Pharmacologically active microcarriers delivering brainderived neurotrophic factor combined to adult mesenchymal stem cells : novel approach for the treatment of spinal cord injury

Saikrishna Kandalam

UNIVERSITE BRETAGNE LOIRE

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Saikrishna KANDALAM

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Dr. Anne Baron-Van Evercooren,
Team leader, ICM Brain and Spine Institute Paris, France.
Thesis Directors:
Dr. Anne des RIEUX,
Professor, Université catholique de Louvain, Brussels, Belgium.
Dr. Claudia MONTERO-MENEI,
Associate Professor, University of Angers, INSERM U1066, France.
Co-director:
Dr. Jérôme GUICHEUX,
Research Director, INSERMU791, Université de Nantes, Nantes, France.

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Dr. Véronique PRÉAT,
Professor, Université catholique de Louvain, Brussels, Belgium.
Examinator:
Dr. Marie MORILLE,
Associate Professor, University of Montpellier, France.
Thesis Directors:
Dr. Claudia MONTERO-MENEI,
Associate Professor, University of Angers, INSERM U1066, France.
Dr. Anne des RIEUX,
Professor, Université catholique de Louvain, Brussels, Belgium.
Co-director:
Dr. Jérôme GUICHEUX,
Research Director, INSERMU791, Université de Nantes, Nantes, France.

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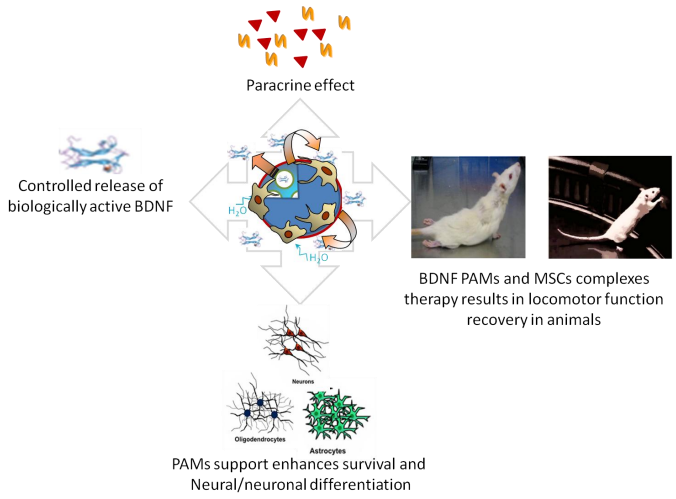

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RÉSUMÉ

Un traumatisme de la moelle épinière (TME) est une condition dévastatrice entraînant la perte permanente de fonctions neuronales. L'objectif de cette thèse est de formuler de microsupports pharmacologiquement actif (MPAs) avec une surface de fibronectine (FN), libérant le « brain-derived neurotrophic factor » (BDNF) de façon controlée. Nous voulons combiner ce système avec des cellules souches mésenchymateuses (CSMs) pour la réparation de TME. Le BDNF nanoprécipité a été encapsulé dans les FN-MPAs et le profil de libération *in vitro* a été évaluée. Elle a montré une libération bi phasique et prolongée de BDNF bioactifs. Nous avons combinés des cellules souches humaines mésenchymateuse issues de la moelle osseuse adulte (cellules MIAMI) et FN-MPAs avec un hydrogel non-toxique silanisés-hydroxypropylméthylcellulose (Si-HPMC). Nous avons démontré que les FN-MPAs et le Si-HPMC augmentait l'expression de marqueurs neuraux/neuronaux de cellules MIAMI après 1 semaine. En outre, l'environnement 3D (hydrogel ou FN-MPAs) a augmenté le sécrétome thérapeutique de cellules MIAMI. Pour avoir un système facile à appliquer en clinique, nous avons choisi d'utiliser les cellules souches de la papille apicale (SCAP) et FN-MPAs libérant ou non du BDNF pour la thérapie du TME. Plus de 90 % du SCAP complexée avec FN-MPAs (libérant ou pas BDNF) demeurent viables pendant 7 jours et il y a augmentation de l'expression des gènes neuronaux/oligodendrogliaux *in vitro*. La récupération de la fonction locomotrice a été significativement améliorée après la transplantation du SCAP complexée avec FN-MPAs-BDNF avec une coordination cohérente du membre postérieur après 28 jours de traitement.

Mots-clés: libération du BDNF; cellules souches mésenchymateuses; Si-HPMC hydrogel; réparation neuronal; microspheres; moelle épinière; récupération motrice

ABSTRACT

Traumatic spinal cord injury (SCI) is a devastating condition resulting in permanent loss of neural functions. The objective of this thesis is to develop pharmacologically active microcarriers (PAMs) with a fibronectin (FN) surface that deliver biologically active brain derived neurotrophic factor (BDNF) in a controlled manner. We want to combine this system with adult mesenchymal stem cells (MSCs) for SCI repair. The nanoprecipitated BDNF was encapsulated in FN-PAMs and the *in vitro* release profile was evaluated. It showed a prolonged, bi-phasic, release of bioactive BDNF, without burst effect. We combined human marrow-isolated adult multilineage-inducible (MIAMI) stem cells and FN-PAMs with an injectable non-toxic silanized-hydroxypropyl methylcellulose (Si-HPMC) hydrogel. We demonstrated that FN-PAMs and the Si-HPMC hydrogel increased the expression of neural/neuronal differentiation markers of MIAMI cells after 1 week. Moreover, the 3D environment (FN-PAMs or hydrogel) enhanced the therapeutic MIAMI cell secretome. To have a clinically translatable system, we chose to use stem cells of the apical papilla (SCAP) and FN-PAMs releasing or not BDNF for SCI therapy. More than 90% of SCAP complexed with FN-PAMs (releasing or not BDNF) remained viable for 7 days and an increased neuronal-oligodendroglial gene expression *in vitro*. The recovery of locomotor function was significantly improved after transplantation of SCAP complexed with FN-PAMs-BDNF with frequent to consistent forelimb-hindlimb coordination after 28 days of treatment.

Keywords: BDNF drug delivery; mesenchymal stem cells; Si-HPMC hydrogel; neural repair; microspheres; spinal cord injury; locomotor function recovery;

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Not a day passes by, dad that you don't cross my mind.

Not all of you departed when you left our earth behind.

In my heart there is a place that only you can hold.

Filled with loving memories more priceless than gold.

I know you still hear me Dad, so please know this is true that

everything I am today is because of YOU.

THANK YOU & I MISS YOU DAD.

DEDICATED TO MY LOVING PARENTS

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ABBREVIATIONS

3D: 3-Dimension

A

ADSCs: Adipose derived stem cells

AGRP: Agouti related neuropeptide

ALS: Amyotrophic lateral sclerosis

B

BBB: Basso-Beattie-Bresnahan

BDNF: Brain derived neurotrophic factor

bFGF2: Basic fibroblast growth factor

BmMSCs: Bone marrow derived mesenchymal stem cells

BSA: Bovine serum albumin

BSCB: Blood-spinal cord barrier

C

CNS: Central nervous system

CNTF: Ciliaryneurotrophic factor

CSF : Cerebro spinal fluid

CST: Corticospinal tract

D

dbcAMP: dibutyryl cyclic adenosine monophosphate

DRG: Dorsal root ganglion

DSCs: Dental stem cells

E

ECM: Extra cellular matrix

EGF: Epidermal growth factor

ELISA: Enzyme-linked immunosorbent assay

EMA: European Medicines Agency

F

FDA: Food and Drug Administration

FN: Fibronectin

G

GAP-43: Growth-associated protein 43

GDNF: Glial derived neurotrophic factor

GF: Growth factors

GFR α-1: GNDF family receptor alpha-1

GRO-alpha: Growth-regulated protein alpha

H

HA: Hyaluronic acid

HAMC: Hyaluronan/methyl cellulose

HGF: Hepatocyte growth factor

I

GFBP-3: Insulin-like growth factor-binding protein 3

IL-8: Interleukin 8

IP-10: IFN-gamma-inducible protein 10

K

KGF: Keratinocyte growth factor

L

LIF: Leukemia inhibitory factor

LST: Lateral spinothalamic tract

M

MAG: Myelin associated glycoproteins

MASCIS: Multicenter Animal Spinal Cord Injury Study

MCP-1: Monocyte chemoattractant protein-1

MIAMI cells: Marrow isolated adult multi-lineage inducible cells

MIP-1 β: Macrophage inflammatory protein-1β

MIP-1α: Macrophage inflammatory protein-1α

MSCs: Mesenchymal stem cells

N

NASCIS: National Acute Spinal Cord Injury Studies

NGF: Nerve growth factor

NSCs: Neural stem cells

NSPCs: Neural stem/progenitor cells

NT: Neurotrophic family

NT-3: Neurotrophin-3

O

OECs: Olfactory ensheathing cells

P

P188: Poloxamer

PAMs: Pharmacologically active microcarriers

PBS: Phosphate-buffered saline

PC: Portable computer

PDGF: Platelet-derived growth factor

PDGF-A: Platelet derived growth factor-A

PDGF-AA: Platelet-derived growth factor AA

PDGF-BB: Platelet derived growth factor-BB

PEG: Poly(ethylene glycol)

PHEMA-co-MMA: Poly 2-hydroxyethyl methacrylate

PIGF-1: Placenta growth factor-1

PLGA: Poly(lactic-co-glycolic acid)

PNS: Peripheral nervous system

PTSD: Post-traumatic stress disorder

R

RANTES: Regulated upon activation normal T-cell expressed and secreted

RNA: Ribonucleic acid

ROS: Reactive oxygen species

RT-qPCR: Quantitative reverse transcription polymerase chain reaction

S

S/O/W: Solid-in-oil-in-water

SC: Spinal cord

SCAP: Stem cells from apical papilla

SCF: Stem cell factor

SCI: Spinal cord injury

SCs: Schwann cells

SDF-1alpha: Stromal cell-derived factor 1 alpha

SEM: Scanning electron microscopy

Si-HPMC: Silanized hydroxypropyl methylcellulose

T

TGF-β3: Transforming growth factor, beta 3

TNFα- Tumor necrosis factor-α

TRH: Thyrotropin releasing hormone

TrkB: Tropomyosin receptor kinase B

U

US: United States

V

VEGF-A: Vascular endothelial growth factor-A

VEGF-D: Vascular endothelial growth factor-D

VPL: Ventral postero-lateral

1. GENERAL INTRODUCTION

1. The Spinal cord injury (SCI):

1.1. Spinal cord anatomy.

The spinal cord (SC) is a long, thin, tubular bundle of nervous tissue and supporting cells, which goes through the hard vertebral canal and stretches out from the medulla oblongata in the brainstem to the lumbar section. Beneath that level, nerve roots from the lumbosacral portions grow in the vertebral canal as the cauda equine [1, 2]. The human SC is gathered into 31 SC segments, from every segment a couple of spinal nerves emerge out and transmit the information to and from the peripheral nervous system (PNS). The spinal nerves (Figure-1) are ordered into 8 cervical nerves which control the muscles and glands and get sensory input from the neck, shoulder, arm and hand; 12 thoracic nerves connected with the chest and abs; 5 lumbar nerves connected with the hip and legs; 5 sacral nerves connected with the genitals and lower digestive tract; and 1 coccygeal nerve supply the skin over the coccyx [1, 2]. Despite the fact that the SC of rodents include 34 spinal nerves – 8 cervical, 13 thoracic, 6 lumbar, 4 sacral and 3 coccygeal, the general related functions are comparative [3].

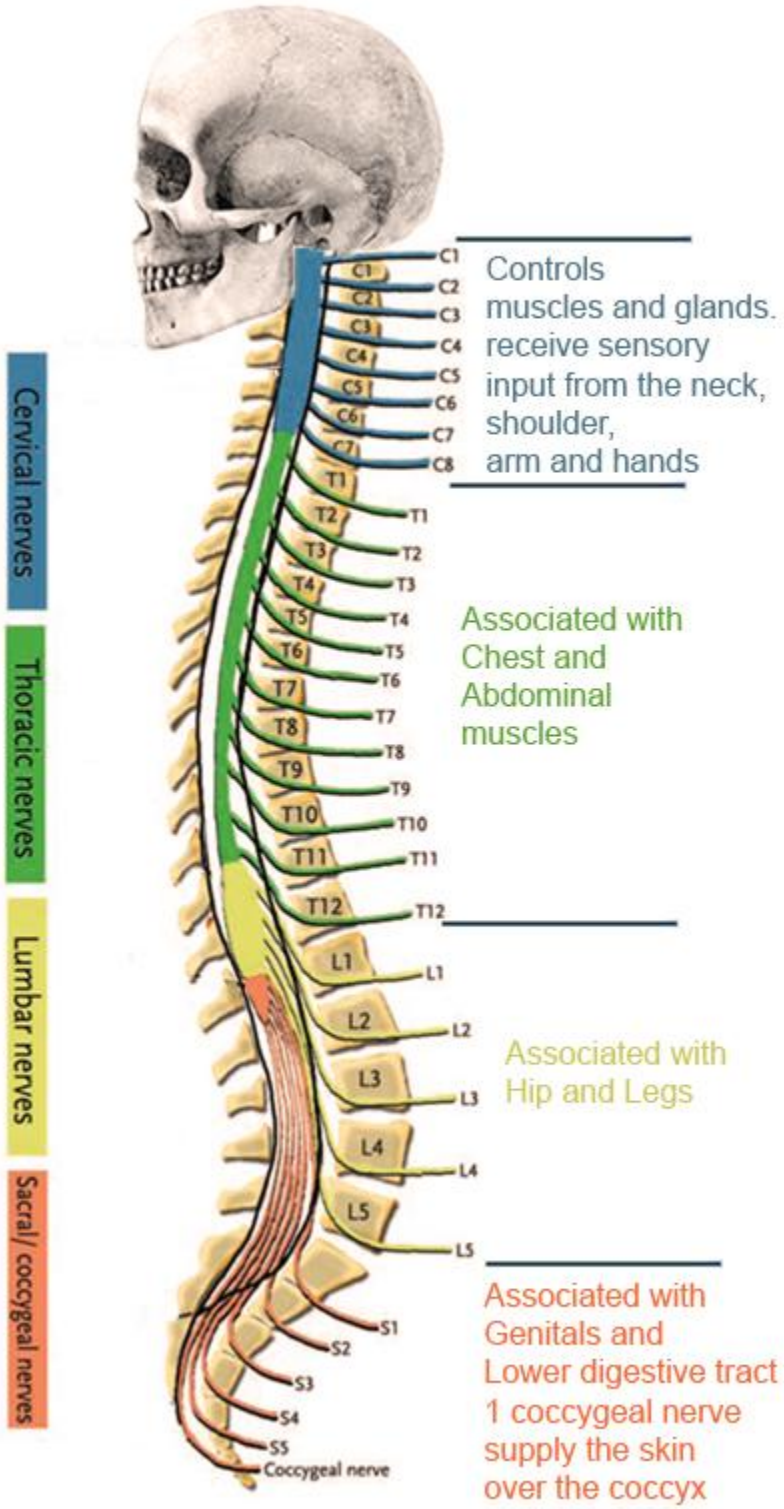

Figure-1: Depicting 31 segments of human SC and different types of spinal nerves and their associated functions.

The inner part of the SC consists of butterfly-shaped gray matter majorly constituted with cell bodies, dendrites, and axons. This is surrounded by the three funiculi (anterior, lateral and posterior) of the organized white matter. The ascending and descending axonal tract (Figure-2) run through the white matter funiculi and convey sensory, motor and autonomic information between PNS and cerebral regions. The ascending tracts (Figure-2) of SC consist of the lateral spinothalamic tract (LST) that has free nerve endings as sensory receptors, which mainly deals with sensations of pain and temperature. The primary synapse of this tract is in the posterior root ganglion and the final connection in the ventral postero-lateral nucleus of the thalamus (VPL). The ventral spinothalamic tract mainly deals with the sensations of either light touch or pressure, gathered by similar free nerve endings. The ventral and lateral spinothalamic tracts carry the same course of neurons and end up in the posterior central gyrus of the brain.

The descending tract (Figure-2) of the SC consists of the reticulospinal tract, which originates in the reticular formation and crosses various levels and branches continuously as the fibers descend. They finally synapse on the alpha and gamma motor neurons to produce the initiation or inhibition of voluntary movements, where the hypothalamus has control of the sympathetic and parasympathetic outflows. The major descending pathway, which controls the voluntary movements in humans, is corticospinal tract (CST) which originates in the primary and secondary motor cortices as well as the parietal lobes of the brain. The majority of the fibers cross over to the contralateral side at the level of the chiasm of the pyramidal cells in the cerebral cortex, become lateral CST, while the rest become anterior CST, crossing at the level of the

pathways final destination. Meanwhile, branches are given to the cerebral cortex, the basal nuclei, the red nucleus, the olivary nuclei and the reticular formation. At the pathways destination, it synapses upon the alpha motor neurons and pass along the information from the central nervous system (CNS) to the periphery which in turn creates rapid, skilled and voluntary movements, especially at the distal ends of the limbs.

The rubrospinal tract starts in the red nucleus and crosses over before descending to reach the alpha and gamma motor neurons. The adult human rubrospinal tract comprises of a small number of large myelinated fibers. Its clinical importance is uncertain, but it may participate in taking over functions after corticospinal injury. It may also take part in flexor posturing of the upper extremities, which is typically found with lesions above the level of the red nuclei. The vestibulospinal tract and olivospinal tract are two other descending spinal tracts, and finally the superior colliculus contributes fibers to a tectospinal tract involved in reflex postural movements concerning sight.

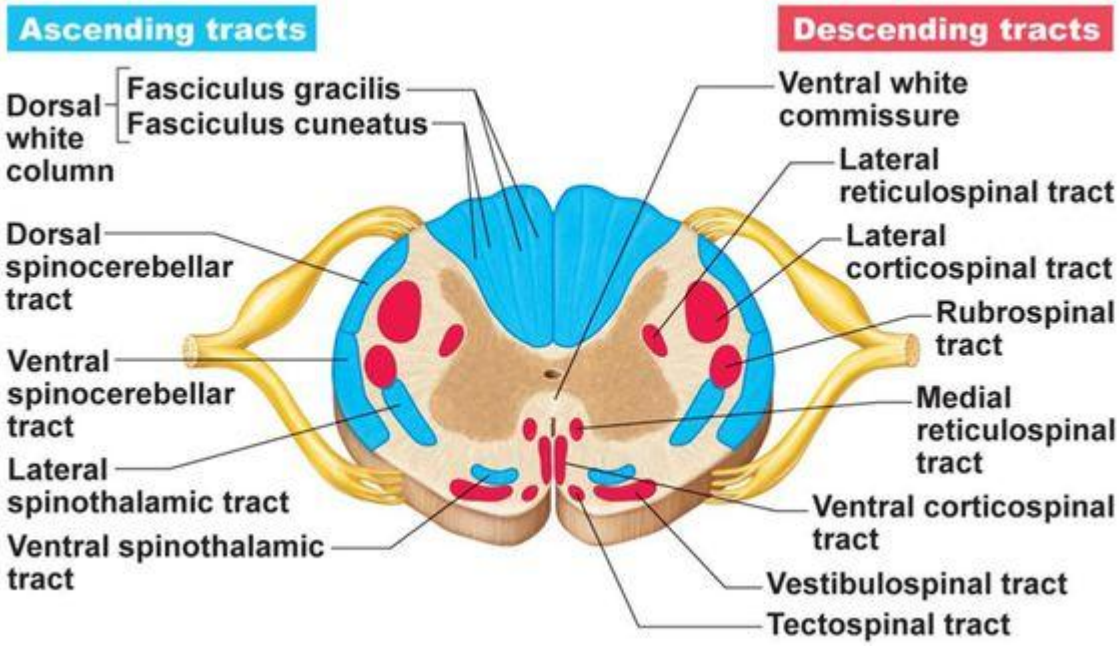

Figure-2: A schematic representation of the SC in cross-section and the ascending and descending axonal tracts location in the white matter tracts [4].

1.2. Spinal cord injury (SCI).

SCI is a devastating condition created by direct mechanical harm to the SC that prompts to complete or incomplete loss of neural functions identified with sense and mobility [5]. The SCI at cervical segments lead to tetraplegia/quadriplegia, resulting in functional impairment of all limbs, trunk, and pelvic organs. The SCI at thoracic, lumbar or sacral segments lead to paraplegia, based on the degree of the lesion, it may involve the trunk, legs and pelvic organs functioning (Figure-3).

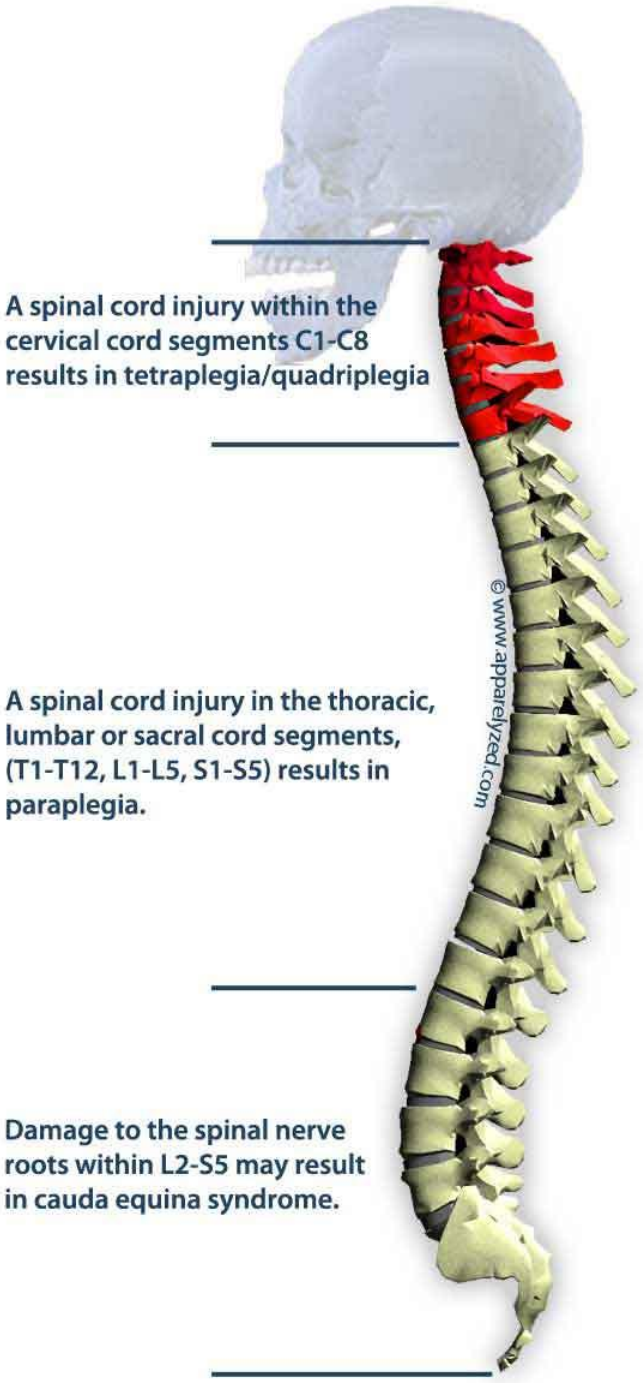

Figure-3: Levels of SCI impacts the extend of deficits [6].

1.2.1. Epidemiology

The incidence of SCI is about 15 to 40 in a million in the world [6]. The yearly occurrence of SCI is 40 cases per million in the United States [7], young men being the most affected (female to male ratio being 2.5:1 [7]). In a study Cripps *et al.* reported that the global prevalence of SCI is between 236 and 1,009 per million with 12,000 new cases per year in the United States (US) [8]. Thirty percent of patients die on the way to the hospital and 10% die during their hospitalization [6].

1.2.2. Societal impact

Despite the fact that the SC is well protected with the vertebra, damage can happen in an assortment of ways which incorporates motor vehicle accidents, falls, sports and violence (Figure-4) [9]. SCI not only affects the patients physically but also affects behavioral, psychological and social functioning [10]. The life expectancy of SCI patients can approach to normal with rehabilitation and good follow up. However, the secondary disabilities are not fatal but impair independent living and are costly to treat [11]. The treatment cost after damage depends on the level and seriousness of the injury, for instance, tetraplegia is associated with higher expenses than paraplegia [12]. Recent data from National Spinal Cord Injury Statistical Center in the USA assessed that in 2013, lifetime costs for a man harmed at age 25 are US$ 4.6 million for high tetraplegia contrasted with US$ 2.3 million for paraplegia [13, 14]. SCI patients face some common neuropsychological deficits including poor concentration ability, poor attention span, limited initial learning ability, impaired memory function, and altered problem-solving ability and these deficits may interfere with rehabilitation after SCI [15]. The individual with SCI is at high risk for experiencing the "Four D Syndrome," i.e.

dependency, depression, drug addiction and if married – divorce [16]. In a study conducted by Kennedy *et al.* assessing the anxiety and depression after SCI; 20-25% of patients experience an anxiety disorder and 30-40% develops a depressive disorder and 10-40% patients develops Post-traumatic stress disorder (PTSD) [17]. Moreover, SCI affects both genital and sexual functioning [18] and sleep [18].

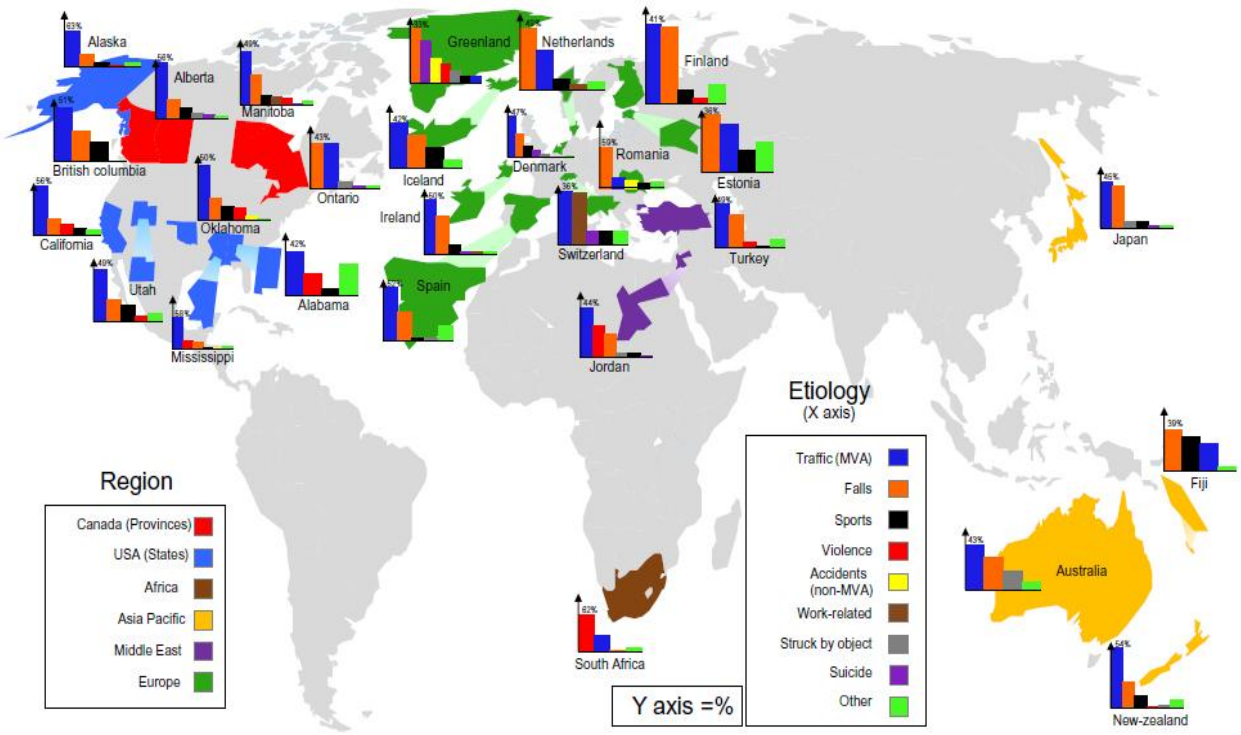

Figure-4: Illustration of SCI causations in all over the world. Blue bars in the graphs representing the major causes for SCI which is motor vehicle accidents and orange bars represents second major cause which is falls following sports and violence [9].

1.3. SCI models, Pathophysiology and potential targets to repair

1.3.1. Models of injury

The need of exact portrayals of physical factors that precisely reflect the physics of the lesioning procedure have prompted to create models of SCI [19]. Several animal models of SCI have been developed to adapt with the human SCI. It is important to create dependable, predictable and reproducible models since they have to recreate a portion of the significant pathological features of human SCI [20]. Besides, the models should permit assessment of a portion of the mechanistic features of damage and recovery after injury. The models should be compelling, and the impact measures should be generally simple. In view of these contemplations, researchers built up various SCI models in rats, cats, dogs and monkeys [20]. Among various models, the computer (PC) controlled weight-drop contusion model exhibits unwavering quality and reproducibility in assessing preclinical treatment for SCI [20]. A weight drop lesion created by the Multicenter Animal Spinal Cord Injury Study (MASCIS) contusion device and other comparative models, for instance, the Ohio State University electromechanical model [21-23], are described by the advancement of focal hemorrhagic necrosis that spreads radially, and rostrocaudally after some time (days to weeks). The last outcome is an ellipsoidal, loculated cystic cavity or cell-filled injury site [21] [24] [25]. A PC controlled impactor comprises of an animal (mouse or rodent) trap gadget that reproducibly conveys a weight to the exposed SC. This gadget permits an investigator to measure the level of injury utilizing settled distinct biomechanics, for example, impulse force, the speed of probe movement, power, and vitality. Additionally, literature proposes that contusion type lesions made by PC controlled weight impact

appear to precisely mimic those found in humans [26] and contusion-injured rats respond to early pharmacologic medications like humans [27]. Reproducibility, ability to measure the level of injury and broadly published reports make them an appealing technique for assessment of intense SCI management procedures [28]. The major pathological features in contusion lesions are discussed in the following section and illustrated in Figure-5.

1.3.2. Pathophysiology

SCI cases are majorly due to compressive or impact injuries [29]. In early 1900s, Allen *et al.* presented the idea of two-step mechanism for SCI and demonstrated that the SC damage is progressive after injury in rodent models [30]. The two mechanisms of damage to the SC after injury are i) primary injury induced by mechanical impact, ii) secondary injury induced by different damage forms including free radical-induced cell death, and glutamate excitotoxicity (Table-1) [31]. The primary injury is locally confined to the area of the vertebral fracture and is characterized by acute hemorrhage and ischemia and the secondary injury that takes place the first week after primary injury is portrayed by further death of neuronal and glial cells, prompting to huge development of the damage, extending paralysis to higher segments [32].

Table-1: Summary of pathophysiological events following SCI

Acute (minutes to hours)
Hemorrhage and hemorrhagic necrosis [33, 34]; Edema [33]; Ischemia [33, 35]; Production of reactive oxygen species (ROS) and lipid peroxidation [36, 37]; Excitotoxicity [33, 36, 38]; Necrotic and apoptotic cell (neuron and glial) death [39]; Neutrophil invasion, Microglial activation, Release of inflammatory factors e.g. IL-1β, TNFα, blood-spinal cord barrier (BSCB) permeability [36] Neurogenic shock (systemic), Axonal swelling [33]; Rupture of myelin sheaths and onset of axon demyelination [33, 36];
Subacute (days to weeks)
Infiltration and proliferation of macrophages [40]; Reactive astrocytosis and initiation of glial scar [36]; Development of small cystic cavities [33, 36]
Chronic (weeks to months)
Stabilization of lesion, progress in the formation of large cystic cavity (post-traumatic syringomyelia), “marshy cord syndrome” [33]; Continued formation of glial scar [36]; Wallerian degeneration of axons [36, 39]; Ongoing axon demyelination [33]; Limited plasticity and axonal sprouting [36, 41]

The compression of SC (Figure-5) results in immediate hemorrhage especially in gray matter due to damage of capillaries and venules of the microcirculation [33, 34]. After initial SCI in humans as well as in animal models, hemorrhaging and edema are the progressive events that spread to farther rostral and caudal regions. Ischemia is another vascular event that occurs immediately after injury due to the combination of hemorrhaging, edema, vasospasm, and thrombosis [33, 35, 36]. Ischemia and reactive oxygen species (ROS) release leading to lipid peroxidation induce neuron and glial death [36, 37]. Glutamate-induce excitotoxicity and subsequent disruption of ionic homeostasis are the additional acute biochemical modifications after SCI [36, 38]. Following SCI neuronal and glial cells thus die by both necrosis and apoptosis [39]. Other pathophysiological changes during the acute phase of SCI include inflammation, disruption of the blood-spinal cord barrier (BSCB), neurogenic shock, axonal swelling and demyelination [33, 36].

Inflammation after SCI starts with the invasion of neutrophils, activation of microglia and the release of inflammatory factors like interleukin-1β (IL-1β) and tumor necrosis factor-α (TNFα) [36]. The permeability of BSCB in rat models after injury peaks at 24 h due to mechanical tissue disruption besides the effect of inflammatory cytokines and ROS [33]. Axonal swelling starts 15 min after SCI and progresses for the next 24 h, myelin sheath rupture, and oligodendrocyte cell death leads to demyelination of spared axons near the injury [33, 36]. Axon demyelination is maximum at 24 h after SCI in rats but the evidence of demyelination of axons in human SCI is still undetermined [36]. Systemic neurogenic shock is also a contributor to the secondary injury, particularly in

cervical injuries and makes alterations to heart rate, blood pressure, and cardiac output [33].

Following the acute phase of SCI, progressive tissue degeneration takes place in sub-acute phase, which includes infiltration and proliferation of macrophages. The phagocytic response to the injury fosters the axonal regeneration by cleaning the myelin debris [36, 40]. Astrocytes play a key role in re-establishing ionic homeostasis and BSCB during sub-acute phase. In rat models, astrocytes restored the BSCB to normal permeability within 2 weeks after SCI. However, reactive astrocytosis also occurs and initiates the formation of an astroglial scar bordering the lesion. In sub-acute phase, the development of cystic cavities takes place at the lesion site besides distal sites of infarct [33]. The fluid-filled cystic cavity named “post-traumatic syringomyelia” surrounding the glial scar is more common in rat models, but in humans only 10% of post-traumatic syringomyelia and formation of discontinuous small cysts or “marshy cord syndrome” progress in chronic phase after SCI [33].

The chronic phase of SCI is a slow and continuous process that aims at stabilizing the injury. Arriving reactive astrocytes together with surrounding connective tissue border a glial scar, a physical hindrance for regrowing axons [42]. The glial scar continues to develop over 6 months after injury and progresses to more extensive glial scarring in compressive SCI rat models [36]. A suppressing environment of the glial scar arrests axon development and myelination of demyelinated axons pinpointing the critical role of the lesion environment in CNS regenerative failure (Figure-6) [3]. Moreover, the glial scar has been proved not only a mechanical obstruction but also add

to regeneration failure by synthesizing various inhibitory molecules [43, 44]. Most of the cells like macrophages, microglia cells, oligodendrocytes precursors, and astrocytes contributing to the glial scar formation appear to be inhibitory to the regenerating axons [3].

Neurite growth inhibitory molecules delivered by oligodendrocytes includes myelin associated glycoproteins (MAG) and tenascin-R. The tenascin-R appears to arrest axonal growth *in vitro* by interfacing with cell surface F3/11 molecules [45] and limit functional recovery after SCI *in vivo* [46]. The mechanisms of MAG-dependent inhibition are not yet completely comprehended [3]. A second significant kind of CNS inhibitory molecules is proteoglycans, which are produced generally by reactive astrocytes and are exceedingly up-regulated inside the scar. Various reviews have officially shown their neurite outgrowth inhibitory properties with chondroitin sulfate proteoglycans as the fundamental players (Figure-6) [3]. Eventually, after the glial scarring is finished SCI enters the chronic phase. The Subpial rim of spared axons is the common characteristic feature of chronic compressive SCI in both animals and humans. Wallerian degeneration of damaged axons is a slow process that continues for up to 1-2 years following injury [36]. In rat models, there is a limited regeneration of axonal sprouting observed from 3 weeks to 8 months in the CST and from 3 to 8 months in the reticulospinal tract [41]. However, in animals and human cases of SCI, the endogenous regenerative capacity of the SC is deficient to restore normal tissue and function and SCI results in permanent dysfunction and disability.

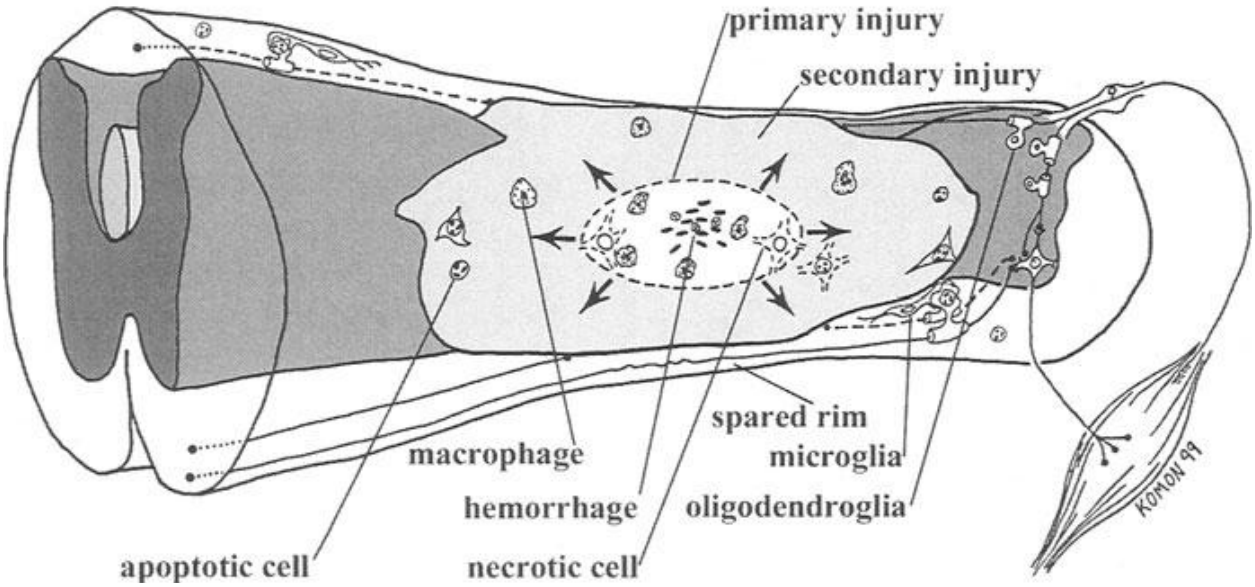

Figure-5: SC contusion injury model. After damage (primary injury) to the SC causes hemorrhagic necrosis which amplifies overtime due to apoptosis of cells, a characteristic event of secondary injury process. A saved rim of tissue at the periphery is still present after the primary injury. Recruitment of immune mediators takes place immediately after injury. Within few days more numbers of macrophages are available at the lesion site, and activated microglia proceed by the edges of the lesion rostrocaudally among the fiber tracts having wallerian degeneration. Microglia takes part in the apoptosis of oligodendrocytes, which additionally happens by the degenerating tracts, expanding into locales far inaccessible from the lesion. The association between the caudal and rostral destinations of the injury is denervated from loss of descending and ascending axons beginning reorganization in those systems [20].

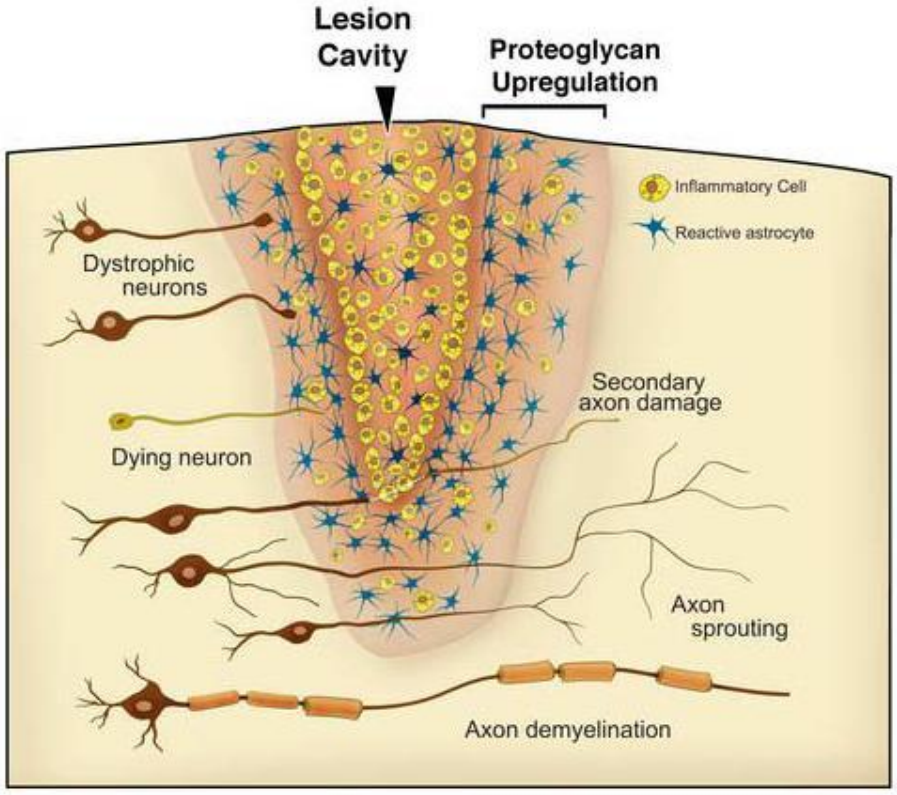

Figure-6: Glial scaring. The lesion cavity of the SCI grows as inflammatory cells associate with the encompassing reactive astrocytes and other reactive glial cells. This area of glial scarring is connected with up-regulation of inhibitory extracellular molecules, for example, proteoglycans, that are dispersed in an expanding concentration from the lesion penumbra to the lesion center. This extreme inflammatory reaction prompts to a course of secondary damage to axons at first saved from direct injury, and demyelination of contiguous axons that are not promptly re-myelinated by adult oligodendrocytes and precursor cells. The concentration of inhibitory molecules up-regulated in the zones of extreme inflammation gives a domain that is non permissive for regeneration, and dystrophic neurons build up the traditionally portrayed sterile end-balls with clubbed endings that are normal for failed endeavors at regeneration [43].

1.3.3. Potential targets for SCI repair

To minimize damage and promote repair and regeneration of SC after injury several targets for intervention have been proposed based on pathophysiology of SCI (Figure-7) [47, 48]

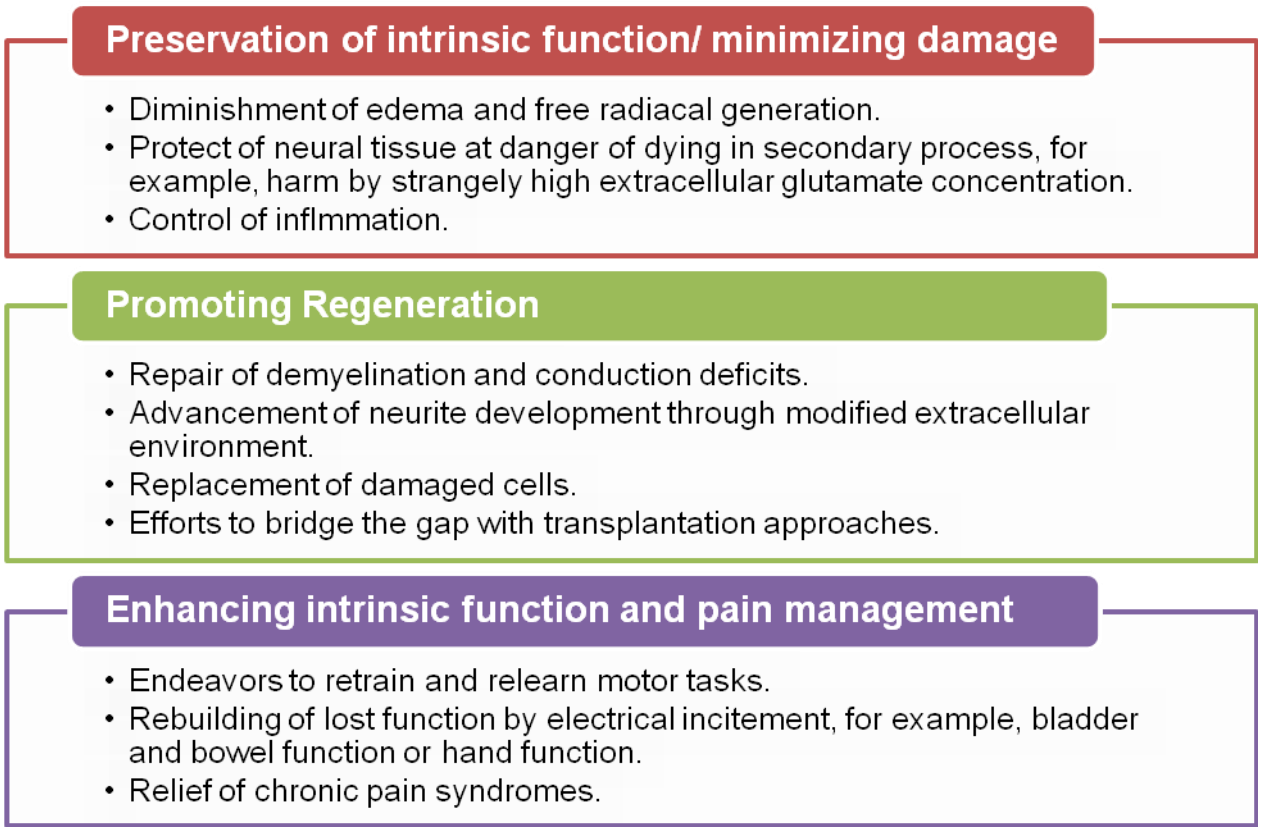

Figure- 7: Targets for treatment after SCI [49].

1.4. Current available treatments and clinical trialss

There are limited clinical treatment options available for SCI repair. During cervical SCI, decompressive surgical intervention shows clinical benefits [50, 51]. Within 30 days after SCI, rehabilitation therapy improves patient's health condition, but the optimal time and intensity of rehabilitation has not been studied yet [52]. So far there were no effective treatment options available for neuroprotection, regeneration and functional recovery after SCI. Since two decades a large number of clinical trials have been conducted with pharmacological agents for SCI treatment [50] [53] [3]. However none of them have successfully translated into current clinical practice (Table-2).

Methylprednisolone, a synthetic glucocorticoid, and its related compounds have been used in several clinical trials [3]. National Acute Spinal Cord Injury Studies (NASCIS) conducted 3 clinical trials with methylprednisolone [54] and in the first trial (Phase I/II) no significant difference in functional outcome was observed between high dose and low dose after six months [55] or one year [56] of treatment (Table-2). In a phase-II study methylprednisolone administered within 8 hours of injury showed significant functional improvement [57] and in a Phase-III study the delivery of methylprednisolone with onset of administration within 8 hours and delivery up to 48 hours in the patients showed significant improvement in motor recovery [58]. Even though methylprednisolone shows positive outcome in SCI repair the serious adverse events like pneumonia and severe sepsis after administration make its use controversial [59] [54]. Different pharmacological treatments showed promising results in pre-clinical studies but poor results in clinical trials (Table-2). In a Phase-II clinical trial conducted by Bracken *et al.* Naloxone (an opiate receptor antagonist) administered along with

methylprednisolone showed no motor scores improvement in patients [57]. Pitts *et al.* carried clinical trials by administering thyrotropin releasing hormone (TRH) which was found in the terminals of the dorsal, lateral, and ventral horns of the SC and facilitates neuronal excitability in animal models [60], demonstrated significant improvement in motor and sensory scores after the treatment in patients [61]. However this study was limited by its small sample size, patients died to subsequent follow-up and the 12-month data were not highly informative. A large multicenter clinical trials study is now required to prove TRH can show functional recovery after SCI.

Dalfampridine or fampridine-S, potassium channel blockers currently approved for the treatment of multiple sclerosis demonstrated beneficial effect on spasticity in phase-II trials [62] but failed in phase-III trials [63]. Riluzole and minocycline which reduces the secondary injury successfully cleared the phase-I trial and are undergoing phase-II/III trials [50]. Other molecules like magnesium chloride in combination with poly(ethylene glycol) (PEG) [50] [64] and basic fibroblast growth factor (FGF2) [50] [3] after SCI have also been investigated in respective individual trials but the reports are not yet published.

Table-2: Clinical Trials of Pharmacological Treatments for SCI

Treatment	Mode of action	Trial Type	Year of Trial	Outcome
Methylprednisolone (MP) [55-57]	attenuation of secondary injury	Phase I/II	1979-1981 (NASCIS I)	no differences between low and high doses
		Phase II	1985-1990 (NASCIS II)	improvements only when given <8 h post-injury
		Phase II	(NASCIS III)	improvements for 48 h MP delivery given <8 h post-injury; adverse events with 48 h delivery
Naloxone [57]	opiate antagonist	Phase II	(published 1990)	no improvements in motor score
Thyrotropin-releasing hormone (TRH) [61]	facilitates neuronal excitability	Phase I/II	1986-1988	improvement only in patients with incomplete SCI
4-aminopyridine (Dalfampridine, fampridine-SR) [62, 63]	potassium channel blocker (improved conduction in demyelinated axons)	Phase II	2000-2001	no adverse events; improvement in secondary outcomes
		Phase III	2002-2004 2002-2004	no adverse events, no improvement in primary outcomes
Riluzole[50]	neuroprotective drug that blocks	Phase I	2010-2011	no adverse events, significant improvements

	glutamatergic neurotransmission			in motor score
		Phase I/III	2014-present	ongoing
Minocycline [50]	inhibition of apoptosis and inflammation	Phase I/II	2004-2010	no adverse events, no improvements in functional outcomes
		Phase III	2013-present	ongoing
AC105 (MgCl2 + PEG) [50]	inhibition of excitotoxicity, lipid peroxidation, and BSCB permeability	Phase I	2009	no adverse events
		Phase II	2013-2015	not yet reported
SUN13837 (FGF2) [3, 50]	inhibition of excitotoxicity, promotion of neuron survival	Phase II	2012-2015	not yet reported

Researchers continue working on different strategies to develop a successful and safe treatment for SCI. Among many strategies neurotrophic factors [65], mesenchymal stem cells [66, 67] and biomaterial scaffolds [68] demonstrated neuroregeneration and functional recovery after SCI in preclinical studies.

2. Neurotrophins role in SCI repair.

Neurotrophic factors play an important role in increasing survival of neurons, axonal sprouting, axonal growth and/or recovery of function after SCI [65]. Moreover, SCI itself increases expression of neurotrophins [69]. Widenfalk *et al.* studied the mRNA expression of different neurotrophins after weight drop SCI model in rats [69]. They observed that mRNA expression of neurotrophins like nerve growth factor (NGF) and glial derived neurotrophic factor (GDNF) was up-regulated in meningeal cells and schwann cells migrated near the lesion; GDNF, GNDF family receptor alpha-1 (GFR α-1) and Tropomyosin receptor kinase B (TrkB) expression was increased in astrocytes and the levels of brain derived neurotrophic factor (BDNF) and p75 neurotrophin receptor expression was increased in neurons [69]. However, this increased expression is not enough to facilitate axonal regeneration. Traditionally, neurotrophic factors have been administered to the injured SC by direct injection [70], placement of growth-factor saturated gel foam, [71] but long-term, localized, controlled dose of neurotrophic factor delivery cannot be achieved by these methods. The route and the concentration of neurotrophic factor administration influence the response after SCI. Several neurotrophic factors cause a chemotropic effect, leading the growth of axonal populations to the areas of highest growth factor concentration. Thus, to promote axonal growth specifically into a site of SCI and functional recovery, initially, the highest concentrations of growth factors should be delivered particularly to the injury site [72]. Promotion of neuronal survival by the administration of exogenous neurotrophic factors to counteract secondary injury is a promising approach to treatment of SCI and

members of the neurotrophin family particularly BDNF have been shown to have potential therapeutic effects on the prevention of neural damage after SCI.

2.1. BDNF for SCI treatment.

BDNF and TrkB receptors are widely expressed in adult brain and SC [73]. BDNF plays an important role in neuroplasticity after SCI [74] and some studies reported that BDNF increases survival of neurons, axonal sprouting, and/or functional recovery when delivered intrathecally [75-77], intraspinally via viral vectors [78] or via transplantation of stem cells genetically manipulated to deliver BDNF [79, 80]. Moreover, BDNF possess axonal growth promoting actions on a variety of neurons, including dorsal root ganglion cells [81], that also extend axons in the SC. Beneficial effects of BDNF delivery in SCI animal models are summarized in Table-3. BDNF interceded neuroprotective result after SCI have been accounted following cervical injuries of rubrospinal projections. The greater part of axotomized rubrospinal neurons generally encounter atrophy on the first weeks after injury, with a few reports detecting a neuronal loss of around 50% in the red nucleus at week five after a cervical injury [82]. Kobayashi *et al.* observed the protection of considerable number of rubrospinal neurons when BDNF was infused in the region of the red nucleus the second week after cervical injury [83]. Similarly Novikova *et al.* also observed the same effect after infusion of BDNF into the lumbar subarachnoid space after cervical injury [82] however the rescuing started at a delay of BDNF infusion 5 weeks post injury. Intraparenchymal use of BDNF for 7 days completely prevented from atrophy or apoptosis of the axotomized upper motor neurons present at CST [84]. Similarly grafting of BDNF secreting human mesenchymal stem cells (MSCs) into a thoracic (T9) dorsal transection site effectively

decreases an obvious loss of axotomized CST neurons [85]. BDNF has extraordinary potential for increasing the number of existing and healthy neurons after axotomy, however the dependability and the level of its activity relies on the individual neuronal [74].

BDNF treatment not only protects the neurons but also stimulates the expression of regeneration-associated genes, such as growth-associated protein 43 (GAP-43) and T-alpha-1-tubulin [83, 86]. In addition, BDNF/TrkB signaling (Figure-8) through ERK pathway increases the levels of second messenger molecule cyclic AMP [87, 88] which may enable axons to overcome myelin inhibition [89], and is thought to partly mediate the axonal growth-promoting effects of conditioning lesions [90]. Adaptive neuronal re-arrangements, collectively called plasticity, can help to rewire neuronal networks at various physiological levels after damage to the SC thereby restoring function [91]. BDNF plays an important role in promoting neurite outgrowth and is also useful for increasing axonal plasticity. Hiebert *et al.* reported that axotomized CST neurons sprouted within healthy tissue rostral to the injury after infusion of BDNF into motor cortex neurons [92]. However, the CST neurons did not grow into a peripheral nerve graft that bridged a thoracic lesion. Following the same treatment, Vavrek *et al.* confirmed this result and additionally reported an increase in contacts between CST fibers and propriospinal interneurons above the lesion [93]. Similarly Fouad *et al.* observed the increasing CST sprouting above a lesion when TrkB agonist was delivered intrathecally [94]. Implantation of gelfoam soaked with BDNF into a cervical lesion cavity was reported to increase the regeneration of axons of several origins into a peripheral nerve graft [77] or into a fetal tissue transplant [75]. The most reactive axons were

serotonergic, noradrenergic, rubrospinal and reticulospinal fibers. There are reports of reasonably increased CST regeneration succeeding BDNF delivery to the lesion site following a thoracic (T3) clip compression model where the sensorimotor cortex was retrogradely labeled from below the lesion [95]. Whether these effects of BDNF are due to direct effects on CST axon growth or reduced axon retraction from the lesion site remains to be determined.

Axonal remyelination is one of the challenges during the repair of injured SC. This applies not only to regenerating fibers [96], but also to spared projections that have been demyelinated throughout the course of secondary injury [97]. McTigue *et al.* observed an increase of oligodendrocyte and Schwann cell proliferation and promotion of axonal myelination after implantation of BDNF producing fibroblasts graft into a contusion injury site [98]. Intrathecal BDNF infusion after 3 days of contusion SCI in rats influence myelination by suppressing delayed oligodendroglial cell death and up-regulating myelin basic protein (MBP) expression [99]. Likewise Girard *et al.* observed increased proliferation and differentiation of oligodendrocytes associated with accelerated and enhanced functional recovery after implantation of BDNF expressing Schwann cells grafts into demyelinated nude mice [100].

An important factor in the pathophysiological events that follow an insult to the SC is secondary injury. As discussed earlier, secondary injury is constituted of a multitude of processes, among them inflammation and the generation and release of reactive oxygen species [101]. Although BDNF is not well known for influencing these factors, there is also some evidence for potent anti-oxidant effects. In particular, pre- and post-injury applications of BDNF can attenuate the up-regulation of nitric oxide

synthase thereby reducing the production of potentially harmful nitric oxide levels [102]. Additionally, a reduction of BSCB damage, edema and overall cell injury was also observed in this study. Likewise, local application of BDNF to a dorsal spinal cord transection site reduced lipid peroxidation around the lesion at 48 hours post injury [103]. BDNF can modulate inflammation on a cellular, cytokine and transcriptional level by suppressing TNF-alpha expression while increasing expression of IL-10 mRNA [104]. Wong et al observed that Pro-BDNF had a negative effect in regulating inflammation by reducing infiltration of ED-1 positive macrophages into the injured SC [105].

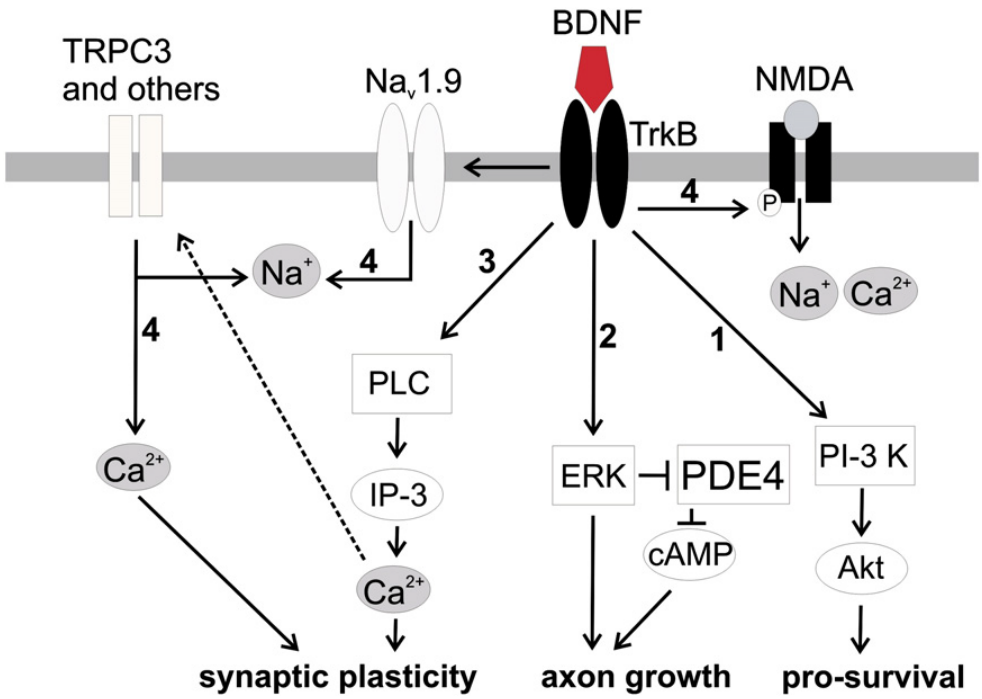

Figure-8: BDNF-induced TrkB signaling, influence synaptic plasticity, axon growth, survival and intracellular cation balance via four main intracellular pathways. Via PI-3 kinase and Akt (1), pro-survival signals dominate, via ERK and an ERK/PDE4-dependent increase in cAMP, axonal growth is promoted (2). Via PLC and IP-3, calcium is released from intracellular stores, resulting in numerous effects including the promotion of synaptic plasticity (3). Elevation of intracellular calcium in turn may open calcium-dependent channels such as TRPC channels, thereby further elevating the intracellular calcium concentration as well as the sodium concentration (4). TrkB activation may also result in the phosphorylation of NMDA channels, leading to calcium and sodium influx (4). It may also interact with the sodium channel Nav1.9, leading to rapid sodium influx (4). The BDNF-induced increase in intracellular sodium levels contributes to the excitatory effects of BDNF. Akt=serine–threonine-specific protein kinase B, BDNF=brain-derived neurotrophic factor, cAMP=cyclic adenosine-monophosphate, ERK=extracellular signal regulated kinase, IP-3 = inositol-3-phosphate, NMDA = N-methyl-D-aspartate, PDE 4 = phosphodiesterase 4, PI-3K = phosphatidylinositol-3 kinase, PLC = phospholipase C, TrkB = tropomyosin-related kinase B, TRPC channel = transient receptor potential cation channel [74].

Table-3: Beneficial effects of BDNF application *in vivo* during SCI by various delivery modes and the time of delivery [74].

Function	Neuronal structure	Mode of delivery	Delivery time
Neuroprotection	Rubrospinal tract, axotomized cervically	Infusion to red nucleus	For 7–14 days [83]
	Rubrospinal tract, axotomized cervically	Sub-arachnoidal infusion into cisterna magna or lumbar cord	Immediately post-injury for 5–8 weeks [82]
	upper motor neuron axotomized at internal capsule	Local infusion	For 7 days [84]
	Corticospinal tract, axotomized thoracically	Graft of BDNF producing cells	Immediately post-injury[85]
Regeneration	Rubrospinal tract, axotomized cervically	Infusion to red nucleus	For 7–14 days[83]
	Corticospinal tract axotomized thoracically	Infusion to motor cortex	Immediately post-injury for 14 days [92]
	Various, axotomized cervically	Gel-foam soaked with BDNF	Immediately post-injury [77]
	Corticospinal tract, rubrospinal tract	BDNF administered to lesion site	Immediately post-injury [75]

	axotomized cervically or thoracically, graft		
	Rubrospinal tract, corticospinal tract axotomized thoracically	Intramedullary infusion	Immediately post-injury for 14 days [95]
Neural plasticity	Corticospinal tract axotomized thoracically	Infusion into motor cortex	Immediately post-injury for 14 days [92]
	Corticospinal tract axotomized cervically	intrathecal infusion of TrkB agonist	Immediately post-injury for 4 weeks [94]
Promotion of myelination	In the SC lesion site	implantation of BDNF producing fibroblasts graft	Immediately post-injury for 10 weeks [98]
	Near SC lesion site	intrathecal infusion of BDNF	3 days after surgery for 2 weeks [99]
Reduction of secondary injury events like nitric oxide levels, BSCB damage, edema.	Near SC lesion site	BDNF applied topically by repeated doses	30 mins before surgery and 30 min after injury [102]
	dorsal SC transection	local application of BDNF	Immediately post-injury for 48hrs [103]

The mode and timing of BDNF delivery also plays a key role for therapeutic effect after injury. *Oudega et al.* reported that continuous infusion of BDNF through osmotic pumps promotes the regeneration of ascending sensory axons in the adult rats but fewer axons were able to grow farther into the spinal tissue. Even though high dose of BDNF were administered, the poor diffusion capacity of BDNF in CNS limited the therapeutic effect [106]. Following a mid thoracic contusion SC lesion, continuous high concentrations of BDNF, infused over 4 weeks into the intrathecal space, enhanced the growth of cholinergic motor axons at the site and stimulated functional improvement [107]. Unfortunately these behavioral effects were no longer present when the infusion pump was removed and the effects reversed after 2 weeks. This was due to loss of BDNF activity, decreased responsiveness of the receptors and changes due to plasticity of spinal cord circuits. Furthermore, reports have shown that the biological efficacy of BDNF may be diminished by as much as 60% over 2 to 3 weeks when stored at body temperature [108].

To achieve local and biologically active BDNF delivery, Liu *et al.* implanted the fibroblasts genetically altered to produce BDNF into the cervical hemisection lesion site of SC [109]. They promoted renewal of injured rubrospinal tract axons into and distal to the lesion site, terminating in the SC grey matter and there was increased use of the affected forelimb in these grafted animals within 1-2 months [109]. However, the limitation for this delivery methodology (i.e. the expression of BDNF through gene modification of cells) is uncontrollable BDNF delivery due to uncontrolled grafted cell survival. Moreover, previous studies of BDNF infusion into intrathecal space of SC failed to prove that the BDNF had an effect on sensory axons [110, 111], contrary to Liu *et al.*

study. Additional studies are required to investigate whether mechanisms other than regeneration of rubrospinal axons (i.e. BDNF effects on local neural plasticity) may generate functional recovery. The development of effective methods to deliver BDNF in a controlled manner to the SC therapy is a necessity for their translation to clinical use. Moreover, this highlights the importance of achieving optimum-dose, localized, controlled and biologically active BDNF delivery in order to elicit significant pharmacological effect for SCI repair.

3. Cell therapy in SCI repair.

Many preclinical studies demonstrated the beneficial use of exogenous cells in SCI repair and even translated to clinical trials (Table-4). However none of these studies have gone beyond phase-II clinical trials. Two clinical trials with olfactory ensheathing cells (OECs), a specialized glial cell that assists in the growth of axons of the olfactory sensory neurons, demonstrated different results. Patients reported reduced sensory function (light touch and pin prick pain) in one study [112] and in two other studies OECs were proven safe but not efficient [113, 114]**.** Similarly, anti-inflammatory macrophages shown to be neuroprotective, demonstrated safety in phase-1 trials [115], nevertheless they were not effective in phase-II trials [116]. Human neural stem cells isolated from fetal CNS tissue [117] and purified and transplanted in the form of neurospheres were tested recently in a phase-I trial and results have not been published yet [3]. Likewise Schwann cells, which play a crucial role in endogenous repair and promote axonal regeneration and myelinate axons, demonstrated to be safe in phase-I trial [118, 119] and progressed to phase-II trials [3]. As a source of exogenous cells, embryonic stem cells also have shown promising results in preclinical

studies and a few clinical trials have also started [53]. For instance, the well-publicized Geron trial was started with embryonic stem cell-derived oligodendrocyte precursor cells and terminated (due to financial issues) without publication of any result [53]. However a second phase –I/IIa trial has begun in cervical SCI patients after acquisition by a company called Asterias Biotherapeutics [53]. However, whatever the results, ethical considerations significantly limit the use of embryonic stem cells as a therapy. In order to overcome the ethical concerns related to embryonic stem cells, induced pluripotent stem (iPS) cells has been investigated as candidates for SCI research [3]. iPS demonstrated to have many of the characteristics of embryonic stem cells, such as tri-lineage differentiation and generation of viable chimeras [120]. Moreover, they allow autologous transplantations of pluripotent cells. However, iPS and embryonic stem cells also share similar disadvantages such as the risk of teratoma formation [120, 121]. An increasing number of experiments are being conducted with iPS cells in SCI models. Recently, two experiments from the Okano lab demonstrated that NSCs derived from iPS cells implanted in immunodeficient mice subjected to a SC contusion, differentiated into in neural lineage, functional recovery and no tumor formation [122, 123]. Nevertheless, additional studies are required in order to confirm the safety and feasibility of iPS cells before clinical trials can be initiated.

Table-4: Clinical Trials of cell treatments for SCI

Treatment	Mode of action	Trial Type	Year of Trial	Outcome
Olfactory ensheathing cells (OECs) [112-114]	promotion of axonal regeneration	Phase I/IIa	2004 (published 2005,2008)	no adverse events, no significant improvements
		Phase I	2001-2003	1/7 patients with reduced sensory function
		Phase I	2008-2010	no adverse events
Activated (M2) macrophages [115, 116]	growth factor production and inhibition of excitotoxicity	Phase I	2000-2002	no adverse events
		Phase II	2003-2009	no adverse events, no significant improvements
Human central nervous system stem cells (huCNS-SC®) [3]	tissue repair	Phase I/II	2011-2015	not yet reported
		Observational	2012-present	ongoing
		Phase II	2014-present	ongoing
Schwann cells (SCs) [118, 119]	promotion of axonal regeneration	Phase I	(published 2008)	no adverse events
		Phase I	2012-present	ongoing
Embryonic stem cell-derived oligodendrocyteprecursor cells (OPCs) [53]	tissue repair and remyelination	Phase I	2010-2013	prematurely stopped; not yet reported
		Phase I/IIa	2015-present	ongoing

3.1. Mesenchymal stem cell (MSCs) in SCI repair.

The major challenges faced by cell-based therapies are survival, appropriate cell differentiation and host immune rejection. The mode of implantation in the SCI models and tracking of implanted cells is also to be considered [124]. MSCs are a promising source of cells which are isolated from different origins including bone marrow, adipose tissue, dental tissues, placenta, fetal membrane and umbilical cord [66]. They are easy to isolate and posses self-renewal properties, with a minimal or no immunoreactivity and graft-versus-host reaction of transplanted allogeneic MSCs [67]. MSCs have a high potential of multilineage differentiation with the capacity to differentiate into several distinct cell lineages including osteogenic, chondrogenic, adipogenic, and stromal cells [125]. Moreover, recent studies demonstrated that MSCs can differentiate to neural/neuronal phenotype when treated with specific cytokines in culture [126, 127]. Recently, MSCs have been advocated as a promising cell source in the treatment of CNS disorders [128]. Preclinical studies indicate that MSCs maintain their beneficial effects via immunomodulatory and paracrine mechanisms [129]. MSCs secrete wide range of tissue repair factors like growth factors, which modulate the lesion environment and promote angiogenesis, decrease inflammation, and enhance tissue repair [130]. MSCs are mainly used for regeneration of axons, prevention of apoptosis and replacement of lost cells, to facilitate the remyelination of spared axons and functional recovery after SCI [67]. Among different sources of MSCs bone marrow and dental derived stem cells demonstrated promising results in the treatment of SCI.

3.1.1. Bone marrow derived mesenchymal stem cells (BmMSCs) in SCI repair.

Among many uses BmMSCs demonstrate significant anti-inflammatory and anti-apoptotic properties in experimental brain ischemia and in animals with brain or SCI [131]. Many preclinical studies have reported that BmMSCs possess immunosuppressive properties [132, 133]. Reduced acute inflammatory response in the SCI was reported after their injection, leading to a minimized cavity formation, and reduced astrocyte and microglia/macrophage reactivity [134-136]. Abrams *et al.* observed neuronal protection and cellular preservation through the reduction of injury-induced sensitivity to mechanical trauma [136]. They also observed a beneficial effect on hind limb sensorimotor function via BmMSCs through the attenuation of astrocyte reactivity and chronic microglia/macrophage activation. This was confirmed by Hofstetter *et al.* upon transplanting BmMSCs after SCI that led to the attenuation of the acute inflammation response and promoted functional recovery in animal models [137]. These findings demonstrate that BmMSCs can serve as attenuators of the inflammatory response in SCI. Recent studies reported that BmMSCs, following transplantation enhance axonal elongation across the lesioned SC through secreting a variety of growth factors like BDNF, NGF, VEGF [138]. Similarly, Novikova et al. observed that differentiated BmMSCs provided neuroprotection for axotomized rubrospinal neurons and increased the density of rubrospinal axons in the injury site [139]. They suggested that BmMSCs induced along the Schwann cell lineage increased expression of trophic factors and had neuroprotective and growth promoting effects after SCI. Autologous BmMSCs implantation in a primate SCI model elicited neurogenesis, and illicit a functional recovery as well as normal sensory responses 3 months after

transplantation[140]. Similarly Zurita *et al.* also observed progressive functional recovery 3 months after SCI in paraplegic pigs injected with autologous BmMSCs [141]. The acute pathological improvements after BmMSCs implantation in SCI models are summarized in Table-5.

Table-5: Overview of effects of BMSCs after SCI.

MSC source	SCI model used	Transplant	Main pathological features improved/repaired	Limitations/recommendations/ conclusions
Human	Cervical hemi section in female rats	gelfoam immersed in growth medium containing a suspension of MSC (5×10^5) was implanted into the cavity, followed by injection of another 5–10 µl of undifferentiated MSCs (5×10^4/µl) suspended in growth medium onto the gelfoam	Axonal growth, partial recovery of function	Differences in donor or lot-lot efficacy of MSCs [134]
Human	Contusion model in female rats	Mild injury: single injection (10 µl) of 5×10^5 undifferentiated MSCs in epicenter of the injury Severe injury: single injection (10 µl) of 5×10^5 undifferentiated MSCs in epicenter of the injury Moderate injury: three injections of 1×10^6 undifferentiated MSCs; 10 µl into epicenter, 5 µl each at rostral and caudal extent of	Axonal growth, significant behavioral recovery	Survival of BmMSCs grafts for longer duration [135]

		injury (2 µl/min)		
Rat	Contusion model in female rats	Undifferentiated MSCs suspensions were delivered at a rate of 1 µL/minute amounting to an estimated 3×10^5 cells at the lesion site	No allodynia, anti-inflammatory, increase in white matter volume and decrease in cyst size, sensorimotor enhancements	Survival of MSCs [136]
Rat	Contusion model in female rats	5 µL of injection media containing 3×10^5 neuronal differentiated MSCs was delivered at the injured center	MSC form bundles bridging the lesion epicenter, functional recovery	Neuron-like MSC lacked voltage-gated ion channels for generation of action potentials [137]
Rat	Cervical hemi section in female rats	1.5 µL of injection media containing 1.2×10^5 Schwann cell differentiated MSCs was delivered at the o the lesion site	Extensive in-growth of serotonin-positive raphaespinal axons and calcitonin gene-related peptide-positive dorsal root sensory axons, attenuation of astroglial and microglial activity	Production of trophic factors support neuronal survival and axonal regeneration [139]
Monkey	Contusive injury in male rhesus	0.2 mL of PBS containing 2.5×10^6 neuronal differentiated MSCs injected directly into the	*De novo* neurogenesis and functional recovery	Synergetic effects of MSCs implantation and locally

	monkeys	injured sites	in rhesus monkeys	delivered neurotrophic factors in rhesus SCI models [140]
Pig	Compression by application of two surgical Heifetz's clips for 30 min in female pigs	0.1 mL of plasma containing 15 × 10^6 undifferentiated autologous MSC injected into the centromedullary cavity of injured sites	Improvement in somatosensory-evoked potentials, functional recovery in pigs	Possible utility of BmMSCs transplantation in humans suffering from chronic paraplegia [141]

A first clinical study conducted to evaluate the safety of autologous BmMSCs implantation in SCI patients demonstrated that they are safe and provided a modest functional improvement [142]. A Phase-II clinical trial is on going to test the efficacy of the BmMSCs. In another clinical trial BmMSCs showed neuroprotective effects and exhibited safety in several phase-I trials [143-145]. Phase II/III studies are ongoing, however other studies have been suspended or are of unknown status [3] (Table-6). The use of BmMSCs in SCI includes certain limitations like low survival of grafted cells, inter-donor variability in efficacy and immunomodulatory potency that are reflected in variable clinical outcomes [134].

Table-6: Clinical Trials of BmMSCs treatments for SCI

Treatment	Mode of action	Trial Type	Year of Trial	Outcome
Bone marrow mesenchymal stem cells (bmMSCs) [3]	secretion of anti-inflammatory cytokines and neurotrophic factors	Phase I	(published 2006)	no adverse events, no significant improvements
		Phase I/II	2001-2003	no adverse events, no long-term improvements
		Phase I	2010-2012	no adverse events
		Phase II/III	2008-present	ongoing
		Phase II	2012-present	ongoing

3.1.2. Marrow isolated adult multi-lineage inducible cells (MIAMI cells)

One of the limitations of human MSCs isolated from bone marrow is varying differentiation properties due to heterogeneous population of mixed cells [146, 147]. To overcome this limitation, Marrow isolated adult multi-lineage inducible cells (MIAMI cells), a homogenous sub population of MSCs expressing pluripotency markers (Oct4, Sox2, Nanog, SSEA4) are interesting candidates [148]. MIAMI cells secrete a range of cytokines that stimulate angiogenesis (MCP-1, IL-8, VEGF), have anti inflammatory properties (HSP27, TSG-6), are involved in neuroprotection or other cell survival (GDNF, HGF, Fractalkine, HSP27, VEGF), and in progenitor cell migration (Gro, MCP-1, IL-8). All have a potential role in the treatment of neurodegenerative disorders [149,

150]. MIAMI cells possess a wide range of differentiation potential *in vitro*. They are capable of differentiating into some phenotypes from 3 embryonic germ layers (ectoderm, endoderm and mesoderm) on a fibronectin substrate and can be directed towards a neural-like phenotype. It was observed that after a neuronal induction protocol using NGF, BDNF and Neurotrophin-3 (NT-3), MIAMI cells expressed neuronal markers and presented some electrophysiological characteristics similar to those observed in mature neurons [151]. Moreover, when pre-treated with epidermal growth factor (EGF), bFGF the cells first acquired a neural phenotype and better responded to the neuronal induction protocol [127]. In animal models of neurological disorders like Parkinson's disease [152, 153] and cerebral ischemia [154], rats receiving an intra-cerebral transplant of MIAMI cells transported by microcarriers with a biomimetic surface of extracellular matrix proteins and delivering or not a growth factor, showed functional recovery. MIAMI cells alone or conveyed by microcarriers have however never been tested for SCI repair.

3.2. Dental stem cells on SCI repair.

Recently, dental stem cells (DSCs) gathered attention for the treatment of SCI because of their neural crest origin, high accessibility, proliferation rate and possibility of autologous transplantation [155]. Gronthos *et al.* first reported that adult human dental tissue contains MSCs, which are a highly proliferative cell population and share many features with BmMSCs in terms of gene expression as well as the ability to regenerate tissues [156, 157].

DSCs not only differentiate into adipogenic, osteogenic, and chondrogenic lineage, but also express some neural stem cell and epithelial markers [158]. DSCs

secrete a variety of cytokines that have autocrine and paracrine action, and can have a profound effect on facilitating injury repair [159]. For example DSCs secrete high levels of hepatocyte growth factor (HGF) [160], which exerts a neurotrophic effect on CNS neurons, particularly spinal motor neurons. It has a chemo-attractant effect on spinal motor axons [161, 162] and enhances endogenous repair and promotes functional recovery after SCI [163]. Studies reported that DSCs secrete high levels of angiogenic growth factors VEGF [164] which are crucial for endogenous regenerative response after injury [165, 166]. Moreover recent studies highlighted that VEGF also plays an important role in neurogenesis and neuroprotective action [167, 168]. DSCs not only secret growth factors but also secrete chemokines like stromal cell-derived factor-1 (SDF-1) which is responsible for cell migration and endogenous repair after SCI [169]. Recent studies reported that SDF-1 plays an important role in repair and regeneration via MSCs recruitment [170] as well as neurite outgrowth promotion [171, 172]. Moreover SDF-1 can increase expression of BDNF and GDNF [173]. DSCs also secrete platelet-derived growth factor (PDGF), a key cytokine that can induce mitogenic, differentiation and chemotactic responses in oligodendrocytes and their progenitors [174-177]. Moreover DSCs secrete a wide range of pro- and anti inflammatory factors [159].

Recent studies reported that DSCs express NTs and their receptors for example NT-3 [178, 179], GDNF [180] and demonstrated neuroprotection as well as neurito-/axogenesis *in vitro* and *in vivo* [181]. These actions were mediated through paracrine mechanisms which are superior (in terms of neuroprotection, neuritogenesis, and secretome) to BmMSCs and adipose derived stem cells (ADSCs) [182]. Nosart *et*

al. demonstrated that DSCs derived NGF, BDNF, and GDNF could promote the survival of sensory and dopaminergic neuron through trophic support and save the motor neurons after SCI [183, 184]. Similarly Sakai *et al.* reported that after DSCs transplantation in a completely transected rat SC, the hind limb function was markedly recovered within 8 weeks. Three underlying mechanisms involved were: i) inhibition of SCI-induced apoptosis of neurons, astrocytes, and oligodendrocytes, leading to preservation of neuronal filaments and myelin sheaths; ii) promotion of regeneration of transected axons by paracrine mechanisms; iii) and oligodendrocytes differentiation of DSC [185]. The locomotor recovery after DSCs injection was majorly associated with their neuroregenerative effects including suppression of early inflammatory response, inhibition of neural cell apoptosis and cell replacement by DSC differentiation [186]. The overview of DSCs beneficial effects for SCI repairs strategies are illustrated in Figure-9.

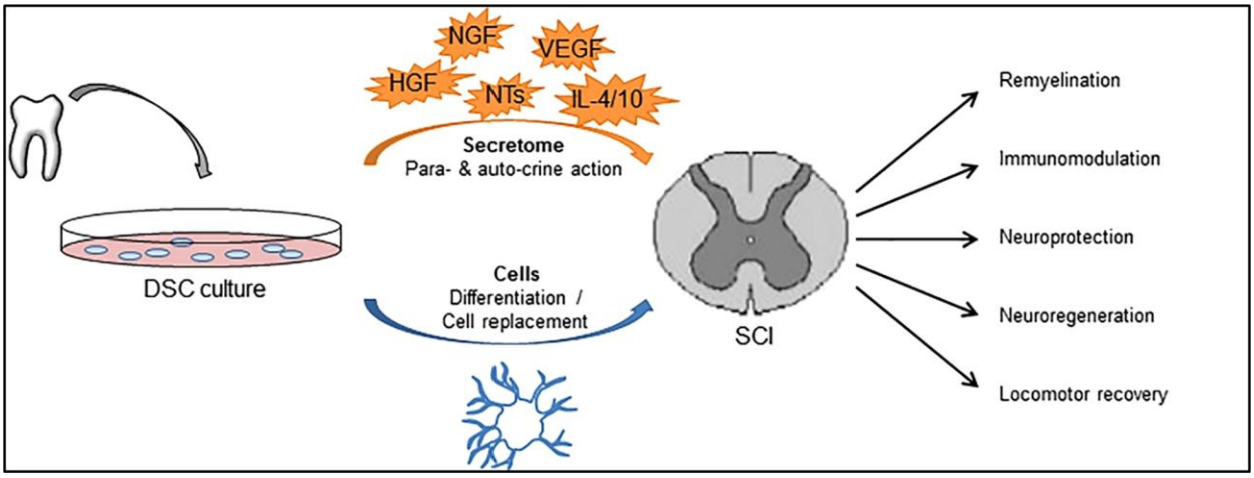

Figure-9: DSCs beneficial effects for SCI repairs strategies. After implantation following SCI, DSC show positive outcome through secretome and cell differentiation [159].

3.2.1. Stem cells from apical papilla (SCAP)

Among different types of DSCs, stem cells of the apical papilla (SCAP) isolated from the immature permanent wisdom teeth are an interesting cell type because they demonstrated higher proliferation rate in culture and utmost regenerative capacity [187]. SCAP demonstrated *in vitro* trigeminal nerve outgrowth regulation and increase peripheral innervations *in vivo* in a BDNF-dependent manner [188]. Studies reported that SCAP conserve their function (immunological properties, viability, proliferation rate, multilineage differentiation potential) after cryopreservation [189]. SCAP are highly adaptable during critical microenvironmental conditions and able to secrete pro- angiogenic factors (angiogenin, IGFBP-3, VEGF) and have therapeutic potentiality in treating neurological disorders [190]. Recently De Berdt *et al.* demonstrated that whole apical papilla can improve the functional recovery and reduce glial reactivity in the hemi section rat models of SCI [191]. Even though SCAP possess a high therapeutic potentiality they are currently less explored in the treatment of SCI.

4. Polymeric scaffolds in SCI repair

While the axons of injured spinal cord have regenerative potential, they are hindered by various pathophysiological changes and complications following an injury. As a remedy to overcome this hurdle, biomaterial engineering for neural repair has received considerable attention in recent years. A biomaterial scaffold synthesized from either a natural or synthetic polymer can help prevent the formation of scar tissue and concentrate neurotrophic growth factors, while promoting axonal regeneration between the two ends of the injured neural tissue [68, 192].The sprouting axons from either damaged or spared pathways may grow through the scaffolds to reconnect with the

neurons on the caudal side of the lesion and reconstitute the circuitry. Many strategies have been investigated to functionalize the scaffolds to create a permissive microenvironment for axonal regeneration.

4.1. Design of biomaterial scaffolds

The scientific community faces a great challenge in delivering an ideal biomaterial to repair an injured spinal cord. The following parameters must be considered when fabricating implantable scaffolds to treat SCI;

i) Biocompatibility- The main criterion of any scaffold for tissue engineering is that it must be biocompatible; cells must adhere, function normally, and migrate onto the surface and finally through the scaffold and start to proliferate before setting down new matrix. After implantation, the scaffold or tissue engineered build must elicit a negligible cell reaction in order to prevent it causing such a severe inflammatory response that it might reduce healing or cause immune rejection [193].

ii) Biomimetic scaffolds- Biomimetic material is a critical element for axonal development since contact-mediated steering of scaffolds advances axonal regeneration. To improve axonal growth, biological molecules of the extracellular matrix, for example, full-length proteins or shorter peptide chains have been conjugated on the surface of scaffolds to mimic a natural extracellular matrix [68].

iii) Biodegradability- Biomaterial scaffolds temporarily affirm axonal regeneration and ought to degrade after some time once that purpose has been met. The degradation rate of scaffolds ought to be controlled in such a way that they are digested by existing

enzymes or simple hydrolysis in the body as the nerves regenerate. The requirement for additional surgery to remove scaffolds may bring about further complications [194].

iv) Mechanical strength- Insistent compressive force and degradation, by means of endogenous cells, act like an outer mechanical stress to scaffolds. Before disintegration scaffolds should support axonal regeneration and also withstand those forces produced from the spine and surrounding muscles. Different strategies, for example, cross linking, and the advancement of the material arrangement have been considered to enhance the mechanical properties of biomaterials [68, 195]. Because of delicate and flexible mechanical properties, like those of the SC, hydrogel has been utilized as a part of SCI and has appeared to bring about minimal mechanical stress to the surrounding tissue, which will be discussed in the following section 4.2.

v) Scaffold morphology- Neural development can be coordinated by means of the assistance of aligned fibers, axially situated pores and channels [196]. A persistent, pours structure that looks like a natural matrix offers a positive domain for axonal regeneration. High porosity is essential for cell attachment, takes into consideration for tissue vascularization, and will enhance implant stability via the incorporation of scaffold and surrounding tissue [197].

4.2. Role of natural polymer-based scaffolds in SCI.

Hydrogels allow cell migration and nutrient exchange with the environment and they mimic the native extra cellular matrix (ECM) and can be constructed with altering elasticity and degradation rates to balance proliferation and differentiation of transplanted cells and/or in growth of host cells [68, 197]. Collagen is an extracellular

matrix protein that forms a gel at physiological temperature and is widely used biomaterial for tissue engineering [198]. Spilker *et al.* demonstrated that the bridging of collagen tubes in completely transected rat SC, allowed growth of axons within the tube lumen and decreased glial scar formation [199]. In a completely transected rabbit SC model, aligned collagen filaments were grafted and the axons regenerated across the distal and proximal ends of the implants and significantly improved locomotor function was seen compared to the control group [200]. Fibrin, has been used extensively as a biopolymer scaffold in tissue engineering [201]. Hydrogel fabricated from fibrin, delivering neurotrophic growth factors, were applied in the treatment of a rat hemisection SCI model and showed improved axonal growth [202]. Fibronectin (FN) an extracellular matrix protein was fabricated into mats and implanted into a sectioned SC (1 mm laterally from the midline and 1 mm ventrally from the surface of the spinal cord) and observed the mats supported myelinated axonal growth [203]. Moreover, FN displays a better engogenous cell attachment than fibrin and showed neuroprotective properties. However, FN does not aggregate into a gel as easily as fibrin. Interestingly, a blend of fibrin and FN achieved *in situ* gel formation and improved cell attachment and proliferation [204]. The injured SC, treated with this mixture, showed improved tissue integration and axonal growth.

Another biocompatible and biodegradable natural polymer, which recently attracts the attention for tissue engineering is a high molecular weight hyaluronic acid (HA) hydrogel [205]. After implantation of this hydrogel in rat SC with dorsal hemi section injury, astrocyte proliferation reduced, thus it helps to attenuate the inflammatory response and gliosis in the surrounding tissue [205]. A similar effect was also observed

by Wei *et al.* after implantation of HA-based hydrogels [206]. Furthermore Park *et al.* have demonstrated that HA based scaffolds containing BDNF promote axonal re-growth following SCI [207]. Agarose, a biocompatible natural polymer, can be fabricated as a scaffold with guidance pores, which are stable under physiological conditions without the need for cross-linking. Agarose scaffolds containing BDNF have been used to treat complete transected spinal cord and have shown significant axonal regeneration [208]. Freeze dried agarose scaffolds with uniaxial channels were implanted into an injured rat SC [209]. Agarose scaffolds were well integrated into the host tissue, and aligned axonal growth was observed in the scaffolds 1 month after surgery [209]. It is very important to process the natural polymers before use as a combined strategy for SCI repair. For instance, some disadvantages in using collagen or other natural polymers reside in their potential to elicit an alteration of cell behavior along with immunogenic response [210] .

4.3. Role of Synthetic polymer-based scaffolds in SCI.

Synthetic polymers are highly useful in biomedical application due to their properties (e.g., porosity, degradation time, and mechanical characteristics), which can be tailored for specific applications. Moreover synthetic polymers are often cheaper than biologic polymers and can be produced in large uniform quantities. Poly(lactic-co-glycolic acid) (PLGA), a FDA and EMA approved synthetic copolymer of polylactic acid and polyglycolic acid is biocompatible and biodegradable by simple hydrolysis. The degradation rate of the copolymer can be controlled by altering the ratio of polylactic acid and polyglycolic acid. Teng *et al.* have shown that PLGA-based scaffolds, associated with neural stem cells (NSCs), promote long-term functional recovery in

adult hemi-sectioned rats by reducing glial scarring, preventing tissue loss, and promoting neuronal repair by NSCs [211]. Similarly the implantation of PLGA fabricated neural conduits in completely transected rat SC induced axonal regeneration in the channels [212, 213]. However, it was noted that the breakdown of PLGA produced glycolic and lactic acids, lowering the local pH and potentially hindering the tissue-repair process. In a xeno-transplantation study, a PLGA/small intestinal submucosa scaffold seeded with human BmMSCs was implanted into completely transected rat SC [214]. The researchers observed improved hind limb locomotor function and motor-evoked potential 8 weeks post-surgery. Furthermore, histology showed MSCs survival until 8 weeks post-surgery.

Polycarbonate a thermoplastic polymer, degrades to non-acidic products has been used for SCI treatment. Due to its hydrophobic nature, cells do not easily adhere but in combination with poly-L-lysine coating this polymer demonstrated a positive outcome in SCI treatment [215]. Poly-L-lysine-coated polycarbonate tubes were seeded with Schwann cells, and were implanted into the wounded thoracic SC of rats [216]. Two months after implantation, axons growing through the tubes were observed. However the only limitation for the polycarbonate tubes was lack of cell adhesion properties. Nomura *et al.* fabricated Poly 2-hydroxyethyl methacrylate (PHEMA-co-MMA) hydrogel scaffolds and implanted between the stumps of a completely transected SC; the hydrogel not only supported axonal regeneration but also reduced the formation of necrotic tissue [217]. However, the cytocompatability of this hydrogel is highly questionable. In spite of the beneficial effects on cell survival, proliferation, and differentiation seen with polymer biomaterials, it has been recognized that the

transplantation of cells in biomaterials alone is not suitable for cell integration and tissue regeneration. Therefore, recent work has focused on using biomaterials to combine cells and growth factor delivery to promote SCI repair [218].

5. Cells and bioactive molecules combined therapies for SCI repair

SCI is a multifactorial problem thus, the combination of different therapeutic approaches to achieve functional recovery after SCI is very promising. The combination of controlled and sustained delivery of bioactive molecules and cells using biomaterial-based scaffolds will be able to regenerate damaged tissue after SCI. The delivered therapeutic molecule could be beneficial to transplanted cells (e.g. to improve cell viability or direct cell differentiation). Transplanted cells into the injury site can integrate into the host tissue and secrete paracrine factors that promote the regeneration of the damaged tissue. The ability to control and sustain the delivery of both cells and therapeutic molecules is vital to ensure that they reside in the target tissue for a sufficient period of time to interact with each other and also with the injured tissue.

Strategies for using biomaterials to co-deliver cells and growth factors into the CNS include directly immobilizing growth factors to the biomaterials, or encapsulating them within particles. For instance, differentiation of neural stem/progenitor cells (NSPCs) into oligodendrocytes has been achieved by culturing NSPCs in a hyaluronan/methyl cellulose (HAMC) biopolymer composite loaded with recombinant platelet derived growth factor-A (rPDGF-A) [219]. Delivery of NSPCs in rPDGF-A-modified HAMC into a rat SCI (clip compression) model showed improved behavioral function and improved tissue repair compared to cells delivered in media alone [219]. Johnson *et al.* used fibrin-based hydrogels covalently altered with heparin-binding

peptides to bind heparin and subsequently the heparin-binding growth factors platelet-derived growth factor AA (PDGF-AA) and NT-3 [220]. When this scaffold was combined with embryonic stem cell- derived neural progenitor cells, functional recovery and tissue repair was observed [220]. However, tumor formation was also observed, underlying the importance of controlling cell phenotype before combining with scaffolds. Genetically modified cells that over express neurotrophic factors have also been pursued in a variety of combinatorial strategies [68]. Encapsulation of therapeutic molecules into stable microparticles offers another method for sustained release. Rooney *et al.* encapsulated dibutyryl cyclic adenosine monophosphate (dbcAMP) in PLGA microspheres, which were embedded in oligo[(polyethylene glycol) fumarate] hydrogels containing BmMSCs or Schwann cells (SCs), and grafted this multifaceted system in transected SC [221]. The sustained release of dbcAMP inhibited axonal regeneration in the presence of SCs but rescued MSC-induced inhibition of axonal regeneration. The reason for these conflicting results is unclear. However dbcAMP may be exerting varying effects on the SCs and MSCs, thereby altering their regenerative potential. The authors speculate that the regenerative potential of SCs in the injured CNS required maintenance of the cells in a dedifferentiated state.

When combined with cell grafts, biopolymer scaffolds may enhance survival, and if delivering growth factors enhance or maintain the differentiation of cells. Pharmacologically active microcarriers (PAMs), combine in a unique injectable microcarrier these properties. They are biodegradable, biocompatible poly(lactic-co-glycolic acid) (PLGA)-based microparticles coated with extracellular matrix proteins such as fibronectin or laminin to provide a biomimetic 3D support and also releasing in a

controlled manner a neurotrophic factor [222]. The combined effect of the biomimetic surface and the growth factor enhanced the survival and maintained the pre-differentiation of the grafted cells in various models of neurological disorders [223-225] (Figure-10). For instance, NT-3-loaded PLGA PAMs with laminin and poly-D-lysine covered surface were used to deliver MIAMI cells into hemi-Parkinsonian rats. After 8 weeks post-transplantation, MIAMI cells delivered by PAMs in the absence or in the presence of NT-3 increased MIAMI cells survival 2- or 3-folds, respectively, relative to the cells delivered alone [153]. Importantly, rats treated with NT-3 PAMs and MIAMI cells showed significant behavioral improvements compared to the other treatment groups. This strategy might be useful for BmMSCs survival and modification of the microenvironment after intra spinal transplantation (one of the limitations of cell strategy) during SCI.

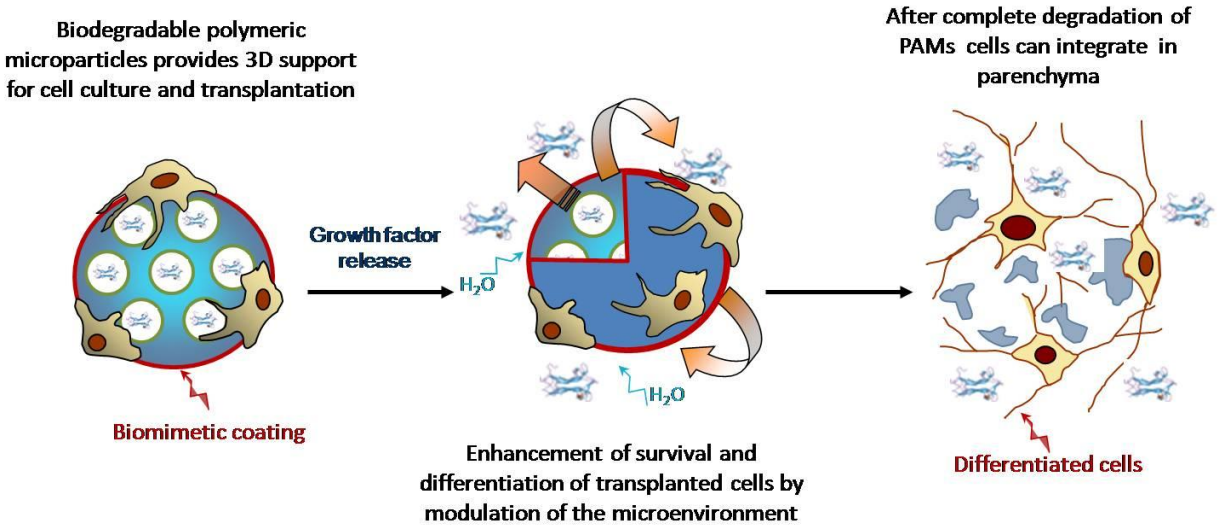

Figure-10: Representation of PAMs concept. Microspheres coated with cell adhesion molecules conveying and delivering growth factors are used as cell carriers which can then be grafted. Released growth factors and adhesion molecules influence the survival and differentiation of transported cells and the microenvironment. PAMs are biodegradable and degrade eventually allowing the cells integrate within the parenchyma [222].

6. OBJECTIVES OF THIS THESIS

The literature review on SCI repairing strategies clearly identifies that combinatorial strategies are of great interest compared to individual approaches. Hence, we want to develop an effective combinatorial tool to transplant in the damaged SC to obtain an effective repair.

The main objective of this thesis is to develop a controlled delivery of biologically active BDNF using PAMs and to combine this system with adult MSCs for SCI repair.

The first chapter concerns the preparation of PAMs, which releases continuously a bioactive BDNF for several weeks. The effect of PAMs releasing BDNF on MIAMI cells differentiation was investigated. According to the literature MSCs show functional recovery mainly through paracrine mechanism. Hence the effect of PAMs releasing BDNF on MIAMI secretome was then investigated. As hydrogel scaffolds may confine the injected cell/particles near the lesion site, an injectable hydrogel has been combined with PAMs conveying MIAMI cells. The neural differentiation and secretome of the cells in this combined system was investigated.

Literature review clearly identifies that stem cells of the apical papilla (SCAP) possess a high therapeutic potential in treating neurological disorders but they are currently less explored in the treatment of SCI. The second chapter concerns the validation of BDNF releasing PAMs on SCAP survival, neural differentiation and investigates the combinatorial approach of PAMs releasing BDNF conveying SCAP in the SCI animal models.

A general discussion comparing the existing strategies and major achievements in the present approach will close this work and open new prospects.

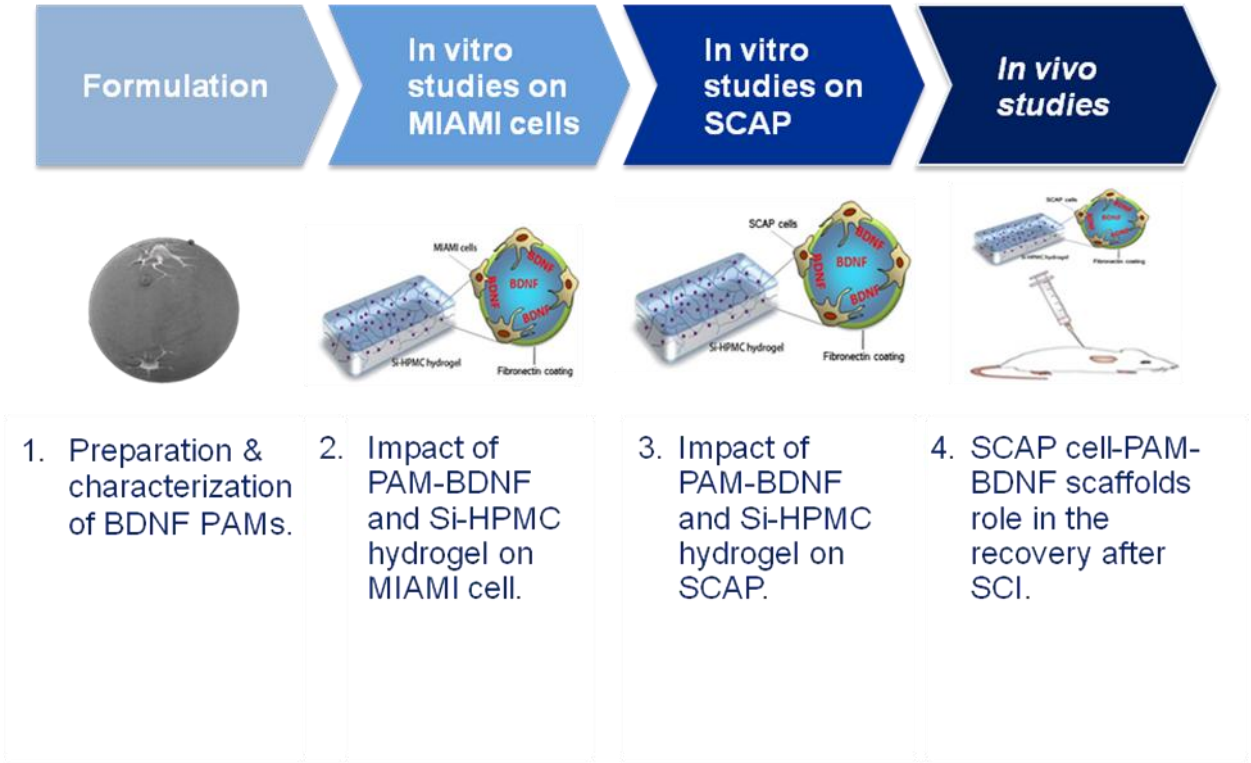

Figure-11: Objectives of the present thesis**.**

7. CHAPTER-1

Pharmacologically active microcarriers delivering BDNF within a hydrogel: novel strategy for human bone marrow-derived stem cells neural/neuronal differentiation guidance and therapeutic secretome enhancement

Scientific context

Recently, a new regenerative medicine strategy has been suggested using smart biomaterials able to carry and deliver cells and release the encapsulated growth factors (GF) in the damaged spinal cord. BDNF is a homodimeric polypeptide protein which shares about 50% amino acid identities with the other member of the neurotrophic (NT) family (NGF, NT-3) [226] entailed in neural cell growth and differentiation [81, 179]. Even though the majority of neurons in the mammalian brain are formed prenatally, parts of the adult brain retain the ability to grow new neurons from neural stem cells through neurogenesis. In this process neurotrophins in particular BDNF help to stimulate and control neurogenesis [227, 228]. In the spinal cord, Dougherty *et al.* have demonstrated that BDNF is expressed in astrocytes, microglia/macrophages and oligodendrocytes in normal adult spinal cord as well as in the spinal cord after injury. This study suggests that in the spinal cord these cells play a role in local BDNF availability and also in BDNF-mediated healing directly within the injury site as well as surrounding wound site [229]. However due to inhibitory environment at the lesion site the expression of BDNF by the endogenous cells is not sufficient enough for repair. It requires additional BDNF support to facilitate axonal regeneration and functional recovery.

Namiki and group reported that intramedullar infusion of BDNF at a rate of 625 ng/h into the SCI site significantly improved clinical neurological functions after SCI in comparison with other neurotrophins, NT-3 and NGF, in rat clip compression SCI models [95]. BDNF increased the proliferation of Schwann cells and their migration along with the production of peripheral myelin at the lesion site. They identified that NT-

3 administrated for the first 14 days after injury, may not be useful for neuroprotection and the prevention of motor tract damage after SCI in adults. A programmed, sequential delivery of neurotrophic factors, for example an initial introduction of BDNF for neuroprotection, followed by NT-3 for regeneration, may be a useful approach to the treatment of SCI [95]. However, in this study the authors observed that the continuous infusion of neurotrophic factors into the spinal cord, at a flow rate of 0.5 pL/h for 14 days, created additional damage and cavity formation, which extended rostrally and caudally from the cannula tip [95]. The number of corticospinal neurons was significantly decreased compared to controls due to the implanted cannula. The other potential therapeutic uses of BDNF in spinal cord injury were discussed in the general introduction section 2.1. Along with that, the mode and timing of BDNF delivery also plays a key role for therapeutic effect after injury (discussed in the general introduction section 2.1). Altogether these findings highlight the importance of a controlled delivery system of biologically active BDNF for the spinal cords functional recovery after injury.

In the present chapter we developed and characterised a system that releases biologically active BDNF in a controlled manner from PAMs and we combined this tool with hydrogel scaffolds and observe their impact on MIAMI cells (discussed in general introduction section 3.1.2). Based on the literature when the protein is in a nanoprecipitated form it preserves the protein structure, integrity and facilitates prolonged release of biologically active protein from the PAMs [230]. In order to find the best conditions to nanoprecipitate BDNF we used a model protein (α-chymotrypsin) and D-optimal experimental design to depict the best experimental conditions for the maximum nanoprecipitation yield. In this way we were able to choose the optimum

conditions to apply to the costly therapeutic protein BDNF. Additionally, an additive poloxamer (P188) is co-precipitated with the protein and these nanoprecipitates are then encapsulated by using s/o/w emulsion extraction technique developed in our laboratory.

It is progressively realized that cell or drug therapies alone will not be enough for successful tissue engineering in many CNS disorders and insults. For this reason, engineered scaffolds have gained greater interest in the last years. Among the wide field of emerging materials, research has been focused on hydrogels, three-dimensional polymeric networks able to convey and confine the cells and drug delivery devices at the injury site. Moreover these scaffolds slowly degrade in the physiological environment, leading the growing tissue to replace the former filled site. In this study we choose silanized hydroxypropyl methylcellulose (Si-HPMC) hydrogel to combine with the PAMs releasing BDNF and MIAMI cells. Si-HPMC hydrogel is a self-cross linkable cellulose-based hydrogel made of 98% (approximately) of water. Promising results were obtained with this hydrogel when combined to stem cells for tissue engineering in terms of cytocompatibility both *in vitro* and *in vivo* [231, 232]. It has been reported that Si-HPMC hydrogel in combination with mesenchymal stem cell (from different origin) was used in bone regeneration [233], cartilage tissue repair [231], but not explored in neurological application. In this study for the first time we want to explore the usage of Si-HPMC hydrogel for neurological application based on its potentiality to reticulate at pH 7.4 which will match the cerebro spinal fluid (CSF) pH (7.3) as well as plasma pH (7.3-7.4). After formulating the PAMs with the nanoprecipitated BDNF, physicochemical characterization was evaluated. PAMs surface characterization and encapsulation yield

of BDNF was determined. We studied the mechanical properties and cytotocompatibility of Si-HPMC hydrogel and the impact of combined strategies include hydrogel and BDNF releasing PAMs on MIAMI gene expression as well as cytokines secretion.

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Full length article

Pharmacologically active microcarriers delivering BDNF within a hydrogel: Novel strategy for human bone marrow-derived stem cells neural/neuronal differentiation guidance and therapeutic secretome enhancement

Saikrishna Kandalam [a,b], Laurence Sindji [a,b], Gaëtan J.-R. Delcroix [c,d,e], Fabien Violet [a,b], Xavier Garric [h], Emilie M. André [a,b], Paul C. Schiller [c,d,e,f,g], Marie-Claire Venier-Julienne [a,b], Anne des Rieux [i,j], Jérôme Guicheux [k,l,m], Claudia N. Montero-Menei [a,b,*]

[a] INSERM U 1066, 'Micro et Nanomédecines biomimétiques-MINT', Angers, France
[b] Université Angers, UMR-S1066, Angers, France
[c] GRECC and Research Service, Veterans Affairs Medical Center, Miami, FL, USA
[d] Department of Orthopaedics, University of Miami Miller School of Medicine, FL, USA
[e] Interdisciplinary Stem Cell Institute, University of Miami Miller School of Medicine, Miami, FL, USA
[f] Department of Biochemistry & Molecular Biology, University of Miami Miller School of Medicine, FL, USA
[g] Geriatrics Institute, University of Miami Miller School of Medicine, FL, USA
[h] Artificial Biopolymers Department, Institut des Biomolecules Max Mousseron (IBMM) UMR5247, CNRS, University of Montpellier, ENSCM, France
[i] Louvain Drug Research Institute, Advanced Drug Delivery and Biomaterials, Université Catholique de Louvain, 1200 Bruxelles, Belgium
[j] Institute of Condensed Matter and Nanosciences, Université Catholique de Louvain, 1048 Louvain-la-Neuve, Belgium
[k] INSERMU791, Laboratory for Osteo-Articular and Dental Tissue Engineering LIOAD, groupe STEP, 44042 Nantes, France
[l] University of Nantes, UFR Odontologie, 44042 Nantes, France
[m] CHU Nantes, PHU 4 OTONN, 44042 Nantes, France

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ABSTRACT

Stem cells combined with biodegradable injectable scaffolds releasing growth factors hold great promises in regenerative medicine, particularly in the treatment of neurological disorders. We here integrated human marrow-isolated adult multilineage-inducible (MIAMI) stem cells and pharmacologically active microcarriers (PAMs) into an injectable non-toxic silanized-hydroxypropyl methylcellulose (Si-HPMC) hydrogel. The goal is to obtain an injectable non-toxic cell and growth factor delivery device. It should direct the survival and/or neuronal differentiation of the grafted cells, to safely transplant them in the central nervous system, and enhance their tissue repair properties. A model protein was used to optimize the nanoprecipitation conditions of the neuroprotective brain-derived neurotrophic factor (BDNF). BDNF nanoprecipitate was encapsulated in fibronectin-coated (FN) PAMs and the *in vitro* release profile evaluated. It showed a prolonged, bi-phasic, release of bioactive BDNF, without burst effect. We demonstrated that PAMs and the Si-HPMC hydrogel increased the expression of neural/neuronal differentiation markers of MIAMI cells after 1 week. Moreover, the 3D environment (PAMs or hydrogel) increased MIAMI cells secretion of growth factors (b-NGF, SCF, HGF, LIF, PlGF-1, SDF-1α, VEGF-A & D) and chemokines (MIP-1α & β, RANTES, IL-8). These results show that PAMs delivering BDNF combined with Si-HPMC hydrogel represent a useful novel local delivery tool in the context of neurological disorders. It not only provides neuroprotective BDNF but also bone marrow-derived stem cells that benefit from that environment by displaying neural commitment and an improved neuroprotective/reparative secretome. It provides preliminary evidence of a promising pro-angiogenic, neuroprotective and axonal growth-promoting device for the nervous system.

Statement of Significance

Combinatorial tissue engineering strategies for the central nervous system are scarce. We developed and characterized a novel injectable non-toxic stem cell and protein delivery system providing regenerative

* Corresponding author at: INSERM U1066, MINT "Micro et nano médecines biomimétiques", F-49933 Angers, France and Université Angers, UMR-S1066, Angers, France.
E-mail address: claudia.montero-menei@univ-angers.fr (C.N. Montero-Menei).

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cues for central nervous system disorders. BDNF, a neurotrophic factor with a wide-range effect, was nanoprecipitated to maintain its structure and released in a sustained manner from novel polymeric microcarriers. The combinatorial 3D support, provided by fibronectin-microcarriers and the hydrogel, to the mesenchymal stem cells guided the cells towards a neuronal differentiation and enhanced their tissue repair properties by promoting growth factors and cytokine secretion. The long-term release of physiological doses of bioactive BDNF, combined to the enhanced secretion of tissue repair factors from the stem cells, constitute a promising therapeutic approach.

1. Introduction

Stem cell-based therapy is promising for preventing neuronal damage and promoting tissue repair in neurological disorders. Adult multi-potent mesenchymal stromal cells (MSCs) are good candidates as they can be differentiated into neural-like cells and secrete a wide variety of growth factors and cytokines involved in immunomodulation and tissue repair [1,2]. However, secretome studies allowing the understanding of their tissue repair properties remain scarce. Clinically, autologous or allogenic MSC administration into the CNS is feasible, ethically acceptable, appears to be safe in human subjects [3,4] and is not hindered by major tissue rejection-related concerns. However, inconsistent results have been reported in the literature [5]. This could be attributed to the heterogeneity of MSCs as a result of both donor and isolation technique variability.

*M*arrow *I*solated *A*dult *M*ultilineage-*I*nducible (MIAMI) cells are a more homogeneous subpopulation of human MSCs exhibiting pluripotent embryonic stem cell gene expression with the potential to differentiate into cells from all three embryonic germ layers [6–8]. It was recently demonstrated that MIAMI cells significantly secrete high levels of anti-inflammatory and angiogenic cytokines and pro-survival chemokines/growth factors *in vitro* [1,9]. MIAMI cells are capable of differentiating into immature neuron-like cells exhibiting neuronal ionic channel activity *in vitro* on a fibronectin substrate [7] while pre-treatment with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) enhanced their neural specification and response to neuronal commitment *in vitro* [10].

Biomaterial-based scaffolds have been developed to enhance cell survival and engraftment after intracerebral injection [11–13]. They can serve as local delivery vehicles for therapeutic molecules and for cells directly at their site of action. Indeed, as the brain is isolated from the systemic circulation by the blood-brain barrier and is composed of structurally and functionally different regions a local stereotaxic administration is necessary. Pharmacologically active microcarriers (PAMs) are biodegradable, biocompatible poly(lactic-co-glycolic acid) (PLGA)-based microparticles that release growth factors in a controlled manner while providing a biomimetic 3D support of extracellular matrix molecules to the transported cells [14–16]. These combined properties joined in a single small-sized injectable carrier enhanced the survival and maintained the pre-differentiation of the grafted cells in different animal models of neuronal, cardiac and cartilage disorders [14–17]. Particularly, PAMs coated with fibronectin improved the survival of MIAMI cells in a global ischemia animal model [18]. Moreover, MIAMI cells conveyed by PAMs releasing neurotrophin-3 provided functional improvement in an animal model of Parkinson's disease [19], probably due to their paracrine activity [20].

Brain-derived neurotrophic factor (BDNF) is widely distributed throughout the adult central nervous system (CNS) and acts through TrkB receptors. BDNF plays an important role in the regulation of neuronal survival and differentiation, axonal growth, connectivity, and synaptic plasticity *in vitro* and *in vivo* [21,22]. However, delivery of recombinant human BDNF to attain therapeutic efficacy is hindered by its low stability, short *in vivo* half-life, inability to pass through blood-brain barrier and to achieve an appropriate pharmacokinetic profile [22]. The delivery of BDNF into the brain by mini pump creates a steep concentration gradient originating from the point of infusion, which could lead to alteration of the infused tissue and the development of adverse effects, such as edema [23]. Encapsulation of BDNF in biodegradable and biocompatible nano- or micro-carriers is one strategy to protect the BDNF from the environment and obtain a controlled and sustained delivery at the site of injection [11,13]. Their small size allows their implantation in the brain by stereotaxic injection, at one or different sites, and the subsequent local sustained release of physiological doses of BDNF *in vivo*. The sustained and complete delivery of growth factors from polymeric vectors at physiological doses still remains a technological challenge. We propose that nanoprecipitated BDNF encapsulated in PAMs constituted of a more hydrophilic polymer than PLGA, a triblock PLGA-poloxamer (P188)-PLGA polymer, will preserve its structure and integrity, thus maintaining biological activity [19,24,25].

Research on combinatorial therapeutic strategies are needed for CNS disorders as single strategies are not efficient and the "magic bullet" was not yet found for these disorders. In the present study, we have optimized BDNF nanoprecipitation using a model protein and encapsulated it in PAMs. BDNF encapsulation, *in vitro* release profile and bioactivity were assessed. EGF-bFGF pre-treated MIAMI cells, thereby specified towards the neural/neuronal lineage, were adhered onto PAMs covered with fibronectin and releasing or not BDNF. These PAMs may not only further stimulate E/F MIAMI cell neural/neuronal differentiation as the released BDNF act on the surrounding neuronal environment after transplantation. MIAMI cells adhered onto PAMs were incorporated in an injectable hydrogel with two goals in mind: to maintain them at the desired site of action after transplantation as well as to further improve their neural specification [26]. Silanized-hydroxypropyl methylcellulose hydrogel (Si-HPMC HG) is a biodegradable and injectable hydrogel that is non-toxic, has low swelling properties and forms a gel *in situ* at physiological pH [27,28]. The impact of PAMs on Si-HPMC hydrogel rheological properties was evaluated. Finally, we investigated the impact of all these biomaterials on the gene expression and secretome profile of MIAMI cells. Indeed, as MSCs mainly contribute to tissue repair by their paracrine activity it is essential to understand the secretion profile of these MIAMI cells associated to the injectable scaffolds for future clinical applications.

2. Materials and methods

2.1. Materials

PLGA–P188–PLGA was synthesized by the IBMM-DBA CNRS UMR 5247 laboratory (Montpellier, France). Polytetrafluoroethylene (PTFE) filters Millex®-FH (pore size 5 μm) were obtained from Millipore (Millipore SA, Guyancourt, France), Polyvinyl

alcohol (Mowiol® 4-88) from Kuraray Specialties Europe (Frankfurt, Germany), P188 poloxamer or Pluronic® F68 from BASF (Levallois-Perret, France), culture mediums and collagenase from Lonza (Levallois, France), BDNF from Peprotech (Paris, France), EGF, bFGF and DuoSet ELISA Development kit from R&D Systems (Lille, France), biotinylated anti-mouse IgG antibodies from Vector laboratories (Burlingame, USA), streptavidin–fluoroprobe 547 from Interchim (Montluçon, France), Costar ultra-low cluster plates from Corning (Avon, France), 96-well flat-bottom culture plates from Nunc® (Dutscher, Strasbourg, France), Total RNA isolation Nucleospin® RNA II from Macherey Nagel (Hoerdt, France), Qiaquick PCR purification kit from Qiagen (Courtaboeuf, France), iQ SYBR Green Supermix Biorad (Fermentas), anti-aggrecan polyclonal rabbit antibody from Millipore (Molsheim, France), and anti-type II collagen monoclonal mouse antibody from Interchim (Montluçon, France), LIVE/DEAD™ assays (Thermo Fisher, France). All others reagents were purchased from Sigma Aldrich (Saint-Quentin Fallavier, France).

2.2. PLGA-P188-PLGA synthesis

PLGA–P188–PLGA polymer (Mn88, 400 g.mol-1) synthesis and characterization was performed as previously described [17]. Briefly, PLGA–P188–PLGA polymer was prepared by ring-opening polymerization (ROP) of DL-lactide and glycolide using P188 as an initiator, and stannous octoate [$Sn(Oct)_2$] as a catalyst at 140 °C. After removal of water and oxygen by 15 vacuum-nitrogen purge cycles, flasks were frozen at 0 °C, sealed under dynamic vacuum at 10^{-3} mbar and polymerization then took place at 140 °C under constant agitation. After 5 days, the products were recovered by dissolution in dichloromethane, precipitated with 500 mL of ethanol, filtered, washed and dried overnight at 45 °C under reduced pressure, until stabilization of the final product mass.

2.3. BDNF nanoprecipitation: experimental design

For nanoprecipitation, the protein powder was first dissolved with a non-buffered aqueous salt solution and this solution added to a cold glycofurol solution, with poloxamer (P188) containing or not a salt solution (additive). Thirty minutes later, the protein nanoparticles were recovered by centrifugation (10,000g, 30 min, 4 °C). To determine the conditions that will ensure the highest nanoprecipitation efficiency of BDNF (Mol.wt- 27 kDa, Number of amino acids-240, Isoelectric point-9.59), D-optimal experimental design [29] was implemented using α-chymotrypsin (Mol.wt-25 kDa, Number of amino acids-241, Isoelectric point-9.1) as a model protein. Four parameters known to influence protein precipitation were evaluated within an experimental domain: i) concentration of protein: 5.85 or 10 mg/mL (2 levels), ii) the molar concentration of NaCl in the protein solution: 0.00, 0.16 or 0.30 M (3 levels), iii) the molar concentration of $ZnCl_2$ in the protein solution: 0.000, 0.008 or 0.016 M (3 levels), iv) addition of poloxamer (P188) with salts (ratio P188:protein 20:1): without P188 (ø), P188 with 0.3 M NaCl, P188 with 0.008 M $ZnCl_2$ (3 levels) (Supplementary file: Table-1). Cold glycofurol was added to reach a final volume of 1 mL (1.04 g/mL). Nanoprecipitated α-chymotrypsin was centrifuged, the pellets collected and the protein quantified using a NanoOrange® Protein Quantitation Kit (Molecular Probes, Eugene, OR, USA). Nanoprecipitation efficiency was calculated as the percentage of protein recovered after precipitation and dissolution versus the initial amount of protein. The effects of the four studied parameters and the three interactions ([NaCl]/P188 with salts; [$ZnCl_2$]/P188 with salts and [NaCl]/[$ZnCl_2$]) on α-chymotrypsin precipitation yield were determined using the following mathematical model corresponding to the experimental design:

$$
\begin{aligned}
Y_{ij} &= \beta_0 + \beta_1 P_i + \beta_2 NaCl(0/0.30)_i + \beta_3 NaCl(0.16/0.30)_i \\
&+ \beta_4 ZnCl_2(0/0.016)_i + \beta_5 ZnCl_2(0.08/0.016)_i \\
&+ \beta_6 P188(\varnothing/ZnCl_2)_i + \beta_7 P188(NaCl/ZnCl_2)_i \\
&+ \beta_8 (NaCl(0/0.30)_i \times ZnCl_2(0/0.016)_i) + \beta_9 (NaCl(0/0.30)_i \\
&\times ZnCl_2(0.08/0.016)_i) + \beta_{10} (NaCl(0.16/0.30)_i \\
&\times ZnCl_2(0/0.016)_i) + \beta_{11} (NaCl(0.16/0.30)_i \times ZnCl_2(0.08/0.016)_i) \\
&+ \beta_{12} (NaCl(0.16/0.30)_i \times P188(\varnothing/ZnCl_2)_i + \beta_{13} (NaCl(0/0.30)_i \\
&\times P188(NaCl/ZnCl_2)_i) + \beta_{14} (NaCl(0.16/0.30)_i \times P188(\varnothing/ZnCl_2)_i \\
&+ \beta_{15} (NaCl(0.16/0.30)_i \times P188(NaCl/ZnCl_2)_i) \\
&+ \beta_{16} (ZnCl_2(0/0.016)_i \times P188(\varnothing/ZnCl_2)_i + \beta_{17} (ZnCl_2(0/0.016)_i \\
&\times P188(NaCl/ZnCl_2)_i) + \beta_{18} (ZnCl_2(0.08/0.016)_i \\
&\times P188(\varnothing/ZnCl_2)_i) + \beta_{19} (ZnCl_2(0.08/0.016)_i \\
&\times P188(NaCl/ZnCl_2)_i) + u_j + \varepsilon_{ij}, \\
&\text{where } u_j \sim \mathcal{N}(0,\tau^2),\ \varepsilon_{ij} \sim \mathcal{N}(0,\sigma^2) \text{ are independent}, \\
&1 \leqslant i \leqslant 31, \text{ and } 1 \leqslant j \leqslant 3, \text{ and } Var(Y_{ij}) = \tau^2 + \sigma^2.
\end{aligned}
$$

NEMROD-W software (2000, LPRAI, Marseille) was used for the generation and exploitation of the statistical experimental design illustrated in Fig. 1 (Figure generated by the NEMROD-W software). The D-optimal experimental design was composed of a set of 31 distinct experiments well distributed in the experimental domain (n = 2 or n = 3). According to the results of the experimental design, BDNF (10 mg/mL) was nanoprecipitated in 0.3 M NaCl in P188 with glycofurol. After nanoprecipitation and centrifugation, the pellet was collected and BDNF was quantified by ELISA according to supplier instructions. Nanoprecipitation efficiency was calculated as the percentage of BDNF recovered after precipitation and dissolution versus the initial amount of BDNF.

2.4. BDNF encapsulation in PAMs

PLGA-P188-PLGA microspheres were prepared using a *solid/oil/water* (s/o/w) emulsion solvent evaporation–extraction process as previously described [17,24]. The total protein loading used was 0.6% w/w (0.1% BDNF and 0.5% human serum albumin) as previously reported [19,25]. NaCl and glycofurol were used to precipitate human serum albumin and BDNF separately. HSA was nanoprecipitated as described [17,25]. Regarding BDNF nanoprecipitation, 1.077 g of cold glycofurol was added to 5 µl of a solution containing 50 µg of BDNF, 1 mg of P188 and 0.3 M NaCl. After 30 min at 4 °C, the nanoprecipitated proteins were recovered by centrifugation and dispersed in the organic phase (50 mg PLGA–P188–PLGA polymer was dissolved in 670 µL of 3:1 methylene chloride : acetone solution). The suspension was emulsified in a poly(vinyl alcohol) aqueous solution (30 mL, 6% w/v at 1 °C) and mechanically stirred at 995 rpm for 1 min. After addition of 33 mL of deionized water and stirring for 10 min, the emulsion was added to 170 mL water and stirred until evaporation of the organic solvent. Microspheres were then filtered on a 5 µm Millipore Durapore Membrane Filter (SVPP), washed and freeze-dried. Microspheres without BDNF were prepared following the same process, and called blank-microspheres, or blank-PAMs when coated with fibronectin. Protein encapsulation yield was determined by measuring the entrapped BDNF after dissolution in dimethylformamide (n = 4). This solvent did not cause alterations

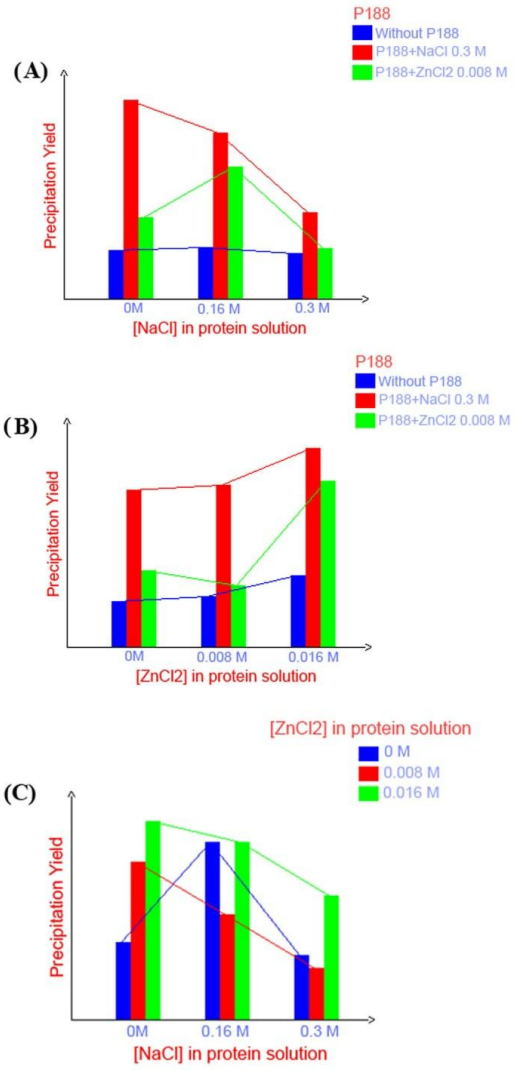

Fig. 1. D-optimal experimental design and interactions among parameters that influence α-Chymotrypsin nanoprecipitation yield. The connecting lines help to visualize the interactions between the different parameters. A) Interaction between [NaCl] in protein solution and P188 with salts depicts that the effect of [NaCl] on nanoprecipitation yield depends on the presence of P188 (no effect when there is no P188 (blue bars) or negative effect (reduce the precipitation yield) when it is associated to P188 with NaCl (red bars) or $ZnCl_2$ (green bars). B) Interaction between $[ZnCl_2]$ in protein solution and P188 with salts: the effect of $[ZnCl_2]$ on nanoprecipitation yield depends on the presence of P188 (the effect is positive (increase protein precipitation) when it is associated to P188 with $ZnCl_2$. C) Interaction between [NaCl] and $[ZnCl_2]$ portrays that the effect of [NaCl] in protein solution depend on $[ZnCl_2]$ in protein solution: the effect of [NaCl] is negative (decrease protein precipitation) and it is more marked when it is associated to 0.008 M $ZnCl_2$ (red bars) compared to 0.016 M $ZnCl_2$ (green bars). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of the protein as detected by ELISA (data not shown). Controls were performed with blank-microspheres. To obtain PAMs, microspheres were coated with fibronectin and poly-D-Lysine as previously described [17]. Microspheres were suspended in the solution containing fibronectin and poly-lysine (at 16 μg/mL and 24 μg/mL, respectively, at a 40:60 ratio) and rotated at 15 rpm at 37 °C during 1h30. PAMs were then washed 3 times in sterile distilled water, lyophilized and kept at −20 °C.

2.5. Characterization of PAMs

The average diameter and size distribution of PAMs were evaluated using a Multisizer® Coulter Counter (Beckman Coulter, Roissy, France) (n = 3). PAMs and microspheres were also imaged by scanning electron microscopy (SEM) as described [14]. The fibronectin coating was characterized by immunofluorescence as described previously [17,25]. Zeta potential was measured using a Malvern Zetasizer® (Nano Series DTS 1060, Malvern Instruments S.A., Worcestershire, UK) in 1 mg/mL of PAM in NaCl 1 mM at pH 7.

2.6. In vitro release of BDNF

In vitro release of BDNF from microspheres or PAMs (5 mg) was performed in 500 μL of 0.01 M citrate buffer (pH 5) containing 0.1% w/v BSA at 37 °C under agitation (125 rpm) (n = 3). At different times, the tubes were centrifuged for 5 min at 2800*g* and the supernatant was collected for analysis and replaced by fresh buffer. The concentration of BDNF in the release buffer was evaluated by ELISA. The percentage of cumulative release was calculated based on the total amount of protein encapsulated. Samples were observed for changes in the surface morphology over time using scanning electron microscopy (SEM) [14].

2.7. BDNF bioassay

Activity of released BDNF in 0.01 M citrate buffer with 0.1% BSA (pH 5) was assessed using the rat dorsal root ganglia (DRG) assay. Animal care and uses were in strict accordance with the regulations of the French Ministry of Agriculture and of the animal experimentation ethic committee of Pays de la Loire (2015-30). DRG were collected from new born Sprague Dawley rats (P2-P5) in Hank's Balanced Salt Solution (HBSS) containing antibiotics and mechanically triturated in dissection medium containing HBSS, antibiotics and 2 mg/mL collagenase. Debris was let to sediment and the supernatant containing the cell suspension was collected. DRG cells were seeded on poly-lysine (5 $\mu g/cm^2$) coated 24 well-plates at 10^4 cells/cm^2 and cultured in Neurobasal media, 1% B27, 0.5 mM glutamax, 1% antibiotics. BDNF collected from *in vitro* release assay at the different time-points cited in 2.6 and diluted to a final concentration of 5 ng/mL (based on quantification by ELISA), was used for the bioassay. This concentration gave the best response from DRG neurons using recombinant BDNF (positive control). 0.5 mg of PAMs (blank or loaded with BDNF), were also added directly to the DRG cells (n = 3). After 7 days, immunofluorescence against β3-tubulin was performed to observe BDNF-induced neuron-like differentiation as previously described [20]. All β3-tubulin positive cells exhibiting extended neurite outgrowth (at least 5 times longer than the cell body) were taken in consideration and their number evaluated by image analysis (Metamorph®, Molecular Devices Corp., Sunnyvale, CA). Bioactive BDNF results were expressed as ng/mL and the cumulative release profile was compared with ELISA estimation.

2.8. Si-HPMC hydrogel synthesis and rheological properties

Si-HPMC hydrogel was prepared as previously described [27]. Briefly, the synthesis of Si-HPMC was performed by grafting 14.24% of 3-glycidoxypropyltrimethoxysilane (3-GPTMS) onto E4 M® in heterogeneous medium. Si-HPMC polymer (4% w/v) was dissolved in 0.2 M NaOH aqueous solution (pH 12.8); followed by two dialyses in 0.09 M NaOHaq with a 6–8 kDa molecular weight cut-off. To obtain the reticulated hydrogel solution (pH 7.4, allowing cells or PAMs incorporation), 1 vol of Si-HPMC basic solution was mixed with 0.5 vol of acidic buffer solution at pH 3.6. The viscoelastic properties of Si-HPMC hydrogel (2% final concentration at pH 7.4) with or without addition of PAMs were measured with a Kinexus® rheometer (Malvern Instrument S.A., UK) using cone/plate geometry (diameter 40 mm, angle 2°), at day 0 and day 7 (n = 3). Storage modulus (G′) and loss modulus (G″) were monitored as a function of time at constant frequency and strain, 1 Hz and 0.1% respectively, as previously described [25].

2.9. Culture of MIAMI cells

MIAMI cells were obtained from iliac crest aspirates from a human male post-mortem organ donor (Anonymous N° 32885, 65 years, male). This protocol was performed in collaboration with the «Coordination Hospitalière de prélèvements d'organes, de tissus ou de cellules » and has the agreement of the French Agency of Biomedicine. The cells are stored in the University Hospital of Angers under declaration number DC2011-1467. Isolation and culture of MIAMI cells was performed in DMEM low glucose supplemented with 3% fetal bovine serum at low oxygen conditions (3% O_2) and low density (120 cells/cm^2) as previously described [6,10]. To induce neuronal specification, MIAMI cells were pre-treated for 10 days with 20 ng/mL of epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and 5 µg/mL of heparin under low oxygen tension (E/F MIAMI cells).

2.10. Formation of E/F MIAMI cells-PAM complexes and incorporation in Si-HPMC hydrogel

The biocompatibility of the hydrogel with E/F MIAMI cells was first confirmed with a LIVE/DEAD™ assay kit. The cells (0.6×10^6 - cells/mL) dispersed in hydrogel solution right after preparation were then incubated at 37 °C under a 5% CO_2 humidified atmosphere. The percentage of living cells (green) and dead cells (red) relative to the total number of cells was calculated after observation with a confocal laser scanning microscope at 1, 3 and 7 days using Image J (NIH, public domain software) (six random fields, n = 3).

E/F MIAMI cells (0.2×10^6 cells) were incubated for 4 h with 0.5 mg PAMs (blank or BDNF) in 24 well Costar ultra-low cluster plate as previously described [17,19]. Cell adhesion on PAMs was assessed using light microscopy and SEM as described [14]. E/F MIAMI cells alone (0.2×10^6 cells) or E/F MIAMI cells-PAM complexes were centrifuged and dispersed in the hydrogel solution (0.3 mL) within 5 min following its preparation. The samples were incubated at 37 °C in 5% CO_2 for 7 days.

2.11. E/F MIAMI cells gene expression

To evaluate the impact of PAMs incorporated or not in the Si-HPMC hydrogel on E/F MIAMI cells, gene expression of several markers (Table 1) has been studied by RT-qPCR. Human sequences were determined using PubMed nucleotide search (www.ncbi.nlm.nih.gov) and Ensembl (www.ensembl.org) websites. The online freeware Primer blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used for primer modeling, Clustal W (www.ebi.ac.uk) to align nucleotide sequences, and nucleotide blast (www.ncbi.nlm.nih.gov) to confirm the specificity of the defined primer sequences. They were validated using cDNA from human adult or fetal brain and commercial qPCR Human Reference cDNA (Clontech, Takarabio, Saint-Germain-en-Laye, France) as described [10,20].

Table 1
Sequence of primers validated in RT-qPCR.

Gene	Full name	NM accession number	Sequences
NFM	Neurofilament, medium polypeptide	NM_005382	F = 5′- GACCTCAGCAGCTACCAG – 3′ R = 5′- CTAGTCTCTTCACCCTCCAG – 3′
GFAP	Glial fibrillary acidic protein	NM_002055	F = 5′- AGTTGCAGTCCTTGACCTG – 3′ R = 5′- CTCGTCCTTGAGGCTCTG – 3′
Olig2	Oligodendrocyte lineage transcription factor 2	NM_005806	F = 5′- GAAGCAAATGACAGAGCCG – 3′ R = 5′- TGGTGAGCATGAGGATGTAG – 3′
TrkB	Neurotrophin tyrosine kinase, receptor, type 2,	NM_006180.3	F = 5′- TTGTCTGAACTGATCCTGGTGGGC – 3′ R = 5′- AGGTTAGGTGCGGCCAGATTTGC – 3′
Nes	Nestin	NM_006617	F = 5′- AGAAACAGGGCCTACAGAG – 3′ R = 5′- AAAGCTGAGGGAAGTCTTG – 3′
β3-TUB	Homo sapiens tubulin, beta 3	NM_006086	F = 5′- CCAGTATGAGGGAGATCG – 3′ R = 5′- CACGTACTTGTGAGAAGAGG – 3′
Col2a1	Collagen type II alpha 1	NM_033150	F = 5′- GAGGGGATCGTGGTGACAAAGG – 3′ R = 5′- TTGCATTACTCCCAACTGGGCG – 3′
RUNX2	Runt-related transcription factor 2	NM_001024630–NM_004348	F = 5′- ACAAATCCTCCCCAAGTAGC – 3′ R = 5′- GACACCTACTCTCATACTGGG – 3′
BGLAP	Osteocalcin	NM_199173	F = 5′- GGCGCTACCTGTATCAATGG – 3′ R = 5′- TCAGCCAACTCGTCACAGTC – 3′
FABP4	Fatty acid binding protein 4	NM_001442	F = 5′- ACAGCACCCTCCTGAAAACTGC – 3′ R = 5′- TGTTAGGTTTGGCCATGCCAGC – 3′
GAPDH	Glyceraldehyde-3-phosphate deshydrogenase	NM_002046	F = 5′- CAAAAGGGTCATCATCTCTGC – 3′ R = 5′- AGTTGTCATGGATGACCTTGG – 3′
ACTB	Beta-actin	NM_001101	F = 5′- CCAGATCATGTTTGAGACCT – 3′ R = 5′- GGCATACCCCTCGTAGAT – 3′
SOX2	SRY-box 2	NM_003106.3	F = 5′- AACATGATGGAGACGGAGC – 3′ R = 5′- CATCTTGGGGTTCTCCTGG – 3′
Nanog	Nanoghomeobox	NM_024865.3	F = 5′- GATCCAGCTTGTCCCCAAAG – 3′ R = 5′- GCTGTCCTGAATAAGCAGATCC – 3′

RT-qPCR experiments were performed following the guidelines of the PACEM core facility (“Plateforme Analyse Cellulaire Et Moléculaire”, Angers, France) [13,25] with sense and antisense primer pairs at 5 μM (Eurofins MWG Operon, Ebersberg, Germany) (Table 1). Total RNA was extracted using RNeasy Micro Kit from QIAGEN (Courtaboeuf, France), purified using Qiaquick PCR Purification Kit (QIAGEN, Courtaboeuf, France) and concentrations determined using a ND-2000 NanoDrop (Thermo Fisher Scientific, Wilmington, Delaware USA). First strand complementary DNA (cDNA) synthesis was performed with a SuperScript™ II Reverse Transcriptase (Invitrogen), in combination with random hexamers using 0.5 μg RNA. Purified 2.5 ng of cDNA was mixed with Maxima™ SYBR Green qPCR Master Mix (Fermentas) and primer mix (0.3 μM) and amplification (95 °C for 10 min and 40 cycles of 95 °C for 15 s, 60 °C for 30 s) was carried out on a Chromo4 thermocycler (Biorad) or LightCycler 480 (Roche) (N = 2, n = 3). The GeNorm™ freeware (http://medgen.ugent.be/-jvdesomp/genorm/) was used to determine that GAPDH and ACTB were the two most stable housekeeping genes. The relative transcript quantity (Q) was determined by the ΔcT method $Q_{\text{normalization}} = [Q_{\text{gene of interest}}/NF]$ where as $Q_{\text{gene of interest}} = 2^{\Delta Ct}$, $\Delta Ct = (Ct_{\text{minimal cycle}} - Ct_{\text{Sample}})$ and NF = geometric mean of $[(\Delta Ct_{HSK1} = (Ct_{\text{minimal HSK1}} - Ct_{\text{Sample}}), [(\Delta Ct_{HSK2} = (Ct_{\text{minimal HSK2}} - Ct_{\text{Sample}})]$ described in Vandesompele et al. [30].

2.12. Immunostaining

E/F MIAMI cells alone or adhered on Blank PAMs or BDNF releasing PAMs were stained for NFM immunocytofluorescence based on a previous publication [25]. A rabbit anti-NFM (1:50, Abcam, Paris France) was used and IgG1k was used as isotypic control (1:50, BD Biosciences, Rungis, France). Detection was performed with a secondary biotinylated anti-mouse (1:200, Eurobio, Courtaboeuf, France) and streptavidin– rhodamine (1:500, Dako) and DAPI (1:1000, Sigma). Images were acquired with a confocal microscope (Axioskop, Carl Zeiss, Le Pecq, France).

2.13. MIAMI secretome analysis

A Luminex® Multiplex secretome assay was used to quantify cytokines and growth factors secreted by MIAMI cells under different conditions. The secretome of MIAMI cells was compared to the secretome of E/F pre-treated MIAMI cells cultured for 7–10 days. The secretome of E/F MIAMI cells was then analyzed after exposure to different conditions for 7 days using 0.2×10^6 cells in 1 mL: (i) E/F MIAMI cells in corporate in 0.3 mL of Si-HPMC hydrogel, (ii) E/F MIAMI cells attached onto 0.5 mg blank-PAMs, (iii) E/F MIAMI cells attached onto 0.5 mg BDNF-PAMs, and (iv) E/F MIAMI cells-BDNF PAM complexes incorporated in 0.3 mL Si-HPMC hydrogel. Twenty-three human growth factors and chemokines (Table 2) were quantified using 3 Luminex® Multiplex assay panels: (1) ProcartaPlex® Human Chemokine Panel I 9 Plex (#EPX090-12187-901, Affymetrix, Santa Clara, CA, USA), (2) ProcartaPlex® Human Chemokine Panel 11Plex (#EPX090-12187-901, Affymetrix), (3) Milliplex MAP Kit Human Pituitary Magnetic Bead Panel 1 (#HPTP1MAG-66 K, Millipore, Billerica, MA, USA). Samples were centrifuged at 4 °C for 10 min at 10,000*g* and prepared as per the manufacturer’s recommendations using a Bio-Plex Pro wash station (Bio-Rad, Hercules, CA). No sample dilution was performed. Quantification of growth factors was performed on a Magpix apparatus (Bio-Rad) and analyzed with the Bio-Plex Manager Version 3.0 software (Bio-Rad). Background subtraction was performed with MIAMI expansion media, or with E/F media for E/F MIAMI cells (n = 3).

Table 2
Luminex® Multiplex secretome assay.

Growth factors	Brain-derived neurotrophic factor (BDNF), beta polypeptide – Nerve growth factor (b-NGF),stem cell factor(SCF), Leukemia inhibitory factor (LIF), Ciliaryneurotrophic factor (CNTF), Epidermal growth factor (EGF), Fibroblast growth factor 2 (FGF-2), Hepatocyte growth factor (HGF), Platelet derived growth factor-BB (PDGF-BB), Placenta growth factor-1 (PlGF-1), Stromal cell-derived factor 1 alpha (SDF-1α), Vascular endothelial growth factor-A (VEGF-A), Vascular endothelial growth factor-D (VEGF-D)
Chemokines	Monocyte chemoattractant protein-1 (MCP-1), Macrophage inflammatory protein-1α (MIP-1α), Macrophage inflammatory protein-1β (MIP-1 β), Regulated upon activation normal T-cell expressed and secreted (RANTES), Interleukin 8 (IL-8), IFN-gamma-inducible protein 10 (IP-10), Growth-regulated protein alpha (GRO-α), Eotaxin
Neuropeptide	Agouti related neuropeptide (AGRP)

2.14. Statistical analysis

Data are presented as the mean value of three independent experiments ±standard deviation (SD). Significant differences between samples were determined using an ANOVA test, followed by Dunnett’s multiple comparison tests, unless otherwise stated. Threshold P-value was set to 0.05, unless otherwise stated. In the figures, *indicates significant difference at $p < 0.05$, **at $p < 0.01$, ***at $p < 0.001$.

3. Results

3.1. Optimization of BDNF nanoprecipitation

To determine the optimal nanoprecipitation conditions for BDNF, the model protein α-chymotrypsin was used. As the efficiency of nanoprecipitation varies with the process conditions, the composition of the medium was varied within a defined experimental domain. The results allow determining the most important parameters affecting the nanoprecipitation yield and also show which factors interact with each other to influence the nanoprecipitation yield. We first observed that the protein concentration greatly influenced protein precipitation (Supplementary file: Table-1). Out of two concentrations tested (5.85 mg/mL and 10 mg/mL), based on our previous results [24,25], we observed that 10 mg/mL gave the higher precipitation yield of α-chymotrypsin. The studied interactions are significant (Supplementary file: Table-2); the effects of NaCl concentration, $ZnCl_2$ concentration and presence of P188 solution with salts on the protein precipitation yield are dependent (Fig. 1). According to Fig. 1A, B, C the effects of NaCl concentration or $ZnCl_2$ concentration on the protein precipitation yield, depend on P188. Without P188, [NaCl] and $[ZnCl_2]$ have no effect but they have an impact on the precipitation yield when they are associated to P188 + NaCl or P188 + $ZnCl_2$ (Fig. 1A and B respectively, red and green bars). Moreover the effects of [NaCl] and $[ZnCl_2]$ on protein precipitation yield are also dependent (Fig. 1C). So taking into account the interaction between the studied parameters, the best conditions for α-chymotrypsin nanoprecipitation is obtained by the combination of a 10 mg/mL protein concentration without NaCl in the protein solution (Fig 1A, red), or with $ZnCl_2$ in the protein solution (Fig 1B red, Fig 1C green) and with P188 + NaCl (Fig 1A and B red). We decided to use only one type of salt, preferably NaCl, to precipitate the BDNF. Thus, the best condition to nanoprecipitate BDNF would be to dilute BDNF at 10 mg/mL in water and to add a solution of P188 (200 mg/mL) in 0.3 M NaCl. In this condition BDNF nanoprecipitation yield was 92.2 ± 10%.

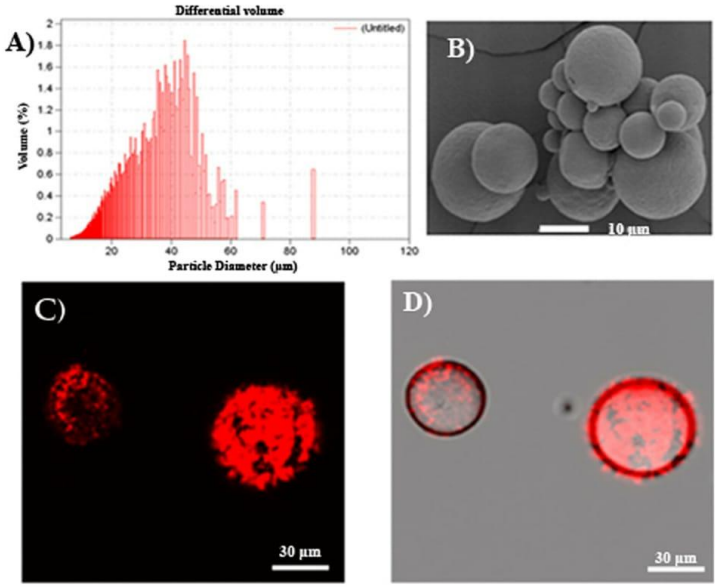

Fig. 2. Characterization of PAMs. (A) Average diameter and size distribution of BDNF loaded PLGA–P188–PLGA MS. (B) SEM imaging of PLGA–P188–PLGA MS showing their rough surface. (C) Fibronectin (FN) surface coating of PLGA–P188–PLGA PAMs observed after FN immunofluorescence by confocal microscopy shows that they are well covered by FN. (D) Overlay of FN immunofluorescence image on differential interference contrast (DIC) microscopic image.

3.2. Characterization of PAMs

PAMs had a mean diameter of 33 ± 12 µm (Fig. 2A) and a spherical shape with a rough surface (Fig. 2B). Fibronectin coating can be detected at the surface of PAMs (Fig. 2C). The coating was more or less homogenous but present in all the PAMs (Fig. 2C & D). In addition, microsphere zeta potential shifted from −23 ± 1.2 mV to 41 ± 6.6 mV when they were incubated with fibronectin and poly-D-lysine, confirming the surface modification of PAMs with poly-lysine. The encapsulation yield of BDNF in PLGA-P188-PLGA microspheres was 76.9 ± 3.3%.

3.3. BDNF in vitro release

Preliminary data indicated that protein release was similar whether the microspheres were coated (PAMs) or not; there was only a slight difference during the first hours of release. Therefore, the presented release study data were obtained with microspheres. BDNF release medium (0.01 M citrate buffer + 0.1% BSA at pH 5) was selected to reduce BDNF re-adsorption at the surface of microspheres or PAMs after being released (data not shown).

The BDNF release study for 40 days showed a controlled and sustained cumulated BDNF release of 96.3 ± 0.5%. BDNF release profile was bi-phasic with a linear sustained release for the first two weeks with no burst effect. Over 29.7 ± 4.1% of encapsulated BDNF was released (Fig. 3A). Then, from 2 to 6 weeks, the release accelerated to reach 96.3 ± 0.5% of released BDNF after 5 weeks. During the first two weeks, the release rate was about 0.42 ± 0.05 %/day/mg microspheres which corresponded to a dose 32.49 ± 0.93 ng/day/mg to then increase to 1.01 ± 0.05 %/day/mg or 78.06 ± 2.1 ng/day/mg microspheres.

As the release rate is associated with the degradation of microspheres, their degradation was studied by SEM at different time-points. At day 14, the rounded shape of the microspheres was preserved but the surface seemed less smooth than at day 0 (Fig. 3B). After 3 weeks, the rounded shape was still maintained but the

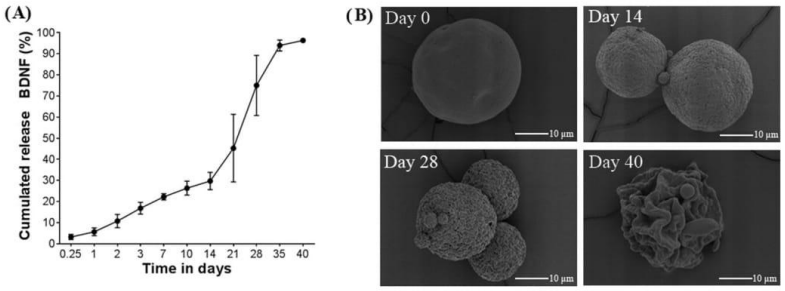

Fig. 3. *In vitro* release study of BDNF (A) Cumulated release of BDNF (%) from 5 mg microspheres using 0.01 M citrate buffer (pH 5) containing 0.1% of BSA (n = 3). (B) SEM imaging of PLGA-P188-PLGA microsphere degradation at different time intervals in 0.01 M citrate buffer-BSA at pH 5 (n = 3).

surface was very rough and a few pores were visible. At the end of the release (40 days), microspheres collapsed and lost their spherical shape.

3.4. Bioactivity of released BDNF

We investigated the bioactivity of BDNF released from microspheres in the *in vitro* release study (diluted to 5 ng/ml of BDNF according to ELISA results for the different time-points: 1, 7, 21, 28, 35 days) as well as from PAMs, using a bioassay based on rat DRG cell process extension. Based on the results of cell counts and comparing to the standard, we estimated the active BDNF concentration (in ng/mL) released in the *in vitro* release assay for all the different aliquots collected (taking into account the dilution factor) and plotted the graph against ELISA estimated BDNF release profile. The cumulative release profile of the bioactive BDNF overlapped over time the curve of BDNF measured by ELISA (Fig. 4A). Indeed, after incubation with release medium from BDNF-releasing microspheres (collected from days 1, 7, 21, 28) the number of DRG neurons presenting neurites at least 5-times longer than the cell body was similar to recombinant BDNF (5 ng/mL): 93 ± 20 cells/well compared to the control with no BDNF (43 ± 12 cells/well) (Fig. 4B) (n = 9). A slight reduction was observed at day 35 (Fig. 4A). When DRG neurons were incubated directly with 0.5 mg of BDNF-releasing PAMs 101 ± 20 cells/well exhibited extended neurites, which is significantly different from the controls (culture medium, blank PAMs (Fig. 4B)). These results suggest that the BDNF released from PAMs is bioactive.

3.5. Impact of PAM incorporation on Si-HPMC hydrogel rheological properties

The moduli (G′: elastic modulus and G″: viscous modulus) of 2% Si-HPMC hydrogel increased with time and showed little variation when blended with PAMs. After 1 h of incubation at 37 °C, Si-HPMC hydrogel had a G′ modulus of 60 Pa and a G″ modulus of 11 Pa regardless of supplementation with 0%, 0.25% or 0.5% of PAMs, respectively. After 7 days, G′ was around 250 Pa and G″ around 15 Pa. Whatever the condition, G″ was always lower than G′, illustrating the low elasticity of the gel (Supplementary file: Fig. 1).

3.6. Characterization of E/F MIAMI cells with PAMs in the Si-HPMC hydrogel

E/F MIAMI cells incorporation and culture in Si-HPMC hydrogel had no strong impact on their viability for 1 week, which was around 85% of viable cells (Supplementary file: Fig. 2). The cells in the gel remained spherical and dispersed within the hydrogel (Supplementary Fig. 2C). All the E/F MIAMI cells attached to blank PAMs as well as to PAMs releasing BDNF and formed 3D complexes within 4 h (Fig. 5A). These 3D complexes are better observed by SEM (Fig. 5B & C). After 7 days of culture, E/F MIAMI cells on PAMs formed bigger aggregates (Fig. 5D). Moreover, we showed in a previous study [17,25] that cell association with PAMs maintained their viability after 7 days *in vitro*. E/F MIAMI cell-PAM complexes were maintained, after incorporation in Si-HPMC hydrogel (4 h) (Fig. 5E). Small cell-PAM complexes were still visible in the hydrogel after 7 days, while bipolar shaped cells and cellular extensions emerged around the complexes (Fig. 5F).

3.7. Impact of PAMs and hydrogel on E/F MIAMI gene expression and immunostaining

The method used to evaluate MIAMI gene expression [30] allows comparing the expression of the different genes. No expression of pluripotent genes Nanog, SOX2, nor glial genes GFAP and Olig2 was observed in E/F MIAMI cells in any of the tested conditions. On the contrary, the neural/neuronal genes (Nestin,

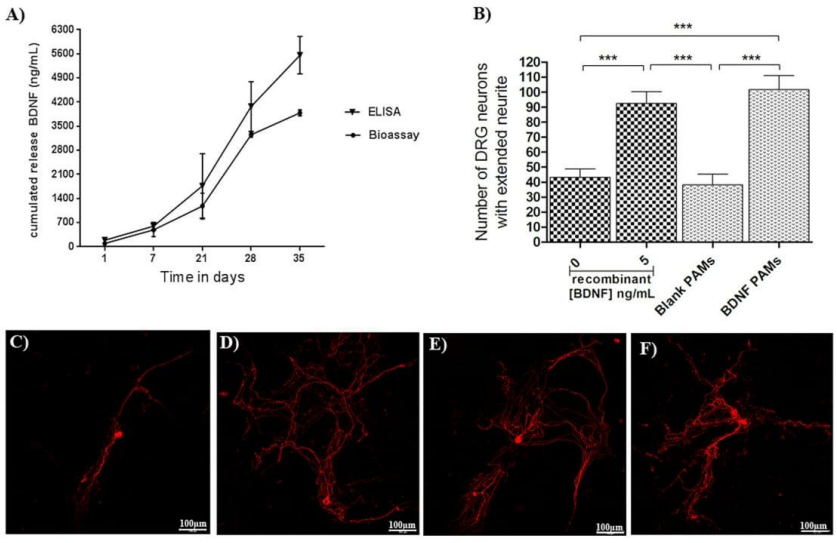

Fig. 4. BDNF bioactivity assay. A) The cumulated *in vitro* released bioactive BDNF (ng/mL) levels estimated by DRG bioassay was approximately correlated with ELISA estimated BDNF levels. B) Graphical presentation of DRG neurons (in number) with extended neurite growth shows that BDNF released from PAMs and added directly to the DRG culture acts on DRG neurons in a similar way than the recombinant BDNF (5 ng/ml positive control). The effect observed is also significantly different to the recombinant negative control (0 ng/mL) and to blank PAMs. C) Illustrative DRG neuron observed by immunofluorescence against β3-tubulin in non-treated controls. D) Illustrative DRG neuron cultured with 5 ng/mL of recombinant BDNF exhibiting extended processes. E) Illustration of DRG neuron cultured with BDNF collected at day 1 from *in vitro* release media (similar extended neurite processes was observed for day 7, 21, 28). F) Illustration of DRG neuron cultured with PAMs BDNF also showing similar extended neurite processes (n = 3).

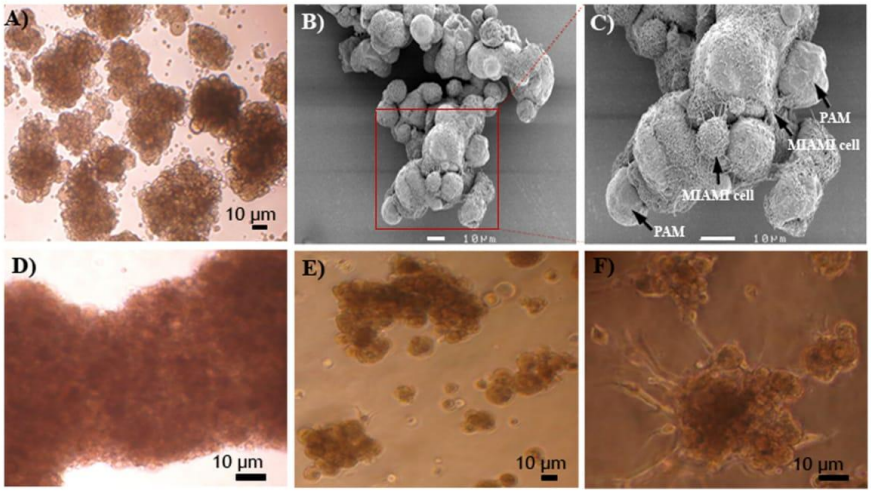

Fig. 5. Morphology of MIAMI cells/PAM complexes. A) Adherence of E/F MIAMI cells (0.2×10^6) seeded with 0.5 mg of PAMs on ultra low cluster plates was observed by optic microscopy. B & C) SEM images show 3D complexes of MIAMI cells and PAMs after 4 h. D) Optical microscopy images show these complexes aggregated together to form larger complexes after 7 days in culture E) Cell/PAM 3D complexes blended within Si-HPMC hydrogel were also observed by light microscopy after 4 h. F) 3D cell/PAM complexes remained in place and bipolar shaped cells with cellular extensions were observed after 7 days.

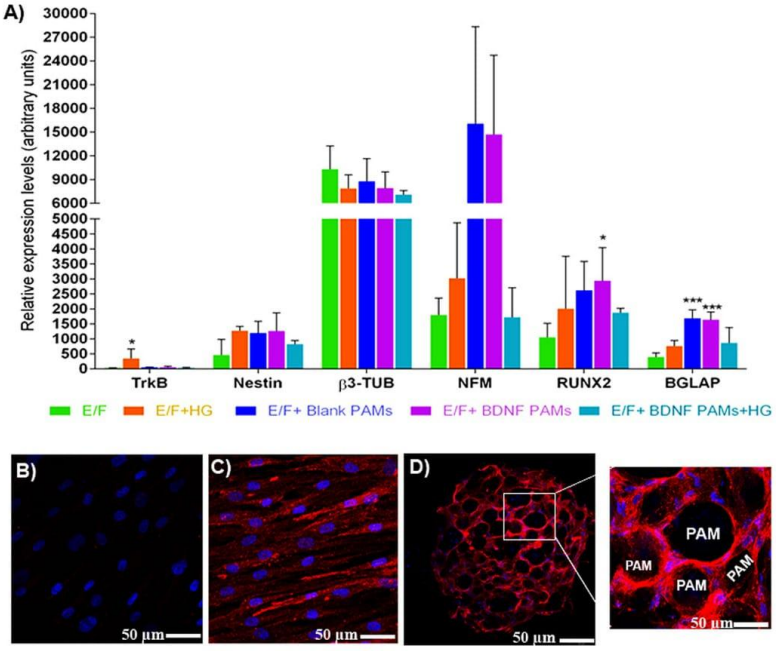

Fig. 6. Transcript expression of neural/neuronal and osteoblastic markers in E/F MIAMI cells adhered on PAMs releasing GFs and embedded in Si-HPMC hydrogel. A) The mRNA relative levels of BDNF receptor (TrkB), neural/neuronal markers (Nestin, β3-tubulin, NFM) and osteoblastic (RUNX2, BGLAP) markers were evaluated at week-1. Values are expressed as Mean ± SD of qRT-PCR arbitrary units, normalized with each other and in respect to the reference housekeeping gene expression [30]. Statistical analysis was carried out in comparison with E/F MIAMI cells (Control). *indicates significant difference p = 0.05, **p = 0.01, ***p = 0.001. (N = 2, n = 3). E/F stands for E/F pre-treated MIAMI cells. Protein expression of mature neuronal marker NFM in E/F MIAMI cells adhered to the PAMs (Blank or BDNF) and cultured for 1 week. B) E/F MIAMI cells cultured in 2D (isotypic control), (C) E/F MIAMI cells in 2D slightly expressed NFM (D) and this expression is enhanced when complexed with PAMs (Blank or BDNF).

β3-tubulin and NFM) were expressed, highlighting the neuronal commitment (Fig. 6).

TrkB gene expression (BDNF receptor), was low but above detection levels in E/F MIAMI cells and increased after culture in the Si-HPMC hydrogel. E/F MIAMI cells cultured in the hydrogel showed an increase in Nestin and a slight increase in Neurofilament expression compared to E/F MIAMI cells, while β3-Tubulin gene expression remained high and overall unaffected. When E/F MIAMI cells were complexed with blank PAMs or BDNF releasing PAMs, Nestin was again also increased when compared to E/F MIAMI cells, TrkB levels and β3-Tubulin levels remained the same, while NFM gene expression increased 8–9 folds compared to E/F MIAMI cells. However, E/F MIAMI cells adhered onto BDNF PAMs blended in Si-HPMC hydrogel showed no major change in TrkB, β3-Tubulin and NFM gene expression when compared to E/F MIAMI cells alone (Fig. 6).

To determine whether culture conditions stimulated MIAMI cell differentiation to other lineages, gene expression of chondrogenic (Col2a1), adipogenic (FABP4) and osteoblastic (RUNX2 and BGLAP) markers was assessed (Fig. 6A). The chondrogenic Col2a1 and adipogenic FABP4 markers were not detected whatever the condition tested. The expression of the early osteoblastic marker, RUNX2, was increased in the E/F MIAMI cells cultured in hydrogel and on PAMs with or without hydrogel compared to 2D conditions. There was a slight expression of the mature osteoblastic marker BGLAP by E/F MIAMI cells, the expression was significantly higher when E/F MIAMI cells were cultured on PAMs (blank and BDNF) but not when grown in the hydrogel.

To confirm the higher NFM gene expression of E/F MIAMI cells complexed with PAMs, immunostaining against NFM was performed (Fig. 6B, C & D). After immunostaining we observed that the protein was only slightly expressed in the cells cultured in 2D (Fig. 6C). The expression of NFM protein was enhanced when the cells were complexed with PAMs (Blank or BDNF) (Fig. 6D).

3.8. Impact of PAMs and hydrogel on E/F MIAMI cell secretome

The secretome profile of E/F MIAMI cells was compared to naïve MIAMI cells (Fig. 7A & B), and the effect of PAMs and hydrogel on the secretome profile of E/F MIAMI cells was also assessed (Fig. 7C & D). MIAMI cells secreted growth factors (Fig. 7A) like BDNF, SDF-1α and VEGF-A (between 7 to 600 pg/mL) as well as chemokines (Fig. 7B) like RANTES, MIP-1α, IL-8, MIP-1β and MCP-1(between 4 to 120 pg/mL). Pre-treatment with E/F significantly increased the secretion of VEGF-A (2 folds), but also specifically induced the production of PIGF-1, b-NGF, LIF, SCF and HGF (between 1 to 1100 pg/mL) (Fig. 7A). VEGF and HGF were secreted at particularly high levels. However, E/F pre-treatment had a limited impact on chemokine secretion, MCP-1 being the only one that significantly increased (3 folds) upon E/F treatment (Fig. 7).

When E/F MIAMI cells were incorporated in the hydrogel and cultured for 7 days, cells were able to secrete growth factors like

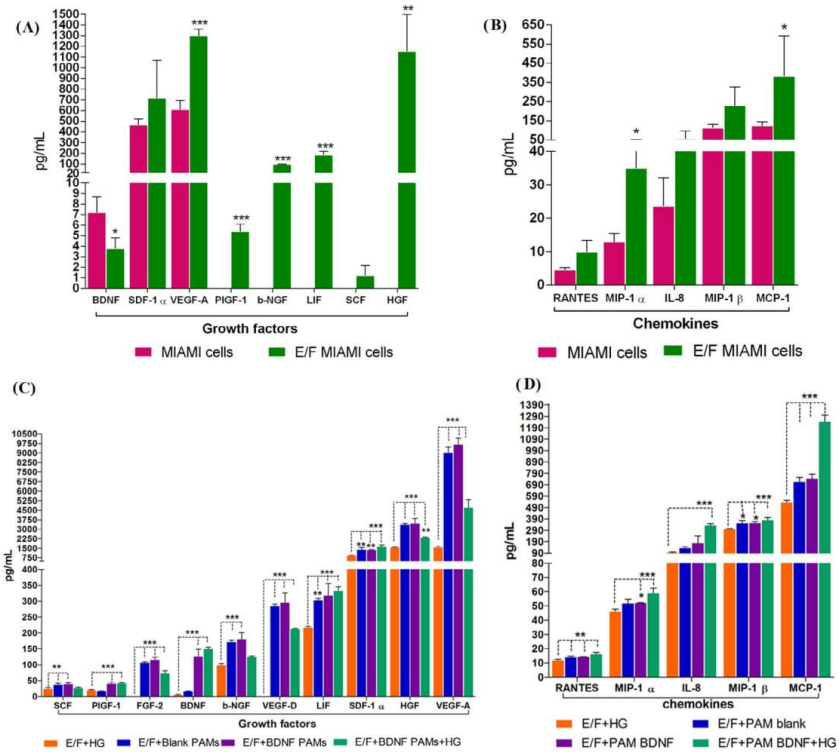

Fig. 7. Secretome profile of MIAMI cells and E/F pretreated MIAMI cells cultured in different conditions. Growth factor (A) and chemokines (B) secretion of MIAMI cells and E/F MIAMI cells cultured in 2D. Growth factor (C) and chemokines (D) secretion of E/F MIAMI cells cultured in 3D environment includes Si-HPMC hydrogel, PAMs (blank or BDNF) and combination of both. E/F in the graphs means E/F pre-treated MIAMI cells. Values are expressed as mean ± SD in pg/mL (n = 3) and statistical significance was compared with E/F MIAMI cells blended in Si-HPMC hydrogel for (C&D). *p < 0.05; **p < 0.01; ***p < 0.001.

SCF, PlGF-1, BDNF, b-NGF (between 5 to 100 pg/mL) and LIF, SDF-1α, HGF and VEGF-A (between 200 to 1500 pg/mL) (Fig. 7C). In addition to this, the secretion of chemokines like RANTES, MIP-1α, IL-8, MIP-1β and MCP-1 (between 12 to 500 pg/mL) was also observed (Fig. 7D). When the E/F MIAMI cells were complexed with PAMs (blank and BDNF) the secretion of growth factors like SCF, PlGF-1, BDNF, b-NGF (between 30 to 200 pg/mL) and LIF, SDF-1α, HGF and VEGF-A (between 300 to 9500 pg/mL) was significantly increased compared with E/F MIAMI cells culture alone in the hydrogel. Very high levels of VEGF-A were detected (around 9500 ng/mL). The amounts of BDNF detected in the media, was probably also due to its release from the PAMs, masking any other differential expression. In addition, VEGF-D (average of 290 pg/mL) and FGF-2 (average of 111 pg/mL) were secreted only when E/F MIAMI cells adhered onto PAMs (Fig. 7C). Moreover, all the growth factors, except b-NGF and all the chemokines were significantly increased, when E/F MIAMI cells complexed with PAMs (BDNF) were incorporated in the hydrogel compared with cells alone in the hydrogel (Fig. 7C and D). Particularly high levels of the chemokine MCP-1 (around 1200 ng/mL) were measured in this condition. Small quantities of IP-10 (<4 pg/mL) were detected in all the conditions. Noteworthy, EGF, CNTF, PDGF-BB, GRO-α, Eotaxin, AGRP and GH were not detected whatever the condition.

4. Discussion

Easily accessible adult bone marrow-derived MSCs secrete many factors that could stimulate tissue repair and more particularly CNS repair. Their potential for cell therapy in the scope of neurological disorders is currently being assessed in clinical studies [31]. Combining them to injectable biomaterial-based scaffolds and growth factors to induce and maintain their neuronal commitment is important for their safe use in neuroregenerative medicine. Furthermore, a hydrogel matching the mechanical properties of the tissue may help maintain the cells and PAMs within the lesion cavity and could be useful for certain applications such as spinal cord lesions. Finally, the long-term release of physiological doses of BDNF, a neurotrophic factor with a wide-range effect, constitutes a promising therapeutic approach to promote tissue repair in neurological disorders [21]. In this study, we designed a novel well-characterized non-toxic injectable biomaterial-based carrier delivering MSCs and BDNF, enhancing cell neuronal commitment and the secretion of tissue repair factors that could positively impact neuronal regenerative medicine and be safely implanted in the brain. In this paper we showed for the first time i) a complete and sustained release of bioactive BDNF for around 40 days from PLGA-based PAMs, that are able to convey cells on their surface; ii) an enhanced secretion of several growth factors and chemokines from E/F pre-treated MIAMI cells thus specified towards the neuronal lineage; iii) the induction towards a neuronal commitment and the secretion of higher levels of biofactors as well as novel cytokines when these cells adhered onto PAMs releasing or not BDNF, compared to the cells in the Si-HPMC hydrogel alone; iv) PAM/cell complexes placed within a non-toxic hydrogel with an elasticity similar to nervous tissue, conveying cells with an elongated morphology tending to release similar to lower levels of growth factors and more chemokines when compared to cells on PAMs.

Efficient and sustained release of proteins from FDA approved PLGA-based scaffolds remains a challenge due to protein instability during the formulation process and release [24,32,33]. In previous studies, we have shown that solid-state proteins are better preserved upon encapsulation and during release as they are less sensitive to denaturation and exhibit restricted conformational flexibility [17,19,24,25]. In the present study, the model protein α-chymotrypsin, chosen for similar physico-chemical properties to BDNF, was nanoprecipitated with either zinc or sodium salts. Moreover, the presence of poloxamer (P188) with sodium salt increased the nanoprecipitation yield. The conditions which gave the maximum nanoprecipitation yield for the model protein were translated to BDNF and achieved nearly 100% nanoprecipitation yield. It has been reported that poloxamer P188 when blended with protein or PLGA enhances protein stability [34]. Moreover, studies reported that P188 possesses neuroprotective properties [35,36]. In previous studies, 100% nanoprecipitation has been obtained with other proteins without the poloxamer [24,25]. These results show that the nanoprecipitation yield is protein-specific and depends on the molecular weight and isoelectric point among other protein properties and should be set-up first with appropriate model proteins that are cost-effective before applying the process to therapeutic proteins.

The more hydrophilic triblock-in-triblock polymer, PLGA-P188-PLGA, was selected to encapsulate the nanoprecipitated BDNF. Based on previous studies, [17] we had reasons to believe that it would allow a complete release of BDNF within a reasonable window (around 1 month). And indeed, we demonstrated for the first time that a sustained and complete release (>90%) of BDNF could be achieved after encapsulation in microspheres. The amount of BDNF released per day, during the first and second phase, was in the range of BDNF therapeutic dose (33 ng/day) for an easily injectable dose of PAMs (0.5 mg). In a previous study using different PLGA-based copolymers, either a very strong initial burst (more than 50% of the encapsulated BDNF) was obtained or on the contrary less than 10% release occurred during the first 2 weeks, followed by a sustained and complete release of BDNF [37]. Moreover, in our study BDNF maintained its bioactivity throughout the release, and more importantly, released BDNF from injectable amounts of PAMs in physiological conditions (added directly to the culture) induced the survival and differentiation of DRG neurons.

The mechanical properties of the surrounding or underlying environment influence the stem cells viability and differentiation. The E/F pre-treated MIAMI cell specification towards a neural/neuronal lineage was previously reported [10]. In our study, the cells were further induced toward the neuronal lineage due to the 3D environment provided by the PAMs, which was evidenced by the expression of mature neurofilament protein. The rheological characterization of the Si-HPMC hydrogel showed that, at 7 days, its stiffness was around 500 Pa, which may evolve over time to a modulus slightly higher than 1 kPa [27,38], and was not influenced by the incorporation of PAMs. Recently Saha et al. reported that a synthetic hydrogel with a similar stiffness (</=500 Pa) facilitated neural stem cell (NSCs) differentiation, with hard surfaces favouring astrocytic differentiation and soft surfaces promoting neuronal differentiation [39]. Similarly, MSCs in a soft 3D environment (1 kPa) differentiated toward a neuronal lineage while stiffer substrate promoted glial differentiation [40]. Moreover, the 2% Si-HPMC hydrogel gels at pH 7.4, which is close to the CSF pH (7.3) as well as to plasma pH (7.3–7.4). Recently Emily R. et al. reported that when the pH of the hydrogel matches the pH of the injection site, the incompletely polymerized hydrogel solution can subsequently continue polymerizing in response to the *in situ* pH which allows the hydrogel to fill an irregularly shaped lesion site or coat the lesion surface [12]. It has also been shown that fibronectin on stiffer substrates stimulates the formation of neurites, suggesting that as the neuronal differentiation progresses a stiffer substrate is required [41]. Accordingly, the fibronectin-covered PAMs could stimulate the neuronal differentiation of MIAMI-derived neuronal committed cells expressing high levels of the neurofilament gene. Fibronectin distribution on the surface of PAMs may also affect mesodermal cell fate. It has been shown that large fibronectin islands on top of microcarriers offered an appropriate anchorage

for MSCs stimulating osteogenesis, while inhibiting adipogenesis and chondrogenesis [42]. We could thus hypothesize that the heterogenous fibronectin coating of the PAMs could be responsible for the lack of adipogenic and chondrogenic markers expression, while osteogenic markers were detected. Although RunX2 is a well-known master regulator of bone development, recently Runx2 expression in the brain has been reported [43] suggesting a possible role of RunX2 in neuronal function. It should also be noted that the primer-pair used to detect BGLAP transcripts was the best we could use; however, this primer-pair can also detect naturally occurring read-through transcript variants not encoding a functional BGLAP transcription. Further studies are necessary to clearly conclude on osteoblastogenesis.

Cell morphology also differed depending on the 3D environment, round and dispersed in the hydrogel, forming spheres with the PAMs, and with some cellular extensions when the cell/PAM complexes were embedded in the hydrogel. These cell extensions although not reminiscent of thin neurites do merit further attention, as changes in cell morphology concomitant with cytoskeletal reorganizations are required for neuronal differentiation. Further studies *in vivo* or in *ex vivo* organotypic brain cultures modeling neurological disorders [20] should be performed to better understand the long-term behavior of the cells within these biomaterials. Finally, biodegradability of the Si-HPMC hydrogel should be assessed after implantation in the central nervous system as we can now only hypothesize that it could be biodegraded by reactive microglia/macrophages in a similar way than what was described after implantation in bone [44].

It is widely admitted that the tissue regenerative properties of adult stem cells are mediated, at least partly, by the large variety of factors they secrete [2,4]. Secretion by MIAMI cells of high levels of some tissue repair factors like VEGF and fractalkine were previously reported [1,9]. The secretome analysis in this study clearly identifies that E/F pre-treatment of MIAMI cells further increases the secretion of a variety of cytokines that are able to induce angiogenesis, neurogenesis, and affect cellular migration and immunosuppression. After E/F pre-treatment, MIAMI cells secrete growth factors like b-NGF, SDF-1α and HGF, which are further increased with the PAMs, combined or not to the hydrogel. These factors have been reported to be involved in MSC differentiation towards a neuronal lineage and in their neuroprotective effects [45–47]. The increase in LIF secretion further strengthens the neural repair capacities of the E/F MIAMI cells in the combined scaffold. Indeed, it was reported that LIF augments corticospinal axon growth and expression of NT-3 after spinal cord injury [48] and that the addition of LIF to cells treated with NGF increases neurite length and induces neurite restructuration *in vitro* [49]. Secretion of angiogenic factors like VEGF-A and PlGF-1 were also increased. It was more pronounced with PAMs combined or not to the hydrogel. In addition to its classical role in angiogenesis, VEGF-A also promotes a wide range of neuronal functions, both *in vitro* and *in vivo*, including neurogenesis, neuronal migration, neuronal survival and axon guidance [50]. Accordingly, previous results showed that these cells combined to PAMs preserve blood vessels near their site of transplantation, probably increasing their survival but also participating to enhance their neuroprotective role [20]. Furthermore, in our study, FGF-2 and VEGF-D are only secreted by cells on PAMs combined or not to the hydrogel, confirming a previous report that the expression of FGF-2 and VEGF by MSCs is enhanced on microcarrier cultures compared with 2D cultures [51]. It has also been shown that a stiffer substrate stimulates VEGF production compared to a softer one [52].

Immunological responses triggered after central or peripheral nervous system injury are very complex and some studies have shown that a certain degree of inflammation has beneficial effects after injury influencing axonal outgrowth [53]. The secretion of pro inflammatory cytokines like IL-8, MIP-1 β were similar for MIAMI cells pre-treated or not with E/F except for MIP-1 α, and MCP-1. The secretion of these cytokines was further increased when the cells were combined to PAMs and particularly with BDNF PAMs combined with the hydrogel. Interestingly, MIP-1 α and MCP-1 are able to promote cellular migration of MSCs and the latter also has cell protective effects under hypoxic conditions [2]. BDNF release had no major effect on the secretion of the other cytokines. It could be due to the short time of exposure (7 days). Nevertheless, the prolonged delivery of BDNF by the PAMs will be essential to enhance the nervous tissue repair capacities of the combined device. Indeed, the neuroprotective and lesion repair capacities of BDNF delivery has been demonstrated in many animal models of neurological disorders [54–57]. The E/F MIAMI cells combined to PAMs delivering BDNF could be used alone or associated to the hydrogel, as different cell phenotypes as well as tissue repair properties are observed. Further *in vivo* evaluation of Si-HPMC hydrogel biodegradability in the central nervous system and comprehension of the mechanisms involved in neural repair of the combined system (E/F MIAMI cells + BDNF PAMs + Si-HPMC hydrogel) is needed to assess its functional potential for neural regeneration and functional recovery.

5. Conclusion

In conclusion, these results showed that PAMs with a biomimetic coating of FN and releasing bioactive BDNF in a sustained manner combined or not with Si-HPMC hydrogel could be used as a therapy to convey E/F MIAMI cells, guide their neuronal differentiation and further improve their protective/reparative properties. The released BDNF and the enhanced secretion of growth factors by the MIAMI cells could potentially have a synergistic action on damaged neural tissues by protecting and repairing neurons, and also by preserving the vascular bed and stimulating axonal growth in the context of neurological disorders.

Conflicts of interest

The authors confirm that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2016.11.030.

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Supplementary file

Table-1: D-optimal experimental design.

Exp N°	[α-chymotrypsine] (mg/mL)	[NaCl] (M)	[$ZnCl_2$] (M)	Additives (P188)	Precipitation Yield (%)
1	10	0	0	0	3.80
	10	0	0	0	2.00
	10	0	0	0	4.20
2	5.85	0.16	0	0	2.00
	5.85	0.16	0	0	5.60
3	5.85	0.3	0	0	3.20
	5.85	0.3	0	0	9.60
	5.85	0.3	0	0	3.30
4	5.85	0	0.008	0	7.00
	5.85	0	0.008	0	6.80
	5.85	0	0.008	0	7.70
5	10	0.16	0.008	0	2.10
	10	0.16	0.008	0	9.10
	10	0.16	0.008	0	3.20
6	5.85	0.3	0.008	0	2.60
	5.85	0.3	0.008	0	4.50
	5.85	0.3	0.008	0	2.80
7	5.85	0	0.016	0	7.50
	5.85	0	0.016	0	7.70
	5.85	0	0.016	0	7.00
8	5.85	0.16	0.016	0	11.40
	5.85	0.16	0.016	0	5.30
	5.85	0.16	0.016	0	15.40
9	10	0.3	0.016	0	7.70
	10	0.3	0.016	0	4.80
	10	0.3	0.016	0	10.30
10	5.85	0	0	NaCl	23.50
	5.85	0	0	NaCl	13.60
	5.85	0	0	NaCl	16.60
11	5.85	0.16	0	NaCl	0.50
	5.85	0.16	0	NaCl	1.90
	5.85	0.16	0	NaCl	0.50
12	10	0.16	0	NaCl	78.30
	10	0.16	0	NaCl	69.60
	10	0.16	0	NaCl	74.10
13	5.85	0.3	0	NaCl	11.40
	5.85	0.3	0	NaCl	9.80

	5.85	0.3	0	NaCl	11.00
14	10	0.3	0	NaCl	15.00
	10	0.3	0	NaCl	14.70
	10	0.3	0	NaCl	15.70
15	5.85	0	0.008	NaCl	1.80
	5.85	0	0.008	NaCl	5.30
	5.85	0	0.008	NaCl	1.40
* 16	10	0	0.008	NaCl	87.80
	10	0	0.008	NaCl	92.70
	10	0	0.008	NaCl	77.00
17	5.85	0.16	0.008	NaCl	17.70
	5.85	0.16	0.008	NaCl	15.10
	5.85	0.16	0.008	NaCl	15.50
18	10	0.3	0.008	NaCl	17.80
	10	0.3	0.008	NaCl	19.50
	10	0.3	0.008	NaCl	18.20
19	10	0	0.016	NaCl	49.90
	10	0	0.016	NaCl	51.40
	10	0	0.016	NaCl	48.50
20	5.85	0.16	0.016	NaCl	20.30
	5.85	0.16	0.016	NaCl	19.70
	5.85	0.16	0.016	NaCl	20.30
21	5.85	0.3	0.016	NaCl	20.40
	5.85	0.3	0.016	NaCl	19.60
	5.85	0.3	0.016	NaCl	19.50
22	10	0	0	$ZnCl_2$	22.70
	10	0	0	$ZnCl_2$	22.20
	10	0	0	$ZnCl_2$	23.10
23	5.85	0.16	0	$ZnCl_2$	14.40
	5.85	0.16	0	$ZnCl_2$	11.00
24	10	0.3	0	$ZnCl_2$	10.50
	10	0.3	0	$ZnCl_2$	12.00
	10	0.3	0	$ZnCl_2$	11.90
25	5.85	0	0.008	$ZnCl_2$	0.30
	5.85	0	0.008	$ZnCl_2$	2.00
	5.85	0	0.008	$ZnCl_2$	0.50
26	10	0	0.008	$ZnCl_2$	17.70
	10	0	0.008	$ZnCl_2$	19.00
	10	0	0.008	$ZnCl_2$	17.80
27	5.85	0.16	0.008	$ZnCl_2$	15.40
	5.85	0.16	0.008	$ZnCl_2$	12.10
	5.85	0.16	0.008	$ZnCl_2$	17.90
28	10	0.3	0.008	$ZnCl_2$	10.60
	10	0.3	0.008	$ZnCl_2$	11.20
	10	0.3	0.008	$ZnCl_2$	11.40
29	5.85	0	0.016	$ZnCl_2$	16.80

	5.85	0	0.016	$ZnCl_2$	16.50
	5.85	0	0.016	$ZnCl_2$	25.20
30	10	0.16	0.016	$ZnCl_2$	33.70
	10	0.16	0.016	$ZnCl_2$	38.90
	10	0.16	0.016	$ZnCl_2$	38.60
31	5.85	0.3	0.016	$ZnCl_2$	12.20
	5.85	0.3	0.016	$ZnCl_2$	10.40
	5.85	0.3	0.016	$ZnCl_2$	27.70

Table-2: Statistical analysis of the coefficients estimated of the mathematical model.

	Coefficient	t.exp.	Significance. %
β0	18.416		
β1	-10.084	-27.05	< 0.01 ***
β2	2.694	5.43	< 0.01 ***
β3	4.023	7.81	< 0.01 ***
β4	-2.868	-5.61	< 0.01 ***
β5	-3.066	-6.18	< 0.01 ***
β6	-9.090	-17.44	< 0.01 ***
β7	10.509	21.74	< 0.01 ***
β8	-6.969	-9.69	< 0.01 ***
β9	5.041	7.48	< 0.01 ***
β10	6.391	8.82	< 0.01 ***
β11	-4.001	-5.59	< 0.01 ***
β12	-2.692	-3.74	0.0418 ***
β13	6.674	9.82	< 0.01 ***
β14	-3.483	-4.64	< 0.01 ***
β15	-1.042	-1.49	14.1
β16	1.142	1.54	12.9
β17	0.187	0.28	78.4
β18	2.190	3.04	0.350 **
β19	1.355	2.00	5.1

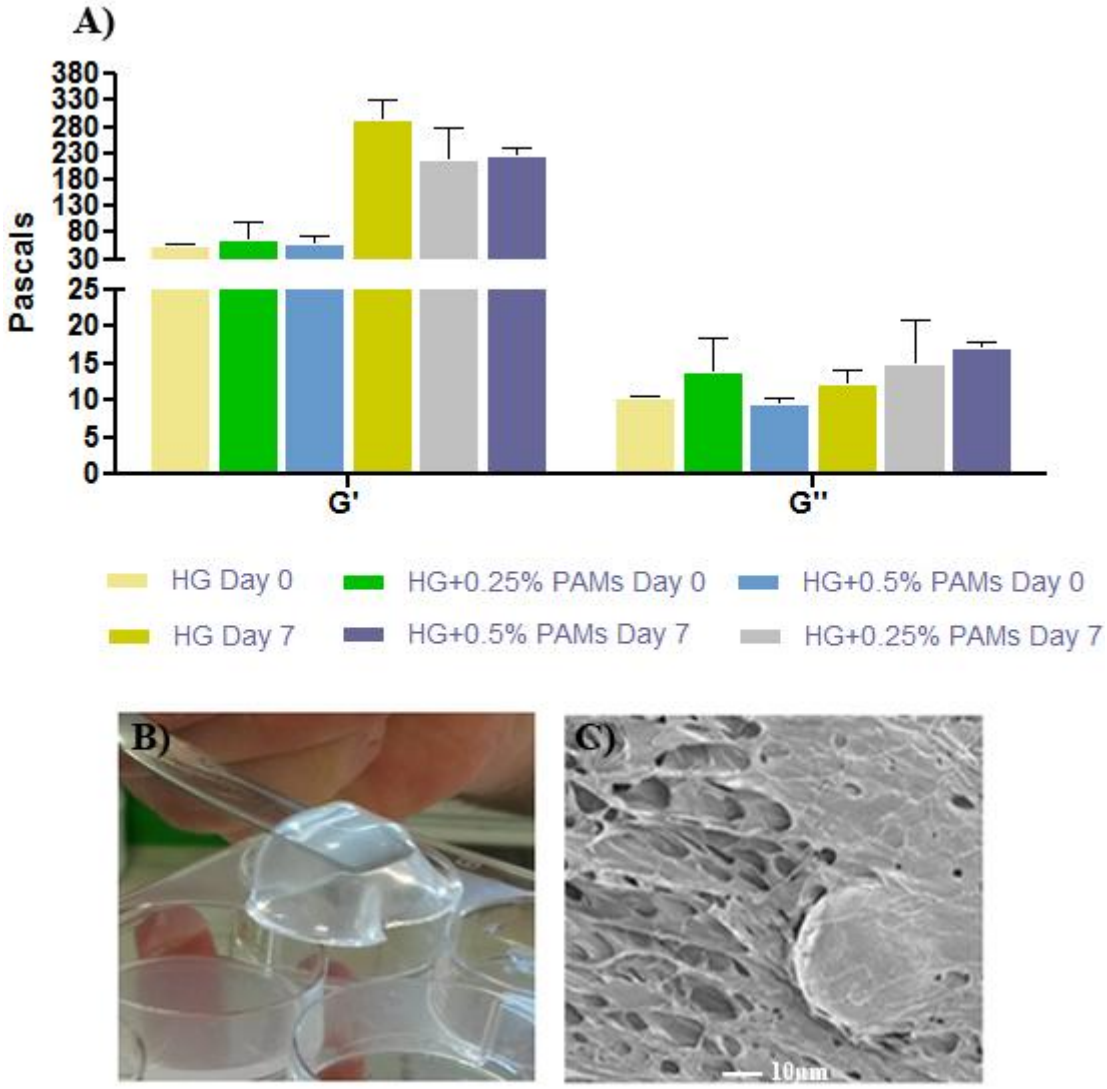


Figure-1: Rheological characterization of 2% Si-HPMC hydrogel loaded with different concentrations of PAMs at different time intervals (A). The viscoelastic moduli of hydrogel increased with time and the addition of injectable doses of PAMs into the hydrogel did not induce significant changes. Morphology of 2% Si-HPMC hydrogel blended with PAMs after 7 days (B) SEM images of Si-HPMC hydrogel bearing PAMs (C).

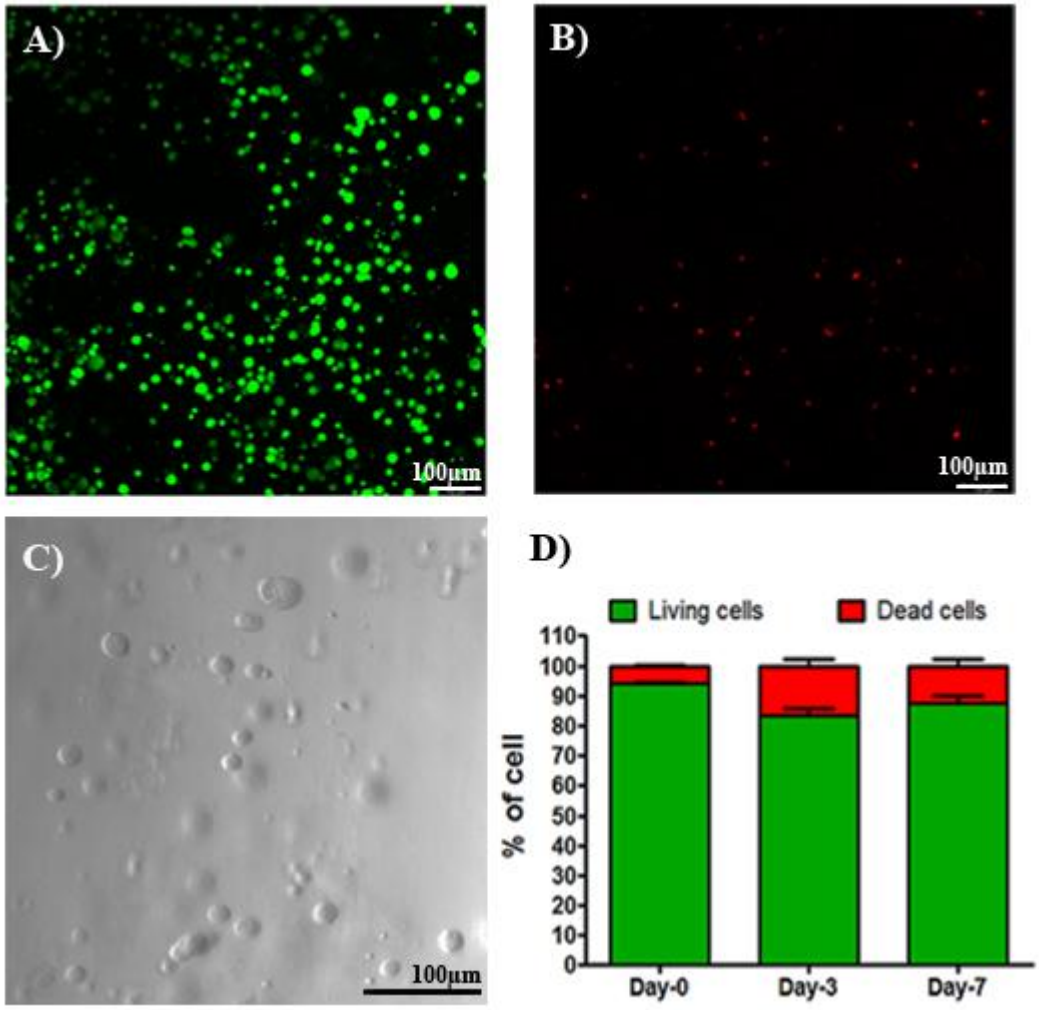


Figure-2: Confocal microscopic images of viable (green) cells (A) and non-viable (red) cells (B) cultured for 7 days in 2% Si-HPMC hydrogel by LIVE/DEAD™ assay. Optical microscopic image (C) shows cells remain spherical in shape. Image analysis of the percentage of living cells vs dead cells cultured in hydrogel at different time-points (0, 3 and 7 days) shows a maximum of 20% of non-viable cells (D) (n=3).

In vitro release study of BDNF

Materials & Methods:

To identify the optimal buffer to preserve BDNF stability during the *in vitro* release study at low concentrations (50 ng/mL) at 37°C and during different incubation periods (6hrs, 1, 3 and 7 days), ultrapure water, ultrapure water with 0.1% bovine serum albumin (BSA), phosphate-buffered saline (PBS) pH 7.4, PBS with 0.1% BSA pH 7.4, or citrate buffer 0.01 M with 0.1% BSA pH 5 were tested. Both PBS and citrate buffers with BSA preserved BDNF stability as measured by ELISA (Supplementary file: Figure-3). Furthermore, *in vitro* degradation of microspheres (5 mg) was evaluated in both buffers after incubation at 37 °C, shaking at 125 rpm at different times (day 0, 14, 28 and 40). Samples were observed for changes in the surface morphology over time using SEM as previously described [234] (Supplementary file: Figure-4). The *in vitro* release of BDNF from PLGA-P188-PLGA microspheres (5 mg) was performed in 500 µL of PBS-BSA (pH 7.4) and citrate buffer 0.01 M (pH 5) containing 0.1% w/v BSA at 37 °C under agitation (125 rpm) (n=3). At different times, the tubes were centrifuged for 5 min at 2800 g and the supernatant was collected for analysis and replaced by fresh buffer. The amount of released BDNF was evaluated by ELISA. The ratio of cumulative release (in percentage) was calculated based on the total amount of protein determined from the encapsulation yield.

BDNF adsorption on microsphere surface during release

To determine whether BDNF adsorbs onto the microspheres during release, blank microspheres (5 mg), in PBS or citrate buffers containing BSA were incubated (37 °C,

125 rpm) with BDNF (50 ng/ml). After 7 days the supernatant was collected and the BDNF was measured by ELISA. The microspheres were freeze dried, washed and BDNF immunostaining was performed to detect BDNF potentially adsorbed on the surface. Anti-BDNF mouse monoclonal antibody (5 µg/mL in DPBS) was incubated with microspheres at 4 °C for overnight under rotation. After washing, samples were incubated with biotinylated anti-mouse IgG antibody (2.5 µg/mL in DPBS) for 1h at room temperature, washed and revealed with streptavidin–fluoroprobe 547 (1:500 in DPBS) before observation under confocal microscopy (Olympus Fluoview^{TM}FV 300, Rungis, France). Isotypic controls were also performed (n=3).

Results

BDNF *in vitro* release

We observed a maximum of 3.0±0.4% cumulated released BDNF after 40 days when PBS-BSA buffer was used for *in vitro* release studies (Supplementary file: Figure-4) even though BDNF stability in this buffer was around 100% (Supplementary file: Figure-3). On the contrary, *in vitro* release studies carried out with 0.01 M citrate buffer + 0.1% BSA at pH 5 for 40 days showed a controlled and sustained cumulated BDNF release of 96.3±0.5% (Supplementary file: Figure-4). To better understand the small amount of BDNF released from the microspheres in PBS-BSA buffer, we studied the morphological changes of microsphere degradation using SEM at different time intervals. We observed that the microspheres retained their shape with few pores forming on their surface over time until 40 days (Supplementary file: Figure-5). In contrast, in citrate-BSA buffer they showed a rough surface with many pores and finally collapsed at 40 days losing their spherical shape (Supplementary file: Figure-5). In

addition, after 7 days of incubation of a known quantity of recombinant BDNF along with blank microspheres, only 0.4±0.05% of BDNF was detected in PBS-BSA buffer compared to 101±1.34% in citrate–BSA buffer. Finally, an immunostaining against BDNF showed some staining on the blank microspheres incubated with BDNF in the PBS-BSA buffer (Supplementary file: Figure-6 A), while no staining was observed on the microspheres incubated in citrate-BSA buffer (Figure-6B). These results suggest that BDNF probably adsorbed to the microspheres in PBS buffer, thereby explaining the low release observed.

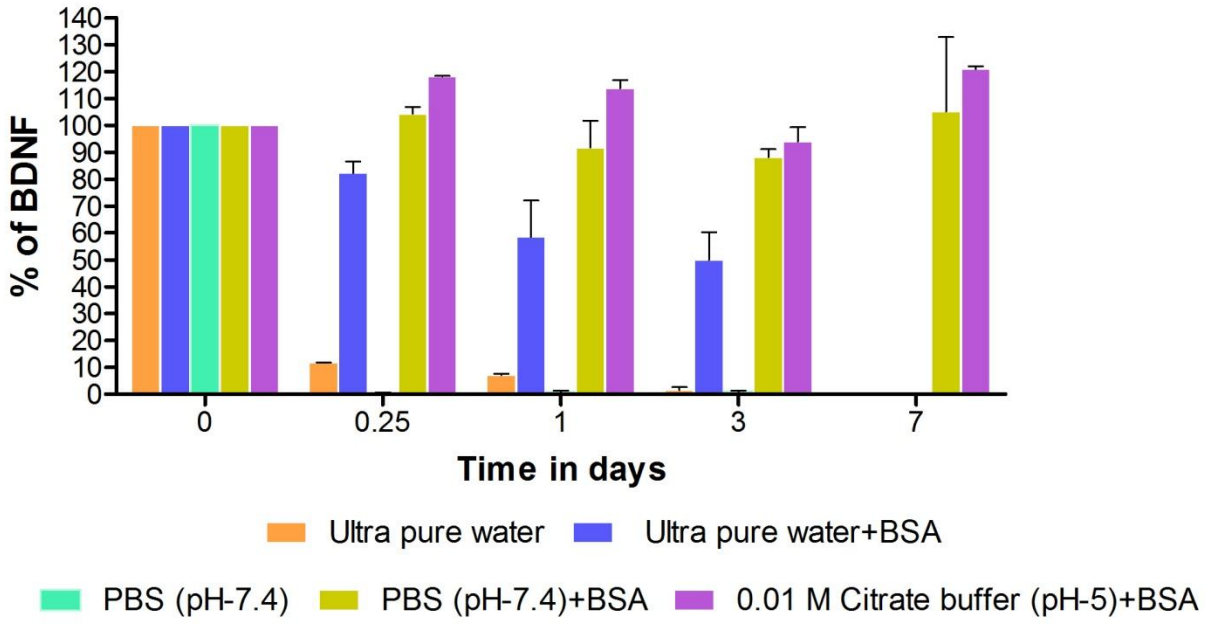


Figure-3: BDNF in vitro release studies buffer selection. Maximum BDNF integrity quantified by ELISA was found in PBS (pH7.4) +BSA buffer and 0.01 M citrate buffer (pH-5)+BSA.

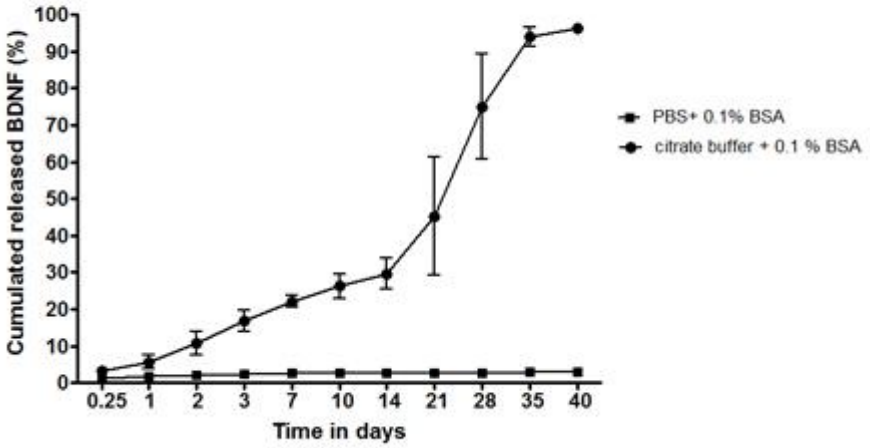


Figure-4:*In vitro* release study of BDNF (A) Cumulated release of BDNF (%) from 5 mg microspheres using 0.01 M citrate buffer (pH 5) and PBS (pH7.4) containing 0.1% of BSA (n=3).

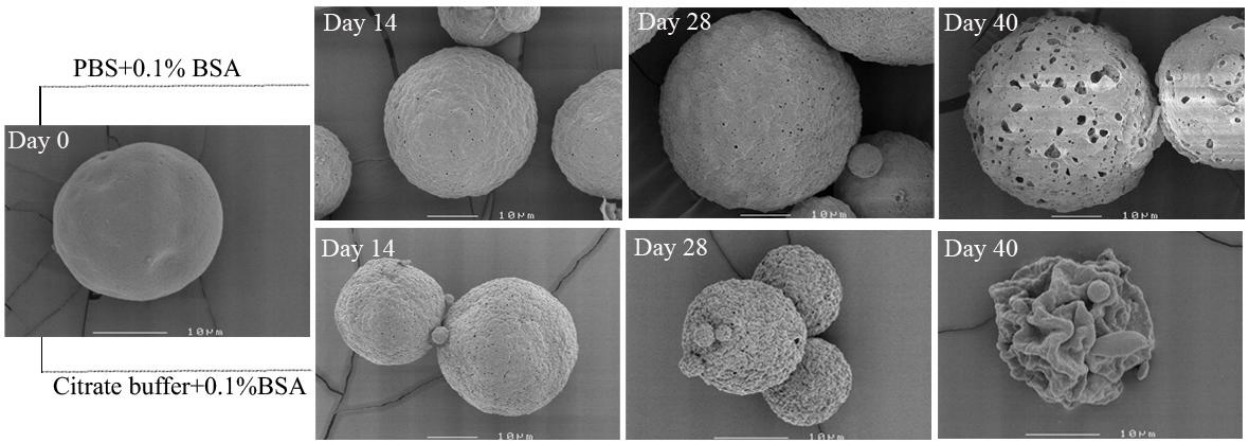


Figure-5: SEM imaging of PLGA-P188-PLGA microsphere degradation at different time intervals in PBS-BSA at pH 7.4 and 0.01 M citrate buffer-BSA at pH 5 (n=3).

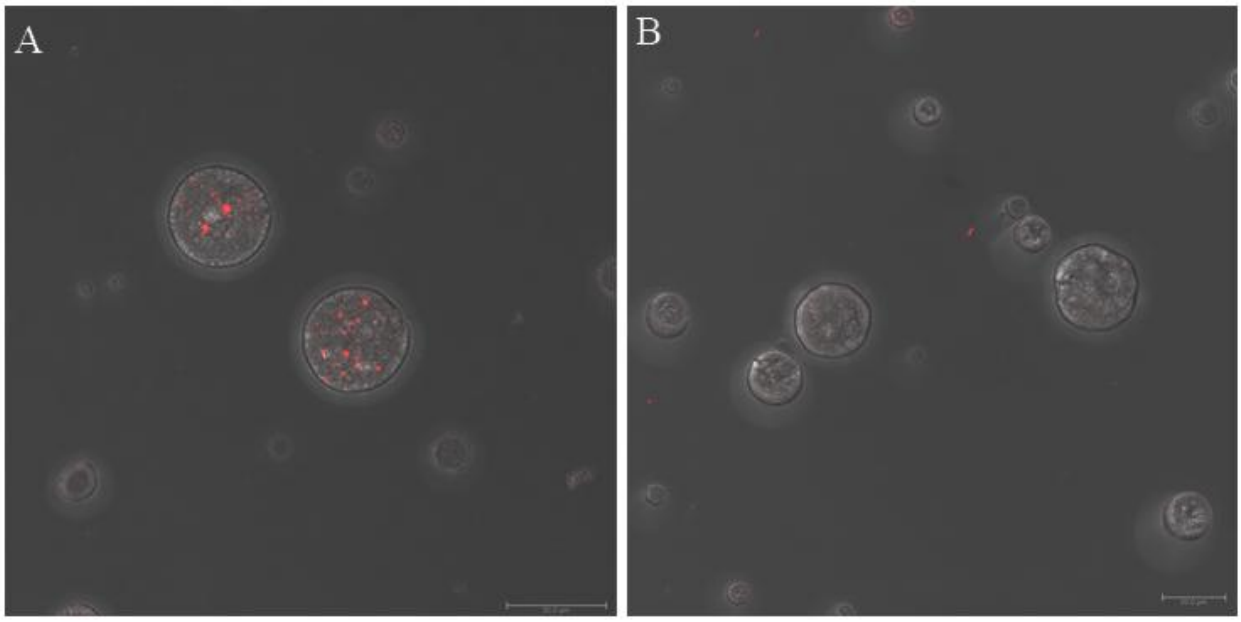


Figure-6:BDNF immunofluorescence image by confocal microscopy after day-7 incubated in A) PBS at pH 7.4 and B) 0.01 M citrate buffer at pH 5 containing 0.1% BSA respectively.

Discussion:

Interestingly, a low and incomplete BDNF release was observed in PBS-BSA (pH 7.4), commonly used for BDNF *in vitro* release studies in the literature [235]. However, a loss of protein activity during *in vitro* release of α-chymotrypsin in PBS has been reported due to its inactivation during prolonged incubation at 37 ^{o}C [236]. BDNF microsphere degradation and immunostaining experiment results suggest that contrary to what was observed in citrate buffer, incomplete degradation of microspheres allows BDNF to stick onto their surface and causes incomplete protein release in PBS-BSA at pH 7.4. Accordingly, it has been reported that the pH and ionic strength of the buffer influences the stability and conformation of the protein, degradation and erosion of the microparticles during *in vitro* protein release studies [237, 238]. Sodium citrate buffer

(pH 5) is also known as a good stabilizer for a large number of proteins such as GDNF, TGF- β1 and keratinocyte growth factor (KGF) [239]. Future studies are needed to clarify the mechanism involved in incomplete BDNF release in PBS. Nevertheless, these results highlight the need to thoroughly set-up the conditions for an *in vitro* release study.

Conclusion

In this article BDNF has been successfully nanoprecipitated by implementing the conditions predicted by the model protein (α-chymotrypsin) and encapsulated within the microspheres. BDNF released from microcarriers in a complete and sustained manner for 40 days following the bi-phasic release mechanism. To identify the optimal buffer to preserve BDNF stability during the *in vitro* release study, different incubation periods (6hrs, 1, 3 and 7 days) and different buffers including ultrapure water, ultrapure water with 0.1% BSA, PBS (pH 7.4), PBS with 0.1% BSA (pH 7.4), or citrate buffer 0.01 M with 0.1% BSA (pH 5) were tested. Both PBS and citrate buffers with BSA preserved BDNF stability as measured by ELISA (Supplementary file: Figure-3). In the present study we used 0.01 M citrate buffer with 0.1% BSA at (pH 5) for *in vitro* release studies of BDNF. The rationality for choosing this buffer was explained in the supplementary file. Moreover, the released BDNF was biologically active and as shown by the DRG cell bioassay. The size, biomimetic surface coating with fibronectin and charge of the PAMs were well characterized. EGF/bFGF pre-treatment (E/F) to the MIAMI cells initiated the neural differentiation and the MIAMI cells adhered onto PAMs releasing or not BDNF, increased the gene expression of neural/neuronal markers. Neural commitment was confirmed by the protein expression of mature neural marker NFM. Moreover, we observed that the growth factors and chemokines responsible for angiogenesis, neurogenesis and cellular migration produced by the MIAMI cells significantly increased with the PAMs. We observed that the Si-HPMC hydrogel is non cytotoxic and its rheological properties were not modified even after the incorporation of the PAMs. Moreover the hydrogel possesses the stiffness favourable for the MIAMI cell

differentiation towards neural phenotype. E/F MIAMI cells cultured in the hydrogel showed an increase in Nestin and a slight increase in Neurofilament expression compared to E/F MIAMI cells alone, while β3-Tubulin gene expression remained high and overall unaffected. However, E/F MIAMI cells adhered onto BDNF PAMs blended in Si-HPMC hydrogel showed no major change in TrkB, β3-Tubulin and NFM gene expression.

Since there were hundreds of growth factors, cytokines, chemokines involved in the tissue repair, exogenous administration of these secretome in an injection form is very difficult to achieve. However, it is becoming increasingly accepted that stem cells secrete a vast array of proteins – including growth factors, cytokines, chemokines, metabolites and bioactive lipids – that regulate their biology in an autocrine or paracrine manner, while orchestrating multiple interactions with the surrounding microenvironment [240]. Hence the use of stem cells for secreting a wide range secretome is a good strategy for tissue repair. In this article we show that E/F MIAMI cells were able to secrete growth factors like SCF, PIGF-1, BDNF, b-NGF, LIF, SDF-1α, HGF and VEGF-A and chemokines like RANTES, MIP-1α, IL-8, MIP-1β and MCP-1 after incorporation in the hydrogel. Moreover these growth factor and chemokine secretion was significantly increased when the E/F MIAMI cells were complexed with BDNF releasing PAMs. These growth factors already demonstrated beneficial effects after SCI. HGF- promotes motor function via a chemo-attractant effect on spinal motor axons, VEGF- promotes endogenous regenerative response through angiogenesis, LIF- promotes locomotor function through the increase of myelinated axons, SDF-1α- promotes neurite outgrowth and NGF- promotes motor function through axon growth. All the growth factors and

chemokines were significantly increased when E/F MIAMI cells complexed with PAMs releasing BDNF were incorporated in the Si-HPMC hydrogel compared with cells alone in the hydrogel. But it was lower than what was observed with PAMs (Blank or BDNF) complexed with the cells. Thus, this study allowed the design of a novel well-characterized non-toxic injectable biomaterial-based carrier delivering MSCs and BDNF, enhancing neuronal commitment and the secretion of tissue repair factors for neuronal regenerative medicine.

Apart from spinal cord injury repair, the formulation of PAMs releasing biologically active BDNF in a controlled manner possesses a wide range of application in neurodegenerative disorders. For example, many studies have reported that the alteration of the BDNF levels in the CNS leads to multiple pathologies, like Alzheimer's, Parkinson's or Huntington's diseases and also in depression [241-245]. Moreover, a few studies related to neurodegenerative diseases demonstrated that recombinant BDNF administration prevented lesion-induced death of neurons in adult rats, mice and primate models [246-249]. However, in these cases, the use of recombinant BDNF directly for the treatment has its limitations like low stability, short *in vivo* half-life, and inability to pass through blood-brain barrier. Even in the clinical trials the infusion of recombinant BDNF in patients with amyotrophic lateral sclerosis (ALS) [250] failed to demonstrate a statistically significant effect on the survival of patients, probably owing to the poor pharmacokinetics associated with the BDNF. This lead to cessation of trials and opened the gateway for investigating new modes of administration [245]. So we believe that PAMs releasing biological active BDNF in a controlled manner can be a hope for certain neurodegenerative disorders. Moreover our group has already started

testing the BDNF releasing PAMs in combination with MIAMI cells in Huntington disease models and observed promising preliminary results (publication in preparation).

8. CHAPTER-2

The role of BDNF releasing pharmacologically active microcarriers combined with stem cells of the apical papilla in locomotor function recovery after spinal cord injury.

Scientific context

Traumatic SCI cases are frequently classified as complete or functionally incomplete. The series of pathological events following contusion injury was well discussed in the general introduction section 1.3.2. At glance, after contusion injury, a central region of necrosis, damaged nerve cells and a spared rim of white matter occurred [251, 252]. The spared tissue is a promising target for treatments to promote functional plasticity and improved recovery [253]. Locomotor function impairment after traumatic injury is mainly due to the disruption of the active interactions between the spinal neuronal network, supraspinal pathways and peripheral sensory inputs [254, 255]. In spite of the fact that the fully removed supraspinal protrusions in the areas beneath the injury result in the stopping of the voluntary movements, some leftover motor function may remain. It emerges from the spontaneous reorganization and recovery of neuronal network excitability within any saved ascending and descending systems [256-258]. However, the endogenous CNS repair mechanisms are not sufficient to reverse paralysis and restore the locomotor function [259, 260]. To restore the voluntary motor function and repair the injured SC several strategies have been employed [261].

The possible treatment strategies majorly include neuroprotective therapies, which prevent or minimize the secondary injuries [262, 263], and neuritogenic approaches, which facilitate functional regeneration at the lesion site [70, 264]. Many studies reported that BDNF possesses neuroprotective actions on spinal cord neurons. For instance, continuous infusion of BDNF administration intrathecally restored the

cholinergic markers or prevents the death of motoneurons after ventral root axotomy in the rat SCI models [108, 265, 266]. The role of BDNF in the treatment of SCI is well discussed in general introduction section 2.1. It is very important to develop a drug delivery system for BDNF for the treatment of SCI. Even though BDNF infusion through minipumps improved clinical neurological function after clip compression model of SCI in rats, there is a significant decrease in corticospinal neurons (control voluntary movements) because of the cannula utilization for BDNF delivery. Moreover, the continuous infusion of a neurotrophic factor through a cannula created additional damage and cavity formation, which extended rostrally and caudally from the cannula tip [95]. Likewise it was also reported that continuous infusion of high concentrations of BDNF via osmotic pumps through cannula in the injured SC promoted the sensory fibers ingrowth into the dorsal SC. However, fewer fibers were able to grow farther into the spinal tissue towards the infusion site because of poor diffusion capacity of BDNF in the SC [106]. Jakeman *et al.* reported that continuous infusion of BDNF in the contusion SC injured rats resulted in behavioural recovery. However this effect was no longer present when the infusion pump was removed. Additionally, recovery was reduced after 2 weeks due to loss of biological efficacy of BDNF [107]. To address these challenges we developed a local BDNF delivery system from PAMs. Upon sterotaxic injection near the lesion site, PAMs release biologically active BDNF in a continuously programmable manner for several weeks (5 weeks at least) to elicit the therapeutic effect without additional damage to the SC tissue [267].

In our previous chapter, we discussed the development of PAMs releasing biologically active BDNF in a controlled manner. We demonstrated that FN coated

PAMs releasing or not BDNF enhances the neural/neuronal commitment of MIAMI cells as well as the secretion of a variety of cytokines responsible for neurogenesis and angiogenesis. Moreover, we incorporated the PAMs/Cell complexes in the Si-HPMC hydrogel and demonstrated that hydrogel has no major effect on the neural differentiation of MIAMI cells but the cytokines secretion was significantly increased [267]. However, there are certain limitations for using this well-characterised system in the therapy of SCI. The first and foremost limitation is MIAMI cell culture.

MIAMI cell require special culture conditions including low oxygen tension requiring hypoxic cell incubators, low density, and pre-treatment with growth factors for their neural commitment, so it may be more difficult to translate this cell type to the clinical study for SCI repair. Even though MIAMI cells expressed high levels of neuronal markers (β3-TUB, NFM), there is no expression of glial markers (Olig-2 and GFAP) by MIAMI cells alone or even when complexed with PAMs or SI-HPMC hydrogel. In order to have a simple, effective and easily translatable system to clinical studies, we used SCAP to complexed with BDNF releasing FN-PAMs and studied their impact on the contusion SCI animal models. SCAP (described in general introduction section 3.2.1) are a subtype of DSCs, can be obtained from wisdom tooth and are easy to amplify in culture. Moreover, SCAP are committed towards a neural/neuronal lineage without the use of pre-treatment with growth factors. SCAP expressed high levels of neuronal markers (NFM) and glial markers (Olig-2 and GFAP) and even their expression is up-regulated in the presence of PAMs. Recent studies also reported that SCAP are highly adaptable under critical microenvironmental conditions and secreted pro- angiogenic factors (angiogenin, IGFBP-3, VEGF) like MIAMI cells. They therefore have therapeutic

potentiality in treating neurological disorders. Hence we choose SCAP instead of MIAMI cells for the in vivo studies.

In the present study, for the first time, we combined SCAP to BDNF releasing PAMs with a FN functionalized-surface and characterized their neural/neuronal differentiation. We further assessed the locomotor function recovery after implantation of PAM/SCAP complexes in a contusion SCI model in rats. Additionally, we also studied the effect of Si-HPMC hydrogel on PAM/SCAP complexes for their viability and gene expression. However, there are certain practical limitations for using Si-HPMC hydrogel in the rat SCI models. It is very difficult to produce small volumes (5-10µL) of Si-HPMC hydrogel where PAMs/cell complexes are incorporated. Finally, more pressure is required to inject the hydrogel in the spinal cord (the reticulation of gel starts immediately within the syringe) which could further damage the spinal tissue. Even though the Si-HPMC showed promising in vitro results (more than 90% of cell viability), an effective method has to be developed for the application of Si-HPMC hydrogel in the SCI models. Hence in the present study, we used polymeric PAMs releasing biologically active BDNF combined to SCAP as a potential therapy of SCI. Hence in the present study, we used polymeric PAMs releasing biologically active BDNF combined to SCAP as a potential therapy of SCI.

The role of BDNF releasing pharmacologically active microcarriers combined with stem cells of the apical papilla in locomotor function recovery after spinal cord injury.

Saikrishna Kandalam [1,3,4], Kevin Vanvarenberg [1], Bernard Ucakar [1], Pauline De Berdt [1], Claudia N. Montero-Menei [3,4], Anne des Rieux [1,2*]

[1] Louvain Drug Research Institute, Advanced Drug Delivery and Biomaterials, UniversitéCatholique de Louvain, 1200 Bruxelles, Belgium;

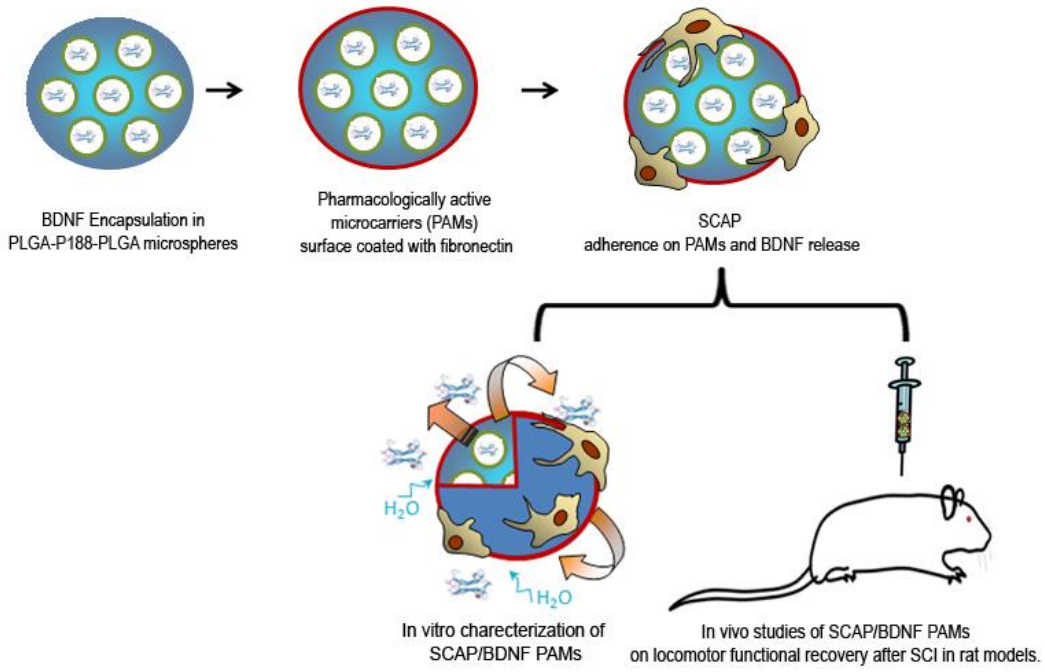
[2] Institute of Condensed Matter and Nanosciences, Université Catholique de Louvain, 148 Louvain-la-Neuve, Belgium;

[3] LUNAM Université, UMR S-1066 F-49933 Angers, France;

[4] INSERM U1066, MINT "Micro et nanomédecines biomimétiques" F-49933 Angers, France;

*** Corresponding author:** Anne des Rieux, anne.desrieux@uclouvain.be

GRAPHICAL ABSTRACT



ABSTRACT

Objective: Traumatic spinal cord injury (SCI) leads to neural and vascular disruption causing ischemia and resulting in the initiation of the secondary pathological events, which defines the extent of locomotor function recovery. The objective of the present study is to evaluate the effect of the transplantation of pharmacologically active microcarriers (PAMs) with a fibronectin (FN) surface coating conveying stem cells of the apical papilla (SCAP), and releasing brain derived neurotrophic factor (BDNF) on locomotor functional recovery in SCI. We hypothesize that this combinatorial approach has a potential therapeutic role for treating SCI.

Design and Setting: *In vitro* SCAP viability and gene expression when complexed with FN-covered PAMs releasing or not BDNF was assessed. Rats were then subject to a contusion of SCI (200kDy, Spinal cord Horizon Impactor) and SCAP alone or conveyed by FN-PAMs releasing or not BDNF were injected around the contusion. The rat motor function was evaluated by the BBB test.

Main Results: More than 90% of SCAP complexed with FN-PAMs releasing or not BDNF and cultured for 7 days *in vitro* remained viable. The expression of neural-oligodendroglial marker Olig-2 increased by 2 folds and the mature neuronal marker NFM increased by 10 folds when the SCAP were complexed with the FN-PAMs releasing or not BDNF compared to cells alone cultured in 2-dimension (2D). The recovery of locomotor function was significantly improved by the SCAP transplanted adhering to BDNF releasing FN-PAMs with frequent to consistent forelimb-hindlimb coordination after 28 days of treatment.

Conclusions: The combinatorial approach of SCAP complexed with BDNF releasing FN-PAMs facilitated locomotor functional recovery after traumatic SCI and can be a promising strategy for human SCI repair.

Key words: BDNF drug delivery; Stem cells of the apical papilla (SCAP); microcarriers, spinal cord injury; transplantation; locomotor functional recovery;

1. INTRODUCTION

Traumatic spinal cord injury (SCI) is a devastating condition caused by mechanical trauma that results in permanent loss of neural functions [1]. Based on the presence or absence of motor function, SCI can be sorted as incomplete or complete. Succeeding incomplete injury some of the functions related to movements and sensation below the level of injury are retained depending on the severity of the injury and the level of axonal preservation. Moreover, spontaneous reorganization and recovery may help to maintain some motor function although the endogenous reparative response is limited. Different strategies have been employed to repair the injured spinal cord and restore the locomotor function. Among many approaches, local administration of brain-derived neurotrophic factor (BDNF) a potential neurotrophic factor is a promising strategy for promoting neuroprotection and functional recovery after injury [2].

Many studies with models of SCI in adult rats reported that continuous intrathecal infusion of BDNF prevented the death of motoneurons after ventral root axotomy [3, 4] and promoted extensive axonal growth from motoneurons after root avulsion [5, 6]. Oudega M et al. observed that a high concentration of BDNF infusion in the injured spinal cord promoted sensory fibre ingrowth into the dorsal spinal cord region, but not many were able to grow farther into the spinal tissue towards the infusion site due to poor diffusion capacity of BDNF in the spinal cord [7]. Similarly Jakeman L.B et al. observed motor axonal growth and functional improvement after continuous infusion of high concentrations of BDNF after SCI in rats. However, these behavioral effects were no longer present when the infusion pump was removed and the pharmacological effects deteriorated within 2 weeks due to loss of BDNF activity [8]. To address these

challenges, we developed pharmacologically active microcarriers (PAMs) with a biomimetic coating of fibronectin (FN) and releasing bioactive BDNF in a sustained manner. PAMs are biodegradable, biocompatible microcarriers made of PLGA based polymers with an adjustable size and surface coating that can deliver a growth factor in a sustained manner [9]. They can serve as a support for cell culture, and can easily be implanted at different sites by simple stereotaxic injection. Their biomimetic 3D surface combined to the programmed delivery of the growth factor can stimulate the grafted cells survival and differentiation [9-11]. Recent studies demonstrated that PAM conveying cells maintained pre-differentiation and enhanced their survival leading to functional recovery in different animal models of neurological disorders [12-14]. They can furthermore modify the host microenvironment by enhancing the cytokines secretion of grafted cells. In our previous study we reported that FN covered PAM releasing BDNF and conveying a subpopulation of MSCs (MIAMI cells) in vitro induced their neural/neuronal differentiation and enhanced the secretion of growth factors and chemokines responsible for neurogenesis and angiogenesis [15].

Many studies reported that transplantation of mesenchymal stem cells (MSCs) from different origins (bone marrow, adipose tissue, umbilical cord) showed functional improvement after SCI [16, 17]. Since the last decade dental stem cells (DSCs) have attracted much attention for the treatment of SCI due to their neural crest origin, high accessibility, proliferation rate and possibility of autologous transplantation [18]. Moreover, human DSCs display superior neural stem cell properties than bone marrow–derived MSCs [19]. De Almeida, F. M. et al. reported that DSCs enhanced white matter preservation, high levels of trophic factors secretion, remyelination and locomotor

improvement after transplantation in the compressed SCI mouse models [20]. Our group recently reported the potential role of DSCs in SCI therapy [21]. Among eight different types of DSCs [21, 22], stem cells of the apical papilla (SCAP) are isolated from apical papilla of human immature permanent teeth. They have a greater tissue regenerative capacity, as well as higher proliferative potential than dental pulp stem cells [23]. Recently De Berdt P et al. reported that transplantation of a whole apical papilla tissue at the lesion site improved gait and motor function recovery and reduced glial reactivity in a hemi section model of spinally injured rats [24]. A combined strategy of neurotrophic factors and MSCs is a promising strategy for the axonal growth and functional recovery after SCI [25]. Thus we hypothesize that SCAP combined to BDNF releasing FN-PAMs was suitable for intra spinal delivery and may elicit motor function recovery after transplantation into a rat contusion model of SCI. In this study we first characterized SCAP in vitro viability and gene expression when complexed with FN-PAMs and proceed to in vivo assessment.

2. Materials and Methods

2.1. Materials

PLGA–P188–PLGA was synthesized by the IBMM-DBA CNRS UMR 5247 laboratory (Montpellier, France). Polyvinyl alcohol (Mowiol® 4-88) from Kuraray Specialties Europe (Frankfurt, Germany), P188 poloxamer or Pluronic® F68 from BASF (Levallois-Perret, France), BDNF from Peprotech (Paris, France), Costar ultra-low cluster platesfrom Corning (Avon, France), CyQUANY cell proliferation assay kit (Molecular Probes, Eugene, OR).General laboratory reagents were purchased from Sigma–Aldrich unless otherwise specified.

2.2. Preparation and characterization of BDNF loaded FN coated PLGA-P188-PLGA PAMs

BDNF was nanoprecipitated and encapsulated in PLGA-P188-PLGA microspheres using a solid/oil/water (s/o/w) emulsion solvent evaporation–extraction process as previously described [15]. The total protein loading used was 0.6 % w/w (0.1% BDNF and 0.5% human serum albumin) as previously reported [12, 26]. Briefly, BDNF was nanoprecipitated with poloxamer (P188), NaCl and cold glycofurol. After incubation and centrifugation the nanoprecipitated BDNF was dispersed in the organic phase containing PLGA-P188-PLGA polymer. The suspension was emulsified in poly(vinyl alcohol) aqueous solution under mechanical stirring. After emulsification the organic solvent evaporation was carried by transferring the emulsion to deionized water. Microspheres were then filtered, washed and freeze-dried. Microspheres without BDNF were prepared following the same process, and called blank-microspheres. To obtain PAMs, microspheres were coated with FN and poly-D-Lysine as previously described and referred to FN-PAMs [15, 27]. Briefly, microspheres were suspended in the solution containing FN and poly-lysine and incubated for 1h30 at 37 °C. PAMs were then washed 3 times in sterile distilled water, lyophilized and kept at −20°C. *In vitro* release of BDNF from FN-PAMs (2.5 mg) was performed in 250 µL of 0.01 M citrate buffer (pH 5) containing 0.1% w/v BSA at 37 ^{0}C under agitation (125 rpm) (n = 1). At different times, the tubes were centrifuged for 5 min at 2800g and the supernatant was collected for analysis and replaced by fresh buffer. The concentration of BDNF in the release buffer was evaluated by ELISA. The percentage of cumulative release was calculated based on the total amount of protein encapsulated.

2.3. Culture of SCAP

Previously characterized stem cells of the apical papilla (SCAP/RP89) which were isolated from immature permanent teeth were cultured (2D) using minimum essential medium (Sigma-Aldrich) supplemented with 10% bovine serum (Gemini Bio-Products), 1% of L-glutamine (Gemini) and 1% of penicillin and streptomycin (Gemini) at 37 °C in 5% CO_2 until they reached 80% confluence as previously described [28]. Mesenchymal stem cell markers (CD90, CD73, CD105) are homogenously coexpressed by RP89 cells (97% of the population) [28] and RP89 cells are multipotent [29]. Then SCAP were used to complex with the FN-PAMs (3D).

2.4. SCAP adherence to PAMs and in vitro viability

SCAP were washed with PBS, detached with accutase (Life Technologies Europe, Gent, BE), and pelleted at 1400 rpm for 10 min. Cells were then suspended in culture medium supplemented with 10% bovine serum. 0.2 x 10^6 cells were seeded in 1.9 cm^2 24 well Costar ultra-low cluster plate with FN-PAMs releasing or not BDNF. Lyophilized FN-PAMs (0.5 mg, releasing or not BDNF) were suspended in coated eppendorf tubes containing α-MEM medium, 10% FBS for 15 min and the suspension was briefly vortexed prior to adding the cell suspension (0.2 x 10^6 cells/ 0.5 mg FN-PAMs releasing or not BDNF). Plates were incubated at 37 °C in 21% O_2, 5% CO_2 to allow cell attachment on FN-PAMs surface as previously described [9, 15]. Cell adhesion on FN-PAMs was assessed using light microscopy at day-0 and day-7. Cell viability was estimated at various time intervals (4h (day-0), day-3 and day-7) and adherent live cells were quantified by DNA quantification using the cyquant cell proliferation assay® following the manufacturer's guidelines.

2.5 SCAP gene expression

To evaluate the impact of FN-PAMs (releasing or not BDNF) on SCAP, gene expression of neural/neuronal and BDNF receptor markers has been studied by RT-qPCR. Total mRNA was extracted by adding 1 mL of extraction buffer containing 0.4 M of Lithium chloride, 0.2 M Tris base, 0.025 M EDTA and 1% of SDS (all the chemical reagents from Sigma-Aldrich) to the samples and sonicated for 15 secs at 10% frequency in ice. To the samples 550 µL of chloroform per tube was added and centrifuged for 5 mins at 12000 RPM at 4 0C. Supernatants were transferred to a new eppendorf tubes and phenol/chloroform extraction method was followed for the RNA extraction. One microgram of mRNA was reverse transcribed using the Reverse Transcription System kit (Promega, Madison, USA) (n=4). The resulting cDNA was used as template for 30 cycles of semi-quantitative polymerase chain reaction in a T100 TM thermo cycler (Bio-Rad, BE). Primer sequences are summarized in Table-1. To amplify the cDNA, SYBR green real time q-PCR (GoTaq q-PCR Master Mix kit, Promega, A6000) were conducted with primers using StepOne Plus Real-Time PCR System (Applied Biosystems, BE). Melting curves were analyzed for each run to assess the presence of unspecific PCR products. StepOne Software V2.1 was used to analyze results. The mRNA expression of neural/neuronal (Nestin, β3-TUB, NFM, GFAP, Olig-2) and BDNF receptor (TrkB) genes were calculated relative to the expression of corresponding housekeeping gene GAPDH, according to the delta-delta Ct method.

Table-1: Sequence of primers validated in RT-qPCR

Gene	Full name	NM accession number	Sequences
NFM	Neurofilament, medium polypeptide	NM_005382	F= 5'- GACCTCAGCAGCTACCAG - 3' R= 5'- CTAGTCTCTTCACCCTCCAG - 3'
GFAP	Glial fibrillary acidic protein	NM_002055	F= 5'- AGTTGCAGTCCTTGACCTG- 3' R= 5'- CTCGTCCTTGAGGCTCTG - 3'
Olig2	Oligodendrocyte lineage transcription factor 2	NM_005806	F= 5'- GAAGCAAATGACAGAGCCG - 3' R= 5'- TGGTGAGCATGAGGATGTAG- 3'
TrkB	Neurotrophin tyrosine kinase, receptor, type 2,	NM_006180.3	F= 5'- TTGTCTGAACTGATCCTGGTGGGC - 3' R= 5'- AGGTTAGGTGCGGCCAGATTTGC - 3'
Nes	Nestin	NM_006617	F= 5'- AGAAACAGGGCCTACAGAG - 3' R= 5'- AAAGCTGAGGGAAGTCTTG - 3'
β3-TUB	Homo sapiens tubulin, beta 3	NM_006086	F= 5'- CCAGTATGAGGGAGATCG - 3' R= 5'- CACGTACTTGTGAGAAGAGG - 3'
GAPDH	Glyceraldehyde-3-phosphate deshydrogenase	NM_002046	F= 5'- CAAAAGGGTCATCATCTCTGC - 3' R= 5'- AGTTGTCATGGATGACCTTGG - 3'

2.6. Surgical procedures and injection of SCAP/PAM complexes for a moderate contusion model of rats

The animal experiments were approved by the ethical committee for animal care of the health science sector of Université catholique de Louvain. Female Sprague Dawley rats (Janvier, Saint Berthevin, France) were divided randomly into 5 groups (n=5 animals per group): 1) Injection media (vehicle control) 2) SCAP (positive controls) 3) FN-PAMs releasing BDNF (positive controls) 4) SCAP complexed with FN-PAMs

(positive controls) 5) SCAP complexed with FN-PAMs releasing BDNF (treatment). All the animals were given cyclosporine (15 mg/kg, subcutaneous) 1 day prior to the surgery and every day during the entire course of the experiment [24, 30]. All the animals were anesthetized using a rodent anesthesia system (Equipement Veterinaire Minerve, Esternay, FR) with vaporized isoflurane (Isoba, Schering-Plough Animal Health, Merck Animal Health, Boxmeer, NL) prior to spinal cord surgery. The absence of a flexor response to a noxious stimulus was taken as an indication of complete anesthesia. Then, the backs of the rats were shaved and disinfected, the vertebral column was exposed by an incision, and a laminectomy was performed at T8 to expose spinal cord segment T9. The spinal column was then secured with clamps. Particular precautions were taken to avoid unnecessary stretch that could affect the severity of injury. The spinal cord was exposed, and a contusion was produced by using the Infinite Horizons impactor (Precision Systems and Instrumentation, Lexington, KY) with a force of 200 kDynes, with no dwell time.

Immediately after contusion, each animal group received the predefined injections once on either side of the lesion. Serum free MEM media containing 0.94% of NaCMC (Sigma-Aldrich) was used as vehicle. Each injection of SCAP ($0.2X10^6$ cells), FN-PAMs releasing BDNF (0.5 mg), SCAP complexes with FN-PAMs (releasing or not BDNF) ($0.2X10^6$ cells+0.5 mg) were suspended in the vehicle (5 µl) and loaded into 10µl Hamilton syringe with SS plunger, 26-G needle. The syringe was positioned into the automated injecting machine, and let the needle tip touching the spinal cord. Piercing the needle into the tissue for 2 mm deep, and then lifted up for 1 mm. The suspension was then injected at the rate of 1 µl/ min for 5 mins using a programmable

injector. After completion of injection the needle was kept at the same position for 4 mins to avoid any leakage. Immediately after injections, paravertebral muscles were sutured on the midline, and the skin was stapled. Rat bladder was emptied twice per day until the bladder function recovered [31]. Rat body weight was recorded for each animal over the duration of the experiment.

2.7. Assessment of locomotor function

The Basso-Beattie-Bresnahan (BBB) test [32] was performed post surgery at 1 and 3 days and every week upto 28 days by 3 independent observers blinded for conditions, for 5 min per rat. The BBB procedure uses an ordinal nonlinear 21-point scale for locomotor evaluation. Scores increasing from 0 (no hindlimb movement) through 7 reflect the return of the isolated movements in the joints of the hip, knee, and ankle. Scores from 8 through 13 indicate improvement in paw placement and coordination with the forelimbs. Scores from 14 through 21 indicate improvement in toe clearance, paw position, trunk stability, and tail position. The subjects scored 21 on the BBB scale as a baseline prior to surgery [32]. Because of the nonlinear nature of the BBB scale, measurements are reported as medians. Left and right hindlimbs were scored individually, and BBB scores were averaged from both hindlimbs for individual subjects.

2.8. Statistical analysis

Statistical analysis was performed using PRISM (GraphPad Software, CA, USA). One-way ANOVA followed by Dunnett's multiple comparison tests was performed; p-values between 0.05 and 0.001 were considered significant (***p < 0.001, **p < 0.01, *p

< 0.05). Error bars represent the standard error of the mean in all figures except for Figure-2D, where they represent the standard deviation.

3. RESULTS

3.1 Characterization of FN-PAMs releasing BDNF

FN-PAMs releasing or not BDNF had a similar size with a mean diameter of 33 ± 12 µm. Encapsulation yield of FN-PAMs containing BDNF was 72.5 ± 2.8%. FN surface characterization, in vitro release and bioactivity assay of BDNF released from microspheres was well characterized in our previous report [15]. In this paper, we studied the *in vitro* release of BDNF from FN-PAMs using release medium (0.01 M citrate buffer + 0.1% BSA at pH 5). The BDNF release study for 40 days showed a controlled and sustained cumulated BDNF release of 80.7 ± 0.1%. BDNF release profile from FN-PAMs was bi-phasic similar to microspheres with a linear sustained release for the first two weeks with no burst effect. Over 26.1 ± 0.6% of encapsulated BDNF was released (Fig. 1A). Then, from 2 to 6 weeks, the release accelerated to reach 80.7 ± 0.1% of released BDNF after 5 weeks. During the first two weeks, the release rate was about 0.37 ± 0.0003 %/day/mg FN-PAMs which corresponded to a dose 27.5ng/day/mg to then increase to 0.84 ± 0.0001 %/day/mg or 62.6ng/day/mg FN-PAMs (n=1).

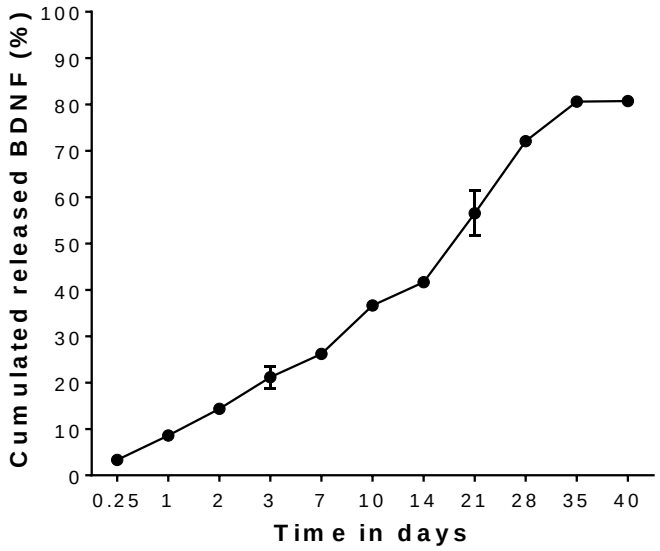


Figure-1: *In vitro* release study of BDNF. Cumulated release of BDNF (%) from 2.5 mg FN-PAMs using 0.01 M citrate buffer (pH 5) containing 0.1% of BSA (n = 1).

3.2. Characterization of SCAP with FN-PAMs and viability

SCAP added to the FN-PAM suspension (Figure-2A) were well attached to FN-PAMs releasing or not BDNF and formed 3D complexes within 4 hours of incubation (Figure-2B). After 7 days of culture, SCAP on FN-PAMs releasing or not BDNF formed bigger aggregates (Figure-2C). Moreover, cells cultured with FN-PAMs releasing or not BDNF showed more than 90% of viability after 1 week of in vitro culture (Figure-2D).

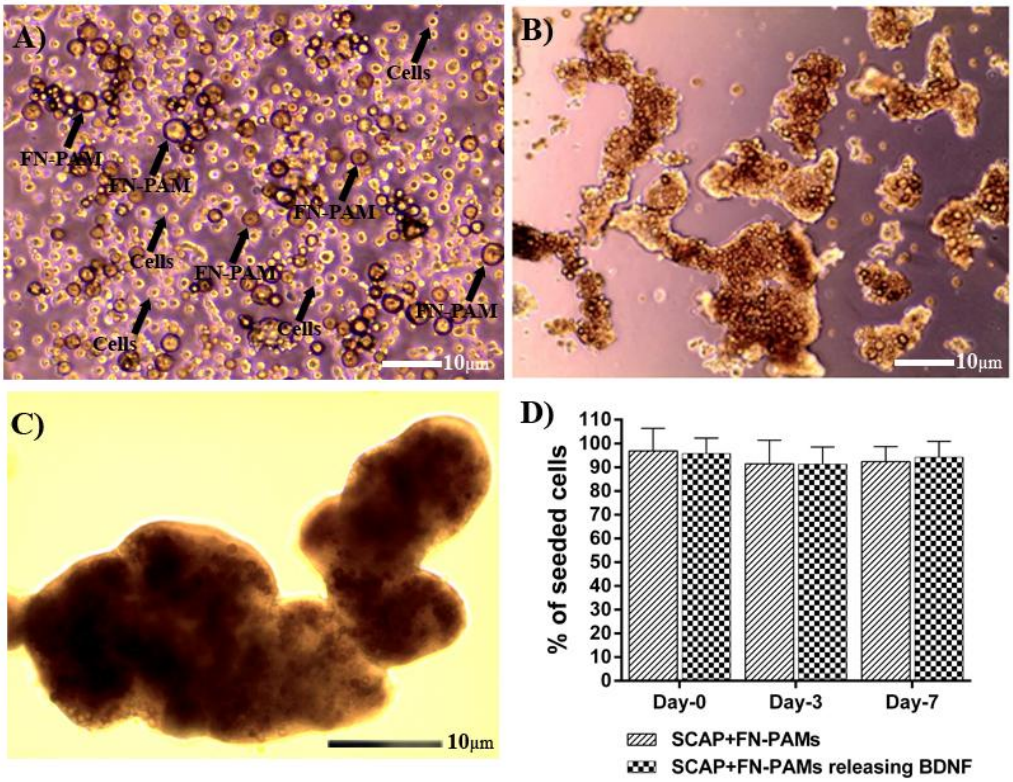


Figure-2: Morphology of SCAP/FN-PAM complexes observed under optical microscopy. A) SCAP ($0.2x10^6$ cells) seeded with 0.5 mg of FN-PAMs on ultra low cluster plates before adhesion. B) SCAP and FN-PAM form complexes after 4 hours of incubation. C) Complexes aggregated together to form larger complexes after 7 days in culture. D) CyQUANT® Cell Proliferation Assays analysis of the percentage of living cells seeded on PAMs and cultured at different time-points (0, 3 and 7 days) shows a minimum of 90% cell viability (n=3).

3.3. Impact of FN-PAMs on SCAP gene expression

To determine whether FN-PAMs releasing or not BDNF enhanced early neural (Nestin), neuronal precursor (β3-TUB) and more mature neuron (NFM), glial (GFAP, Olig-2) and BDNF receptor gene (TrkB) expression in SCAP, RT-qPCR was performed. SCAP cultured for 7 days slightly expressed (1 fold) early neural, neuronal precursor and mature neuron markers (Figure-3). When SCAP were complexed with FN-PAMs releasing or not BDNF, the expression of Nestin and β3-TUB decreased significantly and the expression of NFM dramatically increased (11-18 folds). After 7 days of culture, SCAP slightly expressed glial and BDNF receptor genes (1 fold) in 2D. There was a slight increase in GFAP, significant increase in Olig-2 (2 folds) gene expression and slight increase in TrkB gene expression by SCAP when complexed with FN-PAMs releasing or not BDNF in 3D cultured for 7 days.

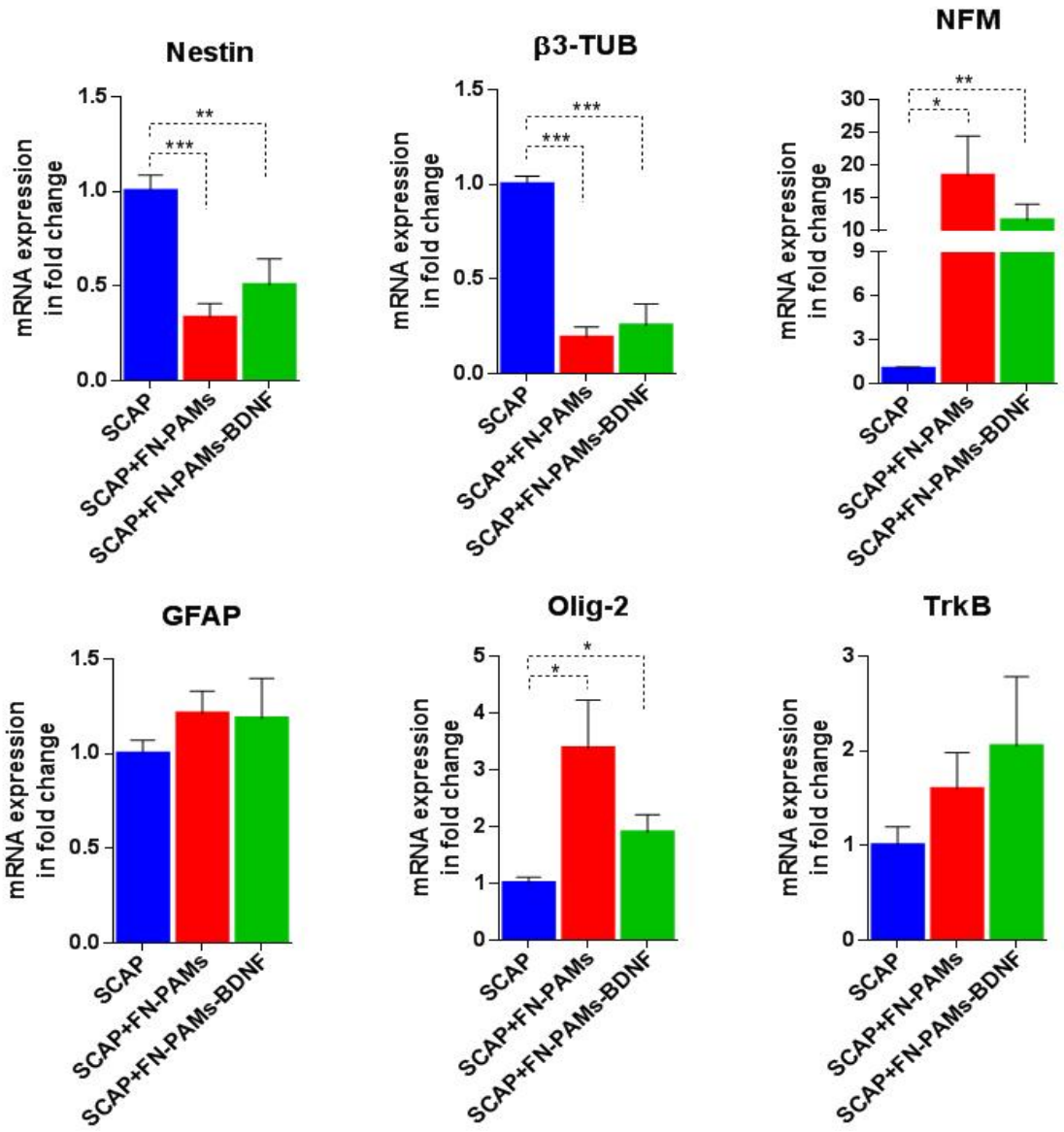


Figure-3: Transcript expression of neural/neuronal and BDNF receptor markers in SCAP adhered on FN-PAMs releasing GFs. Expression of neural/neuronal genes (Nestin, β3-tubulin, NFM, GFAP and Olig-2) and BDNF receptor (TrkB) genes were evaluated at week-1. Values are normalized to housekeeping gene (GAPDH) and expressed in fold change compared to the standard condition (SCAP) which was normalized to 1.* indicates significant difference p = 0.05, ** p= 0.01, *** p= 0.001. (N=3, n=4).

3.4. Impact of FN-PAMs and SCAP complexes on locomotor function of SCI rats

The locomotor function following SCI was assessed using the BBB score. In all animals, the BBB score improved over the 28 days of study, consistent with compressive injury (Figure-4A). At day 14, 21 and 28, animals that received the SCAP complexed with FN-PAMs releasing BDNF showed a significantly higher ($p \leq 0.05$ and $p \leq 0.01$) BBB score in comparison with the vehicle controls (injection media). At day 28 this treated group showed significant difference ($p \leq 0.05$) with SCAP alone-injected rats. They achieved a BBB score of 13.4±0.24 after 28 days of treatment, indicative of frequent to consistent forelimb-hindlimb coordination, in comparison with the control groups (9.8±0.58). The animals treated with SCAP alone, SCAP+FN-PAMs and those treated with FN-PAMs releasing BDNF showed a little improvement in BBB scoring without any statistical significance. Thus, FN-PAMs releasing BDNF together with SCAP were able to elicit modest improvement in locomotor function. Regardless of the group, the rats followed the same weight curve as naive animals, from ±200 g to ±300 g in 4wk (Figure-4B), with only a slight retard for the FN-PAMs releasing BDNF treated group.

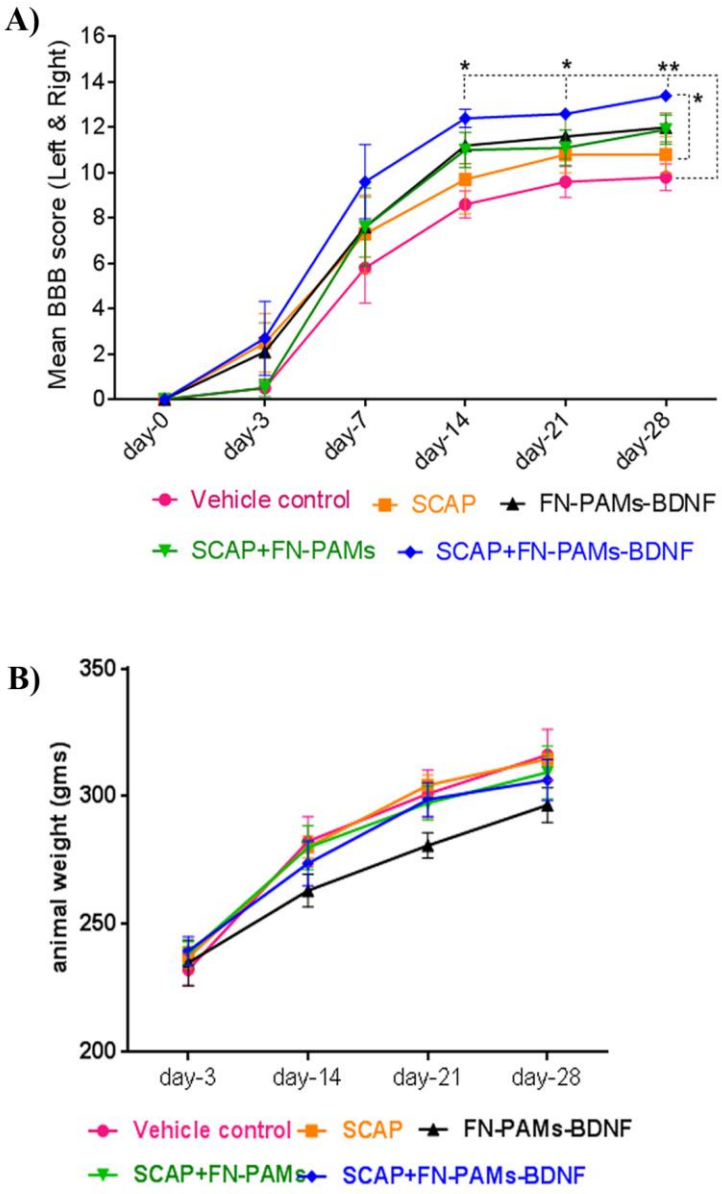


Figure-4: Assessment of locomotor function of rats followed by the Basso, Beattie Bresnahan (BBB) scoring method weekly for 28 days. A) SCAP complexes with BDNF releasing PAMs group had a significantly greater BBB score in comparison with vehicle control ($p \leq 0.05$, and $p \leq 0.01$ n=5 for all groups). B) Weight of the animals (gms) during the treatment (n=5).

4. DISCUSSION

Recent studies demonstrated that stem cells offer an attractive therapeutic option for the repair of injured spinal cord, as grafted cells may replace the lost ones as well as provide neurotrophic benefits to surrounding tissue [33]. However, the microenvironment at the injured site is very poor for transplanted cell survival, neuronal differentiation and maturation. Therefore, to enhance the capability of stem cells in CNS repair, scientist have recently begun to engineer stem cells with biomaterial scaffolds to have better survival, and desired differentiation and maturation properties [34]. Combinatorial strategies of biomaterial scaffolds delivering neurotrophic factors and stem cells have an added value and constitute a promising therapeutic approach for the treatment of spinal cord injury [35-37]. In the present study we combined SCAP to novel well-characterized non-toxic injectable FN-covered biomaterial-based microcarriers delivering BDNF. This is the first study showing that these FN-PAMs or with a sustained release of BDNF enhanced neural/neuronal commitment of the SCAP. Furthermore, FN-PAMs conveying SCAP and releasing BDNF induced motor function recovery after transplantation in a spinal cord contusion injury in rats.

Dental stem cells, and particularly SCAP, which derive from the neural crest are interesting candidates for the treatment of SCI. SCAP express a variety of neuronal markers [22] and secrete large amounts of pro angiogenic factors like VEGF [38]. Recently, De Berdt et al. tested whole human apical papilla tissue (SCAP is isolated from this tissue) combined with fibrin hydrogel in rat hemisection models of SCI. They observed that whole apical papilla tissue show motor function recovery and reduced glial reactivity [24]. However, SCAP delivered in a fibrin hydrogel did not achieve

positive outcomes after SCI due to fast degradation of the hydrogel. Recent studies reported that PAMs enhance cell survival and differentiation of transplanted cells [11, 26], particularly, PAMs coated with FN improved the survival of MIAMI cells in a global ischemia animal models [14] [38]. In a previous study, we showed that FN-PAMs guide MIAMI cell neural/neuronal differentiation and enhance the therapeutic secretome [15]. Accordingly, in this study FN coated PAMs maintain 90% of SCAP viability during in vitro culture for 7 days. In our study, the SCAP were further induced towards the neural/neuronal lineage due to the 3D environment provided by the FN-PAMs (releasing or not BDNF), which was evidenced by the expression of neurofilament gene. The decreased expression of Nestin and β3-tubulin and increased expression of NFM indicates that the SCAP cells were committed towards a neuronal differentiation in the presence of FN-PAMs. It was similarly reported that human adult marrow stromal cells committed to neuronal lineage by expressing more neurofilament and restricting the expression of neural precursor genes especially nestin or Hes as well as of immature neuronal β3-tubulin [39]. A recent study also observed that FN on stiff substrates stimulated the hippocampal neurite outgrowth [40]. Therefore we assume that FN on stiff substrate of the PLGA-based microcarriers could be responsible for facilitating the neural/neuronal differentiation of SCAP, however, further studies are needed to clearly determine the mechanism.

To promote locomotor recovery after SCI, several human cell transplantation paradigms have been reported with limitations in long-term cell survival, evidence of differentiation and functional integration of human cell [41]. Our previous study showed motor function recovery in hemisectioned SCI rats transplanted with whole apical papilla

alone, but no functional improvement was observed after transplantation of SCAP combined with fibrin hydrogel [24]. In the present study we further assessed the impact of SCAP isolated from apical papilla tissue, complexed with FN-PAMs releasing BDNF on locomotor functional recovery after contusion SCI in rats. In our previous study we demonstrated that a sustained and complete release (>90%) of biologically active BDNF could be achieved through PLGA-P188-PLGA PAMs. In this study, we demonstrated that the animals receiving SCAP adhering onto FN-PAMs releasing BDNF show significant improvement in the BBB scoring compared with the vehicle control. Moreover the animals show frequent to consistent forelimb-hindlimb coordination in the treatment group from day 14 until day 28. Accordingly, it was reported that SCAP regulated trigeminal nerve outgrowth in vitro and promoted increased peripheral innervations in vivo in a BDNF-dependent manner [42]. We also assume that this locomotor recovery is due to the long-term survival as well as therapeutic secretome of SCAP in the presence of FN coated PAMs. The reason for this assumption is Garbayo.E et al. demonstrated that FN coated PAMs after transplantation in rat hippocampus following global cerebral ischemia, enhanced the survival of the transplanted MSCs (MIAMI cells) which could lead to increased paracrine secretion resulting in neuroprotection [14]. Moreover, we already demonstrated that FN PAMs releasing or not BDNF enhance the secretion of variety of growth factors and chemokines by bone marrow MSCs (MIAMI cells) adhering onto their surface in vitro [15]. Further studies are needed to identify the secretome of SCAP in the presence of FN-PAMs releasing or not BDNF and ascertain the possible mechanisms for the locomotor function recover.

Even though there are no direct and accurate evaluation methods for the assessment of functional recovery of locomotor capacity in rats after contusion SCI, Basso et al. introduced a simple, easy to use and practical scale (BBB) for the evaluation of functional recovery [32]. This scale predicts the sequential, cumulative scores related to motor functions based on the movements of the animals. Moreover BBB scale has excellent reproducibility and satisfactory sensitivity to evaluate the locomotor capacity in rats after SCI. Further studies, in particular immunohistochemistry analysis are needed to confirm/explain these results.

5. CONCLUSION

In conclusion, SCAP delivered via BDNF releasing FN-PAMs improved the locomotor function after their transplantation in the lesion site of spinal cord injured rats. Moreover FN-PAMs maintain the viability of SCAP in vitro and enhance the neural/neuronal differentiation by increase the expression of neurofilament gene. To our knowledge, this is the first combinatorial work describing BDNF releasing FN-PAMs conveying SCAP for SCI repair. Further work is necessary to explore the underlying mechanisms of SCAP efficiency.

CONFLICTS OF INTEREST

The authors confirm that there is no conflict of interest.

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Conclusion

In this article SCAP were complexed with the FN-PAMs releasing or not BDNF and with 90% of viable cells during *in vitro* culture for 7 days. SCAP adhered onto FN-PAMs (releasing or not BDNF), and increased their gene expression of neural/neuronal cell markers. Particularly the expression of mature neuronal gene (NFM) has been highly up regulated by the PAMs and the expression of oligodendrocytic gene (Olig-2) has also been up regulated in comparison with the cells cultured in 2D. There was a little increase of astrocytic gene expression (GFAP) and BDNF receptor gene (TrkB) in SCAP when complexes with FN-PAMs without any statistical significance. In this paper we demonstrated that SCAP cells complexes with BDNF releasing FN-PAMs treated group shows significant improvement in the BBB scoring compared with the vehicle control. Moreover the treated animals showed frequent to consistent forelimb-hindlimb coordination and expressed mild functional recovery of locomotor capacity in rats after contusion SCI. Further experiments on anatomical analysis (immunohistochemistry) are needed to conclude the effect of BDNF releasing FN-PAMs combined with SCAP on the injured tissue. For instance, immunohistochemistry of human nuclei (hNu) or human mitochondria immune markers to measure SCAP in vivo viability, CD86/ ED1 and arginase-1 markers to evaluate macrophage phenotypes (inflammatory and anti inflammatory respectively), GFAP marker to measure the astrocytes responsible for glial scar formation. Along with that, measuring B3-TUB, MAP-2, NFM markers for neurogenesis and axonal growth, GAP-43 marker for axonal sprouting, PDGFRα,Olig-1, MBP for oligodendrocyte protection and axonal repair and 5-HT marker for motor neuron function can conclude the effectiveness of our combinatorial approach (SCAP complexes with BDNF releasing PAMs) in contusion SCI rats.

9. GENERAL DISCUSSION AND CONCLUSION

The wounded adult mammalian spinal cord microenvironment fails to support tissue repair and axonal regeneration, leading to absent or incomplete functional recovery. This failure has been attributed to various elements including a shortage of growth-supportive cues, an over abundance of inhibitory signals, the absence of an adequately growth permissive substrate, and inability of the SC environment to sufficiently react to the injury. It has been assumed that function would return if adequate repair was accomplished. If a small portion of fibers, in the ventral funiculus are saved, animals can accomplish extensive locomotor activity, including frequent hindlimb weight support and stepping [268, 269]. Due to the various obstacles to neuronal repair that have been identified, numerous SCI investigators believe that combinatorial treatment approaches will offer the best hope for CNS recovery. The combinatorial procedures such as biomaterial frameworks conveying neurotrophic factors and stem cells constitute a promising restorative approach for the treatment of SCI [135, 218].

In order to be successful, this therapeutic strategy must consider several issues, including the choice of neurotrophic factor and the type of stem cells to be used. Consolidating the transplanted cells within a 3D injectable structure, providing the biological cues required for an ideal graft engraftment was the methodology proposed for this study, wherein the development of biologically active BDNF released from PAMs serving as a multifaceted tool combined with MSCs for SCI repair was explored.

Promising leads about BDNF as a promoter of cell survival and neurite outgrowth have spurred an enthusiasm for investigating BDNF's potential in treating SCI [241]. For instance, severed neurons can be saved from degenerative atrophy and apoptotic cell

death when treated with BDNF [83]. Reports demonstrate that BDNF can enhance plasticity and regenerative growth in tracts like CST (responsible for locomotion), which usually appear to be fairly resistant to other growth promoting techniques [92, 93, 95, 270]. Consequently enthusiasm for this neurotrophin in this study appears to be justified in particular because different therapeutic targets might be addressed by a single molecule. Since a hefty portion of BDNF doesn't cross the blood-brain barrier, they should be directly infused into the brain or SC region of interest by implantation of a permanent guide cannula. Recurrent infusions caused significant tissue damage, which limits the treatment duration of the animal studies. It would be worthwhile to convey sustained levels of BDNF specifically to the parenchyma without the utilization of a permanently embedded guide cannula.

The primary objective of this thesis was to develop a system that would allow a controlled release of biologically active BDNF from FN-PAMs. Since a solid-state protein shows confined conformational flexibility, non-aqueous encapsulation approaches have risen to guarantee protein stability upon encapsulation in biodegradable polymeric microspheres [271]. There are a few techniques like spray-drying or spray-freeze drying for the formulation of small protein particles yet they have the disadvantages of low protein recovery and proteins denature during encapsulation [272, 273]. Correspondingly, the freeze-drying technique with PEG was often considered to give fine protein particles with no protein loss [274, 275]. Nevertheless, the remaining amount of PEG in the freeze-dried protein product contributes to an initial burst release [276, 277]. Keeping in mind the end goal to get protein particles without these inconveniences, for the first time, we nanoprecipitated BDNF using salt, glycofurol

and additive P188 to protect the protein structure, integrity and facilitate prolonged release of biologically active protein.

To optimize BDNF nanoprecipitation we used a model protein α-chymotrypsin, which possesses similar physico-chemical properties to BDNF. In the literature it was proposed that model proteins with similar physico-chemical properties can be used to predict their precipitation environment because of the expensive cost of therapeutic proteins [230]. Indeed we observed that the conditions, which gave the maximum nanoprecipitation yield for the model protein, could be adapted to BDNF and allowed a nanoprecipitation yield close to 100%. Sodium chloride was selected for BDNF precipitation because this salt decreased protein solubility with minimal denaturant effects and, it can used in parenteral pharmaceutical formulations [230, 278]. Similarly in the literature a nearly 100% of nanoprecipitation with TGF-β3 [279], and NT-3 [152] was observed in the presence of sodium chloride with this nanoprecipitation technique. Moreover, salt-induced precipitation is a widely utilized strategy in biotechnology for isolating target proteins from multi-component protein solutions as the first purification step [280-282]. The probable mechanism of these 3 components (salt, glycofurol and P188) in nanoprecipitation is: i) salt addition to the protein solution reduced the electrostatic repulsive interactions between the charged proteins, promoting attractive hydrophobic interactions and forms precipitates. ii) P188 being a stabilizing agent, protects the protein and prevents self-association of the protein particles (precipitates) [278], iii) the presence of glycofurol a non-toxic protic solvent induces a liquid–liquid phase separation bringing about a protein-rich phase and a protein-poor phase and

simple centrifugation step easily separate these phases and forms spherical nano-particles of uniform size and proceed for the encapsulation [230].

However, the limitations of this method for BDNF reside on the fact that 100% of the nanoprecipitation is only achieved in the presence of poloxamer (P188) which is contradictory to the previous observations in the laboratory (Inserm U1066) where without poloxamer, 100% nanoprecipitation was achieved with other proteins. We assume that this is due to dissolving the protein powder directly in a non-buffered aqueous solution of sodium chloride in previous studies, while in our study the lyophilized protein (due to manufacturer's conditioning) was dissolved in non-buffered aqueous solution and the salts (NaCl) and additives (P188) were added later to precipitate the protein. In this situation some quantities of BDNF might be folded or denatured when it is in non-buffered aqueous solution and remaining unfolded BDNF is available to the salts for salting out precipitation which results in low precipitation yield. In the presence of poloxamer, it may suppress aggregation and facilitate refolding of denatured proteins in solution [283] leading to more available protein for salts to precipitate, resulting in high precipitation yield. Even though poloxamer is reported as neuroprotective [284, 285], still it is questionable for its use in formulations, due to poloxamer (P188) induced hypersensitivity reactions [286]. For this reason, to avoid the poloxamer in the formulation development, further studies are needed to test non-buffered aqueous solution containing sodium chloride added directly to the powdered BDNF to obtain a high precipitation yield. Nevertheless, the nanoprecipitation yield is protein specific, where the best conditions for one therapeutic protein cannot be utilized

for other therapeutic proteins and every time an experimental setup is required to find out the optimum conditions.

We utilized a solid-in-oil-in-water (s/o/w) procedure to encapsulate the nanoprecipitated BDNF in microspheres, based on the suspension of a protein nanoprecipitate in an organic solvent. Without an aqueous internal phase, the protein is less sensitive to denaturation by adsorption to the organic phase. In addition, the structure of the protein in this organic phase is thermodynamically solidified and the changes of confirmation won't happen [230, 278]. However, some limitations including complex manufacturing process still exists for this technique. During the formulation process it is difficult to get 100% microspheres yield (initial polymer quantity vs final quantity of microspheres) and there is a 30% of loss in the microspheres during the manufacturing process indirectly affecting the protein loss. Further studies are needed to optimize this technique to overcome these limitations. One possible way is prilling process (laminar jet break–up technology) to prepare the microparticles loaded with growth factors, which is easily transferable to the pharmaceutical production, leading to monodispersed and highly controlled microspheres [287].

PLGA is a biodegradable and biocompatible synthetic polymer currently in wide use for drug delivery. PLGA-based implantable devices are approved by the FDA/EMA [234], however their utilization in protein drug delivery has a few limitations including its hydrophobic nature and acidic pH during biodegradation [279]. Recent studies reported that efficient and sustained release of proteins from PLGA-based microspheres remains a challenge mainly because of adsorption, accumulation, and denaturation of protein during the formulation process or incomplete protein release from the microspheres

[288, 289]. Literature review identified that copolymerization of surfactants like P188 with PLGA can facilitate the complete protein release [279]. In the present study, for the first time BDNF was encapsulated in more hydrophilic PLGA-P188-PLGA based microspheres with a sustained and complete release of BDNF (nearly 100%) within 40 days with no burst effect. In some biological applications an initial burst is generally unwanted, since the drug released in this period is not available for prolonged release, and, more importantly, it can result in toxic side effects [290]. In the literature recombinant BDNF was encapsulated in PLGA-polylysine-polyethyleneglycol microspheres with 100% encapsulation and complete protein release over a period of 60 days [235]. However with this polymer, there was either a very strong initial burst (more than 50% of the encapsulated BDNF) or on the contrary less than 10% release occurred during the first 2 weeks, followed by a sustained and complete release. This is due to the encapsulation techniques (double or spontaneous emulsion) used in their studies [235].

Our laboratory previously demonstrated encapsulation efficiency (80%) and complete and sustained release (over 70% in 30 days) of TGF-β3 from PLGA-P188-PLGA microspheres (60µm) with an initial burst effect [279]. The possible reasons for the diminished initial burst effect with biphasic sustained release of BDNF in our study might be due to microsphere small particle size (30 µm). In general, particle size plays an important role in burst release; the larger particles become more porous during drug release than small particles, resulting in higher apparent diffusivities and, thus, higher drug release rates [291]. Further studies on PLGA-P188-PLGA microsphere internal

porosity (high internal porosity, thus, high initial bursts [290]) is required to confirm our sustained release profile of BDNF compared to Morille *et al.* [279] observations.

Sustained delivery of a growth factor is only meaningful if that factor is active upon delivery. We showed that released BDNF from microparticles was bioactive and possessed a strong neuronal differentiation capacity on DRG cells. However, the buffered media used for this study was citrate buffer (pH 5) and this condition is not present either in brain or SC. In order to mimic the *in vivo* application it would be better if this *in vitro* release study has done using artificial CSF (pH 7.3-7.4) as the buffered medium or *in vivo* release studies in animal models. Nevertheless, we also demonstrated that BDNF released from injectable amount of PAMs in physiological conditions (added directly to the culture) prompted the survival and differentiation of DRG neurons. Even though *in vitro* release studies are not a true representation of the *in vivo* protein release, with this information it is clearly identified that there is no conformational change in BDNF and it remains biologically active during the entire process of formulation. Moreover, after the FN coating we observed no major changes in the *in vitro* release of BDNF (nearly 80% of protein released in sustained biphasic manner without initial burst) from PAMs compared to microspheres. This type of results was also observed by Morille *et al.* where TGF-β3 release from PAMs was almost similar to microsphere release pattern [292]. Over all, with some minor limitations, we succeeded in the first objective of developing a controlled delivery of biologically active BDNF from microspheres. This is a multifaceted tool, which can be utilized in the treatment of several CNS and PNS disorders.

The second objective of this study is to determine the impact of BDNF releasing FN-PAMs on MSCs to establish a powerful combinatorial strategy for the SCI repair. Literature review demonstrated that MSCs offer various advantages over alternative origins of transplant tissue and thus a solid possibility for tissue replacement treatment for the SCI repair. MSCs can be obtained through a simple surgery, and could be collected directly from the injured subject, thereby nearly obviating the potential for immune rejection problems. Moreover MSCs are able to differentiate into a wide variety of CNS cells, including astrocytes, myelin shaping cells, endothelial cells, and maybe neurons [293-295]. Several reports demonstrated the positive outcomes in SCI after transplanting MSCs from different sources (bone marrow, adipose tissue, dental tissues, and umbilical cord). However, none of them have been in clinical practice and still the search for potent MSCs is ongoing for SCI therapy.

Despite the fact that MIAMI cells and SCAP are very potential sub types of MSCs they have never been evaluated in the therapy of SCI. The major limitations of MSCs in the therapeutic *in vivo* application for SCI are their low survival rate after graft, the lack of neural differentiation, and the transplantation time-point [296-298]. MIAMI cell transplantation adhering onto PAMs delivering neurotrophic factors has demonstrated functional recovery in animal models of different neurological disorders [152-154, 190]. Regarding SCAP, only one study showed that whole apical papilla tissue transplantation in a hemisection SCI in rats promoted motor function recovery [191]. Ours is the first study to evaluate the therapeutic potential of MIAMI cells and SCAP in the context of SCI repair. We believe that combination of controlled and sustained delivery of BDNF via biomimetic microcarriers providing a 3D support for these MSCs will be able to

enhance cell engraftment and regenerate damaged tissue after SCI. Although we showed 90% cell viability *in vitro on the PAMs* further studies are needed to confirm that PAMs maintain or increase cell survival after transplantation in a SCI model.

Pre-differentiation of MSCs to neuron-like cells is critical for accomplishing the best outcome for functional improvements in SCI [299]. Neuronal differentiation of MSCs require activation by particular exogenous factors [300] as they do not demonstrate a neuronal phenotype after intraspinal transplantation and they differentiated to glial cells [301]. In this study we used E/F pretreatment to initiate the neuronal differentiation of MIAMI cells and studied the impact of FN PAMs releasing or not BDNF on their neuronal differentiation. The E/F pre-treated MIAMI cells specification towards a neural/neuronal lineage was previously reported [127]. In this study, the cells were further induced toward the neuronal lineage due to the 3D environment provided by the FN PAMs releasing or not BDNF, which was evidenced by the expression of mature neurofilament (NFM) protein.

Interestingly, recent studies reported that SCAP without neurogenic stimulation demonstrate positive staining for several neural markers (β3-Tub, NFM) [302, 303]. In this study, SCAP were further induced towards the neural/neuronal lineage in the presence of FN-PAMs releasing or not BDNF similar to E/F pre-treated MIAMI cells. In both cell types, there was a decreased expression of nestin and increased expression of NFM in the presence of FN-PAMs. It was similarly reported that MIAMI cells committed to neuronal lineage by expressing more NFM and restricting the expression of neural precursor genes especially nestin or Hes as well as of immature neuronal β3-tubulin [151, 304]. Recently, Pedram et al. demonstrated that local transplantation, in

compressed SCI rats, of neuron-like cells derived from MSCs (expressing mature neural protein neuron-specific enolase (NSE) and β3-tubulin) induced significant hindlimb locomotion recovery compared to naïve (undifferentiated) MSCs [299]. Therefore the MIAMI/SCAP cells committed towards neural/neuronal phenotype in the presence of FN-PAMs seem to be good candidates for SCI treatment. However, in this study no significant difference was observed between FN-PAMs or BDNF releasing FN-PAMs on neuronal differentiation of MIAMI/SCAP. The possible reason for this might be the short in vitro culture time (7 days). A recent study also observed that FN on stiff substrates stimulates the hippocampal neurite outgrowth [305]. Therefore we assume that FN on stiff substrate of the PLGA-based microcarriers could be responsible for facilitating the neural/neuronal differentiation. However, further studies are needed to identify the signaling pathways responsible for the MSCs neural differentiation.

Although MSCs demonstrate functional recovery upon transplantation in the animal models of SCI, there is no clear evidence about the mechanism by which it repairs the damaged SC and cause functional recovery. One possible mechanism by which MSCs demonstrated its positive outcomes is through paracrine effect. Since, Quertainmont *et al.* demonstrated that MSCs induce repair processes in SCI rats via the paracrine secretion of neurotrophic and pro-angiogenic factors. Even after 21 days of observations, the transplanted MSCs had not integrated into the recovered host tissue due to poor cell viability, but there was a significant improvement in functional recovery from as early as a week after MSC treatment, demonstrating a MSC-interceded paracrine effect [306]. Moreover, they suggested that strategies enhancing the MSCs

survival for longer periods could indirectly enhance the paracrine effect leading to tissue repair.

In this study we observe the impact of FN-PAMs releasing or not BDNF on the secretome of MIAMI cells. We observed E/F pretreated MIAMI cells secreted angiogenic factors like VEGF-A, MCP-1, PIGF-1 and a more marked secretion of these angiogenic factors along with FGF-2 and VEGF-D in the presence of FN-PAMs releasing or not BDNF. This confirmed a past report on secretion of FGF-2 and VEGF by MSCs enhanced on microcarrier culture compared with 2D cultures [307]. A favorable vascular microenvironment is important for recovery from SCI [308] and recent studies demonstrated that MSCs secretome of pro-angiogenic (VEGF, bFGF, angiogenin, MCP-1 or IL-6) factors and neurotrophic factors (NGF, BDNF) lead to functional recovery after SCI [306, 309-311]. Therefore our system, FN-PAMs releasing or not BDNF combined to MSCs, seems to be a promising approach for secreting high amounts of angiogenic factors. Moreover, E/F pretreated MIAMI cells complexed with FN-PAMs releasing or not BDNF enhanced the secretion of NGF, SDF-1α, LIF and HGF, which have been demonstrated to be involved in neurogenesis, cellular migration, immunosuppression, and functional recovery after SCI in animal models. Finally, the prolonged delivery of BDNF by the FN-PAMs will be essential to enhance the endogenous nervous tissue repair capacities.

Recent studies reported that SCAP also secrete high amounts of pro- angiogenic factors like angiogenin, IGFBP-3, VEGF [190] similar to MIAMI cells. We hypothesize that SCAP after being complexed with FN-PAMs releasing or not BDNF, enhanced the secretion of angiogenic factors as well as other factors responsible for neuroprotection

and immunoregulation. Based on our observations with MIAMI cells as well as published reports, FN coated PAMs enhancing the survival of the transplanted MSCs could lead to increased paracrine secretion [154]. However, a secretome analysis is required for the confirmation of this hypothesis.

The final objective of this thesis is to assess BDNF releasing FN-PAMs combined with SCAP on locomotor functional recovery after contusion SCI in rat models. We observed that only the treatment group (SCAP+FN-PAMs-BDNF) showed significant improvement in locomotor recovery within 28 days, compared with the vehicle control group. Moreover the animals showed frequent to consistent forelimb-hindlimb coordination in the treatment group from day 14 until day 28. However, further studies are necessary with a large number of animals (currently studied rats are n=5 per group) to confirm this result. Based on the previous observation, we can assume this motor function recovery might be due to neuroprotection, neuroregeneration, remyelination of damaged axons, neural plasticity because of continuous BDNF release from FN-PAMs together with paracrine effect of neural/neuronal committed SCAP. Indeed, it is interesting to study the secretome of SCAP and it could be assumed that FN-PAMs might enhance the release of certain tissue repair factors as for the MIAMI cells. In addition to this, immunohistochemistry studies are on going to confirm the locomotor recovery results, looking for human nuclei or human mitochondria immune marker to assess SCAP *in vivo* viability, CD86/ ED1 and arginase-1 markers to assess macrophage phenotype (inflammatory and anti inflammatory respectively), GFAP marker to visualize glial scar formation, β3-TUB, MAP-2, NFM markers to assess

neurogenesis and axonal growth, GAP-43 marker to assess axonal sprouting, PDGFRα, Olig-1, MBP to assess oligidendrocyte differentiation.

In conclusion, we have developed FN-PAMs releasing BDNF combined to MSCs, a promising tool for the CNS repair (Figure-12). To our knowledge, this is the first combinatorial work describing BDNF releasing PAMs conveying MSCs for SCI repair. The delivery of a single growth factor is less effective and probably not effective in human SCI. A more appropriate approach in humans would be to deliver a cocktail of neurotrophins (NT-3, BDNF and NGF) or growth factors in a programmed, sequential manner. For example, an initial introduction of BDNF for neuroprotection, followed by NT-3 or NGF for neuroregeneration from the PAMS conveying MSCs might be a very powerful approach for the human SCI treatment. We tried to combine the FN-PAMs and MSCs to the Si-HPMC hydrogel in order to develop a sophisticated tool for SCI repair. However, we encountered some practical limitations like producing small volumes (5-10µL) of hydrogel where PAMs/cell complexes are incorporated to allow in injection in a SCI rat model. An effective method has to be developed for the application of Si-HPMC hydrogel in the SCI models. If we achieve this it could be an ideal combinatorial approach for the SCI treatment. Due to time constrains, anatomical analysis (immunohistochemistry) was not done in this thesis. It is thus difficult to conclude about the effect of BDNF releasing FN-PAMs combined with SCAP on the injured tissue.

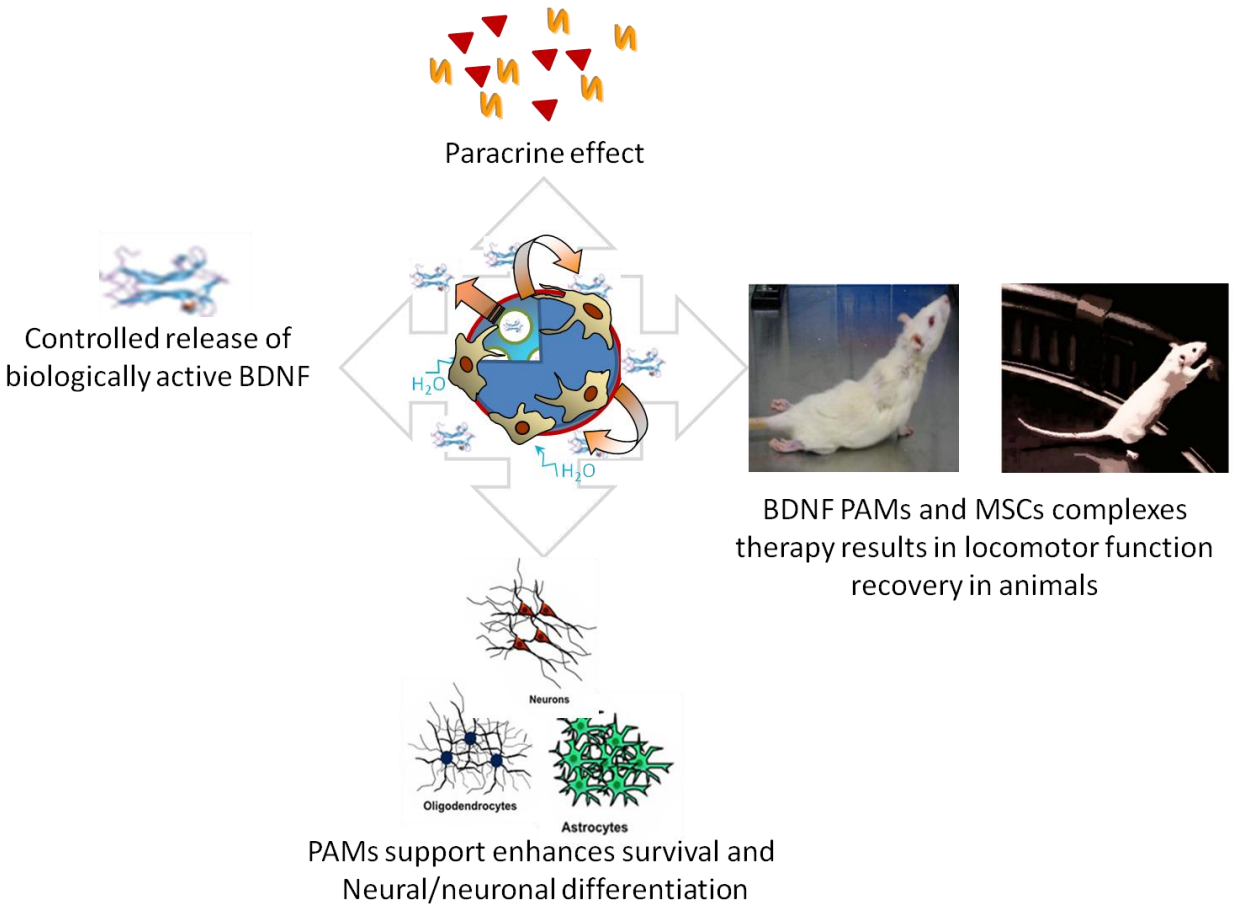

Figure-12: Illustration describes the FN-PAMs releasing BDNF in a controlled manner conveying MSCs for SCI repair. FN-PAMs enhance the cell survival and differentiation of MSCs towards neural/neuronal phenotype. In addition FN-PAMs also enhanced the secretome of MSCs. Together they improves locomotor function after SCI in rats.

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ANNEXES

Saikrishna KANDALAM

Lakanal Hall of Residence, N-18,
25, rue Lamarck, 49045-Angers, France.
DOB: 10/07/1987; Indian
+33(0) 76 91 89 077
kandalamsai@gmail.com

OUTLINE

Currently an Erasmus Mundus Ph.D. in Pharmacy and holds 2 masters (Business & Pharmaceutical sciences), two years of industrial experience and certification in base SAS programming.

COMPETENCES

Personality: **Leadership** potential, Stress resistant (meet up with swift deadlines), **self-planning** and time management, dynamic team player, **diplomatic** interpersonal skills, public speaker, **flexible** and open mindset, problem solving approach, **result oriented**, matrix work environment and networking, mentor.

Scientific skills: Pre-clinical research, Formulations-dosage form design with proteins, Stem cell cultures, Tissue engineering, GMP, Kinetic studies, toxicology, animal and cellular assays, immunochemistry, ELISA, RT-qPCR, western blot, polymer applications, and advanced analytics (HPLC, FPLC, UV-Vis, FTIR, MS, XRD, DSC, TGA).

Project management: Strategy development, planning and execution, **leading cross-functional teams**, Stakeholder engagement and communication, team building and collaboration, high quality meetings and minutes, risk management and contingency plans, prioritization and budget preparation.

Technical skills: MS office (word, PowerPoint, Excel), Linux, Windows, SAS, literature/SOP database.

Languages: English (Expert user), French (Basic user), Telugu (mother tongue), Hindi (Proficient user)

PROFESSIONAL SUMMARY

2013-17 **PhD research,** Inserm U1066, Université d'Angers**, France &** LDRI, Université catholique de Louvain, **Belgium.**

- Developing a controlled drug delivery system for therapeutic proteins using nanotechnology.
- Culture and characterization of human mesenchymal stem cells, combined to 3D scaffolds for Brain or Spinal cord tissue engineering.
- Strong collaborative work with Post-Docs, Physicians, PhDs, technicians as well as master's students and improved relationship management skills.

2016 **Internship (February-June) -** *Gastroentérologie Hépatologie Pédiatrique, Cliniques Universitaires St Luc, Brussels,* ***Belgium***.

- *In vitro* and *In vivo* characterizations of liver stem cells with polymeric 3D scaffolds.
- Networking with physicians, post docs and technicians.

2011–13 **Quality Analyst -** *Aurobindo Pharma Ltd, Hyderabad,* ***India***.

- Practical experience in technical operations, quality spec sheets.
- Coordinated with the regulatory team and prepared documents related to Product Transfer Details (PTD) for Site transfer of the projects.
- Prepared and reviewed In-process Specifications and SOPs.
- Reviewed documents and ensured complied with (cGMP) requirements.

EDUCATION

2013-17 **Ph.D.** at Université d'Angers **(France)** and Université catholique de Louvain **(Belgium)**. Major in Neuroscience, human mesenchymal stem cells, protein drug delivery and hydrogel 3D scaffolds for the treatment of spinal cord injury.

2013-15 **Master's in Business Administration: Mahatma Gandhi University (India), Grade: First class (69.2%).**
Major in project management, international business, corporate strategies, stakeholder management.

2009-11 **Master's in Pharmaceutical sciences -** Andhra University **(India),** Grade: First class with distinction (8.4/10 points).
Major in Pharmacology and toxicology, Pharmacokinetic and drug metabolism, Bioassays and Biostatistics, pre-clinical & clinical research, analytical applications.

2005-09 **Licentiate in Pharmacy -** Jawaharlal Nehru Technological University **(India),** Grade: First class (70%).
Major in Pharmaceutical technology and formulation design, medicinal chemistry, pharmacology and toxicology, pharmaceutical laws, kinetics, pharmaceutical analysis

CERTIFICATIONS

2015 **SAS certified Base SAS 9.2 Programmer.**
(https://www.youracclaim.com/badges/b76ffefe-c8bd-4cf8-9c54-d251200be875)
Import and export raw data files, combining SAS data sets, creating basic detail and summary reports using SAS procedures.

VOLUNTEER ACTIVITIES

NanoFar program and Regional chapter representative at Erasmus Mundus Association: Responsible for public relations, networking, strategic planning-partnerships, program promotion, External stakeholder management, promotion of EU higher education, motivate people to extend the EMA activities, EMA brand representation in student meetings and foster relationships with potential stakeholders.
(http://www.em-a.eu/en/about-ema/regional-chapters/indian-chapter.html)

AWARDS, HOBBIES & INTERESTS

- Erasmus Mundus scholarship by European Commission (EACEA) for PhD research.
- National student merit scholarship by All India council for technical education (AICTE) for Master studies.
- "Best Exhibit Award" for performing isolated Heart technique at Andhra University, during Science fare.
- Short film making, cooking.

PUBLICATIONS

1. Saikrishna Kandalam, et al. "Pharmacologically active microcarriers delivering BDNF within a hydrogel: novel strategy for human bone marrow-derived stem cells neural/neuronal differentiation guidance and therapeutic secretome enhancement" Acta Biomaterialia, 49 (2017), 167–180.

2. K.Saikrishna, et al. "Cardiotonic activity of fresh, alcoholic and Chloroform extracts of flowers of *Bauhinia purpurea* with emphasis on its mechanism of action through inhibition of Na+, K+ ATPase" Journal of Pharmacy Research, 2012 5(6), 3247-3250.

3. K.Saikrishna, et al. 'Simultaneous determination of Piperacillin and Tazobactum in bulk and Pharmaceutical dosage forms by RP-HPLC' Vol 3 Issue 2, April 2011, IJPPS.

For full details, references and volunteering activities please visit: https://fr.linkedin.com/in/saikrishnakandalam

UNIVERSITE BRETAGNE LOIRE

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Thèse de Doctorat

Saikrishna KANDALAM

Pharmacologically active microcarriers delivering brain-derived neurotrophic factor combined to adult mesenchymal stem cells: novel approach for the treatment of spinal cord injury

Résumé

Un traumatisme de la moelle épinière (TME) est une condition dévastatrice entraînant la perte permanente de fonctions neuronales. L'objectif de cette thèse est de formuler de microsupports pharmacologiquement actif (MPAs) avec une surface de fibronectine (FN), libérant le « brain-derived neurotrophic factor » (BDNF) de façon controlée. Nous voulons combiner ce système avec des cellules souches mésenchymateuses (CSMs) pour la réparation de TME. Le BDNF nanoprécipité a été encapsulé dans les FN-MPAs et le profil de libération *in vitro* a été évaluée. Elle a montré une libération bi phasique et prolongée de BDNF bioactifs. Nous avons combinés des cellules souches humaines mésenchymateuse issues de la moelle osseuse adulte (cellules MIAMI) et FN-MPAs avec un hydrogel non-toxique silanisés-hydroxypropylméthylcellulose (Si-HPMC). Nous avons démontré que les FN-MPAs et le Si-HPMC augmentait l'expression de marqueurs neuraux/neuronaux de cellules MIAMI après 1 semaine. En outre, l'environnement 3D (hydrogel ou FN-MPAs) a augmenté le sécrétome thérapeutique de cellules MIAMI. Pour avoir un système facile à appliquer en clinique, nous avons choisi d'utiliser les cellules souches de la papille apicale (SCAP) et FN-MPAs libérant ou non du BDNF pour la thérapie du TME. Plus de 90 % du SCAP complexée avec FN-MPAs (libérant ou pas BDNF) demeurent viables pendant 7 jours et il y a augmentation de l'expression des gènes neuronaux/oligodendrogliaux *in vitro*. La récupération de la fonction locomotrice a été significativement améliorée après la transplantation du SCAP complexée avec FN-MPAs-BDNF avec une coordination cohérente du membre postérieur après 28 jours de traitement.

Mots clés: libération du BDNF; cellules souches mésenchymateuses; Si-HPMC hydrogel; réparation neuronal; microspheres; moelle épinière; récupération motrice

Abstract

Traumatic spinal cord injury (SCI) is a devastating condition resulting in permanent loss of neural functions. The objective of this thesis is to develop pharmacologically active microcarriers (PAMs) with a fibronectin (FN) surface that deliver biologically active brain derived neurotrophic factor (BDNF) in a controlled manner. We want to combine this system with adult mesenchymal stem cells (MSCs) for SCI repair. The nanoprecipitated BDNF was encapsulated in FN-PAMs and the in vitro release profile was evaluated. It showed a prolonged, bi-phasic, release of bioactive BDNF, without burst effect. We combined human marrow-isolated adult multilineage-inducible (MIAMI) stem cells and FN-PAMs with an injectable non-toxic silanized-hydroxypropyl methylcellulose (Si-HPMC) hydrogel. We demonstrated that FN-PAMs and the Si-HPMC hydrogel increased the expression of neural/neuronal differentiation markers of MIAMI cells after 1 week. Moreover, the 3D environment (FN-PAMs or hydrogel) enhanced the therapeutic MIAMI cell secretome. To have a clinically translatable system, we chose to use stem cells of the apical papilla (SCAP) and FN-PAMs releasing or not BDNF for SCI therapy. More than 90% of SCAP complexed with FN-PAMs (releasing or not BDNF) remained viable for 7 days and an increased neuronal-oligodendroglial gene expression *in vitro*. The recovery of locomotor function was significantly improved after transplantation of SCAP complexed with FN-PAMs-BDNF with frequent to consistent forelimb-hindlimb coordination after 28 days of treatment.

Key Words : BDNF drug delivery; mesenchymal stem cells; Si-HPMC hydrogel; neural repair; microspheres; spinal cord injury; locomotor function recovery;