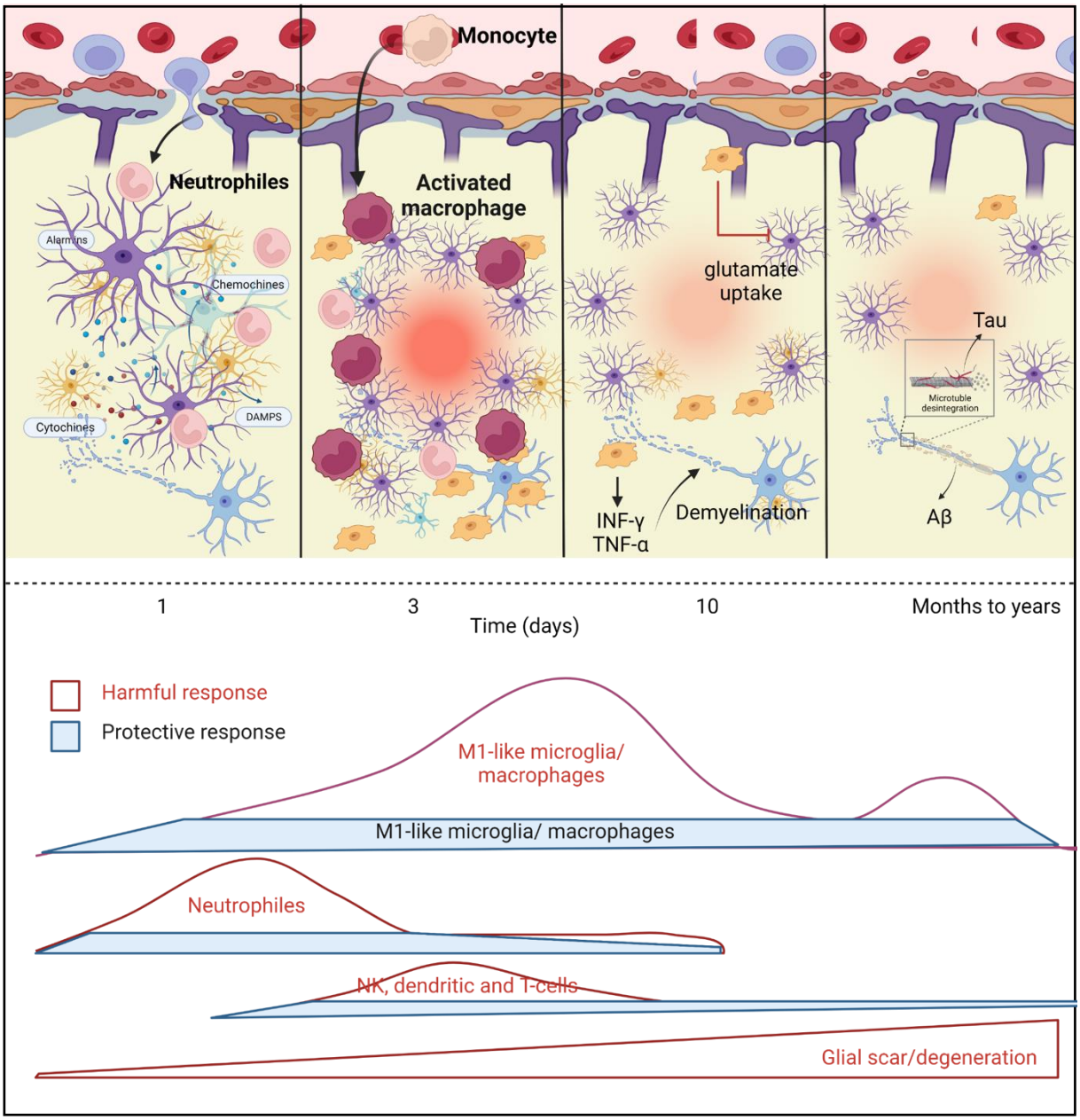


Figure 5: Inflammation following TBI: Primary mechanical injury to the CNS will lead to cell membrane disruption, vascular and BBB damage followed by secondary reactions involving ionic imbalance originating cell death and potentiating the first initial damage. Primary and secondary injury lead to release of DAMPs, cytokines, chemokines, activation of microglia and astrocytes, and recruitment of peripheral immune cells. Both local and peripheral response overlap temporally. The inflammatory response is crucial to clearance of debris, repair, and regeneration after TBI. However, dysregulated inflammation can be detrimental leading to chronic brain injury.

Briefly, the cellular membrane disruption due to the mechanical impact will originate the release of damage-associated molecular patterns (DAMPs) and alarmins from the damaged meninges, glial limitants, and brain parenchyma (**Figure 5**). These cellular signals bind to pattern recognition receptors (PRRs) and cytokine receptors on CNS resident glial cells that initiate a robust neuroinflammatory response by producing more cytokines and chemokines, such as interferon-γ, IL-1, IL-2, IL-6, and tumor necrosis factor (TNF)-α, which coordinate the recruitment of peripheral immune cells to the sites of tissue damage. DAMPs interact with Toll-like receptors (TLRs), NOD-like receptors (NLRs), and scavenger receptors to detect endogenous danger molecules released from damaged or dying cells. DAMPs can also direct inflammatory signaling after TBI via the activation of inflammasomes, intracellular multiprotein complexes involved in activating of caspase-1 and generation of mature IL-1β and IL-18 (Huber-Lang, Lambris, e Ward 2018). Inflammasomes can be assembled in multiple CNS populations, including microglia, astrocytes, neurons, and brain-infiltrating macrophages. In the CNS, the NLRP1 and NLRP3 inflammasomes appear the most relevant in injury and chronic neurodegeneration (Freeman et al. 2017). The next paragraphs explore the main brain resident cellular populations playing an inflammatory role in TBI, as well as the peripheral immune cells that infiltrate from the periphery.

Local inflammatory response

Microglia

Microglia are the resident innate immune cells of the CNS. They act as the first line of defense and play a critical role in neuroinflammatory responses following acute brain injury (X. Huang et al. 2021). Microglia share a common origin with monocytes, deriving from primitive erythromyeloid progenitors during embryogenesis and express common markers such as CD11b, IBA1, CD45, CD68, F4/80, CX3CR1 (Jurga, Paleczna, e Kuter 2020). After TBI, microglia rapidly activate, contract their processes and, change morphology from highly ramified to hypertrophic/bushy to amoeboid shape. Therefore, it becomes difficult to distinguish them from infiltrated macrophages morphologically. Microglia has a dual response in TBI and can acquire classical/inflammatory or non-classical/anti-inflammatory phenotypes (Loane e Kumar 2016) (**Figure 5**). To differentiate from both microglia types in experimental systems, this cellular population was classified as classical M1 and alternative M2 microglia (Franco e Fernández-Suárez 2015). Microglia is classified as M1 when pro-inflammatory and neurotoxic expressing, high levels of cellular markers such as CD16, CD32, CD86, iNOS and MHC class II. In contrast, M2 microglia is geared towards

damping down inflammation promoting tissue repair mechanisms trough scavenging debris. M2 alternative microglia is characterized by the expression of phenotypic markers such as CD163, CD206, Arg-1, Ym-1, and FIZZ-1 (Franco e Fernández-Suárez 2015). However, it is important to keep clear that although helpful to categorize microglia profiles in in vitro models, this is an oversimplified concept and poorly reliable to describe microglial activation in in vivo conditions (X. Hu et al. 2015).

Astrocytes

Astrocytes have a crucial role in maintaining physiological balance within the CNS. They support neuronal activities, glial transmission, and signalling through Ca^{2+} release and uptake (Y. Chen e Swanson 2003). Astrocytes also play a role in maintaining BBB integrity by forming astrocytic end feet around endothelial cells (Risau e Wolburg 1990).

After injury there is an increase in astrocytic reactivity named astrogliosis (Michinaga e Koyama 2021). Reactive astrocytes have two distinct states, termed type A1 (Toxic) and type A2 (Trophic) (Escartin et al. 2021). A1 subtype astrocytes can be induced by inflammation and upregulate the expression of interleukin 1α (IL1-α), complement 1q, and TNF (Clarke et al. 2018). These factors are toxic to neurons, harmful to synaptic plasticity and lack of phagocytosis. Contrarily, the A2 subtype has a beneficial and protective role. A2 subtype astrocytes are induced by ischemia and upregulate neurotrophic factors to promote neuronal survival and synapse repair (Miyamoto et al. 2020; Neal et al. 2018). Again, as in microglia, the A1/A2 classification is superficial and lacks specificity when in vivo. Astrocyte activation is a heterogeneous condition, and it is vital to understand the mechanisms behind each cell subtype activation. Further research is needed to uniformly delineate astrocytes classification, regarding both morphology and secreted factors. Independently of their dual tasks in TBI, astrocytes are essential and play key roles such as sensing the damage, inducing pro and anti-inflammation, promoting neurorepair and neuro-damage effects, enhancing and inhibiting synaptogenesis, increasing and decreasing angiogenesis and blood-brain barrier. Astrogliosis involves changes in morphology, increased expression of both, the glial GFAP protein and gene, and secretion of inflammatory mediators and growth factors (Liddelow e Barres 2017). Studies show that astrocytes undergo extension of their processes and cell body swelling – known as hypertrophic morphology. Morphological changes were found in both lesional and perilesional areas three days post-TBI (Villapol, Byrnes, e Symes 2014). Further morphological changes were evident seven days after injury, and findings supported evidence for enduring reactive

gliosis, with glial scar formation up to 2 months' post-injury, suggesting an ongoing response of these cells and a long-term role in response to brain injury.

Glial scar formation

Astrocytes get activated and reactive after injury. The injury severity affects how extended and intense the hypertrophy and the cells profile. In severe TBI, astrogliosis gets exacerbated and persists overtime originating a glial scar (**Figure 5**). Immediately adjacent to the injury, astrocytes proliferate and interlace to form a glial scar (Sofroniew 2009). Glial scar surrounds and restricts the spread of the intense inflammatory response in the lesion core. Microglia and astrocytes play a role in glial scar formation, together with endothelial cells, fibroblasts and extracellular matrix (H. Wang et al. 2018). The scar creates a physical barrier around the injury site and has proved essential for preventing toxic molecules and DAMPs from seeping into healthy tissues while excluding invading cells after an injury (**Figure 5**). Nonetheless, this valuable process may come at the cost of impeding axonal regrowth and regeneration. Besides protective, the glial scar is also considered a major barrier to axonal regeneration and neuronal connectivity recovery, due to the production of growth inhibitory components and the formation of physical and chemical barriers that hinder axon elongation (T. Yang et al. 2020; J. Li, Wang, e Qin 2021).

Peripheral innate responses following TBI

Neutrophils

Neutrophils are the most abundant cell population in circulation after TBI (Liao et al. 2013). They are the first peripheral immune cells to be recruited to the damaged meninges and brain parenchyma in response to chemokines gradients produced immediately after TBI (Helmy et al. 2011). Additionally, vascular endothelium cell adhesion molecules, such as E-selectin and intercellular adhesion molecule 1 (ICAM1) (Whalen et al. 2000; Pleines et al. 1998) are upregulated after injury and facilitate neutrophil trafficking into damaged brain parenchyma. Neutrophils are recruited within hours of the brain injury and predominate within the lesion microenvironment during the first few days (Clark et al. 1994; Soares et al. 1995). From 5-7 days post-injury neutrophils infiltration is greatly decreased (Clark et al. 1994; Soares et al. 1995). Their functional role is to contain injury progression and remove cellular debris (Y.-W. Liu, Li, e Dai 2018). However, they can also compound brain injury by direct toxic effects of matrix metalloproteinases (MMPs), reactive oxygen species (ROS), and TNFα («Polymorphonuclear Leukocytes Promote

Neurotoxicity through Release of Matrix Metalloproteinases, Reactive Oxygen Species, and TNF-α» s.d.), as well as by increasing vascular permeability and edema formation (Y.-W. Liu, Li, e Dai 2018). Therefore, neutrophils can also contribute to BBB damage.

Monocytes

Monocytes are circulating bone marrow–derived leukocytes that differentiate into macrophages or dendritic cells (DCs) after the invasion of injured tissue. They perform critical innate immune functions, such as phagocytosis, cytokine/chemokine release, antigen presentation, immune modulation, and tissue repair. Following TBI, monocytes enter the perivascular spaces and brain parenchyma within 1-2 days, differentiating into macrophages, which can reside in the brain for several weeks (Beschorner et al. 2002). Blood-derived monocytes are recruited to the injured brain in response to local chemokine gradients which are found in the CSF or plasma of TBI patients (Lumpkins et al. 2008; Semple et al. 2010). Once in the brain, they differentiate into macrophage subpopulations distinguished by relative cell-surface expression of the CCR2 and CX3CR1 (Russo, Latour, e McGavern 2018). On the one hand, monocytes and subsequent macrophage activation may be beneficial, but on the other hand prolonged activation of macrophages may contribute to a state of chronic inflammation and tissue damage. Macrophages can release cytotoxic substances and pro-inflammatory cytokines, exacerbating the injury and potentially causing neurotoxic effects. This chronic inflammation can contribute to secondary injury mechanisms and impair the recovery process after TBI.

B and T-cells

The release of brain antigens into the systemic circulation after TBI may activate an adaptive immune response, including humoral immunity, mediated by B-lymphocytes producing antibodies, and cell-mediated immunity, directed by effector T-lymphocytes (E. J. Needham et al. 2019). However, clinical and experimental studies provide conflicting evidence of both protective and pathogenic autoimmunity mediated by specific CNS antigens. The recruitment of T cells into the traumatically injured brain occurs soon after monocyte infiltration (Holmin et al. 1998) and through the upregulation of cell adhesion molecules and in response to local chemokines (Peter JA Hutchinson, 2011»). Following TBI, there is a massive release of CNS-specific proteins, GFAP, MBP, myelin oligodendrocytes glycoprotein (MOG), and myelin-associated glycopeptide (MAG) (E. J. Needham et al. 2019; Edward J. Needham et al. 2021; Z. Zhang et al. 2014), both directly into

the circulation and to the cervical lymph nodes via the glymphatic and meningeal lymphatic systems (Plog et al. 2015). CNS proteins can be phagocytosed by professional antigen-presenting cells which result in the production of self-reactive T-cells. Once activated, T-cells upregulate the necessary surface molecules to migrate across the BBB. The antigen-specific $CD8^+$ T-cells have been demonstrated to migrate into the CNS and co-localize to their cognate antigen (Ling et al. 2006). In the CNS, autoreactive T-cells are typically considered to be harmful (Daglas et al. 2019), however, autoreactive T cells specific to CNS antigen have also been shown to protect injured axons following damage (Moalem et al. 1999). The discrepancy in experimental literature regarding pathogenic or protective autoimmunity following TBI may be due to differing T-lymphocyte populations contributing to specific detrimental and beneficial injury responses.

2. Experimental models of Traumatic Brain Injury

The increasing availability of TBI experimental models contributed largely to the discovery of potential cellular and molecular mechanisms underlying TBI pathology. There is a vast heterogeneity of mechanical models being used in the literature, both in vitro and in vivo, (**Figure 6**) although none of them completely represents TBI complexity (Zelenika, Grima, e Pessac 1993). Models of TBI are mainly divided into static, dynamic and chemical models. The static models comprise the compressive models in which the intensity, force, and duration of the compression model are crucial features. Compressive models attempt to reproduce similar effects to those where there is a blow and flattening of the skull (Bar-Kochba et al. 2016). Dynamic models represent the impact on the skull, directly, by an accelerative object, or indirectly through shock wave-induced damage (X. Ma et al. 2019). Lastly, chemical models, only used in vitro, reproduce secondary mechanisms such as glutamate excitotoxicity, hypoxia and lack of nutrients (Salvador, Burek, e Förster 2015) (Sabogal-Guáqueta et al. 2019).

2.1 In vitro models of injury

In vitro models offer tight control of experimental variables, allowing repeatable, well-controlled, and environmentally isolated experiments. Additionally, they offer an isolated platform to examine the local brain response to the mechanical impact without systemic confounders, such as systemic inflammation or hypoxia/ischemia. Moreover, using in vitro platforms, pathological alterations may be followed in real-time, within specific brain regions of interest. In addition, these studies pose

fewer ethical problems and are much more cost-effective compared with in vivo, thus offering a potential high-throughput format for the screening of neuroprotective agents.

Despite providing valuable snapshots of trauma evolution under controlled conditions, it is important to keep in mind that in vitro platforms do not fully model brain injury pathology and in vivo tests are crucial before translation to clinics.

Static mechanical injury

Transection models

Penetrating injuries cause tissue laceration which outcomes in localized primary axotomy and subsequent cell loss. Transection in vitro aims at the study of axotomy and penetrating injuries, and it can be modelled by using a plastic stylet (Tecoma et al. 1989)or blades (Mukhin et al. 1998) - Scratch models - ,or a rotating scribe - Shear models (Mukhin et al. 1997) to scrape adherent neurons, or other brain resident cells in culture plates. Different injury intensities may be modelled by increasing the number of lesions and injury severity may be assessed by measuring cell damage and death. These techniques are quite simple, straightforward and not technically demanding. Additionally, their low cost makes them ideal platforms to get quick and simple results on drug testing and discovery. Despite this, a major drawback of transection models is being manually performed and dependent on the operator. To address these issues and increase control over lesion parameters, the microtransection model was developed, where microlesions are performed with a laser on single cells (Gross, Lucas, e Higgins 1983; Lucas et al. 1985). Using a laser exponentially increases trauma precision and control over injury parameters.

Compression

Tissue-level replication of focal injuries takes place with in vitro testing through the use of impactors or weight-drops (Fahlenkamp et al. 2011; Wen Shi et al. 2021). The use of these models will be deeper explored in section 2.2 of this Thesis, ''*In vivo models of TBI*''. The mechanical impact creates direct mechanical damage at the injury site along with additional tissue trauma noted within the secondary injury areas (Church e Andrew 2005; Llufriu-Dabén et al. 2019). Customizing the force intensity, depth, shape of the impactor and duration enables researchers to control injury severity and is a major advantage of these models. While weight drop models have been used extensively in vitro, studies using impactor models in this setup were just recently published in both organotypic slices from cortex and brain organoids (Ramirez, Mukherjee, Sepulveda, Gherardelli,

et al. 2021; Ramirez, Mukherjee, Sepulveda, Becerra-Calixto, et al. 2021; Pischiutta and Cavaleiro et al. 2023)

Hydrostatic Pressure

TBI originates changes in hydrostatic pressure inside the brain (Bernard 2023). In vitro, application of either transient or static pressure to cultures will lead to cell damage. As the pressure wave travels through the tissue, pressure gradients lead to deformation and injury. Since the brain tissue is relatively incompressible, inducing cellular injury typically requires prolonged exposure to high pressures (Sahay et al. 1992; Budday et al. 2020). Scientists have used different techniques to generate hydrostatic pressure on cultured cells and organotypic slices, with the most common methods being quasistatic barotrauma chambers that exert consistent levels of pressure over designated periods of time (MURPHY and HORROCKS 2009); modified fluid percussion systems; and weight dropped from a given height on a piston fluid-sealed chamber (Wallis and Panizzon 1995).

Hydrostatic pressure models opened a window to study blast injury, which is the most prevalent type of TBI experienced by soldiers serving military duty. Recently, researchers have developed new methods using hydrostatic pressures within an in vitro model, to study this type of injury (Campos Pires et al. 2018; D. Xu et al. 2022)

Dynamic mechanical injury

Accelaration/decelaration

In human TBI, acceleration/deceleration forces are one of the main mechanisms driving neuronal death, as they cause shear forces that originate contusions and diffuse axonal injury (Mesfin et al. 2023). To better replicate these phenomena was developed a user-friendly method that exposes brain tissue to tangential accelerations at high G forces. Specifically, after medium removal, cultured cells are impacted with a moving pendulum with a specific acceleration (Lucas e Wolf 1991). To ensure accurate results strain guidelines must be applied based on physical models that represent clinically relevant post-injury outcomes. For instance, Margulies and collaborators tested filling non-human primate skulls with materials simulating the properties of the brain and exposing them to inertial loading forces characteristic of diffuse axonal injuries (Margulies, Thibault, e Gennarelli 1990). This approach yielded valuable findings that could then guide further in vitro research endeavors.

Hydrodynamic

Inertial loading of the brain, causing shear or stretch forces, can be studied through hydrodynamic models of TBI. The in vitro cell shearing injury device consists of a parallel plate viscometer that has cells growing on one plate and a variable speed rotating plate. The speed of the rotating plate, as well as the gap between the two plates, may be adjusted. With this, a range of hydrodynamic forces is applied to the cells cultured on the growing plate (LaPlaca e Thibault 1997). Individual cell strain can be accurately calculated, as the cells can be observed directly by light microscopy, and indirectly by measurements on microbeads attached to the plates (Bottlang et al. 2007; Begonia et al. 2021, 201). Hydrodynamic shear strain-based TBI modelling might be more relevant to the pathological processes in human TBI than models based on acceleration/ deceleration shear stress.

Stretch injury Models

Stretch injury models focus on studying axonal injury. They were developed to injure cell processes while circumventing cell body deformation. More specifically, stretch models allow to study microtubule damage and axonal injury (Yap et al. 2017; Nakadate et al. 2017; Yap et al. 2014), BBB disruption (Rosas-Hernandez et al. 2018) and activation of proinflammatory cytokines (Y. Yu et al. 2017; Abdul-Muneer, Bhowmick, e Briski 2018), and mitochondrial dysfunction (F. Wang et al. 2014). In stretch models, cells or organotypic slices are grown on a flexible membrane, which is deformed to induce cell damage. The extent of stretch injury can be modified by altering the amplitude and duration of trauma. An advantage of such a system is that the mechanical force is applied directly to the cells (Rosas-Hernandez et al. 2018; Sturdivant et al. 2016). Cells cultured on membranes may be deformed by compressed gas (Ellis et al. 1995), which does not induce a homogeneous substrate strain, or by a controlled vacuum pulse (Arundine et al. 2003).

Chemical injuries

Chemical injuries lack the mechanical component of trauma. In these models, generic neuronal and tissue damage is induced through the application of neurotoxins or by depriving the tissue of oxygen and glucose.

Glutamate excitotoxicity plays a crucial role in secondary injury post-trauma. *In* vitro glutamate administered to neuronal tissue or organotypic brain slices has been used to study cell death in acute phases after TBI (Kuebler et al. 2015; Lahtinen et al. 2001). Similarly, free-radical injury is

a key feature in the propagation of secondary injury in TBI. Researchers attempting to model secondary injury mechanisms, such as ischemia, might expose tissues to hydrogen peroxide that activates cellular-level responses via oxidative stress (Armogida et al. 2011; Yaka et al. 2013). Another feature of TBI is low tissue vascularization and consequent lack of oxygen and nutrient supply. This condition is mainly due to ICP leading to hypoxia and ischemia. To mimic such an environment on a dish Oxygen Glucose Deprivation (OGD) models are widely used (Ryou e Mallet 2018; Pischiutta et al. 2016). In the OGD model, cell or organotypic slices are deliberately exposed to a controlled reduction in oxygen and glucose supply, mimicking the in vivo conditions of reduced blood flow and nutrient availability that occur during ischemic events. Importantly, a chemical injury may be studied alone or together with a mechanical injury model. This would result in a more complete model of TBI, mimicking both primary and secondary injury events (Salvador, Burek, e Förster 2015). Nevertheless, it is important to have in mind the fragility of in vitro setups, and their limited recovery. Two different types of lesions to the same cells or tissue may be too intense and cell viability should be closely followed.

2.1.1. In vitro tissue surrogates

Cell cultures (Primary cultures, dissociated cultures, immortalized cell lines)

Primary cell cultures refer to cell cultures derived directly from fresh tissues or organs. These cells can retain many of their in vivo characteristics and functionalities (Z. Li 2019). Dissociated primary cell cultures allow the study of the injury effect on both single cell type, or between different cells in mixed cultures (Sherman et al. 2016; S. M. Ahmed et al. 2000).

A drawback of primary cultures is that cells are commonly harvest from embryonic or young postnatal tissue. Age at harvest can significantly affect cellular phenotype and, consequently, injury response (Y.-C. Lin et al. 2002). For instance, without long-term culture, freshly-dissected neurons harvested from early developmental stage tissue lack excitotoxic reactions triggered by glutamate, usually seen in mature neurons (Y.-C. Lin et al. 2002). Another negative point is that most cultures are performed on monolayers, yet 3D constructs better mimic the in vivo microenvironment (Gruber e Hanley 2000).

Immortalized cell lines are easy to obtain, have low-maintenance, robust nature and consistency across experiments and laboratories when compared to the primary cell cultures. These cell lines are also well characterized and are easy to scale up for large studies, but immortalization usually translates into the disruption of normal intracellular signalling cascades and apoptotic mechanisms

(Zhan et al. 2016). Also, immortalized cells can suffer from poor adhesion and disruption of contact dependency, which may be a concern in case of weight drop and stretch models, for instance. Some of the most used immortalized cell lines in TBI research differentiate into neurons or other brain cells. For instance, PC2/PC12 cells, SH-SY5Y neuroblastoma cell line, NG108, among others. Cell line analogues of other brain cell types include endothelial cells (ECV304) and microglia (BV-2).

Acute preparations

Acute preparations are slices (<475 μm) of CNS tissue, cultured in the laboratory. Experiments using acute slices last no longer than 8h, before tissue begins to deteriorate. Experimental TBI studies have been performed on acute preparations of the brain from the rat frontal cortex (Koshinaga et al. 2000), guinea pig cerebellum (Etzion e Grossman 2000), and rat hippocampus (Panizzon et al. 1998). Acute preparations have several advantages. First, as there is no restriction on the age of the harvested tissue, the translation of results is straightforward. Furthermore, acute preparations preserve anatomical structure, heterogeneous cell populations and the neural circuits found in vivo, but it causes disassembly of microtubules and eliminates dendritic spines in neurons (Kirov et al. 2004). Thus, a significant limitation of this set up is the associated damage during to tissue isolation process, even though by comparing injured and uninjured tissues it is not clear whether isolation alters responses to trauma. Furthermore, secondary injury mediators released into the bathing medium may affect in vitro responses to trauma.

Organotypic cultures

Organotypic cultures are thin explants of brain tissue, where the hippocampus has been the most widely used brain area for this purpose. Since the organotypic brain slice cultures preserve the basal cellular, cell function and connective organization, these culture system allows researchers to investigate neuron-glia interactions intricately through individual cell studies amongst heterogeneous cell population. Furthermore, organotypic brain slices allow to study multiple processes crucial for TBI, such as cell death, neurogenesis, cell migration and synaptic properties (Q. Li, Han, e Wang 2016; Humpel 2015). Organotypic cultures can be expensive and technically challenging due to their thinness (often around 250-500 μm) or small cubic size (around $1mm^3$) which demands expertise for manipulation. Their low thickness is critical for ensuring constant

diffusion of essential nutrients in the medium and oxygen flow to prevent hypoxia, ischemia or necrosis.

Organotypic slices are suitable for most of the injury models mentioned above and have been crucial to understanding the mechanisms behind TBI. For instance, this setup has been used to reproduce weight drop (Llufriu-Dabén et al. 2019), OGD (Pischiutta et al. 2016) and CCI models (Pischiutta and Cavaleiro 2023).

Brain organoids

Brain organoids, also known as cerebral organoids or mini brains, are three-dimensional (3D) structures derived from induced Pluripotent Stem Cells (iPSCs). Generally, an organoid is formed by organ-specific cell types (stem cells, neurons and glial cells in the case of brain organoids); it can recapitulate vital functions of the organ (capable to produce neurotransmitters); and it can self-organize to resemble the in vivo situation (Lancaster e Knoblich 2014). In sum, brain organoids consist in multiple cell types that self-organize spatially, displaying enhanced cellular maturation and functionality (Kelava e Lancaster 2016; Lai et al. 2020). To generate brain organoids in vitro there are two main techniques: i) unguided methods, based on spontaneous morphogenesis and intrinsic differentiation ability of pluripotent stem cell aggregates, and ii) guided methods, which require external patterning factors to induce differentiation in specific structures (Qian, Song, e Ming 2019). As compared to simplistic two-dimensional (2D) models, brain organoids present several significant advantages resulting from their capacity to self-organize in 3D that replicates authentic aspects of the brain tissue. Additionally, human brain organoids provide the opportunity to model pathology within a human genome. This is a point of great interest since TBI pathology involves a variety of genetic networks (chapter 1) (Dardiotis et al. 2010). Despite their potential, brain organoids lack the full complexity and functionality of a mature human brain, as for instance the microglial cells and vasculature at the human brain proportions. Nonetheless, several research groups are focusing on drawing protocols aiming to cover this drawback (Jgamadze et al. 2020; H. Kim et al. 2019). Furthermore, mini brains are an isolated system and do not capture the influence of other body systems. Only recently brain organoids utility started to be explored on TBI research (Ramirez, Mukherjee, Sepulveda, Gherardelli, et al. 2021). Ramirez and colleagues, exposed for the first time brain organoids to the CCI model (Ramirez, Mukherjee, Sepulveda, Becerra-Calixto, et al. 2021; Ramirez, Mukherjee, Sepulveda, Gherardelli, et al. 2021). The authors proved that human brain organoids, providing from iPSCs recapitulate pathological hallmarks of

TBI. Namely, after exposed to CCI the brain organoids show neuronal loss and damage as well as astrogliosis (Ramirez, Mukherjee, Sepulveda, Becerra-Calixto, et al. 2021; Ramirez, Mukherjee, Sepulveda, Gherardelli, et al. 2021).

2.2 In vivo models of injury

As mentioned above, TBI is a multifaceted and heterogeneous condition, regarding both causes and disease mechanisms. Therefore, the animal models to study different TBI are many. Rodents are widely used in TBI research due to their low cost, small size and practicality in measuring outcomes, among other advantages. For more advanced research the pig is a valuable organism to understand TBI mechanisms.

The existent models of TBI focus on increasing the knowledge of the detrimental and complex molecular cascades that are initiated by trauma. Among them, four specific models are widely used in TBI research: cortical impact injury (CCI) (P. Zhao et al. 2022; Ye et al. 2021), weight drop injury (X.-S. Zhang et al. 2021), fluid percussion injury (FPI) (Ghosh et al. 2021), and blast injury (Bradshaw et al. 2021).

Controlled Cortical Impact (CCI)

One of the most widely used models of experimental TBI is CCI. CCI is a mechanical model that was developed nearly 30 years ago aiming to create a testing platform to determine the biomechanical properties of brain tissue exposed to direct mechanical deformation (Lighthall 1988). CCI is produced using a device that rapidly accelerates a rigid impactor. Two different types of devices are available for CCI research. As so, a pneumatic or an electromagnetic device may be used to drive a rigid impactor into the exposed intact dura. The first device to be produced was the pneumatic impactor, which is still used nowadays (Lighthall 1988; X. Chen et al. 2022). The electromagnetic apparatus was developed later but is gaining popularity due to its lower cost and greater portability. Both types of impactor devices are discussed and compared in detail in the Osier and collaborators study (Osier e Dixon 2016).

The impactor tip can be varied in size and geometry to accommodate scalability to different species. Additionally, the CCI device allows for control of dual time, speed and depth of the impact, increasing trauma precision and reproducibility (Osier e Dixon 2016). The previous features are

unique to CCI model, compared with others such as weight drop and FPI, allowing to test of different injury severities.

The impactor tip hits directly on the exposed brain tissue, which causes blood vessel breakage (blood-brain barrier damage) (Fang et al. 2022), cortical tissue loss, acute hematoma, brain cellular injury, and glial activation (Loane et al. 2014). Moreover, many secondary injury cascades seen in clinics have also been reported after CCI, as evidenced by histological markers of several processes, including apoptosis (Raghupathi, Graham, e McIntosh 2000; Yakovlev et al. 2001), inflammation (Schaible et al. 2013), and oxidative stress (Schwarzbold et al. 2010; L. Zhang et al. 2012). Thus, the CCI model induces significant cortical contusion, deep brain injury, and neurodegeneration around the impact site mostly at ipsilateral cortex, as common in focal injuries (Fournier et al. 2021). Besides that, the CCI model has been expanded to closed skull injury to investigate mild and repetitive TBI (Moro et al. 2023).

CCI animals develop both functional and cognitive impairments closely related to both depth of deformation and concussion speed (Di Sapia et al. 2021; Zanier et al. 2013). These cognitive deficits may persist up until one-year post-CCI and be associated with brain atrophy and progressive decline of cerebral blood flow (Mao et al. 2020). Along with this, emotional deficits were observed amongst rodents inflicted with CCI, as measured through forced swim test and elevated-plus maze (Washington et al. 2012).

Weight drop

In weight drop (WD) models the skull is exposed (with or without a craniotomy) to a free-falling guided weight, to generate a focal (Flierl et al. 2009) or a diffuse brain injury (Büchele et al. 2016). Injury severity in these models can be altered by adjusting the mass of the weight and the height from which it falls. One example of a weight drop model is *Feeney's model,* characterized by a weight-drop to the dura after a craniotomy causing focal cortical damage (Feeney et al. 1981). These injuries progress from haemorrhages in the white matter beneath the contused cortex during the first few hours after injury to necrotic cavity development within 24 hours. The cavitation expands over two weeks (Feeney et al. 1981; Sutton, Lescaudron, e Stein 1993). Even though rats recover most of the functional deficits induced by this model within the initial two weeks, persistent deficits can persist beyond 90 days (Feeney et al. 1981; Sutton, Lescaudron, e Stein 1993). Another focal weight drop model for closed head injury (CHI) was introduced by Shohami's group. Specifically, a weight drop is delivered to one side of the unprotected skull of a rodent (Shapira et

al. 1988) having the head placed on a hard surface. This injury produces BBB disruption, cerebral edema, and cell death. The injured animals show neurological impairments, revealing motor function and cognitive deficits in the neurological severity score (NSS) test, which correlates closely with the pathological severity of brain damage (Ng et al. 2012).

Marmarow's WDI model produces shear stress and diffuse axonal injury, unlike prior TBI models that produce focal brain contusion. It results from accelerated motion to the rodent head that resembles human diffuse TBI from falls or car accidents (Spurlock et al. 2017). Marmarow's WDI model also induces motor and cognitive deficits as evaluated by both the NSS and the beam walk test, and these deficits correlate with injury severity (T.-H. Hsieh et al. 2017).

Overall, WDI models provide a simple way to mimic clinical conditions ranging from focal to diffuse injuries, while being inexpensive and capable of reproducing graded DAI. However, one disadvantage of the weight-drop models is the relatively high variability in injury severity related to the risk of rebound.

Fluid percussion injury

In Fluid Percussion Injury (FPI) models, the insult is inflicted by a pendulum striking the piston at the end of a tube filled with fluid. This generates a fluid pressure pulse to the intact dura at the place of a previously made craniotomy. Injury may be made either centrally, around the midline, or laterally over the parietal bone, between bregma and lambda. The percussion produces brief displacement and deformation of brain tissue, and the severity of the injury depends on the strength of the pressure pulse (T. K. McIntosh et al. 1989; Wahab et al. 2015; Katz e Molina 2018).

FPI model has been used to replicate the human TBI without the skull fracture that generates edema, intracranial haemorrhage, and progressive gray matter damage (San Martín Molina et al. 2020). The injured area grows overtime, and originates a cavity with cell death and activated glia (I. Yu et al. 2010). FPI reproduces neurobehavioral and cognitive deficits such as difficulties with movement and memory that are commonly seen in patients with TBI (Bray et al. 2022; Witcher et al. 2021).

As drawbacks, FPI models have highly associated mortality compared with other TBI models (Cernak 2005). Additionally, it allows minimal biomechanical control of the insult, since the only adjustable parameter is the height of the pendulum. To solve this issue and improve reproducibility, a microprocessor-controlled pneumatically driven instrument was associated with the standard FPI

device in rats (Kabadi et al. 2010). Another reliable improvement to the model includes computer-controlled FPI devices, which permit to monitor the fluid percussion waveforms.

Penetrating ballistic-like and blast injury models

Penetrating ballistic-like and blast injuries are both frequent in military personnel. Penetrating ballistic-like brain injuries (PBBI) may also affect policeman or civilians involved in gun fights impacted by lost bullets or random projectiles at high speed. PBBI injury is displayed by a temporary cavity in the brain caused by the transmission of high-energy projectiles and their consequent shockwave on brain tissues (Lichte et al. 2010). To model PPBI in rodents, a novel non-fatal model for low velocity using a modified air rifle that accelerates a pellet directed to the brain was developed (Williams et al. 2006; Thangavelu et al. 2020). This model causes temporary cavity formation, white matter degeneration, haemorrhage, edema, and gliosis (Williams et al. 2006). Additionally, exposure to these models triggers cognitive and sensorimotor impairments in animals (Spurlock et al. 2017) The PBBI model is ideal to experimentally represent several unique temporal aspects of a ballistic brain injury, but it also causes extensive intracerebral haemorrhage throughout the primary lesion, due to the penetrating nature of the injury and because of the temporary cavity formation.

Regarding blast injuries, to elucidate the effects of primary blast waves on the brain, various methods such as open field blasts, blast tubes and shock tubes were designed through the years (Clemedson e Criborn 1955; de Lanerolle et al. 2011; Bauman et al. 2009). Shock tubes were used throughout the times to investigate shock-wave-related issues in a wide range of fields, from fundamental to applied technology. More recently, they started being used in biomedical research (Rutter et al. 2021). Anaesthetized animals are restrained in a specimen holder inside or outside the propagation tube, and the pressure is released through the tube, reaching the animal head. Blast TBI models resemble a closed skull injury and damage to the brain tissue is originated by the rapid movement of the brain inside the skull. Consequently, inertial forces may distort and shear tissue which created widespread axonal injury, cerebral edema or swelling throughout the brain and ischemic damage across areas of brain deprived of normal blood flow (J.-H. Zhang et al. 2018; Abdul-Muneer et al. 2013). Genes involved in neurogenesis and synaptic transmission were found to be downregulated (Risling et al. 2011).

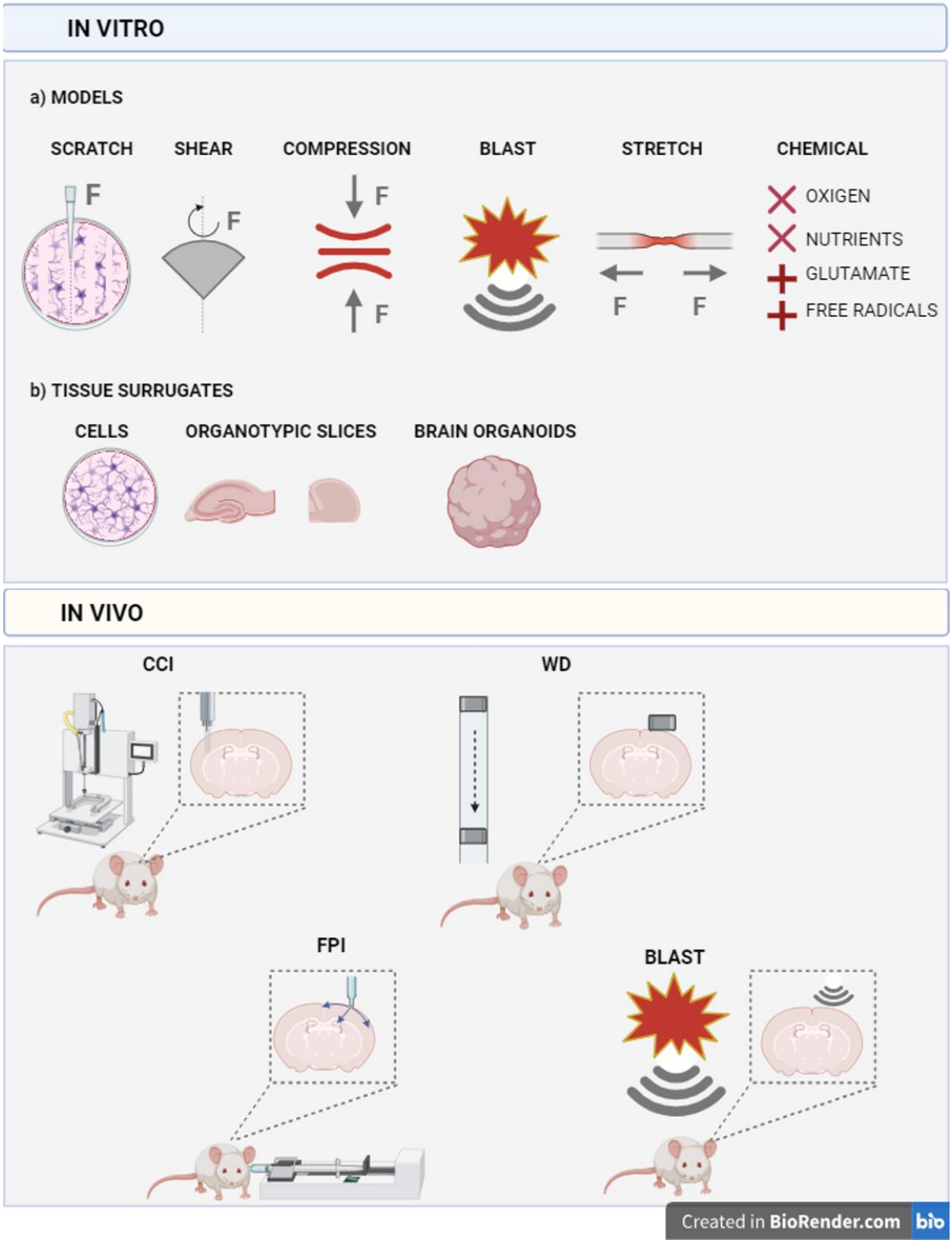


Figure 6. In vitro and in vivo TBI models: In a laboratory TBI may be vastly represented depending on the specific lesion type aiming to be studied. In vitro models, are simpler, allowing to look closely to specific mechanisms. Mostly used in vitro TBI models are transection models, including, starch and shear models; compression, mostly performed using impactors or weight droppers; hydrostatic pressure models, being blast injury the most common of this type; stretch; and chemical injuries including oxygen and glucose

deprivation, glutamate and hydrogen peroxide induced lesions. In vivo models represent focal injuries, such as CCI and FPI, diffuse injury, induced by WD models, and blast injuries.

3. Cell therapy for Traumatic Brain Injury

Cell therapy is the use of cells for medical purposes (Yasuhara et al. 2019; Shimizu, Ntege, e Sunami 2022; Berebichez-Fridman et al. 2017). These cells may be isolated either from the patient's own body (autologous) or from another person, a donor (allogenic). Cell therapy includes non-stem cell and stem cell-based, unicellular, and multicellular therapies. The first studies using cell therapy for TBI focused on replacing damaged neurons to promote tissue recovery and outcome improvements. One of the earliest studies, on cell therapy for TBI tested post-mitotic neurons in rodents with inconsistent therapeutic success (Muir et al. 1999). Gradually, stem cells started to be studied in TBI models.

Stem cells are present in different tissues in the body and have the capacity to differentiate and renew themselves (Bahmad et al. 2021). As reviewed by others, several stem cell types were tested as therapy for TBI, including, neural stem cells (NSCs), induced pluripotent stem cells (iPSCs) mesenchymal stem cells (MSCs), epithelial or skin stem cells (A. I. Ahmed et al. 2016; Borlongan e Rosi 2022). In a TBI mouse model, NSCs delivered 3 days post injury, survived, differentiated into neurons or glia, migrated to the lesion site, and improved motor and cognitive function of TBI animals (Riess et al. 2002). In another study it was demonstrated that NSCs delivered directly at the lesion core, enhance expression of synaptic markers such as synaptophysin and GAP-43, while promoting functional recovery (H. Ma et al. 2011). Likewise, embryonic stem cells were shown to improve functional outcomes after TBI in rodents, but originated tumors formation (Blum e Benvenisty 2008; Gordeeva e Khaydukov 2017). For this reason, later studies involved pre-differentiating stem cells into more lineage-restricted precursors in order to reduce the likelihood of tumorigenesis (Le Belle, Caldwell, e Svendsen 2004). In an effort to reduce reliance on embryonic or fetal tissue, there have been a number of studies more recently focused on induced pluripotent stem cells (iPSCs) derived from adult somatic cells. iPSCs have shown promise in reducing cognitive and motor deficits (Imai et al. 2023), and to be able to survive and migrate to the injured brain tissue (Imai et al. 2023; Furmanski, Nieves, e Doughty 2019). Despite success in experimental TBI models, the use of iPSCs is still complex due to issues in obtaining them, high therapy costs and technical limitations. Mesenchymal Stem Cells (MSCs) is another promising

source that has received a great deal of attention in preclinical studies of TBI and it will be deeper explored in the following sections.

3.1 Mesenchymal Stem Cells

MSCs are multipotent stem cells with self-renewable capacity and a high ability to proliferate. MSCs were isolated for the first time from bone marrow (BM) by Alexander Friedenstein, that described them as fibroblast shape-like cells with clonogenic potential and plastic culture adherence (Friedenstein et al. 1974). More than 50 years of research on MSCs allowed their isolation from other tissues besides BM, such as adipose tissue (ASCs), skin, dental pulp, corneal limbus, peripheral blood, umbilical cord tissue (UC) , muscles, lungs, menstrual blood, placental tissues, and neonatal tissues (Wright, Arthaud-Day, e Weiss 2021; Merimi et al. 2021).

In 2005, the International Society for Cellular Therapy (ISCT) established specific criteria to define in vitro human MSCs as being plastic-adherent displaying a fibrobast-like morphology, simultaneously expressing CD105, CD90, and CD73 simultaneously, and lacking expression of CD34, CD45, CD14, CD19, CD11b, CD79a, and HLA-DR surface molecules. These markers can nevertheless change, depending on specific aspects. For instance, CD34 expression will change depending on donors, passages, and culture conditions (C.-S. Lin et al. 2012; Bellagamba et al. 2018). As last criteria, MSCs must be capable to differentiate (in vitro), into osteoblasts, chondroblasts, and adipocytes (Dominici et al. 2006). In 2019, new topics were established to better characterize MSCs. Those topics include obligatoriness to know the cells' tissue origin; unless there is rigorous evidence for stemness, the use of stromal cell term shall be used; functional assays must be used to define the therapeutic mechanism of action, although no tissue-specific guidelines were addressed (Viswanathan et al. 2019). Nevertheless, despite efforts to give MSCs a consistent characterization, it is important to keep in mind that they are a heterogeneous cell population, influenced by their origin properties (species, tissue source, and donor), as well as by culture conditions such as expansion media and passage number (Merimi et al. 2021).

3.2 Mesenchymal Stem cells to treat TBI

In 2001, the first experimental study assessing MSC protective effects in TBI was published (D. Lu, Mahmood, et al. 2001). Since then, interest has grown, attracting many research groups worldwide, and MSCs are now the most frequently used stem cells for therapy in experimental TBI (**Table 1**).

Table 1: Mesenchymal stem cell therapy in TBI animal models

MSCs							Animal model			Study
MSC source	**specie of origin**	**amount**	**admin route**	**time of transplant**	**multiple admin**	**modification**	**specie**	**TBI model**	**Major improvements**	**Author, year**
BM	rat	$3x10^6$	IV	24 h	no	labelled	rat	WD	Improved neurological function	Anbari F, 2014
	rat	$2x10^6$	IV	24 h	no	labelled	rat	CCI	Functional behavioral improvements	Bakhtiary M, 2010
	rat	$5x10^6$	cavity	60 d	no	naive	rat	WD	Functional improvements; Neural and astroglial differentiation; Increased neurogenesis	Bonilla C, 2009
	rat	$15x10^6$	IV	60 d	no	naive	rat	WD	No neurological or brain tissue improvements	Bonilla C, 2012 a
	rat	$5x10^6$	cavity	60 d	no	naive	rat	WD	Functional improvement in mild TBI	Bonilla C, 2012 b
	rat	$2x10^6$	subarc	60 d	no	naive	rat	WD	Cells migrated to the lesion site; no functional or brain tissue improvements	Bonilla C, 2014
	rat	$5x10^6$	cavity	60 d	no	naive	rat	WD	Functional improvements	Bonilla C, 2018
	rat	$1x10^6$	IC	48 h	no	labelled	rat	WD	Functional improvements	Chen L, 2014

MSCs							Animal model			Study
MSC source	**specie of origin**	**amount**	**admin route**	**time of transplant**	**multiple admin**	**modification**	**specie**	**TBI model**	**Major improvements**	**Author, year**
	rat	$3x10^6$/kg	IV	24 h	no	pre-conditioning	rat	WD	Lesion volume reduction; Improved neurological function	Danilina TI, 2017
	Human	$1x10^6$	IV	6h	no	naive	rat	CCI	Prevented anhedonic behavior; improved depression and working memory	Darkazalli A, 2016
	human	$3x10^6$	cavity	7 d	no	labelled: inside collagen	rat	CCI	Collagen improved cell survival; improved spatial learning; improved functional behavior	Guan J, 2013
	human	$1x10^6$	IC (CC)	24 h	no	naive	rat	FPI	Functional improvements; Reduced astroglial activation	Han EY, 2013
	rat	$2x10^6$	IV	24 h	no	labelled	rat	CCI	No motor or funtional improvements	Harting MT, 2009
	rat	$1x10^6$	IC	24 h	no	labelling (genetic)	rat	WD	Reduction of lesion cavity; Neurological function improvement	Hu J, 2019
	rat	1,E+06	icv	12 h	no	naive	rat	WD	Increase in endothelial progenitor cells; Increase of peripheral angiogenic markers;	Hu W, 2018

MSCs							Animal model			Study
MSC source	**specie of origin**	**amount**	**admin route**	**time of transplant**	**multiple admin**	**modification**	**specie**	**TBI model**	**Major improvements**	**Author, year**
									increase of neuronal markers	
	rat	$3x10^6$	cavity	7 d	no	matrix	rat	CCI	Increase fractional anisotropy; decreased lesion volume; improved functional recovey	Jiang Q, 2011
	human	$2x10^6$	IV	24 h	no	naive	rat	CCI	Decreased caspase 3 cleavage; improved neurological functional outcome; increased neurothrophic factors; decreased neuronal apoptosis	Kim HJ, 2010
	Human	$1x10^6$ /kg	IV	72 h	no	naive	rat	CCI	Decreased BBB permeability; reduced microglia; enhance neurogenesis	Kota DJ, 2016
	human	$3x10^6$	IV	5 d	no	labelled	rat	CCI	Preserved cerebral blood flow, reduced brain atrohpy, improved functional outcome	Li L, 2011

MSCs							Animal model			Study
MSC source	**specie of origin**	**amount**	**admin route**	**time of transplant**	**multiple admin**	**modification**	**specie**	**TBI model**	**Major improvements**	**Author, year**
	Human	$3x10^6$	IV	6 h	no	naive	rat	CCI	Reduced cerebral atrophy; enhanced cerebral tissue perfusion, enhanced neurogenesis; improved functional and cognitive outcome	Li L, 2012
	Human	$3x10^6$	IV	6 h	no	naive	rat	CCI	Enhanced white matter reorganization	Li L, 2017
	rat	$2x10^6$	IV	24 h	no	labelled	rat	CCI	MSCs migrated to the brain; MSCs expressed neuronal and astrocytic markers	Lu D, 2001
	human	$3x10^6$	cavity	4 d	no	naive inside collagen	rat	CCI	Reduced lesion volume; improved spacial learning; improved sensoriomotor function	Lu D, 2007
	rat	$2x10^6$	IV	24 h	no	naive	rat	CCI	Improved motor and neurological deficits; MSCs expressed neuronal and astrocytic markers	Mahmood A, 2001

MSCs							Animal model			Study
MSC source	**specie of origin**	**amount**	**admin route**	**time of transplant**	**multiple admin**	**modification**	**specie**	**TBI model**	**Major improvements**	**Author, year**
	rat	$1x10^6$	IC	24 h	no	labelled	rat	CCI	Improved motor function; MSCs expressed neuronal and astrocytic markers	Mahmood A, 2002
	human	$1x10^6$	IV	24 h	no	naive	rat	CCI	Improved neurological and locomotor function; MSCs expressed neuronal and astrocytic markers	Mahmood A, 2003
	rat	$1x10^6$	IC	24 h	no	naive	rat	CCI	MSCs expressed neuronal markers; Improved functional behavior	Mahmood A, 2004b
	human	2,E+06	IV	24 h	no	naive	rat	CCI	Improve functional outcome; MSCs remained in the lesion boundary until 3 months post injury	Mahmood A, 2005
	rat	$2x10^6$; $4x10^6$; $8x10^6$	IV	7 d	no	naive	rat	CCI	Improved functional outcomes in mice that received 4 and $8x10^6$, but not in those receiving $2x10^6$; increased expression of brain BDNF	Mahmood A, 2006

MSCs							Animal model			Study
MSC source	**specie of origin**	**amount**	**admin route**	**time of transplant**	**multiple admin**	**modification**	**specie**	**TBI model**	**Major improvements**	**Author, year**
	mice	$4x10^5$	IC	10 d	no	labelled (genetic)	rat	CHI	MSCs expressed neuronal and astrocytic markers; cogntive dysfunction	Maruichi K, 2009
	human	$3x10^6$	cavity	7 d	no	naive inside collagen	rat	CCI	MSCs inside collagen were more effective than MSCs alone; Increasead axonal sprouting at spinal cord; Improved sensoriomotor function; Improved spacial learning;	Mahmood A, 2013
	rat	$8x10^5$	IC	36 h	no	labelled (genetic - to overexpress IL-10)	rat	CCI	Reduction od GFAP ans CD86; Increased microglia activation; recovered the number of foot faults TBI-induced	Peruzzaro ST, 2019
	human	2,E+05	icv	24 h	no	naive	mouse	CCI	Improved sensoriomotor deficits; improved cognitive deficits; reduced lesion volume; rescued vessel density; reduced the glial scar; induced neurogenesis	Pischiutta F, 2014

MSCs							Animal model			Study
MSC source	**specie of origin**	**amount**	**admin route**	**time of transplant**	**multiple admin**	**modification**	**specie**	**TBI model**	**Major improvements**	**Author, year**
	mouse	3,E+05	IV	24 h	no	naive	mouse	CCI	Improved neurological function; increased vascular density	Qu C, 2008
	Human	$0.3x10^6$	IV or cavity	7 d	no	naive inside collagen	mouse	CCI	MSCs inside collagen were more effective than MSCs alone; improved spatial learning; reduced lesion volume; increased vascular density	Qu C, 2009
	human	3,E+06	cavity	7 d	no	naive inside collagen	rat	CCI	Decreased functional deficits, enhances VEGF expression of astrocytes	Qu C, 2011
	mouse	$1.5x10^4$	ICV	24 h	no	genetic	mouse	CCI	Improved spatial learning and memory; promoted neurogenesis; improved dendritic arborization of hippocampal neurons	Sharon RA, 2020
	rat	$1x10^5$	IC	7 d	no	naive	rat	CCI	Improved memory, increased neurogenesis, supressed apoptosis in the hippocampus	Shin MS, 2016
	rat	$1,5x10^5$	IV	24 h	no	naive	rat	WD	Improved neurological function; improved motor function	Silachev DN, 2015

MSCs							Animal model			Study
MSC source	**specie of origin**	**amount**	**admin route**	**time of transplant**	**multiple admin**	**modification**	**specie**	**TBI model**	**Major improvements**	**Author, year**
	human	$3x10^6$	IV	6 d	no	naive	rat	CCI	Neurite reorganization; improved functional behavior; improved cognitive behavior	Wang S, 2013
	rat	$1x10^6$	icv	24 h	no	transfected with BDNF	rat	FPI	Increased BDNF at the cerebrospinal fluid; improves neurological dysfunction;	Wang Z, 2013
	FVB mouse	$4\text{-}5x10^5$	IV	24 h	no	CXCR4 engineered MSCs	mouse	CCI	CXCR4 enhanced MSCs migration and activates Akt kinase pathway; treatment with MSCs-CXCR4 enhanced vasculogenesis; enhanced neuroprotection; improved behavioral outcome;	Wang Z, 2015

MSCs							Animal model			Study
MSC source	**specie of origin**	**amount**	**admin route**	**time of transplant**	**multiple admin**	**modification**	**specie**	**TBI model**	**Major improvements**	**Author, year**
	rat	3,00E+05	IC	24 h	no	NT3 $^{P75-2}$ gene-modified MSCs	mouse	CCI	NT3 $^{P75-2}$ gene improved survival of MSCs after transplant; improved neurological function; reduced glial activation; reduced brain water content, reduced lesion volume	Wu K, 2019
	rat	2,E+06	cavity	72 h	no	labelling inside collagen-chitosan matrix	rat	WD	Improved neurological function; improved memory; increased VEGF at the transplantation site and sorroundings; The protective effect was higher when cells were transplanted inside collagen matrix	Yan F, 2019
	mouse	?	cavity	7 d	no	Naive inside Gelatin-Hydroxyphenyl matrix	rat	WD	Improved learning and memory; increased neurothrophic factors expression; supressed cell apoptosis	Yao M, 2019

MSCs							Animal model			Study
MSC source	**specie of origin**	**amount**	**admin route**	**time of transplant**	**multiple admin**	**modification**	**specie**	**TBI model**	**Major improvements**	**Author, year**
	mouse	2,E+06	IV	24 h	no	hypoxic pre-conditioning	mouse	CCI	Improved cognitive function; reduced lesion volume; increased neural glial antigen 2 positive cells	Yuan X, 2020
	human	150 000	icv	24 h	no	labelled	mouse	CCI	Induced M2 profile of microglia in vitro and in vivo; increased neurological function; improved locomotor function	Zanier ER, 2014
	rat	$4x10^6$	IV	2 h	no	naive	rat	WD	Reduced microglia and macrophages at the brain; reduced peripheral infiltrating leukocytes; reduced pro-inflammatory cytokines	Zhang R, 2013

MSCs							Animal model			Study
MSC source	**specie of origin**	**amount**	**admin route**	**time of transplant**	**multiple admin**	**modification**	**specie**	**TBI model**	**Major improvements**	**Author, year**
	rat	100ug	IV	24 h	no	exosomes	rat	CCI	Improved spatial learning; improved neurological deficites; improved locomotor function; increased newly endothelial cells; increased newly formed neurons; reduced inflammation	Zhang Y, 2015
	human	100ug	IV	24 h	no	exosomes	rat	CCI	Improved spatial learning; improved neurological deficites; improved locomotor function; increased newly endothelial cells; increased newly formed neurons; reduced inflammation	Zhang Y, 2017
	Human	50ug	IV	24 h	no	exosomes	rat	CCI	Improved sensoriomotor and cognitive function; reduced hippocampal neuronal loss; promoted angiogenesis and neurogenesis; reduced neuroinflammation	Zhang Y, 2020

MSCs							Animal model			Study
MSC source	**specie of origin**	**amount**	**admin route**	**time of transplant**	**multiple admin**	**modification**	**specie**	**TBI model**	**Major improvements**	**Author, year**
UC	human	$2x10^6$	IV	24 h	no	naive	rat	CCI	Reduced motor and neurological deficits; MSCs that migrated to the brain expressed NeuN, MAP-2 and GFAP	Lu D, 2002
	human	1,50E+05	icv	24 h	no	naive	mouse	CCI	Improved memory, reduced lesion volume; increased CD11b; decreased CD68; decreased GFAP astrocytic marker at the lesion scar	Zanier ER, 2011
	human	$25x10^5$	IC	36 h	no	naive	rat	FPI	Trans-differentiation of Th17 into Treg Neurological recovery Learning and memory improvement	Chong Chen, 2022
ASCs	Human	1,00E+06	IV	3 h	no	naive	rat	CHI	Lower levels of TNF-α; Lower levels of β-amyloid protein	Kappy NS, 2018

MSCs							Animal model			Study
MSC source	**specie of origin**	**amount**	**admin route**	**time of transplant**	**multiple admin**	**modification**	**specie**	**TBI model**	**Major improvements**	**Author, year**
	rat	2,00E+05	IC	24 h	no	naive	rat	WD	Improved locomotor function; increased cell density in the hippocampus; enhanced neurogenesis in the hippocampus	Mastro-Martinez I, 2015
	Human	3.000.000/ kg	IV	3 d	no	naive	rat	CCI	Improved spatial learning; improved memory; Inhibition of microglial activation; reduced activated astrocytes	Ruppert KA, 2020
	Human	$4x10^6$	IV	3 h	no	CM	rat	CCI	Improved motor and cognitive function; reduced cortical damaged area; decreased hippocampal cell loss	Tajiri N, 2014

The administration dose of MSCs used in experimental TBI models in rodents varied from 1×10^5 (J. Hu et al. 2019) to 5×10^6 cells (Bonilla et al. 2009). Lower doses were reserved for intracerebral injection (Galindo et al. 2011; Hong et al. 2011; Asim Mahmood, Lu, e Chopp 2004), internal carotid artery (D. Lu, Li, et al. 2001), or lateral ventricle delivery (Zanier et al. 2011; Pischiutta et al. 2014). Other delivery routes include, intranasal (Shahror et al. 2019), and intraperitoneal, being the most frequent intravenous (Pischiutta et al. 2021). In studies with intravenous administration, higher cell dose was associated with higher cell survival rate, but not with better functional improvement (Asim Mahmood et al. 2005, 2; 2006). In TBI research, MSCs were mainly isolated from rat and human, and the used sources were mostly, bone marrow, adipose tissue, placenta, umbilical cord blood, and peripheral blood (Pischiutta et al. 2021). Despite studies pointing differences in MSCs depending on their isolation tissue (Pischiutta et al. 2021), no comparative study was performed in TBI using MSCs from different origins. Indeed, our team has demonstrated that human BM-MSCs secrete more anti-oxidative proteins, while human UC-MSCs and ASCs are more suitable to downregulate excitotoxicity (Pires et al. 2016). Of note, MSCs release different factors depending on the external stimuli. Therefore, a variety of preconditioning stimuli were used to prime MSC reactivity and potentiate the release of bioactive factors. These include, exposure of MSC to pro-inflammatory cytokines (e.g. tumor necrosis factor-α, TNFα; interferon-γ, INFγ) (Jha et al. 2018; 2019; 2021), hypoxia (E. Chang et al. 2013; C. Xu et al. 2020) or brain extract from traumatized rodents (X.-Y. Liu et al. 2020). Also, when cultured with NGF and BDNF, it is observed an increase in microtubule-associated protein-2 (D. Lu, Mahmood, et al. 2001; Asim Mahmood et al. 2002). Bang and colleagues, delivered FGF-2 with BM-MSCs and found higher expression of neuronal and astrocytic markers together with functional improvements in locomotor activity of treated animals (Bhang et al. 2007). Others have exposed MSCs to hypoxic conditions before transplant into animals, showing an increased expression of hepatocyte (HGF) and vascular endothelial growth factor (VEGF) than those cultured in normoxia. Animals treated with conditioned cells under hypoxic conditions performed significantly better in motor and cognitive tests and showed less neuronal death at the brain (E. Chang et al. 2013). With the discovery of important genes for the repair of the damaged brain, MSCs genetically modified with those genes hold improved therapeutically potential. For instance, when transfected to produce the neuroprotective substance glucagon-like peptide (GLP-1), MSCs revealed a more pronounced therapeutic effect in reverting hippocampal neuronal cell loss and glial abnormalities in the cortex of TBI animals(Heile et al. 2009). Also, MSCs engineered to overexpress IL-10, reduced inflammation to a greater extent and lead to better functional outcomes in a TBI in vivo model than MSCs alone (Peruzzaro et al. 2019). In general, MSCs have shown angiogenic, immunomodulatory, anti-apoptotic and

neuroprotective effects which may be helpful to promote TBI recovery (Pischiutta et al. 2016; Salgado et al. 2010b; Teixeira et al. 2015). Additionally, when tested in animal models of TBI these cells ameliorated functional outcomes, regarding both, sensorimotor and cognitive performance. In addition, MSCs reverted into some extent the histological damage induced by experimental TBI (Pischiutta et al. 2014; Zanier et al. 2011; Feng et al. 2017). For instance, protection against TBI pathology was induced by BM-MSCs when delivered acutely, 24h post injury. Functional activity was measured by Neuroscore test which is a composite of motor, sensory, reflex and beam walking tests (Mahamood et al 2005). Mahamood study reveals improvements in functional outcome of 3 different doses of BM-MSCs which persisted until 3 months' post injury (Mahamood et al 2005). Also Zanier and colleagues, revealed that TBI animals receiving UC-MSCs from blood 24h post injury, revealed improvements in sensorimotor functions accessed by neuroscore and beam walk tests. Specifically, 7 days post injury, treated animals, showed reduced foot faults and a higher classification on neuroscore, compared with TBI animals treated with PBS, indicating recovery of TBI induced impairment in sensoriomotor activivty. Sensoriomotor improvements induced by MSCs persisted until 4 weeks post injury (Zanier et al. 2011). In Morris water maze test TBI animals treated with UC-MSC performed significantly better than TBI mice treated with PBS, with lower latency times to find the hidden platform. Additionally, in this study, Zanier and colleagues, show that UC-MSCs from blood delivered after TBI, significantly reduced the lesion cavity volume at 7 and 35 days post injury and attenuated the gliotic scar (Zanier et al. 2011).

Above we have discussed some studies using MSCs as a therapeutic approach for TBI conditions. One may observe variability on the followed protocols by different groups. For instance, cell dose, delivery route, cells source, cells preconditioning before transplant, may influence the results obtained and standardization is a must when in attempt to find a feasible translational therapy. In a recently published meta-analysis we examined the efficacy of MSCs in improving functional and cognitive outcomes, having into consideration their source and type of transplant, timing, dose, and route of administration as well as model-related variables (species, sex, and TBI models). Last, we analyzed the effects of MSC manipulation as potential efficacy boosters. Our meta-analysis revealed that UC-MSCs show the best effect on TBI behavior, and cells lead to a better therapeutic effect when inside of protective scaffolds. Additionally, no protective effect was observed when using ASCs (**Figure 7**). Accordingly, a combinatorial approach to treat TBI, using UC-MSCs encapsulated in a protective matrix would be an interesting approach as a TBI therapy. It is also important to mention that, the therapeutic potential of MSCs in TBI was shown either by direct contact, by secretion of bioactive molecules in situ (Pischiutta et al. 2014; 2021) or by using their bioactive molecules as conditioned medium, commonly termed MSCs secretome

(Pischiutta et al. 2016; 2022). In fact, first, it was believed that MSCs protective effect was due to cell replacement. Today, there are proof that protection is also conferred by paracrine effects, by the factors these cells secrete (Pischiutta et al. 2016; 2022; Salgado et al. 2010a; Fraga et al. 2013). MSCs release a mix of components, including cytokines, chemokines, growth factors, lipids and extracellular vesicles, that act in unison to protect the brain and promote tissue regeneration (L et al. 2019). As mentioned previously, the factors released by MSCs, change, depending on their source and on external stimuli and environment. Thus, we hypothesize, that having the cells, in situ, secreting specific factors regarding the lesion environment, would be advantageous for TBI compared with the secretome of these cells. Following we better describe, BM, ASCs and UC-MSCs 3 of the most used MSCs sources that may hold promise for TBI therapy.

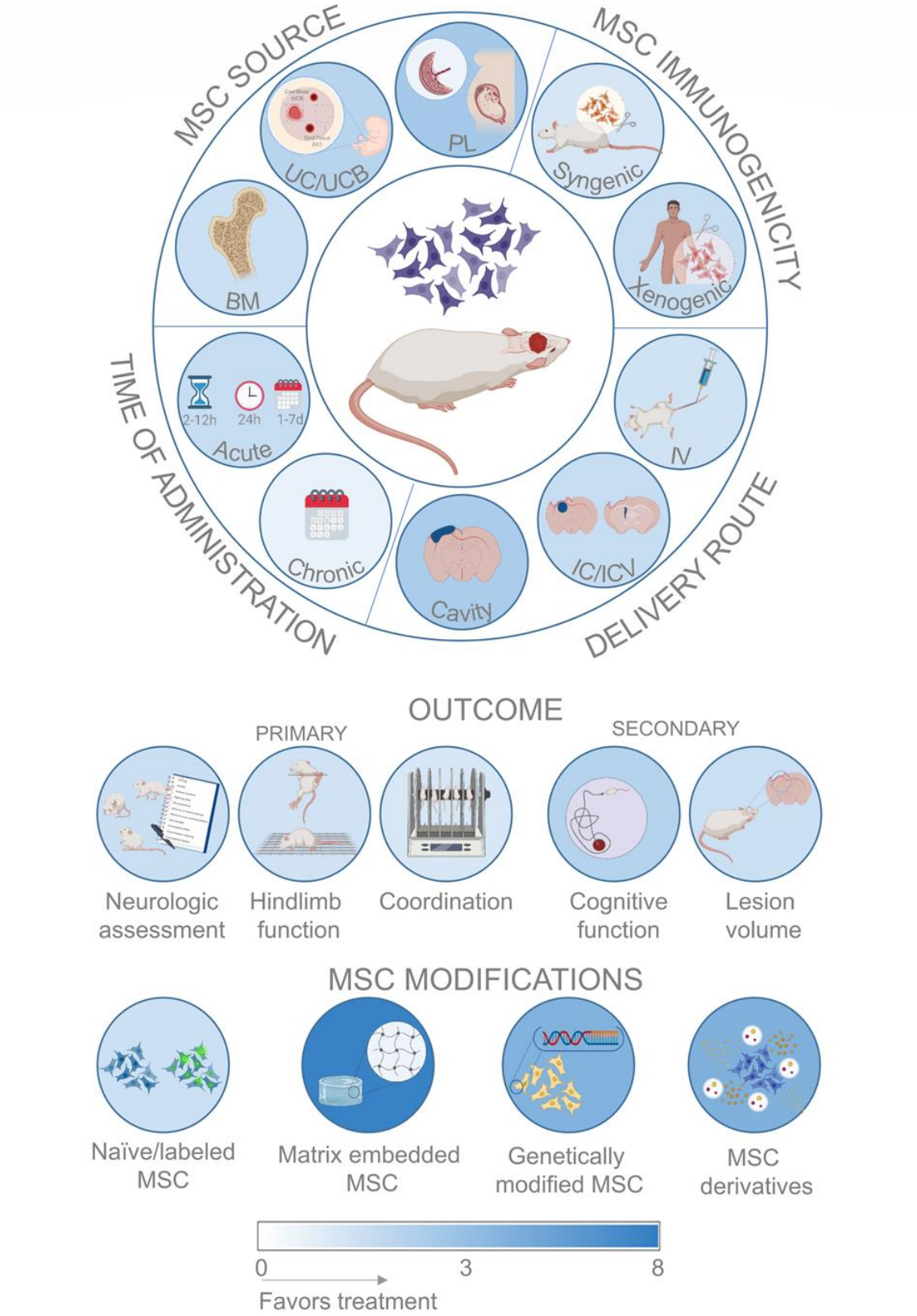


Figure 7: MSCs effect on TBI functional outcome. Graphical summary of the results obtained a recently published meta-analysis (Pischiutta et al. 2021), illustrating the main categories of MSCs sources (ASCs were excluded due to lack of works showing their effectiveness in locomotor function), time of administration, delivery route, and MSCs immunogenicity, used in TBI pre-clinical works and their favorable effect on functional and

cognitive outcome .The background colors of the circles are indicative of the effect size (as absolute SMD) as in the color scale at the bottom. Image created with BioRender.com.

Bone Marrow Mesenchymal Stem Cells

Being the first discovered, and due to their interesting properties, BM-MSCs have been the most studied from all MSCs. Harvesting these cells involves drilling a hole, usually in the person hip bone and using a syringe to extract the bone marrow. Most of the stem cells found in bone marrow are hematopoietic stem cells, platelets and white blood cells. BM-MSCs account for ~ 0.001–0.01% of bone marrow mononuclear cells. Due to its low abundance, extensive in vitro culture and expansion are necessary to ensure quantities for research or clinical use (Pittenger et al. 1999).

The research group of Mahmood provided numerous evidences of BM utility as TBI therapeutics, independently of the administration route. Specifically, their studies show that TBI rats treated with BM-MSCs, administered intracerebral, intravenously, or intra-arterially, show major improvements regarding functional and histological outcomes (Y. Zhao et al. 2016; Osanai et al. 2012; J. Hu et al. 2019; A. Mahmood et al. 2001). BM-MSCs are able to produce neurotrophic factors that promote neuronal viability and survival and stimulate axonal growth. Additionally, these cells show a high release of anti-inflammatory cytokines, and extracellular matrix molecules to the extracellular media (Zou, Liu, e Zhang 2018). Accordingly, BM have shown to inhibit apoptosis, promote angiogenesis, and stimulate host progenitor cells to differentiate toward neurons and astrocytes. In this manner, they showed the ability to repair the injured tissue, by ameliorating the neuronal dysfunction and reducing inflammation leading to a recovered function in animal models of TBI (.X. Shi et al. 2018). A study by Feng et al.(Feng et al. 2017) found that administration of BM-MSCs via tail vein injection in a rat TBI model promoted neuromotor recovery via up-regulation of endothelial and neurotrophic factors (VEGF and BDNF) and synaptic proteins (synaptophysin) in the brain. Therefore, BM-MSCs besides promoting neuroregeneration, also restored the synaptic function of newly formed neurons(Feng et al. 2017). Additionally, re-establishing blood flow to the damaged brain regions is essential to promote tissue recovery and outcome improvements. BM-MSCs have shown angiogenetic potential, by secreting factors such as VEGF and vascular growth factors (Y. Huang et al. 2020; Merckx et al. 2020). BM transplantation increased the number of endothelial progenitor cells in the peripheral blood of rats with TBI as well as the expression of angiogenic and neuronal markers while improving the neurological function of these animals(W. Hu et al. 2018). Additionally, in other study, besides the upregulation of angiogenic factors such as angiogenin-1 e VEGF, it was observed the formation of micro-vessels (W. Hu et al. 2018).

Adipose Stem Cells

ASCs are isolated after enzymatic digestion of fat lipoaspirates, discarded from weight lost surgery or plastic surgery (Lamblet e Ferreira 2022; Ohta et al. 2018). Accordingly, these cells are very attractive for regenerative medicine applications due to their simple harvesting procedure, while they maintain most of BM and other MSCs characteristic(Bunnell 2021). As umbilical cord, ASCs have the advantage to be maintained in vitro for extended periods, keeping stability during passages and low levels of senescence. Besides their multipotent potential, ASCs are known for their paracrine activity, secreting several growth factors, cytokines, ECM molecules, among others (Trzyna e Banaś-Ząbczyk 2021). For instance ASCs release neurothrophic factors with neuroprotective properties such as BDNF and GDNF Salgado et al. 2010a). The secretome of ASCs has immunoregulatory properties being effective to mitigate TBI neuroinflammation, reducing neuronal death and contusion volume and increasing neurogenesis(Tajiri et al. 2014). Other studies show that ASC secretome delivered after TBI improves neurological function while decreasing neuroinflammation and consequent edema, neuronal apoptosis and nerve fiber damage (C. Xu et al. 2020). Although, it is worth to mention inconsistency of ASCs therapeutic effects on TBI functional deficits. Kappy and collaborators, for instance, despite observing a decreased inflammation and less neuronal damage, in TBI animals treated with ASCs 3h post injury found no differences in functional recovery (Kappy et al. 2018).

Umbilical Cord Stem Cells

There is a growing interest in using MSCs derived from the human umbilical cord (UC-MSCs). In fact, several recent reviews highlight the therapeutic efficacy of UC-MSCs and their potential advantages over other sources (Joerger-Messerli et al. 2016; Kalaszczynska e Ferdyn 2015; Nagamura-Inoue e He 2014). From the many advantages one may specify the noninvasive collection and easiness to obtain, stronger self-renewal and differentiation abilities comparing with other MSCs (J.-Y. Hsieh et al. 2010), low immunogenicity and less risk of rejection after transplantation (Fong et al. 2011; Romanov, Svintsitskaya, e Smirnov 2003). Further, from a technical point of view they grow quickly in vitro and reach higher confluence levels (Sarugaser et al. 2005; Troyer e Weiss 2008). Additionally, our recent meta-analysis, shows that, following the gold standard, BM, UC-MSCs is the most used cell source in TBI research (Pischiutta et al. 2021). They are collected from the discarded UC at the child birth and, for this reason lack ethical concerns (Baudin et al. 2007). The UC is composed by two umbilical arteries and one umbilical vein, both embedded in a specific mucous of connective tissue known as wharton's jelly

UC-MSCs may be harvested form UC blood, UC Wharton's Jelly or UC perivascular tissues (Gökçinar-Yagci, Özyüncü, e Çelebi-Saltik 2016; Bongso e Fong 2013; Davies, Walker, e Keating 2017) (**Figure 8**). Previous studies shown that there are differences on MSCs, provided from different anatomical structures within the cord. For instance umbilical perivascular cells (HUCPVCs) have a different phenotype and increased proliferative capacity, compared with the remaining tissue of the core (Nanaev et al. 1997). To add, perivascular cells from the UC render a vastly increased number of, MSCs, comparing with UC blood (Ennis et al. 2008).

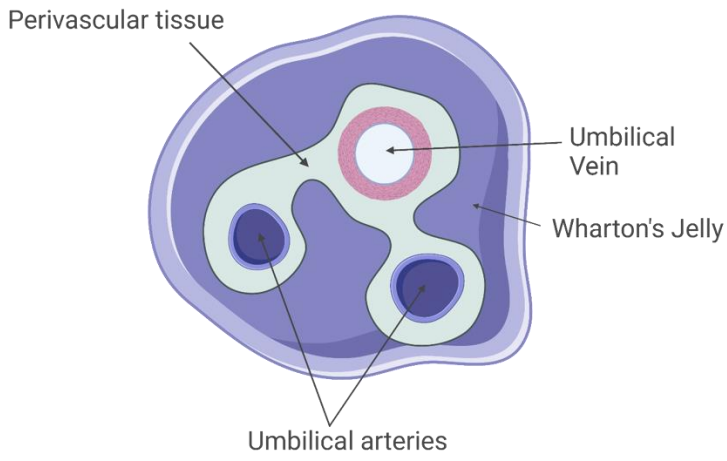


Figure 8. Umbilical cord. Umbilical cord transversal cut with the main structures that compose it. The cord has 2 arteries and 1 vein involved in perivascular tissue. All these structures are inside the mucous of connective tissue that fulfills the umbilical cord.

In 2011, Zanier, showed that UC-MSCs delivered intraventricularly, 1day post injury, can effectively restore brain injury and improve TBI neurological outcomes (Zanier et al. 2011). Specifically, human UC-MSCs evoked trophic events, microglia/macrophage phenotypical switch, and inhibited glial scar formation (Zanier et al. 2011). Trophic support was conceded by secretion of neurotrophin-3 (NT-3), neutrophil activator, BDNF, VEGF and fibroblastic grow factor (FGF), that shown to induce neuronal regeneration and vascularization in the damaged area (Pires et al. 2016). Others studies, show comparable results when treating TBI animal with a cell free therapy, using UC-MSC secretome. Liu et al, got significant improvements in cognitive function of TBI animals 4 weeks post TBI, when treated with UC-MSCs secretome. Associated to behavioral improvements, Liu and collaborators, observed increased neurogenesis and reduced apoptosis at the lesion core (X.-Y. Liu et al. 2020). In addition, UC-MSCs from

perivascular tissues proved to provide axonal survival cues, leading to improved axon morphology of TBI treated animals compared with non-treated animals (Barretto et al. 2020). UC-MSCs have as well anti-inflammatory properties that may prevent and decrease secondary brain injury (G. Wang et al. 2022). In traumatic brain injury rat models, the transplantation of UC-MSCs triggers the trans-differentiation of T-helper 17 into T regulatory, which in turn repairs neurological deficits and improves learning and memory function (C. Chen et al. 2022).

Clinical trials

Despite the promising outcomes observed in pre-clinical studies just a few clinical trials have assessed the safety, feasibility, and efficacy of MSC therapy for TBI patients. Currently, some clinical trials are in recruiting phase (NCT02525432 NCT05951777, NCT05018832, NCT04063215, NCT04744051).

Kawabori and colleagues conducted double-blinded phase two study (NCT02416492) to assess the effectiveness, safety, and tolerability of a stereotatic intracranial implantation of allogenic modified BM-MSCs (SB623) (Kawabori et al. 2021). These cells were modified with a plasmid containing Notch 1 domain to reduce the differentiation potential and enhance trophic factors secretion. The study involved a total of 63 patients with stable chronic motor deficits TBI related (1.4-28.4 years post-TBI). Patients were randomized to control or SB623 treatment groups, at doses of 2.5, 5, or 10 M cells, and followed for 6 months. These study reported safety and preliminary improvement of several neurological parameters.

The non-random, open-label interventional cohort study by Tian and colleagues (97 patients) evaluated the safety and efficacy of 1M autologous BM-MSCs transplanted by lumbar puncture in TBI patients, one month or more after injury (Tian et al. 2013). Within a period of 14 days some patients with persistent vegetative state and motor disorders demonstrated varying degrees of improvement in consciousness or motor function (37 %). Younger patients exhibited higher chances for improvement.

Wang et al (S. Wang et al. 2013) conducted a randomized and blind controlled trial using umbilical cord mesenchymal stem cells to address TBI sequelae, in chronic patients. Over a period of 57 days 20 patients (with 20 controls) received injections via lumbar puncture with $1x10^7$ cells (four times). This treatment led to improvements in motor function, sensation, and balance scores six months after transplantation. Additionally, the patients experienced advancements in their mobility, self care abilities, communication skills and sphincter control.

4. Hydrogels for cell-based therapies

As observed in the previous chapters, besides promising, MSCs therapy is hampered by a wide variability of results. Conceptual issues, methodological differences between laboratories, TBI models, administration dose, and others, may affect the outcome of a specific cell therapy. The administration route is one of the parameters that could lead to a different outcome using the same cell therapy. Despite the higher permeability of the BBB observed after injury, this barrier stills poses an obstacle in the administration of peripheral therapeutic agents. Although some targeted alternatives to bypass this barrier are being explored their efficacy was not proven. Therefore, local administration of therapeutic agents seems the best approach to guarantee the highest cell survival at the injury site (C.-T. Lu et al. 2014). Unfortunately, poor cell survival at the lesion core (Sortwell, Pitzer, e Collier 2000; Müller-Ehmsen et al. 2006), cell aggregation, and lack of cell integration into the host tissue constitute major challenges for cell transplantation approaches. Specifically, in traumatic CNS lesions few surviving cells proliferate at or near their injection point due to the hostile environment of the injured tissue which complicates integration. Several mechanisms may contribute to cell death during and post-transplantation. The main include mechanical forces exerted during cellular injection, anchorage-dependent cell death without sufficient growth factors, as well as limited host tissue support, which also includes restricted vasculature accessibility.

Biomaterials successfully tackle these challenges, enhancing cells survival and performance after transplant (Rocha, et al 2020). From the available biomaterials for cell therapies, hydrogels, are crosslinked polymer chains with high biocompatible properties and water content, that resemble living tissues, and thus are suitable for tissue regeneration applications (Lee J et al 2021). Following we describe how hydrogels may counteract the main cell death triggers during or post- cell transplantation into host tissues.

Mechanical stress: Mechanical stress during cell injection is a major reason of cell death (Aguado et al. 2012; Amer et al. 2016). The needle diameter is much thinner than the syringe, which increases pressure and cell membrane break with consequent cell death. In addition, the needle diameter is too small for the cells size (Aguado et al. 2012; Amer et al. 2016). Mechanical stress during the injection process may be overcome trough incorporation of shear-thinning biomaterials. These biomaterials behave as viscous fluids under shear stress and recover solid like properties upon stress elimination. For instance, alginate or hyaluronic acid (HA), have the previous described behavior and will lubricate the syringe upon cells delivery, leading to uniform flow rates during the injection process (Wagner, Marks, e Bhatia 2014). Another aspect is that, the injected hydrogel will polymerize at the injection site, limiting the cells to that

region and increasing the remaining cells in the tissue after transplantation. Otherwise, interstitial pressure of the tissue would push the cells out. This is a well-known phenomenon and is called ''cellular backflow after injection''(Ballios et al. 2015). For instance, when MSCs are delivered in an alginate hydrogel, cells retention at the injury site is 60% of the starting population, while the animals injected with cells in saline, without any protection matrix, only kept 9% of the injected cells, 24h post injury (Roche et al. 2014).

Cell death of anchorage dependent cells: The majority of cells, excepting hematopoietic cells, need to adhere to matrix to survive (Ishikawa et al. 2015). When delivered as a treatment, cells lack the adherence molecule that guarantees their survival. Integrins are cell surface receptors which bind to extracellular matrix components and promote cell survival (Mishra e Manavathi 2021). Biomaterials with integrins or integrin ligands may overcome this drawback. Specifically, molecules from the extracellular matrix that act as integrin ligands such as collagen, laminin, fibronectin or HA, can prevent cell death induced by anchorage lack (anoikis)(Åberg, Edén, e Siegbahn 2020; Benoit et al. 2007). For instance, the use of a collagen I and laminin scaffold in an animal model of TBI, increased more than 5 times cells survival compared with cells injected without scaffold. Additionally, only the animals treated with cells and biomaterial reached behavioral recovery (Tate et al. 2009). As for integrin binding domains, the tripeptide RGD (arginine-glycine-aspartic acid) is the best well-known and studied example. This domain binds to, at least, eight different integrin complexes(Pierschbacher e Ruoslahti 1984; Gahmberg e Grönholm 2022). The use of RGD-alginate (S. S. Ho et al. 2016) and RGD-gellan-gum hydrogels (Gomes et al. 2020; 2016) resulted in a marked increase in cell survival in vitro (Silva et al 2012) and improved SCI animals outcome and histological deficits (Gomes et al. 2020).

Limited host tissue support: In degenerating tissues, secreted cell factors are lost. Cell factors are crucial for transplanted cells survival. Biomaterials may, besides acting as cell carriers, work as a matrix for controlled growth factors release, offering support for the transplanted cells to survive at the lesion site environment (J. Parker, Mitrousis, e Shoichet 2016). For example Ma and collaborators added stromal cell-derived factor (SDF-1), to a sodium alginate and collagen matrix, aiming to improve cells viability inside the gel and resistance after transplant into a TBI animal model(S. Ma et al. 2021). SDF-1 is a key factor that regulates cell survival, recruitment and differentiation. From all the groups in this study, the matrix with SDF1 and cells showed the best results regarding cell survival and proliferation, behaviour outcomes and tissue deficits resolution(S. Ma et al. 2021).Additionally, due to their porous characteristics,

hydrogels are ideal materials for cells or drug loading. Specifically, they fit MSCs therapies, as they allow cell secreted factors to diffuse through the porous membrane to the extracellular space and play their function at the lesion core. The rational design of hydrogels to transplant cells requires an understanding of molecules diffusion throughout the hydrogel. By tuning the crosslinking and density of hydrogels, the diffusion rate may be controlled(T.-C. Ho et al. 2022). Therefore, these biopolymers may be adapted for both cells or secretome delivery(Weber, Lopez, e Anseth 2009; D. Silva et al. 2023). To find the ideal hydrogel for each purpose, properties such as, thermal sensitivity, biodegradability, and injectability should be taken into consideration. These properties are vital for cell-hydrogel and hydrogel–tissue interactions.

4.1 Hydrogels and MSCs for TBI

With the purpose to treat traumatic events, such as TBI with skull perforation, and cavity formation, hydrogels offer the possibility to fill the irregular lesion sites, resulting in a better coverage of the damage tissue (Ulndreaj, Badner, e Fehlings 2017). Another important concern to have in mind when idealizing hydrogels as a local therapy for TBI is the intracranial pressure they might induce. Thus, in TBI, locally applied treatments should mostly respect the soft nature of the tissue, offering physical support and adapting to the lesion site while impeding exacerbated inflammatory reactions. It is also important to guarantee hydrogel porosity for the factors and cells to migrate to the lesion core and play their function. Hydrogels counteract a strong drawback of cell therapy, offering cells protection and reducing cell death after transplant. Furthermore, in TBI animal models hydrogels have been shown to fill the lesion cavity, promoting both tissue and functional recover (S. Ma et al. 2021).

Hydrogels may be classified as synthetic or natural **(BOX 3)**. Synthetic hydrogels offer the advantage to manipulate their physical and chemical properties, as well as, degradation rates, which allows to better adapt the polymer to specific scientific needs (Belanger et al. 2016). As synthetic hydrogels we can mention Poly(lactic acid) (PLA) and Poly(lactic-co-glycolic acid) (PLGA), Methacrylate-Based Hydrogels, Poly(ethylene glycol) (PEG), etc. **(BOX 3)**. Integration into the host tissue is crucial when idealizing a hydrogel therapy. Therefore, formulations to use in biomedical field tend to use natural polymers or molecules from living tissues. For neuroregeneration, natural-based hydrogels are commonly composed by ECM proteins or molecules with features that attract brain cells. This facilitates the scaffold integration within the host (A. J. Salgado et al. 2008). As natural hydrogels, we mention, for example Agarose, Sodium Alginate (SA), collagen, Hialuronic acid (HA), and gellan gum (GG) **(BOX 3)**.

BOX 3 I Hydrogels classification

Synthetic

Poly(lactic acid) (PLA)

Poly(lactic-co-glycolic acid) (PLGA)

Methacrylate-Based Hydrogels

Poly(ethylene glycol) (PEG)

Natural

Agarose

Sodium Alginate (SA)

Collagen

Hialuronic acid (HA)

Chitosan

Gellan gum (GG)

Despite there are works using natural (Alvarado-Velez et al. 2021) or synthetic hydrogels (Townsend et al. 2018) separately, there are some that use a combination of both in order to reach specific features. For instance, Jeong da Un(Jeong et al. 2021) and Macks, C.(Macks et al. 2022), in different studies, combined the synthetic hydrogel PEG and the natural hydrogel HA to deliver a steroidal anti-inflammatory drug, dexamethasone, in a TBI rat model. In both works the hydrogel treatment, successfully reduced the cavity size, inflammatory response and apoptosis induced by TBI. In addition, Macks, C. and co-workers, observed significantly improved motor and cognitive functions after treatment (Macks et al. 2022).

The natural hydrogel agarose modified with Fas ligand was used to transplant BM-MSCs to TBI animals. Agarose-Fas hydrogels, dramatically reduced CD8+ T cells and enhance MSCs survival at the injury site. Additionally, the authors found increased neurotrophic factors at the lesion site of TBI animals but not in animal treated only with cells (Alvarado-Velez et al. 2021).

Other work in 2021, took advantage of two natural biopolimers, to produce an alternative neural regeneration scaffold for brain injury repair. Ma et all joined together sodium alginate (SA) and collagen with BM-MSCs inside, to treat TBI rats. Additionally, this scaffold was modified with the stromal cell-derived factor 1 (SDF-1), an essential factor to promote MSCs survival, recruitment and differentiation(S.

Ma et al. 2021). Animals treated with SA/Collagen/MSCs, showed reduced brain cavity volume, neuronal death and inflammation. Furthermore, this therapy, significantly improved motor and cognitive function of TBI rodents(S. Ma et al. 2021). Importantly, this beneficial effects were always higher when compared with the BM-MSCs delivered alone (S. Ma et al. 2021).

SA together with HA was also proposed as a promising combination to treat TBI (K. Zhang et al. 2018). SA/HA used to load UC-MSCs induced a therapeutic effect on TBI animals. Specifically, animals that received SA/HA/MSCs revealed motor recovery and improved learning and memory abilities. Additionally, the study shows higher expression of differentiation, proliferation and tissue regeneration markers in animals that received the scaffold with UC-MSCs, comparing with other tested models (K. Zhang et al. 2018).

Sultan synthesized silk fibroin-based hydrogels for the encapsulation of human MSCs (Sultan et al. 2021). Here the encapsulated BM-MSCs were engineered to overproduce BDNF. In vitro, it was confirmed that BDNF released by MSCs was inducing neurite growth of co-cultures neuronal cells. In this study, TBI rats were treated with the biopolymer by trans-septal transplantation. Bioengineered MSCs inside the silk matrices, induced a significant increase in BDNF levels 7 days post implantation. This treatment promoted neurological function recovery via significantly reducing neuronal death in the hippocampus 7 days post-injury, as well as reducing lesion area (Sultan et al. 2021).

Injectable hydrogels based on self-assembled peptides have shown great potential for regenerative medicine. For instance, Wang et al. have successfully developed peptide hydrogels using the sequences RADA16 and SVVTGLR resulting in nanofiber formation and gel like scaffolds (T.-W. Wang et al. 2017). Importantly, the presence of the SVVYGLR motif improves angiogenic performance of these peptide based hydrogels. Specifically, it enables the formation of a connected network of endothelial cells which, in turn, enhances migration, proliferation, and differentiation of endogenous neural stem cells. In vivo, RADA16-BDNF peptide scaffold, (R-B-SPH scaffold), a self-assembling peptide hydrogel, were used to seed UC-MSCs co-cultured with activated astrocytes (W. Shi et al. 2016). When transplanted into TBI animals, this scaffold repaired moderate-sized lesion cavities, although no protective effect was induced on large cavities(W. Shi et al. 2016). Additionally, co-culture of astrocytes and MSCs promoted BDNF secretion which may benefit to both neural differentiation of MSCS and endogenic neurogenesis (W. Shi et al. 2016).

4.2. Gellan Gum

GG is a natural polysaccharide produced by Pseudomonas elodea, composed of L-rhamnose, D-glucuronic acid and two D-glucose subunits (Giavasis, Harvey, e McNeil 2000) **(Figure 9).**

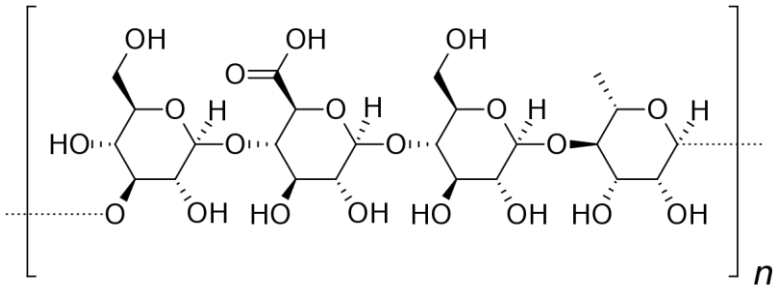


Figure 9. Gellan gum chemical structure. GG is a high molecular weight polysaccharide composed of repeating units of monosaccharides. The primary monosaccharides present in GG are rhamnose, glucuronic acid and glucose.

Commercially, GG is usually produced by batch fermentation using the Sphingomonas paucimobilis strain, since these species originate higher yields and purity of GG (Bajaj et al. 2006). One of the defining properties of the GG polysaccharide is the ability to form hydrogels. Specifically, when in presence of Ca^{2+} or Mg^{2+}, the junction zones of GG mesh are stabilized which start the gelation process, forming a stable hydrogel structure (Miyoshi, Takaya, e Nishinari 1995). GG has high water content, is porous and, being soft-like material, will adapt easily different tissue lesions, while allowing the factors to diffuse lesion site. GG is particularly interesting for tissue engineering due to their easiness for blending and chemical modification (Ferris et al. 2013). Despite cytocompatible, GG lacks specific cell-binding sites, which may be solved by adding specific anchorage motifs to this matrix. A wide range of modifications are available aiming to modify GG properties, reviewed here: ''Tissue engineering with Gellan Gum''(Stevens et al. 2016). For instance, arginyl-glycyl-aspartic acid (RGD) sequence, is one of the most well-known peptides for chemical modification. The covalent attachment of RGD to GG induces cells to actively bind with the polysaccharide as they would for natural ECM, improving growth and differentiation behaviors of neural stem cells (N. A. Silva et al. 2012), primary cortical neural tissues (Lozano et al. 2015), and mesenchymal stem cells (N. A. Silva et al. 2013). Similar to RGD, the laminin-derived peptides IKVAV and YIGSR and the collagen-derived peptide GFOGER can be used to improve cell adhesion and survival, associated with biomaterial therapies (Negishi e Nomizu 2019; Sawicki et al. 2019). In 2012, Silva et al. investigated GG on rat adult NSCs/PCs (N. A. Silva et al. 2012). The study demonstrated the crucial impact of RGD

peptide to provide interface sites for cell anchorage, enhance neurite extension and prevent cell apoptosis (N. A. Silva et al. 2012).

In vitro, ASCs and OECs co-cultured within GG-GRGDS hydrogels led to higher neurite outgrowth of dorsal root ganglions when compared to controls. When transplanted to SCI rat models this scaffold led to significant motor improvements, less inflammatory cells infiltration and astrocytes, together with increased expression of neurofilament (Gomes et al. 2016) In 2015, Lozano et al. showed that GG-RGD supported cell viability, differentiation, and promoted neurite invasion into neighboring layers, in a 3D mammalian brain cortex tissue model(Lozano et al. 2015). The model consisted of rat primary cortical neurons arranged in six layers within GG-RGD. This works highlights that RGD-modified gellan gum protected the cells during the bioprinting process. In a different research, Koivisto and collaborators proposed an alternative method for GG crosslinking using bioamines. This studied showed that the hydrogels crosslinked with bioamines have similar mechanical and rheological properties comparable to the naïve rabbit brain. Additionally, these hydrogels were biocompatible with NSCs derived from hIPSCs (Koivisto et al. 2017). When laminin was added to the crosslinking gels, it was noticed a significant increase in neurites length and number on top of gel surfaces(Koivisto et al. 2017). Other in vitro work, performed in 2020, used a modified structure of GG in which a cysteine group was added to the conventional GG chain to form a thiolated gellan gum (TGG). TGG modified with laminin and loaded with NGF, and NSC show to have excellent biocompatibility features. The NSC adhered to tightly to the scaffold and differentiated into neurons (W. Li et al. 2020).

Notably, several studies, had demonstrated GG cytocompatibility in vitro. Few in vivo studies on CNS pathological conditions, confirmed the biocompatibility and delivery scaffold features of GG. Although, the published works show interesting results and hold great potential. Gomes and colleagues showed the therapeutic effect of GG-GRGDs to deliver MSCs into both lumbar (Gomes et al. 2016) and cervical (Gomes et al. 2020) SCI lesions. The scaffold of GG-GRGDs with a co-culture of ASCs and Olfatory ensheating Cells (OECs) was delivered into a SCI animal model. Treated animals showed significant motor improvements compared to control and TBI animals treated with empty gel or the cells alone. These beneficial results were accompanied by a decrease in GFAP and Cd11b labeling, pointing to reduced inflammatory levels. Additionally, treated animals with GG-GRGDs and cells showed increased NF intensity values (Gomes et al. 2016). Other works, attempted GG in a different approach than local delivery to reach the brain. Intranasal administration is a widely recognized approach for drug delivery into the brain. Zhou S. and colleagues formulated an efficient drug delivery approach, using glutathione modified gellan gum (GSH-GG) encapsulated on galanthamine hydrobromide loaded liposomes (GH-Lipo@GSHGG), to

reach the brain via nasal delivery(Zhou et al. 2022). In accordance, using the same delivery method, Rajput and co-workers incorporated a resveratrol nanostructures carrier into scaffolds of a mixture of gellan gum and zanthan gum. The hydrogel matrix increased the permeation across the nasal membrane when compared to resveratrol delivered freely (Rajput et al. 2018). When delivered in rodents the hydrogel with resveratrol significantly improved their memory function evaluated in Moris Water Maze test (Rajput et al. 2018).

5. Conclusion

TBI is a complex and multifactorial disorder affecting millions of people worldwide, significantly decreasing their quality of life, as a consequence of the increasing disability caused by disease progression. Due to the dual nature of most of the mechanisms triggered during trauma, modulating the secondary cascade induced by the first mechanical impact to the tissue, appears beneficial in detrimental to silence specific mechanisms.

Cell therapy, specifically MSCs, have shown promising results in counteracting TBI deficits, reducing inflammation and promoting protective and regenerative mechanisms. Despite this, cell therapeutic effect is highly influenced by the lesion site environment, which changes MSCs secreted factors. Research throughout the years has revealed that MSCs delivery, directly at the lesion core, may be improved as most of the cells die, losing the capacity to engraft and play their roles in situ. Additionally, TBI cavities must be filled with a physical support simultaneously allowing the engraftment of transplanted cells and the restoration of the tissue cytoarchitecture.

Biomaterials, specifically hydrogels, have come a long way in regenerative medicine and now constitute an indispensable component of a successful cell transplantation strategy. Combinatorial approaches that aim to increase cell survival, decrease inflammation and improve host integration have been developed. GG, specifically, is an FDA approved, natural based hydrogel, that meets CNS physical and chemical properties. Modifications with GRGDS have shown beneficial to enhance cell survival and engraftment. GG-GRGDS has been tested in SCI models as a successful therapy when combined with ASCS and OECS. In this sense, MSCs factors released in situ, when these cells are encapsulated in GG-GRGDs matrices may be used as a novel approach to successfully counteract TBI secondary cascade.

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CHAPTER II

A novel organotypic cortical slice culture model for traumatic brain injury: molecular changes induced by injury and mesenchymal stromal cell secretome treatment

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CHAPTER II | A novel organotypic cortical culture model for traumatic brain injury: molecular changes induced by injury and mesenchymal stromal cell secretome treatment

Abstract

Traumatic brain injury (TBI) is a major worldwide neurological disorder with no neuroprotective treatment available. Three-dimensional (3D) in vitro models of brain contusion serving as a screening platform for drug testing are lacking. Here we developed a new in vitro model of brain contusion on organotypic cortical brain slices and tested its responsiveness to mesenchymal stromal cell (MSC) derived secretome.

A focal TBI was induced on organotypic slices by an electromagnetic impactor. Compared to control condition, a temporal increase in cell death was observed after TBI by propidium iodide incorporation and lactate dehydrogenase release assays up to 48h post-injury. TBI induced gross neuronal loss in the lesion core, with disruption of neuronal arborizations measured by microtubule-associated protein-2 (MAP-2) immunostaining and associated with MAP-2 gene down-regulation. Neuronal damage was confirmed by increased levels of neurofilament light chain (NfL), microtubule associated protein (Tau) and ubiquitin C-terminal hydrolase L1 (UCH-L1) released into the culture medium 48h after TBI. We detected glial activation with microglia cells acquiring an amoeboid shape with less ramified morphology in the contusion core. MSC-secretome treatment, delivered 1h post-injury, reduced cell death in the contusion core, decreased NfL release in the culture media, promoted neuronal reorganization and improved microglia survival/activation.

Our 3D in vitro model of brain contusion recapitulates key features of TBI pathology. We showed protective effects of MSC-secretome, suggesting the model stands as a tractable medium/high throughput, ethically viable, and pathomimetic biological asset for testing new cell-based therapies.

Keywords: “traumatic brain injury”, “3D in vitro model”, “organotypic cortical brain slices”, “mesenchymal stromal cells”, “secretome”

1. Introduction

Traumatic brain injury (TBI) is defined as an alteration in brain function, or other evidence of brain pathology, caused by an external force (Menon et al., 2010). About 50-60 million people sustain a TBI each year, with a high impact on individuals, their families, and the society (GBD 2016 Traumatic Brain Injury and Spinal Cord Injury Collaborators, 2019; Maas et al., 2022). There have been advances in the clinical management of TBI patients, but no neuroprotective or regenerative drugs are available to prevent or mitigate injury progression and chronic neurological deficits.

Animal models have been successful in identifying pathological mechanisms induced by TBI and they are a fundamental step for therapeutic development. However, 3D in vitro models recapitulating brain structural complexity are needed as a viable platform for drug screening to address the need to replace, reduce, and refine animal testing (Neuhaus et al., 2022; Wadman, 2023). Organotypic brain slices fit these features well, preserving anatomical architecture, cellular diversity and connectivity, as well as extracellular matrix composition (Nogueira et al., 2022). So far, organotypic hippocampal brain slices have been used to model TBI by stretch (Di Pietro et al., 2013; Dollé et al., 2013; Kang et al., 2015; Omelchenko et al., 2019), blast (Vogel et al., 2016, 2017b) or compressive injuries (Adamchik et al., 2000; Adembri et al., 2008; Coburn et al., 2008; Harris et al., 2013). However, a purely contusive model on cortical areas mimicking a TBI focal lesion is still missing. Thus, we developed a new in vitro model of brain contusion by exposing murine cortical organotypic slices to a controlled impact (CCI) delivered by an electromagnetic impactor as largely characterized for in vivo TBI studies in rodents.

In vivo TBI studies across a range of different models and injury severities show that mesenchymal stromal cells (MSCs) improve functional outcome and anatomical damage (Pischiutta et al., 2021). The pleiotropic protective and reparative events induced are mainly mediated by released bioactive factors contained in the MSC-secretome (Tajiri et al., 2014; Mendes-Pinheiro et al., 2019; Pischiutta et al., 2022). We previously found a protective effect of MSC-secretome on ischemic organotypic brain slices (Pischiutta et al., 2016) and here we tested its activity after a biomechanical impact. We observed that MSC-secretome treatment reduced cell death, restored the network complexity, and promoted microglia response, providing evidence for its application as an effective tool for drug testing.

2. Methods

Study approval

The IRFMN adheres to the principles set out in the following laws, regulations, and policies governing the care and use of laboratory animals: Italian Governing Law (D.lgs 26/2014; Authorisation n.19/2008-A issued March 6, 2008 by Ministry of Health); Mario Negri Institutional Regulations and Policies providing internal authorization for people conducting animal experiments (Quality Management System Certificate – UNI EN ISO 9001:2008 – Reg. N° 8576-A); the NIH Guide for the Care and Use of Laboratory Animals (2011 edition) and EU directives and guidelines (EEC Council Directive 2010/63/UE). They were reviewed and approved by the Mario Negri Institute Animal Care and Use Committee which includes ad hoc members for ethical issues and by the Italian Ministry of Health (Decreto no. D/07/2013-B).
C57BL/6J mice were bred (one male and two females per cage) in a specific pathogen free vivarium at a constant temperature (21±1°C) and relative humidity (60±5%), with a 12h light–dark cycle and ad libitum access to food and water. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Prefrontal cortex slices collection

Organotypic cortical brain slices were obtained under sterile conditions from the prefrontal cortex of C57BL/6J or Cx3Cr+/GFP (expressing green fluorescent protein in microglial cells) mouse pups (P1-P3), as previously described (Dossi et al., 2013; Pischiutta et al., 2016) and as represented in Figure S1. Briefly, the brain was removed from the skull and embedded into an agarose solution (3% w/V, Promega). Agarose blocks containing mesencephalic and forebrain areas were fixed onto the specimen stage of a vibratome (Leica, VT 1000S), with super glue. The fixed brain was kept in ice-cold (4°C) artificial cerebral spinal fluid (ASCF, NaCl = 87mM, NaHCO3 = 25mM, NaH2PO4 = 1.25mM, MgCl2 = 7mM, CaCl2 = 0.5mM, KCl = 2.5mM, D-glucose = 25mM, sucrose = 75mM, Penicillin = 50U/ml, Streptomicin = 50µg/ml) and oxygenated with 95% of oxygen and 5% CO2. Prefrontal cortex slices with 200µm were cut and transferred onto petri dishes with cold ACSF (4°C). Intact slices were transferred to membranes of tissue culture inserts (Millicell Culture insert, 0.4 µm pore size, Merk-Millipore). The slices were maintained in culture (37°C, 5% CO2), two by two per insert in a 6-well plate. Each well was filled with 1 ml of culture medium (MEM-Glutamax 25%, basal medium eagle 25% [Invitrogen], horse serum 25% [Euroclone], glucose 0.6%, Penicillin 100U/ml, Streptomicin 100µg/mL [Euroclone]; pH=7.2) for two

days, and then cultured in neurobasal medium supplemented with B27 (NB/B27, B27 1:50, L-glutamine 1:100, penicillin 100U/mL, streptomycin 100µg/mL) with medium changes every other day.

In vitro controlled cortical impact (CCI)

After one week in culture, cortical slices were subjected to controlled cortical impact (CCI) delivered by an electromagnetic impactor device (Impact OneTM, Leica) to model TBI. A stereotactic apparatus, connected to the impactor was used to set the trauma depth. Each insert with slices was taken from the plate and placed under the impactor, with the proper medium supply. The damage was driven by a 1 mm diameter impactor tip, at 1.5 m/s of speed and 0.1 sec dwell time. At first, we tested three different deformation depths: 0 µm (the piston just touched the slice, without penetrating it), 100 µm or 200 µm, the latter corresponding to the thickness of the whole slice (experimental design in Fig. 1a). The 100 µm depth induced a significant increase in PI incorporation with no tissue perforation, thus it was selected for the next experiments (experimental design in **Fig. 1c**). Control slices were subjected to the same protocol, without injury.

Propidium iodide incorporation

CCI induced cell death was measured by propidium iodide (PI) incorporation assay at tissue level. At 24h and 48h after injury, slices were incubated with PI (2 µM diluted in NB/B27) for 30 minutes, washed with PBS and photographed using an Olympus IX71 inverted microscope. Fluorescence intensity per slice was measured using ImageJ's Integrated Density function and the value was normalized over the slice area. In order to evaluate cell death in a region-specific manner, the fluorescence intensity was also evaluated in specific regions of interest (ROIs), drawn in a concentric manner, spaced 0.5 mm, starting on the lesion site to the slice periphery.

Lactate dehydrogenase assay

Cell death was evaluated by a Lactate dehydrogenase (LDH) assay in the culture medium collected at time 0 (before injury), 4, 8, 24, and 48h after injury and stored at −20°C. LDH release was measured using Cytotox 96 Non-Radioactive Assay (Promega) following manufacturer instructions. Absorbance was read at 490 nm (TECAN, Infinit®200 PRO).

Quantification of injury biomarkers released in the culture medium

To assess neuronal damage, we quantified the release of three different neuronal markers in the culture medium: the neurofilament light chain (NfL), the tubulin associated unit (Tau) and the Ubiquitin C-terminal hydrolase L1 (UCH-L1). Injury to astrocytes was assessed by measuring the release of the glial fibrillary acidic protein (GFAP). Culture medium was collected 48h post-injury, and stored at −20°C. Biomarker releases in the culture media were measured by single molecule array (simoa) immunoassay (Quanterix, Billerica, MA). Analyses were run using commercial simoa assays on a SR-X Analyzer: NfL advantage assay (catalog #103400) and Neurology 4-Plex B Kit (catalog # 103345). Samples were diluted 1:200 in diluent buffer. A single lot of reagents was used for all samples. All samples were examined in duplicate.

Immunofluorescence

Forty-eight hours post-CCI, the slices were fixed 1h in paraformaldehyde (PFA) 4%, dehydrated two overnights in a 30% sucrose solution, rapidly frozen in N-pentane (-45°C) and stored at -80°C. For immunofluorescence staining, the slices were detached from the transwell and incubated overnight in free-floating with the primary antibodies: anti microtubule-associated protein (MAP-2, 1:500 Abcam, ab11267), glial fibrillary acidic protein (GFAP, 1:2000, MERK, AB5804), and ionized calcium binding adaptor molecule 1 (Iba-1, 1:1000, Wako, 019-19741), followed by 1h incubation with secondary antibodies anti-mouse alexa Fluor® 488, or anti-rabbit alexa Fluor®594 (1:500, Invitrogen).

Confocal microscopy and digital image analysis

Confocal microscopy was done on a Nikon A1 confocal scan unit, managed by NIS elements software. Whole cortical brain slices were imaged at laser excitations of 488 or 594 nm with a sequential scanning mode to avoid bleed-through effects. An overview was obtained with a 10x 0.5 NA and a concentric grid centered in the lesion site and distanced 500 µm was used to define concentric areas: the lesion, perilesion and periphery (Fig. 2a). Within each area, 4 ROIs were acquired with a 40x 0.75 NA objective. Each image was 1024 x 1024, having a pixel size of 0.21 µm and a step size of 0.85 µm for MAP-2 and a pixel size of 0.31 µm and a step size of 1µm for GFAP and Iba-1. In order to standardize the image acquisition and minimize inter-sample variation, all acquisition parameters were kept constant throughout microscopy sessions.

We quantified the complexity of neuronal arborizations in terms of number, size and density of branches on MAP-2 stained slices, by using an originally developed ImageJ algorithm to segment and skeletonize the signal as previously described (De Paola et al., 2023). Briefly, the background was normalized

imposing a minimum gray value of 1200, and the image was corrected with a median filter with a radius of 5 and binarized over the z stack by the Li method (Li and Tam, 1998). An extended focus image of the binary image was obtained and the skeletonize function was applied. Then the 'Analyze Skeleton 2D/3D' ImageJ plugin was applied with no endpoint pruning.

GFAP and IBA1 % stained area was measured on 5 focal plans using image J. Microglia morphology was analyzed, as described (Zanier et al., 2015; Moro et al., 2021). A size threshold (40-500 μm2) was used to select cells to be analyzed for the following shape descriptors: area, perimeter, Feret's diameter and circularity. Mean single-cell values for each parameter were used for statistics.

Results within lesion, perilesion and periphery are shown as the mean values of the 4 ROIs. Since no differences were present between concentric areas in CTRL slices, results are shown as the average of the 12 ROIs.

Gene expression analysis

Forty-eight hours after CCI, slices were collected, and total RNA was extracted by using the Ribospin II extraction kit (GeneAll) following the manufacturer's protocol. The RNA concentration was determined with a Nanodrop spectrophotometer and 200 ng/μl RNA from each sample was reverse transcribed to cDNA using TaqMan® qPCR RT Master Mix (Applied Biosystems by life technologies) following the manufacturer's protocol. Real-time RT-PCR was performed, and the relative gene expression to the reference gene RPL27 was determined by ΔΔCt method. Data are expressed as $\log_2$ of the fold difference from the CTRL group. The primers used are listed in Table 1. Primers were designed using the Primer-BLAST tool (NCBI, United States, https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi), and Prime3 open-source software (https://primer3.ut.ee/).

Table 1: Primers used for real-time reverse transcription polymerase chain reaction

	NCBI reference	Forward Primer	Reverse Primer
RPL27	NM_011289.3	TCATGAAACCCGGGAAAGT	GAGGTGCCATCGTCAATGT
MAP2	NM_0010399341	TCAGCTGACAGAGAAACAGCA	TTGTGTTGGGCTTCCTTCTC
GFAP	NM_001131020	GAAACCGCATCACCATTCC	TCGGATGGAGGTTGGAGA
CD11b	NM_010562.2	GAGCAGCACTGAGATCCTGTTTAA	ATACGACTCCTGCCCTGGAA
BDNF	NM_007540	AGGCACTGGAACTCG	AAGGGCCCGAACATAC

Secretome treatment

Mesenchymal stromal cells (MSCs) were isolated from human umbilical cord perivascular cells (Fraga et al., 2013) and the secretome was collected as a conditioned medium in passage 8 (P8) as previously described (Mendes-Pinheiro et al., 2019). Briefly, cells were seeded at a density of 4000 cells/cm2 and incubated with α-MEM medium (Gibco, USA), supplemented with 5% human platelet lysate (HPL) (α-MEM-Glutamax (Gibco®), 1% penicillin-streptomycin, 0,04% heparin. After 3 days in culture, cells were washed 4 times with PBS without Ca^{2+}/Mg^{2+} (Invitrogen) and incubated for 24h with Neurobasal-A medium (Gibco, USA) supplemented with 1% of penicillin-streptomycin (Fraga et al., 2013). On the next day, the conditioned medium containing the MSC-secretome was collected and centrifuged at 1,200 rpm (Thermo Electrocorporation, EUA) for 5 minutes to remove any cell debris. Then, secretome was snap frozen in liquid nitrogen and stored at -80°C. Before treating the slices, the collected secretome was supplemented with 2% B27 (Euroclone, IT) and 1% L-glutamine (Euroclone, IT), and applied to the organotypic culture. Treatment was applied 1h post-CCI and no medium changes were performed during the following 48 hours.

Pathway Analysis

For the interactome pathway analysis, we used Elsevier's Pathway Studio version 10.001 (https://www.elsevier.com/solutions/pathway-studio-biological-research). To establish the various relationships among the different altered validated proteins, ResNet Pathways Studio Propriety database was utilized to infer all the interactions. Interactome network was generated using a "direct interaction" algorithm for cellular processes, biological process mapping as well as the proposed pathway interactions.

Statistical analysis

Cortical slices were allocated to injury and treatments by a list randomizer (http://www.random.org/list). All evaluations were done blinded to injury/treatment status. Data are presented as mean ± SEM. The choice between parametric or non-parametric tests was based on passing the Shapiro–Wilk normality test, and data distribution was inspected by QQ plot. For each experiment, the figure legend reports the statistical analysis of data. For statistical analyses we used GraphPad Prism (GraphPad Software Inc., USA, version 9.2.0). For the statistical analysis, we used Fisher's statistical test to determine if there are nonrandom associations between two categorical variables organized by specific relationship (protein

interaction and biological process). The algorithm compares the sub-network distribution to the background distribution using a one-sided Mann-Whitney U-Test, and calculates a p-value indicating the statistical significance of the difference between two distributions.

3. Results

In vitro TBI model set-up: definition of the optimal injury depth

TBI severity was tailored by impacting cortical slices with CCI deformation depth of 0, 100 or 200 µm (**Fig. 1a**). PI incorporation was highest after CCI at 100 µm deformation depth ($p<0.01$, Fig. 1b). CCI at 200 µm depth resulted in the complete perforation of the brain tissue and loss of the lesion core (**Fig. 1b**) leading to lower overall PI integrated density values. Thus, CCI at 100 µm depth was selected for the subsequent experiments (Fig. 1c).

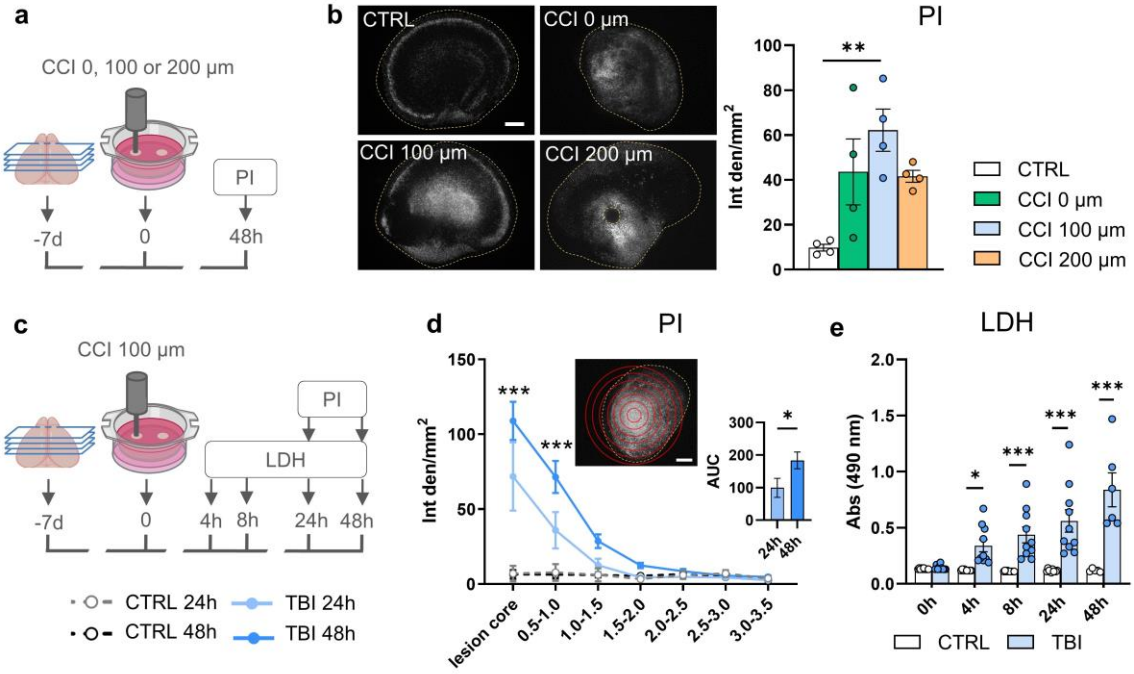


Figure 1: In vitro TBI model set-up. a) Experimental design of TBI grading by increasing CCI deformation depth from 0 to 200 µm. b) Representative images and quantification of PI incorporation at 48 h in CTRL and TBI slices. c) Experimental design for spatio/temporal cell death characterization after TBI. d) Quantification of regional-specific PI incorporation on concentric ROIs (top insert) and as AUC (bottom insert). e) Temporal LDH release in culture medium after TBI. Data are mean ± SEM; b) One-way ANOVA followed by Tukey's multiple comparisons test; d-e) Two-way ANOVA followed by Tukey's multiple comparison test; *p<0.05, **p<0.01, ***p<0.001. d) n=6-8. b-d) bars = 500 µm.

Spatial and temporal characterization of TBI induced cell death

We quantified regional PI incorporation by drawing concentric ROIs centred in the lesion core (Fig. 1d). PI incorporation in TBI slices showed the maximum value in the lesion core ($p<0.001$) and a gradual decrease towards peripheral ROIs at both 24 and 48h post-injury (Fig. 1d). AUC in TBI slices was higher at 48 compared to 24h ($p<0.05$) indicating a spatial and temporal increase in cell death (Fig. 1d right insert).

After TBI, LDH release progressively increased up to 48h with a significant difference already observed at 4h ($p<0.05$) compared to controls (Fig. 1e) thus confirming the increase of cell death over time. CTRL slices showed a low PI incorporation and LDH release uniform over space and time.

Evaluation of neuronal and glial damage after TBI

To investigate TBI-induced modifications of neuronal cytoarchitecture we quantified MAP-2 immunofluorescent signal on whole-mounted slices at 48 h post injury, selecting ROIs at lesion, perilesion and periphery (**Fig. 2a**). Compared to CTRL (**Fig. 2b**), TBI slices showed a strong decrease of MAP-2 staining in the lesion and perilesional areas (**Fig. 2c**). MAP-2 morphometric parameters showed that TBI decreased the complexity of neuronal arborizations, with significant reduction in the number of branches ($p<0.001$, **Fig. 2d**), in the size of the longest branch ($p<0.001$, Fig. 2e) and in the network density ($p<0.001$, **Fig. 2f**) at the lesion core, while showing values comparable to controls at the periphery. In accordance, gene expression analysis at 48h indicated a TBI-induced downregulation of MAP-2 and the neuronal associated growth factor BDNF ($p<0.01$, **Fig. 2g**). When analyzing culture media, we found a significant increase of NfL ($p<0.05$), Tau ($p<0.01$) and UCH-L1 ($p<0.01$) levels after TBI, confirming neuronal death (**Fig. 2h**).

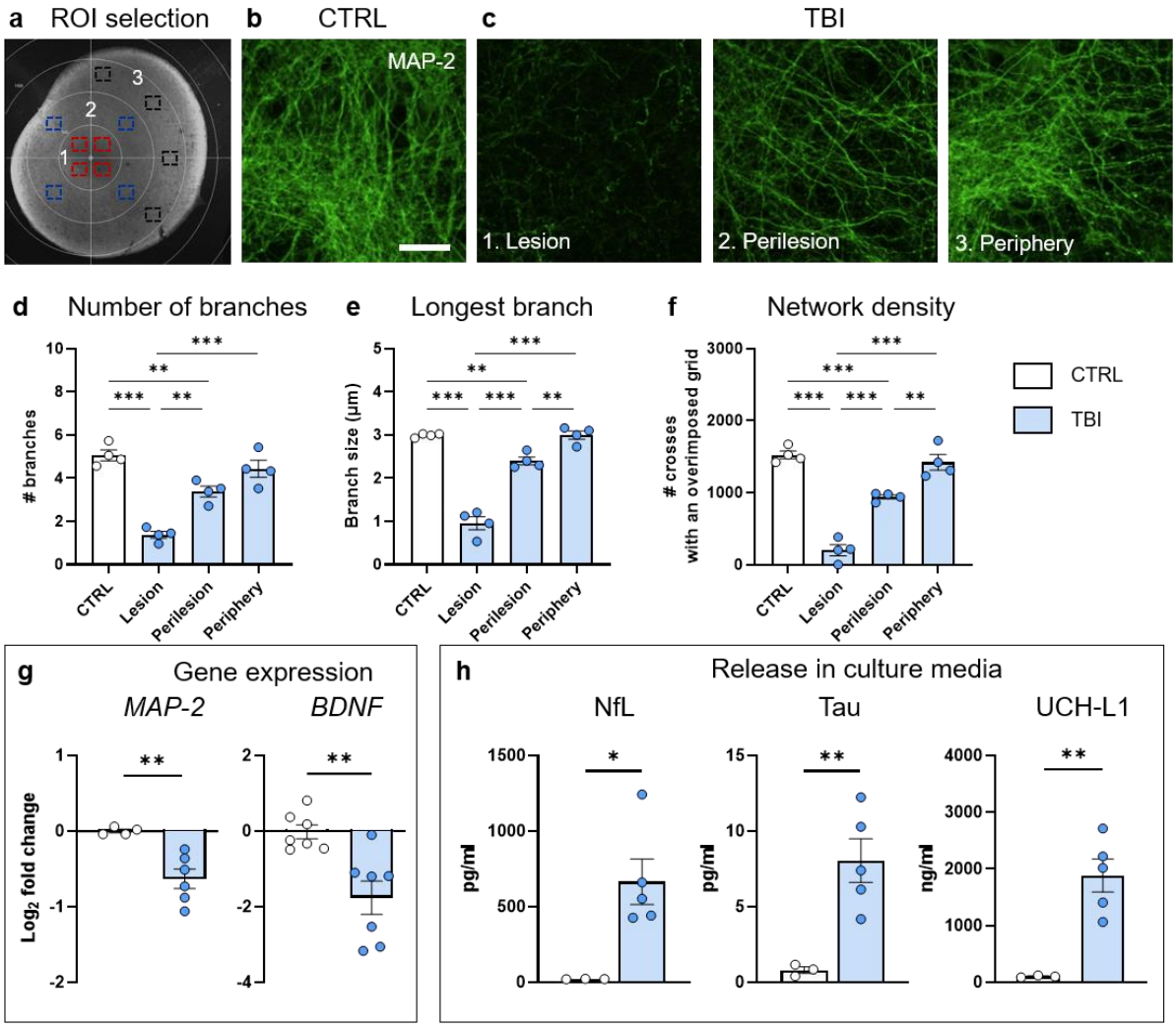


Figure 2: Evaluation of TBI induced neuronal damage 48h after injury. a) Representative image of ROIs in the lesion (1, red squares), perilesion (2, blue squares) and periphery (3, black squares). Since MAP2 density was uniform in CTRL slices throughout concentric areas, results are shown as the average of the 12 ROIs. b) Representative confocal images of MAP-2 staining in CTRL slices and c) in the lesion, perilesion, and periphery of TBI slices. d) Quantification of the number of neurite branches, e) the size of the longest branch and f) the network density of MAP-2 immunostaining. g) Gene expression analysis of neuronal-associated MAP-2 and BDNF markers in cortical slices. h) Levels of neuron-associated biomarkers released in culture media. Data are mean ± SEM. d-f) One-way ANOVA, followed by Tuckey's multiple comparisons test, g-h) t-test. *p<0.05, **p<0.01, ***p<0.001. Bar = 25 µm

Histological analysis of GFAP+ astrocytic marker, showed a uniform labeling in CTRL slices, cells displayed the typical morphology of cortical astrocytes with defined cell somata and long processes protruding outward (**Fig. 3a**). At 48h post injury, GFAP stained area significantly decreased in all ROIs

(p<0.001), with the highest effect in the lesion core (Fig. 3b-c). TBI also induced a significant release of GFAP in culture media 48h post-injury (p<0.01, **Fig.3d**), concurrent with an upregulation of GFAP gene expression (p<0.001, **Fig.3e**).

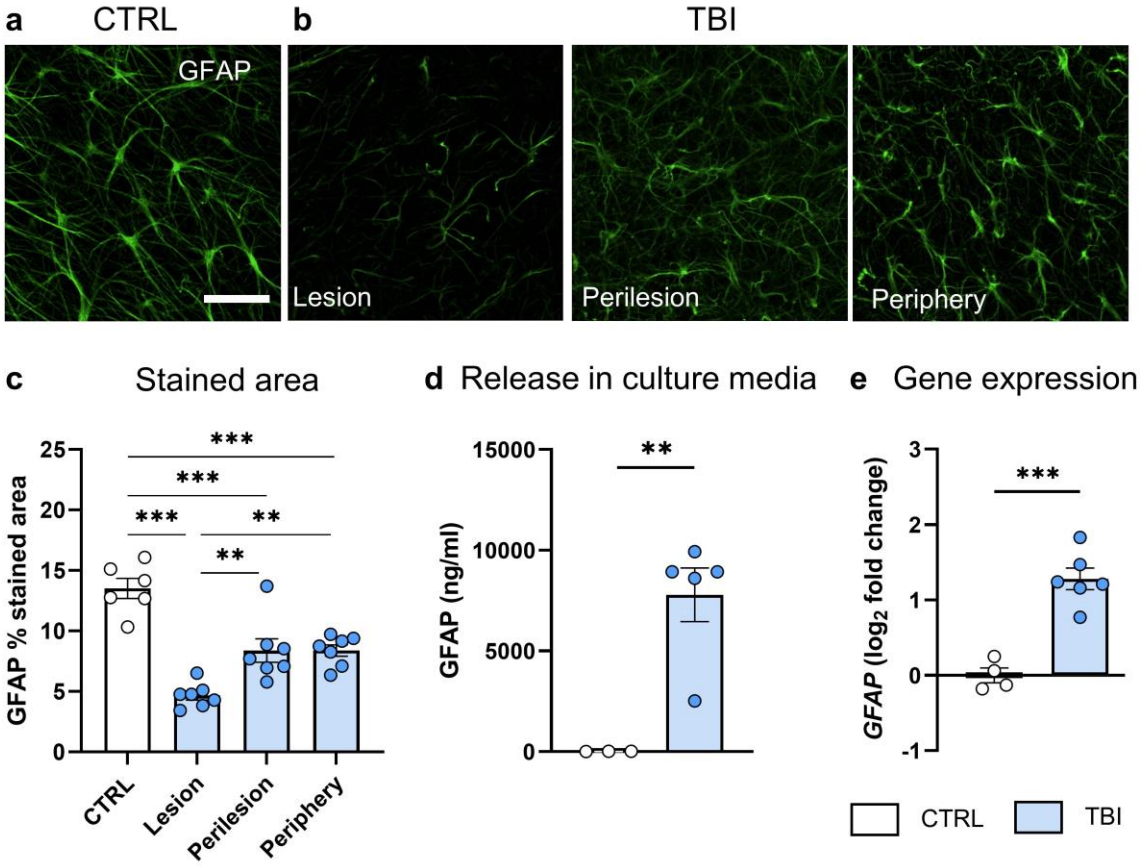


Figure 3: Evaluation of TBI-induced astrocytic damage/activation 48h after injury. a) Representative confocal images of GFAP staining in CTRL slices and b) in the lesion, perilesion and periphery of TBI slices. Since GFAP staining was uniform in CTRL slices throughout concentric areas, results are shown as the average of the 12 ROIs. c) Quantification of the GFAP % stained area. d) Levels of GFAP biomarker released in culture media. e) Gene expression analysis of GFAP marker in cortical slices. Data are mean ± SEM. c) One-way ANOVA, followed by Tuckey's multiple comparisons test, d-e) t-test. **p<0.01, ***p<0.001. Bar = 50 µm

We found a reduction of IBA1+ stained area in the lesion area of TBI slices compared to CTRL (p<0.01, **Fig. 4a-c**) that progressively recovered toward the periphery. Analysis of shape parameters (Fig. 4d-g) showed a decreased cell area (p<0.05, **Fig. 4d**), perimeter (p<0.01, **Fig. 4e**) and Feret's diameter (p<0.05, **Fig. 4f**) in the lesion core of TBI slices, indicating the polarization of microglial cells towards an amoeboid shape with a less ramified morphology. Accordingly, an increase in circularity value was

observed in the lesion core ($p<0.01$, **Fig. 4g**). In the periphery, morphometric parameters were comparable to controls. Gene expression analysis revealed that TBI-induced an upregulation of CD11b microglial pan marker ($p<0.01$, **Fig 4h**).

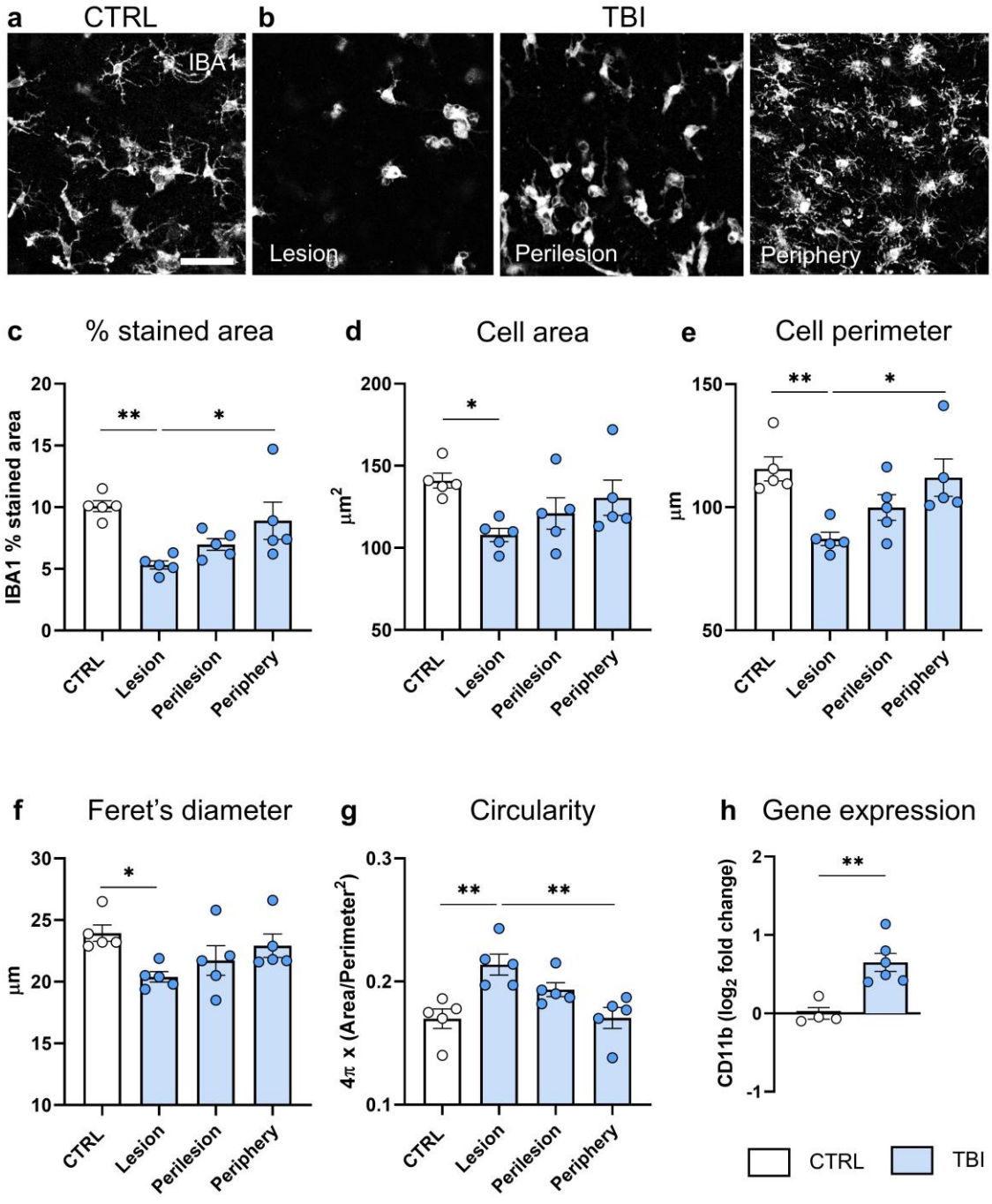


Figure 4: Evaluation of TBI induced microglia changes 48h after injury. a) Representative confocal images of IBA-1 staining in CTRL slices and b) in the lesion, perilesion, and periphery of TBI slices. Since Iba1 staining was uniform in CTRL slices throughout concentric areas, results are shown as the average of the 12 ROIs. c) Quantification of Iba-1 % stained area, and morphometric cell parameters: d) mean cell area, e) perimeter, f) Feret's diameter and g) circularity. h) Gene expression analysis of microglial CD11b

marker in cortical slices. Data are mean ± SEM. c-g) One-way ANOVA, followed by Tuckey's multiple comparisons test, i) t-test. *p<0.05 **p<0.01, ***p<0.001. Bar = 50 µm

Systems biology analysis

Using the differentially expressed mRNA and proteins released in the culture media, system biology analysis was conducted to identify cellular processes represented in our in vitro TBI model. Altered proteins (NfL, Tau, UCH-L1, MAP2, GFAP) were related with other two TBI well established biomarkers (S100-B and neuron specific enolase, NSE) (**Fig. S2**). Pathway analysis revealed an association with neuronal death, nerve injury and degeneration, as well as glial reaction and astrocytosis (**Fig. S2**).

MSC-secretome rescues TBI induced damage

We assessed the effects of MSC-derived secretome administration, delivered to TBI slices 1h after injury in the culture media (experimental design in **Fig. 5a**). Cell death analysis at 48h by PI incorporation assay revealed a significant protection induced by MSC-secretome in the lesion core and the adjacent regions (p<0.001, **Fig. 5b**), resulting in a global reduction of cell death (AUC TBI vs TBI + MSC-sec: p<0.01, **Fig. 5b** insert). Analysis of MSC-induced effects on neurons showed a rescue effect on TBI-induced downregulation of MAP-2 (p<0.05) and BDNF (p<0.01, **Fig. 5c**), a decrease of NfL release in the culture media (**Fig. 5d**), and an increase of neurite branches in the periphery of the slice (**Fig. 5e, h**), while no changes were detected for the longest branch size and network density (**Fig. S2**). Analysis on glial cells revealed no differences on GFAP and an up-regulation of microglial CD11b expression (p<0.05) induced by MSC-secretome compared to TBI untreated slices (**Fig. 5f**). Histological analysis of microglia activation revealed an increased stained area in MSC-secretome treated vs untreated TBI slices (**Fig. 5g, h,** Two-way ANOVA p of treatment <0.01), but no changes in shape descriptors (**Fig. S3**).

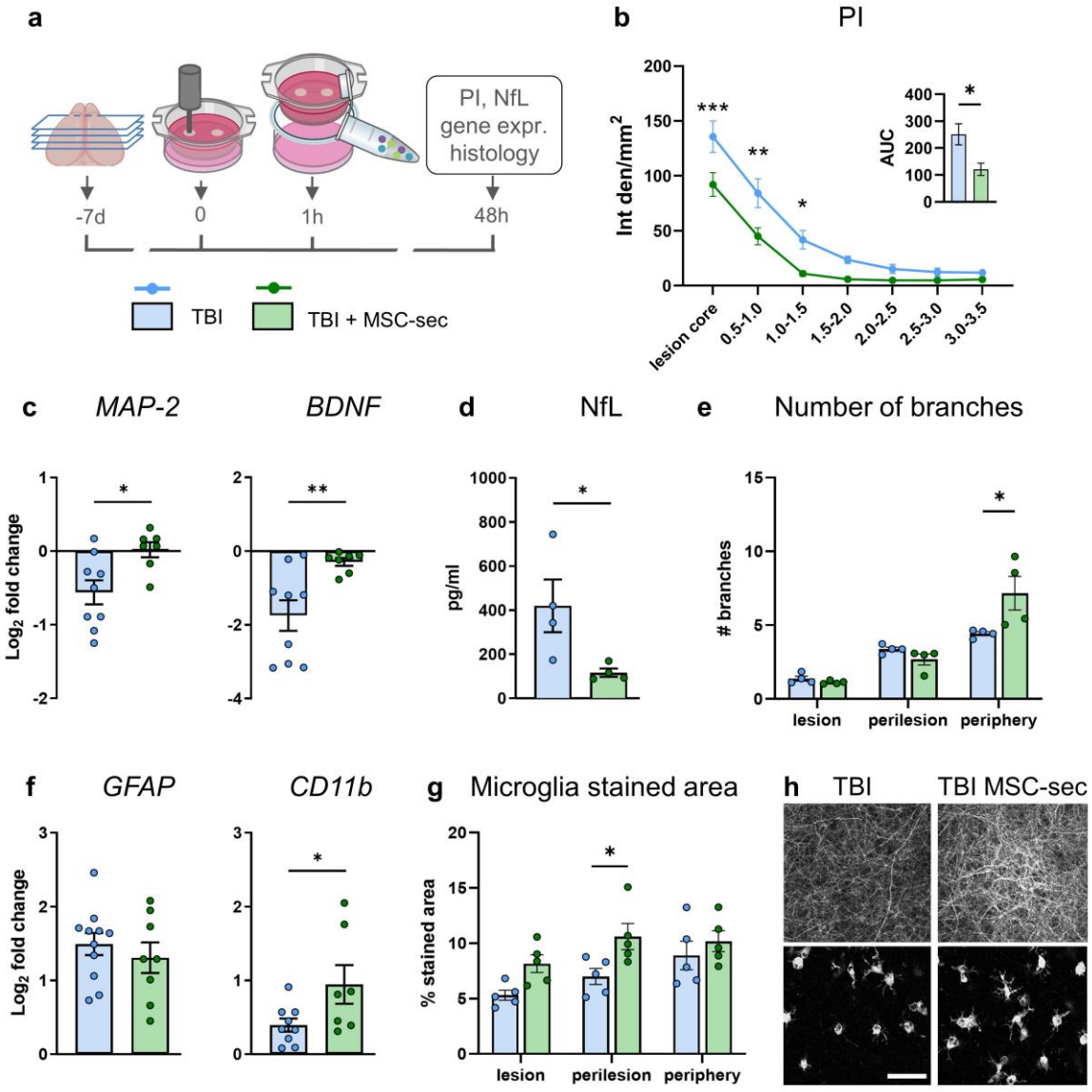


Figure 5: MSC-sec protects brain tissue after TBI. a) Experimental design. b) Quantification of PI incorporation at 48 h after injury in concentric ROIs and as AUC (insert). c) MAP-2 and BDNF gene expression analysis in cortical slices. d) Levels of NfL biomarker released in culture media. e) Histological quantification of the number of neurite branches. f) GFAP and CD11b gene expression analysis. g) Histological quantification of microglia % stained area. h) Representative confocal images of MAP-2 staining and microglial cells in TBI and MSC-sec treated slices. Data are mean ± SEM. b) Two-way ANOVA, followed by Tukey post test, n=8-10. c-d-f) t-test, e-g) Two-way ANOVA, followed by Sidak's post test, *p<0.05, **p<0.01, ***p<0.001. Bar = 50 µm

4. Discussion

In the present work, we developed a new 3D in vitro TBI model on organotypic cortical brain slices subjected to a biomechanical impact (CCI). The model recapitulates key features of in vivo CCI in mice, including a focal injury characterized by neuronal, microglial and astrocytic cell death, and the release of neuronal and astrocytic proteins in the cultured media. When treated with MSC-secretome, TBI slices showed reduced cell death and neuronal damage, demonstrating the potential of this model as a valuable tool to test for therapeutic candidates.

Failures in translating preclinical results in the clinical setting call for new experimental models to test therapeutic candidates (Kumaria, 2017). Our work aimed to establishing a reliable in vitro TBI model on 3D brain tissue, allowing a close representation of the post-traumatic cellular and molecular events. To date, TBI modelled in organotypic slices used hippocampal tissues (Kang et al., 2015; Effgen and Morrison, 2017; Vogel et al., 2017a), an area that, in vivo, is mainly damaged by secondary injury mechanisms (Osier and Dixon, 2016; Siebold et al., 2018). To better reflect the in vivo condition, we decided to model brain contusion on cerebral slices obtained from cortices. As impactor, we used the electromagnetic-driven piston, commonly used in in vivo TBI studies on rodents. The system has the advantage of a fine control of the impact parameters (velocity, dwell time and deformation depth) and a localized injury, while the existing stretch, blast or compression models provide a more diffuse damage. When tailoring injury severity, 100 µm injury depth induced a consistent focal lesion, with PI accumulating at the lesion core and progressively decreasing toward the periphery. Cell death increased overtime, indicating that brain pathology is progressing, thus mirroring the injury evolution reported in TBI animals (Siebold et al., 2018). By 0 µm deformation depth, with the piston only touching the slice, tissue damage was also observed, likely due to the perturbation air wave during the strike, with high variability and no difference from controls. 200 µm deformation depth destroyed the strike area with clear tissue perforation thus preventing the evaluation of tissue damage.

One of the hallmarks of TBI is neuronal injury, with the disruption of network connections and function. Compared to 2D neuronal cell cultures, the proposed model has a 3D structure preserving the cytoarchitecture of the in vivo cerebral microenvironment in terms of cell-cell and cell-extracellular matrix interactions, neuronal network and synaptic organization (Humpel, 2015; Croft et al., 2019; AlaylioĞlu et al., 2020), thus the assessment of neuronal network alterations has translational value. Histological analysis of MAP-2 stained neurites showed an altered network organization after TBI, with a higher effect in the lesion core associated with a reduced number and size of branches and network density. Of note, these parameters follow a gradient distribution with values in the periphery comparable to uninjured

slices. Accordingly, gene expression analysis showed MAP-2 downregulation in conjunction with a decrease of trophic support (BDNF). Biomarker evaluation in cell culture media confirmed high neuronal damage, with massive release of proteins from axonal (NfL and tau) and cell body (UCH-L1) structures (Wang et al., 2018). Those are the biomarkers found to be elevated in TBI patients in blood with injury severity dependency (Whitehouse et al., 2022) and showing prognostic value (Shahim et al., 2016; Graham et al., 2021; Helmrich et al., 2022; Korley et al., 2022; Moro et al., 2022) thus supporting the utility of the developed in vitro model.

We also explored glial susceptibility/activation. Astrocytes and microglial cells are essential contributions to several homeostatic functions that could directly influence neuronal survival and tissue integrity after TBI (Karve et al., 2016; Michinaga and Koyama, 2021). Histological analysis revealed a TBI-induced astrocytic damage with greater effect in the lesion core. Concordantly, GFAP released in the culture medium indicated astrocyte death. Gene expression analysis showed discordant results, pointing to a GFAP mRNA up-regulation after TBI. During highly dynamic adaptation processes, as in response to injury, post-transcriptional events may lead to deviation from an ideal mRNA-protein correlation and a delay timeframe is likely to occur (Liu et al., 2016).

Similar to what was observed in astrocytes, we observed a decrease in microglia stained area within the lesion and a concomitant up-regulation of CD11b gene expression. A more detailed analysis of microglia morphometric parameters demonstrated a clear transition from a ramified to an amoeboid cell morphology in the lesion core, as demonstrated by the reduction of cell area, perimeter, Feret's diameter, and increased circularity. These results recapitulate findings in TBI mice (Zanier et al., 2015; Kumar et al., 2016; Caplan et al., 2020) emphasizing the value of the model. In the peripheral areas, morphometric parameters were not different from control slices.

To establish a link between the in vitro and in vivo complexity, we used a system biology approach to integrate changes at the protein and gene levels into biological processes. Altered validated proteins (NfL, Tau, UCH-L1, MAP2, GFAP) were studied in relationship with other two established TBI-biomarkers (S100-B and NSE) showing their implication in astrogliosis and astrocytosis, neuronal injury and degeneration, associated with learning and memory deficits (Figure S2). This analysis further confirms that our in vitro TBI model integrates well into complex biological processes otherwise accessible in in vivo TBI models only.

We next tested our model response to the therapeutic efficacy of MSC-based approach. MSCs have shown large beneficial effects in preclinical TBI models (Pischiutta et al., 2021) and now their secreted bioactive factors (i.e. secretome) stand as the main drivers of protection, paving the way for a potential cell-free

approach. MSC-secretome treatment of TBI-injured slices induced a reduction of PI incorporation in the lesion core and surrounding regions, pointing to a protection against cell death by MSC-secretome. MSC-secretome induced neuronal protection was confirmed at both gene and protein levels. In particular, MSC-secretome reduced the release of the axonal injury maker NfL in the culture media, providing evidence of the NFL validity as pharmacodynamics biomarker in monitoring treatment response in the TBI model. In addition, histological analysis revealed an increased number of branches in treated slices, in accordance with previous findings from our group, showing MSC-secretome promotion of axonal outgrowth in primary cultures of rat embryonic hippocampal and cortical neurons, with a major role of BDNF in the observed effects (Martins et al., 2017). MSCs have been shown to induce a microglia protective phenotype (Zanier et al., 2011, 2014; Pischiutta et al., 2016, 2022; Kota et al., 2017; Xu et al., 2020) and reduce pyroptosis, a type of programmed cell death associated with inflammatory response (Feng et al., 2022). We found that MSC-secretome promoted microglia activation calling for in-depth analysis of microglia functional state.

Limitations of this model include the lack of a functional vasculature and of systemic infiltrating immune cells playing an important role in the evolution/resolution of brain damage after TBI (Plesnila, 2016). At the same time our model has the major advantage of allowing the specific study of microglial role after TBI, without the presence of infiltrated monocyte/macrophage cells whose distinction from round-shape microglia after in vivo TBI is challenging (Jurga et al., 2020). The isolation of brain pathological mechanisms without confounding aspects can help verifying tissue-specific punctual hypothesis.

In addition, our model cannot reproduce the influence of cerebrovascular autoregulation, intracranial pressure and neurovascular coupling (Li et al., 2016), which are critical in TBI pathophysiology, and is not suitable to assess chronic neurodegenerative processes (such as those due to proteinopathies and amyloid accumulations), for which in vivo rodents TBI models remains the best options.

Further implementation of the model could include the assessment of the functional neural connections, by a fine characterization of the synaptic integrity and by electrophysiological measurements. In addition, we obtained slices from postnatal donors, because of high survival in culture compared to adult donors (Humpel, 2015). Our method, as described by Dossi et al., (2013) shows a progressive maturation of cortical brain slices reaching a synchronized electrical activity by 5-7 days in culture, comparable to adult mouse brain. The recent set-up of long-term organotypic culture from adult rodents (Humpel, 2019; Mayerl and Ffrench-Constant, 2021) will allow future studies to address the impact of the donor age in injury evolution and response to treatment.

5. Conclusions

In the present study, we developed a new TBI model on organotypic cortical slices, a 3D tissue in which the proportion of cerebral populations and their anatomical architecture are representatives of the in vivo structure. The injury recapitulates key features of TBI pathology, including neuronal damage, network changes, and glial activation with the possibility to assess injury and therapeutic responses by outcome measurements, with direct translational value, like fluid biomarkers. Our findings provide the first evidence that this new 3D in vitro TBI model stands as a tractable medium/high throughput, ethically viable, and pathomimetic biological asset for testing new cell-based therapies.

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SUPPLEMENTARY MATERIAL

A novel organotypic cortical slice culture model for traumatic brain injury: molecular changes induced by injury and mesenchymal stromal cell secretome treatment

Figure S1: Schematic drawing of the process used to obtain cortical brain slices.

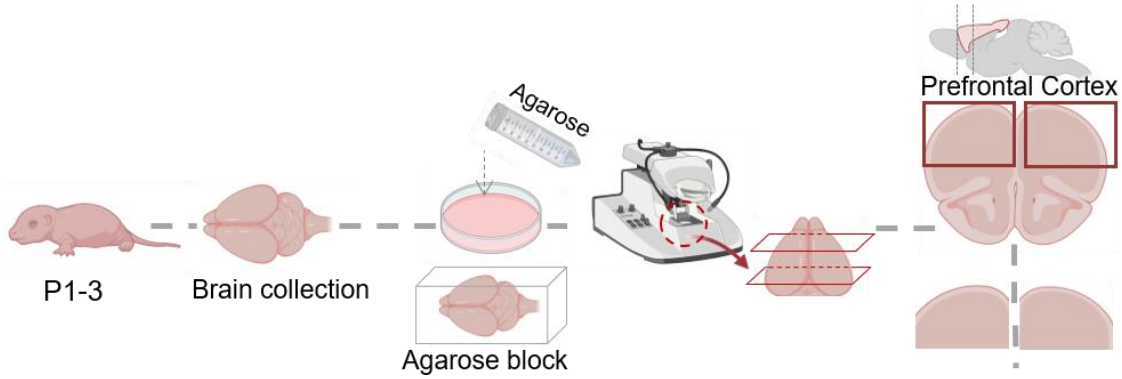
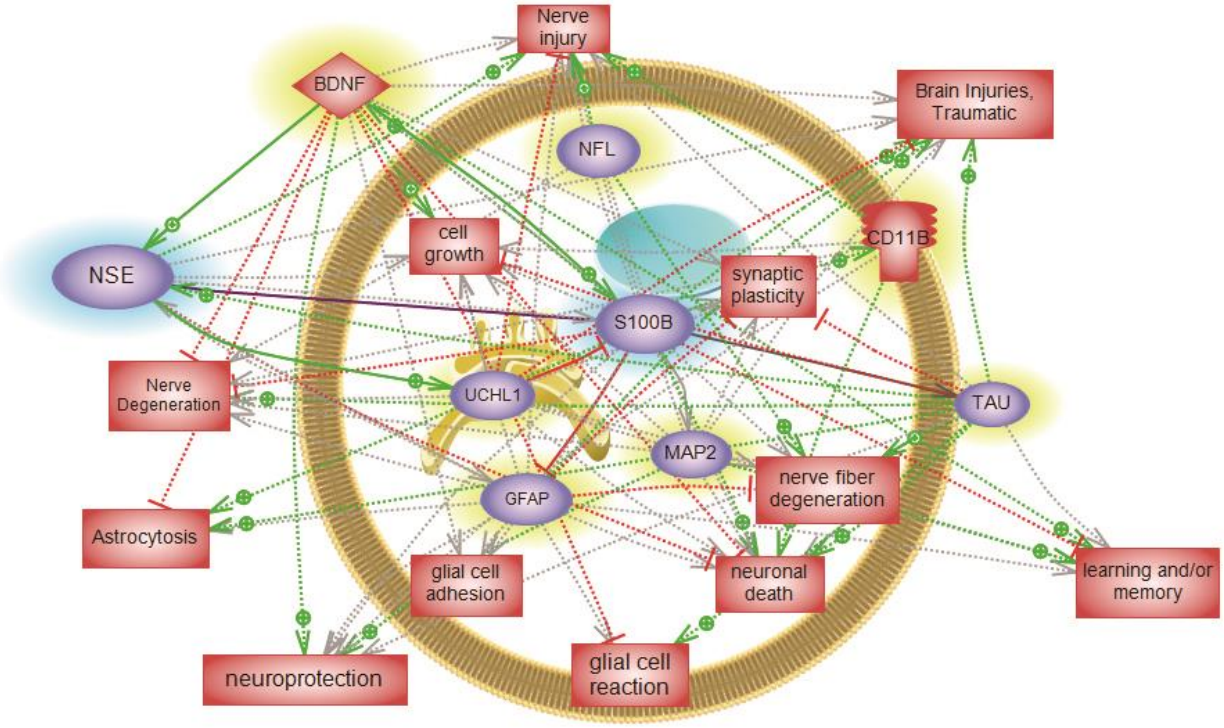


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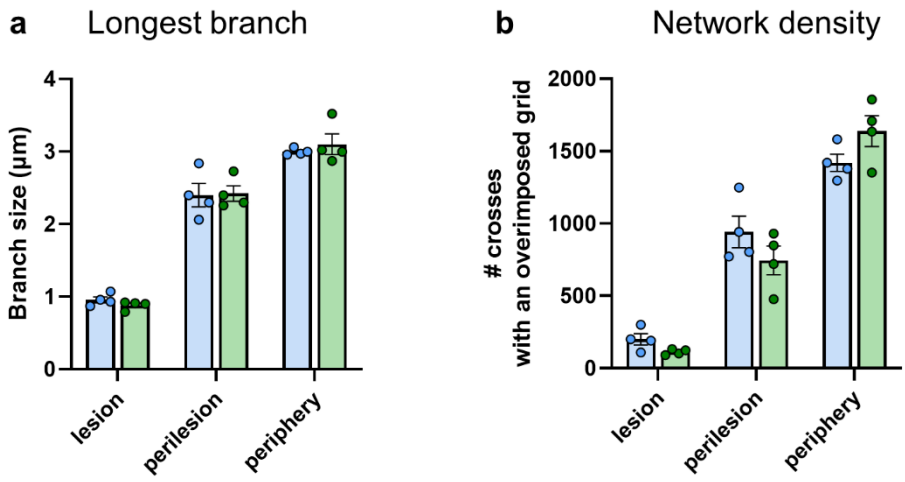
Figure S2: Systems biology analysis of altered proteins which revealed several statistically significant molecular functions and biological process pathways



Pathway	p-value
Neurodegenerative Diseases	p<0.001
Nerve injury	p<0.001
Nerve fiber degeneration	p<0.001
Traumatic Injuries	p<0.001
Neuronal death	p<0.001
Learning and/or memory	p<0.001
Neuroprotection	p<0.001
Synaptic plasticity	p<0.001
Glial cell reaction	p<0.001
Astrocytosis	p<0.001
Cell growth	p<0.001
Glial cell adhesion	p<0.001

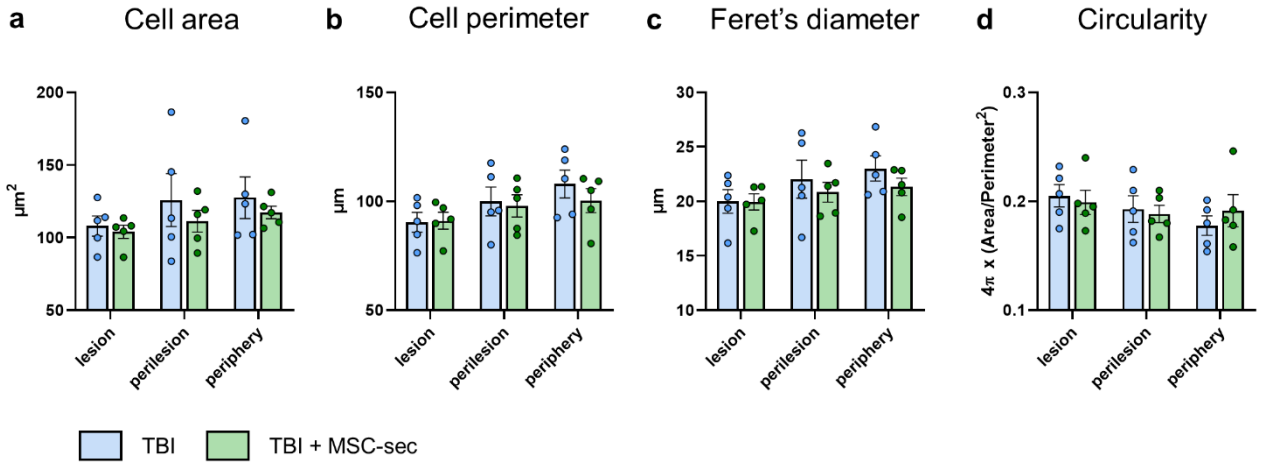
Figure S2: Systems biology analysis of altered proteins which revealed several statistically significant molecular functions and biological process pathways. Picture shows the interaction map of altered protein and their corresponding relation to neural injury development; these statistically significant proteins include UCH-L1, GFAP, tau, Map2 (yellow hallow) as well as the two established TBI proteins S100B, and Neuron Specific Enolase (NSE) (Blue hallow).

Figure S3: Morphometric analysis of neuronal network after MSC-secretome treatment.



Quantification of the size of the longest branch (**a**) and the network density (**b**) of MAP-2 immunostained slices after MSC-secretome treatment. Data are mean ± SEM.

Figure S4: Microglial shape descriptors after MSC-secretome treatment.



Quantification of morphometric microglia cell parameters after MSC-secretome treatment: mean cell area (**a**), perimeter (**b**), Feret's diameter (**c**) and circularity (**d**). Data are mean ± SEM.

CHAPTER III

Locomotor and cognitive function of Traumatic Brain Injury mice are rescued by Human Umbilical Cord Perivascular Cells in GG-GRGDS Hydrogels

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CHAPTER III | Locomotor and cognitive function of Traumatic brain injury mice are rescued by Human Umbilical Cord Perivascular cells inside Gellan Gum scaffolds

Abstract

Traumatic brain injury (TBI) is a major cause of death and disability worldwide. Despite promising preclinical data, no effective treatment was found to counteract disease progression. Mesenchymal Stem Cells (MSCs) have been shown to revert TBI pathological hallmarks. From the existent MSC sources, the umbilical cord is particularly interesting due to the rapid proliferation of cells with an MSC phenotype from the Wharton Jelly, as well as the neuroprotective character of its secretome. In addition, the perivascular regions from the umbilical cord render the most abundant source of MSCs, compared with other cord areas. In this study, we tested the efficacy of human umbilical cord perivascular cells (HUCPVCs) combined with gellan gum (GG) based hydrogels, modified with GRGDS (GG-GRGDS) to treat a controlled cortical impact (CCI) model of TBI. Cells inside GG-GRGDS hydrogels were delivered to the injured animals 3 days after injury. The sensorimotor function was evaluated at various time points after TBI by neuro score and SNAP tests. Cognitive behavior was evaluated by the Y-Maze test. Treatment with HUCPVCs formulated in GG-GRGDs improved the sensorimotor behavior of TBI mice from 4 weeks after trauma, getting significant statistical differences at 13 weeks after TBI. Additionally, treated animals revealed a better performance on Y-Maze test, at 8 weeks post-TBI. The combinatory approach delivered directly at the lesion cavity reduced the activation of GFAP induced by trauma at the cortical region, 13 weeks post-injury. Additionally, at the cortex were observed, a slight decrease on Iba-1 activation, 13 weeks post-injury and an increase in the number of NeuN-positive cells at both 4 and 13 weeks after treatment, despite not significant. Taken together, this data suggests that the combinatory delivery of HUCPVCs within GG-GRGDS hydrogels delivered directly at the injury site can reduce TBI-induced damage proving therapeutic potential for TBI.

1. Introduction

Traumatic brain injury (TBI) is a major cause of death and disability among young adults (Peeters et al. 2015). It is a major public health problem with a high economic burden (Peterson et al. 2021). Cerebral contusions occur in up to 35% of TBI severe cases with patients showing complicated locomotor and long-term cognitive impairments (Lobato et al. 1983). The first mechanical injury leads to cell loss and initiates an ongoing pathophysiological cascade that exacerbates over time. The majority of therapeutic attempts aim to stop the secondary detrimental mechanisms and to promote neurorestorative

processes, such as neurogenesis, axonal sprouting, and angiogenesis. Despite the efforts, nowadays no pharmacological treatment has been successful in clinical trials.

Mesenchymal stem cells (MSCs) have shown interesting results in different pre-clinical models of CNS injury and degeneration (Gomes et al. 2016; Zanier et al. 2011; Gomes et al. 2020; Andrzejewska et al. 2021; Pischiutta et al. 2021). Nowadays it is well accepted that in addition to repopulating injured tissues (Hellmann et al. 2006; Gordon et al. 2010; Zhao et al. 2022; Danielyan et al. 2020), MSCs exert their therapeutic effect largely through their paracrine effects (Salgado et al. 2015; Pinho et al. 2020). Additionally, different MSCs sources release different factors that may influence their therapeutic function (Pires et al. 2016). Human umbilical cord perivascular cells (HUCPVCs), have previously shown to have an important neuroprotective impact in an *in vitro* model of TBI (Pischiutta et al. 2023). Additionally, they also exhibit some interesting advantages over other MSC sources, particularly lower immunogenicity, high abundance (Sarugaser et al. 2009), and are easy to obtain (La Rocca et al. 2012). Previous studies by Fraga and colleagues (Fraga et al. 2013) studying HUCPVCs secretome, identified proteins highly involved in the regulation of neuronal survival, proliferation, and differentiation. Specifically, in the previous study were found UCHL-1, hsp70, 14-3-3 protein, and peroxiredoxin 6. Indeed, in vitro studies using different in vitro and in vivo models have shown that HUCPVCs paracrine interactions positively impacted glial cell viability, proliferation, and differentiation, as well as axonal growth (Salgado et al. 2010; Ribeiro et al. 2012; Martins et al. 2017; Pires et al. 2014; Teixeira et al. 2015). Moreover, Barreiro and colleagues showed that, when co-cultured with neurons, HUCPVCs interact preferentially with axons and reduce axonal degeneration (Barretto et al. 2020). Despite the previous, effectively transferring and keeping viable cells at the lesion site is still a technical challenge. Therefore, biomaterials are a useful approach to confer cells protection and improve their survival at the lesion site (Roche et al. 2014; Gomes et al. 2016).

At the present study, HUCPVCs were engrafted on natural gellan gum scaffolds chemically modified with GRGDS (GG-GRGDS), a fibronectin synthetic peptide (Silva et al. 2012) before direct transplantation into the lesion site. We evaluated whether HUCPCVs are able to improve TBI locomotor and cognitive sequelae and restore brain function after TBI. Our results show that HUCPVCs, improved locomotor impairments from 4 weeks to 13 weeks post TBI. Additionally, HUCPVCs significant reduced of cognitive deficits 8 weeks post-injury, together with a tendency to increase NeuN positive cells and reduce of GFAP and Iba-1 percentage of stained area, in the cortex of TBI treated animals.

2. Material and Methods

Study design

Figure 1 shows the experimental timeline. Traumatic Brain Injury (TBI) was modeled using the controlled cortical impact (CCI) model performed by a pneumatic impactor device – red circle. Three days' post-injury animals were treated with human umbilical cord perivascular cells (HUCPVCs), that were expanded in vitro inside of a Gellan Gum matrix modified with GRGDS (GG-GRGDS) for seven days. Locomotor behavior was accessed by Simple Neuroassessment of Asymmetric Impairment (SNAP) and Neuroscore tests at 2 days, 1,2,3,4,7 and 13 weeks post CCI – blue circle. Cognitive behavior was evaluated by the YMaze at 8 weeks post TBI – yellow circle.

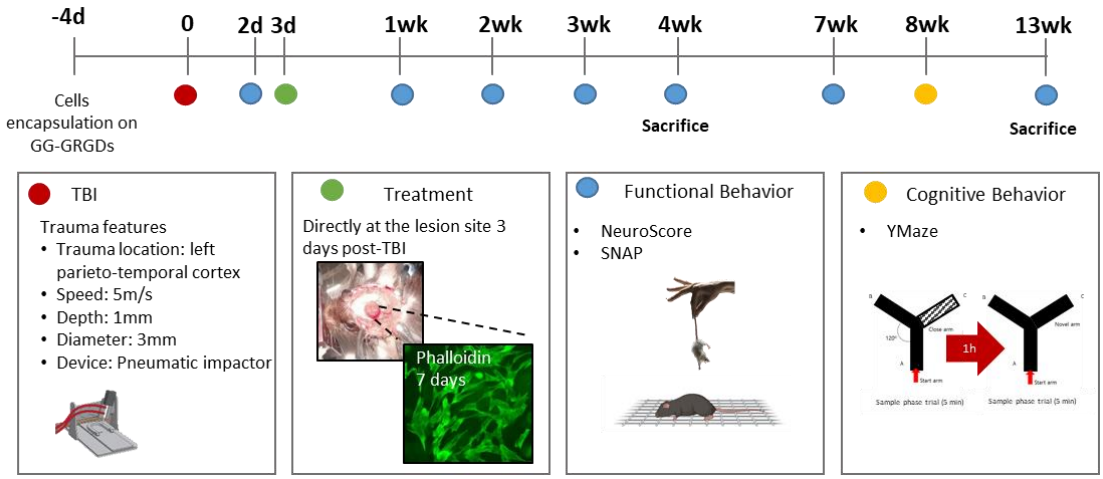


Figure 1: Experimental time-line. TBI was modeled using a pneumatic impactor device (red circle). 3 days after injury, animals were treated with HUCPVCs, directly at the injury site (green circle). HUCPVCs were engrafted in gellan gum matrices for 7 days. Locomotor Behavior (blue circle) was accessed by Neuroscore and SNAP tests, every week until 4 weeks post-trauma, and at 7, and 13 weeks. Cognitive behavior (yellow circle) was accessed by the Ymaze test at 8 weeks after injury.

Animals

Male C57BL/6J mice (9 weeks of age) from Envigo (Horst, Netherlands) were used. Animals were housed in a pathogen-free vivarium with constant temperature (21 ± 1°C) and relative humidity (60 ± 5%), with a 12h light–dark cycle and ad libitum access to food and water. All efforts were made to minimize animal suffering and to reduce the number of animals used.

The IRCCS-Istituto di Ricerche Farmacologiche Mario Negri (IRFMN) adheres to the principles set out in the following laws, regulations, and policies governing the care and use of laboratory animals: Italian Governing Law (D.lgs 26/2014; Authorization n.19/2008-A issued March 6, 2008 by Ministry of Health); Mario Negri Institutional Regulations and Policies providing internal authorization for persons conducting

animal experiments (Quality Management System Certificate – UNI EN ISO 9001:2008 – Reg. N° 6121); the NIH Guide for the Care and Use of Laboratory Animals (2011 edition) and EU directives and guidelines (EEC Council Directive 2010/63/UE). For statistical validity 10 mice were assigned to the different groups, using randomization lists (https://www.random.org/lists/). Investigators who did behavioral and post-mortem analysis were blinded to the treatment allocation.

Experimental traumatic brain injury

Before the surgical intervention mice were anesthetized with isoflurane (induction 2,8% vol; maintenance 1,5% vol) in an N_2O/O_2 (70%/30%) mixture and placed in a stereotaxic frame. Mice were then subjected to a craniectomy, as earlier described (Zanier et al. 2016; 2018). CCI was induced using a 2-mm-diameter rigid impactor driven by a pneumatic controlled impact device rigidly mounted at an angle of 20° to the vertical plane and applied vertically to the exposed dura mater. The impact was done between bregma and lambda, over the left parieto-temporal cortex (antero-posteriority 2,5 mm; laterality 2,5 mm), at 5 m/s of speed and deformation depth of 1 mm. The craniotomy was then covered with a cranioplasty and the scalp sutured. Body temperature was maintained at 37 °C during all surgical procedures.

HUCPVCs isolation and culture

HUCPVCs were obtained from Prof. John E. Davies (University of Toronto, Toronto Canada). Cells were isolated from umbilical cords, with the consent of parenting entities. The isolation of HUCPVCs was performed based on the protocol established by Sarugaser et al. (Sarugaser et al. 2009). Briefly, the umbilical cord was cut into small segments (4–5 cm) to expose both arteries and vein, rejecting the epithelium. Once isolated the vessels, with a sterilized suture, the ends of the vessels were fixed creating a short loop in order to reduce the contamination by erythrocytes. Vessels were then placed in a collagenase solution (0.5 mg/ml for arteries and 0.75 mg/ml for veins; Sigma, USA) and incubated at 37 °C for a period of 16–18 h. Completed this period, the vessels were removed from the suspension and diluted in PBS without Mg^{2+}/Ca^{2+} (Invitrogen, USA). Finally, HUCPVCs were re-suspended in Alpha-Mem medium (Invitrogen, USA) supplemented with 1 % of antibiotic/antimycotic (Invitrogen, USA) and 5 % of human platelet lysate (HPL) and plated on culture. Subsequently, the culture medium was renewed every 3 days and the culture was maintained at 37 °C,5 % CO_2, 95 % air, and 90 % relative humidity until confluence.

HUCPVCs encapsulation in GG-GRGDS hydrogel

The synthesis of GG-GRGDS hydrogel was performed according to the protocols described by Silva et al. (Silva et al. 2012). After synthesis, GG-GRGDS was lyophilized and stored at 4˚C until the experiment day. GG-GRGDS lyophilized powder was sterilized by exposure to UV lights for 15 min, a method previously used without affecting the material properties GG-GRGDS (Silva et al. 2013) was dissolved in ultra-pure water at 1,25% (w/V) concentration and mixed overnight at 37˚C. Cells (600 cells/µl of gel), were re-suspended in cell medium. Subsequently, cellular suspension (at 4:1, cells: biomaterial) was added to the biomaterial. With a pipette, the solution was mixed, during 2 minutes, until homogeneous. Drops of 20µl of gel with the cell suspension were plated into a 24 well plate. After 5 minutes, 250µl of cell medium was added until full cover of the GG-GRGDs. HUCPVCs in passage 8 (P8) were maintained in culture, embedded in the GG-matrices for 7 days, before transplanting into the TBI cavity, with medium changes every two days.

Treatment

3 days, post-TBI animals were anesthetized and the cranioplasty previously attached to the animal's skull was removed. HUCPVCs (1200 cells) inside 20µl GG-GRGDS drops were transferred to the lesion cavity. The cranioplasty was rebuilt and the skin closed. Vehicle animals underwent the same procedure without no treatment being applied.

Behavioral tests

Sensory motor deficits were evaluated by neuroscore (NS) (Pischiutta et al. 2014) and simple neuroassessment of asymmetric impairment (SNAP) test at 2 days, and 1, 2, 4, and 7 weeks post-TBI (Moro et al. 2021). For neuroscore, animals were scored from 4 (normal) to 0 (severely impaired) for each of the following indices: 1) forelimb function; 2) hind limb function; and 3) resistance to lateral pulsion as previously described. The maximum score per animal is 12. The SNAP test assesses eight parameters exploring vision, proprioception, motor strength and posture. Scores range from 0 (normal) to 5 (severely impaired); the worst score is 40.

Cognitive deficits were evaluated in the Ymaze test. The Y maze is a two-trial spatial recognition memory test based on the innate propensity of rodents to explore new environments. The Y maze apparatus consisted of three arms joined in the middle to form a “Y”. The insides of the arms are identical, providing no intra-maze cues. Visual cues were placed around the perimeter of the maze, and kept

constant during the behavioral test. During the acquisition phase (trial 1), one arm of the Y maze was closed. The position of the closed arm was chosen randomly among the three arms. Each mouse was placed in one of the other two open arms ("starting arm"), and allowed to visit the two accessible arms of the maze for 5 min. At the end of the trial, mice were placed in their home cage. After an inter-trial interval of 1 h, mice were placed in the same "starting arm" as in trial 1, with free access to all three arms for 5 min (trial 2, retrieval phase) (Dellu et al. 2000; Conrad et al. 1997). During each trial, the number and the duration of visits to each arm were recorded (Ethovision XT 5.0, Noldus).

Tissue processing for histological analysis

At 8 and 13 weeks post-treatment, mice were deeply anesthetized (10% ketamine + 10% medetomidine + 80% saline; 10 ml/kg, i.p.) and perfused intracardially with phosphate buffer saline (PBS) 0,1 mol/L, pH 7.4, followed by chilled paraformaldehyde (4%) in PBS. The brains were removed from the skull and post-fixed for 6h at 4°C, and then transferred to 30% sucrose in 0.1 mol/L phosphate buffer for 24h. The brains were frozen by immersion in isopentane at -45°C for 3 minutes and stored at -80°C until use. Twenty µm thick serial sections were cut using a cryostat from bregma +1mm to bregma -4mm.

Immunofluorescence

Brain sections were washed three times in PBS, being each wash 5 minutes. The slides were blocked with a solution of 10% FCS in PBS for 1h. After that, the samples were incubated overnight with the following primary and respective secondary antibodies, diluted in PBS 0,5% with 5% FCS: i) mouse anti-GFAP (Sigma, USA, ref: G3893); ii) rabbit anti-rat Iba-1 (wako, china, ref: 019-19741); iii) mouse anti-NeuN (Merck-Millipore, USA, ref: mab377). On the next day, samples were incubated for 2 h with the respective secondary antibodies, alexa fluor 488 goat anti-mouse for GFAP, alexa fluor 594 goat anti-rabbit/anti-mouse for Iba-1 and NeuN respectively. All samples were counterstained with DAPI (Sigma, USA) for 15 min. Finally, the slides were mounted in Immuno-Mount® (Thermo Scientific, USA) and observed at Olympus Widefield Inverted Microscope IX81. All images were treated using ImageJ software.

Inflammatory Cytokine Profile

Blood was collected from the facial vein in awake mice, transferred to BD microvacutainer tubes (Becton Dickinson, Milan, Italy), and centrifugated at 4°C for 5 min at 5000 rcf. After, centrifugation plasma was collected and stored at -80°C until analysis.

The expression of inflammatory cytokines was quantified in blood plasma samples from sham, TBI and treated animals at 13 weeks' post-injury. Plasma cytokines were assessed using the LEGENDplexTM Mouse Inflammation Panel kit (Biolegend, Cat. No. 243 740150) according to the manufacturer's instructions. This kit includes the following analytes: IL-1α, IL-1β IL-6, IL-10, IL-12p70, IL-17A, IL-23, IL-27, MCP-1, IFN-β, 245 IFN-γ, TNF-α and GM-CSF. Briefly, reagents were prepared from the stocks provided, and standard serial dilutions were prepared to generate a standard curve. Assay buffer (25 247 µL) was added to the standard and sample wells at a 1:1 ratio. Then, 25 µL of mixed beads and 25 µL of detection antibodies were added to each well, and the plate was incubated for 2h at RT with continuous agitation at 800 rpm. Streptavidin-phycoerythrin (SA-PE) (25 µL) was added directly to the previous solution, and the plate was incubated for 30 min at RT with agitation at 800 rpm. After washing with 150 µL of 1× wash buffer, the samples were ready to read using the flow cytometer. The samples were vortexed, and beads were acquired using a cytometer (BD LSR II Flow Cytometer 254 (RRID:SCR_002159)).

Data analysis was conducted in Biolegend's LEGENDplexTM software web site. Analytes with concentrations below the detection limit (LOD) were excluded from the analysis. The experimenter was blind to the experimental groups when performing the protocol, sample acquisition, and analysis.

Membrane-based Antibody Array

A membrane-based antibody array (Human Neuro Discovery Array C1, AAH-NEU-1–2, RayBiotech, USA) was used, following manufacturer's instructions, to evaluate the factores secreted by HUCPVCs when plated in 2D conditions or encapsulated in GG matrices. Briefy, after 30 min of blocking, the membrane array was incubated ON, at 4°C with HUCPVCs secretome from each condition. On the next day, the membrane was incubated with the biotinylated detection antibody cocktail, followed by incubation with streptavidin conjugated HRP. Chemiluminescence detection was performed using Sapphire Biomolecular Imager and spot intensity was measured using AzureSpot software (Azure Biosystems).

3. Results

HUCPVCs delivered at the lesion site rescued sensorimotor and cognitive dysfunction of TBI mice

To evaluate sensorimotor performance of TBI animals, Neuroscore (NS) and simple neuroassessment of asymmetric impairment (SNAP) were used. Sham animals revealed a good and constant performance at both, NS and SNAP,during all the experimental time (**Fig. 2A** and **2B**). NS indicated significant improvement of sensorimotor function, of animals treated with HUCPVCs, 50 days post-TBI (**Fig.2A**). SNAP test revealed improvements, slightly sooner, with animals treated with HUCPVCS/GG-GRGDS showing better performance 25 days post-injury compared with vehicle animals. Significant results in locomotor function tests were also observed, 70 days post-injury in both NS ($p < 0,05$; **Fig. 2A**) and SNAP ($p < 0,05$; **Fig. 2B**). Significant differences were maintained until the end of the experimental course for both NS and SNAP ($p < 0,05$; 100 days post-TBI; **Fig. 2A**. $p < 0,01$; 100 days post-TBI; **Fig. 2B**). Fifty-six days (8 weeks) after TBI, Y Maze was performed to evaluate spatial learning and memory function. On the probe trial, HUCPVCs treated animals performed significantly better showing a higher percentage of time exploring the new arm compared with vehicles ($p < 0,05$, **Fig. 2C**).

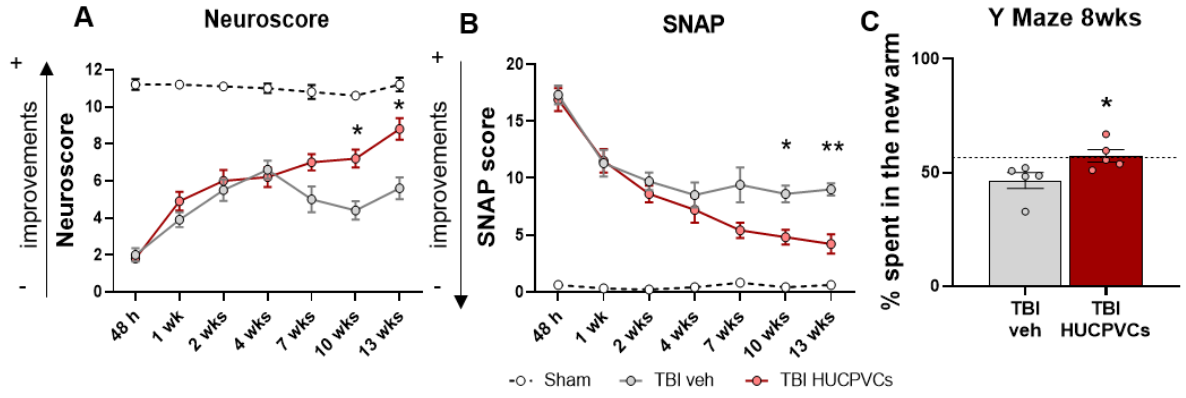


Figure 2: Sensorimotor and cognitive assessment. The evaluation of sensorimotor function was evaluated from the injury time until 13 weeks post trauma using **A.** Neuroscore and. **B.** SNAP tests. From 7 weeks post trauma, animals treated with HUCPVCs (red line) showed better locomotor performance that became statistical significant 10 weeks post TBI. **C.** Cognitive function of TBI animals, specifically short term spatial memory was measured by Y Maze test. Treated animals (red bar) recovered their spatial memory showing high % of time spent in the new arm, comparable to sham animals (dotted line). Data are the mean ± SEM. a, b) Two-way ANOVA, followed by Sidak's post test. c) One-way ANOVA, followed by Tukey's multiple comparisons test. n=5-10. *$p < 0,05$; **$p < 0,01$.

Lesion site delivered HUCPVCs reduced GFAP and Iba-1 in TBI and increased NeuN positive cells

To study astrocytic changes after TBI, the percentage of GFAP stained area was measured at 1.6 mm of distance from bregma, at the cortical region, and distanced from 400 μm from the cortex edge (**Fig. 3B**). Compared to sham animals (**Fig. 3C**), TBI showed a strong increase of GFAP stained area, at both 4 and 13 weeks post-TBI. Treatment with HUCPVCs, caused a reduction on GFAP stained area percentage at both 4 and 13 weeks (**Fig. 3C**).

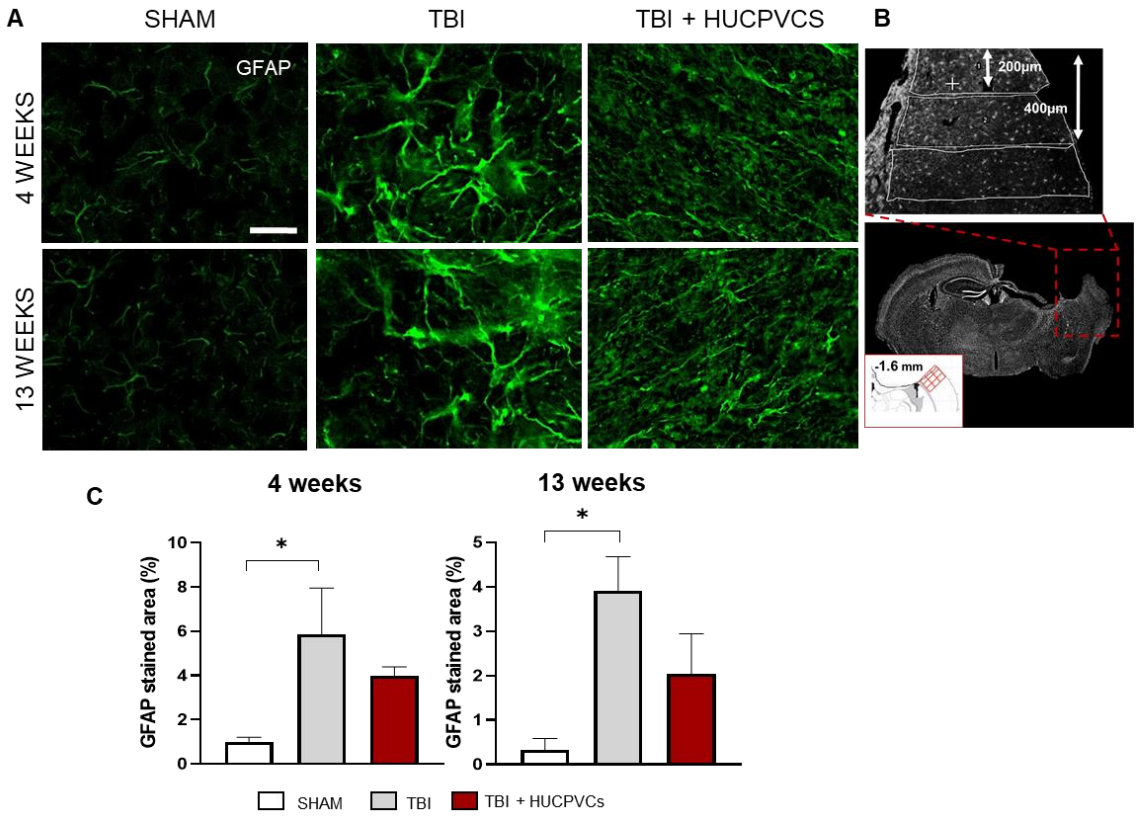


Figure 3: Evaluation of HUCPVCs effect on astrocytes 4 and 13 weeks after injury. **A.** Representative microscopy images of SHAM, TBI, and TBI + HUCPVCs animals. **B.** Representative image of the ROI evaluated. Immunofluorescence quantifications were performed in the cortical region, 400um distant from the tissue edge. **C.** Quantification of GFAP stained area at 4 and 13 weeks after TBI. Data are the mean ± SEM. c) One-way ANOVA, followed by Tukey's multiple comparisons test. n=3-5, for each time-point. *p < 0,05. Bar = 20μm

Microglia stained area was increased in TBI animals 4 and 13 weeks after injury (**Fig. 4A** and **4B**). No effect was observed in IBA-1 stained area 4 weeks after injury in animals treated with HUCPVCs (**Fig. 4B**). After treatment with HUCPVCs 3 days post-TBI, Iba-1 stained area was significantly reduced at the cortical region of these animals at 13 weeks post injury (**Fig. 4B**).

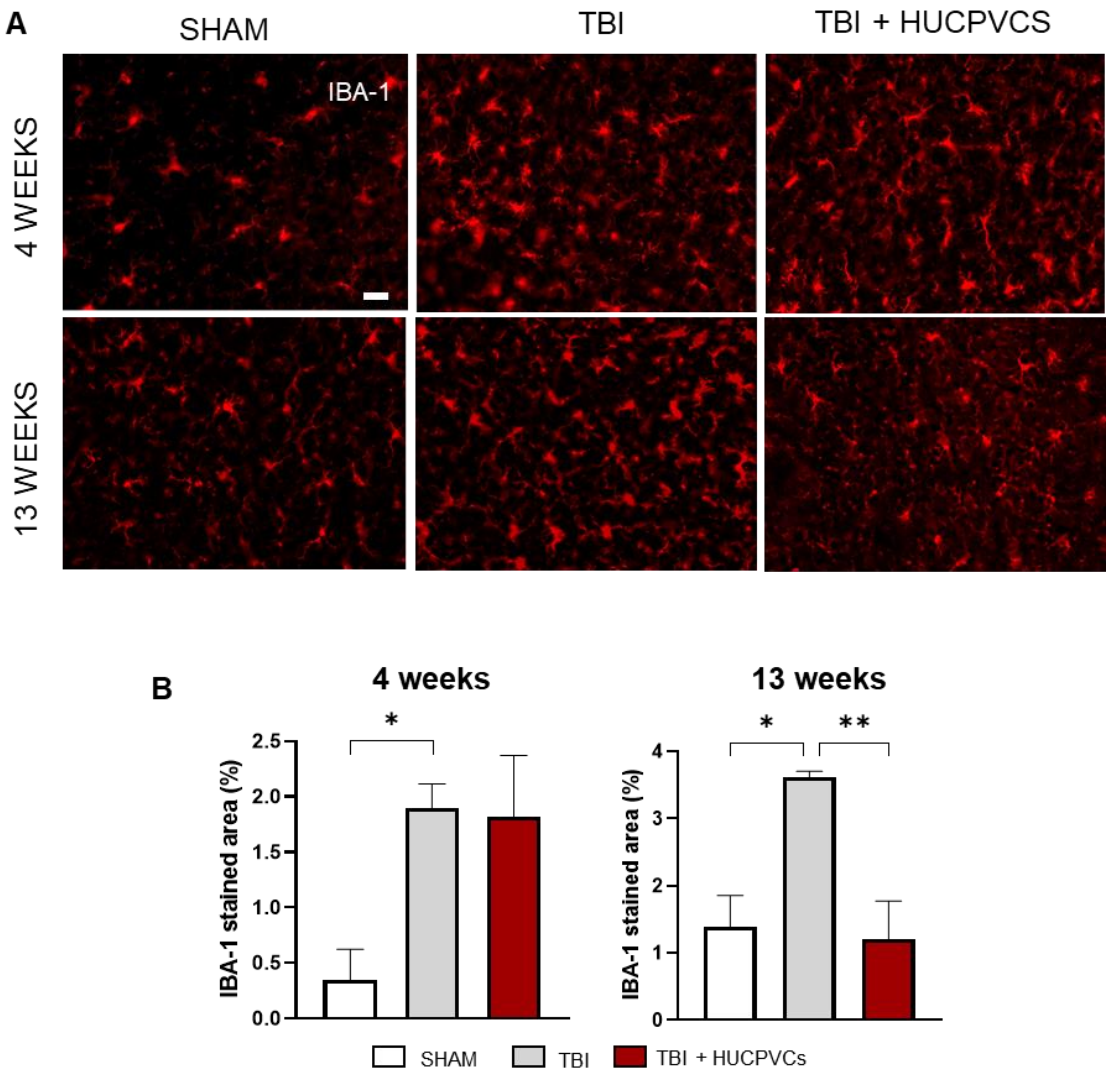


Figure 4: Evaluation of HUCPVCs effect on microglia 4 and 13 weeks after injury. **A.** Representative microscopy images of SHAM, TBI, and TBI + HUCPVCs animals. **B.** Quantification of IBA-1 stained area at 4 and 14 weeks after TBI. Data are the mean ± SEM. b) One-way ANOVA, followed by Tukey's multiple comparisons test. n=3-5, for each time-point. *p < 0,05; **p < 0,01. Bar = 20μm

NeuN marker was used to identify neuronal cell bodies (**Fig. 5A**). 4 and 13 weeks after injury the number of NeuN positive cells was decreased in the cortex of TBI animal (**Fig. 5B**). Neuronal loss, induced at the cortex of TBI animals was partially recovered, without significant results, after treatment with HUCPVCs, at 4 and 13 weeks post-TBI (**Fig. 5B**).

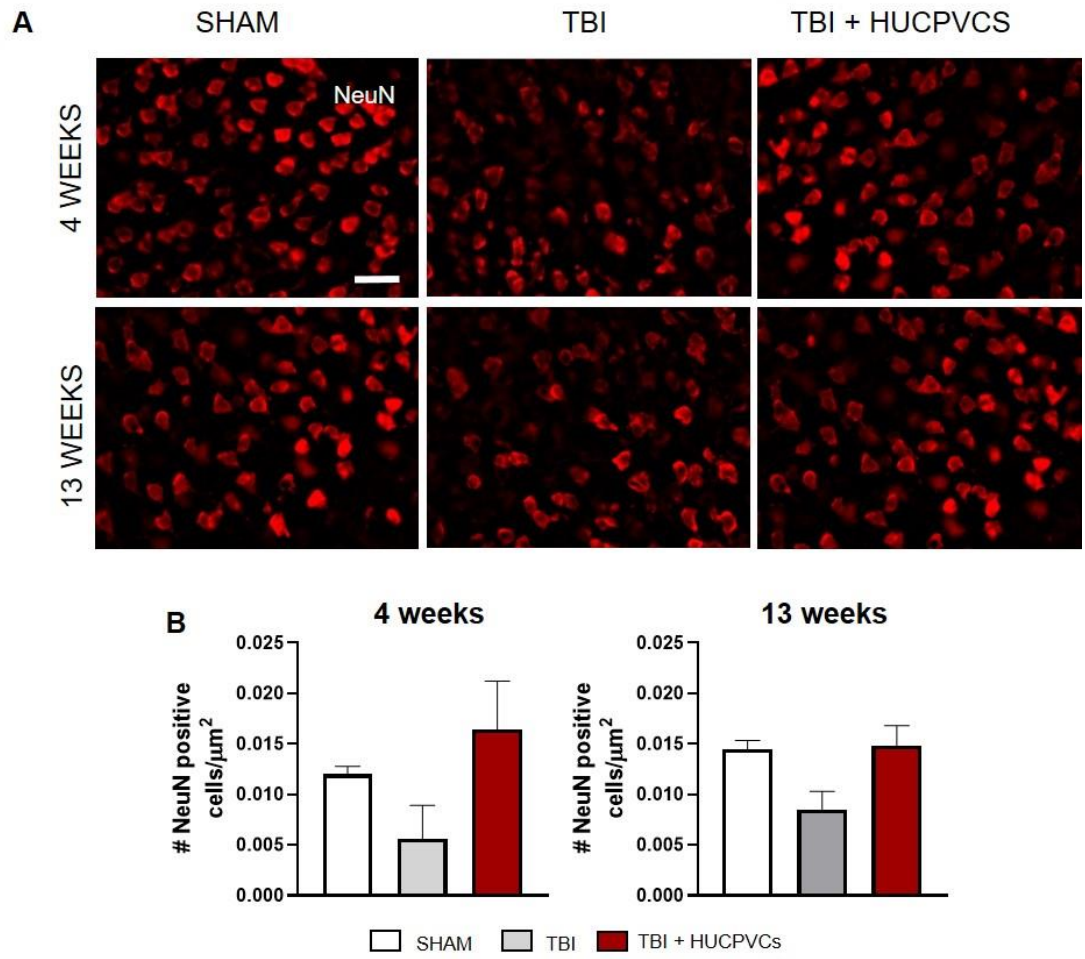


Figure 5: Evaluation of HUCPVCs effect on neuronal bodies at 4 and 13 weeks after injury. **A.** Representative microscopy images of SHAM, TBI, and TBI + HUCPVCs animals. **B.** Quantification of $NeuN^+$ cells at 4 and 14 weeks after TBI. Data are the mean ± SEM. b) One-way ANOVA, followed by Tukey's multiple comparisons test. n=3-5, for each time-point. Bar = 20µm

Lesion site delivered HUCPVCs reduced pro-inflammatory profile after TBI

The pro-inflammatory profile of the TBI animal's plasma was assessed 13 weeks post-trauma. No significant statistical results were found, although there is a general trend for a decrease in pro-inflammatory cytokines concentration, after treatment with HUCPVCs, with the exception of IL-1β (**Fig. 6**). IL-1β shows a tendency to increase after treatment with cells, with treated animals showing similar values to sham. All the other evaluated cytokines, namely, IL-1α, IL-6, IL-17, and IFN-γ, show higher concentrations after trauma, compared to sham animals, but these values are rescued after treatment, with animals treated with cells, showing lower cytokines concentration comparing to non-treated TBI animals (**Fig. 6**).

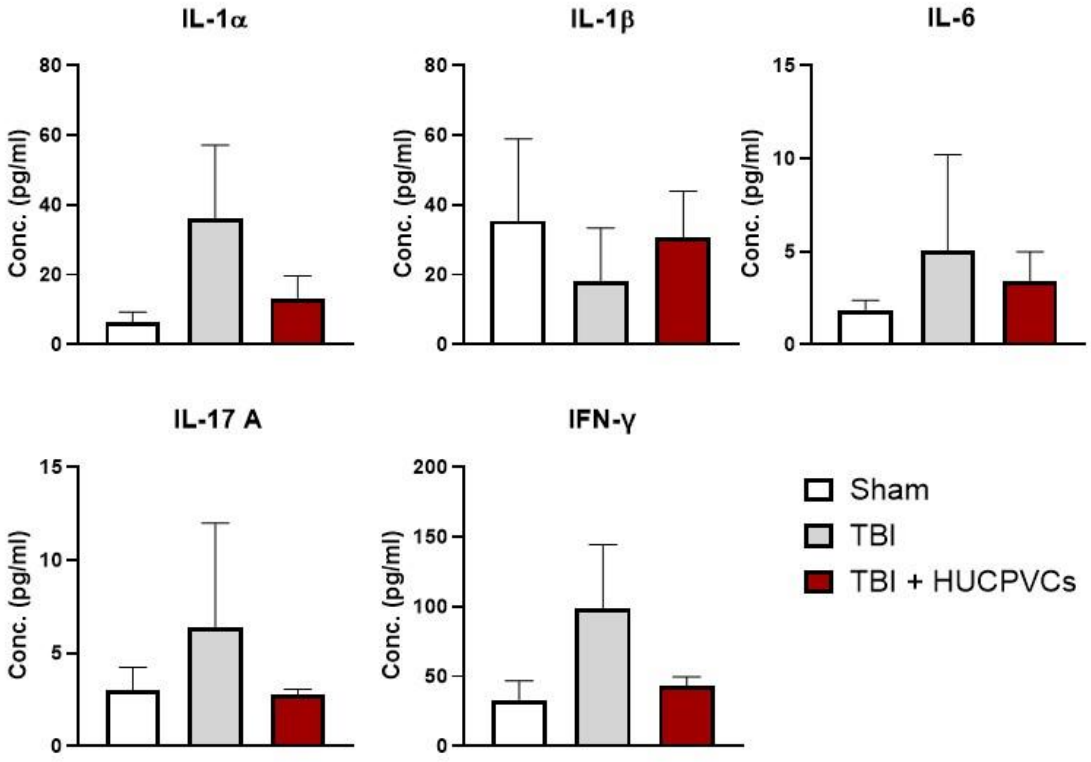


Figure 6: Inflammation markers measured in TBI and treated animals 13 weeks post injury. HUCPVCs tendentially decreased the levels of IL-17 A, IL-6, IL-17A and IFN-γ comparing to TBI animals levels 13 weeks post-TBI. Data are Mean±SEM. One-way ANOVA, followed by Tukey's multiple comparisons test. n=5

Assessment of HUCPVCs secretome profile

To address which molecules were implicated in the previously described protective effects, we compared the secretome content of HUCPVCs when plated in 2D conditions or when engrafted in GG-GRGDS matrices. Proteins such as β-NGF, GCSF, TGF-β and TGF-α showed residual values in both conditions under study. Proteins supporting neuronal survival and growth were found significantly enhanced when cells were encapsulated inside GG-GRGDS matrices. For example, BDNF, HB-EGF, and GDNF (**Fig. 7C**) were highly expressed by cells engrafted in GG-GRGDS, compared with plated cells. Additionally, when inside GG-GRGDS matrices, HUCPVCs released higher amounts of proteins responsible for astrocytic and vessel support, such as S100B and VEGF-A, respectively (**Fig. 7C**). The expression of IL-8 and MCP-1 is significantly higher when the cells are encapsulated within GG, although, these proteins show significant expression levels in both conditions. Additionally, MMP-2 is also release in higher concentration when cells are inside matrices (**Fig. 7C**).

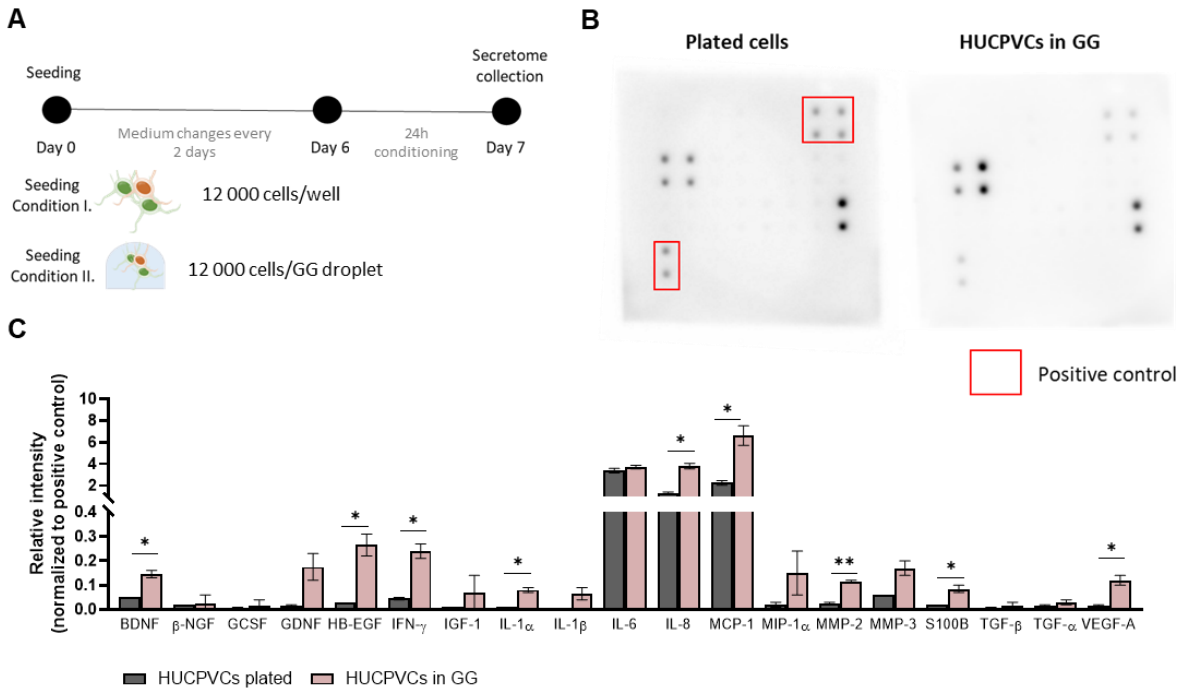


Figure 7: Profile of neuronal proteins released by HUCPVCs plated or engrafted in GG matrices. **A.** Experimental design. **B.** Membrane arrays depicting the proteins detected in the conditioned medium of plated and encapsulated cells. **C.** Quantification of array spot intensity for each condition. Data are mean ± SEM of relative intensity for each protein, normalized to positive control intensity. c) t-test for each protein. *p < 0,05; **p < 0,01

DISCUSSION

MSCs have been widely used as a promising therapy in pre-clinical (Pischiutta et al. 2016; Lu et al. 2001) and clinical trials for TBI (Tian et al. 2013; Wang et al. 2013; Kawabori et al. 2021). There is a growing interest in using MSCs derived from the human umbilical cord (UC-MSCs). In fact, recent reviews highlight their therapeutic efficacy and potential advantages over other sources (Joerger-Messerli et al. 2016; Kalaszczynska e Ferdyn 2015; Nagamura-Inoue e He 2014). Additionally, perivascular tissues from the UC showed a higher yield of MSCs (HUCPVCs) compared with other regions of the cord like, Wharton's jelly or umbilical cord blood (Ennis et al. 2008).

Biomaterials are a remarkable system for cell encapsulation and subsequent transplantation. GG hydrogel is a biomaterial with soft-like brain structural properties (Stevens et al. 2016). When modified with the fibronectin synthetic peptide GRGDS, GG proved cytocompatible with different cells, such as Neural Stem cells, and induced pluripotent stem cells and MSCs (Silva et al. 2012; 2013; Lozano et al. 2015). Studies from our group provide evidences that GG-GRGDS, enhances MSCs proliferation rate and metabolic activity (Silva et al. 2013). Moreover, when delivered into neuronal cultures, the secretome from MSCs engrafted in GG-GRGDS lead to higher viability of these cultures and increased neuronal density, comparing with secretome of cells plated in 2D conditions (Silva et al. 2013).

In the present work, we developed a combinatorial approach to TBI repair based on the use of HUCPVCs inside GG-GRGDS hydrogels. To better understand the effect of GG-GRGDS scaffold on the HUCPVCs secreted factors, we compared the concentration of neuronal proteins released by HUCPVCs when plated in 2D conditions and when inside GG-GRGDS drops. This analysis revealed, that cells encapsulated secreted higher amounts of neurotrophic factors, such as BDNF, GDNF and HB-EGF (Oyagi e Hara 2012). Previous works from our group demonstrated BDNF in HUCPVCs secretome to be crucial, mediating axonal outgrowth in CNS neurons (Martins et al. 2017). Also, VEGF-A was released in higher amount when cells were inside GG-GRGDS drops. VEGF signaling pathway has a key role in angiogenesis (Shibuya 2011) but also has been shown to promote adult neurogenesis and neuronal cell migration by stimulating endothelial cells in vascular niches to release cues for neural stem cells (Zhang et al. 2003). IFN-γ and IL-8 besides their activity as pro-inflammatory cytokines have been shown to display pleiotropic effects in the central nervous system, including neuronal protection and regeneration (Abd-El-Basset, Rao, e Alsaqobi 2020; Araujo e Cotman 1993; Saas et al. 2002). Rather than addressing the potential role of each identified molecule, it is important to acknowledge that these signaling molecules act together activating a set of different signaling pathways. One of such pathways is the Akt signaling pathway, which is activated by most of the growth factors and cytokines identified on the array and regulates diverse

functions, including glucose metabolism, proliferation, cell growth, transcription and cell survival (Shioda, Han, e Fukunaga 2009).

In line with previous studies, we found early significant impairments in sensorimotor functions, with partial improvements as the time progressed (Pischiutta et al. 2018; Zanier et al. 2011). When transplanted into TBI cavities, our combinatorial approach led to significant functional and cognitive improvements 10 weeks after TBI. Other studies, using UC-blood cells, show earlier functional improvements. We hypothesize, this may be due to low density of transplanted cells, compared with other works. For instance, Chen C. and colleagues, delivered into the lesion site of TBI rats $5x10^5$ non-encapsulated cells, and observed functional improvements at the NS test as soon as 7 days post injury (Chen et al. 2022).

In our work, the treatment with HUCPVCs and GG-GRGDS, slightly increased the number of NeuN positive cells in the cortex. One hypothesis to explain this tendency in NeuN increase after treatment is that, the HUCPVCs secreted factors are stimulating neurogenesis, leading to maturation and differentiation of new neurons. This tendency may explain the gain of function observed at SNAP and NS tests. Although, our results suggest that the observed functional improvements were not directly connected to regeneration or preservation of neuronal circuits, but more likely, linked to modulation of the inflammatory environment at the cortical regions of these animals. Additionally, it is widely recognized that neuronal apoptosis and degeneration in the hippocampus are closely correlated with the impairments of neurological function (Komoltsev et al. 2023). Therefore, it would be of great interest to study neuronal or apoptotic markers in the hippocampus of these animals.

Previous studies have suggested that microglia and astrocyte activation generally occur after TBI. These activated glial cells increase the detrimental neurotoxic effects by releasing numerous cytotoxic substances (Loane et al. 2014). Here, we found that TBI induced by CCI increased microglial activation, as evidenced by the increased percentage of area fraction. After treatment, we observed a marked decrease of IBA-1 marker 13 weeks after TBI, at the cortex of TBI animals. We did not see differences in any other analyzed markers, but strong trends, pointing into tissue recover after treatment. Specifically, GFAP was slightly decreased in treated animals compared with non-treated. In fact, a reduction of inflammation and astrogliosis was associated in previous studies with improvements in locomotor function (Homsi et al. 2010). Other markers such as β 3-Tubulin, Neurofilament, and Microtuble associated protein-2 (MAP-2), should be accessed in further studies to better understand the therapeutic effect of HUCPVs and their impact on neurons and neuronal circuits regeneration.

Peripheral neuroinflammation was shown to influence brain mechanisms and interfere with disease progression. In addition, chronic inflammation is highly detrimental and not favorable to the survival of neuronal cells and implanted stem cells (Corps, Roth, e McGavern 2015). In the present study, we found that the administration of HUCPVCs inside GG-GRGDS could lessen chronic peripheral neuroinflammation, which was reflected by the slight decrease of pro-inflammatory cytokines measured in the plasma.

The routes of delivering stem cells to the host, which commonly include intravenous, intra-arterial and intracranial delivery methods, play an important role in the success of stem cell therapies. The intravenous method seems to be the most attractive for clinical applications, although many of the cells may get entrapped in peripheral organs. Here we propose an intracranial delivery, directly at the lesion site. Intracranial transplant represents a practical clinical approach in ambulatory conditions when accidents result in skull opening or during surgery procedures. Specifically, in a high number of TBI cases a craniotomy is needed for the relief the intracranial pressure induced by hematoma. In this cases, local therapy may be easily applied without causing any extra inconvenient to the patient.

Despite promising there are some limitations to this study. At 13 weeks post-injury we could not encounter any trace of evidences of the GG-GRGDs at the lesion cavity. Previous studies, reveals that, comparing with other hydrogels, GG has a slow degradation rate and it can be found as late as four weeks following injury (Silva et al. 2010). Additionally, in a SCI model, Gomes E. suggests that the degradation rate of GG in vivo, occurs between 4 to 8 weeks (Gomes et al. 2016, 201). Importantly, independently, of the hydrogel availability in situ, it seems that it remained at the injury site sufficient time to allow tissue remodeling and functional improvements. Therefore, it would be interesting to understand if HUCPVCs integrated the host tissue and characterize their availability at different times post-injury. Additionally, from the clinical point of view it would be of great interest to validate the translational value of our work by measuring blood biomarker through the disease progression. Biomarkers are a promising diagnostic tool and would allow to better characterize the therapeutic potential of our combinatorial approach overtime.

Conclusions

Overall, this work reveals the therapeutic effect exerted by HUCPCVs when delivered to a contusion TBI animal model, directly at the lesion site, inside of a GG-GRGDs scaffold. Our combinatorial approach was able to promote functional and cognitive recovery. This recovery was associated with a robust decrease of microglia markers at the cortical region of TBI animals. A strong tendency indicating a decrease of

astrocytic activation as well as an increase in neuronal positive cells. Our promising results were probably induced by the HUCPVCs secreted factors that were richer in neuronal and angiogenic proteins when encapsulated in GG-GRGDS.

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CHAPTER IV

General discussion and Future perspectives

General discussion and future perspectives

TBI is a devastating disease that affects people of all ages all over the world (GBD 2019 Ageing Collaborators 2022). Of all injuries that may affect the brain, TBI is the major cause of disability and death, burdening individuals, families, health systems, and societies (Maas et al. 2022). TBI is heterogeneous throughout all the disease stages; from the first initial moment of the accident to the complex cascade of neurodegeneration that follows, and the vast number of symptoms that may develop from there.

Based on GCS, and complementary diagnosis methods, TBI is classified regarding severity. Severe TBI may lead to death, and survivors often experience physical, cognitive, or emotional disabilities. Therefore, severe TBI is a major concern. Detailed characterization of the disease phenotype is crucial for TBI management and for predicting outcomes in individual patients. However, diagnosis techniques still need to be improved. Methods among hospitals and healthcare institutions should be standardized to improve service quality. Biomarkers measurement in cerebral spinal fluids is a major step in TBI management since it offers a non-invasive and reliable approach to follow TBI progression over time (Czeiter et al. 2020; Wang et al. 2021).

Despite major advances in TBI research, and in medical support to the victims, an effective therapy in counteracting disease progress is still lacking. Here, we point to the absence of reliable in vitro models as one of the contributors to the failure of therapies' translation into clinics. In vitro models are simpler than in vivo models, allowing us to restrict the area of study and to look closer into specific targets. Additionally, in vitro models have reduced cost, higher throughput, and reduced animal use, compared to in vivo models (Kumaria 2017). Specifically, in vitro models allow us to tackle two important aspects. On one side, a good TBI model will allow to understand specific biological alterations triggered by the mechanical impact and to deeper understand the disease mechanisms. On the other side, a representative model will better track a drug or therapy effect revealing specific mechanisms of action. Organotypic slices are a promising platform as a 3D model in vitro, since they keep cells function and the tissue cytoarchitecture (Humpel 2015). Although organotypic slices offer unprecedented opportunities for understanding the mechanisms underlying TBI and developing new therapies, it is undeniable the key role of animal models in finding new therapeutic approaches.

Stem cell-based therapies are promising to develop regenerative treatments and have provided hope for a significant number of patients. Initially, neural stem cells (NSCs) were considered the best choice to treat CNS disorders. However, research has shown that MSCs may be more promising and yield better

therapeutic outcomes. MSC-based treatments have demonstrated strong protective effects in different animal models of neurological diseases in experimental studies (Pischiutta et al. 2016; Nih et al. 2018). Preclinical works using different experimental models of TBI, show that MSCs may attenuate a wide range of TBI-related pathological aspects. Specifically, MSCs have shown results in reducing TBI-induced motor and cognitive deficits, in animal models (Zanier et al. 2011; Pischiutta et al. 2016; 2021). Moreover, they have been found to promote neurogenesis provide neuroprotection, and facilitate repair in rats and mice with TBI (Anbari et al. 2014). Furthermore, MSCs have demonstrated effects in modulating inflammation by downregulating proinflammatory genes and upregulating antiinflammatory genes, in the brains of rats with TBI (Galindo et al. 2011).

In this thesis, we evaluated HUCPVCs as a source of therapeutic factors with neuroprotective potential for severe TBI focal lesions, both in vitro and in vivo conditions. Given the increasing promise of MSCs as a therapeutic agent, there is a need to establish alternative and higher yield sources, compared to the gold standard from bone marrow. HUCPVCs are harvested from the umbilical cord perivascular tissues and own as the main advantage their perinatal origin and thus their youth low differentiation state. To add, previous studies show that, umbilical perivascular tissues render a vastly increased number of MSCs compared with umbilical cord blood (Ennis et al. 2008).

To represent TBI, we used the controlled cortical impact (CCI) model, in vitro and in vivo. CCI is the most widely used model to mimic TBI in rodents, but is the first time it has been used to injure cortical organotypic slices. CCI is performed using an impactor device which allows injury parameters manipulation, thus offering the possibility to induce a wide variability of injury severities, depending on the specific aim of the study, simultaneously allowing for trauma reproducibility (Siebold, Obenaus, e Goyal 2018). Additionally, CCI in rodents triggers pathological hallmarks of human TBI, like inflammation, astrogliosis, and neuronal and glial death. In Chapter II, we used the secretome of HUCPVCs to treat, a newly implemented model of CCI in organotypic brain slices from the cortex. Our innovative CCI in vitro model closely reproduced key hallmarks of human TBI, namely neuronal death, network changes, and glial activation. Our model offered the possibility to assess injury and therapeutic responses, with direct translational value. Delivered in acute time points after injury, HUCPVCs released factors successfully attenuated CCI features in organotypic cortical slices. Our CCI in vitro model opens new possibilities to a deeper study TBI pathology and brings a strong method to screen new drugs and therapeutics before proceeding into animal models. Importantly, our CCI in vitro model may be deeper characterized. For instance, oxidative stress species, metabolic imbalances, and demyelination may be assessed. Simultaneously, it would be a major achievement to evaluate the electrophysiological function of neuronal

cells in the organotypic tissue, before and after treatment. Accordingly, in 2016, Gong and collaborators developed an innovative microelectrode array system to cultivate and record electrical activity from organotypic brain slices (Gong et al. 2016). This system offers the chance to record continuously the neuronal network activity over time, offering the possibility to study the chronic impact of induced diseases or treatments (Gong et al. 2016).

Although having attractive features, organotypic slices show limitations. One major limitation of this system is the loss of connectivity with other brain areas. Despite one advantage of organotypic slices is the possibility to study specific regions in an isolated way, it is important to keep in mind that certain brain processes and interactions rely on connectivity, and may be disrupted or negatively influenced by this disassociation. Studying deeply neuronal networks, for instance, would be challenging in these conditions. In accordance with the previous, it is important to keep in mind that the results obtained do not fully represent brain reality and do not have a direct translation. In fact, previous works have shown that in vitro, neurons organize differently, and have more branches, dendrites and more complicated synapses (De Simoni, Griesinger, e Edwards 2003). Also, microglia and astrocytes behave differently and show different morphology (Czapiga e Colton 1999) in organotypic tissues. Limited lifespan of these cultures is another drawback, which typically goes from few days to weeks. Therefore, the study of TBI chronic effects or long term outcomes of specific treatments would be compromised. For instance, to study in vitro, chronic complications after TBI, such as Tau and myeloid β accumulation, would be challenging in organotypic brain slices (Johansson et al. 2006; Frozza et al. 2009; Suh et al. 2008). Another lack of organotypic slices is peripheral blood flow and peripheral infiltration of immune cells and signals from other organs of the human body, which may influence the brain response after trauma (Plesnila 2016; Hanscom, Loane, e Shea-Donohue 2021). In fact, a chronic and maladaptive peripheral neuroinflammatory response is a major contributor to progressive neurodegeneration and long-term neurological impairments (Simon et al. 2017).

Regardless MSCs satisfying results, several works have demonstrated that MSCs survival and engraftment at the lesion site is typically too short. In fact, works show that just 1% of transplanted MSCs survive after transplant (Roche et al. 2014; Li et al. 2016). To date, several strategies have been studied to increase the migration of cells to target tissues and to retain them and specific delivery sites of interest. These strategies include 1) in vitro modifications; before cells transplantation into host tissue, by changing the in vitro cell culture conditions. 2) Cells genetic modifications. 3) Cell membrane engineering; by attaching new molecules to the cell membrane or modifying existent elements in their membranes. 4) Modifying the host environment. 5) Using protective matrices.

In Chapter III, we used a combinatorial approach to treat a CCI in mice. Our treatment consisted in a natural hydrogel (GG), modified with a fibronectin synthetic peptide (GRGDS) with HUCPVCs encapsulated. The GG-GRGDS hydrogel has been extensively studied by our group, demonstrating in vitro to be a suitable biocompatible matrix for the growth of different types of cells (Silva et al. 2013, 201; 2012; Assunção-Silva et al. 2015; Oliveira et al. 2017). In addition, this hydrogel has been demonstrated to improve the abilities of encapsulated cells leading to neuronal growth and increased expression of neurotrophic-related genes (Silva et al. 2013; Oliveira et al. 2017). Interestingly, here we measured the concentration of neuronal proteins released by HUCPVCs plated in 2D conditions or engrafted in GG matrices. Results show, that when inside GG drops these cells, increases the release of important growth factors such as BDNF, GDNF, HB-EGF and the angiogenic factor VEGF-A. Previous studies from our group show a major role of BDNF secreted by HUCPVCs in inducing axonal growth of CNS neuronal cultures (Martins et al. 2017). When depleting BDNF using a TrkB Fc molecule, that specifically binds and neutralizes the growth (Leal, Afonso, e Duarte 2014), the enhancing effects on axonal growth previously induced by HUCPVCs secretome was lost to basal levels (Martins et al. 2017). An increasing body of evidence suggests that S100B, one of the factor enhanced on GG-GRGDs condition, might have a role during neurogenesis, participating in astrocyte maturation (Raponi et al. 2007).

As mentioned previously, in addition to their multipotent differentiation potential, MSCs paracrine capacity is pointed as the main mechanism responsible for tissue repair. GG is a porous matrix and, besides offering cell protection, at the lesion site, will allow tissue-cell contact and the release of their secreted factors in situ, in a controlled manner. Our TBI animals showed locomotor deficits and cognitive deficits, highlighting the severity of the induced trauma. In line with previous studies, TBI mice presented early significant impairment in sensorimotor functions, with partial recovery at latter time-points (Pischiutta et al. 2018; Zanier et al. 2011; 2016). The HUCPVCs delivered 3 days post-TBI, using the previously described combinatorial approach led to functional and cognitive recovery of TBI animals, 13 weeks after trauma. Functional recovery is probably due to immunomodulation and tissue repair indicated by, low astrocytic and microglia activation. Previous studies, using MSCs have shown earlier improvements (Zanier et al. 2011; Barretto et al. 2020). We hypothesize that our chronic therapeutic effect may be due to the low number of cells engrafted on GG-GRGDs matrices or with the low diffusion rate of cells and secreted factors into the host tissue. Additionally, 8 weeks after TBI, our combinatorial approach resulted in a significantly better performance of treated animals on Y-Maze test. Y-Maze measures the spatial memory. Specifically, animals' performance is not related with memorizing a rule, but in their innate capacity to recognize new places and explore new environments. Treated animals,

spent higher percentages of time, exploring the new arm, indicating improvements of their spatial memory after treatment. Additionally, the Y maze test rely on hippocampal dependent assessments of short term spatial memory. Cognitive amelioration induced by our treatment is probably due to glial modulation and neuronal regeneration on hippocampus, and would be an addition in next studies to further explore this aspect and other regions far from the lesion site.

Overall, we can state that the work performed at this thesis provided promising results regarding the potential use of HUCPVCs to attenuate TBI deficits. Although more research needs to be done regarding MSC standardization and culture, and characterization of the molecules by them secreted in normal or ''TBI-conditions''. Some other studies have shown that exposing MSCs to hypoxic and inflammatory stimuli alters their secreted factors and would be interesting to further explore this using our combinatory treatment. Additionally, this work has further limitations. One of the main setbacks is the lack of tracing of MSCs inside the matrix in vivo, and their capacity to adhere grow and bridge into TBI lesions, which has been widely reported. Also, HUCPVCs secreted factors into the host tissue, and the evaluation of GG-GRGDS degradation both in vitro and in vivo would be of great interest. Another drawback in our work is the lack of some controls. To evaluate the effect of GG-GRGDS alone in TBI cavities would help to better elucidate, the effect of this scaffold regarding intracranial pressure.

In sum, a neuroprotective or disease-modifying therapy that can immunomodulate or stop disease progression is a major unmet medical need for TBI treatment. One of the major hurdles to define a neuroprotective therapy for TBI includes disease heterogeneity, and the uncertainty behind the trauma causes and the specific target for each case. Accordingly, it is evident the need for a personalized management. For that unifying efforts between experimental and clinical are needed to develop and implement an effective therapy

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