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Assessment of the Therapeutic Effects of Adipose Mesenchymal Stem Cells Secretome on a Mouse Spinal Cord Injury Model

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Avaliação dos Efeitos Terapêuticos do Secretoma de Células Estaminais de Tecido Adiposo em Lesões Vertebro-Medulares

Master Thesis in Health Sciences

Under the supervision of:

Dr. António Salgado

Dr. Nuno Silva

July 2018

Statement of Integrity

I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration. I further declare that I have fully acknowledge the Code of Ethical Conduct of the University of Minho.

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Resumo

As lesões vertebro-medulares são atualmente uma condição devastadora e sem tratamento. Todos os anos surgem novos casos (57.8 novos casos por ano em Portugal), com uma incidência bastante elevada em jovens do sexo masculino. A causa mais comum de lesão é devida a eventos traumáticos, tais como acidentes de viação, quedas, violência e atividades desportivas, enquanto que os eventos não-traumáticos (tumores, doenças neurodegenerativas e infeciosas) são menos prevalentes. Geralmente, as lesões vertebro-medulares causam perdas de função motora e sensorial abaixo do nível da lesão. Tudo isto conduz a uma redução da qualidade de vida, tanto económica como socialmente, quer para os pacientes quer para os familiares. Este tipo de lesão na medula espinal desencadeia uma cascata de eventos moleculares e celulares, conhecida por lesão secundária, que aumentam o dano neuronal e criam um ambiente desfavorável para que a regeneração nervosa ocorra. Atualmente, na clínica apenas são usados glucocorticoides, sendo também realizada a descompressão da espinal medula por via cirúrgica. Contudo, estas abordagens apresentam uma eficácia limitada para recuperação da patologia. Neste sentido é urgente desenvolver novas terapias que possam solucionar este problema. Assim, aqui pretendemos avaliar o efeito terapêutico do secretoma de células estaminais derivadas de tecido adiposo. Estas células são caracterizadas por produzir uma vasta gama de fatores de crescimento (anti-apoptóticos, angiogénicos, neuroprotetores e imunomoduladores) que poderão modular o ambiente desfavorável após a lesão para um estado neuroprotetor e regenerador. Para além disso, a aplicação de secretoma é independente de transplantação celular, evitando os problemas associados com esta técnica. Em primeiro lugar, foi implementado um novo modelo de lesão vertebro-medular no laboratório por compressão em ratinho e verificou-se que a severidade de lesão é dependente do tempo de compressão. Depois avaliou-se o potencial terapêutico do secretoma das células estaminais derivadas de tecido adiposo neste modelo. Foram utilizados três grupos experimentais: (1) administração sistémica de secretoma; (2) administração local de secretoma; (3) Controlo - administração de meio de cultura. Os resultados mostram um efeito positivo da administração sistémica de secretoma comparativamente com o controlo, com a observação de uma tendência consistente nos diferentes testes para uma redução no tamanho da lesão, melhoria na função locomotora, comportamento exploratório, no equilíbrio e na coordenação fina. Estes resultados revelam que o secretoma de ASCs pode ter uma contribuição terapêutica importante para o tratamento de lesões vertebro-medulares.

Abstract

Spinal cord Injury (SCI) is a dramatic pathology, with a high number of new cases emerging every year (57.8 new cases per year in Portugal) and a typical higher incidence is found in younger males. The most common cause of injury comes from traumatic events, such as traffic accidents, falls, violence and sports activities, while the non-traumatic events (tumours, neurodegenerative and infectious diseases) are less prevalent. Usually, SCI causes motor and sensory functional loss below the level of the spinal cord. It leads to high debilitating conditions having a strong effect on patients' physiologic, psychologic and social behaviour. The injury itself triggers several biological events such as the activation of apoptotic pathways, the release of inflammatory cytokines and also the formation of a glial scar that primarily contains further damage, but also releases biomolecules that inhibit axons outgrowth. Currently, there is no effective treatment for SCI and the acute clinical management of these patients relies only in cord decompression and, in some cases, methylprednisolone infusion. For this reason, it is urgent to find new treatments that specifically target this condition. With this in mind, herein we aimed to assess the therapeutic value of Adipose Stem Cells (ASCs) secretome on a newly-established mice SCI model at our lab. ASCs are reported to secret several factors, including: anti-apoptotic and angiogenic factors, neuroprotectants and immunomodulators which may prime the unfavourable environment to a more neuroprotective/neuroregenerative one. Furthermore, this is a cell-transplantation-free therapy without the disadvantages associated with the transplantation of cells. Firstly, it was implemented a compression SCI mouse model with two different severities depending on compression time. Then, it was evaluated the therapeutic value of ASCs secretome after spinal compression. Mice were subjected to a laminectomy and consecutive compression at T7-T8 and divided in three experimental groups: (1) systemic administration of ASCs secretome; (2) local administration of ASCs secretome; (3) administration of culture media as control. The results showed a positive effect of systemic administration of ASCs secretome. It was also observed a consistent trend among tests to a reduction of the lesion volume, improvement of locomotor function, exploratory behaviour, balance and fine motor coordination. Altogether, these results demonstrate the therapeutic value of ASCs secretome administration for SCI.

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List of Figures and Tables

List of Abbreviations

µl- microliter

10s- 10 seconds

2s- 2 seconds

AD-Alzheimer disease

ASCs- Adipose stem cells

ASIA- American Spinal Injury Association

BBB_1- Basso, Beattie and Bresnahan

BBB_2- Blood Brain Barrier

BDNF- Brain-derived neutrophic factor

bFGF- Basic fibroblast growth factor

BMS- Basso mouse scale

Ca^{2+}- Calcium

CADH2- Cadherin-2

CCL22- chemokine ligand 22

CCL5- chemokine ligand 5

cm- centimeter

CM- Conditioned media

CNS- Central Nervous System

CO_2- carbon dioxide

CSF- Cerebral Spinal Fluid

CXCL1- chemokine ligand 1

CXCL12/ SDF-1- stromal cell derived factor 1

CYPA- Cyclophilin A

CYPB- Cyclophilin B

CYSC- Cyclophilin C

DJI-1- Protein deglycase

DNA- Deoxyribonucleic acid

dpi- days post injury

EES- Epidural stimulation

FBS- Fetal bovine Serum

FDA- Foods and Drugs administration

Gal-1- Galactin-1

HBO- Hyperbaric oxygen therapy

HGF- Hepatocyte growth factor

HSP-27- Heat shock protein 27

IL-1- Interleukin 1

IL-10- Interleukin 10

IL-1ß- Interleukin 1ß

IL-6- Interleukin 6

iPSCs- induced pluripotent stem cells

M1- pro-inflammatory macrophages

M2- anti-inflammatory macrophages

MCP-1-Monocyte chemoattractant protein 1

Mg^{2+}- Magnesium

MIP-1 alpha- macrophage inflammatory protein 1 alpha

MIP-1beta- macrophage inflammatory protein 1 beta

MMP1- Matrix metalloproteinase-1

MMP2- Matrix metalloproteinase-2

MP- methylprednisolone

MSCs- Mesenchymal stem cells

MST- Motor swim test

NaCl- Sodium Cloride

NGF- Nerve growth factor

NSCs- Neural stem cells

OECs- Olfactory ensheathing cells

P6- Cells passage 6

PAI-1- Plasmiogen activator inhibitor-1

PBS- Phosphate-buffered saline

PD- Parkinson Disease

PDEF- Prostate-derived Ets transcriptor factor

PEDF-Pigment epithelium-derived factor

PGE- Prostaglandin E

PRDX-1- Peroxiredoxin-1

RNA- Ribonucleic acid

SCF- stem cell factor

SCI- Spinal Cord Injury

s-seconds

T5, T8, T9, T12- Thoracic level

TGF-ß- Transforming Growth factor beta

TNF- α- Tumor necrose factor α

VEGF- Vascular endothelial factor

α-MEM- α Minimum Essential Media

CHAPTER 1 – INTRODUCTION

1-The Central Nervous System – A Master Controller

The Central Nervous System (CNS) is responsible to control every single cell, tissue and organ and orchestrates everything together so the whole body works perfectly. CNS comprises two main structures, Brain and Spinal Cord, in which the main building blocks are the neurons. Any disturbance/damage on them such as infections, inflammatory diseases, neurodegenerative pathologies, traumatic injury or even cancer, may have dramatic consequences leading patients to poor health and life conditions.

1.1-CNS structures, characteristics and basic anatomy

The adult human brain weights approximately 1,30 kg – 1,40kg and it is constituted by different compartments. The largest one is the *Cerebrum* which is a wrinkled structure divided into left and right hemisphere connected by the *Corpus Callosum,* a mass of white matter. The brainstem lies underneath and behind it is located the *Cerebellum* [1]. The Brain receives, integrates, controls, and transmits information to any part of the body through the optical nerve, the Spinal Cord, the Peripheral Nervous System (PNS), as well as. through the endocrine system through the release of hormones.

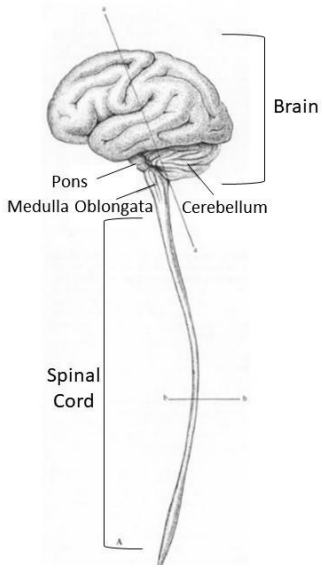

Figure 1- Illustration of the main Structures of the CNS, Brain and Spinal Cord.
Figure adapted from[172]

The Spinal Cord is physically continuous connected to the Brain through the lower part of the Brainstem, named *Medulla Oblongata* (Fig.1). Moreover it has essential functions such as regulating breathing, heart rate, blood pressure or digestion[1]. The Spinal Cord in the adult is a continuous cylinder of central nervous tissue, divided into segments determined by the emergence of spinal nerves and responsible for controlling voluntary muscles of the limbs and trunk as well as bringing sensory information from these regions. The average length in males is 45 cm and 42-43 cm in women. It is surrounded by the meninges and it is enclosed inside the vertebral canal at the backbone[2].

1.2-Anatomy of the spinal cord

The spinal cord in humans only occupies 2/3 of the dorsal column, below of which there is only spinal nerves. It is slightly flattened dorsoventrally, with a 3-mm longitudinal midline fissure named anterior median fissure (Fig.2). Looking to a transverse section of the spinal cord, in the middle, there is a circular structure termed the central canal remnant of the embryological ventricular system. It runs along all length of the spinal cord being filled with cerebrospinal fluid.

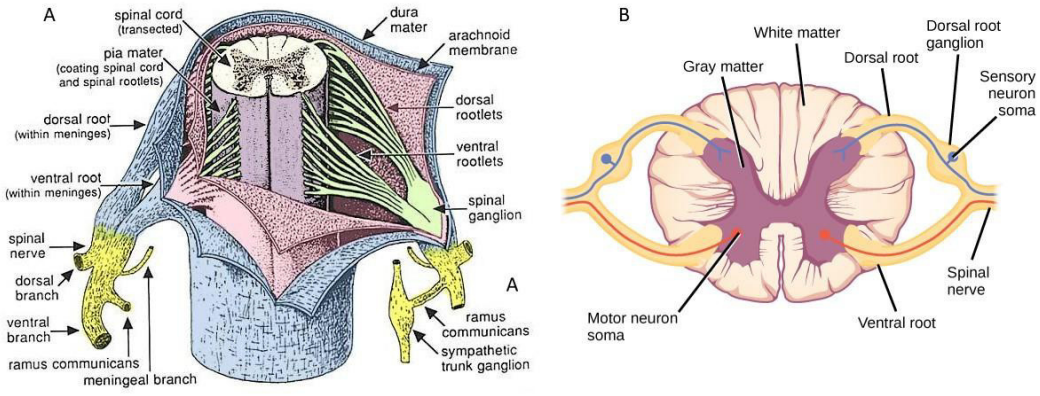

Figure 2-(A) Illustration of the Human Spinal cord**; (B)** Spinal Cord Cross Section**;** Figures adapted from[173,174]

Nonetheless in human adults the central canal is usually closed or blocked with cellular debris[2]. In humans the spinal cord is made up of 31 segments 8 cervical (C1 to C8), 12 thoracic (T1 to T12), 5 Lumbar (L1 to L5), 5 Sacral (S1 to S5) and 1 coccygeal segment, the division is based on where the pairs of spinal nerves arises.

Pairs of nerves leave the spinal cord trough the intervertebral space of the vertebral column. They are made up of 6-8 dorsal and ventral rootlets emerging from the spinal cord on each side, and bundle together forming the dorsal and ventral root. It is known that the dorsal roots contain sensory fibers, while the ventral roots contain motor fibers (fig.2).

Similar to the brain, the spinal cord is also composed of grey and white matter. A layer of white matter surrounds the gray matter except when the gray matter touches the margin of the spinal cord. This kind of organization resembles the shape of a butterfly or a capital letter H in the spinal cord, depending on the spinal level. The dorsally projected arms are called dorsal horns, and the ventrally project arms are called ventral horns, where motor neurons are located. The region between the dorsal and ventral horn is named intermediate gray matter and the left and right side of the gray matter are connected by the commissural gray matter (Fig.2). The gray matter consists of neuronal cell bodies, dendrites, axons, and glial cells. On the other hand, the white matter is made up longitudinally running axons and glial cells[2]. Its name is due the white colour given from the myelin which coats the axons. The white matter is perfectly structurally organized. A large group of axons in a certain region is called a funiculus, at the same time a smaller bunch of axons which share a close function is a fasciculus.

Tract is a term for a group of axons which hold the same origin, course, termination and function while pathways refer to a group of tracts with associated functions[2]. Tracts could be ascending (sensory fibres) or descending (motor fibres). Common examples of spinal tracts are the Spinothalamic tract (pain and temperature sensation), spinocerebellar tract (proprioception sensation), corticospinal tract (voluntary control of skeletal muscles), subconscious tract (balance, muscle tone, eye, hand and upper limb position)[3].

This structural organization is partially shared among species. However, the cortical spinal tract seems to be the unique major tract to have a different location between rodent models (dorsally located) and humans (laterally located) [4]. Since the cortical spinal tract is associated with voluntary movement, the location can represent a significant variable on rodents SCI models.

1.3-The Spinal Cord Cells

At the cellular level the spinal cord is populated by neurons and glial cells (fig.3) [5]. The first one to describe neurons as the basic structure of the CNS was Santiago Ramón y Cajal (1852-1934), who postulated that the nervous system is built of networks of neurons. Working in synergy with neurons, the glial cells act as supporting cells.

The neuron is the communicating cell and is classified based on its function, shape, neurotransmitter synthetized/released and its shape. It is morphologically divided into three different domains: the cell body (nucleus and organelles), a floating number of dendrites and the axon.[5] While dendrites receive neural information the axon is responsible for transmission. Neurons are by nature communicating cells, they conduct electric impulses along the axon and transmit the information between each other's at the synapses.

However, neurons need support of glial cells to function in a proper manner. Glial cells are morphologically distinct from neurons and are divided into astrocytes, oligodendrocytes and microglia. Astrocytes are star-shaped specialized glial cells that play essential functions on the healthy CNS. They are known to aid in the formation and stabilization of the Ranvier nodes[6], act at the synaptic gap as regulators/stabilizers[7], communicate with neurons in a Ca^{2+}-dependent manner releasing neuroactive compounds[8], and maintain CNS homeostasis . Astrocytes also respond to any insult to the CNS in a process called reactive astrogliosis. Oligodendrocytes are myelinating specialized cells. The myelin sheathing of axons results from years of evolution, with the needs of broadcasting rapid impulses from the brain to the executer muscles. It allows a 10 to 100- fold increase in conduction of nerve impulse efficacy. In sum, myelin consists in the plasma membrane

of oligodendrocytes wrapped around axons which promote rapid propagation of the electric impulse and consecutively of information[5].

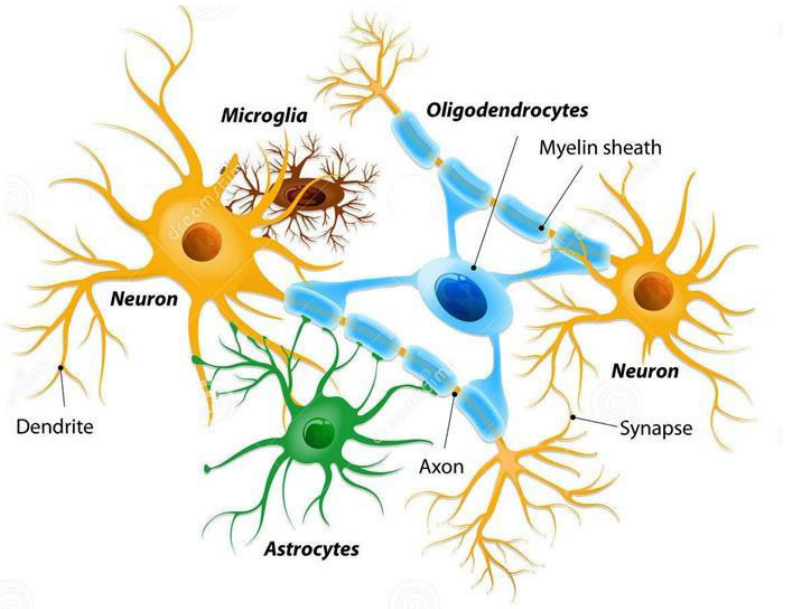

Figure 3- Neurons and Glial Cells: a close relationship. Figure adapted from [175]

Microglia is commonly known as a mediator of the immune response at the spinal cord. It was firstly described by Franz Nissl (1899), referring them as the leukocytes of the CNS and usually compared to macrophages. Microglia could assume two different states: 1) resting microglia and 2) reactive microglia. Upon abnormal changes detected in the CNS, pathological conditions or in case of neurodegenerative diseases, it becomes "activated" changing its morphology and producing immunoregulatory molecules[5].

1.4-Spinal cord protection

Beyond the support to the CNS homeostasis provided by glial cells, protection is also provided to the spinal cord by the vertebral column, meninges, cerebrospinal fluid (CSF) and brain

blood barrier (BBB). The spinal cord lies inside the vertebral canal, being the vertebral column the first physical line of defence[2]. The meninges surround and protect the spinal cord consisting of three layers: 1) Dura mater - it is the closest to the vertebral column, consisting in a ticker and fibrous membrane; 2) Arachnoid mater – with a spider web appearance this middle layer provides cushioning to the spinal cord; 3) Pia mater – firmly attached to the spinal cord it gives support to the vascular system. Regarding the CSF, it is located between the arachnoid and pia mater acting like a cushion to the spinal cord. The last protection layer is the BBB, consisting of a barrier made up by endothelial cells with tight junctions preventing the free entrance of solutes from the blood, such as toxins, into the CNS.

2- Spinal cord injury

Despite the physical protection of the barriers mentioned above, the spinal cord is not immune to internal or external insults. In fact, any form of trauma, disease or degeneration to the spinal cord could lead to a debilitating living life condition usually with permanent sequels to patients. The highly devastating and permanent condition is mainly due to the low capability of the CNS to regenerate itself. In this sense, there are emerging therapeutic strategies that aim to repair the injured spinal cord. Therapeutic approaches mainly focus on remyelination, neuronal protection, excitotoxicity clearance, cellular therapies, axonal regeneration, recruitment of endogenous host stem cells, epidural stimulation and brain- machine interfaces, reviewed by Silva et al[9].

2.1- SCI, incidence metadata and causes

SCI is now seen as a public health challenge that needs to be addressed in multiple perspectives. The incidence could vary between developed and developing countries, the level of education or the existence of preventive strategies or even the individual age.

For instance, in Portugal a SCI epidemiology study published in 1998 by Martins *el al.* indicates an incidence of 57.8 new cases per year between 1989 and 1992. The main causes identified were road traffic incidents and falls, at two age peaks 15-24 and 55-75[10]. Yet, despite the apparently high occurrence of SCI in Portugal, there is a lack of a database and a missing space in the literature about the numbers of the pathology in the country.

The analyses on the global SCI frequency revealed that the annual incidence could fluctuate between 83 per million in Alaska to 8 per million in Spain[11], but different studies clearly corroborate that the higher incidence is found in younger males and typically the most commonly cause of SCI are traffic accidents followed by falls and violence[11].

Moreover, people who suffer from SCI have 2 to 5 times more probability to die prematurely compared with healthy people. This pathology causes high losses to the patients and their relatives at personal, economic and social levels[12].

In 1997, a meta-analysis study by Marcel Dijkers reported that people who suffer from SCI have lower subjective well-being than non-disabled people[13]. SCI also is connected to a higher cases of depression[14] and anxiety prevalence[15] when compared with non-SCI patients.

Injuries in the spinal cord can be divided in traumatic and non-traumatic. The traumatic injury is the most prevalent cause of spinal cord injury, which includes traffic accidents, violence, falls and sport activities as cause of trauma. While the prevalence of non-traumatic spinal cord is unknown, with multiples and heterogenous causes as degenerative CNS disorders, infectious diseases, inflammatory states of the spinal cord, toxicity or tumours[16].

2.2- SCI Consequences

SCI usually leads to deficits in the motor function, sensation loss and impairments in the basic autonomic functions. The degree of paralysis will depend on the injury level and severity of

the damage caused to motor neurons, interneurons and sensory neurons[17]. SCI motor consequences is commonly divided in tetraplegia (arms, trunk, legs and pelvic region affected) or paraplegia (trunk, legs and pelvic area affected)[14]. Usually, the higher the lesion, the more severe it will be. For instance, a C3 lesion is more severe than in T10, since the C3 lesion will affect more spinal cord segments and consequent affect more parts of the body (Fig.4).

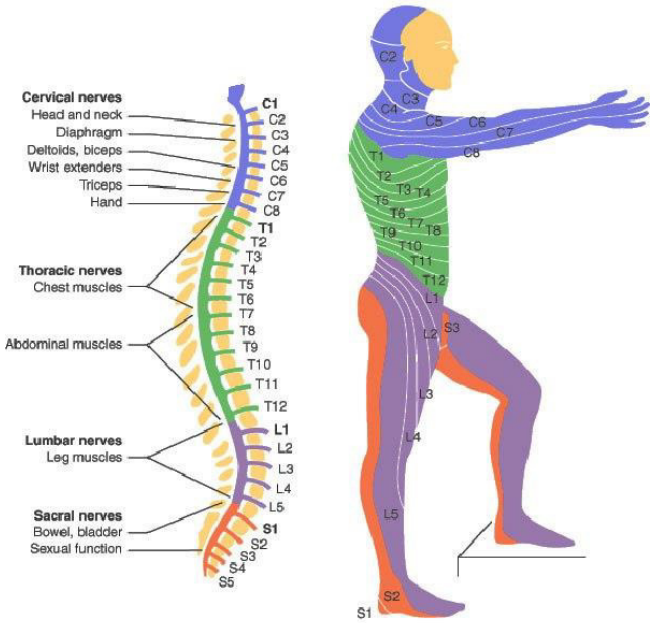

Figure 4- Illustration of the body parts affected after SCI, dependent of the injury level. Figure Adapted from[176]

Clinically, accordingly to the ASIA (American Spinal Injury Association) committee, the lowest healthy part of the spinal cord is referred as the neurological level of the injury. Moreover, injuries are also classified as complete or incomplete. An injury is considered to be **Complete** if all sensory and motor function are lost bellow the spinal cord injury, and **Incomplete,** if there is some sensory and motor function bellow the injury site[18].

Furthermore, there is also impairments in the autonomic nervous system (ANS) that includes: compromised cardiovascular, urinary and respiratory functions, as well as dysfunction on bowel movements and sexual activities[19].

As starting measure, there is the necessity to create awareness among the population about the debilitating conditions of this pathology and on prevention strategies.

2.3- Spinal Cord Injury Pathophysiology

The severity of SCI pathophysiology is manly caused by the inability of damaged tissue to regenerate. This kind of lesion triggers a cascade of biological events that leads to further neuronal damage, such as the activation of apoptotic pathways, microglia activation, recruitment of leukocytes, production of reactive oxygen species (ROS) and release of inflammatory cytokines[9]. There is also the formation of a glial scar by reactive astrocytes aiming to restore the integrity of CNS[20]. However, these reactive astrocytes release proteoglycans that are involved in the suppression of axons outgrowth, which also contributes to spinal cord regeneration impairment[20].

Overall, the traumatic SCI comprises three main injury phases: the primary injury, secondary injury, and the chronic phase described below.

2.3.1- Primary injury

The primary injury comprises essentially the mechanical trauma caused to the spinal cord by physical deformation. It can include: (i) impact plus persistent compression; (ii) impact alone with transient compression; (iii) distraction; and (iv) laceration/transection[21]. The mechanical impact leads to blood vessels damage, axons disruption and neuronal death. There is also disruption of the blood flow pressure, glucose/ oxygen supply and acidosis[22].

Moreover, two distinct events may occur after trauma, neurogenic shock and/or spinal shock. The neurogenic shock happens only in high injury levels and is a consequence of vasomotor tone and cardiac sympathetic innervation loss resulting in hypotension and bradycardia. The spinal shock is a initial loss of sensation, motor paralysis and muscle flaccidity with gradual recovery[23].

2.3.2- Secondary Injury

Minutes upon the primary injury, ischemia, edema, cell death, ionic imbalance, accumulation of neurotransmitters and membranes disruption triggers a secondary injury, exacerbating the consequences of the primary injury[24]. These events are interconnected, initiating

a cascade where a mechanism triggers another one, perpetuating the secondary injury for several weeks[24].

During this phase there is an expansion of the edema to the adjacent areas, fluid accumulation and a worsening of the ischemia[25]. Moreover, a lower oxygen and glucose supply translates into an energy failure of neuronal cells, conducting them to premature dead[21].

Massive neuronal death leads to the release of several compounds from the disrupted cells resulting in ionic imbalance. Moreover, loss of membrane potential causes influx of Na^+ and Ca^{2+} [26] that exacerbate the poor energetic balance causing mitochondrial dysfunction [27]. Furthermore, abnormal levels of Ca^{2+} leads to the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) due to mitochondrial dysfunction. That in turn, conducts to lipid peroxidation and damage to nucleic acids and proteins. These events lead to damage to cytoskeleton proteins which conduct in last case to cellular dead and neuronal tissue loss[21,24].

Another typical event of the secondary injury is glutamate excitotoxicity. Glutamate by itself is the main excitatory neurotransmitter of the CNS. However during SCI there is release around the lesion site due to disruption of neuronal membranes [28]. Extracellular glutamate binds to calcium dependent receptors leading to excitatory cell death [24]. Neurons and oligodendrocytes are the main targets of the neuronal excitotoxicity, resulting in the loss of neurons and the myelin sheathing of axons[29].

The CNS is an immune-privileged site, in homeostasis leukocytes are absent from the CNS parenchyma, being the microglia the only responsible cell for CNS immune surveillance [30]. The aim of the immune response is to remove damaged tissues and dangerous molecules after SCI. In first place, there is only activation of the microglia due to the release of several Damage-Associated Molecular Patterns (DAMPs), from dying cells (neurons, oligodendrocytes) like ATP, DNA, glutamate and cytokines. Activated microglia produces a wide array of cytokines and chemokines (IL-1β, TNF, IL-6, MIP-1alpha and MIP-1beta) which act to promote leukocytes recruitment[31,32]. The first leukocytes to arrive at the injury site are neutrophils, arriving 6 hours post injury, peaking in the first days and decreasing by 96 hours [33,34]. However, neutrophils are known to produce several cytotoxic factors, proteases, ROS and nitric oxide[34], contributing for further tissue damage.

Monocytes are recruited 2-3 days after injury, being differentiated in macrophages[34]. Macrophages can assume several distinct phenotypes, being at one pole the most inflammatory macrophages termed M1 and at the other pole the anti-inflammatory macrophages (M2). M1

secrete pro-inflammatory cytokines (TNF-α, IL-1, and IL-6) contributing for amplifying the inflammatory response and tissue damage of the spinal cord, while the M2 macrophages secrete anti-inflammatory cytokines (IL-10, TGF-β0), suppressing the inflammatory response and promoting wound healing and tissue restoration. A mix of both phenotypes can be found at the injury site, being M1 macrophages predominant over M2. [35,36] The detrimental side of the inflammatory macrophages was demonstrated by the depletion of the peripheral macrophages during SCI, which resulted in hindlimb function improvement, decreased cavitation and enhanced preservation of myelin[37].

T cells are also reported to be recruited with the increase of BBB permeability, but only in reduced numbers[34]. The role of the T lymphocytes during the lesion is still controversial, with both beneficial and detrimental effects. On one side, they are known to produce inflammatory cytokines, stimulate macrophages activation into M1 and induce necrotic and apoptotic cells death. On the other hand, T cells are reported to provide neuroprotection, since they are also reported to produce neutrophins and IL-10 which polarize macrophages into M2, inducing a regenerative environment[38].

The immune response after SCI, is built primarily in order to remove toxic molecules and death cells fragments. Although, the exacerbation of the immune response contributes to an increase of the secondary injury and induces deleterious effects in CNS.

2.3.3- Chronic injury

After stabilization of the acute phase there is the onset the chronic phase which goes from days to years after injury. At this point the apoptosis and cell death continues in both orthograde and retrograde directions including some brain regions, grey matter dissolution, cystic cavity formation, connective tissue deposition and reactive gliosis that leads to the formation of the glial scar[9,39]. The latter is composed essentially by reactive astrocytes, microglia, macrophages and inhibitory molecules (proteoglycans and myelin-associated molecules). Its formed upon the breakdown of the BBB, surrounding and containing the lesion site.

3- Management and treatment perspectives of SCI

Currently, there is no effective clinical treatment for SCI condition. The clinical approaches used are based on surgical procedures that decompress and stabilize the spinal cord, as well as the use of methylprednisolone (MP)[9,39] , a glucocorticoid that reduces inflammation at the injury site and protects neuronal cells. However, its use is associated with severe harmful side effects. Therefore, there is the need for effective novel therapies to target SCI.

3.1- SCI - Clinical approach

Once the patients arrive at the hospital, the main focus is to maintain the ability to breathe and prevent vascular damage. For this purpose, the spinal column is immediately stabilized in order to prevent further damage to the spinal cord. Upon evaluation the patient undergoes surgical decompression, as there are evidence that early decompression prevents neuronal loss and improves outcomes[40]. A clinical study which compares early (<24h) versus delayed (>24h) decompression in SCI patients demonstrates that early decompression improves neurological outcomes[41]. In clinical context the neurological status evaluation is performed using the ASIA impairment scale (table.1)

Table 1- ASIA Impairment Scale to evaluate neurological status of patients suffering from SCI in Clinical context

A	Complete	No sensory or motor function is preserved in the sacral segments S4-S5.
B	Sensory Incomplete	Sensory but no motor function is preserved bellow the neurological level and extend through sacral segments S4-5.
C	Motor Imcomplete	Motor function is preserved at the most caudal sacral segments for voluntary anal contraction and more than half of key muscles bellow the neurological level have a muscle grade <3.
D	Motor Incomplete	Motor function is preserved bellow the neurologic level, and most key muscles bellow the neurologic level have muscle grade >3.
E	Normal	Sensory and motor functions are normal

In some clinical contexts, methylprednisolone (MP) is used to treat SCI. It is a glucocorticoid used in diverse pathologies as potent anti-inflammatory agent. MP is also known to have beneficial effects during SCI, such as facilitation of the spinal cord impulse generation and synaptic transmission, enhancement of spinal cord blood flow and decreased lipidic peroxidation[42]. However the use of MP is arguable, since two randomized clinical trials at the end of the 90´s showed two differents results. While one indicates a beneficial effect from the use of MP (NASCISIII)[43], the other one strongly contest the benefits of this corticosteroid during acute SCI[44], suggesting that the use of MP is accompanied by harmful side effects. Furthermore, in 2013 the American Association of Neurological Surgeons/Congress of Neurological Surgeons developed a level 1 recommendation against the use of MP: "Administration of methylprednisolone (MP) for the treatment of acute spinal cord injury (SCI) is not recommended. Clinicians considering MP therapy should bear in mind that the drug is not Food and Drug Administration (FDA) approved for this application. There is no Class I or Class II medical evidence supporting the clinical benefit of MP in the treatment of acute SCI. Scattered reports of Class III evidence claim inconsistent effects likely related to random chance or selection bias. However, Class I, II, and III evidence exists that high-dose steroids are associated with harmful side effects including death"[45].

Overall, a recent guideline by Fehlings *et al.* do not recommend a 48-hour infusion of high dose MP to adult patients, sugesting the use of MP during 24 hours may have some benefits[46]. Fehlings et al. also sugest the use of anticoagulant thromboprophylaxis within the first 72 post injury, in order to minimize the formation of blood clot and prevent venous thromboembolism (VTE).

Due to the limited therapeutic options a vast number of SCI clinical trials have emerged since the last century, including pharmacologic compounds, molecular therapies, cellular transplantation and the use of technologies (computer interfaces and electric stimulation devices) that will be discussed in the next section.

3.2-In search for a cure: from bench to the bed side

Currently several strategies are in different stages of development to promote neuronal growth, protect the tissue from further damage, all of them with the objective to promote functional improvement.

3.2.1 Molecular therapies

Riluzole: It is a sodium channel blocker reported to increase glutamate uptake and reduce glutamate release, acting as a neuroprotective drug. This potential therapeutic effect was demonstrated by several authors and recently revised in Wistar Han rats with an improved behavioural recovery and reduced lesion size compared with saline controls[47]. This drug has already been through a clinical trial phase I (safety and pharmacokinetic) where was also described an increased motor score in the Riluzole group[48]. Currently, is on course a Riluzole clinical trial phase II/III on patients with SCI (clinicaltrials.gov, identifier: NCT01597518).

Minocycline: This drug is a commonly prescribed antibiotic, associated with neuroprotection, improving the survival of oligodendrocytes[49] and with evidences of functional improvements in adult rats[50]. However, a clinical trial phase I involving minocycline administrated to SCI patients did not showed promising results. The result of the clinical trial phase I goes in accordance with another work where it was not found the claimed neuroprotective effect after treating SCI rats with monocycline[51].

Anti-NogoA: Nogo-A is a protein of CNS myelin which exert inhibition over neurite growth, induce the disruption of the growth cone leading to growth arrest and reduction of CNS plasticity[52]. In the end of the 80s, Caroni and Schwab identified two antibodies (IN-1; IN-2) against these protein that revert the role of NogoA protein, allowing axonal outgrowth of neurons in culture[53]. In rats, intrathecal delivery of anti-NogoA antibodies enhance the regeneration of the corticospinal tract axons and produce significant improvements in motor recovery due to the restauration of spontaneous plasticity[54]. Similar results were also observed in adult monkey treated with anti-NogoA antibody, with enhanced corticospinal axonal sprouting and observed functional recovery[55]. Recently, it was also demonstrated that anti-NogoA antibody

administration allied with locomotor training translates into a superior motor recovery[56]. Regarding the clinical application, taking in account the promising results from the animals, a phase I clinical trial coordinated by Novartis was already performed without side effects to the patients (clinicaltrials.gov, identifier: NCT00406016). A phase II clinical trial is now ongoing to evaluate the therapeutic potential of anti-Nogo-A antibody in SCI patients.

Chondroitinase ABC (ChABC): This enzyme follows the same strategy of the anti-NogoA antibodies, aiming to degrade the inhibitory chondroitin sulphate proteoglycans (CSPGs) present in the glial scar, supressing the axonal growth inhibitory properties of it. This beneficial therapeutic potential was already demonstrated in *in vivo* SCI models, where axonal sprouting in the lesion area after ChABC treatment, was observed[57]. Functional improvements were also detected on chronic SCI after combination of ChABC with rehabilitation in rats[58].

Axonal Growth factors: One of the main obstacles to axonal growth is the intrinsic low capability of adult neurons to growth and the lack of neurotrophic factors to promote axonal growth after SCI. Several factors were already tested aiming to promote axonal outgrowth, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF) and neurotrophin 3 (NT3). Usually, the use of neutrophins translates in axonal sprouting and growth in ascending and descending tracts of the lesioned spinal cord[59,60,61,62]. However, the restoration of axons lost after SCI was not sufficient to promote functional improvements. In fact this could only be achieved after induction of neuronal plasticity such as training or electrical stimulation[63].

3.2.2-Physical Therapy and External Stimuli

Physical methods are also used to promote recovery and minimize the secondary damage to the spinal cord after injury. The most common physical approaches are based on hypothermia, physical exercise and hyperbaric oxygen (HBO) therapy.

Hypothermia is based on the temperature reduction around the injury site or systemically, and it is reported to reduce the glutamate in the CSF, edema, neutrophils evasion, oxidative stress, haemorrhage and apoptosis[64]. Several experimental results have demonstrated the potential role of hypothermia after SCI, including promotion of neuroprotection and gain of function[65,66]. Furthermore, there are clinical reports which have reported physiological improvements in some patients[64]. The Miami project to cure paralysis has already completed a phase I clinical trial in which "there were no serious safety concerns and there was a trend for more people to recovery more function after 1year post-injury than would normally occur spontaneously". They have currently a phase II/III clinical trial on going to evaluate the safety of different durations of hypothermia[67].

Physical exercise and training has been related with motor recovery. Several strategies to improve motor training in injured animals were developed since the beginning of the century. Experimental designs using cages with environment enrichment, are reported to improve gross and fine motor recovery[68,69] Also, forced training is shown to improve functional improvements during SCI[70], on both animals models and in clinical settings[71], being more consistent when given a combinatory therapy, such as epidural stimulation, pharmacologic compounds and cells transplantation. This kind of outcome, seems to be related with the capability of the spared tissues to reorganize, form new intraspinal circuits and bypass the lesion with consequent restoration of function[72].

HBO therapy is based on the increase the oxygen tension which confers neuroprotection under the follow mechanisms: 1) decreasing apoptosis; 2) reducing oxidative stress; 3) diminishing inflammation; 4) promoting angiogenesis; 5) reducing spinal cord edema; 6) increasing autophagy[73]. Actually, the HBO therapy is reported to promote functional recovery in rats with an increase in the BBB score after treatment[74,75]. A couple of clinical studies revealed a mix of conflicting results in patients with SCI and HBO treated[73].

3.2.3-Technologic and innovative approaches

Electrical stimulation, brain machines interfaces, brain spinal cord interfaces, use of delivery systems, nanoparticles and implementation of biodegradables matrices have been developed to reduce the deleterious effects of SCI.

Epidural electric stimulation (EES), through the delivery of electric stimulus into the spinal cord is related with induction of hindlimb stepping[76], apparently by activation of central pattern generator (CPG)[71]. In the clinical context was already demonstrated the potential therapeutic role of ESC[77–79]. Currently a clinical trial is taking place by Courtine-Lab in patients with chronic incomplete spinal cord injury (clinicaltrials.gov, identifier: NCT02936453).

Courtine-Lab has recently developed a brain-spinal interface which consist in a brain motor cortex implant that decode the neural information, transmitting then in real time to an epidural implant, producing regulated electric stimulation to the spinal cord. This mechanism, was able to restore the disrupted communication between the brain and the spinal cord, reducing gait deficits after SCI in primates[80].

The use of brain machine interfaces (BMI) is another strategy, based on recording signals from the brain to operate a brain computer interface to produce movement of the affected members. Using these concepts Nicolelis lab build a BMI that reproduce the movement of the arm of a monkey on a robotic arm in real time[81]. Later was shown the monkey operating a brain machine interface plus receiving artificial tactile feedback[82]. More recently, it was demonstrated that long-term training with BMIs induces neuronal recovery, with the patients recovering the control of key muscles an at sensory level[83].

Another innovative strategy to revert the spinal cord injury deficits is the use of biomaterials, which work as a matrix to fill up the damage tissue or even the cystic cavity in the chronic phase. The main target of these materials is to restore the tissue integrity and promote tissue repair. They should be biodegradable, biocompatible and have physical and trophic characteristics with capability to ensure a perfectly functional regenerated tissue[84]. Biomaterials could be derived from natural or synthetic sources. The natural source include agarose, collagen, fibrin or gellan-gum, being the synthetic polymers derived from poly(ethylene glycol) (PEG), poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA) for instance[85]. Despite the fact that these polymers are able *per se* to play a positive role during SCI events, the most interesting fact is their ability to be modified

with peptides, be combined with cellular or drug deliver. Peptides modification is used to improve cellular adhesion, neuronal differentiation and growth. Biomaterials are used as vehicle to cellular transplantation, since the lesion microenvironment gives poor change to survival, growth or differentiate, these materials could be built to offer a more desirable environment for the transplanted cells. The release of drugs, growth factors or proteins enclosed in the biomaterials could be controlled to a constant diffusion flow, extending the molecules effects by weeks[84,86]. Our group has already demonstrated the positive role of encapsulation of Adipose stem cells (ASCs) and Olfactory stem cells (OECs) on gellan-gum hydrogels modified with peptides upon transplantation into a spinal cord hemisection injury model[87]. A different strategy to fill up the gap of complete transection of the spinal cord is the use of microconnector system to restore the tissue continuity, which consists up in a rough surface disc to promote spinal cord tissue adhesion. This system alone is able to promote axonal growth and functional recovery[88].

One of the main obstacles to tackle the SCI is the delivery method of the drugs or biological compounds to the spinal cord. The use of nanoparticles could represent an alternative to current methodologies. For instance, the slow release of MP loaded nanoparticles showed to be therapeutically superior than bare MP[89]. In another perspective, immune-modified nanoparticles have been used to specifically target, sequester and promote apoptosis of circulating monocytes in the spleen, which reduce the fibrotic scar formation and produce functional recovery.[90] Moreover, new ways to tackle the SCI events, could take advantage of nanoparticles developed in the cancer research field, who developed a nanoparticle based in transferrin (TF) which cross the BBB[91].

3.2.4-Cell Therapies

The transplantation of cells in the human injured spinal cord seems to be feasible and,in some cases, a safe process[92,93]. However, there are several questions that still need to be answered, namely adequate transplantation methods, evaluate how cells interact with the host spinal cord and the mechanism which confers neuroprotection, as well as how they lead to tissue repair and functional recovery. Additionally, it is also essential to study how cells survive in the host and the capability of the cells to respond to environmental cues. A wide array of cells is being currently tested to treat SCI pathology, such as, embryonic stem cells, induced pluripotent stem

cells (iPSCs), neural stem cells (NSCs), mesenchymal stem cells (MSCs), olfactory ensheathing cells (OECs), Schwann cells and immune cells[94]. They can act as players to the replace neurons and white matter lost, give support to neuronal growth and provide factors which modulate the lesion environment[94].

Stems cells are pluripotent undifferentiated cells with self-renewing capabilities, originating specialized cells when submitted to different *stimuli*, with different functions. In adult human, they are responsible to replace and repair damaged tissues[95].

NSCs have demonstrated to be able to replace the lesion site with neurons and glial cells[96], form a functional synaptic connection between the grafted neurons and the spared host neurons[97,98], promote functional recovery in both rodent[96,97] and non-human primate SCI models[99]. Furthermore, NSCs promote axonal growth through secretion of neurotrophins molecules[100]. Altogether, in SCI context they are reported to be able to differentiate into neurons, oligodendrocytes and astrocytes, also secrete neutrophic, neuroprotector and immunomodulatory molecules.

On the other hand, MSCs are considered to be one of the most promising sources of cellular therapies to SCI. It includes a wide array of cellular types with different origin (adipose tissue, bone marrow or dental pulp)[9,101]. Several studies suggests a beneficial role of MSCs transplantation [102]. The mechanisms underlying the therapeutic potential are largely discussed. While some studies indicate that MSCs cellular differentiation into neural phenotypes as the therapeutic mechanism, it seems to be more related with the molecules secreted by these cells (HGF, VEGF, BDNF, NGF, IL-6)[85,103]. More detailed information about the MSCs can be find in the section 3.3, since it is the main focus of this thesis.

Another cell types that have attracted the attention for a possible role on the SCI regenerative process, include glial cells (OECs, Schwan cells) and immune cells. OECs are responsible to support regeneration and guide the axonal growth of olfactory neurons, making them suitable to promote regeneration of axons lost after SCI[85]. Schwann cells are related with the capacity of the PNS to regenerate, during SCI they have the ability to migrate in the injury site, differentiate, secrete growth factors and participate in the myelinization of the axons[104], showing potential to repair the injured spinal cord.

As mentioned in the section 2.3.2, the immune system has a prominent role during the secondary injury events, contributing for exacerbating SCI outcomes. Macrophages are one of

the cellular types recruited into the injury, where they essentially develop into an inflammatory profile (M1) inducing tissue damage. On other side, macrophages could also assume an anti-inflammatory phenotype, assuming regenerative characteristics. Taking advantage of the potential to macrophages assume distinct phenotypes, the right profile could exert wound healing and regenerative profile into the injured spinal cord. Rapalino et al. demonstrated that implantation of pre-stimulated homologous macrophages with peripheral nerve explants improve recovery of paraplegic rats[105]. A clinical trial phase I involving the administration of macrophages was performed without adverse effects observed[106]. More recently, the proCord project, sponsored by Proneuron Biotechnologies, started a clinical trial phase I involving macrophages administration, however it was stopped due to financial problems (clinicaltrials.gov, identifier: NCT00073853).

Moreover, several clinical trials involving cells transplantation already showed preliminary results and others are currently ongoing. Regarding OECs, is currently on going a clinical trial phase I to assess the transplantation feasibility and safety (clinicaltrials.gov, identifier: NCT01231893). The Schwann cells transplantation seems not to produce complications to the patients, accordingly to a clinical trial phase I integrated in the Miami project to the paralysis cure, with some minor sensory changes[93]. NSCs transplantation in humans demonstrated to be safe, however without recovery effects detected[107].

3.3 - Adipose stem cells and secretome

Adipose stem cells (ASCs) are a source of mesenchymal adult stem cells (MSCs). MSCs can be obtained from a wide array of multiple sources, including umbilical cord or bone marrow [108,109], being the ASCs easily isolated from adipose tissue[110]. Nowadays, due to the recent high interest in biomedical application of diverse MSCs, and with a great increase of clinical trials employing MSCs (fig. 5)[111], a guideline from the International Society for Cellular Therapy stated that MSCs need to fill some requirements. Besides the mesoderm embryonic origin and the self-renewal capability, MSCs should follow 3 minimal inclusion criteria: 1) must be plastic adherent in standard culture conditions; 2) Express CD105, CD73 and CD90, and be negative for CD45, CD34, CD14 or CD11b, CD79 and HLA-DR surface molecules; 3) capable to differentiate to osteoblasts, adipocytes and chondroblasts[112].

ASCs were firstly isolated and identified in 2001 by Zuk et al. Comparatively to the others MSCs populations, they can be easily obtained and isolated in large scale through a simple local liposuction from human adipose tissue[110]. Several applications of ASCs in regenerative medicine have arisen in a diverse variety of pathologies, from cardiovascular diseases to a neuro-regenerative approaches. ASCs are reported to play a positive role in muscle injuries, vascular and cardiac repair, mainly through the secretion of soluble factors which support the regenerative process and mediate cellular protection [113,114,115].

Adipose derived stem cells were also described to play a role during inflammatory events. ASCs exosomes are known to reduce the systemic inflammation on both molecular and cellular aspects[116]. During colitis, ASCs plays a role decreasing the inflammasome activation and modulating the macrophages transition from M1 to M2, controlling the inflammatory response[117]. In addition, during bacterial and leishmanial infections, ASCs modulate and control the immune response in order to combat and eliminate the infectious agent[118,119].

During neuropathological events such as: Stroke, Parkinson disease (PD), Alzheimer disease (AD) and SCI, ASCs have neuroprotective effects. Indeed both cells, as well as their secretome, have important neuroprotective effects in PD models, attenuating microglia activation and decreasing cellular death[120,121]. Neuropathological deficits produced by AD, are attenuated with increased memory and learning abilities after systemic or local ASCs treatment. This is the result of ASCs-mediated alternative activation of microglia, reduction of proinflammatory cytokines and

amyloid plaques[122,123]. Also, ASCs and their secretome promote functional recovery and brain repair after stroke injury, with reduction of cell death and increased angiogenesis[124,125,126]. Beyond the soluble factors secreted that induce vascular remodelling, microRNAs provided by ASCs exosomes are described to promote angiogenesis[127].

Regarding SCI, ASCs transplantation within a matrix or alone have been reported to produce functional recovery, neuroprotection and axonal regeneration after injury[87,102,128,129]. These outcomes are related with the immunomodulatory, neuroprotective and neurotrophic molecules secreted by the ASCs, that is their secretome.

ASCs secretome is constituted by soluble factors, micro vesicles, exosomes and apoptotic bodies[130]. Recently our group have identified several factors secreted by ASCs. Some of these factors are known to protect against oxidative stress: DJ-1, TRX, CYPA, CYPB, PRDX1, AS and HSP27 or factors that mediate neuroprotection trough the anti-apoptotic activity: CYPA, CYPB, CYSC, IL-6, Gal-1 and HSP27. Another known factors present in secretome modulate glutamate excitotoxicity: IL-6, EDF and PAI-1. ASCs secretome also contains factors involved in neurite outgrowth (SEM7A, GDN, HUCPVC) and neuronal differentiation (PEDF, CADH2, IL-6)[131]. Another study has focus on the evaluation of the cytokines and growth factors profile. ASCs secrete GRO—α (CXCL1), IL-6, IL-8, MCP-1, MCSF, MDC (CCL22), RANTES (CCL5), SCF, SDF-1 (CXCL12). These molecules work as chemoattracts and modulators of the immune response. Growth factors which facilitate the angiogenesis process (angiogenin, bFGF, VEGF, NGF and HGF) are also shown to be secreted by ASCs[132,133] as well as proteins involved on ECM formation and remodelling (ex. MMP1, MMP2, collagen, laminin)[134].

A couple of studies reported the ASCs secretome per se could act as a potential neuroregenerative tool. It is reported to induce *de novo* vascularization [132] and stimulate regeneration of peripheral nerves through the BDNF secretion[135]. Actually, our group has been working on ASCs and has associated the interaction of these cells with neuronal cells through the paracrine factors released by them (secretome). It was demonstrated that ASCs secretome increase hippocampal neurons density and induce cellular viability [136]. Regarding the SCI context, our group proposed a combinatory therapy using modified gellan gum hydrogels combined with ASCs and OECs, which have shown to promote motor improvements, with a reduced inflammatory state of the spinal cord, apparently through the release of paracrine factors than by cellular

diferentiation[87]. The combinatory strategy of OECs and modified peptides with ASCs is supported by previous studies which stated a favourable role of these combination[137,138]. Moreover, it has also been shown that ASCs secretome has potent effect on neurite outgrowth[88], surpassing the effects produced by MSCs isolated from other sources (bone marrow and Wharton Jelly) *(unpublished data)*.

ASCs are also related with modulation of the immune response. Recently it was shown a paracrine anti-inflammatory effect exerted by ASCs secretome on monocytes. Including reduction of migratory activity even in the presence of CCL2, reduction of production of TNFα by M1 macrophages and enhanced production of IL-10 and TGFß$_1$ by M2 macrophages. A decrease of M1 macrophages when treated with conditioned media was also reported[139]. This suggest a control of the inflammatory response and a switch to a wound healing environment. Furthermore, another reports have related the ASCs with the macrophages polarization from M1 to M2 by secretion of PGE2[140]. The same role is given to the exosomes releases by ASCs, which are recruited to the injured spinal cord and up taken by macrophages converting them from M1 to M2[141]. Also, in the previously year a group demonstrated that the switch from M1 to M2 in macrophages treated with ASCs secretome is improved by all fraction of the secretome (soluble and vesicular), but appear to be mostly favoured by the extracellular vesicle part[142]. An important note to state is that the ASCs secretome beyond the soluble factors is also rich in exosomes, micro vesicles and apoptotic bodies that seem to play a regenerative role, presumable by the content of microRNA, DNAs and proteins[130].

However, the ASCs secretome therapeutic role on SCI remains unclear. A couple of studies have recently emerged using the paracrine factors of a wide array of cells. Two studies have tested the intrathecal delivery of bone marrow stromal cells secretome, and both reported improvements in motor performance[143,144]. Another study administrated only the exosome fraction of the secretome intravenously, achieving neuroprotection trough the attenuation of apoptosis, inflammation and promoting angiogenesis, with slight motor recovery[145]. Despite the several studies using secretome of others MSCs (total fraction or only components) with beneficial functional improvements, the potential role of ASCs secretome on SCI remains to be described.

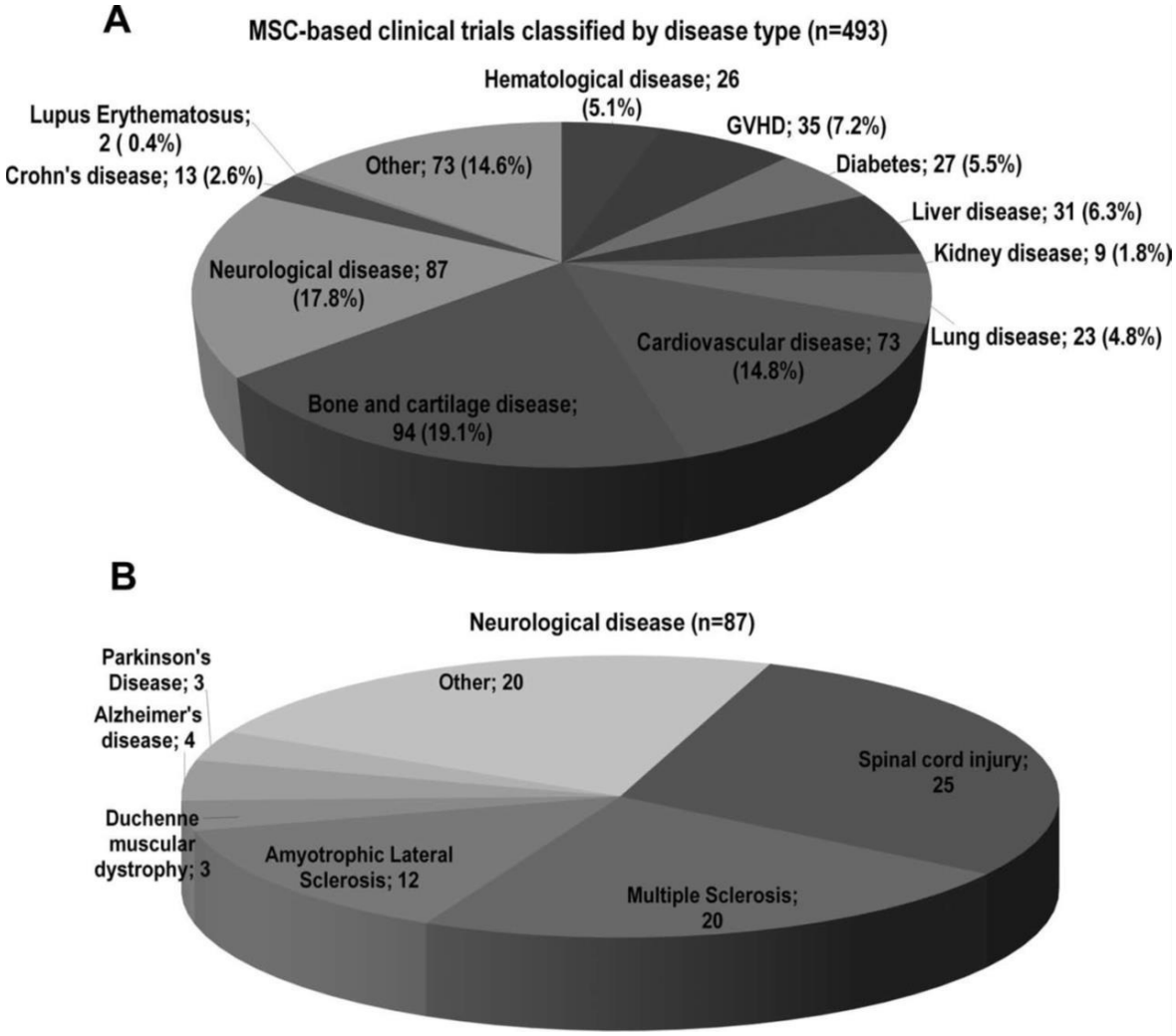

Figure 5- Current state of clinical trials with mesenchymal stem cells[177]

CHAPTER 2 -OBJECTIVES

Objectives

The main goal of this thesis was to develop a novel therapeutic strategy for SCI based on the ASCs secretome. In order to do that, we first aimed to implement a new SCI mice model in our lab, as a new tool to better understand SCI pathology. The mice model was chosen due to be anatomical, physiological and genetically similar to humans, very cheap to maintain and have a short lifespan. There are several of genetically modified strains, including immunodeficient and knockout mice, which could be used to better understand the SCI pathophysiology. The objective of the second part of the thesis was to assess the therapeutic effect of the ASCs secretome on SCI pathology. Two distinct administration routes were tested: 1) Locally into the spinal cord parenchyma; 2) Systemically, by tail vein injection. The choice of ASCs secretome was mainly due to the extensive work performed by our group using Adipose stem cells that identified a potential positive role during SCI, due to their neuroprotective, anti-apoptotic and neuroprotective effects, mainly due to the release of paracrine factors.

CHAPTER 3 – METHODS

3-Cell culture and secretome collection

3.1-Adipose Stem Cells expansion and secretome collection

Adipose Stem Cells (ASCs), were collected from human donors under a simple abdominal surgical procedure. Human Adipose-derived Stem Cells (ASCs) were kindly provided by Professor Jeffrey Gimble and isolated according to the protocol described by Dubois et al[146].Briefly, adipose tissue is obtained by liposuction aspiration, followed by mechanical and enzymatic dissociation. The stromal and vascular fraction resultant from this process go through several centrifugation steps, before ASCs are isolated and platted [147]

3.2-ASCs Culture

Human Adipose stem cells in Passage 3 (P3) from our cell bank were cultured in completed α-MEM [Invitrogen, USA; supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin- Streptomycin (P/S)] in 5 % CO_2 incubators at 37°C and 4 000 cells/cm^2. Culture medium was renewed every two days until confluence. Once confluence established, cells were trypsinized and counted. The previous steps were successively repeated until P6 ASCs obtained, passage in which secretome collection occurs.

3.3-Conditioned Medium (Secretome) Collection

Conditioned media was collected as described previously[148]. After the procedure described in **3.2,** the ASCs were seeded at a density of 12.000 cells/ cm^2 in T175 flasks during 72h in α-MEM (Gibco, USA) supplemented with 10% FBS and 1% de P/S, which work as antibiotic, blocking the growth of microorganisms. Then, the cells adhered to the culture flask are washed five times with PBS (1x) without Ca^{2+}/Mg^{2+}, and washed twice with Neurobasal A medium. Following the washing step, neurobasal A media (Thermo Fisher, USA) was added, supplemented with 1% of kanamycin. 24h later, the conditioned medium (CM) is collected, concentrated 100x and frozen in liquid nitrogen (Fig.6). Briefly, the medium is 100x concentrated using a 5Kda cut-of concentrator (Vivaspin™, GE Healthcare), divided in vials, snap frozen in liquid nitrogen and store at -80°C.

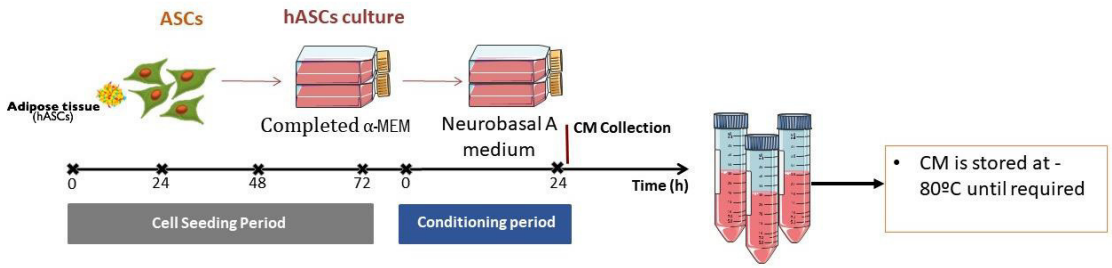

Figure 6- Schematic representation of the steps to obtain secretome from an initial culture of human ASCs.

3.4- In vivo protocols

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3.4.1- Animals and Groups

C57BL/6 mouse (Charles River, USA) were maintained in the animal facilities of Institute of Life and Health Sciences (ICVS, Braga, Portugal), under sterile conditions, in light, humidity and temperature controlled rooms, food and water was provided *ad libitum.* C57BL/6J adult females (10-15 weeks of age) were used. Experimental protocols involving mice were performed according the European Union Directive 86/609/EEC, and according to the animal care guidelines[149].

3.4.2- Model Implementation

For the implementation of the mice SCI compression model we explored three distinct groups: **1)** laminectomy (n=5) and two groups of graded spinal cord severities, **2)** compression for 2 seconds (n=10) resulting in a moderate injury and **3)** 10 seconds (n=10) resulting in a severe injury.

3.4.3- Spinal cord injury surgery procedure

All animals were anaesthetized with an intraperitoneal injection of Imalgene (ketamine, 75mg/kg, Merial, France), Dormitor (medetomidine, 1mg/kg, Pfizer, USA). Bupaq (0,1mg/kg, Ritcher pharma, Austria) was also administrated subcutaneously for analgesia. Vaseline was applied into the eyes to prevent drying and consequent blindness.

Once anesthetized, they were shaved in the lumbar region and disinfected with chlorohexidine. After this step, they were placed in a warmer blanket in a prone position. A dorsal midline incision was made at the level of the thoracic spine (T5-T12). The paravertebral muscles were retracted and the spinous processes and the laminar arc of T8-T9 removed, exposing the spinal cord. The spinal cord was then compressed using a fine forceps, **2 seconds** or **10 seconds** depending on the group. All surgical procedures were performed under sterile conditions, mice were randomized and the lesions were performed always by the same person.

3.4.4- Post operative care

Immediately after the surgical procedure, the wound was disinfected with chlorohexidine, the muscles sutured and the skin closed with three 9mm autoclip (Braintree Scientific, USA) and anti-sedan (atipamezole, 1mg/kg) administrated subcutaneous to the animals. The animals were placed under red light to prevent hypothermic condition. A combo solution combining four distinct compounds was also administrated subcutaneously; **1**) the antibiotic Baytril (enrofloxacina, 5mg/kg), to avoid infections, **2)** the analgesic Bupaq (burprenorphine, 0,05 mg/kg) to reduce the animal pain, **3)** vitamins (Duphalyte/Pfizer, USA) and **4)** 0.9% NaCl in order to supress dehydration problems. This combo was administrated two times a day until the animals show autonomy to eat and drink and no infections are detected (5 days at least). Solid drink and food were also provided on floor during the first days, due to their movement restrictions. Supplementation with oral administration of caloric food (Nutrical) was given to the animals that lost more than 10% weight. Furthermore, the bladder voiding was manually performed during the four weeks, two times a day.

3.4.5- Therapeutic evaluation of ASCs secretome

Concerning the *in vivo* proof of concept, where we evaluated the therapeutic effect of ASCs secretome on mouse spinal cord compression injury, there was 3 independent groups: **1)** conditioned medium (CM) intraspinal **2)** CM systemic and **3)** culture medium injection (local and systemically). The volume of injection was: **Local:** 2µl rostral and caudally to the epicentre and **Systemic:** 100 µl through repeated tail vein administration.

The conditioned medium obtained on the section **4.3** was given to the mice, locally or systemically.

Intraspinal administration: performed immediately after the injury, before the wound closure. Two injections with the Hamilton syringe were performed, adjacent to the epicenter, rostrally and caudally. In each was delivered 1 µl of CM making a total of 2 µl injected.

Systemic administration: CM was systemically administrated intravenously in the mouse tail lateral vein, once a day in the first 72 hours post injury. Subsequent injections were given once a week. A schematic overview of the treatment schedule is represented on **figure 7.**

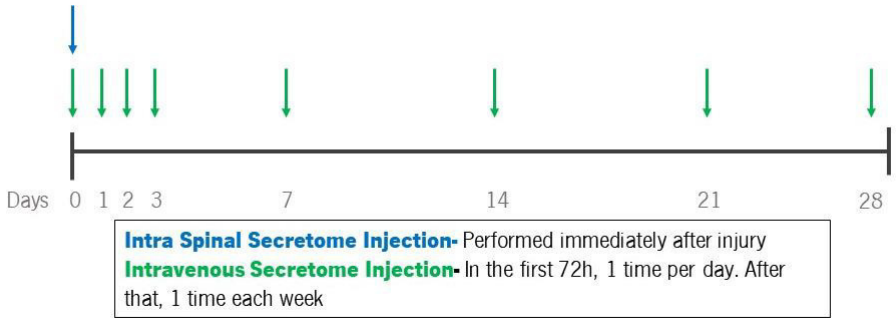

Figure 7– Schematic representation of the injection protocol, systemic in green and local in blue.

3.5- Behaviour analysis

To determine the impact of the different injury severity on functional recovery over time several behavioural tests were performed: **1)** Basso mouse scale (BMS),**2)** Activity Box Test (ABT), **3)** Beam Balance, **4)** Gait analysis and **5)** Motor Swim test, **6)** Von Frey

3.5.1- Basso Mouse Scale (BMS), locomotion score

The locomotion assessment was performed using the BMS motor scale[150]. This test was optimized to be a reliable alternative to the widely used Basso, Beattie, Bresnahan Locomotor scale (BBB) developed for SCI rats. BMS test evaluates several motor parameters such as: ankle movement, plantar placing of the paw, coordination, paw rotation (internal/external), weight support or even the stability of the trunk.

The BMS scale has a score ranging from 0 to 9 (table 2)**.** For instance: 0 means no ankle movement; 3: Plantar placing of the paw with or without weight support; 9: Plantar stepping, mostly coordinated, paws parallel at initial contact and lift off, normal trunk stability and tail always up.

Briefly, the mice were placed in an open area arena, for 4 minutes and their locomotor function was scored according to the BMS scale by two independent observers, blind to the experimental groups. BMS test was performed 3 days post-injury and once a week for 4 weeks.

Animals presenting a BMS score superior to 1 in the first BMS assessment (3dpi) were excluded due to incomplete compression of the cord.

Table 2- Basso Mouse scale to evaluation of locomotor behaviour[150]

Scores and Operational definitions for the Basso Mouse Scale for Locomotion (BMS)	
0	No ankle movement
1	Sight ankle movement
2	Extensive ankle movement
3	Plantar placing of the paw with or without weight support -OR- Occasional, frequent or consistent dorsal stepping but no plantar stepping
4	Occasional plantar stepping
5	Frequent or consistent plantar stepping, no coordination -OR- Frequent or consistent plantar stepping, some coordination, paws rotated at initial contact and lift off
6	Frequent or consistent plantar stepping, some coordination, paws parallel at initial contact -OR- Frequent or consistent plantar stepping, mostly coordinated, paws rotated at initial contact and lift off
7	Frequent or consistent plantar stepping, mostly coordinated, paws parallel at initial contact and rotated at lift off -OR- Frequent or consistent plantar stepping, mostly coordinated, paws parallel at initial contact and lift off, and severe trunk instability
8	Frequent or consistent plantar stepping, mostly coordinated, paws parallel at initial contact and rotated at lift off, and mild trunk instability -OR- Frequent or consistent plantar stepping, mostly coordinated, paws parallel at initial contact and lift off, and normal trunk stability and tail down or up & down
9	Frequent or consistent plantar stepping, mostly coordinated, paws parallel at initial contact and lift of, and normal trunk stability and tail always up

Slight: Moves less than half of the ankle join
Extensive: Moves more than half of the ankle joint excursion
Plantar Placing: Paw is actively placed with both thumb and the last toe of the paw touching the ground
Weight support: (dorsal or plantar): The hindquarters must be elevated enough that the hind end near the base of the tail is raised of the surface and the knees do not touch the ground during the step cycle.
Occasional: Stepping less than or equal to half of the time moving forward.
Frequent: Stepping more than half of the time moving forward.
Consistent: Plantar stepping all of the time moving forward with less than 5 missed steps (due to medial placement at initial contact, butt down, knee down, skiing, scoliosis, spasms or dragging) or dorsal steps.
Coordination: For every forelimb step a hindlimb step is taken and the hindlimbs alternate during an assessable pass. For a pass to be assessable, a mouse must move at a consistent speed and a distance of at least 3 body lengths. Short or halting bouts are not assessable for coordination. At least 3 assessable passes must occur in order to evaluate coordination. If less than 3 passes occur then the mouse is scored as having no coordination.
Some coordination: Of all assessable passes (a minimum of 3), most of them are not coordinated.
Most coordination: Of all assessable passes (a minimum of 3), most of them are coordinated.
Paw position: Digits of the paw are parallel to the body (P), turned out away from the body (external rotation) or turned inward toward midline (internal rotation).
Severe trunk instability: Severe trunk instability occurs in two ways.
(1) The hindquarters show severe postural deficits such as extreme lean, pronounced waddle and/or near collapse of the hindquarters predominantly during the test.-OR-**(2)** Five or more of any of the following *events* stop stepping of one or both hindlimbs

- Haunch hit: the side of hindquarters rapidly contacts the ground
- Spasms: sustained muscle contraction of the hindlimb which appears to immobilize the limb in a flexed or extended position
- Scoliosis: lateral deviation of the spinal column to appear "C" shaped instead of straight

Mild trunk instability: Less than 5 events listed above and some sway in the hindquarters. Mild trunk instability is scored when the pelvis and haunches predominantly dip, rock, or tilt from side-to-side (tilt). If the tail is up, the swaying of the pelvis and/or haunches produces side-to-side movements of the distal third of the tail which also indicates mild trunk instability (side tail).
Normal trunk stability: No lean or sway of the trunk, and the distal third of the tail is steady and unwavering during locomotion. No severe postural deficits or events and less than 5 instances of mild instability.

3.5.2- Activity Box Test (ABT)

Activity Box test was used to assess the gross motor parameters and general health of the animals[151]. The animals were firstly acclimatized to the room testing conditions and then placed in the middle of the ABT chamber (med associates Inc, USA) for 5 minutes. The total distance travelled and the number of rearings were measured the test was performed 4 weeks post injury.

3.5.3- Beam Balance test

Beam balance test was used to evaluate the balance and the fine motor coordination of the mice[151]. The ability to cross the narrow beams (10mm & 17 mm round square beams) was measured using an adapted scale from Metz et al, 2000[151]. The scale ranges from 0 to 2; **0:** inability to walk on the beam, animals fell down immediately ; **0.33:** mice are able to cross 1/3 of the beam; **0.66:** mice are able to cross 2/3 of the beam; **1:** able to cross the whole length of the beam; **1.5:** when stepping with the hindlimbs were partial possible; **2:** full length with normal weight support and accurate foot step[151]. This test has a duration of 5 days and was performed in the last week of the experiment. Animals were trained for 3 days in order to learn how to cross the beam without help, and then in the following two days the performance on the beams was evaluated. Four distinct beams were used.

3.5.4 Gait analysis

The gait of the animals treated with ASCs secretome and vehicle was analysed by the footprint pattern. Hind and fore paws were coated with blue and pink non-toxic paints, and the animals walked over a sheet of white paper in a runway with 100 cm long and 10 cm wide. The footprint patterns were analysed such as: stride length, hind-front footprint overlap, base of support and paw rotation. As it can be seen in the section 4, due to the high severity of the lesion the intended parameters to be evaluated become difficult to be determined.

3.5.5- Motor Swim Test

Swimming test was performed in a transparent rectangular pool (100 x 15 x 40 cm) filled with water. The temperature was maintained at 23°C and monitored using a thermostat. The animals were trained to swim in the direction of a platform placed in the end of the pool. The assessment was performed evaluating the time which the animas took to cross the pool. However, due to the inability of these animals to swim in a straight line (lesion results in coordination lost), a qualitative scale to analyse the swimming behaviour was used[152]. One more time, as seen in the results section this test failed to be valid during the mice SCI model.

3.5.6- Von Frey test

Von Frey test was used to determine tactile sensitivity and allodynia by measuring how much force is required to elicit movement of the paw fingers, using the up-and-down method with Von Frey monofilaments[153]. The experimental setting consists in the placement of the mice in an elevated mesh, restrained inside a standard perforated box. Before the test start, each animal was habituated to the testing conditions. A total of 8 monofilaments were used, ranging from 0.02 to 1,4 g. Both paws were stimulated with the central monofilament. If the animal moves the fingers of the paw, a weaker monofilament was used, if not, a stronger one was applied. The test was performed until observed response to the 0,4g, didn't respond to 15g monofilaments or after a total of 6 measures around the threshold. 50 % threshold was calculated using the formula:

50% g threshold $=\frac{10^{(x_f+k\delta)}}{10000}$; x_f is the value of the final monofilament used (log units), K is the tabular value for the pattern of positive/negative responses and δ is the mean difference between stimuli (0.267).

3.6- Histological analysis

After 4 weeks post-injury, in the end of the behaviour analysis, the animals were sacrificed to perform a histological analysis of the spinal cord.

3.6.1 – Tissue preparation

Mice were deeply anesthetized with intraperitoneal injection of Imalgene (ketamine, 150 mg/kg, Merial, France), Dormitor (medetomidine, 2mg/kg, Pfizer, USA). After that, a needle was inserted in the ascending aorta and perfused with PBS (1x) followed by 4% PFA. A rough dissection of the vertebral column is performed, and the tissues fixed in 4% PFA for 24 hours. Then, the spinal cord was isolated from the vertebral column and leaved in a cryopreservation solution of 30% sucrose. After 24 h, 2 cm length of spinal cord centered in the lesion epicentre, were involved Optimal Cutting Temperature compound (OCT, Thermo Scientific), snap frozen in liquid nitrogen and stored at -20°C. Lastly, transversal sections of 20 μm thickness of each spinal cord were cut in a Leica CM1900 cryostat.

3.6.2- Fluoromyelin staining

Fluoromyelin™ is a commercial fluorescent dye, that is used to selectively stain myelin in tissue sections[154]. Briefly, each lamina containing the spinal cord sections were placed at room temperature and were rehydrated in PBS (1x) for 20 minutes and then incubated with Fluoromyelin™ Green fluorescent myelin stain solution (Molecular Probes, Invitrogen, USA) diluted 1:300 in PBS for 1 hour and co-stained with DAPI. Slides were washed three times in PBS (1x) for 10 minutes and mounted in Immu-Mount® (Thermo Scientific, USA). Pictures were taken in Olympus IX53 microscope, and the lesion volume analysed following the protocol in the section 3.6.3.

3.6.3- Lesion volume quantification

The lesion area was analysed in every section spaced by 120 µm. Briefly, the images of the lesion were obtained as show in the figure 8, and two areas was taken; **1)** Total section area are in the figure 8B; **2)** Total lesion area (fig. 18C). The figure 8A is representative of histological injury provoked by the compression 30 days post lesion, where the lesion parameters analysed were: demyelination, necrotic tissue and architecture modification.

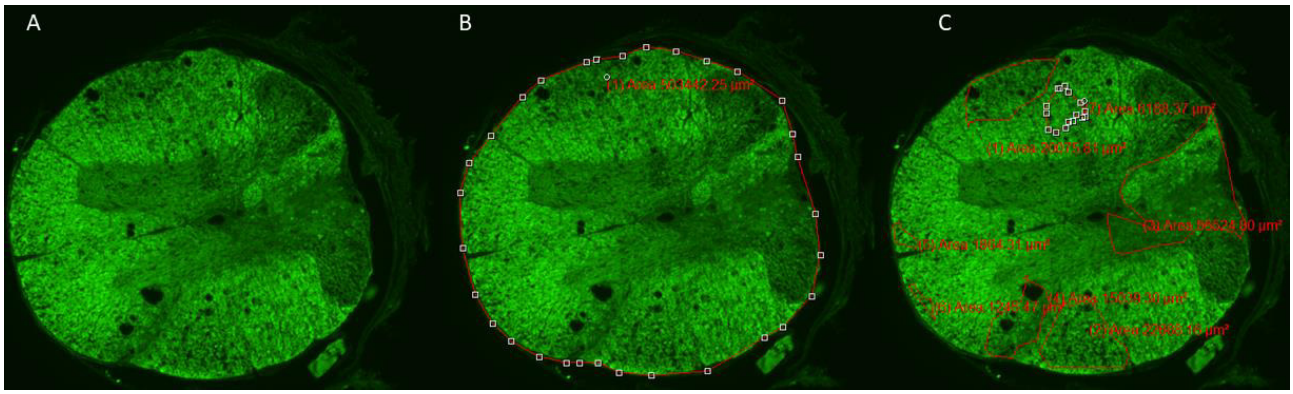

Figure 8- Lesion area quantification protocol. **A)** represent a transversal snapshot of the spinal cord; **B)** Red contour represent the total cross-section area; **C)** Each red circle represents spots of lesion (demyelination, necrotic tissue and architecture modification)

3.7- Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.00 (GraphPad Software, USA). To determine differences between the groups, from all behaviour tests and histological analysis were have used the one-way analysis of variance (ANOVA, coupled with Tukey´s post-hoc test, except for the results of the BMS in which two-way ANOVA repeated measures. Statistical significance was defined for $p<0.05$. Data is show as mean + standard error mean (SEM).

CHAPTER 4 – RESULTS

4 – Results

4.1- SCI mice model implementation – a new tool in house

The initial objective of the present thesis was to implement a compressive model of SCI in mice. For this purpose, forceps were used to compress the spinal cord and two intervals of compression were used: 1) 2 seconds and 2) 10 seconds, in order to create a moderate and a severe injury. In order to evaluate the impact of these lesions at the motor and sensory level, the BMS motor score, Activity Box test and Von Frey test, were used.

BMS test

With the objective to evaluate the locomotor recovery after different injury times, we used the BMS test (Fig.9). As it can be seen, the difference between the both type of injuries and the laminectomy is statistically significant in all time points ($p<0.001$), indicating a clear motor deficit of SCI animals Laminectomy group, which presented always the maximum BMS score, showed a normal motor behavior. The BMS score of 9 in the laminectomy group shows that the surgical procedure of removing the lamina from the lesion area did not affect the spinal cord, indicating that only the compression contributes to the deficits observed in the lesion groups (fig.9).

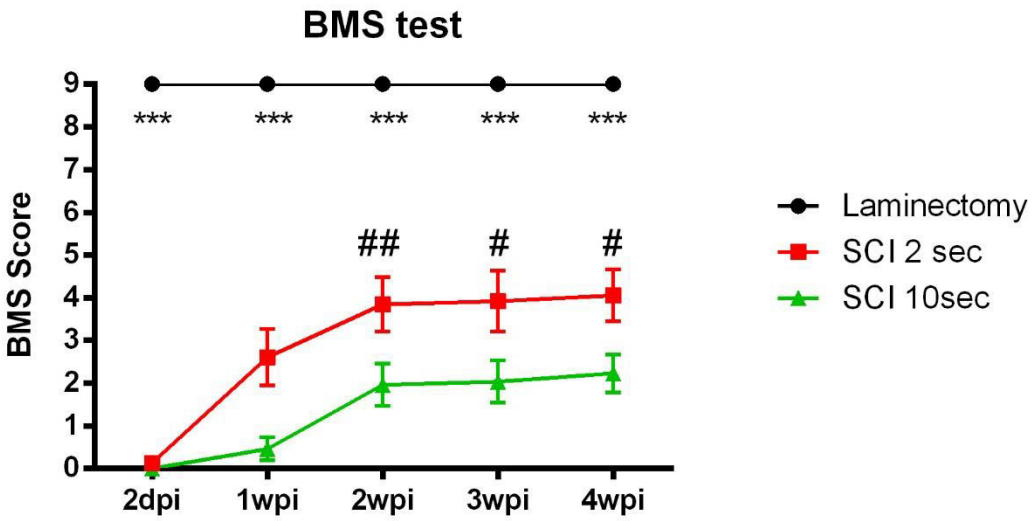

Figure 9-Evaluation of the locomotor behaviour through of SCI animals with distinct compression times: 2 seconds(n=19); 10 seconds (n=13) and animals only subject to laminectomy (n=10). Values are shown as mean ± standard error of mean. *-differences between laminectomy and SCI groups; #-differences between SCI groups. (#P<0.05; ##P<0.01; ***P<0.001).

Regarding the lesioned groups, both start at 3 days post injury in BMS scores close to 0 with practically non-function observed in the hindlimbs. Both lesion severity, 2 seconds and 10 seconds show a natural recovery during the first two weeks, stabilizing after that until the final week. The final score of the both SCI groups indicates a more severe locomotor outcome in the 10 seconds compression model. It evolves from a score of 0, 3 days post injury, to a maximum score of 2.23, which translate in extensive ankle movement only, 4 weeks post injury. On the other hand, 2 seconds compression setting achieves a maximum score of 4, showing occasional plantar steeping 4 weeks post injury. The motor recovery between the SCI groups are statistically significant at 2, 3 and 4 weeks. This indicates that the locomotor recovery in this type of lesion is dependent of the time of compression.

Activity Box test

In order to evaluate the total distance traveled and the number of vertical counts at 4 weeks post injury, both measures of gross motor behavior[151], the activity box was used. Laminectomy animals travel more than both injury compression times ($p<0.01$). Indeed, laminectomy animals have a total distance of 2465 ± 376.6 cm, while 10s injury ran only 796.7 ± 187 cm similar to the distance walked by 2s injury animals 776 ± 201.3 cm (fig.10).

Regarding the number of vertical counts, the groups follow the same profile as the distance travelled. Laminectomy animals performed significantly better than both SCI groups ($p<0.01$). While the laminectomy animals have 32 vertical counts, the injury ones tend to do not show any vertical activity (Fig.10).

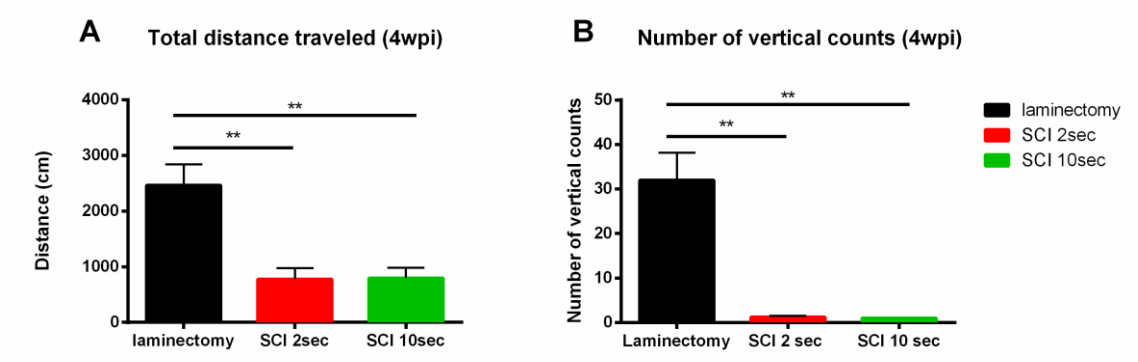

Figure 10- Total distance travelled (A) and total number of vertical counts (B) in the activity box test, 4 weeks post distinct injury levels. Values shown as mean ± standard error mean. (laminectomy =5; SCI 2sec=4; SCI 10 sec=5). (**P<0.01)

Von Frey test

The Von Frey test was performed to evaluate the sensory level of the animals, being and important indicator of sensory deficits[155]. Laminectomy group, performed better than the SCI animals ($p<0.01$) (Fig 11). Laminectomy animals have a normal threshold 50 % of 1 g, performing statistically significant better than the SCI 10 seconds group (0.030 g ± 0.006) (0<0.01). SCI 2

seconds group also performed worse than laminectomy, although have performed 10 times better than 2 seconds animals (0.30 g ± 0.006). This result indicates that SCI animals evoke a response to an external stimulus that normally, non-injured ones do not show.

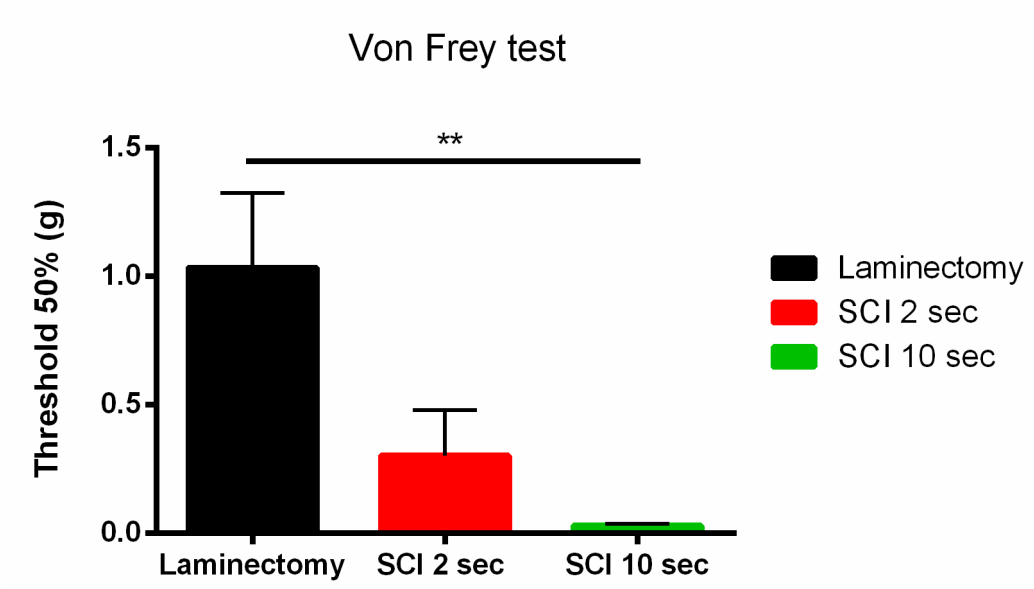

Figure 11- Von Frey test to evaluate sensory deficits in distinct injury severities (4 weeks post injury). Values are plotted as mean± standard error of mean. (Laminectomy=5; SCI 2 sec=4; SCI 10 sec =4; **P<0.01)

Animal welfare post injury

Another parameter that we also looked was the animal welfare after the injury. As a palpable parameter we have measure the weight during the experimental period. We observed that none of the groups went bellow the human end point to the weight lost. Indeed, none of the groups lost more than 10% from the original weight, as seen in figure 12**.** It is possible to observe, the laminectomy never goes under the initial weight, indicating that the simple surgical procedure of removing the lamina, does not affect the quality of life. On the other side, SCI groups never recover the weight lost along the 4 weeks of the experiment. In fact, the laminectomy in the final week have gained 4 % of weight from the baseline measure, while the 2 seconds and 10 seconds SCI groups, lost 4% and 10% of weight respectively. The observed weight difference between the

laminectomy group and the 10 seconds injury, were statistically significant every week post injury ($p<0.001$), while the 2 seconds SCI group is only statistical significant in the last two weeks ($p<0.001$).

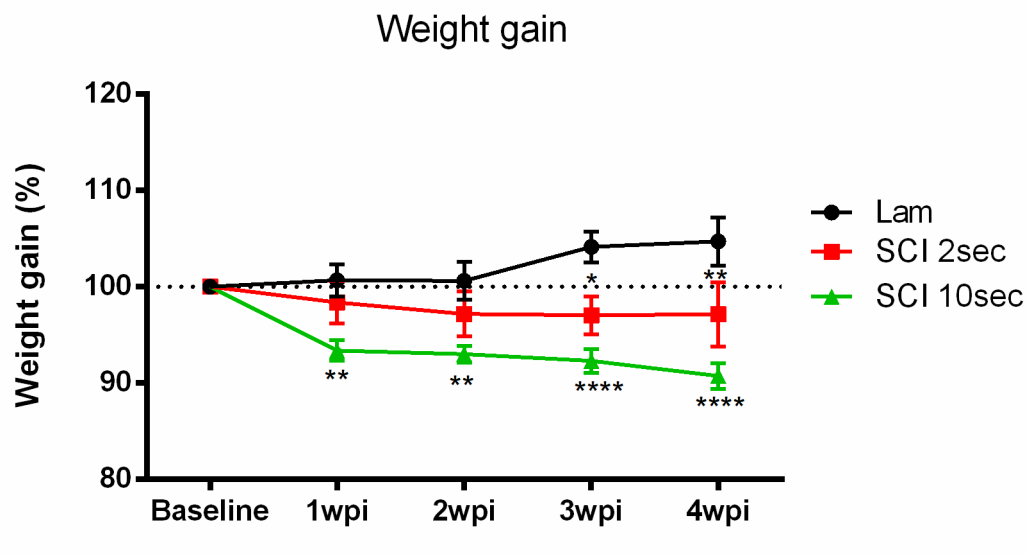

Figure 12- Weight variation as measure of general animal welfare after spinal cord compression; *,**,***,****- refers to differences between SCI groups and Laminectomy. Values shown as mean ± standard error mean (Laminectomy=10; SCI 2sec=18; SCI 10 sec=13; *P<0.05; **P<0.01; ***P<0.001; ****P<0.00001)

It is also interesting to refer, that both injury settings caused deficits at bladder control level and none of the animals recover the full functional control. The laminectomy animals have disclosed a normal bladder control.

The implementation of this new spinal cord injury model in the laboratory was a crucial step before we could advance to another experimental design. The BMS score shows similar evident natural recovery of the animals after the injury in the both compression times, during the first two weeks post injury. Although, the lesion produced by 10 seconds compression seems to be more severe since the first week post injury, 0.46 BMS score against 2.6 of 2 seconds group. From the week two to beyond, the differences between these SCI groups are statistically significant

($p<0.05$). Thus, the BMS score suggests that the locomotor outcome of this SCI model is inversely related to duration of compression.

Furthermore, the injury even though it is performed manually presents a low variability as it can be seen in figure 9. In addition, the laminectomies do not present any locomotor deficit, demonstrating that the surgical procedure of the lamina removal did not cause any kind of damage to the spinal cord, being the motor deficits total related to the compression time.

Concerning the activity box test where we evaluated the total distance traveled and the vertical activity, both tests present similar results. The total distance traveled by both injury groups is identical and significantly different from the laminectomies (fig.10). Regarding the fact that the maximum score achieved was occasional plantar stepping by the 2 seconds SCI group, and since this test evaluate the gross motor performance, and be highly dependent of the walk activity, its seems to plausible that the animals do not have the ability to easy move. This behavior test, do not appear to be sensible enough to detect differences in these severity levels. However, it could be important when higher locomotor BMS score levels are detected. Considering the vertical counts, follow the same pattern of the total distance. Since, this activity is highly dependent of the weight support (BMS score: 3), the lower activity level goes in accordance with the BMS score. One more time, in superior locomotor levels it could be useful.

The Von Frey test, which analysis the minimum force required to elicit a response 50% of times (threshold 50%), shows that sensitivity levels vary according with locomotor score (fig.11). In other words, a lesion which produces higher BMS scores (lower locomotor deficits), tend to respond fewer times to a stimulus that usually does not. This finding suggests that the nociceptive inputs to the spinal cord, elicits a maladaptive plasticity which translate into more functional and sensitive problems after SCI[156]

Globally, even though the only statistically significant difference in the behavior tests was detected in the BMS score, all results together suggests that the model implemented here is time-severity dependent. We hypothesize that differences detected are due to the primary injury, in which differential numbers of cell death exacerbate in different way the secondary injury and produce different histological outcomes and lesion areas, as was seen before in another graded compression study[157].[157].

4.2 – Assessing the therapeutic effect of the ASCs secretome in SCI mice model

Upon the implementation of the model an experimental setup was planned to assess the therapeutic potential of ASCs secretome in mice after spinal cord injury. For this purpose, we selected the moderate injury model (2 seconds compression) for the following reasons: 1) general animal welfare, animals submitted to this type of lesion have enhanced general recovery from surgery such as minor weight loss and less occurrence of autophagic behaviour and 2) because this type of injury results in more surviving neurons, which is crucial for testing the neuroprotective effects of ASCs secretome.

Weight gain

In order to assess the potential deleterious effect of administration of ASCs secretome local and systemically on the animal welfare, we have measured the weight every week post injury. As it can be seen in figure 13, none of the animals lost more than 10% of weight, actually both administration routes have a weight variation profile slightly better than the control group.

The mean weight gain in the first week of the systemic group was 102 %, while the others have lost weight to 92% from the initial weight. This timepoint, was the only one, where the difference between the systemic administration and the other groups were statistical significant ($p<0.01$). In the following weeks, local and the control group regain the weight lost until the final week, where both show weight values similar to the baseline (100%). The systemic group was the only one who gained weight in the last week of the experiment, comparatively to the baseline. Overall, the weight variation, indicates the safety of use secretome of ASCs, injected local or systemically. Since the both treatments do not lose more weight than the control group.

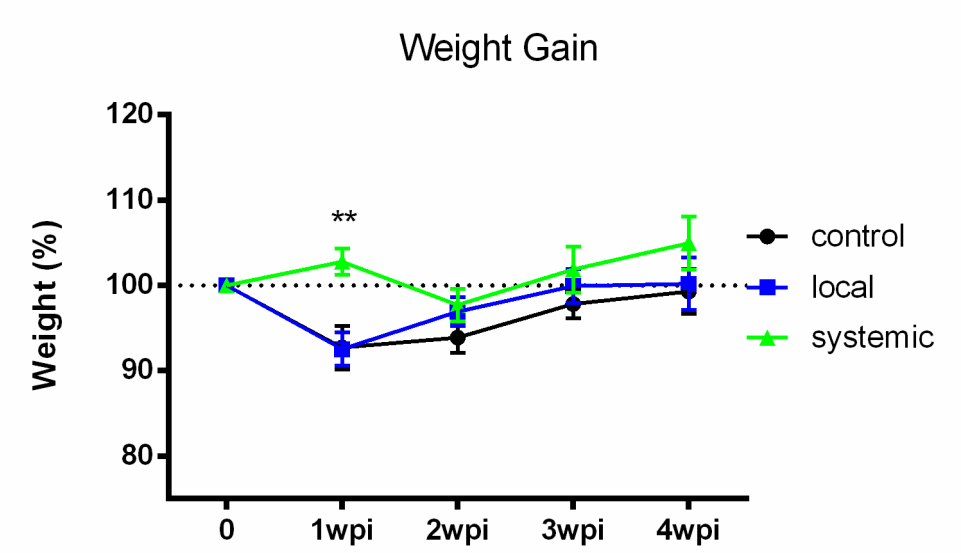

Figure 13- Weight gain as assessment of potential deleterious effects of administration of ASCs secretome. Data show as mean ± standard error mean. (control=5; local=8; systemic=4; **P<0.01.

BMS test

Locomotor evaluation using the BMS score started 3 days after injury, and was subsequently repeated once a week for 4 weeks to assess the level of functional locomotor recovery (Fig.14). Three days after the compression injury, all groups demonstrate decreased locomotor function, control and local treatment (group score=0) and systemic treatment with a score of 0.625 ± 0.239, indicating that the hindlimbs function is severe impaired as only slight movement of the ankle was detected.

The control group, control medium injection, showed a natural locomotor recovery over time, stabilizing after 3 weeks post-injury reaching a maximum score of 2.1±1, which translate in extensive ankle movement. Local treatment with secretome demonstrates a recovery slightly superior to the control group, stabilizing at week 3 and reaching a maximum score of 3.35 ± 0.94 (Plantar placing of the paw with or without weight support, or occasional, frequent or consistent dorsal stepping but no plantar stepping).

In the systemic-treated group, BMS score achieved a maximum score of 6 ± 0.93, which represent frequent or consistent plantar stepping, *some* coordination, paws *parallel* at initial

contact, or frequent or consistent plantar stepping, *mostly* coordinated, paws *rotated* at initial contact and lift off. Furthermore, there is a significant statistical difference between this group and the control at 1, 3 and 4 weeks post injury ($p < 0.05$). Moreover, the systemic group also score higher than the local treatment (fig.14).

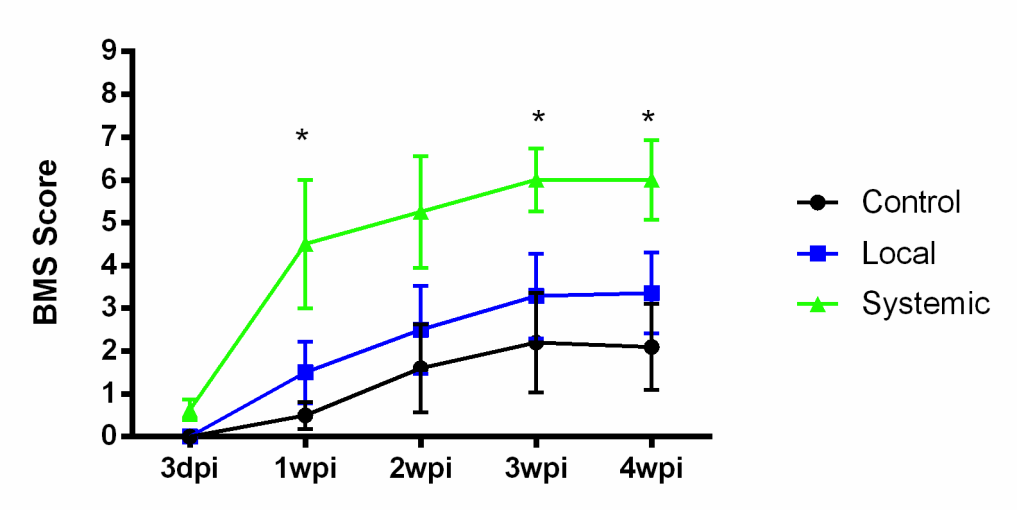

Figure 14- Locomotor behaviour evaluation using the BMS test of animals treated with culture medium and ASCs secretome injected local and systemic. Data show as mean ± standard error mean. (Control=6; Local=9; Systemic=4; *P<0.05)

Activity box test

Gross motor behaviour was performed at 4 weeks post-injury, to evaluate the total distance and also the number of vertical counts by the SCI animals (fig.15). The distance results showed a better performance of the systemic group (2221.4 cm ± 621.4) compared with the control (1120.6 cm ± 352). Both the local treatment (1286.5 cm ± 351) and the control group present similar total distance traveled. The results are not statistically different, however, there is a trend to the systemic group performed better than the others. Regarding the number of vertical counts, the results follow the same trend as the distance traveled. The control group and the local treatment produce similar results, 2.6 times ± 1.6 versus 1.75 times ± 0.4 respectively. While the systemic group, one more time show a trend to perform better in this parameter (32±x55).

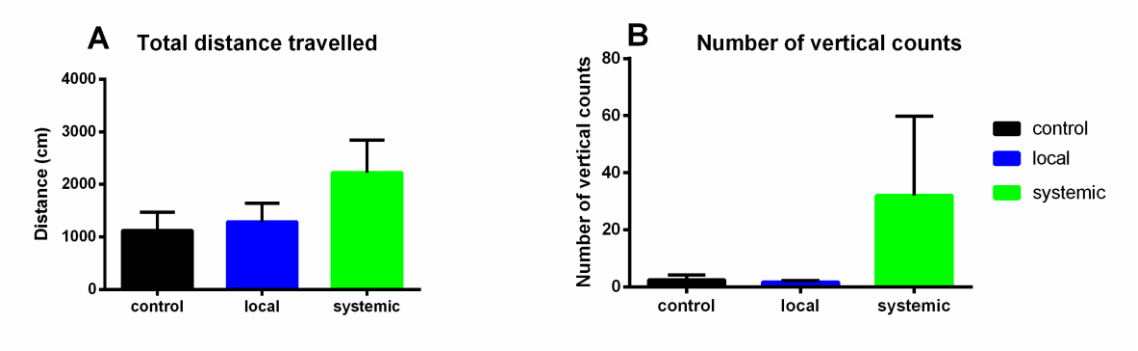

Figure 15- Total distance travelled (A) and total number of vertical counts (B) after 4 weeks in animals treated with secretome local, systemic and with culture media. Data show as mean ± standard error mean. (control=5; local=8; systemic=4)

In order to have a better assessment of the animal's recovery after treatment, and additional three behavioural tests were performed: 1) Beam balance; 2) Gait analysis and 3) Motor swim test (MST), adding to the ones established in the section 4.1. However, the last two failed to be a reliable test to be used, at least for this SCI model. Regarding the gait analysis, the objective was to analyse several parameters of the stepping profile, such as: stride length, limb rotation and base of support, as is stablished to a model of the Machado Joseph disease in our lab[158]. It is possible to observe in the figure 17, some general examples of the wide array of locomotor deficits that we have faced in distinct animals. While in the figures 17A and 17B, that represents animals with BMS score of 5 and 4 respectively, it is possible to determine the gait parameters. However, when the deficits are greater, as seen in the figures 17C 17D, BMS score of 1, it is not possible to measure the gait profile. So, we have decided to exclude this test from our analysis due to the lower number of animals possible to be included in this test.

Beam Balance

Beam Balance test was performed in week 4 to assess fine motor coordination, balance and supraspinal motor control of cortico- and rubrospinal tract. Four distinct beams were used: 10mm and 20mm round and square beams[151]. Figure 16, represent the sum score of 4 beams. The results one more time shows a trend to the systemic group perform better than the control and local secretome administration. Actually, the systemic treatment (3.64 ± 1) was close to the half of the maximum score to be achieved, while the others remain below the total score of 2.

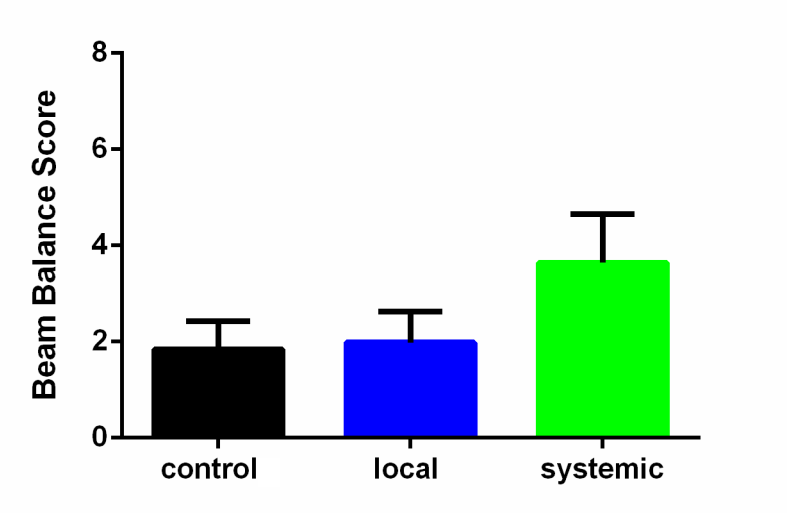

Figure 16- Evaluation of fine motor coordination and balance of animals treated with secretome local and systemic or with culture media(control). Data show as mean ± standard error mean. (control=5; local=8; systemic=4)

Regarding the motor swim test, the main aim was to evaluate the time taken to mice swim a certain distance in a straight line. The high coordination deficits led to a circular swim, instead of a straight line. Schell et al. in 2008, described a novel score which analyse several parameters of the swim motion such as: coordination, inter-hindlimb coordination and the axis of the hind paw, in rat SCI[152]. However, the anatomy of the mice is small and tight, making this analyse difficult using this scale. A future perspective could be the adaptation of the Schnell Swim Test score, to the mice scale.

Figure 17- Typical gait profile of SCI animals. (A)&(B) representative minimal locomotor deficits; (C)&(D) representative of sever locomotor deficits.

Histology

Regarding the histological analysis, here we have evaluated the lesion volume. The figure 18A represent the lesion progression along the spinal cord, the middle point represents the lesion epicentre, being the left and right arm caudal and rostral to it, respectively. The lesion volume was quantified calculating the Area Under the Curve of each treatment. As could it be seen in the figure 18B, in where the lesion volume is plotted by the Area Under the Curve, despite of the mean of the AUC in the systemic treatment (67031±4766), be slightly smaller than the local treatment (78127±11120) and control group (78509±4766), there isn´t statistical difference between them.

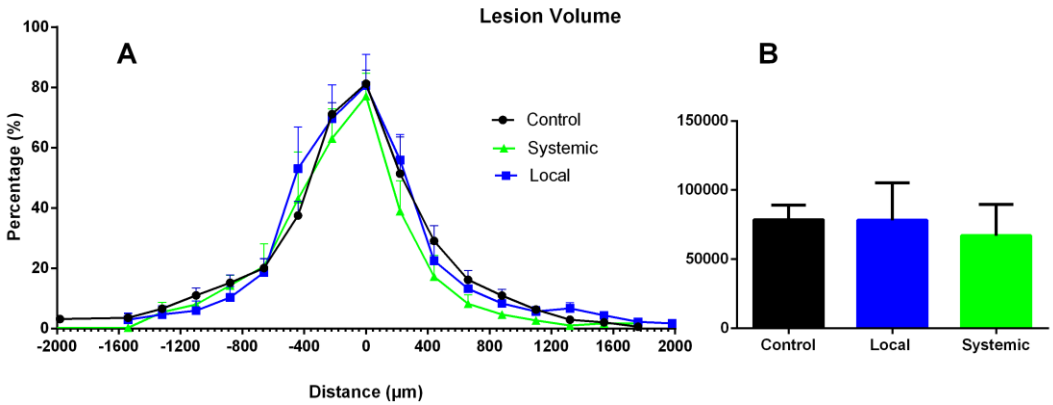

Figure 18- A) Extension of the lesion upon treatment, rostral and caudal with epicentre centre in the maximum percentage of lesion; (B) plot of the mean area under the curve of each treatment representative of the lesion volume). Data show as mean ± standard error mean. (control=5; local=6; systemic=4)

CHAPTER 5 – DISCUSSION

5-Discussion

The spinal cord injury remains a dramatic condition with no clinical treatment to provide a better prognosis to patients suffering from this demanding neurological condition. In this report we have developed and established a clinically relevant model of SCI injury in mice, being a potential tool to dissect mechanisms underlying SCI pathophysiology and test therapeutic agents. The second part was focused on determining the therapeutic effects of ASCs secretome exploring two different routes and schemes of administration using the mice model of thoracic SCI previously implemented.

A mice model presents some advantages compared with other models, since there are plenty of genetically modified strains and knock-outs permitting to better dissect the SCI molecular mechanisms or requiring less volume of pharmacologic agents to test. One important issue to highlight is that mice SCI model is histologically distinct from other mammals. Instead of developing the cystic cavity with reactive astroglia, the inflammatory response recruits a unique cell population only found in mice spinal cord called "fibrocytes" which conduct to develop of fibrous connective tissue over the injury site[159,160]. The comparison of the lesion extension between models at the histological level was not yet explored. In the future stereological analysis of the lesion, together with the study of neuronal loss and cellular recruitment, will help to further characterize this model. Two distinct times of compression were used to mimic a moderate injury and a severe injury, 2 and 10 seconds respectively. Previous report of similar compression found that different times or intensity of compression produces graded locomotor deficits[161,162] It's also important to refer the fact in which this novel SCI model was designed and adapted from a previously described injury model. Before, Plemel et al. have published a compression model with different openings of the forceps, using adapted spacers. They have related different spacers with variable injury severities[163]. This data goes accordingly with what has been demonstrated in this thesis. Herein, we have described that the control of the compression time produces graded injury levels. This data reinforces the reliability of our model.

Significant motor deficits were achieved in both compression times when compared with laminectomy control group (fig.9). Despite of the invasive surgical procedure of removal the vertebral lamina, it is possible to observe that, the control group (laminectomy) presents a normal and consistent motor behaviour during the experiment. Furthermore, looking to the injury groups an important achievement was the significant statistical difference between 2s and 10s in the BMS locomotor score. This indicates that the methodology used to produce the injury is a reliable, resulting in graded injury phenotype, that is closely associated to the compression time. This could be helpful in future works to assess the therapeutic value of novel treatments in different SCI severities, to evaluate different secondary injury events or to observe how the system responds to different injury levels. Moreover, both injury groups show spontaneous recovery during the first 2 weeks, which could be explained by the tissue plasticity and consecutive neuronal reorganization[164,165]. As mentioned in the introduction section the injury cascade is divided in primary, secondary and chronic injury. Taking this into account, it is plausible to speculate that more time of compression will produce an increased primary injury that may exacerbate the secondary events of the SCI and provoke more neuronal death and consecutive greater impairment of the locomotor activity. An important achievement in this work was the establishment of consistent injury model, with low variability within groups.

Furthermore, gross locomotor behaviour was also significantly reduced compared with control, as it can be seen in the activity box text (total distance and number of rearings) (fig.10). This data goes down the same line as BMS. However, the ABT fails to show up statistically significant differences between SCI groups. This lack of statistical significance could be related with the fact that the activity box test, is not sensible enough to detected small motor improvements, especially when comparing severe injuries, as in this case. Although, the two models display different locomotor recoveries, these are subtle differences. In the animals with a more mild phenotype (2s) the animals only produce occasional plantar stepping (BMS=4) while the most severe are able to move externally the ankle (BMS=2). However, the activity box test could be useful when the differences are bigger.

Another important behaviour test already described in the literature is the Von Frey test[166,167]. Most authors only focus on the evaluation of the motor performance. However, more systems are affected post SCI, being this one indicator of sensory deficits. Another hallmark of this

work, was the establishment of functional readouts at the sensorial level, showing that injured animals develop of hypervisibility related issues. Interestingly, some human SCI patients develop neuropathic pain, which reinforces the clinical relevance of these finding in the herein developed animal model. Curiously, animals with better score in BMS (best locomotor score) tend to respond less times to abnormal external stimulus (fig.11). These results can be related with how the both motor and sensory spinal tracts run along the spinal cord, very close within them[4]. As so the lesion cause damage to motor spinal tracts and in same scale to sensory tracts.

We have thus developed a clinically relevant animal model of compression injury. This type of injury by compression is frequently observed on human patients (construct validity). Furthermore, our model display motor and sensory alterations as well as urinary control dysfunction, symptoms similar as the ones observed in human patients (face validity).

The second part of the work was focused on the evaluation of the therapeutic effect of ASCs secretome in SCI. As stated in the introduction section, a couple of reports were performed using secretome of several cells to tackle SCI, but no one has assessed the potential of adipose stem cells secretome[168,144,145]. For this purpose, the two seconds compression was chosen, since this is moderate injury setting of our model, presenting more surviving neurons. This is particular relevant, due to the ASCs secretome known composition of neuroprotective factors[129,135] .

Firstly, we have demonstrated that both administration routes, local and systemic, and per consequent the secretome, apparently do not affect the animal's welfare or cause any kind of abnormal event to the mice(fig.13).

Clinically relevant locomotor recovery was only achieved with the systemic injection of secretome. Four weeks post injury, these animals display, coordinated plantar stepping, although rotating the paws during the stepping cycle(fig.14).

Furthermore, the gross motor behaviour, fine and balance performance follow the same pattern from locomotor behaviour. However, the systemic administration only tends to be better than the other groups, while the local injection in every locomotor and behaviour test was only slightly better than control and worse than systemically administrated secretome (fig.15).

Regarding the histological analysis, the obtained results do not completely match with the locomotor performance, with minor differences observed between groups (fig.18). This might be related with the need to optimize how to quantify the total lesion using the Fluro myelin method. Additionally, is still missing further histological analysis to evaluate the immunological response, neuroprotection and regeneration, being this one currently being optimized in our lab to mice.

The major finding of this part of the thesis was that systemic treatment promotes significant locomotor recovery compared with control. Although the mechanism of action, is still to be unravelled. Multiple hypothesis could be proposed to explain this positive effect. Here we suggest a couple of events which in synergy seems to act to revert the neurological deficits caused upon SCI. The ASCs secretome was described to contain paracrine factors which could potentially promote neuroprotection[131]. For instance, it is known that DJ-1 and CYPA protect against oxidative stress, PDEF and IL-6 protect against glutamate excitotoxity and CYPB and Gal-1 are anti-apoptotic agents. These soluble factors and others reported in the introduction section may protect neuronal cells against the secondary injury events, avoiding exacerbated neuronal losses. Previously, was already reported an increased survival of cultured neurons after treatment of CM of ASCs[169]. With a higher number of survival neurons in the injured spinal cord section, there could be an enhancement of the natural plasticity potential of the spinal cord[164,165], allowing neurons to form new communication routes to bypass the injury. This could be supported by paracrine factors present in ASCs secretome which induce neurite growth, such as BDNF and SEM7A[131,135]. Indeed this has already been demonstrated in and an *in vitro* DRG model[171]. One more important question is the reduction of blood supply after spinal cord injury[22]. The presence of angiogenic factors (Angiogenin, bFGF, VEGF)[133,132] in the secretome cocktail, might be of relevance for overcoming these vascular alterations. The last synergic component of the secretome acting, is the immune modulatory role. It is known, that upon injury there is the recruitment of leukocytes to the injury site[159]. Recently, it was shown that ASCs secretome reduces the recruitment of monocytes, simultaneously priming macrophages towards an anti-inflammatory state[139]. This interaction with macrophages had already been show, either by PGE2 in the secretome[140], or by micro RNA containing inside the exosomes[141], with a switch from M1(inflammatory) to M2(anti-inflammatory). Actually, a recent report from our group had already stated that the paracrine factors of transplanted ASCs to the injury site reduced local tissue inflammatory cells recruitment[171].

Summing up, is plausible to hypothesize that the soluble factors and the vesicular fraction of the secretome are acting together to increase cellular survival, switch the injury environment and induce tissue reorganization.

It is important to highlight the capability of the secretome administrated systemically to reach the health or injured spinal cord[141].

Regarding the locomotor differences between the local and systemic injection of secretome (fig.14), two distinct events might be acting at same time. At first, since the local injection is repeated two times immediately after injury in the spinal cord parenchyma, the size of the Hamilton syringe and the hydraulic pressure caused by the fluid injection, might be causing further damage into the tissue. As an alternative we could use intrathecal delivery of the secretome into the CSF, therefore avoiding additional damage to the spinal cord parenchyma. Additionally, we could reduce the fluid flow speed to avoid the formation of additional cell death.

CHAPTER 6– Conclusions and Future Perspectives

6-CONCLUSION AND FUTURE PERSPECTIVES

The objectives of this thesis were to first implement a new SCI model in the lab, and then assess the therapeutic role of ASCs secretome.

In conclusion, we have successfully implemented a new compression SCI model in our laboratory, being the severity of the injuries produced time dependent. Here, we have characterized the locomotor behaviour, however is still missing the histological evaluation. This last point, it is a crucial task to be performed as a follow-up of this thesis. The histological evaluation, could bring additional information on the extension of the neuronal loss, which immune cells are recruited and play a role upon injury, the glial activation state and how the tissue is structurally reorganized during the behaviour recovery.

In the second part of this report, we have found a therapeutic value of the ASCs secretome under systemic injection. This route of administration promotes a better performance in the BMS, activity box and beam balance tests when compared with local injection and control injection. Furthermore, the intravenously administration route is clinically relevant compared with the local injection, it less invasive than local administration and easy to apply to humans immediately after injury.

In the future we aim to perform a detailed histological evaluation of the spinal cord after treatment, including optimized approaches for analysing lesion volume. This is a crucial step to prove our hypothesis, related with reduction of the inflammatory state and an increased neuronal survival. It will be also interesting to better characterize the secretome, particularly the exosome fraction. Indeed, the emergent data related with the exosomes present in the secretome, seems to be a crucial target to be dissected in a near future. It would also be interesting to report which and how the micro-RNAs contained in exosomes favour the spinal cord regeneration.

On the other hand an *in vitro* experimental culture of neurons stress treated with ASCs secretome, could give some insights to reveal the mechanism of action behind the possible neuroprotection role. In same way, the secretome could be used to assess the monocytes/macrophages polarization from different sources: CNS tissue resident macrophages, circulating and those recruited from lymphoid organs.

To increase the value of this therapeutic approach, a new *in vivo* study with chronic administration could be interesting to increase the possible clinical potential of this methodology, as the diagnostic and treatment of the spinal cord injury in patients, most of the times is not performed immediately after injury.

CHAPTER 7– BIBLIOGRAPHY

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