Chapter 5

Olfactory Mucosa Mesenchymal Stem Cells and Biomaterials: A New Combination to Regenerative Therapies after Peripheral Nerve Injury

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http://dx.doi.org/10.5772/intechopen.68174

Abstract

The peripheral nerve injury after trauma is a common occurrence in both human and veterinary medicine and has severe consequences for the survival and quality of life of the patients. Despite the continuous efforts and the creation of diverse medical and surgical techniques, the harmful effects of this type of injury are far from being overcome. Regenerative medicine has been growing in the scientific milieu as a new therapeutic approach for different situations. Among the cell-based therapies explored, the mesenchymal stem cells are evidenced by their features, versatility and potential applications. The olfactory mucosa mesenchymal stem cells, components of the olfactory system and identified in the *lamina propria*, were newly identified and are still undergoing characterization, appearing as a new promise in the regenerative therapy of several tissues but with special emphasis on the nervous system in general and the peripheral nervous system in particular, for which they appear to have special regenerative aptitude.

Keywords: stem cells, mesenchymal stem cells, olfactory mucosa mesenchymal stem cells, peripheral nerve injury, regenerative medicine, biomaterials

1. Introduction

Peripheral nerve injuries (PNI) lead to serious consequences in the life of the injured, impairing the performance of physiological functions and occupational activities [1]. The causes of

PNI are multiple and varied and may include traumatic events and iatrogenic interventions. In this second group, the peripheral nerve can be injured during manipulation of the nerve with different surgical instruments, due to poor nerve exposure during the procedure, during removal of tumours or lymph nodes, due to the inexperience of the surgeon and even during removal of osteosynthetic devices [2]. The main consequences of this type of injury are the loss of motor, sensory and autonomic function in the denervated body segments, resulting in substantial functional deficits [3].

When compared to the central nervous system (CNS), the peripheral nervous system (PNS) presents a higher reparative and regenerative activity. This contrast in the regenerative capacity depends on the intrinsic characteristics of the injured neurons of CNS and PNS and also on the physiology and functional environment of the two systems [4]. The ability of regeneration also depends on the age of the injured, the mechanism of injury and, particularly, survival and functional status of neural cell bodies [5]. Nevertheless, poor functional recovery is common due to chronic Schwann cell denervation, chronic neuronal axotomy and misdirection of regenerating axons into wrong endoneurial tubes. The effect of muscle denervation atrophy is secondary to the nerve injured and most of the times, implies fibrosis and neurogenic atrophy of the muscle [6].

Peripheral nerve damage not only removes a source of sensory input from the somatosensory system but also triggers a set of modifications in the neural circuits that lead to long-term changes in spinal somatosensory functions [7]. In fact, one of the worst consequences of PNI is the development of neuropathic pain characterized by allodynia and pain hypersensitivity in the partially denervated region [7, 8].

Peripheral nerve lesions can vary widely in severity and in most cases do not show complete recovery with the injured individual suffering from chronic lifelong disabilities. Satisfactory outcomes are usually limited to relatively minor injuries [9]. Even rapidly intervened patients are likely to undergo prolonged denervation in the distal segment of the injured nerve due to the slow rate of regeneration [10].

Achieving better outcomes depends on the advancements in microsurgical performances, introduction of new techniques into clinical practice and improvement of the therapeutics options already in use [11]. Tissue cell therapy and mesenchymal stem cells (MSCs) has been proposed as a promising alternative to treat a variety of neurologic injuries. The use of MSCs that can differentiate into appropriate cell types in the affected area or can secrete important growth factors that promote the regeneration process and positively modulate the local inflammatory response has developed rapidly in the last years [12]. Although MSCs' functional mechanisms are still poorly understood, nasal olfactory mucosa mesenchymal stem cells (OM-MSCs) stand as a promising competitor for therapeutic application due to its advantages [13].

This chapter will focus on the phenomena of PNI and its nuances, on the characteristics of OM-MSCs, their secretome and current applications. Finally, the potential use of these MSCs associated with biomaterials in cases of nerve damage, a tissue engineering technique that has not been applied until today, will be explored.

2. Peripheral nerve injury

2.1. Nerve functional anatomy

The peripheral nerve is composed by sensory and motor neurons whose long axons communicate with distant target organs [14]. The cell bodies of sensory neurons are located in the dorsal root ganglion while those of the motor neurons are found within the CNS, into the spinal cord or brainstem [15]. Its coating is complex and consists of three distinct layers (**Figure 1**). The axons are directly involved by a connective tissue sheath named *endoneurium* whose mechanical load is reduced. A fine network of capillaries exists in association with the *endoneurium*. Groups of axons involved by *endoneurium*, which together form the nervous fascicles, are covered by *perineurium*, a thin but dense epithelial layer. The perineurium offers strength in tension, and also maintains the blood-nerve barrier and endoneurial homeostasis [16]. Groups of fascicles are contained within the peripheral nerve surrounded by a connective tissue layer called *epineurium* that comprises 50% of the total cross-sectional area of the peripheral nerve. The inner epineurial layer separates fascicles, contains the vessels supplying and coursing through the nerve and a small amount of adipose tissue. The external layer surrounds all the fascicles, protects and defines the nerve anatomically [14, 17]. The *endoneurium* is longitudinally oriented while the *perineurium* and *epineurium* are circumferential (**Figure 1**) [18].

2.2. Nerve injury: pathophysiology

Mechanisms of PNI can be divided into three categories: mechanical (traumatic), vascular (ischemic) or chemical (neurotoxic) [19]. Mechanical processes can occur due to a sufficiently aggressive trauma, iatrogenic or not, due to perforating injuries with needles or due to administrations,

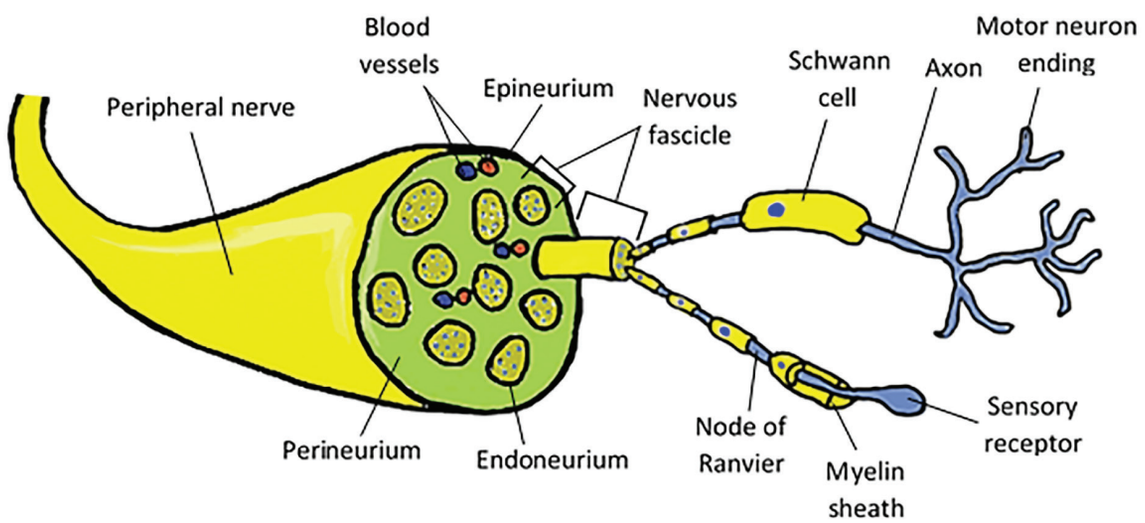

Figure 1. Schematic representation of the peripheral nerve structure and anatomical overview of the PNS. Axons, surrounded by myelinating Schwann cell sheaths, are enclosed by *endonerium*. Next, the *perineurium* binds individual axons together to form fascicles. Several axons are contained in each fascicle. Lastly, *epineurium* groups fascicles to one another, forming the nerve cable. *Endoneurium*, *perineurium* and *epineurium* present a tubular shape.

such as anaesthesia, on the periphery or into the nerve itself [20]. Nerve compression may trigger a blockage in nerve conductivity and, if prolonged, cause focal demyelination of the axons, ischemic phenomena, increased neuropeptide production [21] and increased spinal dorsal horn circuits activity that are involved in sensory information processing, including pain perception [22]. Stretch lesions are generally associated with intense exercise and fractures in the extremities where there is a close contact between the bone and nerves. Peripheral nerves exhibit intrinsic elasticity due to its collagen content at the *endoneurium*, but a strong enough force can lead to stretch injuries, resulting in a complete loss of continuity with nerve avulsion. Despite this, in some cases, continuity is maintained [21, 23]. Nervous lacerations or transections, caused by sharp objects, are common and represent about 30% of the identified cases. These lesions can result in complete transections or maintenance of structural continuity [24].

Vascular damage during nerve injury can lead to local or diffuse ischemia, occlusion of the arteries from which the *vasa nervorum* is derived or haemorrhage occurring within the nerve sheats. This vascular dysfunction and consequent hypoxia, contributes to the manifestation of neuropathic pain [25]. The epineural circulation is constituted by plexuses of microvessels running longitudinally in the *epineurium* that sends transverse branches through the *perineurium* to form a vascular network consisting primarily of capillaries in the *endoneurium*. It is of central importance and any alterations can reduce the nervous blood supply to residual levels. The connective tissue of the internal epineural layer makes the vessels less susceptible to compression since the forces are not directly transmitted to the epineural vessels. Sufficiently intense traumatic forces increase the permeability of epineural vessels and even larger forces or prolonged compressions can also injure endoneurial vessels, leading to intrafascicular oedema and secondary nerve damages [21]. Local anaesthetics and adjuvants also reduce blood flow, depending on both the agent used and its concentration [26].

Chemical lesions originate in the toxicity of solutions injected directly into the nerve or adjacent tissues, with development of acute inflammatory reactions and chronic fibrosis involving the nerve [27]. The site of administration (extraneural, intraneural, interfascicular or intrafascicular) determines the degree of toxicity and the same substance administered at different sites or portions of the nerve can cause different toxicity and lesions [28].

Before regeneration begins, a series of degenerative processes must occur, a direct prelude to the regenerative process. Regenerative success depends on the severity of the lesion and subsequent degenerative changes [21]. Any structural change or defect in the axon or its phospholipid bilayer leads to a programmed cascade of cell death that is interrupted only if there is rapid repair. Axonal degeneration follows a sequence of events that proliferate both proximally and distally to the site of injury. Axons disconnected from their cell bodies undergo degeneration through phenomena of chromatolysis [29]. Once the nerve is injured, its distal portion begins to degenerate due to the activity of proteases and the functional disruption of metabolic resources of the nervous cell body, in a calcium-mediated process known as Wallerian degeneration that involves invasion by myelomonocytic cells and results in the destruction of myelin and the onset of mitosis in Schwann cells (**Figure 2**). The degeneration of the distal axonal endings occurs due to autolytic mechanisms. The proximal end of the nerve swells but suffers minimal damage associated with

http://dx.doi.org/10.5772/intechopen.68174

retrograde degradation. The cytoskeleton starts to breakdown, followed by the dissolution of the cell membrane [30]. Once the cytoskeleton and membrane degrade, the Schwann cells that surround the distal portion of the axon shed their myelinated lipids. Axonal and myelinic debris are then removed by cells with phagocytic activity such as macrophages and Schwann cells which also release interleukins-6 to stimulate other Schwann cell and fibroblast proliferation (**Figure 2**) [31].

After removal of the debris, the regenerative process begins at the proximal end of the injured nerve and extends to the distal end. The new axonal buds (50–100) emanate from the most distal Ranvier nodes, the non-myelinated areas of the axon localized between the Schwann cells; these, in turn, guide the new cytoplasmic axonal extensions between basal membranes of the two nerve ends [32]. Proteases are also released from the growth cone to aid axonal regeneration through tissue. Several axonal extensions develop from the growth cone to contact the receptor at the distal end. The remaining neurites are abraded; otherwise, they continue to grow disorganized and may lead to neuromas that manifest clinically as painful nodules [14]. This process, however, is not free of complications. Uncontrolled branching or misdirecting of growing axons and dysfunctional innervation of target organs are common occurrences (**Figure 2**) [8]. Disruption of motor or peripheral targets secondary to PNI decreases the cortical representation of this zone in the ipsilateral cerebral hemisphere. Thus, adjacent regions in the ipsilateral hemisphere and regions of the contralateral hemisphere overgrow to compensate for deficits. The interpretation of the stimuli becomes unpredictable between regions associated with the lesion and healthy regions, which may lead to phenomena of neuropathic pain and phantom limbs [33]. In humans and rodents, axon regeneration occurs at a slow rate of 1–2 mm/day. Thus, significant injuries can take months to heal [34].

2.3. Nerve injury grading system

Success in regenerative processes after PNI depends directly on the severity of the lesion. Grading systems were developed in order to correlate the microscopic changes of the injured nerve with the clinical manifestations and prognosis. The first classification system of nerve injuries in three categories was proposed by Seddon in 1943 [35]. In this system, neuropraxia

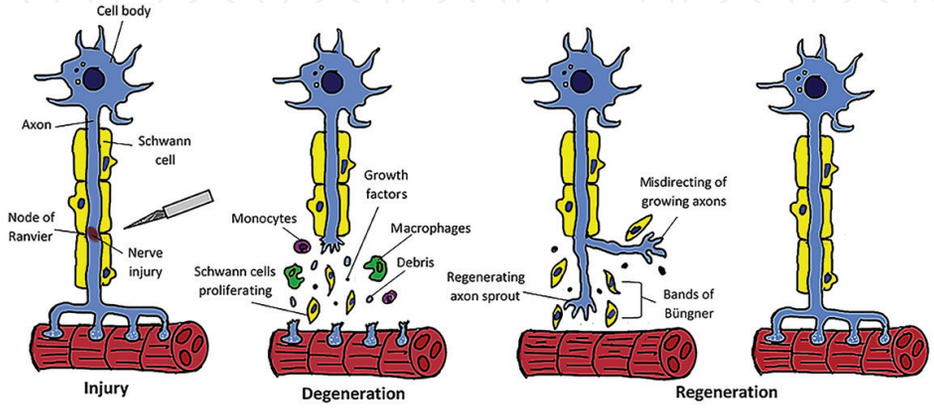

Figure 2. Schematic representation of the Wallerian degeneration.

is the least severe injury, without loss of nerve continuity: axons are anatomically intact, but nonfunctional. Since the affected nerve is unable to transmit impulses, the corresponding body regions become paralyzed. The lesion is followed by a temporary paralysis due to a local ion-induced conduction block and ischemia at the injury site, with a consequent recovery. Usually, no signs of Wallerian degeneration or regeneration are identified, but subtle alterations in myelin structure can be found and lead to motor and sensory loss due to segmental demyelination. Functional changes remain until re-myelination occurs. If decompression disappears, complete recovery is expected, without any intervention, within 3–6 months. Axonotmesis, the second level of injury, is characterized by a complete disruption of the axon and surrounding myelin while structures of supporting connective tissue, namely the *perineurium* and *epineurium*, remain intact. Axon and myelin degeneration occur distal to the point of injury by Wallerian degeneration, causing complete denervation (**Figure 2**). Despite this, once the integrity of the collagenous structures involving the nerve and that function as guides to growth of new axonal buds is maintained, the prognosis of recovery is excellent with a recovery rate of 1 inch per month [35, 36]. Neurotmesis results in a total disconnection between the two ends of the injured nerve. The functional loss is complete and recovery without surgical intervention or any other alternative is unlikely due to the intense scarring phenomena and loss of the collagen coatings and their guide function to axonal regrowth [35, 37].

In 1951, Sunderland proposed the existence of five categories in PNI according to its severity (**Figure 3**) [38, 39]. First- and second-degree injuries are equivalent to Seddon's neurapraxia and axonotmesis respectively. Third-degree lesions refer to a total disruption of the axon (axonotmesis) but are associated with partial lesions of the *endoneurium*. This category is placed between axonotmesis and neurotmesis in Seddon´s classification. The recovery prognosis depends on the extension of the endoneurial lesion and usually occurs over many months with conservative treatment or surgery to release the entrapment sites over the swollen nerve with or without limited neurolysis. Sunderland further divides neurotmesis into fourth or fifth-degree lesions. In fourth-degree lesions, all portions of the nerve undergo disruption with the exception of *epineurium* and internal haemorrhage and fibrous tissue imprisons the growing nerve sprouts due to fascicular discontinuity, inhibiting the axonal growth and originating neuromas-in-continuity. In fifth-degree lesions, the *epineurium* is also injured and the formation of end-bulb neuromas is observed. In both cases, recovery without surgical or similar intervention is impossible (**Figure 3**) [8, 36, 39].

Finally, Mackinnon and Dellon described a sixth-degree for mixed lesions. This degree is based on the evidence that a single trauma can affect different regions of the nerve transverse session variably, causing different degrees of injury at different points of the same nerve. Presumably, this is the most common type of injury, especially in perforating lesions, and is associated with bone fractures. Recovery and treatment vary according to the degree of injuries observed [17, 39].

2.4. Nerve repair and therapeutic options

Primary nerve repair through micro sutures is still the standard method in cases of axonotmesis and neurotmesis. The procedure should be performed immediately after the injury

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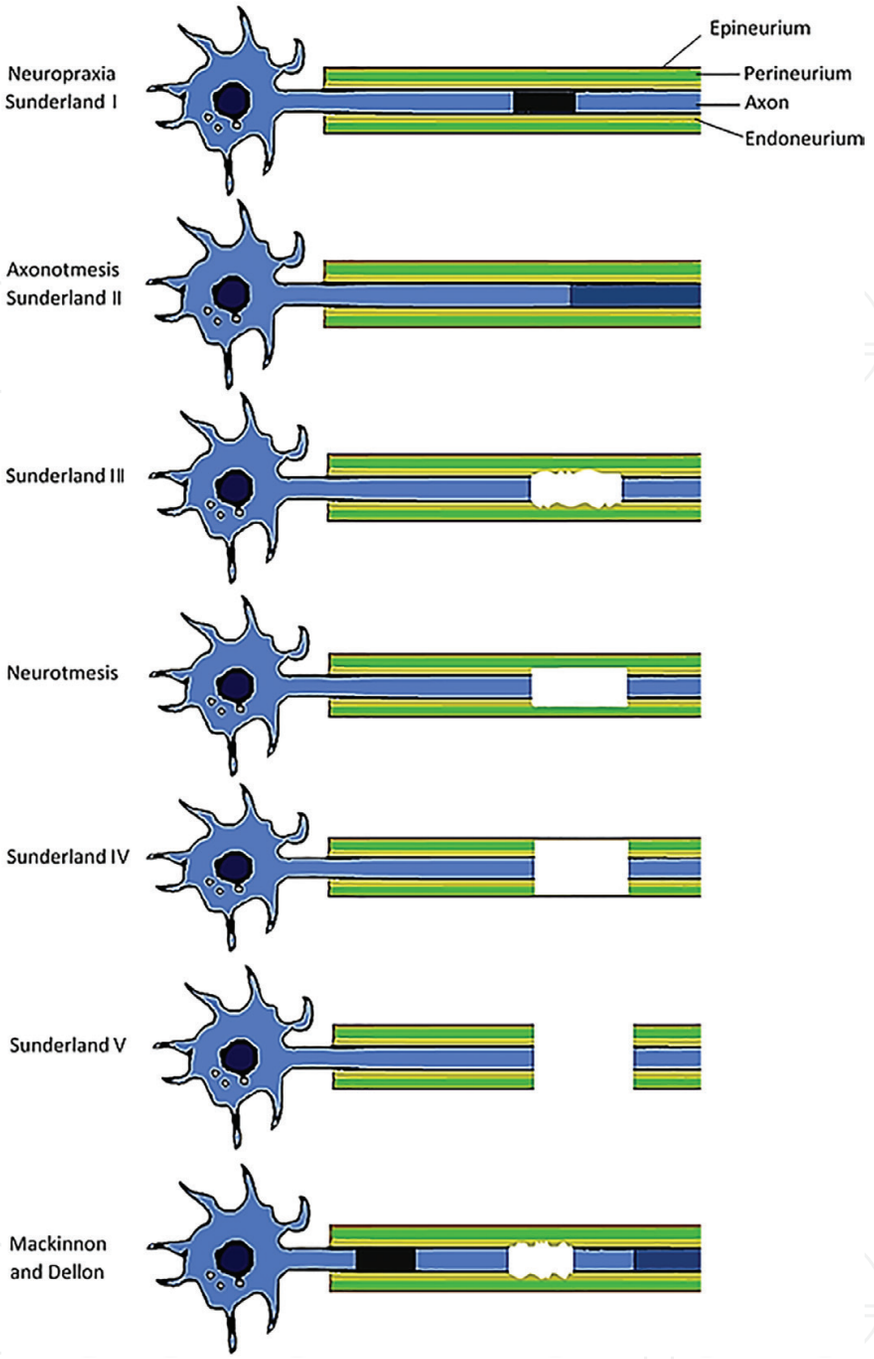

Figure 3. Schematic representation of the Sunderland classification of peripheral nerve injuries. More recently, Mackinnon and Dellon have proposed a sixth-degree for mixed lesions, based on the evidence that a single trauma can affect different regions of the nerve transverse session variably, causing different degrees of injury at different points of the same nerve.

or within a short period of time. Epineural repair is performed when a tension free coaptation in a well-vascularized bed is achieved. The surgical procedure may be divided into distinct phases. In the preparation phase, the nerve ends are prepared to get viable nerves without necrotic tissue. In the approach phase, the nerve ends are coaptated in order to leave a minimum gap between them. This gap will be rapidly filled with blood clots, and a fibrin

matrix containing macrophages serves as a transport medium for Schwann cells between the proximal and distal nerve segments. Axons of the proximal segment grow in association with Schwann cells. In order to maintain the coopted position, interrupted microstructures are performed (neurorrhaphy) in the *epineurium*, always ensuring the physiological position of the segments after suturing and avoiding rotation of the nerve ends [40]. Another surgical technique, more suitable for larger nerves includes an intranerve dissection and suture of fascicular groups. This technique allows a better fascicular alignment but causes more trauma and intranerve scarring due to the permanent presence of the suture [41].

Procedures connecting uninjured nerves to the distal portion of an injured nerve (neurotisation) are sometimes employed if direct repair of the injured nerve is not possible. A healthy nerve transfer and coaptation in cases of nerve root avulsions, as well as reimplantation of an avulsed nerve root, are also possible techniques [11].

When the injury originates a gap too large to perform a neurorrhaphy, a graft or nerve conduit may be used to provide a guidewire for the growing axons. Autologous nerve grafts are most indicated since they have all the microstructural components that facilitate axonal migration and have no antigenic components, but the collection of nerves with adequate diameter and the consequences of sacrificing a healthy nerve are important limitations [42]. Allografts, generally from cadaveric donors, despite providing the necessary cellular structures, require immunosuppressive treatments for long periods of time in order to prevent rejections in the receptor [43]. The allografts can be enzymatically processed to become acellular, thus alleviating the need for immunosuppression, presenting high success rates compared to other techniques. Even so, inflammatory reactions can, even rarely, lead to scarring phenomena that preclude normal nerve regeneration [44, 45].

Nerve guidance conduits (NGCs) have been used as viable alternatives to the grafts in a technique called entubulation or tubulisation [45] that allows the entrapment of the fibrous tissue around the injury site and the local maintenance of the neurotrophic and neurotropic factors secreted in the damaged nerve ends [44]. Since they do not have the microstructure of the nerves, can only be used in defects with no more than 10 mm if these tube-guides are not associated with cell-based therapies or growth factors local delivery. Due to this fact, more attention has recently been paid to its effectiveness in assisting coaptation than to its direct function in repairing the gap. They are usually applied to smaller nerves and overcome the disadvantages of the organic options [45]. To ensure its functionality, the characteristics of the NGCs used must comply with all the criteria established for this type of biomaterials: the material used must be (i) biocompatible with the regenerating tissue where it will be applied and should never trigger any local or systemic inflammatory response [46]; (ii) biodegradable, while ensuring mechanical and architectural stability during the regenerative process and resisting to the application of sutures and to inflammatory tissue reaction [47]; (iii) flexible and resistant in a balanced way in order to avoid compression of the regenerating axons and to limit tissue inflammation [48]; (iv) capable of preventing the growth of excessive fibrous tissue associated with the site of injury and reducing the loss of neurotrophic factors secreted by damaged nerve endings [44]; (v) capable to provide an orientation line to the growth cone through a 3-D tubular structure, thereby diminishing misdirection phenomena

[49]; (vi) semi-permeable and with pores of adequate diameter that allow the influx of oxygen and interstitial nutrients to nourish the growing axon that simultaneously prevent the entry of inflammatory cells and the loss of growth factors [50]; technically efficient, ensuring requirements related to production, sterilization, storage and handling [51]. Adapted in each case, these biomaterials must have appropriate dimensions that allow the connection of the nerve defects without tension, and the diameter of the conduit and the thickness of the wall should be sufficient to accommodate the two stumps at the nerve ends without any compression being exerted. In fact, these dimensional variations seem to have an influence on the rate of nerve regeneration [52]. Various materials can be used in nerve conducts, which can be divided into non-resorbable devices, natural resorbable devices and synthetic resorbable devices.

Non-resorbable devices with synthetic origin, or polyvinyl alcohol hydrogels, consist of water in proportions identical to those observed in biological tissues and in polyvinyl alcohol (PVA) that guarantees mechanical structural stability and facilitates sterilization [53]. In contrast, the nature of these materials creates problems related to compression and tension at the suture lines, even after nerve regeneration has occurred. In addition, there are still few clinical studies evaluating the efficacy of these materials in controlled and randomized models [44].

Resorbable devices with natural origin include type I collagen based devices, a natural and abundant organic component that can easily be isolated and purified to reduce its antigenicity [54]. Its reabsorbability can be defined to varying degrees and its adhesiveness allows cell survival and proliferation for long periods of time [55]. With proven biocompatibility and ability to support and guide tissue regeneration *in vivo*, these devices have already demonstrated efficacy in large gaps as recorded in the literature [56]. The main disadvantages related to the use of these materials are discrepancies observed between the different products available in the market with respect to the months needed to complete biodegradation and the observation of immune responses that require the use of immunosuppressive drugs. [57]. Furthermore, there is evidence that materials of different lots can lead to different results, hindering reproducibility, degeneration intervals can be increased and the regenerative supporting ability of the stored nerve may be compromised [58].

Synthetic scaffolds have recently been developed with cellular guidance channels that facilitate propagation of Schwann cell processes, which may improve the chances of successful nerve regeneration (**Figure 4**) [11]. Poly(L-lactide): poly (glycolide) (PLGA) and poly(DL-lactide-ε-caprolactone) (PLC) subgroups may be included in this group of resorbable devices with synthetic origin. The biomaterials of the first group are characterized by good levels of degradability, mechanical properties and associated cellular viability, having good performance in clinical trials with gaps of considerable dimensions. In contrast, high rates of acidic degradation and their products, rapid changes in mechanical properties and low solubility are important limitations [59]. PCL biomaterials are characterized by being transparent, which brings great clinical advantages during surgical application across the nerve gap defect [60], besides demonstrating efficacy in cases of large gaps [44]. Among the limitations, it is worth mentioning its high rigidity, which requires immersion in saline solution prior to use. In addition, it is necessary to use needles of larger dimensions and more resistant during the

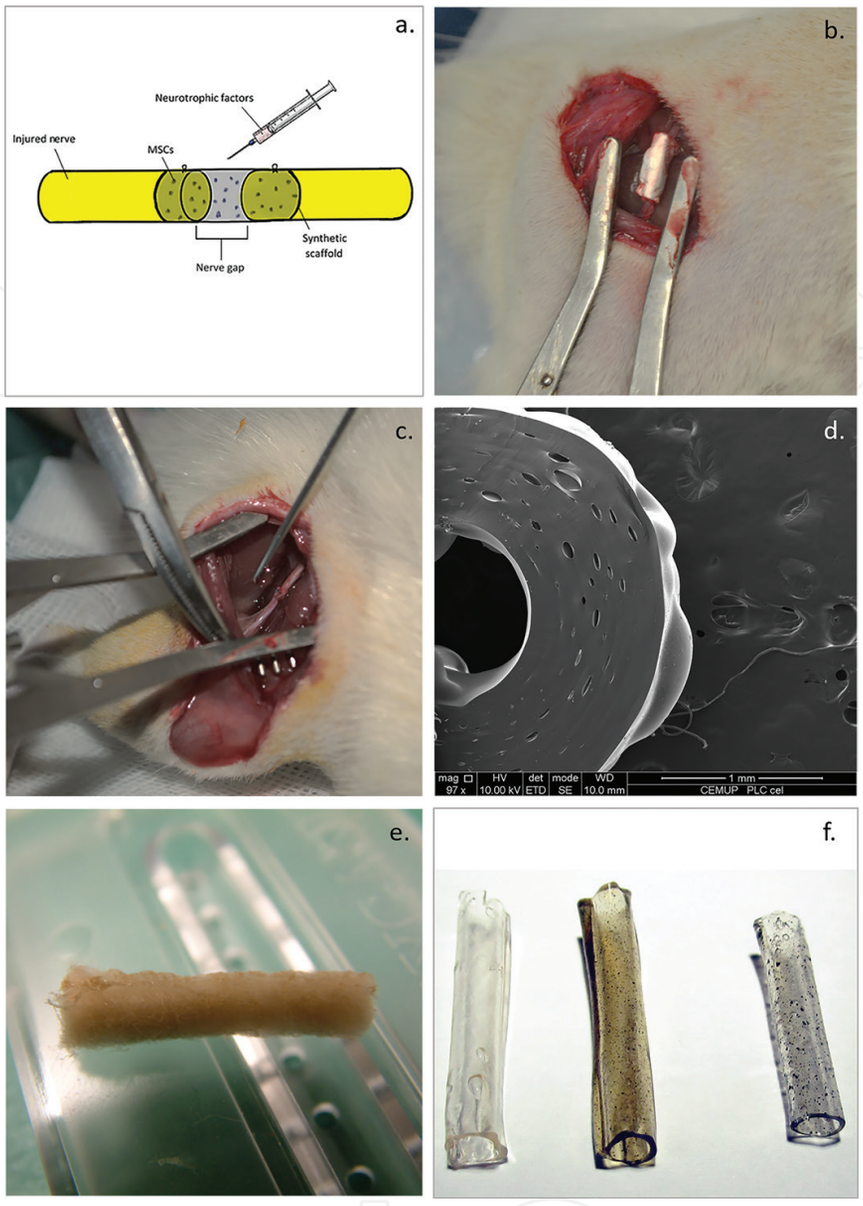

Figure 4. Schematic representation of a tube-guide that can be implanted in neurotmesis injuries (a). Tube-guide made of poly(lactic acid) (PLA)-gelatin piezoelectric material implanted the sciatic nerve of a rat, used as animal model for studying the nerve regeneration process (b). The sciatic nerve of the rat where the neurotmesis injury was reconstructed with an end-to-end suture (c). Scanning Electron Microscope (SEM) image of the inner and outer diameter of a tube-guide made of PLC (transversal section; 1500×) (d). A tube-guide made of PLGA (e). PVA tube-guide; PVA tube-guide loaded with carbon nanotubes (CNTs), and PVA tube-guide loaded with poly-pyrrole (PPy) (f).

application of sutures [50]. Other complications include foreign body reactions, severe swelling with possible device lumen obstruction, fragmentation due to incomplete degradation, early collapse of the device with possible formation of neuromas, and reduced number of myelinated nerve fibres connecting the gap defect [61, 62].

Nerve sheaths constituted by collagen extracellular matrices, acellular and animal-derived, namely with origin in swine intestinal submucosa, have already been used with relative success in the regeneration of different tissues and as nerve guidance channel for regeneration of the peripheral nerve [63]. This technique supports early neovascularization and acts as scaffold in

axonal regeneration without immunogenicity problems. In addition, its use guarantees the presence of several growth factors and cytokines that also aid in neuronal survival and growth [64].

Neurotrophic factors, secreted by neuronal or non-neuronal cells in the proximal and distal nodes of the injured nerve, are essential in the conduction of the regenerative process. The addition of these factors to the wall or lumen of the conduits and their slow release by diffusion at the lesion site are techniques currently applied and without which the synthetic conduits may fail to aid the regenerative process over longer graft lengths [65]. Among the neurotrophic factors identified and commonly used are transforming growth factor, beta superfamily, nerve growth factor, insulin-like growth factors, neurotrophins 3, 4, and 5, ciliary neurotrophic factor, neuregulin-1, brain-derived neurotrophic factor, glial cell line-derived neurotrophic factor [63] and platelet-rich plasma [66].

Cell-based therapies have been proposed as a promising alternative to treat a variety of neurologic injuries and the use of stem cells that can differentiate into appropriate cell types in the affected area has developed rapidly in the last years [67]. Stem cells are undifferentiated cells capable to proliferate and produce both new stem cells and different types of cells and tissues [68]. More specifically, MSCs are multipotent, heterogenic stromal cells derived from the mesoderm [69], one of the two populations of bone marrow progenitors (bone marrow stromal progenitors), and were initially characterized as presenting adherence to plastic culture dishes, fibroblast-like morphology and a unique ability to differentiate into multi-lineage MSCs, phenotypes and specialized tissues [70]. Due to the attention that these cells have received in recent years, it became necessary to create a more precise definition that unified the basic characteristics of the MSCs, which emerged in 2006 by the International Society for Cellular Therapy. Thus, MSCs [71]:

- are plastic-adherent under standard culture conditions (α minimal essential medium plus 20% fetal bovine serum);
- express non-specific markers CD105, CD90 CD73 and CD44, and lack the expression of hematopoietic lineage markers CD45, CD34, CD14 or CD11b, CD79α or CD19 and major histocompatibility complex- (MHC-) II/human leukocyte antigen- (HLA-) DR
- are capable to in vitro differentiate into at least three different cell types, like osteoblasts, adipocytes and chondroblasts.

While this initial definition is broad enough to cover the most obvious features of MSCs, several studies over the years have shown that these cells are able to differentiate not only in osteoblasts, adipocytes and chondrocytes but also in other cells and tissues with mesodermal origin (ligaments and tendons, cardiomyocytes, muscle) and also ectodermal and endodermal origins (skin, retinal epithelial pigment, lungs, hepatocytes, renal tubules, pancreatic islets, sebaceous gland ducts and neural cells) [68]. MSCs can be obtained from a vast array of tissues that include adipose tissue, lungs, bone marrow, umbilical cord (Wharton's jelly and umbilical cord blood), *synovium*, amniotic fluid, fetal blood, dental pulp, skeletal muscle, circulatory system [68, 69] and olfactory mucosa [13]. Applied to regenerative medicine, MSCs present exceptional features that make them great options, such as easy expansion, differentiation into different cell types, immune-privileges and immune modulation, tropism to injured sites, trophic stimulation and modulation of tissues functions [46]. In addition to being able to secrete neurotrophic factors and provide

an environment conducive to neurogenesis and proliferation of Schwann cells in nerve injury sites, they can themselves differentiate into cells with Schwann cell phenotype and modulate the local inflammatory process and the Wallerian degeneration [72], being a precious addition to the use of biomaterials and growth factors in therapeutic techniques after PNI.

3. Nasal olfactory mucosa mesenchymal stem cells

3.1. General features

The olfactory mucosa (OM), as a component of the olfactory system, consists of different types of cells. Among these are olfactory neurons (ON**)** that are able to regenerate continuously throughout adult life. This regenerative capacity is attributed to olfactory stem cells and supporting cells of OM, together promoting axonal regeneration [73]. In addition to the bipolar ON or olfactory neurosensory cells, several cell types can be identified in the OM: horizontal basal stem cells (HBCs) and globular basal stem cells (GBCs) in the olfactory epithelium, MSCs in the lamina propria, olfactory ensheathing cells (OECs) and support cells (**Figure 5**). New nerves generate from GBS in the olfactory epithelium, which are guided to their correct position in the olfactory bulb by OECs in the olfactory mucosa. GBCs, derived from HBCs, were initially thought to be the exclusive source of ON and support cells [74] but it is currently known that MSCs are also capable of producing neurons *in vitro* [75].

The OM-MSCs were initially identified from an embryonic rat OM culture [76] and the first characterization studies evidenced the expression of mesenchymal-specific markers such as CD90, CD105, STRO-1 and differentiation into the three main cell lines [74, 77]. OM-MSCs have important characteristics such as neural crest origin, high versatility, vast distribution, advantageous localization and are not susceptible to chromosomal abnormalities or tumorigenicity [78]. They exhibit high mitotic activity when compared to the bone marrow MSCs (BM-MSCs) (nearly three times higher) and are able to self-renew in long-term cultures (over 15 weeks) by maintaining telomerase activity and lack apoptotic activity [79]. The olfactory mucosa itself is a great cell source since its renewal continues throughout life and OM-MSC potency is not even affected by the age of the donor [80]. Different from BM-MSCs, OM-MSCs promote CNS myelination and induce the differentiation of neural stem cells into oligodendrocytes and oligodendrocyte maturation. All that suggest an easy and rapid propagation to sufficient levels that allow transplantation and that MSCs from a more neurogenic niche may have different properties to the classical BM-MSCs [81, 82].

Due to its origin from ectoderm (resulting from the interaction between cranial neural crest and olfactory placodes) and its high expression of neural cell-related genes, it was proposed that OM-MSCs be renamed as ectomesenchymal stem cells [81]. Even its origin highlights the predisposition for OM-MSCs to differentiate into neural lineage cells.

3.2. Isolation, characterization and differentiation

The properties of the OM-MSCs are still far from being fully understood. Located in the olfactory region of the nasal cavity, OM-MSCs are primarily derived from neural crest cells, have

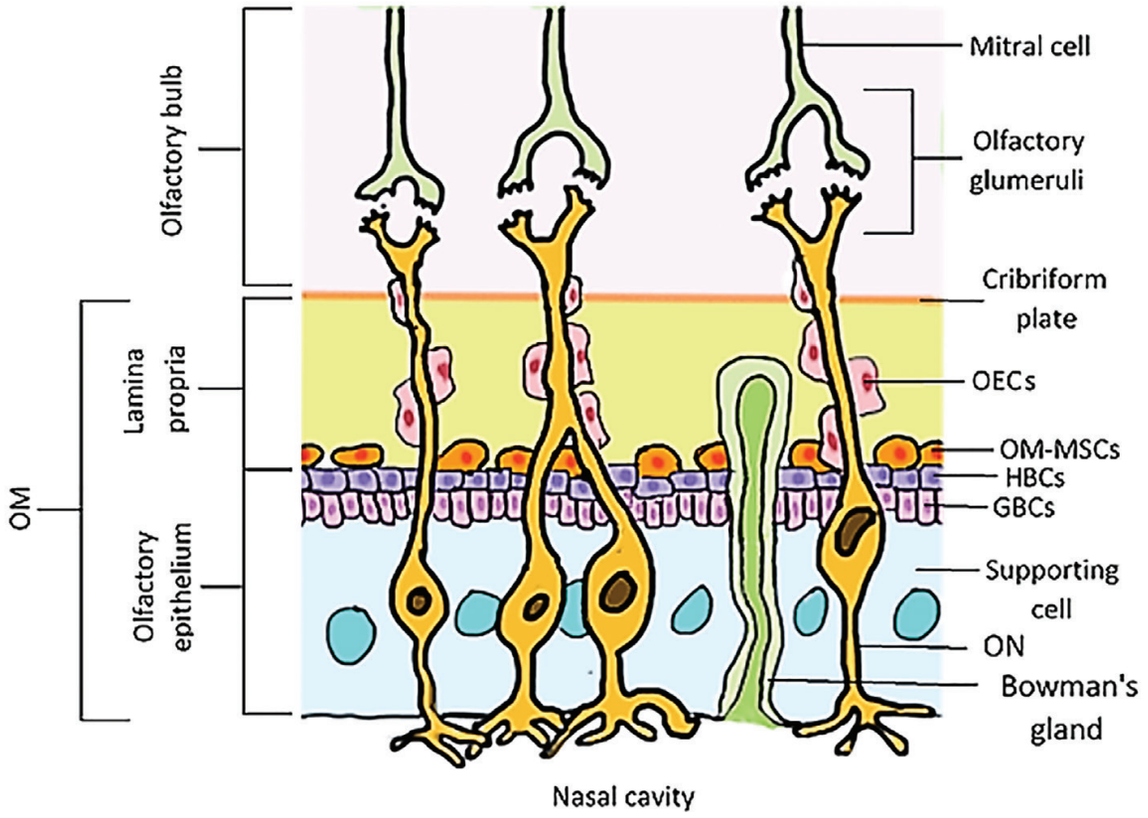

Figure 5. Schematic representation of the olfactory mucosa and relative location of its cells.

a high proliferation rate, self-renewal ability and multiple lineage differentiation capabilities [83]. These cells have already been isolated from human and mice [84], rat [85], rabbit [86] and dog [87], both with *in vivo* [84] and post-mortem protocols [86].

The described collection methods include a delicate discarding of the turbinates [82, 88] or complete collection of the olfactory bulb [80], being the harvested material taken in balanced solutions containing antibiotics and antifungals [82]. The olfactory epithelium or olfactory bulb tissue sample is then cut into small pieces, digested with collagenase and DNase [80, 82] or dispase [78] to separate the cells from their extracellular matrix. Then, they are cultured in flasks containing appropriate medium like Dulbecco's modified Eagle's medium or HAM´s F-12 supplemented with fetal calf serum or fetal bovine serum, antibiotics, antifungals and growth factors [75]. After removal of non-adherent cells, the remaining cells can be trypsinized, expanded [82, 88] and then banked in liquid nitrogen or induced to form for differentiation. [89]. Multilineage differentiation can be achieved by culturing under induction conditions and staining with specific dyes [88].

Determination of the phenotypic markers is achieved through flow cytometric analysis where single cell suspensions are stained with specific fluorochrome-conjugated antibodies [78, 80], RT-PCR [80] or by immunocytochemistry [82]. In addition, the identification of specific proteins of these cells can be performed using immunofluorescence microscopy [88].

In colonies formed after culture, OM-MSCs exhibit mostly fibroblast-like morphology [78, 80, 88, 90]. Phenotypic analysis reveals the presence of Stro-1 [75–77], CD29 [80, 88], CD44 [80, 88], CD49b

[90], CD54 [13, 77, 82], CD73 [77, 82, 90], CD90 [13, 75–78, 80, 82, 88, 90], CD105 [13, 75–78, 80, 82], CD106 [77, 78] and CD166 [77, 78, 80, 82, 90] markers and the absence of haematopoietic stem cell markers such as CD34 [13, 75–78, 80, 88, 90], CD45 [13, 75–78, 80, 88] and CD11b [88]. Strangely, one work indicates lack of Stro-1 [82]. Immunofluorescence microscopy provides evidence for the expression of neural stem cell–related proteins such as vimentin [88], nestin [75–77, 81, 82, 88] and also NG2 [77, 81], a marker whose presence indicates the ability to form neurospheres and to generate neurons in vivo and in vitro [81]. They also express p75-NTR and SMA [77]. Consistent with the characteristics of multilineage differentiation capacity of MSCs, OM-MSCs can be differentiated into osteocytes and adipocytes [13, 82, 88], chondrocytes [78, 90], neuron like cells [13, 78, 81] and myocardial-like cells [80] when cultured under appropriate conditions. They, therefore, present abilities to differentiate into mesodermal and ectodermal cell types.

3.3. Secretome and metabolome

It is known that the regenerative effect of MSCs, as well as OM-MSCs, is not exclusively related to their differentiating ability but also to paracrine factors. These are important in the creation of a support microenvironment that allows cellular survival, differentiation, activation of endogenous neural stem cells, reduction of the inflammatory reaction and induction of angiogenesis [91]. MSCs have also the ability to produce potent protective factors that promote tissue repair and immunomodulation, reducing fibrosis and cell death [92]. The determination of the characteristics and components of the secretome and metabolome of a given cell is essential to uncover the essential needs for its success in regenerative processes [93]. Similar cell types with different secretome profile can reflect their cellular niches and local function. The only study of the determination of the secretome and metabolome of OM-MSCs allowed to identify 274 proteins in OM-MSCs conditioned medium and the identification of some processes that are usually associated to transplantation processes such as biological regulation, cellular processes, metabolic processes, development processes and response to stimuli [13]. These processes promote repairing by facilitating migration to injured sites, remodelling the extracellular matrix and increasing metabolism and cellular activity. In the OM-MSCs, genes related to cell growth and migration, angiogenesis and blood circulation, inflammatory and immune regulation and neurotrophy, the major components of transplantation and regenerative promotion, were identified and they can also produce cytokines that promote hematopoietic stem cell survival, proliferation and differentiation [90]. In addition, it has also been identified the secretion of important molecules in neural differentiation such as Dystroglycan that can organize axon guidance cue location which is critical for nervous system development and plays important roles in perisynaptic and axonal matrix formations, contributing to synaptic homeostatic plasticity [94]. Proteins like Dermcidin, retinoic acid induced 1 and cadherin 13 that contribute to cell cycle related events and play roles in neural differentiation were also identified. Dermcidin acts as a neural growth factor [95]; retinoic acid is involved in neurobehavioural disorders and plays a role in normal neural development [96] and Cadherin acts as a regulator of neural cell growth [97]. OM-MSCs were identified as secreting high levels of the chemokine CXCL12 [98] that is known to be important in the promotion of endogenous myelinations [99]. Evidences that OM-MSCs are capable to alter the biological properties of the precursors of OECs and oligodendrocytes and of increasing the myelination of the oligodendrocytes in the CNS [63], in conjunction with the other findings referred, demonstrate the

enormous potential of these cells to be applied in regenerative medicine of the nervous system in general and in the PNS in particular.

3.4. Applications

The studies and characterizations carried out to date suggest that the OM-MSCs have phenotypes and differentiation characteristics similar to other MSCs and can efficiently proliferate in culture. However, studies of direct clinical application of these cells in regenerative therapies are still few and the results obtained need more research and deeper approaches.

Several studies have demonstrated the improvement of locomotor function in animal and human patients with spinal cord injury after implantation of entire OM grafts and OECs [100] but isolated OM-MSCs have never been used singly in this type of cell therapies [73]. In these cases, the effect of OM-MSCs may be masked by the use of OM as a whole, making it impossible to determine the direct effect of MSCs on motor recovery. Despite this, it has been already demonstrated that OM-MSCs promote rat CNS myelination *in vitro* [77]. A study on the use of cell-based therapy in cases of deafness revealed that OM-MSCs present repair efficiency in the spiral ganglion neuron after lesion induction in the cochlea [101]. Transplantation of OM-MSCs into a brain after injury led to a partial reconstitution of the hippocampus, with observation of important phenomena such as migration of stem cells to the inflamed region, *in situ* neuronal differentiation and local stimulus to neurogenesis. The injured individual presented reversal of learning deficits, recovered memorization capabilities and enhanced physiological function. All these events are also observed if the OM-MSCs were transplanted directly into the cerebrospinal fluid. These results open precedents for the use of OM-MSCs in patients with post-traumatic memory loss [102]. Another study has shown that OM-MSCs generated dopaminergic cells and reduced the asymmetries resulting from the ablation of dopaminergic neurons when transplanted into a rat brain model of Parkinson's disease, also opening precedents [103] for its use in neurodegenerative diseases [104]. Recently, immunoregulatory properties of OM-MSCs have been identified, which can exert immunosupressive functions and modulate T-cell responses. These findings indicate a potential use of these cells targeting autoimmune diseases [88, 105].

It is possible that in some studies in which olfactory mucosa cells were used without an exact determination of the cell types present in the heterogeneous cell mix, OM-MSCs were part of the group used and performed specific actions. In these cases where there is no detailed determination of the composition of the cell matrix used, it is impossible to know which type of cell has dominant function or effectively reparative properties. For instance, a study in which transplantation of OM to rat hearts after infarction led to differentiation into cells resembling cardiomyocytes, but failure to specify the types of cells included in the transplantation makes it impossible to attribute regenerative function to OM-MSCs and makes it difficult to optimize future procedures [106]. The use of unpurified olfactory mucosal cells cannot be directly compared to purified OM-MSC and it is essential that each type of transplanted cell is characterized prior to the procedure. Even the preliminary study of the use of OM-MSCs in the treatment of Parkinson's disease resulted from the use of the OM as a whole and not from the MSCs isolated [103]. The combined use of cells is not, obviously, a wrong procedure and it has already been shown that the combined use of OECs and OM-MSCs has beneficial effects of inter-stimulation between cells with respect to their functionality and secretome [106, 107].

It is, however, important to define exactly the role of each of them in the regenerative process before its use on a larger scale. At this point, and given the existing knowledge about the functional characteristics of OM-MSCs, the experience obtained in the few realized studies and the potential that has been established for these cells, is important to set specifc goals and start more focused works to determine the importance of these cells in the future of regenerative medicine, in the approach to lesions of the peripheral nerve as well as to nervous system in general and to other organic systems.

4. Conclusions and further directions

Although we have known for some time that there are multipotent cells in the olfactory epithelium and the olfactory bulb, only recently OM-MSC was identified at the *lamina propria* of OM. Since its discovery, most of the studies that addressed OM-MSC focused on its complete characterization and few studies have yet applied these cells in order to determine their regenerative capacity. The use of these cells presents clear technical advantages due to their location and their collection can be easily made in the donors under anaesthesia and practically without any side effect. In addition, besides its high versatility and clonogenic activity, OM-MSC may be used for autologous transplants, circumventing possible rejections at the site of application and ethical issues. Since MS-MSCs are submitted to an environment with continuous regenerative activity, it is understandable that they secrete higher levels of neurotrophic and myelinating factors when compared to MSCs with other origins. Thus, OM-MSCs appear as a robust candidate for the approach to PNI cases when compared to other MSCs that do not achieve significant success in promoting nerve growth through the glial scar. The regeneration of the peripheral nerve has been the subject of multiple studies and there are many therapeutic techniques currently under development. Despite this, and considering the restrictions still observed in these approaches, regenerative medicine is one of the options with higher potential to achieve success in this type of lesions. Although several types of stem cells and MSCs are already under study, the OM-MSCs, their identified characteristics and preliminary results observed after their application, make them a promise in regenerative medicine in general and, specifically, a revolutionary approach to PNI lesions or demyelinating diseases and in situations in which neuroprotection or neurite outgrowth is important for repair. In this moment of rapid expansion of the knowledge we have about OM-MSCs, the next steps will have to include a complete and unambiguous characterization of these cells, their secretome and their metabolome and a precise determination of their regenerative potential in different tissues, specifically in the peripheral nerve, after isolation from the remaining OM cells. Finally, it will be necessary to explore their use associated with different biomaterials (something that has not yet been done), growth factors and even other cells whose associations have already been shown to be effective, such as OECS.

Acknowledgements

This research was supported by Programa Operacional Regional do Norte (ON.2 – O Novo Norte), QREN, FEDER with the project ‘iBone Therapies: Terapias inovadoras para a

regeneração óssea', ref. NORTE-01-0247-FEDER-003262, and by the program COMPETE—Programa Operacional Factores de Competitividade, Projects PEst-OE/AGR/UI0211/2011 and PEst-C/EME/UI0285/2013 funding from FCT. This research was also supported by Programa Operacional Competitividade e Internacionalização (P2020), Fundos Europeus Estruturais e de Investimento (FEEI) and FCT with the project 'BioMate—A novel bio-manufacturing system to produce bioactive scaffolds for tissue engineering' with reference PTDC/EMS-SIS/7032/2014 and by COMPETE 2020, from ANI—Projectos ID&T Empresas em Copromoção, Programas Operacionais POCI, by the project 'insitu.Biomas—Reinvent biomanufacturing systems by using an usability approach for in situ clinic temporary implants fabrication' with the reference POCI-01-0247-FEDER-017771. The research was also supported by the research project 'BEPIM III—Microdispositivos médicos com capacidades osteintegradoras por micoPIM', with the reference POCI-01-0247-FEDER-017935, from Fundo Europeu de Desenvolvimento Regional (FEDER), by the Programa Operacional da Competitividade & Internacionalização. Ana Rita Caseiro (SFRH/BD/101174/2014) acknowledges FCT, for financial support.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this chapter.

Abbreviations

BM-MSCs	Bone marrow MSCs
CNS	Central nervous system
CNTs	Carbon nanotubes
GBCs	Globular basal cells
HBCs	Horizontal basal cells
MSCs	Mesenchymal stem cells
NGCs	Nerve guidance conduits
OECs	Olfactory ensheathing cells
OM	The olfactory mucosa
OM-MSCs	Olfactory mucosa mesenchymal stem cells
ON	Olfactory neurons
PLA	Poly(lactic acid)
PLC	Poly(DL-lactide-ε-caprolactone)
PLGA	Poly(L-lactide):poly(glycolide)
PVA	Poly(vinyl alcohol)
PNI	Peripheral nerve injuries
PNS	Peripheral nervous system
PPY	Poly-pyrrole
SEM	Scanning Electron Microscope

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