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STUDY OF NEURAL PLASTICITY AND REGENERATION IN SENSORY SOMATIC AND AUTONOMIC NERVOUS SYSTEM AFTER PERIPHERAL NERVE INJURY

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1. INTRODUCTION

1.1 Functional Organization of Peripheral Nervous System

The peripheral nervous system (PNS) is functionally divided in two components: the somatic nervous system and the autonomic nervous system. The somatic nervous system is involved in skeletal muscles and skin innervations. The autonomic nervous system (ANS) is involved in visceral functions, controlling visceral organs of the thoracic, abdominal, and pelvic cavities. The ANS affects heart rate, digestion, respiratory rate, salivation, pupillary dilation, urinary and sexual functions. From the same organs also receives sensory information directed to neuroaxis centers.

Most of its actions are involuntary, others work in tandem with the conscious mind. Since the late nineteenth century, it has been common divide the ANS into three subsystems: the sympathetic nervous system (SNS), the parasympathetic nervous system (PSNS) and the enteric nervous system which differ in organization and structure but are functionally integrated (Swenson Rand 2006).

1.1.1 Sympathetic Nervous System

The sympathetic nervous system affects the peripheral transmission of the visceral organs through nerves and ganglia. Sympathetic trunks are two ganglionated nerve cords that extend on the other side of the vertebral column from the cranial base from the coccyx.

The efferent pathway involves therefore two neurons: the preganglionic and the post ganglionic neuron.

Anatomically preganglionic neurons arise from the thoracolumbar region of the spinal cord at T1 to L2-L3 level and reach the paravertebral ganglia in which they synapse with a postganglionic neuron. From there, the long postganglionic neurons axons across most of the body reaching different target organs. In general one preganglionic neuron may synapse with 15-20 postganglionic neurons, allowing the wide diffusion of many autonomic effects (Figure1). Preganglionic axons are myelinated with an organization that resembles the somatic nerves, while postganglionic axons are unmyelinated and organized in small diameter bundles surrounded by a single Schwann cell (type C fibers).

The sympathetic nervous system is involved in responses that would be associated with fighting or fleeing, they reached the viscera causing vasoconstriction, bronchial

and bronchiolar dilatation, papillary dilatation, inhibition of gastrointestinal muscle contraction, etc. (Saper 2002; Furness 2006; Swenson Rand 2006).

1.1.2 Parasympathetic Nervous system

Preganglionic parasymphatetic neuronal cell bodies are located in cranial nerve nuclei of brainstem and in the grey matter of the second to fourth sacral segment of the spinal cord.

Postganglionic parasympathetic neurons are located distant from the CNS, in ganglia near the innervated structure, in the wall of viscera, their fibers are usually unmyelinated and shorter than the sympathetic fibers.

Parasympathetic nerves arise with cranial nerves III, VII, IX and X, as well as from the sacral segments S2-S4, they have been termed the "craniosacral outflow." Parasympathetic neurons corresponding to the cranial nerve III synapse in the cilliary ganglion and are involved in pupillary constriction and accommodation for near vision, parasympathetic neurons in cranial nerve VII synapse in the pterygopalatine ganglion (lacrimation) or the submandibular ganglion (salivation) while those in cranial nerve IX synapse in the otic ganglion (salivation from parotid gland). The vagus nerve follows a long distance to supply the thoracic and abdominal organs and synapse with ganglia very close to (or within) the organ walls (Figure1). The pelvic parasympathetics segment activate bladder contraction and also supply lower abdominal and pelvic organs innervations (Furness 2006).
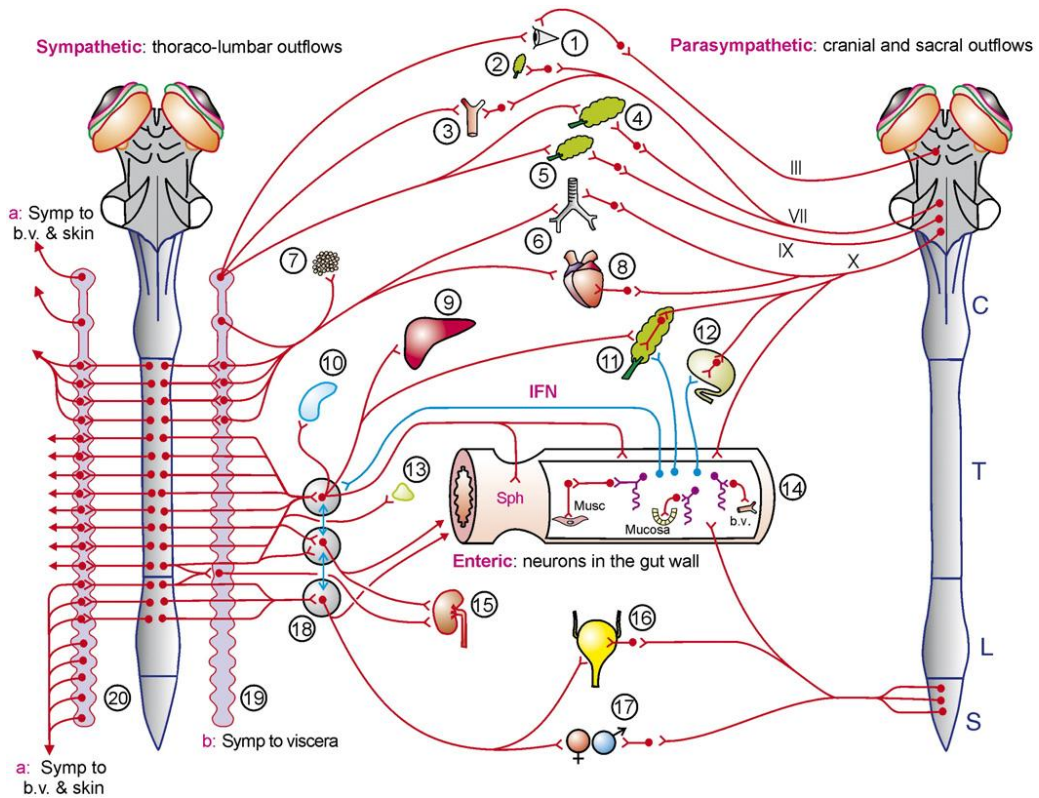
Figure 1*: Representation of pathway of ANS and its connection (Furness, J.B. 2006).*

1.1.3 Somatic nervous system

The somatic nervous system is the part of the PNS carrying motor and sensory information both to and from the CNS, controlling all voluntary muscular system within the body and also mediate involuntary reflex arcs. This system is made up of nerves and sensory ganglia that connect and bring information to the skin, sensory organs, and all skeletal muscles. It is also responsible for sensory information that arrives by external stimuli including hearing, touch, and sight.

The somatic nervous system contains two major types of neurons: the sensory neurons, also known as "afferent" and carrying sensory information from the periphery to the CNS, and the motor neurons, also known as "efferent", responsible for carrying information from the brain and the spinal cord to muscle throughout the body (Kandel, Schwartz "Principles of Neural Science).

1.1.4 Peripheral nerve anatomy

Peripheral nerves are cord tube-shaped branched anatomical structures, formed by bundles of myelinated and unmyelinated nerve fibers, that connect the CNS to the periphery of the body and vice versa. Peripheral nerves are responsible of the sensory and motor stimuli transmission, reaching skeletal muscles, myocardium and different organs that belong to the digestive, respiratory, excretory and circulatory systems (Lundborg 2005; Geuna, Raimondo et al. 2009; Siemionow and Brzezicki 2009) .

Peripheral nerves include, cranial nerves (12 pairs) and spinal nerves (33 pairs). The spinal nerves are divided into 8 cervical nerves, 12 thoracic nerves, 5 lumbar, 5 sacral and 3 coccygeal. The spinal nerves connect the brain and spinal cord to the peripheral tissues. Each nerve is a complex structure formed by of cellular and tissue elements, and surrounded by three connective tissue layers: the epineurium, the perineurium and the endoneurium (Lundborg 2005; Geuna, Raimondo et al. 2009; Siemionow and Brzezicki 2009). Starting from the periphery of the nerve, the most external connective layer is the epineurium, that surrounds the nerve structures during movements and against external trauma. It consists of loose connective tissue of collagen (types I and II) fibers, fibroblasts and adipocytes, and it's penetrated by blood and lymph vessels which branch out in the perineurium (Flores, Lavernia et al. 2000; Geuna, Raimondo et al. 2009; Siemionow and Brzezicki 2009). The perineurium is characterized by an outer layer named circumferential, and an inner layer named interfascicular, consisting of collagen fibers. The perineurial membrane is not only a resistant barrier to mechanical trauma, but also an active barrier, that protects the below endoneurium providing a metabolic filter action. The capillaries that cross through the perineurium continue into the endoneurium, ensuring the necessary oxygen and substance supply to single axons and Schwann cells (SCs) (Diao and Vannuyen 2000; Flores, Lavernia et al. 2000; Lundborg 2005). The endoneurium represents the inner layer consisting of loose connective tissue and collagen type I fibers, which run parallel to the axis of the axons; in addition to these elements, fibroblasts, macrophages and mast cells are still present. The endoneurium surrounds the single nerve fiber, made of axon and SCs. (Diao and Vannuyen 2000; Lundborg 2005) (Figure 2). The smallest functional unit of a peripheral nerve is the nerve fiber, and is responsible of the motor and sensory impulse conduction. Anatomically, depending on the strategy adopted from SCs to enclose axons, nerve fibers can be distinguished in two subgroups: myelinated and unmyelinated.

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Myelinated nerve fibers consist of a single axon that is enveloped individually by a single SC (Figure 3A). The SC membrane wraps around the nerve fiber to form a multilaminated myelin sheath.

Unmyelinated nerve fibers are composed of a group of several axons enveloped by a single Schwann cell (Flores, Lavernia et al. 2000) (Figure 3B). In myelinated fibers axons are enveloped by a chain of SCs, arranged in longitudinal sequences along the length of the axon. Between each SC, there is an interspace of axon known as "node of Ranvier". This area allows extracellular ions to reach the axon, inducing the saltatory conduction of the impulses along the nerve fiber. (Flores, Lavernia et al. 2000; Geuna, Raimondo et al. 2009).

Furthermore the nerve fibers can be also classified according to the type, direction of the stimulus, and to the diameter.

Considering the type and direction of the pulse, nerve fibers can be distinguish between: effector, sensitive and sensitive specific fibers.

The effector (efferent) fibers, lead centrifugal impulses from the CNS to the periphery. They could be subdivided into somatic fibers, that supply voluntary movements of skeletal muscle, and visceral fibers, providing the innervation of smooth muscles of visceral organs, vessels, myocardium and glands.

The sensitive (afferent) fibers, supply centripetal information from the periphery to the CNS and are classified into: somatic fibers, leading to nervous centers sensory impulses derived from sensitive receptors skin, muscles and joints; visceral fibers, that supply sensory impulses from sensory receptors located into the visceral organs and the sensitive specific (or special sensory) fibers, providing information from sense organs to the nervous centers.

Fiber diameter is directly proportional to the size of the cell body to which the fiber belongs, and to the length of the fiber itself. For this reason, nerve fibers could be classified in three different groups: A-fiber, myelinated fiber with a diameter of 3-22 µm; B-fibers, myelinated with 1.5-3 µm diameter; C-fibers, unmyelinated with 0.3 to 1.5 µm diameter.
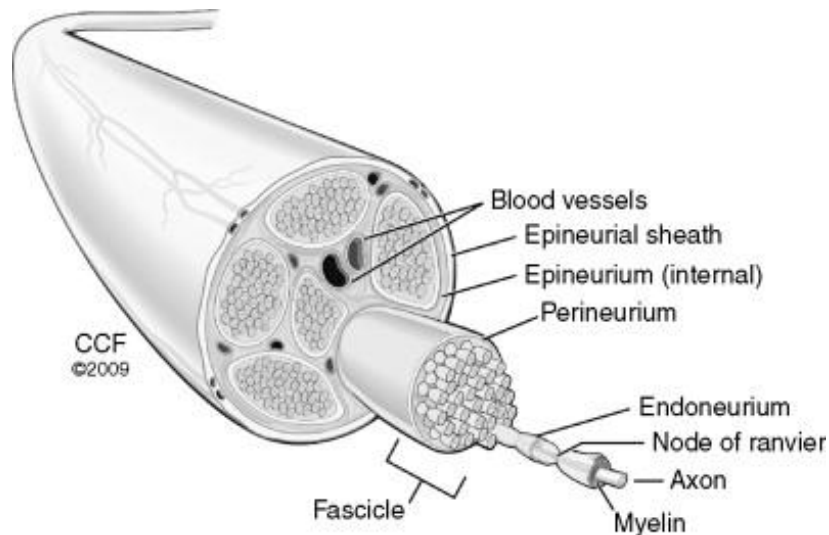
Figure 2: *Anatomy of peripheral nerves (Siemionow and Brzezicki 2009).*
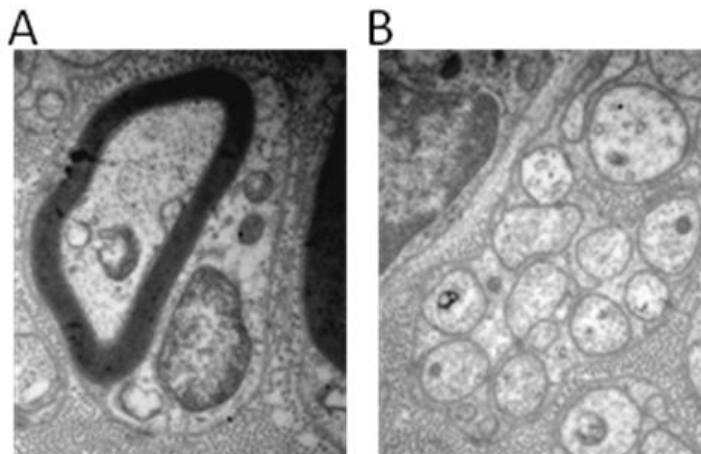
Figure 3: *Transmission electron microscope (TEM) showing myelinated nerve fiber (A), and unmyelinated nerve fibers (B).*

1.1.5 Dorsal root ganglia anatomy

The dorsal root ganglion (DRG) is composed by a cluster of neuronal cell bodies located in the dorsal root of a spinal nerve and surrounded by a connective tissue capsule. During development neural crest cells extensively migrate ventrally between the neural tube and the dermomyotome giving arise the DRG. It is possible to distinguish the ganglion cell population in pseudounipolar sensory neurons and satellite cells, (Sapunar, Kostic et al. 2012).

The satellite cells represent the supportive glial cell that envelop neurons within the ganglion making extensive contacts. They are located in the connective interstices between the neurons and exert many essential and complex functions, such as trophic

and supporting functions (Nascimento, Santiago et al. 2008; Procacci, Magnaghi et al. 2008).

Satellite glial cells show a similar role to astrocytes in the CNS, supplying nutrients to the surrounding neurons and also have some structural function as protective, and cushioning cells. Furthermore they express a variety of receptors that allow a range of interactions with neuroactive chemicals molecules (Hall and Landis 1992; Shinder and Devor 1994; Hanani 2010; Hanani 2010).

Sensory DRG neurons are characterized by the presence of a voluminous pyrenophore and a central nucleus. From the pirenophore a neurite branches off, which after a short path divides into T, resulting in a central branch and a peripheral branch with a cellulite conduction (Fornaro, Lee et al. 2008); for this reason neuronal population within the ganglia show a pseudounipolar morphology, typical of sensory ganglia both associated with spinal and cranial nerves. These neurons are responsible for sense touch, pain, cold and warm sensation, limb movements and spatial position (Marmigere and Ernfors 2007).

Based on morphological and ultra structural features DRG neurons can be also classified into two neuronal subpopulations: large-light neurons and small-dark neurons (Figure 4).

The different neurons are specialized to a specific function and the functional type presents unique molecular characteristic, unique set of ion channels and respond to unique set of sensory stimuli (Marmigere and Ernfors 2007).

The location and the presence of the connective capsule surrounding the DRG define this structure as an isolated peripheral station of neuronal bodies for this reason, easily identifiable and represent a valid model for many experimental study (Sugawara, Atsuta et al. 1996; Cho, Shin et al. 2002; Sapunar, Kostic et al. 2012).
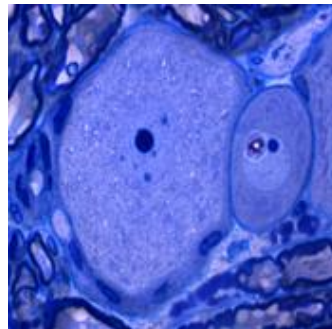
Figure 4: *subpopulation of dorsal root ganglia neurons "large and light" and "small and dark".*

1.2. Peripheral nerve injury classification

Nerve injury can be classified as a defect, that results in a lesion of a nerve, impairing the action potential transmission. Peripheral nerve lesions are common injuries, that may result in partial or complete loss of motor or sensory function, leading to long-term disability (Grinsell and Keating 2014). A large number of injuries types and severities have been classified, in 1943, Sir Herbert Seddon introduced a classification of 3 discrete types of nerve injury: neurapraxia, axonotmesis, and neurotmesis (Seddon 1942).

(i) Neurapraxia is a mild injury characterized by local myelin damage. Axon continuity is preserved, and the nerve does not undergo Wallerian degeneration. It may result from exposure to a wide range of conditions such as heat, cold, irradiation or electrical injuries, but is most commonly due to mechanical stress, such as concussion, compression or traction injuries. Recovery may occur within hours, days, weeks, or up to a few months.

(ii) Axonotmesis involves additional damage to peripheral axons, but connective tissue structures remain intact. The interruption of axons is often the result of nerve pinching, crushing or prolonged pressure. Wallerian degeneration occurs, but subsequent axonal regrowth may proceed along the intact endoneurial tubes. Recovery depends upon the degree of internal disorganization in the nerve as well as the distance to the end organ.

(iii) Neurotmesis is the most severe injury, equivalent to physiologic disruption of the entire nerve. Functional recovery does not easily occur because of the extent of endoneurial tube disruption. Nonetheless, successful regeneration might result with surgical intervention.

In 1951, Sunderland expanded Seddon's classification to five degrees of peripheral nerve injury instead of three (Figure 5) (Sunderland 1951). He divided Seddon's axonotmesis grade into three types, depending on the degree of connective tissue involvement.
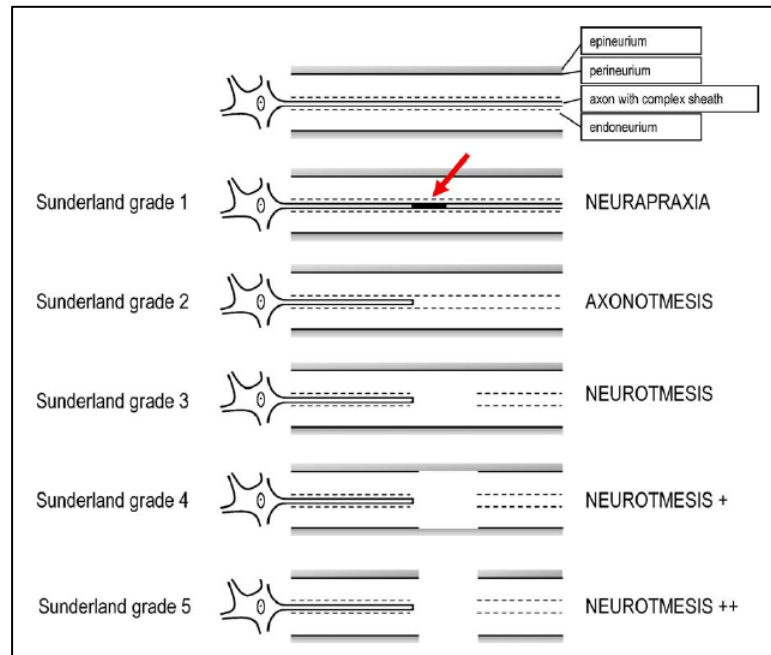
Figure 5: *Schematic representation of the five degrees of nerve injury according to Sunderland (Sunderland 1951). (Deumens, Bozkurt et al. 2010)*

(i) Type 1 injury corresponds to Seddon's neurapraxia with conduction block and completely intact stroma.

(ii) Type 2 injury corresponds to Seddon's axonotmesis. The endoneurium, perineurium, and epineurium are still intact, but the axons are physiologically disrupted. Recovery can occur by axonal regrowth along endoneurial tubes, and complete functional recovery can be expected. The time for recovery depends on the level of injury, usually months.

(iii) In type 3 injury, the endoneurium is also disrupted, but the surrounding perineurium and epineurium are intact. Recovery is incomplete and depends upon how well the axons can cross the site of the lesion and find endoneurial tubes.

(iv) In type 4 injury individual nerve fascicles are transected, and the continuity of the nerve trunk is maintained only by the surrounding epineurium. This type of injury requires surgical repair or reconstruction of the nerve.

(v) Type 5 injury is equivalent to Seddon's neurotmesis (complete nerve disruption), and spontaneous recovery is negligible.

Although Sunderland's classification provides a concise and anatomic description of nerve injury, the clinical utility of this system is debatable since a nerve may undergo a combination of different degrees of injury.

Therefore, in 1988 Susan E. Mackinnon and A. Lee Dellon describe a 6th degree of nerve injury to address a mixed nerve injury. They use the term "neuroma in continuity" to describe a combination of the degrees of injuries per fascicle (Mackinnon 1989).

1.3 Plasticity after peripheral nerve injury and regeneration

In the CNS the term "plasticity" is refers to the ability of neurons to changes and reorganize themselves in response to different stimuli that lead to new connections (Kolb and Gibb 2011). Plasticity (in response to stimulus) and regeneration (in response to injury) are based on adaptive changes in neural circuitries and synaptic reorganization of cortical neurons that can change their synaptic organization and function (Mohanty, Bhat et al. 2015).

In the PNS instead, plasticity is predominantly based on axonal (re)growth (Geuna, Fornaro et al. 2010).

It is well known that PNS and CNS are functional integrated regarding the consequences of a nerve injury in which nerve lesion result in long-lasting central modifications and reorganization (Kaas 1991; Wall, Xu et al. 2002; Kaas and Collins 2003; Navarro, Vivo et al. 2007). Recent research has discovered that peripheral nerves trauma induce a cascade of events, at cellular and molecular levels involving also plastic changes at different central levels such as spinal cord, brainstem nuclei, thalamus and brain cortex (Sharma, Classen et al. 2013).

Furthermore recent evidences show that plastic changes involve also the dorsal root ganglia neurons as a consequence of peripheral nerve injury (Muratori, et al. 2014).

In the following paragraphs the plasticity phenomenon will be described with particular reference to the changes occurring at cellular, axon, and molecular level after peripheral nerve injury.

1.3.1 Neuronal reaction and survival

The success of nerve regeneration and functional recovery depends on several processes such as the ability of axotomized neurons to survive and shifts to the regenerative state.

Whereas the Wallerian degeneration occurs, the soma reacts to the injury with substantial metabolic changes necessary for regeneration and axonal elongation. Changes in neuronal cell bodies can be seen several hours after injury with the Nissl bodies dissolution (chromatolysis) followed by nuclear eccentricity and enlargement, cell swelling, dendrites retraction (Lieberman 1971).

Studies show that sympathetic neurons of the superior cervical ganglia undergo dramatic changes after axotomy of the post ganglionic fibers with a deep neuronal loss 3 days after injury (Hou, Lundmark et al. 1998).

The severity and the time course of the neuronal response are mainly influenced by the severity of the injury, the distance of lesion to cell body, the type of neuron involved in lesion, and age: chromatolysis started 8 h following cranial nerves transaction and was not fully reversed by 3 months after axotomy. The reaction was more intense and longer lasting following axotomy without reinnervation than with reinnervation (Guntinas-Lichius, Neiss et al. 1996). Intense chromatolysis affects motoneurons before undergoing apoptosis suggesting that this process could promote either survival/regeneration or apoptosis of neurons (Martin, Kaiser et al. 1999). When neurons starts the apoptotic process after chromatolysis, accumulation of metabolically active mithocondria within the soma and oxidative products occur.

Chromatolytic changes are due to the alteration of the metabolic machinery, and gene expression of growth factor receptors, transmitters, transcription factors (c-Jun, c-Jun N-terminal kinase (JNK), activating transcription factor 3 (ATF3)), and cytoskeletal components (Fornaro, Lee et al. 2008; Dahlin, Johansson et al. 2009). Such changes, switch the neurons function from a "signalling mode" to a "growing mode" (Fu and Gordon 1997), by the expression of axon regeneration associated proteins (Johnson, Zoubos et al. 2005; Dahlin, Johansson et al. 2009; Geuna, Raimondo et al. 2009; Richardson, Miao et al. 2009)

When axons are able to regenerate and reinnervate target organs, neurons showed disorganization of ribosome clusters related to increase of protein synthesis suggesting that chromatolysis represent the most important change associated with an anabolic reaction of the soma (Lieberman 1971; Fu and Gordon 1997).

With the regenerative state, axotomized neurons undergo prominent changes in gene expression that lead to increase the synthesis of growth factors and structural proteins of membrane.

The main metabolic activity of the cell is shifted from synthesizing neurotransmitter-related proteins to the synthesis of structural materials needed for axon repair and growth. For example, choline acetyltransferase (CAT), is downregulated, whereas the neuropeptide, calcitonin gene-related peptide (CGRP), the fast transported growth-associated protein, GAP-43, and the slowly transported cytoskeletal proteins, actin and tubulin, are upregulated (Tetzlaff, Gilad et al. 1988; Haas, Donath et al. 1993). Glucose-6-phosphate dehydrogenase and hydrolytic enzyme are also up-regulated (Fawcett and Keynes 1990; Davis, Taylor et al. 2011). The success of nerve regeneration and functional reinnervation of targets depend at a first instance on the capacity of axotomized neurons to survive and shift towards the regenerative phenotype (Navarro, Vivo et al. 2007).
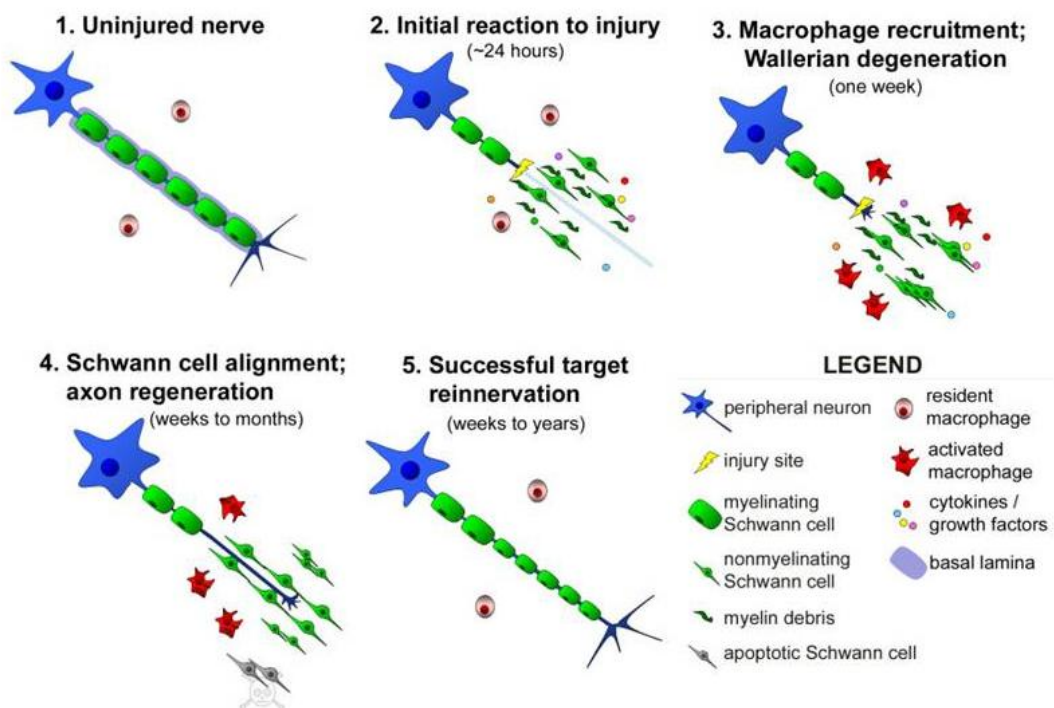
Figure 6: Progression of Wallerian degeneration and axon regeneration after peripheral nerve injury. 1. uninjured nerve fiber consisting of axons, associated SCs and resident macrophages. 2. after peripheral nerve injury, SCs release start to proliferate within their basal lamina tubes, they produce cytokines/trophic factors, and phagocytose detached debris. 3. Wallerian degeneration within

a week of injury. Soluble factors produced by Scs and injured axons activate resident macrophages and lead to recruitment of circulating macrophages that clear myelin and axon debris, and produce factors that facilitate SCs migration and axon regeneration. 4.Injured axons form a growth cone and begin to regenerate along bands of Büngner formed by SCs. 5. axon connects with peripheral targets (Gaudet, Popovich et al. 2011).

1.3.2 Dorsal root ganglia neurons as a model for study the plasticity of PNS

As a consequence of a peripheral nerve injury the trophic support from the periphery is blocked and DRG neuron cell bodies undergo adaptive changes such as the reversal of chromatolysis. Indeed, the nucleus returns to the cell center and nucleoproteins reorganize into the compact Nissl granules. A complex and incompletely understood interaction occurs between the cell body and the regenerating axon tip in which axoplasm arises from the proximal axon segment and cell body.

Results of studies on changes in DRG neurons number following a nerve injury show controversial results. Most of the authors report that after peripheral nerve transaction sensory neurons undergo apoptosis (Himes and Tessler 1989; Vestergaard, Tandrup et al. 1997; Terenghi 1999; Navarro, Vivo et al. 2007), showing that between 7% and 50% of primary sensory neurons (more small than large neurons) die after injury (Himes and Tessler 1989; McKay Hart, Brannstrom et al. 2002). Other authors instead report no significant neuron loss (Swett, Hong et al. 1995) or no detectable loss of dorsal root neurons until 4 months after injury to the spinal or sciatic nerve (Coggeshall, Lekan et al. 1997).

Moreover recent study show that the number of DRG neurons counted in animals that underwent crush injury lesion of brachial plexus nerves was significantly higher compared to controls after crush injury and the data correlate with the increase of DRG volume. These important results allowing to state for that the neuronal population of spinal ganglia after axonotmesis is not static. Furthermore the pattern of expression of precocious neuronal and glial markers lead the hypothesis that new neuronal progenitors may originate from dedifferentiation of satellite glial cells (Muratori, Ronchi et al. 2015).

1.3.3 Cellular and molecular changes that occur during plasticity of the PNS

Peripheral nerve injuries result in partial loss of autonomic and somatic motor and sensory function. The mechanism of plasticity and neural reaction due to axotomy of nerve fibers induce a concurrent cascade of events starting with the degeneration of axons distal to the lesion site. Complex pathophysiological processes, including morphologic and metabolic changes, occur at the injury site almost immediately. The interruption of a peripheral nerve causes significant changes in normal morphology and tissue organization both proximally and distally to the lesion site (Figure 6). The functional significance of the regeneration process is to replace the distal nerve segment reaching the target organ and achieving the functional recovery.As a consequence of nerve injury axons distal to the lesion site are disconnected from the neural body and degenerate; the myelin sheath is degraded by the " Wallerian degeneration" in honor of Augustus Volney Waller, who first characterized this process (Stoll, Jander et al. 2002; Koeppen 2004; Navarro, Vivo et al. 2007). Wallerian degeneration starts immediately after injury and involves myelin degradation of axoplasm, axolemma and myelin sheath that is transformed into neutral lipid compounds by SCs. At the same time infiltrated blood monocytes and macrophages are recruited at the injury site due to an intense inflammatory response that leads an increase of chemokines, cytokines, interleukin 1 (IL-1) and tumor necrosis factor (TNF) (Jessen and Mirsky 2008).

Occurrence of Wallerian degeneration contributes to axonal regeneration due to clearance of myelin debris and growth inhibitors, and subsequent creates a regenerative microenvironment favorable for the axonal regrowth of surviving neurons (Shen, Lassner et al. 2000; Huang, Hu et al. 2010; Cheng, Wang et al. 2017). Scs play a key role during nerve regeneration thanks to their impressive regenerative properties that allow to revert back to a dedifferentiate and proliferative phenotype (Kim, Mindos et al. 2013). Within 48 hours from injury SCs start to dedifferentiate in the distal stump and change the expression of different gene such as myelin proteins (e.g., P0, MAG) and connexin 32 that dramatically decrease as a consequence of axonal degeneration, whereas regeneration-associated genes, and neurotrophin such as NGF, GDNF, BDNF are up-regulated and promote axon growth (Trapp, Hauer et al. 1988; Lee, Shin et al. 2009).

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Between days 1 and 5 after injury, SCs start to proliferate peaking around day 3 post-injury and then decrease during the following weeks. Proliferating Schwann cells align with the endoneurial tubes in columns known as bands of Büngner at the basal lamina level exhibit different adhesion molecules on the plasma membrane, that support and guide the regenerating axons. Axon-SCs attachment is mediated by various adhesion molecules including the immunoglobulin superfamily, e.g. neural cell adhesion molecule (N-CAM), the cadherin superfamily, e.g. N-cadherin and E-cadherin, whereas axonbasal lamina contact is for the most part mediated by laminin (Letourneau, Condic et al. 1994; Ide 1996).

Wallerian degeneration and SCs proliferation processes, are fundamental steps to allow the subsequent axon regeneration (Gaudet, Popovich et al. 2011; Chan, Gordon et al. 2014).

Aligned SCs and their extracellular matrix provide indispensable pathways to guide axonal regrowth (Stoll, Jander et al. 2002; Kim, Mindos et al. 2013).

At this stage of the regeneration process, the presence of an appropriate basal lamina, a specialized and complex network of extracellular matrix proteins capable of supporting axon re-growth, is essential (Figure 6). The columns of Büngner ensure the directional and regenerative substrate for growth cones of sprouting axons (Ann, Mizoguchi et al. 1994; Son and Thompson 1995; Hoke, Redett et al. 2006). During this re-growing phase, SCs are able to produce several neurotrophic factors (NTFs) (Gordon 2010; Richner, Ulrichsen et al. 2014). Growth cones of attracted re-growing axons use these SCs-made tubes as a growing substrate, extending at an average rate of 1-3 mm/day (Radtke and Vogt 2009). At the end of the regeneration process, the interaction between maturing SCs and re-grown axons in the distal nerve stump induces myelin formation with significantly shorter internodes (Luis, Amado et al. 2007; Nave and Trapp 2008; Deumens, Bozkurt et al. 2010; Gordon, Tyreman et al. 2011). Success of axon regeneration and re-myelination results in the target innervation restoration, with neurotransmission recovery at the neuromuscular junction level.

The regeneration process of minor injury or medium-size gap injury start immediately, and the target reinnervation with the consequent functional recovery occurs very quickly (Schmidt and Leach 2003; Deumens, Bozkurt et al. 2010). Instead, in severe injury like neurotmesis, damages can range from a disruption of a subset of endoneurial tubes to a complete transection of the nerve, with high ECM

and cellular architecture disorganization. In this case, fibroblasts and endoneurial SCs proliferate and migrate from both ends of the damaged nerve, shaping a cellular and connective tissue bridge across the lesion site. Because of the extent of endoneurial tube disruption, functional recovery does not always easily occur in this kind of lesions (Battiston, Geuna et al. 2005; Dahlin, Johansson et al. 2009; Deumens, Bozkurt et al. 2010).

1.4 Factors involved during peripheral nerve regeneration: neurotrophin and cytokine

As described above after nerve injury and during regeneration, many interactions between both cellular and the extracellular matrix are involved.

The complexity of nerve regeneration makes a range of elements all essential to the process; among them, neurotrophic factors (NTFs) and cytokines aroused a great deal of interest due to their role as important cell modulators (Sebben, Lichtenfels et al. 2011).

The main difference between these two type of molecules is that cytokines are inducible while NTFs are constitutively expressed. However they are both up-regulated following nerve injury(Terenghi 1999).

Cytokines and neurotrophic growth factors have been classified into families according to their sequence similarity and to the type of binding receptors (Hopkins and Rothwell 1995; Rothwell and Hopkins 1995).

SCs secreted NTFs and play an essential role attracting re-growing axons from the proximal nerve stump, they are involved in chemotaxis, cell migration and support axonal elongation allowing target organs reinnervation (Lewin and Barde 1996; Huang and Reichardt 2001; Gordon 2010; Allodi, Udina et al. 2012; Richner, Ulrichsen et al. 2014).

At the same time, A rapid pro inflammatory response after peripheral nerve injury is required for clearance of tissue debris (Wallerian degeneration) and effective regeneration. Unlike the CNS, this response is rapidly terminated in peripheral nerves starting between 2 and 3 weeks after crush injury (Fregnan, Muratori et al. 2012; Siqueira Mietto, Kroner et al. 2015).

Cytokines and chemokines are important mediators of the immune response, which possesses various functions such as chemotaxis, lymphoproliferation, pro- and anti-

inflammatory activity (Dinarello 1997; Dinarello 2000; Opal and DePalo 2000). Cytokines represent also the main mediators of neuroinflammation in several pathologies and neurodegenerative processes of the nervous system (Cacquevel, Lebeurrier et al. 2004; Franzen, Bouhy et al. 2004) . The up regulation of cytokine and chemokine expression following nerve injury suggests that they play essential roles in both Wallerian Degeneration and axonal regeneration in the peripheral nervous system (Shamash, Reichert et al. 2002).

The main studied growth factors and cytokines are summarized below, with a description of their physiological role during peripheral nerve regeneration.

1.4.1 Neurotrophin

NERVE GROWTH FACTOR (NGF)

The nerve growth factor (NGF) is the first neurotrophin discovered in 1950 by Rita Levi Montalcini and Viktor Hamburger, and most researched among the neurotrophic family, due to its action in Schwann cells migration and neuritis outgrowth (Yip, Rich et al. 1984; Levi-Montalcini 1987; Rich, Luszczynski et al. 1987; Anton, Weskamp et al. 1994; Shakhbazau, Kawasoe et al. 2012; Sarkar, Chaudhary et al. 2013). NGF binds to TrkA receptors promoting neurite outgrowth, SCs migration, survival and differentiation of sensory and sympathetic neurons, (Boyd and Gordon 2003). After nerve injury the NGF expression levels is up regulated in particular in denervated sensory dorsal roots than in motor ventral roots (Allodi, Udina et al. 2012). At the injury site level, NGF is expressed by Schwann cells of the proximal and distal nerve stump in a biphasic trend With a first peak appears at 6 hours after injury, and the second increase is slower and probably stimulated by infiltrating macrophage during Wallerian degeneration (Aloe, Rocco et al. 2012).

NEUROTROPHIN-3 (NT-3) AND NEUROTROPHIN-4/5 (NT4/5)

Other important molecules involved during the regeneration process are *Neurotrophin-3 (NT-3)* and *Neurotrophin-4/5 (NT4/5)*. After nerve transection NT-3 levels have been shown to decrease, returning to normal levels 2 weeks after injury

(Funakoshi, Frisen et al. 1993). Neurotrophin-3 is one of the most important factors for nerve regeneration and remyelination, by supporting denervated Schwann cells survival in the distal nerve stump (Meier, Parmantier et al. 1999; Sahenk, Oblinger et al. 2008).

Neurotrophin-4/5 (NT4/5) is a more recent discovered trophic factor, which exerts its neurotrophic action promoting motor and sensory neuron survival (Schmalbruch and Rosenthal 1995; Stucky, Shin et al. 2002), and axons growth (English, Meador et al. 2005). After peripheral nerve injury, a downregulation of the NT-4/5 mRNA has been reported in the early phases. However, the upregulation of the mRNA level has be found 2 weeks after injury, in the rat distal nerve stump (Funakoshi, Frisen et al. 1993; Griesbeck, Parsadanian et al. 1995). Moreover, it has been reported the involvement of the NT-4/5 in the enhancement of axon regeneration, produced by treadmill training after peripheral nerve injury (English, Cucoranu et al. 2011).

GLIAL DERIVED NEUROTROPHIC FACTOR (GDNF)

The Glial Derived Neurotrophic Factor (GDNF) belongs to the TGF family, and is known to have a trophic effect on motor, sensory and autonomic neurons (Henderson, Phillips et al. 1994; Buj-Bello, Buchman et al. 1995; Trupp, Ryden et al. 1995). GDNF is able to mediates axon-glial interactions, in fact, Schwann cell-derived GDNF is taken up by sensory and motor neurons, anterogradely transported along the axons and released at terminals, acting on glial cells (Allodi, Udina et al. 2012). After nerve injury, expression levels of GDNF and its receptor are upregulated (Hoke, Cheng et al. 2000), suggesting an important trophic role on nerve regeneration.

Furthermore *in vitro* administration of GDNF as free factor or conjugated with iron nanoparticles, induces neurite extension in adult and neonatal DRG explants (Morano, Wrobel et al. 2014); the conjugated form is also responsible of a more precocius appeareance of myelin in an *in vitro* model of DRG organotypic culture (Ziv-Polat, Shahar et al. 2014).

GDNF is considered an important protective factor for motor neurons, as well as for sensory neurons during the regeneration process (Henderson, Phillips et al. 1994).

INTRODUCTION

BRAIN DERIVED NEUROTROPHIC FACTOR (BDNF)

Brain Derived Neurotrophic Factor (BDNF) is expressed in both motor and sensory nerves, promoting axonal outgrowth and neuron survival (Henderson, Camu et al. 1993; Braun, Croizat et al. 1996; Oudega and Hagg 1999; Boyd and Gordon 2002; Allodi, Udina et al. 2012). Moreover, DRG neurons significantly increase the anterograde BDNF transport in both peripheral and central processes (Zhou and Rush 1996; Tonra, Curtis et al. 1998) after injury of the peripheral nerve. Instead, a slow monophasic BDNF mRNA increase production by Schwann cells is observed in the distal nerve segment (Meyer, Matsuoka et al. 1992; Zhang, Luo et al. 2000). The role of endogenous BDNF after peripheral nerve injury is crucial for the induction of the regenerative response in sensory neurons (Geremia, Pettersson et al. 2010), and for motor neuron axonal regeneration and remyelination (Zhang, Luo et al. 2000; Boyd and Gordon 2001; Wilhelm, Xu et al. 2012).

VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)

Vascular Endothelial Growth Factor (VEGF) is a potent angiogenic factor mainly expressed by endothelial cells but also by activated macrophages and during cancer pathogenesis (Berse, Brown et al. 1992) that stimulates endothelial cell migration and proliferation, blood vessel formation and increases vascular permeability (Neufeld, Cohen et al. 1999; Holmes and Zachary 2005).

Since the vascular and the nervous system show similar anatomical features and despite the main role of VEGF as a pro-angiogenetic factor, an increasing number of studies focus the attention on VEGF activity on different neural cell types and recent evidence shows a role for VEGF as a neurotrophic and neuroprotective factor for neurons and glial cells(Ruiz de Almodovar, Lambrechts et al. 2009). In fact, VEGF stimulates the proliferation of neuronal precursors, increasing the BrdU labeling, in *in vitro* and *in vivo* models of neurogenesis (Jin, Zhu et al. 2002). Furthermore it has been reported that VEGF administration enhances axonal outgrowth from dorsal root ganglia adult mice explants promoting the survival of neurons and satellite glial cells (Sondell, Lundborg et al. 1999; Sondell, Lundborg et al. 1999; Hobson, Green et al. 2000; Brockington, Lewis et al. 2004; Pereira Lopes, Lisboa et al. 2011).

Evidence has also been provided that VEGF administration increases the functional recovery after peripheral nerve injury (Haninec, Kaiser et al. 2012) since it was shown that after end-to-end neurorraphy (ETE) and end-to-side neurorraphy (ETS)

of transected muscolocutaneous rats nerves, plasmid VEGF transfection in the distal stumps resulted in a better axon regeneration in terms of fibers density, axons diameter and myelin sheath thickness of regenerated axons (Haninec, Kaiser et al. 2012).

Finally, VEGF may exercise a trophic effect on primary cortical neurons and dorsal root ganglia, promoting neurite outgrowth (Sondell, Lundborg et al. 1999; Sondell, Sundler et al. 2000; Jin, Mao et al. 2006), increases Schwann cell proliferation, migration (Sondell, Lundborg et al. 1999; Schratzberger, Schratzberger et al. 2000) and neuron survival.

1.4.2 REVIEW "ROLE OF INFLAMMATORY CYTOKINES IN PERIPHERAL NERVE INJURY".

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Special Issue

Role of inflammatory cytokines in peripheral nerve injury*☆●

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Abstract

Inflammatory events occurring in the distal part of an injured peripheral nerve have, nowadays, a great resonance. Investigating the timing of action of the several cytokines in the important stages of Wallerian degeneration helps to understand the regenerative process and design pharmacologic intervention that promotes and expedites recovery. The complex and synergistic action of inflammatory cytokines finally promotes axonal regeneration. Cytokines can be divided into pro- and anti-inflammatory cytokines that upregulate and downregulate, respectively, the production of inflammatory mediators. While pro-inflammatory cytokines are expressed in the first phase of Wallerian degeneration and promote the recruitment of macrophages, anti-inflammatory cytokines are expressed after this recruitment and downregulate the production of all cytokines, thus determining the end of the process. In this review, we describe the major inflammatory cytokines involved in Wallerian degeneration and the early phases of nerve regeneration. In particular, we focus on interleukin-1, interleukin-2, interleukin-6, tumor necrosis factor-β, interleukin-10 and transforming growth factor-β.

Key Words

pro-inflammatory cytokines; anti-inflammatory cytokines; inflammatory reaction; peripheral nervous system; nerve injury; wallerian degeneration; Schwann cells; macrophage; axonal regeneration; myelin; neural regeneration

Research Highlights

(1) Inflammatory cytokines activated after nerve injury lead to a characteristic series of cellular and molecular events that facilitate axon regeneration and, eventually, re-innervation of target tissues.
(2) After peripheral nerve injury, pro- and anti-inflammatory cytokines are produced by both immune and non-immune cells resident in the distal part of the injured nerve or recruited from blood circulation.
(3) As surgically repaired nerves still do not give complete recovery of nerve function, better knowledge of these inflammatory events should provide new therapeutic perspectives that improve and accelerate peripheral nerve regeneration and functional recovery.

Abbreviations

WD, Wallerian degeneration; SCs, Schwann cells; TNF-α, tumor necrosis factor-α; TGF-β, transforming growth factor-β; NCAM, neural cell adhesion molecule; SCIP, suppressed cAMP-inducible POU-domain protein.

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INTRODUCTION

Peripheral nerve damage is a common injury; although the fibres retain a considerable regeneration potential in the adult, recovery is usually rather poor, especially in cases of large nerve defects. The clinical outcome of nerve lesions is far from satisfactory and functional recovery is seldom complete. It is necessary to understand more fully the biological mechanisms that underlie the complex sequence of events that follows nerve damage, but also to define the best strategies for optimizing post-traumatic nerve regeneration.

After peripheral nerve injuries, cellular and molecular events that occur in the distal part (Wallerian degeneration, WD) and stimulate axonal regeneration[1-3]. Axons in the distal part of the injured nerve remain intact for some days before granular disintegration of the cytoskeleton[4]. During the first 24 hours following injury and before the arrival of blood-derived macrophages, SCs control demyelination by degrading myelin basic protein[5].

Following the loss of contact of the glia with the axon and the activation of inflammatory events, myelin sheath disintegration, SC proliferation and rearrangement of the band of Büngner occur.

The breakdown of the blood-nerve barrier allows blood factors and cells that facilitate tissue repair to enter the nerve in the first 2 weeks. Permeability of the blood-nerve barrier increases again after 4 weeks in order to regain homeostasis after WD[6].

Immediately after nerve injury, the important phenomenon of macrophage recruitment occurs to aid debris elimination and tissue remodeling[7]. The rapid clearance of degenerated myelin activated by SCs and macrophages is a crucial step for successful nerve regeneration. In fact, during WD, SCs and macrophages phagocytize the degenerated myelin and moreover produce cytokines and neurotrophic factors necessary for axon regeneration.

The inflammatory reaction is mainly due to macrophages. Signaling pathways between primary sensory neurons, SCs and immune cells are highly intertwined, and cytokines and chemokines are central components in this complex network[8].

Recent studies have been focused on inflammatory events occurring during WD[9-11]. There are several different factors produced by macrophages and SCs that play an important role during degeneration and regeneration of a peripheral nerve.

Activation of immune and immune-like glial cells in the injured nerve leads to the release of both pro- and anti-inflammatory cytokines[5]. Pro-inflammatory cytokines [*e.g.* interleukin (IL)-1, -2, -6 and tumor necrosis factor (TNF)] are expressed principally in the first phase of WD, promoting the recruitment of macrophages from 2 to 3 days after the injury, while anti-inflammatory cytokines [(*e.g.* IL-10 and transforming growth factor-β (TGF-β)] are expressed after macrophages recruitment[11] and have the role of attenuating the inflammatory process.

This review focused on the role of the inflammatory cytokines in WD and in the early phases of regeneration of a peripheral nerve.

TNF-α

TNF-α is a pro-inflammatory cytokine expressed as a 26-kDa transmembrane protein that can be cleaved to release a 17-kDa soluble form. It is widely considered as the prototypical pro-inflammatory cytokine because it regulates inflammatory responses after injury to the peripheral or central nervous system[12-14], initiating the activation cascade of other cytokines and growth factors[15]. TNF-α exerts its action *via* its receptors, TNF-α receptor 1 (TNF-R1) and the lower affinity TNF-R2[16], which are expressed in both glia and neurons[17]. TNF-R1 contains an intracellular "death domain" and is responsible for controlling the neuronal death. TNF-R2 contributes to neuroprotection due to his relation to T-cell development and the proliferation[12,13,16-18].

In the peripheral nervous system, the endogenous TNF-α released by SCs, resident macrophages and mast cells occurs after nerve damage. Immediately after peripheral nerve injury, TNF-α expression increases in the site of lesion[19-20] leading to a massive recruitment of macrophages[21-22]. Recruitment is probably mediated by metalloproteinase[23], or through the upregulation of adhesion molecules or cytokines[21].

Shubayev's group, after performing sciatic crush lesions in TNF-α and matrix metallopeptidase 9 (MMP-9) knockout mice, found fewer macrophages in the distal parts of the injury[23]. Furthermore, MMP-9 produced by SCs and endoneurial macrophages after TNF-α activation promote blood nerve barrier degradation and demyelination[23-24].

Macrophages activated during WD phagocytose degenerating myelin, collaborate in the reorganization of the endoneurial space, and help remodel the extracellular matrix[10, 25]. Infiltrating blood-derived cells also release additional TNF-α, resulting in a second peak 3−5 days after injury[26].

The role of TNF-α in peripheral nerve injury has been

thoroughly investigated both *in vivo* and *in vitro* by many groups. Liefner and collegues[21] demonstrated in a transected sciatic nerve of TNF-α deficient mice that poor macrophage recruitment results in delayed myelin removal. Also Uncini and collegues[27] have demonstrated the role of TNF-α in blood-nerve barrier degradation; in fact they showed that the injection of TNF-α in an injured sciatic nerve of the rat resulted in the damage of the blood-nerve barrier and an inflammatory infiltration of the vessel walls, thus supporting the hypothesis of a direct chemotactic effect of TNF-α. Interestingly, two *in vitro* studies revealed the direct effect of TNF-α on SCs[20, 28], showing that TNF-α decreases SC proliferation in a dose-dependent manner without affecting SCs viability.

IL-1

Another very important pro-inflammatory cytokine involved in the peripheral nerve injury process is the IL-1. IL-1, the product of a member of a superfamily of related genes, some of which generate proteins not involved in inflammation[29], causes the accumulation of arachidonic acid metabolites, upregulates inducible nitric oxide synthase, and sustains nitric oxide production. It is a polypeptide produced in 2 molecular forms, IL-1α and IL-1β; despite only a 26% amino acid homology, both forms can determine a wide variety of biological responses. *In vitro*, IL-1 activates T and B lymphocytes and induces a variety of lymphokines, interferons, and other cytokines, particularly TNF, for the induction of inflammatory changes, such as prostaglandin synthesis, activation of endothelial cells, and bone resorption. They are both synthesized as 31 kDa precursors, the processing of these molecules to their final mature form requiring a specific protease. Pro IL-1α remains in the cytosol in association with cytoskeletal structures, *e.g.* microtubules[30]. When the cells die, the pro-protein is released and can be processed by extracellular proteases, but can also be cleaved by the activation of calcium-dependent, membrane associated cysteine proteases called calpains[31]. Following synthesis, pro IL-1β remains in the cytosol and is only partially active. Its active form is secreted from the cell after the cleavage by ICE, the IL-1β converting enzyme[32-33].

Two IL-1 receptors and one accessory protein (IL-1 R-AcP) have been identified. They comprise 3 IgG-like domains, and share a significant homology to each other[30,34]. While the type I receptor (IL-1RI) transduces a signal, the type II receptor (IL-1RII) binds IL-1, but does not exhibit signal transduction; in fact, the receptor IL-1RII acts as a

suppressor of signaling mediated by IL-1 and -3[30]. When IL-1 binds to IL-1RI, a complex is formed that then binds to the IL-1R accessory protein (IL-1R-AcP), resulting in high affinity binding[34]. It is like a heterodimerization of the cytosolic domains of IL-1RI and IL-1R-AcP that can trigger IL-1 signal transduction.

In the intact peripheral nervous system, IL-1α mRNA is constitutively expressed, but IL-1α protein is not synthesized. Due to nerve injury, IL-1α is rapidly upregulated by SCs that have lost their close contact with interrupted axons. This upregulation occurs both at mRNA and protein levels 5 to 6 hours following damage[11].

The fundamental role of IL-1α in this type of injury is to induce the fibroblasts located on site to produce IL-6 and granulocyte macrophage-colony stimulating factor; the production of these molecules is detectable from 2 to 5 hours after injury.

Regarding SCs, IL-1β expression during WD is detected 5−10 hours after injury and the delayed expression might be due to SC-derived TNF-α. Interestingly, the highest levels of TNF-α and IL-1β protein secretion were detected 1 day after the injury, thus before macrophage recruitment; in fact, it has been reported that the administration of a function-blocking antibody against IL-1β into the injured mouse sciatic nerve halts, with a reduction in the recruitment of macrophages and retarded myelin phagocytosis[35].

It is well known that IL-1α, IL-1β and TNF-α determine the first peak of nerve growth factor mRNA within hours after nerve injury in fibroblasts, but not in SCs[11]; moreover, they participate in WD by first upregulating the production of additional inflammatory cytokines and thereafter the production of anti-inflammatory cytokines[25].

IL-6

IL-6 is a glycoprotein belonging to the neurokines family with pro-inflammatory activity, crucial in the acute inflammatory phase of the reaction. It enhances T-cell activation, and acts as a neurotrophic factor for cholinergic and dopaminergic neurons[36]. IL-6 plays a role in neuroprotection[37-38] and modulation of pain, binding a non-signaling α-receptor (IL-6R, also known as CD126), which, after dimerization with gp130, leads to activation of receptor-associated kinases JAK1, JAK2, and Tyk2. In turn, these lead to phosphorylation of proximal tyrosine residues within the intracellular portion of gp130, with subsequent control of STAT1 and STAT3 activity[10, 39-40].

Increased synthesis and release of cytokines, including

IL-6, can directly modulate neuronal activity through the synthesis of neuropeptide transmitters. Furthermore, IL-6 is a key component of the nervous system's injury response. Previous studies have shown that IL-6 plays a role in axon regeneration after peripheral nerve injury and also makes an important contribution in the overall cellular response[41]. IL-6 level is elevated both in neurons and non-neuronal cells, and can increase gene expression of regeneration associated genes (RAGs) in neurons and promote neurite growth together with some neurotrophic factors, such as nerve growth factor[9]. Resident macrophages and fibroblasts of injured peripheral nerves are the major producers of IL6[42]. SC-derived IL-6 increases within 3 hours of nerve lesion and is required for immune cell chemotaxis within 2−5 hours after the injury, with IL-6 secreted by macrophages becoming detectable[11]. IL-6 produced by resident fibroblast is induced by TNF-α and IL-1 produced by SCs.

Several studies show that the expression of IL-6 and IL-6 receptor α-subunit (IL-6Rα) is upregulated in dorsal root ganglion neurons after axonal injury and elongation. However, the biological actions of IL-6 signaling on SCs have not been fully elucidated[43].

IL-10

IL-10 is a 18.5 kDa large immunoregulatory anti-inflammatory cytokine secreted by a variety of cells, including monocytes, macrophages, dendritic cells, T cells, B cells, granulocytes, epithelial cells, and mast cells[44]. The principal routine function of IL-10 may be limited and ultimately terminates inflammatory responses[45]. In fact, IL-10 limits secretion of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6; it deactivates macrophages, inhibits secretion of Th1 cytokines such as IL-2 and interferon-gamma, and controls differentiation and proliferation of macrophages, T cells, and B cells. While keeping pro-inflammatory events under control, it protects against excessive immune responses and tissue damage.

There is evidence showing that IL-10 is involved in nerve healing process after injury. In this context, previous studies have shown that IL-10 plays an important role during nerve regeneration; in particular, the administration of a low dose of IL-10 to a site of sciatic nerve injury reduces scar formation (which is considered one of the main problems in nerve repair), and permits better regeneration of damaged axons that leads to greater myelination[46-47].

Some studies have shown that the effect of IL-10

applied at the repair site is dependent upon the concentration used, with no beneficial effects at high concentration while there was that enhanced regeneration of the nerve at a low concentration[47]. The production and secretion of the anti-inflammatory cytokine IL-10 is induced by resident fibroblasts within 5 hours of injury, but levels are low and ineffective because nerve-resident fibroblasts are poor producers, like SCs. To overcome this deficiency, recruited macrophages produce and secrete IL-10; protein levels increase, peaking at day 7 concomitant with the timing and magnitude of macrophage recruitment. IL-10 then gradually downregulates the production of cytokines, bringing the cytokine network of normal WD to a conclusion 2−3 weeks after injury[11].

OTHERS

In the last part of this review, we wish to focus also on cytokines that remain poorly studied with regard to their role in peripheral nerve degeneration and regeneration. TGF-β family comprises three isoforms in mammals: TGF-β1, -β2 and -β3. Moreover, the TGF-β superfamily has a huge number of homologous proteins[48]. Potentially, every cell in the body, including hematopoietic, endothelial, epithelial, neuronal and connective-tissue cells, produces TGF-β and has receptors for it. TGF-β-related proteins are synthesized as precursor proteins with an amino-terminal residue for identification of the signal target and a varying pro-domain that assists in folding, dimerization and regulation of factor activity[49]. As to the other components, TGF-β1, an anti-inflammatory cytokine, is one that is a secreted signalling molecule mediating many essential events of normal growth and development[50].

TGF-β1 can bind at least three proteins located in the cell surface: type I, type II, and type III receptors, of 53, 70−85 and 200−400 kDa, respectively[51]. Type I and type II receptors are the best binding molecules to transduce signalling pathway. TGF-β1 is the major isoform expressed in SCs, and this rate of expression is modulated by interaction with axons[52]. These authors also found that TGF-β1 has mitogen effects on isolated rat SCs, increasing the expression levels of markers of premyelination, neural cell adhesionmolecule, suppressed cAMP-inducible POU-domain protein, and inhibiting forskolin-induced transition to a myelinating phenotype. In agreement with these early observations, co-culture of axon and SC revealed that administration of TGF-β1 inhibits myelination and the correct formation of the basal lamina[52-53].

TGF-β1 expression and its effects on proliferation are strongly strengthened by the increased protein level immediately after peripheral nerve injury[54-55] and the following downregulation, *i.e.* once the nerve fibers have grown back into the distal stump, thus allowing subsequent ensheathment and myelination.

IL-2 is a 15.5 kDa pro-inflammatory cytokine composed of 133 amino acids[56] that has multiple functions in the inflammatory response, including activation of immune cell effectors and stimulation of the number of white blood cells on the endothelial surface of skeletal muscle. In addition, it is a potent inducer of the proliferation, differentiation, development, survival, memory and regulatory functions of T-lymphocytes[57]. IL-2 exerts its biological activity by binding to the high-affinity IL-2 receptor (IL-2R). Soluble IL-2R is part of a membrane receptor localized on the cell surface of different lymphoid cell lines, including activated T and natural killer cells, monocytes, eosinophils[58].

The IL-2R consists of 3 subunits, the α-chain (also known as CD25), the β-chain (CD122) and the γ-chain (CD132). A major function of IL-2 is to promote proliferation and expansion of CD4$^+$ and CD8$^+$ T cells as well as inducing the production of other cytokines[59]. Previous studies have shown that IL-2 in also present in nervous system; endogenous IL-2 and IL-2Ra occur in different regions of the adult rat brain, such as the frontal cortex, hippocampal formation, hypothalamus and cerebellum. Although the cellular origin of brain IL-2 is unclear, evidence shows that the glial cells, including astroglial and microglial cells, are the possible sources. Evidence has also been found that IL-2 plays multiple roles in the central nervous system. It has trophic functions on both neurons and glia. For example, IL-2, a neurotrophic factor for sympathetic neurons, has been reported to enhance the proliferation and differentiation of oligodendrocytes. Furthermore, IL-2 can modulate neurotransmitter release and affect electrical activity. It is also involved in responses to central nervous system trauma and spontaneous regeneration. Recent studies demonstrated that IL-2 might also play a role in spatial learning and memory[60]. In dorsal root ganglia neurons, IL-2 plays an important role in the modulation of neural and neuroendocrine function; furthermore, there is evidence to show that IL-2 has an antinociceptive (analgesic) effect in dorsal root ganglia by binding to opioid receptors[61].

and molecular events that stimulate axonal regrowth from proximal stumps and support axonal regeneration. Immediately after peripheral nerve damage, SCs, that have lost their contact with axons, and resident macrophages produce TNF-α, IL-1α and -β. These cytokines induce resident fibroblasts to produce IL-6[25]. Concomitantly, IL-6 is expressed by SCs in the early phases of WD; like the other cytokines, it is required for immune cell chemotaxis[25, 59]. Following the activation of these cytokines, metalloproteinase, whose action is very important for damaged tissue remodeling, is responsible for basal lamina degradation, favoring destruction of the blood-nerve barrier and the infiltration of circulating macrophages[62]. Recruited cells (macrophages and T cells) produce inflammatory cytokines, and notably IL-10 which attenuates the inflammatory process by inhibiting cytokine production of activated macrophages, monocytes and other cell types[45, 63]. Initial production of pro-inflammatory cytokines at the site of a peripheral nerve lesion is important in influencing the long-term behavioral outcome of nerve injury. IL-10 may accomplish this by downregulating the inflammatory response of the nerve to injury[63].

TGF-β1 expressed by SCs contributes to the maintenance of the immature phenotype of the cells themselves by stimulating their proliferation and inhibiting the myelination of axons. Only when nerve fibers have grown back during nerve regeneration into the distal stump is TGF-β1 downregulated and myelination is allowed. SC-derived IL-2 promotes proliferation of T cells, has trophic functions on both neurons and glia, and induces production of other cytokines.

Of relevance is the involvement of vitamins in nerve regeneration and their interaction with the inflammatory cytokines in the inflammatory response. Chabas and collegues[64] found that vitamin D potentiates axon regeneration, increasing axogenesis and axon diameter, and improving the responses of sensory neurons to metabolites, *e.g.* KCl and lactic acid.

In vitro studies have demonstrated that vitamin D suppresses pro-inflammatory cytokines and increases anti-inflammatory cytokines[65]. Experimental evidence shows that vitamin D can suppress the release of TNF-α and effectively upregulates the synthesis of anti-inflammatory IL-10[66-67]. Vitamin D reduces the inflammatory milieu and might serve as a new anti-inflammatory agent in future treatment of the disorder.

OVERVIEW

During WD, the orchestrated action of pro-, and anti-inflammatory cytokines regulates the main cellular

CONCLUSION

Peripheral nerve injury leads to a characteristic series of

cellular and molecular events necessary for axon regeneration and re-innervation of target tissues[68]. Inflammatory reaction is one of the crucial events, being important for the orchestration of all the processes that occur during WD.

In this review, the more studied cytokines involved in WD and the early phases of regeneration of a peripheral nerve have been outlined (TNF-α, IL-1, IL-6, IL-10). Attention has also been focused on two cytokines (TGF-α and IL-2) that are poorly described in the literature on peripheral nerve injury to date, which could make them of greater interest in the future.

After peripheral nerve injury, pro- and anti-inflammatory cytokines are produced by both immune and non-immune cells that are resident in the distal part of the injured nerve or recruited from blood circulation. Although nowadays even the most severe nerve injury can be surgically repaired, complete recovery of nerve function does not occur and clinical results remain unsatisfactory. Future research could therefore address the issues to give better knowledge of inflammatory events that might help develop new therapeutic measures that improve and accelerate peripheral nerve regeneration, stimulating the endogenous anti-inflammatory reaction and decreasing pro-inflammatory processes. It is expected that a complete functional recovery could be achieved by coupling surgical repair with a pharmacological approach targeted to the specific molecules that trigger the inflammatory reaction[69-70].

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1.5 Prostate cancer and peripheral nerve regeneration

Prostate cancer (PCa) is the most frequent cancer among men. In Europe is registered an incidence of 214/1000 cases, surpassing the number of the lung and colorectal cancers (Boyle and Ferlay 2005) and representing the second leading cause of cancer mortality (Jemal, Siegel et al. 2008).

The surgical treatment for PCa is represented by the radical prostatectomy, which is the gold standard in the treatment of localized disease and in patients with a life expectancy of at least 10 years (Guidelines on Prostate Cancer updated 2014, European Association of Urology).Unfortunately, in patients who underwent a RP, frequently damage to the peri-prostatic nerve bundles occurs, leading to erectile dysfunction.

The preservation of autonomic nerves involving in continence and sexual potency is a crucial issue in urological postoperative field and new discoveries about the anatomical features of the Neurovascular bundle (NVB) combined with the improvement of surgical technique allowed to enhance the preservation of the nerves fibers responsible of the penis innervations. Here two important aspects of this issue are described: the neuroanatomical structure responsible of prostate innervations, and different strategies to improve the functional recovery in patient undergo RP.

1.5.1 Neuroanatomy of pelvic plexus and prostate innervation

Autonomic innervations of pelvic organs is supplied by the pelvic plexus (synonymus: inferior hypogastric plexus, pelvic ganglion) composed by efferent parasympathetic visceral fibers arising from S2-S4 nerve roots and from sympathetic fibers originating from the toraco-lumbar segment (T1-L2) (Ali, Johnson et al. 2004) (figure 7A). In human pelvic plexus is 4-5 mm in length, and its midpoint is at the tips of seminal vesicles (Schlegel and Walsh 1987). It runs on either side of the rectum and is perforated by numerous vessels going to and from the rectum, bladder and seminal vesicles. The anterior portion of the pelvic plexus goes around the lateral prostatic surface to join the anterior side of the bladder neck (Benoit, Merlaud et al. 1994) while the caudal portion gives rise to a dense innervations difficult to

recognize as well as intramural ganglia that supply the prostatic gland innervation (Walsh and Donker 1982) (Figure 7B).

At this level nerves fibers go across the tips of seminal vesicles and reach the lateral endopelvic fascia near its juncture with Denonvilliers' fascia (Lepor, Gregerman et al. 1985) (Paick, Donatucci et al. 1993; Davies 1997). This structure is called "neurovascular bundle" (NVB) and represent a combination of nerves, arteries, veins, and lymphatics vessels (Figure 7C). Walsh and Donker described the NVB as a well-defined structure containing neural branches for autonomic innervations of corpora cavernosa of penis and blood vessels. Anatomically they are located between the two layers of lateral pelvic fascia , and the prostatic fascia. Most of nerve fibers within the bundle terminate in the prostate after perforating the prostate capsule, other coming from the anterior side of the pelvic plexus reach the lateral prostatic surface to join the anterior side of the bladder neck.

Recent evidences about the morphology of the NVB show that they consists of three different functional compartments: the postero-lateral compartment that supply the rectum, the lateral compartment that supply the levator ani and the most anterior compartment that supply prostate and corpora cavernosa (McVary, McKenna et al. 1998; Costello, Brooks et al. 2004; Stolzenburg, Rabenalt et al. 2007; Schwalenberg, Neuhaus et al. 2010). This important differentiation provide new opportunity to preserve the NVB during RP.

1.5.2 Surgical treatment for prostate cancer resection: nerve sparing radical prostatectomy

As described previously PCa is the most common cancer among men (Boyle and Ferlay 2005; Jemal, Siegel et al. 2008; Jemal, Thun et al. 2008) and typically affects men over the age of 60 years. This tumor arises within the prostate in asymptomatic way until the advancement of the cancer that causes different symptoms such as the interruption of urine flow, urination problems, pain and elevated prostate-specific antigen (PSA) (Kandasamy, Khalid et al. 2017)

Current standard treatment for prostate cancer is represented by radical prostatectomy that involves the removal of the prostate with the surrounding tissues and seminal vesicles. Radical prostatectomy can be performed by different surgical approaches:

-Conventional laparoscopic radical prostatectomy is a minimal invasive approach in which surgery is performed trough incision in the abdomen;

-Robot-assisted radical prostatectomy in which the surgeon manipulates robotic arms of a surgical system that are inserted into the small incision points.

The introduction of a robotic system has revolutionized prostate cancer surgery together with the three dimensional high definition vision system and the miniaturized instruments for microsurgery that allow to perform surgical procedures with an high preservation of the delicate anatomical structuresm (Porpiglia, Bertolo et al. 2017). For this reason, the use of robotic surgery could lead an improvement on the recovery of continence and sexual function in patients undergo radical prostatectomy compared to traditional surgical method (Walsh and Donker 1982; Mistretta, Grasso et al. 2015; Porpiglia, Bertolo et al. 2016).

Depending on different important parameters such as tumor characteristics, patient's age and patient's sexual function, nerve sparing radical prostatectomy can be performed in order to preserve the NVB and the sexual potency.

NVBs are included in a multi-layered fascia that can be fused or not with the prostatic capsule, covering the outer surface of the prostate, known as the "peri-prostatic fascia" (PPF)(Montorsi, Salonia et al. 2005; Walz, Burnett et al. 2010). The relationship between this two compartments has been widely described and in the context of different surgical approach, several dissection plane can be recognized within the PPF allowing different degrees of NS procedures. At this purpose, three possible dissection plane can be performed:

- The **extrafascial dissection** leads to the complete resection of the NVBs.
- **intrafascial dissection** follows an internal plane on PPF capsule and anterior to the fascia covering the seminal vesicles allowing the complete sparing of the NVBs ;
- **interfascial dissection** performed within the thickness of the PPF, allowing a complete or partial NS procedure according to the physiological individual variation of the NVBs;

To further identify the different surgical approaches, a defined terminology was proposed identifying full, partial and minimal nerve sparing corresponding to intrafascia, interfascial and sub-extra fascial dissection respectively (Cathelineau, Sanchez-Salas et al. 2010; Walz, Burnett et al. 2010; Montorsi, Wilson et al. 2012) (Figure 7D).
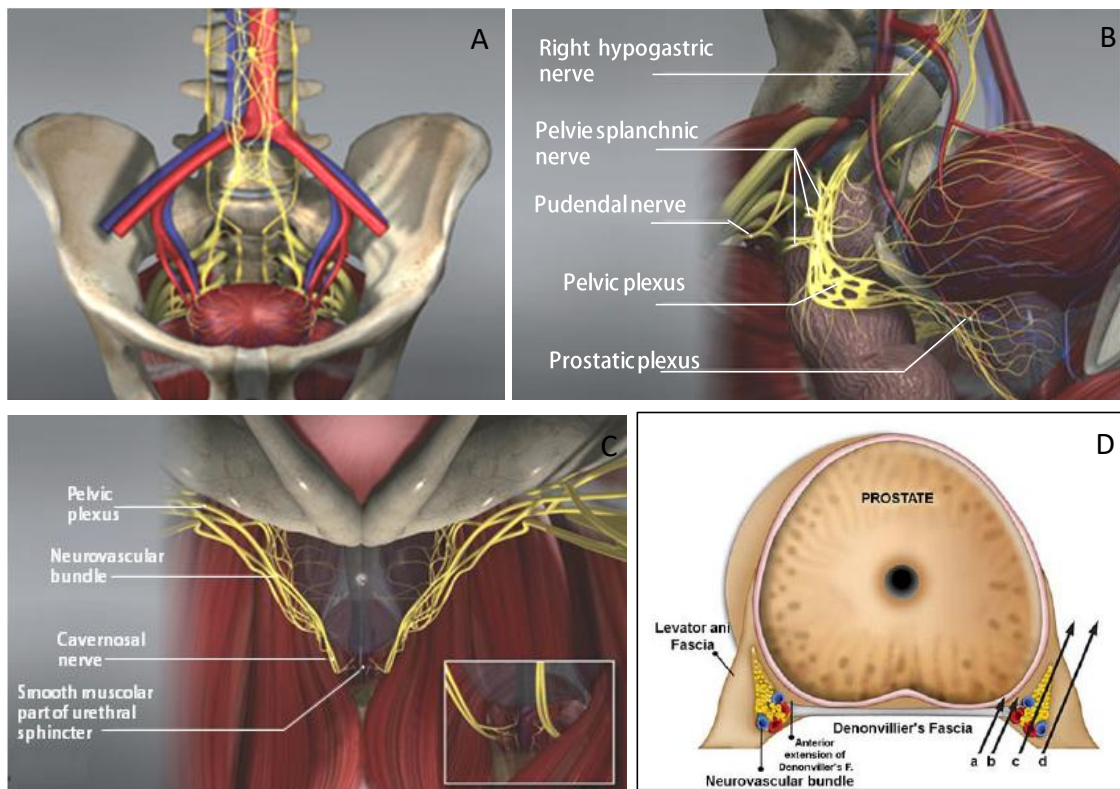
Fig.7: Nerves and vessels of the pelvis (A); Nerves of the pelvis containing sympathetic and parasympathetic fibers (B); Neurovascular bundle representation (C)(Schwalenberg, Neuhaus et al. 2010); degrees of neurovascular bundle preservation during radical prostatectomy (D)(Cathelineau, Sanchez-Salas et al. 2010).

1.5.3 REVIEW "STRATEGIES TO IMPROVE NERVE REGENERATION AFTER RADICAL PROSTATECTOMY: A NARRATIVE REVIEW"

Stefano Geuna , Luisa Muratori, Federica Fregnan, Matteo Manfredi , Riccardo Bertolo,

Francesco Porpiglia.

Strategies to improve nerve regeneration after radical prostatectomy: a narrative review

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Abstract

Peripheral nerves are complex organs that spread throughout the entire human body. They are frequently affected by lesions not only as a result of trauma but also following radical tumor resection. In fact, despite the advancement in surgical techniques, such as nerve-sparing robot assisted radical prostatectomy, some degree of nerve injury may occur resulting in erectile dysfunction with significant impairment of the quality of life.

The aim of this review is to provide an overview on the mechanisms of the regeneration of injured peripheral nerves and to describe the potential strategies to improve the regeneration process and the functional recovery. Yet, the recent advances in bio-engineering strategies to promote nerve regeneration in the urological field are outlined with a view on the possible future regenerative therapies which might ameliorate the functional outcome after radical prostatectomy.

Introduction

Radical prostatectomy is the gold standard surgical treatment for organ-confined prostate cancer. The employment of innovative surgical technique such as nerve-sparing robot assisted radical prostatectomy allowed to magnify the anatomical field leading to a three-dimensional perspective obtained through the robotic lenses and a better anatomical knowledge.

Despite surgical technique advancement, erectile dysfunction rate after radical prostatectomy is still high especially in minimal nerve-sparing technique where frequently iatrogenic damage to the autonomic peri-prostatic nerve bundles occurs(Munding, Wessells et al. 2001; Mullerad, Donohue et al. 2006; Hamidi, Altinbas et al. 2017).

Anatomically, prostate innervations is supplied by peripheral innervation identified in the pelvic plexus with a dense innervations network composed by many nerves difficult to identify as well the intramural ganglia, particularly dense at prostate capsule level and caudal prostate (Benoit, Merlaud et al. 1994; Ali, Johnson et al. 2004).

Concerning the peripheral nervous system, we have to discriminate between somatic and autonomic components that show anatomical and structural differences.

The somatic nervous system includes the sensory and motor nerves that innervate the limbs and body wall. Sensory nerve fibers derive from neurons located in the dorsal root ganglion and supply skin innervations. The motor neurons cell bodies are located in the anterior horn of the spinal cord and supply innervations to the skeletal muscle in which they release acetylcholine that stimulates the voluntary contraction.

The autonomic nervous system consists of two main divisions, the sympathetic and the parasympathetic that affects the peripheral transmission of the visceral organs through nerves and ganglia. The efferent pathway involves two neurons: the preganglionic and the post ganglionic neurons, preganglionic axons are myelinated with an organization that resembles the somatic nerves, while postganglionic axon are unmyelinated and organized in small diameter bundles surrounded by a single Schwann cell (type C fibers). The sympathetic is involved in responses that would be associated with fighting or fleeing, the parasympathetic is involved in energy conservation functions and increases bladder contractility, gastrointestinal motility and secretion (Saper 2002; Furness 2006).

In the parasympathetic nervous system preganglionic fibers are myelinated and arise from different cranial nerves and from the second to fourth sacral spinal nerves. Postganglionic parasympathetic fibers are usually unmyelinated and shorter than the sympathetic fibers, since the ganglia they synapse are in or near the visceral they supply.

The aim of this narrative review is to provide a brief description of the different types of injuries that can occur in the peripheral nerves, and to describe the different steps of the regeneration process.

Somatic nerve lesions are common injuries often caused by trauma or accident at work, for this reason most of the experimental studies described here are performed viewing these nervous components. Despite this, the bases provided in this work have allowed us to understand more in detail the therapeutic possibilities in case of autonomic nerve lesions, and in particular in case of damage of the peri-prostatic bundles after radical prostatectomy.

1. THE BIOLOGY OF NERVE INJURY AND REGENERATION

1.1 Effects of mechanical injury on peripheral nerve fibers

Peripheral nerves represent the main component of the peripheral nervous system forming a complex network that reach the whole body, making them particularly vulnerable to injuries.

Each nerve is a complex structure formed by cellular and tissue elements, and surrounded by three basic protective connective tissue layers: the epineurium that support and surround the whole nerve, the perineurium that surround each nerve fascicle and the endoneurium that protects the nerve fiber (Lundborg 2005; Geuna, Raimondo et al. 2009; Siemionow and Brzezicki 2009).

The smallest functional unit of a peripheral nerve is the nerve fiber, responsible of the motor and sensory impulse conduction. Anatomically, depending on the strategy adopted from Schwann cells to enclose axons, nerve fibers are distinguished in two subgroups: myelinated and unmyelinated. Myelinated nerve fibers consist of a single axon that is enveloped individually by a single Schwann cell.

The Schwann cell membrane wraps around the nerve fiber to form a multilaminated myelin sheath. In myelinated fibers axons are enveloped by a chain of Schwann cells, arranged in longitudinal sequences along the length of the axon. Between each Schwann cell, there is an interspace of unmyelinated axon known as "node of Ranvier" in which the axolemma is exposed to the extracellular space. This area allows to extracellular ions to reach the axon, inducing the saltatory conduction of the impulse along the nerve fiber (Flores, Lavernia et al. 2000; Geuna, Raimondo et al. 2009). On the contrary, unmyelinated nerve fibers are composed by a group of several axons enveloped by a single Schwann cell (Flores, Lavernia et al. 2000).

Peripheral nerve injuries are common conditions with broad ranging symptoms depending on the severity and nerves involved. If not properly treated, nerve traumas could lead to a sensory and motor function deficit. Indeed, despite the spontaneous regeneration potential of peripheral nerve fibers, clinical results of nerve injuries are still unsatisfactory, resulting in not complete functional recovery.

Peripheral nerve injuries can be described and classified using Seddon degree in *neuropraxia, axonotmesis and neurotmesis* (Seddon 1942):

- *Neurapraxia* is a mild injury, characterized by local myelin damage. It may result from exposure to a wide range of conditions such as heat, cold, irradiation or electrical injuries, but it is most commonly due to mechanical stress, such as concussion, compression or traction injuries. Axon continuity is preserved, and the nerve does not undergo Wallerian degeneration. Paralysis of the innervated body part may occur, with possible atrophy due to disuse. The absence of lesion of nerve connective structures makes surgery unnecessary and recovery usually occurs within few days, up to a few weeks.

- *Axonotmesis* is a middle type of injury, in which peripheral axons are damaged or destroyed, but the connective tissue structures remain intact. The axon interruption is often the result of nerve pinching, crushing or prolonged pressure. The distal stump of the nerve undergoes Wallerian degeneration, but the subsequent axonal regrowth may occur along the intact endoneurial tubes. The proximal nerve stump undergoes retrograde degeneration. The transmission of pulses is compromised, as well as the functionality of the connected area of the body. The time of recovery depends upon the internal nerve disorganization as well as the distance of the injury site to the end organ. The absence of lesion of nerve connective structures makes surgery unnecessary.

- *Neurotmesis* is the most severe type of injury. The nerve is completely disrupted with loss of tissue continuity, and the connective tissue is severely damaged. Even in this case the proximal nerve stump undergoes retrograde degeneration, while the distal to Wallerian degeneration. Functional recovery does not easily occur because of the extent of endoneurial tube disruption. Nonetheless, a surgical approach is required to get a proper nerve fibers regeneration.

  A further classification provided by Sunderland in 1951 subdivided the injuries according to the discontinuity of the different connective tissue layers in 5 degree (Sunderland 1951).

- Sunderland type 1 injury correspond to Seddon's *Neurapraxia* with conduction block and completely intact stroma*;*

- Sunderland type 2 injury corresponds to Seddon's axonotmesis represents a severe crush injury with disconnection of axons and Schwann cells sheath but preservation of all connective tissue layers, endoneurium, perineurium, and epineurium are still intact, but the axons are physiologically disrupted. Recovery can occur by axonal regrowth along endoneurial tubes, and complete functional recovery can be expected. The time for recovery depends on the level of injury, usually months.

- In Sunderland type 3 injury, the endoneurial layer is disconnected but the surrounding perineurium and epineurium are intact. Recovery is incomplete and depends upon how well the axons can cross the site of the lesion and find endoneurial tubes.

- In Sunderland type 4 only epineurium continuity is preserved, individual nerve fascicles are transected, and the continuity of the nerve trunk is maintained only by the surrounding epineurium. This type of injury requires surgical repair or reconstruction of the nerve.

- Sunderland type 5 injury is equivalent to Seddon's neurotmesis (complete nerve trasaction), also the epineurium is disconnected resulting in a complete nerve transaction and spontaneous recovery is negligible.

  Although Sunderland's classification provides a concise and anatomic description of nerve injury, the clinical utility of this system is debatable since nerves may undergo a combination of different degrees of injury.

  Therefore, in 1988 Susan E. Mackinnon and A. Lee Dellon described a 6th degree of nerve injury to address a mixed nerve injury. They use the term "neuroma in

continuity" to describe a combination of the degrees of injuries per fascicle (Mackinnon 1989) (Fig.1).

Time of recovery of peripheral nerve depends on the degree of injury and is summarized in Table1.
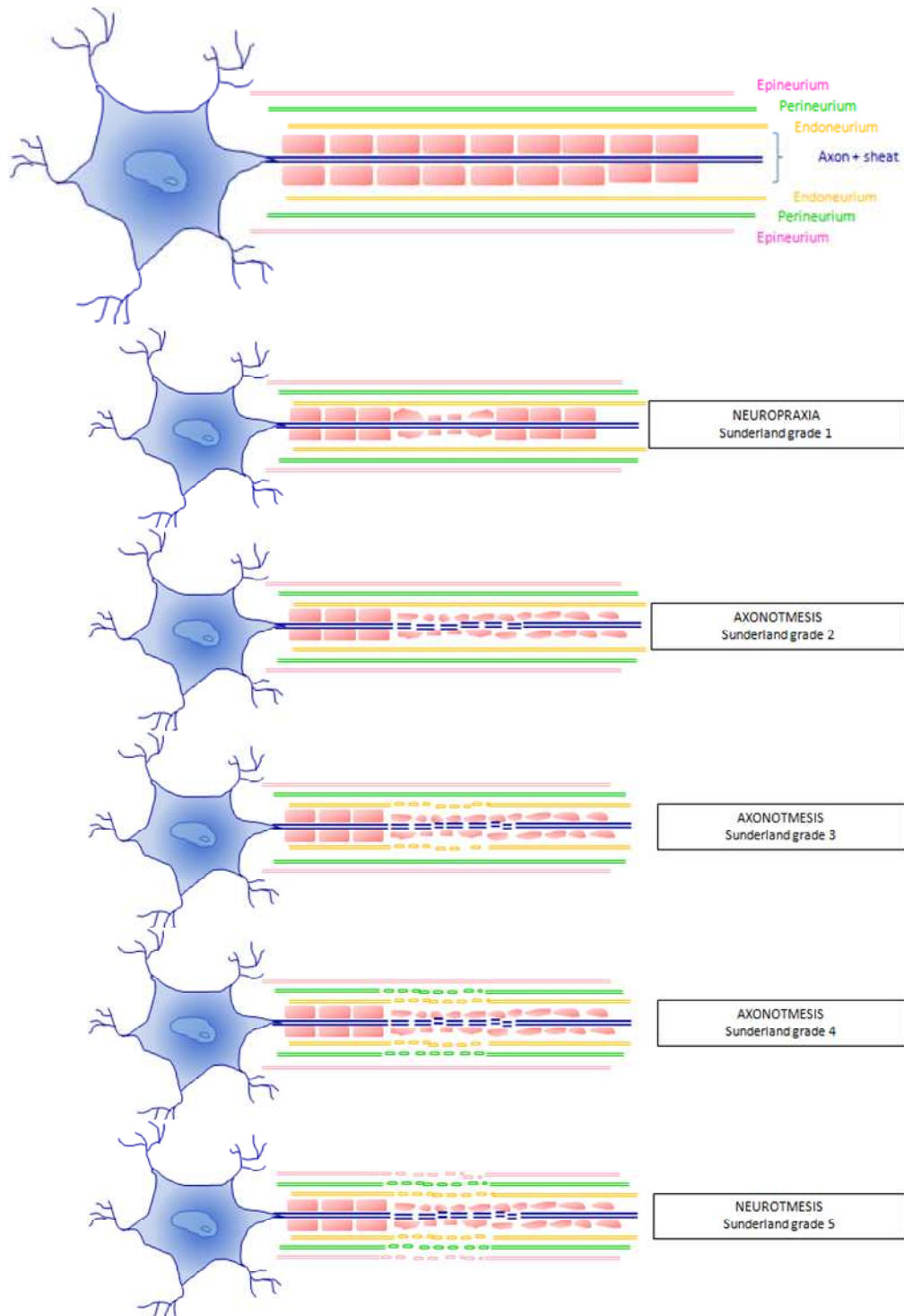

Fig.1: Schematic representation of nerve injuries

Degree of nerve injury	Spontaneous recovery	Time and rate of recovery	Surgery
First: Neuropraxia	Full	Fast: few days, occasionally few weeks	None
Second: Axonotmesis	Full/partial	Slow (1mm/day)	None
Third	Partial or incomplete	Slow (1mm/day)	None or neurolysis
Fourth	None	Regeneration occurs at rate of 1 inch/month	Nerve repair/Graft
Fifth: Neurotmesis	None	Regeneration occurs at rate of 1 inch/month	Nerve repair/Graft
Sixth: mixed injury	Recovery depends on the injury and the combination of different injury degree		

Table 1: Classification and time of recovery of different peripheral nerve injuries

1.2 The biological mechanisms of peripheral nerve regeneration

It is known that, differently to the Central Nervous System (CNS), the Peripheral Nervous System (PNS) is able to regenerate spontaneously in response to injury although the regeneration process is closely related to the severity of the damage. After a nerve injury, several mechanisms occur at the injury site almost immediately including morphologic and metabolic changes (Hall 2005; Campbell 2008; Oliveira, Mostacada et al. 2013).

The interruption of a peripheral nerve causes significant changes in normal morphology and tissue organization both proximally and distally to the lesion site (Deumens, Bozkurt et al. 2010; Oliveira, Mostacada et al. 2013).

The functional significance of the regeneration process is to replace the distal nerve segment reaching the target organ and achieving the functional recovery.

After injury, axons distal to the lesion site are disconnected from the neuronal body and undergo degeneration called "Wallerian Degeneration" in honor of Augustus Volney Waller, who first characterized the disintegration of the frog glossopharyngeal and hypoglossal nerves after axotomy 160 years ago (Navarro, Vivo et al. 2007). This process starts immediately after injury and involves myelin degradation of axoplasm, axolemma and myelin sheath due to proteolysis; myelin is

transformed into neutral lipid compounds by Schwann cells, infiltrated blood monocytes and macrophages are recruited at the injury site (Stoll, Jander et al. 2002; Deumens, Bozkurt et al. 2010). Occurrence of Wallerian degeneration contributes to axonal regeneration due to clearance of myelin debris and growth inhibitors, and subsequent creates a regenerative microenvironment favorable for the axonal regrowth of surviving neurons (Shen, Lassner et al. 2000; Huang, Phillips et al. 2005; Gordon 2010; Cheng, Wang et al. 2017).

Whereas the Wallerian degeneration occurs, the soma reacts to the injury with substantial metabolic changes necessary for regeneration and axonal elongation. The most relevant morphological changes in the neuronal body are the Nissl bodies dissolution followed by nuclear eccentricity and enlargement, cell swelling, dendrites retraction.

In degeneration and regeneration Schwann cells play a critical role: during Wallerian degeneration an intense inflammatory response occurs, mediated by Schwann cells, mast cells, macrophages and activated endothelial cells leading an increase of chemokines, cytokines, interleukin 1 (IL-1) and tumor necrosis factor (TNF) (Chen, Chai et al. 2001; Jessen and Mirsky 2008).

Furthermore, Schwann cells begin to dedifferentiate in the distal stump: within 48 hours of injury, they change their gene expression: myelin proteins (e.g., P0, MAG) (Trapp, Hauer et al. 1988; White, Toews et al. 1989; Lee, Shin et al. 2009) and connexin 32 decrease dramatically as a consequence of axonal degeneration distal to the injury site, whereas regeneration-associated genes, and neurotrophin such as NGF, GDNF, BDNF and IGF, are up-regulated and promote axon growth (Carroll, Miller et al. 1997; Hall 2001; Chen, Yu et al. 2007; Jessen and Mirsky 2008).

Between days 1 and 5 after injury, Schwann cells start to proliferate peaking around day 3 post-injury and then decreasing during the following weeks. Proliferating Schwann cells align with the endoneurial tubes in columns known as bands of Büngner at the basal lamina level, that support and guide the regenerating axons; aligned Schwann cells and their extracellular matrix provide indispensable pathways to guide axonal regrowth (Stoll, Griffin et al. 1989; Griffin and Thompson 2008; Ribeiro-Resende, Koenig et al. 2009).

Nerve regeneration and target reinnervation are complex processes, involving multiple factors. Even if the peripheral nervous system is able to regenerate, nerves can regenerate on their own if injuries are small; indeed, larger injuries must be

surgically treated (Terzis, Sun et al. 1997; Schmidt and Leach 2003; Battiston, Geuna et al. 2005).

Regenerating axons are usually produced at the node of Ranvier located close to the proximal stump of the lesion (Hopkins and Slack 1981; McQuarrie 1985) and extend by attaching themselves to the inner surface of the basal lamina or on the Schwann cell plasma membrane (Fig.2).

The advancement of regenerating axons in the distal segment is promoted by different factors such as fibronectin and laminin together with several cell adhesion molecules through the Schwann cell column(Letourneau, Condic et al. 1994).

Adhesion molecules are no longer detected at around the time that Schwann cells begin to form the myelin sheath on the axon, whereas the mature unmyelinated fibers in which the relationship between axons and Schwann cells is apparently preserved as that during the development, continue to exhibit such adhesion molecules (Ide 1996).

Axons can sprout approaching Schwann cells column or randomly in the connective tissue of the nerve. After few time, regenerated axons that reached the target organs display a close-to-normal diameter, branches that do not reach the target are pruned away (Chaudhry, Glass et al. 1992).

The knowledge of molecular and cellular changes occurring during the degeneration and the regeneration process of a peripheral nerve is of importance when attempting to improve the available strategies for nerve repair.
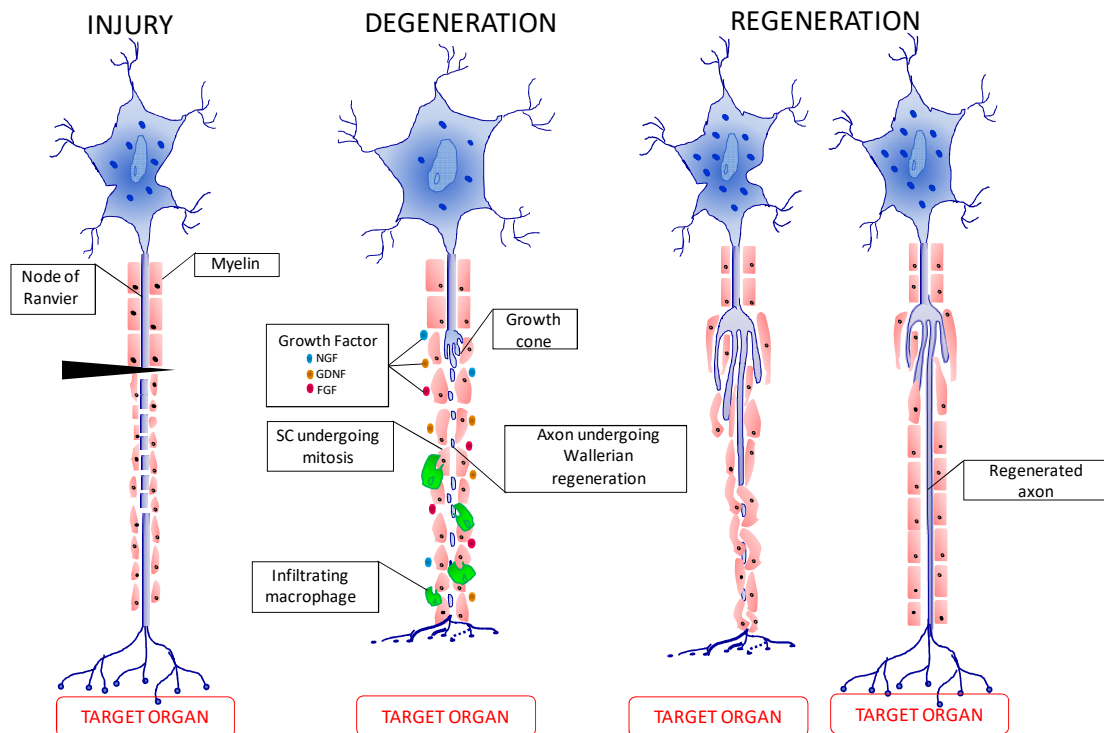
Fig. 2: Schematic representation of degeneration and regeneration processes

2. FACTORS INFLUENCING PERIPHERAL NERVE REGENERATION

In contrast to the central nervous system (CNS), the peripheral nervous system (PNS) is able to regenerate spontaneously after injury. The functional recovery in the PNS can be attributed to different factors, such as the ability of neurons and/or Schwann cells to regenerate neurites, the distal environment supporting axon regrowth, and the target tissues receptive to reinnervation (Scheib and Hoke 2013). In physiological conditions, nerve fibers regenerate after degeneration, and the successful nerve regeneration is considered closely linked to the Wallerian degeneration process.

Functional recovery after nerve injury depends on several extrinsic and intrinsic factors such as: surgeon experience, surgical technique used for the reconstruction, rehabilitation, obese conditions, co-morbidity and not least, age (Montorsi, Salonia et al. 2005; Dubbelman, Dohle et al. 2006; Ayyathurai, Manoharan et al. 2008).

About co-morbidity, it is well know that axonal regeneration following peripheral nerve injury is impaired in diabetic patients and has been documented in experimental diabetic animal models: diabetic conditions induce alterations in the biological properties of neurons and Schwann cells leading to delay in Wallerian degeneration and macrophage infiltration, furthermore reduce the expression of neurotrophic factors and alteration of the extracellular matrix components resulting

in impairment of axonal regeneration (Yasuda, Terada et al. 2003; Sango, Mizukami et al. 2017).

Age is a further factor that influences peripheral nerve regeneration. Whereas it is well reported that young tissue has high regeneration potential, in aged tissue the regenerative capability and functional recovery is significantly reduced. Results about the implantation of nerve conduit in aged rats showed that axonal regeneration after sciatic nerve defects was less effective in aged than in young mice when using either nerve autografts or nerve conduits (Yokoi, Uemura et al. 2017).

Furthermore, morphological and morphometrical studies on crushed sciatic nerve of 6 and 24 month-old mice analyzed 2, 4, and 8 weeks after injury showed that two weeks after axotomy, fascicles of aged mice contained significantly fewer regenerated myelinated fibers than young; 4 weeks later, the difference in the number of myelinated fibers was less. However, measurements of myelinated fibers of aged mice showed that areas of Schwann cell cytoplasm and myelin were significantly reduced at all time points analyzed (Tanaka and Webster 1991; Tanaka, Zhang et al. 1992; Choi, Harii et al. 1996; Larkin, Kuzon et al. 2003; Muratori, Ronchi et al. 2012).

Since the aging population is progressively growing, more studies are needed to improve the regeneration process in aged animals.

3. STRATEGIES TO IMPROVE PERIPHERAL NERVE REGENERATION

Strategies to improve the regeneration of peripheral nerves are proposed to reach the functional recovery (Raimondo, Fornaro et al. 2011; Tos, Ronchi et al. 2013).

A possible approach is represented by the delivery of neurotrophic factor (NTFs) such as NGF, GDND and FGF into the nerve defect. It is known that neurotrophic factors support the different phases of Wallerian degeneration and axonal regeneration. For this reason, different studies report the application and the delivery of NTFs for instance conjugated with iron oxide nanoparticles (IONP), which were supposed to increase the stability of the conjugated NTFs, but also to ensure local and slow release of NTFs (Morano, Wrobel et al. 2014).

Another approach proposed to enhance nerve regeneration is the administration of drugs such as Sildenafil, a selective inhibitor of phosphodiesterase-5 (PDE5) causing intracellular accumulation of cGMP.

Sildenafil is currently used for treatment of erectile dysfunction, and other several conditions such as pulmonary hypertension (Koneru, Varma Penumathsa et al. 2008; Histing, Marciniak et al. 2011).

Recently has been reported that increasing cGMP in cells can also induce neurogenesis, angiogenesis and synaptogenesis in young and old animal models promoting the functional recovery of sciatic nerve (Fang, Shao et al. 2013; Korkmaz, Parlakpinar et al. 2016).

Different materials are currently investigated as device to enhance axonal regeneration, both of biologic or synthetic origin.

According to the material source, they can be classified in different categories: biological nerve conduits or syntethic nerve conduits.

3.1 Biomaterials currently used for peripheral nerve repair

Various materials have been used as gap for peripheral nerves and are classified in: non degradable materials, biodegradable synthetic material and biodegradable materials of natural origin.

Non degradable biomaterials

A wide range of synthetic non degradable materials have been studied and tested for the fabrication of artificial nerve device, they possess many useful chemical and physical properties such as the easy manipulation during the fabrication process.

The most common are represented by: silicone tubes**,** frequently used for nerve repairs empty or filled with collagen, laminin and fibronectin based gel. This material is neither biodegradable nor permeable to large molecules resulting in a possible fibrotic capsule formation around the guide.

Other non degradable materials employed for nerve regeneration are composed by elastomer hydrogel or pourus stainless steel with the disadvantage in strong scar tissue formation and foreign body reaction combined with inflexibility and lack of stability of the material (Siemionow, Bozkurt et al. 2010; Konofaos and Ver Halen 2013).

Biodegradable synthetic materials

Among biodegradable material, the polyglycolic acid (PGA) represent a bioabsorbable material currently used as suture material for wound closure (Anderson and Turmaine 1986).

Polyesters and copolyesters have also been reported as suitable materials for nerve regeneration, to this category belong poly(L-lactide) (PLLA), poly(lactide-co-ε-caprolactone), poly(L-lactide-co-glycolide), poly(1,3-trimethylenecarbonate-co-ε-caprolactone) and poly(ε-caprolactone) (PCL) (Bini, Gao et al. 2004).

Biodegradable materials of natural origin

These materials are able to degrade within a reasonable period proving different useful properties such as flexibility, degradability, porosity and high biocompatibility.

Furthermore these important features can be modified altering the chemical or engineering properties of the materials.

Among these collagen, the major component of the extracellular matrix, has high biologic properties for peripheral nerve regeneration able to enhance the regeneration process but despite the successful results in animal experimental model, no clinical trials have been conducted in human to date (Mackinnon and Dellon 1990; Rosen, Padilla et al. 1990; Lohmeyer, Siemers et al. 2009; Moore, Kasukurthi et al. 2009).

Silk fibroin derived from natural silk has an increased application in biomedical fields thanks to its unique properties such as high tensile strength, elasticity and low immunogenicity (Gu, Ding et al. 2011). In peripheral nerve regeneration, silk fibroin has been used as biomaterial for nerve guides to clarify the biocompatibility with neural tissues *in vitro*, and for bridging nerve defects *in vivo* (Yang, Chen et al. 2007; Madduri, Papaloizos et al. 2010; Sivak, White et al. 2014).

Other natural proteins like keratins have also been tested as nerve scaffold materials (Apel, Garrett et al. 2008; Sierpinski, Garrett et al. 2008) or as filler to support peripheral nerve regeneration (Pace, Plate et al. 2014).

Among the biodegradable materials chitosan, a copolymer of D-glucosamine and N-acetyl-D-glucosamine, obtained from full or partial N-deacetylation of chitin, represents a highly biocompatible, biodegradable, low toxic material. The reasonable of chitosan as a eligible biomaterial for the development of nerve guides resides in its favorable biological properties and in its ability to interact with the ECM molecules. The biocompatibility of chitosan-based biomaterials with CNS and PNS cells has been widely investigated with success (Yuan, Zhang et al. 2004; Freier, Koh et al. 2005; He, Zhang et al. 2009; Simoes, Gartner et al. 2011). Recently, the study of chitosan tubes alone or in combination with other proteins such as poly-L-lysine or with PGA demonstrated the efficiency of such nerve guides for bridging peripheral

nerve defects (Kim, Seo et al. 2008; Ao, Fung et al. 2011; Gnavi, Barwig et al. 2013; Haastert-Talini, Geuna et al. 2013; Biazar and Heidari Keshel 2014; Nie, Deng et al. 2014).

Chitosan is a biodegradable scaffold with very positive effects on nerve regeneration, facilitating nerve healing and improving nerve growth.

For these reasons, chitosan is actually used as scaffold for regeneration of various tissues (nerve, skin, bone, cartilage) (Gnavi, Barwig et al. 2013) (Fig. 3).
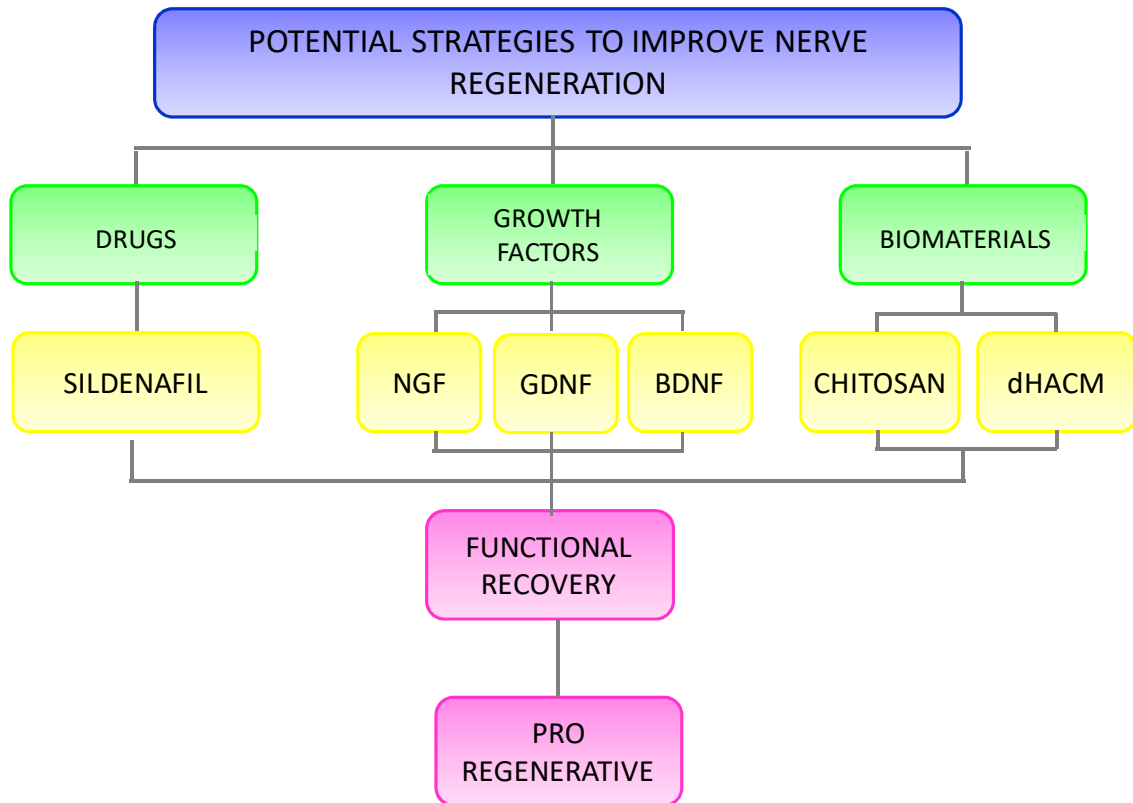

Fig. 3: Schematic representation of the potential strategies to improve nerve regeneration.

4. APPLICATION OF MEMBRANES TO IMPROVE THE REGENERATION OF PERI-PROSTATIC NERVES AFTER NERVE-SPARING ROBOT ASSISTED RADICAL PROSTATECTOMY

Recently, new strategies to improve the regeneration of the prostate nerves are arising to reach the functional recovery in patients who underwent radical prostatectomy. In urological context, the application of different type of membranes is particularly useful to protect and to enhance the regeneration process of the periprostatic nerve inside the neurovascular bundle that surround the prostate. In the following paragraphs a description of the two main clinical membranes applied to neurovascular bundle will be provide.

4.1 Application of dehydrated human amnion/chorion membrane on the neurovascular bundle after nerve sparing radical prostatectomy

some authors described the application of dehydrated human amnion/chorion membranes (dHACM) on the neurovascular bundles after nerve sparing radical prostatectomy as a neuroprotective, pro-regenerative and anti-inflammatory device. Patel et al. reported the clinical use of dHACM as a novel source of implantable neurotrophic factors and cytokines useful to enhance the regeneration process (Quinlan, Nelson et al. 1989; Liang, Liang et al. 2009). This pivotal study was conducted in patients who underwent bilateral nerve sparing radical prostatectomy with the bilateral application of dHACM for a total of 58 patients preoperatively potent and continent. Postoperative evaluation of patients by follow-up information showed that a high percentage of patients recover the urinary continence 8 weeks after surgery. With regard to the potency, considered as the ability to achieved and maintain the erection, they reported a significant shorter mean time period in patients with the dHACM application resulting in a early potency return (Patel, Samavedi et al. 2015).

Another study by Ogaya-Pinies et al. provide encouraging data regarding the enhancement of the functional recovery using dHACM wrap around the neurovascular bundle after radical prostatectomy as an innovation in this clinical field. More specifically, dHACM house and release many important factors involved in tissue repair and growth such as VEGF, TGF-β, FGF, PDGF. Result obtained by follow-up of patients belonging to dHACM group showed a short mean time to return to potency compared with the group of non-graft patients supporting the hypothesis that the application of dHACM is able to accelerate the return to potency. Furthermore, it has been demonstrated that the application of dHACM does not increase the risk of biochemical cancer recurrence (BCR) (Ogaya-Pinies, Palayapalam-Ganapathi et al. 2017).

4.2 Application of chitosan membrane to improve the functional recovery after nerve sparing radical prostatectomy

In order to improve the regeneration process and the functional recovery of peripheral nerves, different artificial devices have been developed and proposed for clinical application (Gu, Ding et al. 2011; Daly, Yao et al. 2012; Meyer, Stenberg et

al. 2016). Interestingly, the application of membranes made of another biomaterial of natural origin namely chitosan has been reported to be safe and effective on the neurovascular bundles after nerve-sparing robot-assisted radical prostatectomy, appreciating also some positive results regarding potency recovery (Porpiglia, Bertolo et al. 2017).

Chitosan a derivative of chitin, obtained from the exoskeleton of crustaceans, is achieving resounding interest both in basic research and in clinical settings due to its biocompatibility, biodegradability, low toxicity and adhesion to the injury site. For this reason, chitosan-based nerve graft have been widely employed for nerve reconstruction as an alternative to autologous nerve graft. The characteristics of chitosan useful in the intra-operative field are (Fig. 4):

(i)   Neuroprotective/neuroregenerative effect (Haastert-Talini, Geuna et al. 2013)

(ii)   Antitumoral activity (Jiang, Ouyang et al. 2011)

(iii)   Antinflammatory and analgesic effect (Wu, Lee et al. 2014)

(iv)   Hemostatic activity (Brown, Daya et al. 2009)

(v)   Antimicrobial activity (Youssef, Abou-Yousef et al. 2015)

In vitro studies on chitosan membrane showed its suitability as a substrate for proliferation, survival of Schwann cells as well as survival and differentiation of neuronal cells.

The direct contact of Schwann cells with the biomaterial proved its good biological properties allowing important cell functions such as ensheathment, myelination and production of extracellular matrix (Yuan, Zhang et al. 2004; Freier, Koh et al. 2005; Huang, Hu et al. 2010; Simoes, Gartner et al. 2011; Gnavi, Barwig et al. 2013).

In addition, several *in vivo* studies showed that chitosan, in form of hollow conduits, achieved promising results improving peripheral nerve regeneration (Ishikawa, Suzuki et al. 2007; Lauto, Stoodley et al. 2007; Lauto, Foster et al. 2008; Matsumoto, Kaneko et al. 2010; Marcol, Larysz-Brysz et al. 2011).

In particular, an *in vivo* study with different degrees of acetylation of chitosan tubes used to reconstruct 10 mm nerve defects in the adult rat displayed functional and morphological nerve regeneration confirmed by stereological analyses. At the same time *in vitro* cytotoxicity was studied to test the biocompatibility of the chitosan tubes showing that the degradation products released by the conduit did not affect negatively the metabolic activity of cells (Haastert-Talini, Geuna et al. 2013; Meyer, Stenberg et al. 2016).

Chitosan nerve conduits have shown promising results not only to bridge somatic nerve defects but also in case of autonomic nerves such as sympathetic and phrenic nerves that following thoracic sympathectomy can be occasionally resected leading to respiratory dysfunction (Matsumoto, Kaneko et al. 2010).

Recent advancements showed that tissue regeneration can be achieved also by chitosan degradation products called "chitooligosaccharides" (COS) deriving from partially hydrolyzed chitosan with high water-solubility. It is reported that COS are able to support the local microenvironment at the injury site and display neuroprotective effects on neurons making them particularly suitable for medical application (Mendis, Kim et al. 2007). COS support nerve cell adhesion and promote neuronal differentiation and neurite outgrowth through upregulating the expression of both neurofilament and N-cadherin factors (Zhou, Yang et al. 2008; Gong, Gong et al. 2009; Yang, Liu et al. 2009).

Furthermore COS display important interactions with Schwann cells that are essential for nerve regeneration (Zhao, Wang et al. 2017).

In addition to its well-known properties useful for tissue repair (biocompatibility, biodegradability, low toxicity), chitosan has also shown excellent potential for supporting three-dimensional organization of regenerating tissues (Ma, Gao et al. 2003; Ho, Wang et al. 2005; Gnavi, Barwig et al. 2013).

Furthermore, another important property of chitosan is represented by its anti-proliferative capability in case of cancer cells.

The mechanism of action of chitosan as an anti-proliferative agent has been well reported, even if the underlying molecular mechanism has not been fully investigated yet. The anti-proliferative properties of chitosan have been tested in several reports with different human cancer cell lines such as breast, gastric carcinoma, melanoma and monocytic leukemia cell lines (Qi, Xu et al. 2005; Ta, Dass et al. 2008; Xu, Wen et al. 2009; Jiang, Ouyang et al. 2011; Salah, Michaud et al. 2013; Gibot, Chabaud et al. 2015).
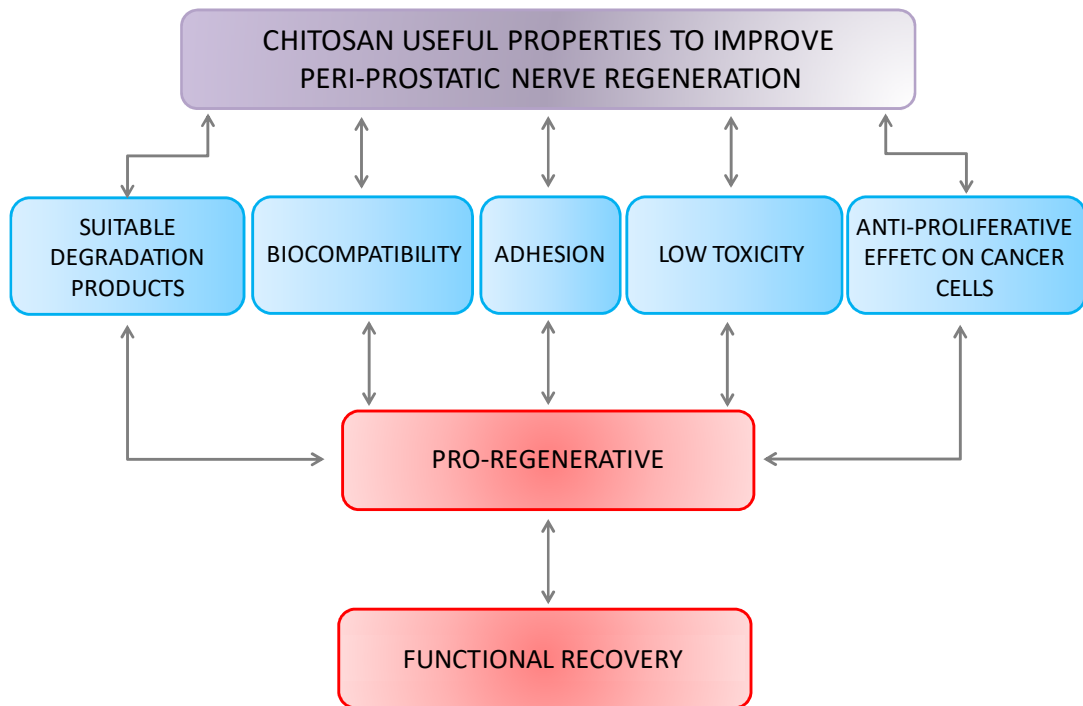
Fig. 4: Schematic representation of chitosan useful properties

DISCUSSION

Erectile dysfunction represents an important impairment in patients following prostate cancer resection, and despite the advancement of the surgical technique such as nerve-sparing robot assisted radical prostatectomy that allowed to preserve the peri-prostatic nerves, the percentage of patients presenting this condition is still considerable.

Furthermore, different pathological conditions such as diabetes can negatively affect the peripheral nerve regeneration, for this reason impairing the functional recovery.

The application of dehydrated human amnion/chorion membranes (dHACM) on the neurovascular bundles has been proposed as scaffold to improve the functional recovery thanks to many growth factors with a neuroprotective, pro-regenerative and anti-inflammatory role. Despite these important properties the application of dHACM has limitations related to the availability and the high clinical cost of the device as well as the controversial use of growth factors in a region in which a tumor has been developed.

The use of a biomaterial such as chitosan, that has already achieved the clinical application for somatic nerve repair, represents a high available and low-cost alternative.

Chitosan has been receiving growing interests among basic and clinical research: results obtained in different *in vitro* and *in vivo* studies showed that chitosan represents an optimal candidate as a neural repair scaffold supporting axonal regeneration.

Its adhesive capability allows the application on neurovascular bundle after robot assisted radical prostatectomy and it can be easily manipulated with the aim to create devices with different structural features.

Moreover, interesting clinical results were obtained by Porpiglia and colleagues reporting a clinical trial in which they tested chitosan membrane, already known for its effectiveness in promoting nerve regeneration (Wrobel, Serra et al. 2014), as protective device for neurovascular bundles following bilateral nerve-sparing radical prostatectomy (Porpiglia, Bertolo et al. 2017).

For this reason chitosan could be a suitable scaffold to improve the regeneration of periprostatic nerve and the functional recovery in the context of peri-prostatic nerves regeneration.

Finally multicentre clinical trials will be carried out to study the efficacy of chitosan membrane in the clinical setting.

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2. AIM OF RESEARCH

AIM OF RESEARCH

Most neuroscientists at the beginning of the twentieth century held the opinion that the human brain lacked any functional capacities for repair, re-adaptation, and response to neuronal damage following degenerative diseases and injuries. This concept has changed radically in the last decades; indeed, recent evidence demonstrates that the brain has a great potential to adapt to various changes in the environment and it is capable of remarkable widespread change and adaptation particularly in response to peripheral injuries.

The plastic behaviour of the brain has essentially been studied with reference to the central nervous system in which the existence of plastic areas has been commonly accepted. Only few studies in the past 10 years have begun to come out evidence that this process occurs also in the PNS.

The issue of adult plasticity in PNS has always been strongly debated especially regarding dorsal root ganglia and the autonomic ganglia.

During my Phd, I focused the attention on different aspects of the plasticity phenomena regarding the sensory somatic and the autonomic neurons within the ganglia.

Dorsal Root Ganglia neurons are used here as a model for the study of adult plasticity assessing if the injury of the peripheral nerve may retrogradly stimulate plastic changes in the corresponding sensory neurons. The aim of this work was to investigated changes in the number of neurons in the time-window of 30 days after a crush lesion of the rat brachial plexus. Furthermore the cellular morphological changes that follow the crush injury were characterized using immunofluorescence and Trasmission Electron Microscopy (TEM).

At the same time the study of long term fibers regeneration was evaluated: it is well known that unlike the CNS, sensory and motor axons of PNS are characterized by spontaneous regeneration after injury, this is mainly due to the permissive extracellular environment, characterized by the presence of growth factors, extracellular matrix components and SCs, that influence and promote peripheral axons regeneration.

While it has been shown that, after a sufficiently long post-traumatic time, the number of regenerated rat peripheral nerve fibers can return to normal levels and animal can regain normal pre-lesion function, no information regarding long-term changes in size parameters of regenerated nerve fibers are still available. To fill this gap, long-term changes (24-week posttraumatic) in myelinated axon such as nerve fiber diameter, myelin thickness and g-ratio (axon diameter/fiber diameter), distal to a nerve crush (axonotmesis lesion) of the rat median nerve were assessed by stereological method.

AIM OF RESEARCH

The expression of Vascular Endothelial Growth Factor (VEGF) and its receptors and co-receptors in regenerating and degenerating peripheral nerve conditions were evaluated in the third part of my PhD project, to deepen one of the main factors involved not only in angiogenesis but also in neuritogenesis.

During this study *in vivo* and *in vitro* analysis were performed and the expression of VEGF and VEGF family members was evaluated in order to study its possible involvement during the regenerative process of the peripheral nerve.

In the last part of my Phd the development of a chitosan membrane able to improve the peri-prostatic nerve regeneration in patients after radical prostatectomy was investigated showing that this biomaterial exhibits suitable characteristics not only in case of somatic nerve repair but also in autonomic peri-prostatic nerves.

3. SCIENTIFIC PUBBLICATIONS

Research Article

Generation of New Neurons in Dorsal Root Ganglia in Adult Rats after Peripheral Nerve Crush Injury

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The evidence of neurons generated *ex novo* in sensory ganglia of adult animals is still debated. In the present study, we investigated, using high resolution light microscopy and stereological analysis, the changes in the number of neurons in dorsal root ganglia after 30 days from a crush lesion of the rat brachial plexus terminal branches. Results showed, as expected, a relevant hypertrophy of dorsal root ganglion neurons. In addition, we reported, for the first time in the literature, that neuronal hypertrophy was accompanied by massive neuronal hyperplasia leading to a 42% increase of the number of primary sensory neurons. Moreover, ultrastructural analyses on sensory neurons showed that there was not a relevant neuronal loss as a consequence of the nerve injury. The evidence of BrdU-immunopositive neurons and neural progenitors labeled with Ki67, nanog, nestin, and sox-2 confirmed the stereological evidence of posttraumatic neurogenesis in dorsal root ganglia. Analysis of morphological changes following axonal damage in addition to immunofluorescence characterization of cell phenotype suggested that the neuronal precursors which give rise to the newly generated neurons could be represented by satellite glial cells that actively proliferate after the lesion and are able to differentiate toward the neuronal lineage.

1. Introduction

The dorsal root ganglia (DRG) are located along the dorsal spinal roots and are surrounded by a connective capsule that isolates this cluster of neurons. The location and the connective capsule define the DRG as an isolated peripheral pool of neuronal bodies that, for this reason, are easily identifiable and represent a valid model for the study of permanent neurons. Therefore, the absence of postnatal cell migration from or to the DRG makes it a particularly suitable model for the study of adult neurogenesis due to the presence of a stem cell niche within the ganglia [1–9].

The generation of new neurons in both central and peripheral adult nervous systems is well acknowledged today [6, 10–15]. Although it is still a controversial matter, for decades several groups have collected data suggesting that,

in different animal species, DRG may undergo a progressive age-dependent increase in neuron number [2, 8, 16–19].

Recently, it has been demonstrated *in vitro* that adult rat DRG and trigeminal ganglia explants are able to give rise to neurospheres that can differentiate into neurons and glia [6, 13, 20]. Moreover, an *in vivo* study demonstrated that, as a consequence of peripheral nerve injury (crush lesion or axonotmesis), DRG neurons undergo adaptive changes [21, 22] enabling them to respond and recover from injury [23–26]. Finally, evidence of satellite glial cells proliferation was demonstrated in adult rats DRG after capsaicin injection [27].

In the present study, we investigated whether the sequence of events that follow peripheral axon damage also included a change in the number of DRG neurons assessed by a means of accurate and unbiased stereological counts.

We considered that the exceptional stimulus represented by massive nerve regeneration, which is characterized by the presence of supernumerary axons distal to the lesion site [28], may retrogradely stimulate plasticity in the corresponding neurons of DRG. For our experiments, we adopted the nerve crush lesion paradigm using a nonserrated clamp [29], which causes axonotmesis without interrupting epineurial continuity and thus posttraumatic axonal regeneration occurs without requiring surgical repair of the nerve.

Our stereological results indicated that a relevant increase in neurons number occurred in the DRG belonging to the brachial plexus during the first month after crush injury of the four main terminal plexus branches. The presence of BrdU-immunopositive neurons expressing the neural progenitor markers supported the stereological evidence of posttraumatic neurogenesis and suggested that the precursor cell population which gives rise to the new-generated neurons may be represented by satellite glial cells.

To test our hypothesis on the role of satellite glial cells (SGCs), we characterized the cellular morphological changes that follow the crush injury using immunofluorescence and transmission electron microscopy (TEM) analyses. Our results supported the view that the neuronal precursors are represented by SGCs that actively proliferate after the lesion and are able to differentiate toward the neuronal lineage.

2. Materials and Methods

2.1. Animals and Surgical Procedure. Adult female Wistar rats (Charles River Laboratories, Milan, Italy) weighing approximately 190–220 g were used for this study ($N = 25$). All procedures were performed in accordance with the Ethics Committee and the European Communities Council Directive of November 24, 1986 (86/609/EEC). Adequate measures were taken to minimize pain and discomfort taking into account human endpoints for animal suffering and distress.

All surgical procedures were carried out under deep anesthesia obtained with tiletamine + zolazepam (Zoletil) i.m. (3 mg/kg). The median, ulnar, radial, and musculocutaneous nerves of the left forelimb were approached from the axillary region to the elbow with a longitudinal skin access. Under operative microscope, nerves were carefully exposed from their origin at the brachial plexus until the elbow. The crush lesion was applied to each nerve using a nonserrated clamp [29]. Animal well-being assessment was carried out using careful animal surveillance to check for passive and active movements, automutilation, skin ulcers, and joint contracture, especially during early postoperative times.

2.2. Sample Collection. Rats were sacrificed by a lethal i.m. injection of tiletamine + zolazepam. The vertebral column was surgically dissected and the vertebral bodies were cut off and removed in order to reach the spinal cord. Briefly, using fine scissors, we accessed the vertebral canal performing a double cut on both sides of the vertebral bodies. Using the dorsal spinal roots as guides, the ganglia that participate in the formation of the brachial plexus (the last 4 cervical, C5–C8, and the first thoracic, T1) were identified and removed.

For the stereological analysis, T1 DRG from both sides were removed after 30 days from the crush injury and processed for the resin embedding procedure. DRG were removed also from 5 healthy rats from both sides, as control.

For the immunohistochemical analysis, animals underwent BrdU injections (see Section 2.6) and DRG were harvested at different time points: 1, 3, 5, 7, 10, 15, and 30 days from the crush injury and processed for the paraffin embedding procedure. DRG removed from healthy rats were used as control.

2.3. Resin Embedding. DRG were fixed by immediate immersion in 2.5% glutaraldehyde for 2 h, washed in Sorensen phosphate buffer 0.1 M (pH 7.4) with 1.5% sucrose, and postfixed in 2% osmium tetroxide for 2 h. After dehydration in ethanol, samples were cleared in propylene oxide and embedded in Glauerts' embedding mixture of resins consisting in equal parts of Araldite M and Araldite Harter, HY 964 (Merck, Darmstadt, Germany), containing 0.5% of the plasticizer dibutyl phthalate and 1-2% of the accelerator 964, DY 064 (Merck, Darmstadt, Germany).

2.4. Stereology Analysis for Sensory Neurons Number. For stereological analysis, only T1 ganglion from both sides was analyzed since it predominantly contributes, in terms of number of sensory fibers, to the crush injured peripheral nerves. All of DRG, randomly oriented, were cut into serial semithin sections ($2.5 \mu m$) using an Ultracut UCT ultramicrotome (Leica, Wetzlar, Germany) and stained with 1% toluidine blue.

The physical disector stereological analysis [30] was performed on T1 DRG divided into 4 experimental groups ($n = 5$/group): (1) left T1s harvested 30 days after the nerve crush lesion (CRUSH); (2) right T1s taken from the same animal of group 1 used as internal control (INTERNAL CTRL); and (3) and (4) left and right T1-DRG taken from healthy animals that did not undergo the crush lesion (NORMAL LEFT and NORMAL RIGHT).

Four/six disector pairs (depending on the size and the orientation of the DRG) were selected by systematic random sampling [30] from each DRG, setting the distance between consecutive disector pairs at $100 \mu m$. The reference section was taken at $5 \mu m$ from the counting section. The determination of neuron number was based on the identification of the top of the nucleus; each nucleus in the reference section was identified, marked, and carefully recognized in the counting section under high-resolution light microscope observation by blinded observers (Figures 1(a) and 1(b)). Only nuclear profiles that were observable in the reference section but not in the counting section (thus suggesting the nucleus ended in the thickness between the pair of sections analyzed) were counted (Figure 1(b)) and the average density (N_v) was calculated. Then, the reference volume (V_{ref}) of the entire ganglion was estimated using the Cavalieri principle; the fibrous portion of the hilum was not included in the reference volume. Finally, the total number (N) of the DRG neurons was calculated as $N = V_{ref} \times N_v$ [31]. The precision of
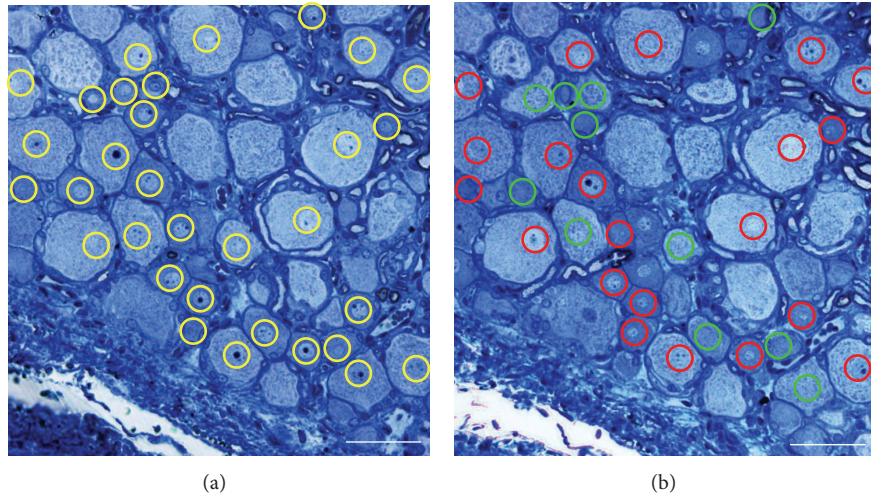
(a)   (b)

FIGURE 1: Example of the toluidine blue stained micrographs of reference (a) and counting (b) sections from which neurons numbers are estimated using the physical disector. All nuclear profiles are recognized in the reference section (yellow circles); nuclear profiles that do not appear in the counting section are counted (green circles). The nuclei of cells that appear in both the counting and reference sections are not counted (red circles). Bars = 50 μm.
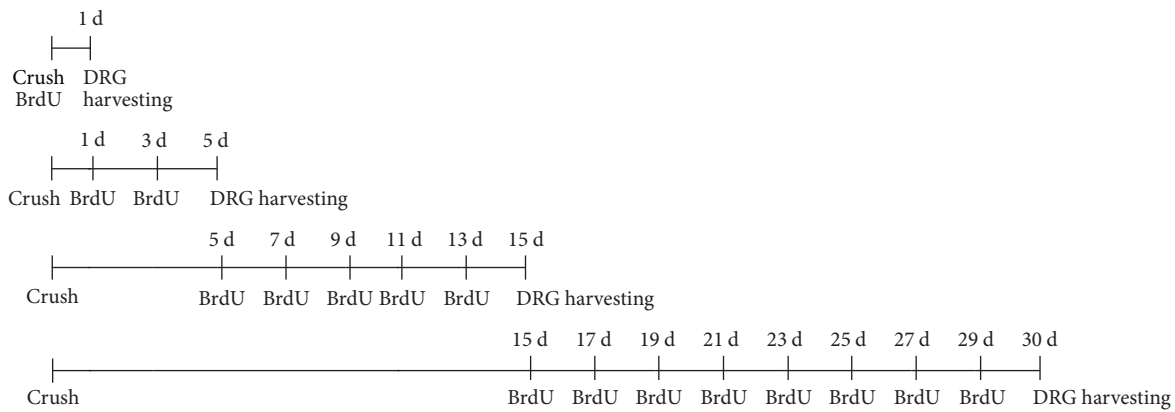

FIGURE 2: Experimental design of BrdU administration.

the estimates was evaluated by calculating the coefficient of error (CE) as described by Schmitz [32] and the sampling scheme was designed in order to keep the CE below 0.10, which assures enough accuracy for neuromorphological studies [33].

2.5. Morphometric Analysis for Sensory Neurons Size.
Left T1-CRUSH DRG and left T1-CTRL DRG were processed for morphometric analysis in order to evaluate the size of the sensory neurons. Morphometric analysis was performed on the same cells counted for the stereological analysis. For this purpose, serial semithin section previously used for the stereology was considered and the neurons area was measured using the unbiased point counting method with a DM4000B microscope equipped with a DFC320 digital camera and an IM50 image manager system (Leica Microsystems, Germany). Areas were converted into diameters and cells size distribution was obtained.

2.6. BrdU-Treatment.
For the qualitative and quantitative evaluation of BrdU incorporation, animals were injected intraperitoneally with 5-bromo-2-deoxyuridine (BrdU, Sigma, St. Louis, MO, 50 mg/kg, made from 10 mM BrdU dissolved in 7 mM NaOH) diluted in PBS. The experimental design of BrdU administration is illustrated in Figure 2. Animals scarified 1 day after crush lesion were subjected to one single BrdU injection on the same day of the surgery. Animals scarified 5 days after the crush lesion were subjected to BrdU injection at days 1 and 3, while rats scarified after 15 days were subjected to BrdU injection every other day starting from the 5th day after the injury. Finally, rats scarified 30 days after crush lesion were subjected to BrdU injection every other day starting from the 15th day after the injury. This protocol was used in order to prevent an overlapping between the different time points, thus allowing us to predict the BrdU incorporation rate in a determined time window. DRG were harvested 1, 5, 15, and 30 days after

the crush injury. T1 DRG were used for BrdU quantification, whereas C5–C8 DRG were used for qualitative evaluation.

2.7. Stereology for BrdU Quantification. Stereological analysis was performed on T1 DRG embedded in paraffin and cut into serial sections (10 μm) to assess the number of BrdU-positive cells, which was counted by blinded observers with a variation of the physical disector method adapted to confocal laser microscopy [34].

BrdU-positive cells were counted [30] as neuronal and nonneuronal cells based on their morphological features. As an additional criterion, nestin immunopositivity (see Section 2.10) was used to distinguish small neurons from satellite cells in case of doubts. Briefly, confocal z-stacks of five random samples for each DRG were taken and considered for count. For each sample, the counting/reference pair of sections was randomly selected at 3 μm distance from each other. The determination of BrdU-positive cells was based on the identification of the top of the nucleus; each nucleus in the reference level was identified and only nuclear profiles that were observable in the counting level but not in the reference section were counted. Finally, to predict the daily rate of cells which incorporate BrdU in the different time windows, the number of BrdU-positive cells was divided by the days of BrdU treatment.

2.8. Statistics. Statistical analysis was performed using both one-way analysis of variance (ANOVA) test and t-test for morphometric data. Parametric tests were adopted assuming that sample mean values for all estimated parameters present a normal distribution. Values are expressed as mean ± standard deviation (SD). The level of significance was set at $P \leq 0.05$ (*), $P \leq 0.01$ (**), and $P \leq 0.001$ (***). All statistical tests were performed using SPSS software.

2.9. High-Resolution Light Microscopy and Electron Microscopy Analysis. For light and electron microscopy analysis, DRG samples of control rats (CTRL) and 1 day and 5 days after crush injury (CRUSH) were embedded in resin (see Section 2.3). Transversal cross sections of 2.5 μm (light microscopy) and 70 nm (electron microscopy) were obtained from the DRG using an Ultracut UCT ultramicrotome (Leica, Wetzlar, Germany). Sections for light microscopy were then stained with toluidine blue and images were taken with a DM4000B microscope equipped with a DFC320 digital camera and an IM50 image manager system (Leica Microsystems, Germany). Sections for electron microscopy were stained with uranyl acetate and lead citrate and examined by a JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan) equipped with a Mega-View-III digital camera and a Soft-Imaging-System (SIS, Münster, Germany) for the computerized acquisition of the images.

2.10. Immunofluorescence. Samples were fixed in 4% paraformaldehyde for 2 h, washed in a solution of 0.2% glycine in 0.1 M phosphate buffer (pH 7.2), dehydrated, and embedded in paraffin. Sections were cut with thicknesses of 10 μm by

TABLE 1: Primary antibodies used for immunofluorescence.

Antibody	Antibody characteristics
	Manufacturer, catalog number, species, dilution.
a-Neurofilament	Sigma, St. Louis, MO, N014 Mouse monoclonal 1 : 200
a-Peripherin	Chemicon International, AB1530 Rabbit polyclonal 1 : 1000
a-S100	Sigma, St. Louis, MO, S2644 Rabbit polyclonal 1 : 600
a-GFAP	Sigma, St. Louis, MO, G9269 Rabbit polyclonal 1 : 500
a-Nestin	Sigma, St. Louis, N5413 Rabbit polyclonal 1 : 1000
a-SOX-2	Santa Cruz Biotechnology, Santa Cruz, CA sc-17320, goat polyclonal, 1 : 50
a-Ki67	Novocastra Laboratories, Newcastle, UK NCL-Ki67-MM1, mouse monoclonal 1 : 500
a-NeuroD	R&D System, Minneapolis, MN Goat polyclonal 1 : 500
a-Nanog	sc-33760, rabbit polyclonal, 1 : 1000
a-BrdU	Sigma, St. Louis, MO, B-2531 Mouse monoclonal 1 : 500

a RM2135 microtome (Leica Microsystems, Wetzlar, Germany).

For BrdU staining, rehydrated sections were incubated with 2 N HCl (in PBS, 0.1% triton X100 solution, 15 min at room temperature), rinsed in PBS, and neutralized with 0.1 M sodium tetraborate. Sections were then incubated with monoclonal antibody anti-BrdU.

For all the other staining techniques, sections were permeabilized, blocked (0.1% triton X-100, 10% normal goat serum (NGS)/0.1% NaN$_3$, 1 h), and processed for an immuno-histochemical protocol. See Table 1 for the list of primary antibodies used.

Sections were incubated overnight in primary antibody or, in case of double immunofluorescence experiments, in a mixture of primary antibodies and visualized using a solution containing the appropriate secondary antibody/ies: goat anti-mouse IgG Alexa-Fluor-488-conjugated (1 : 200, Molecular Probes, Eugene, Oregon), rabbit anti-goat IgG Alexa-Fluor-488-conjugated (1 : 200, Molecular Probes, Eugene, Oregon), and CY3-conjugated anti-rabbit IgG (dilution 1 : 400, Dako, Milan, Italy). The samples were finally mounted with a Dako fluorescent mounting medium and analyzed by a LSM 510 confocal laser microscopy system (Zeiss, Jena, Germany).

3. Results

3.1. Stereological Evaluation of DRG Sensory Neuronal Number and Size. The effect of the nerve crush lesion applied to the radial, ulnar, median, and musculocutaneous nerves on the thoracic T1 DRG sensory neurons was investigated 30 days after nerve damage by quantitative evaluation of DRG neurons total number. For this purpose, the physical disector stereological method, which deals better with the difficulty of
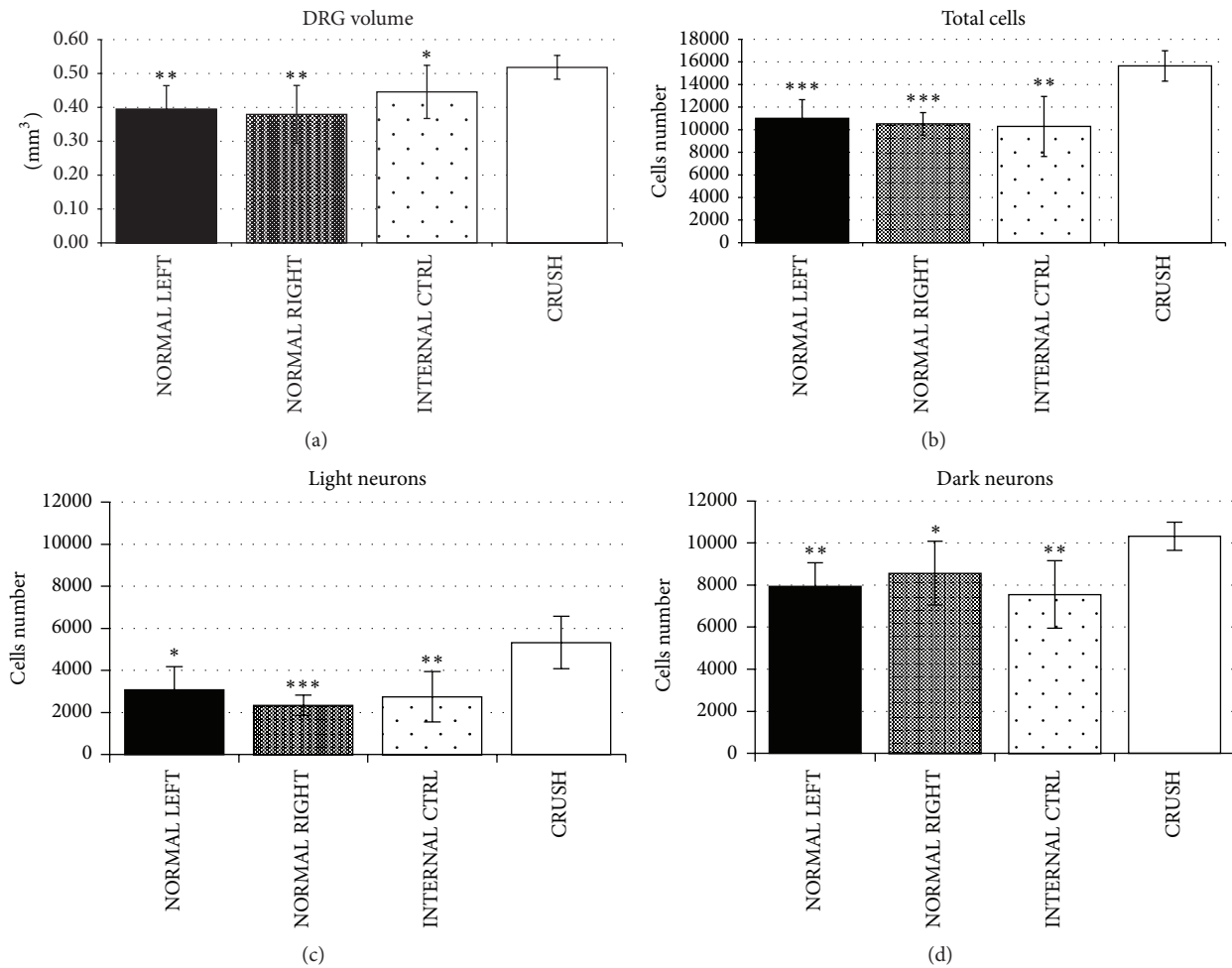
(a)

(b)

(c)

(d)

FIGURE 3: (a) T1s affected by crush lesion showed an evident hypertrophy compared to controls. (b) Physical disector stereological analysis was applied to T1s ganglia. The overpopulation of neurons was distributed among the light and large neurons (c-d). The data were analyzed using both ANOVA and t-test analyses. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$ versus CRUSH group.

counting the number of neurons (considering their different cell sizes) within the ganglia, was applied.

In order to avoid artifacts, as, for instance, tissue shrinkage, thus guaranteeing a more precise stereological analysis, the physical disector method was performed on semithin sections that underwent the embedding procedure utilized for electron microscopy (see Section 2). In addition, for a more complete evaluation of differences within the cell population of the two sets of ganglia (CRUSH and NORMAL LEFT), in consideration of a physiological variability between the pair (left and right) of the same ganglia, the quantification of the neuronal population of the two groups was compared to the contralateral ganglia, which means the right T1s taken from the same animal that underwent crush lesion (INTERNAL CONTROL) and the right T1s from control (NORMAL RIGHT), respectively.

The analysis of the entire DRG volume showed an evident hypertrophy after 30 days from the crush injury compared to all the three control groups (Figure 3(a)). For the total cell number count, in order to discriminate neurons that needed to be included in the count, the quantitative analysis

was carried out by blinded observers. The physical disector stereological method demonstrated a significant increase of neurons within hypertrophic crushed T1s (CRUSH: 15642 ± 1347) compared to controls (NORMAL LEFT: 11006 ± 1649; NORMAL RIGHT: 10512 ± 995; INTERNAL CTRL: 10285 ± 2650) (Figure 3(b)). A neuronal overexpansion of 42% was estimated for the DRG neuronal population in crushed animals compared to nonoperated animals. Moreover, as shown in Figures 3(c) and 3(d), the overpopulation of neurons was distributed among the two neuronal subpopulations: the light and the dark neurons.

Finally, we examined the changes in the size distribution of all sensory neurons in T1s after 30 days from the crush injury (Figure 4) observing a right shift (i.e., hypertrophy) in the neuron-diameter distribution of animals that underwent crush injury compared to controls.

3.2. Morphological Changes Occurring within DRG after Nerve Crush Lesion. Morphological analysis showed that most of the neurons of the crush group appeared different compared
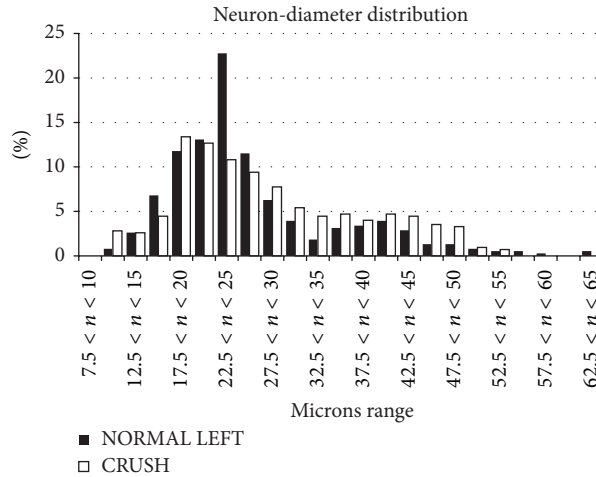
FIGURE 4: Diameter-frequency distribution of pooled T1 DRG neurons belonging to CRUSH and NORMAL LEFT groups. There is a small rightward shift in diameter-frequency distribution in animals that underwent crush injury.
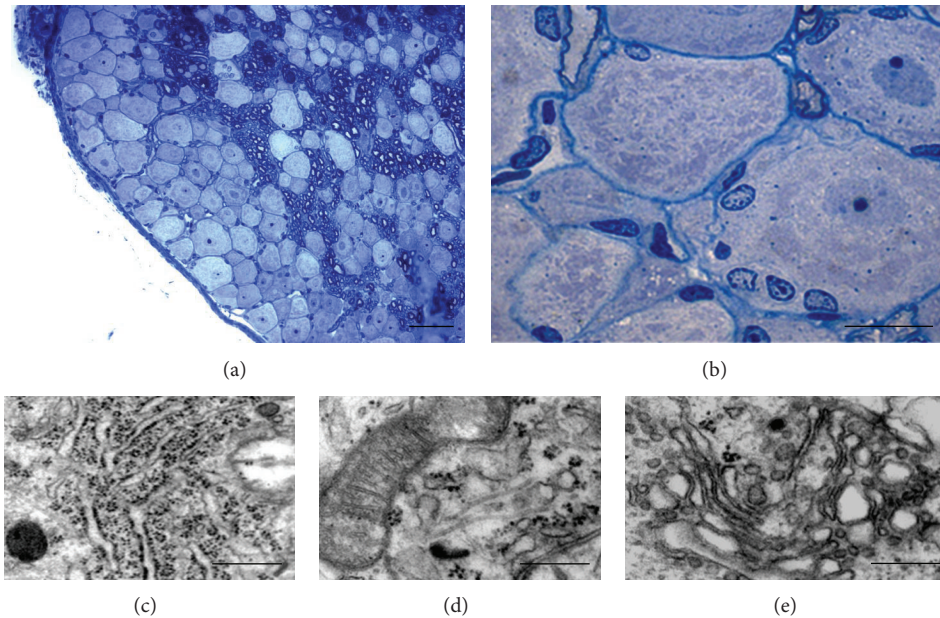

FIGURE 5: Resin-embedded 2.5 μm semithin sections of crushed DRG at low (a) and high (b) magnification stained with toluidine blue. The ultrastructural analysis in electron microscopy shows that sensory neurons are particularly rich in organelles as RER (c), mitochondria (d), and Golgi apparatus (e). Scale bars: a = 50 μm; b = 20 μm; c–e = 0.5 μm.

to controls. The DRG neurons in crushed animals (Figures 5(a) and 5(b)) showed a nonhomogeneous cytoplasm aspect due to an organization of subcellular organelles and neurofilaments, typically seen associated with an increase in metabolic cellular activity. The ultrastructural analysis in electron microscopy showed neurons particularly rich in subcellular organelles such as rough endoplasmic reticulum (RER), mitochondria, Golgi apparatus, and many free ribosomes, with no signs of cell suffering as a consequence of the peripheral nerve lesion (Figures 5(c)–5(e)).

Noteworthy, as a consequence of the crush lesion applied to the peripheral nerves, an increased population of cells with different structural and ultrastructural features appeared within DRG (Figure 6). The shape of neurons changed and many cells, not seen in controls (Figure 6(a)), were seen surrounding, as a clear crown, the neuronal profiles starting from day 1 after lesion (Figure 6(b)). Among the new population of cells, many different morphological features allowed the discrimination of different cell types. Some of these cells were immunopositive for the glial marker anti-S100 (box in Figure 6(b)). The morphological differences were investigated also in electron microscopy. Electron microscopy analysis showed a diffuse electron dense chromatin in the nucleus of some of these cells particularly evident close to the nuclear
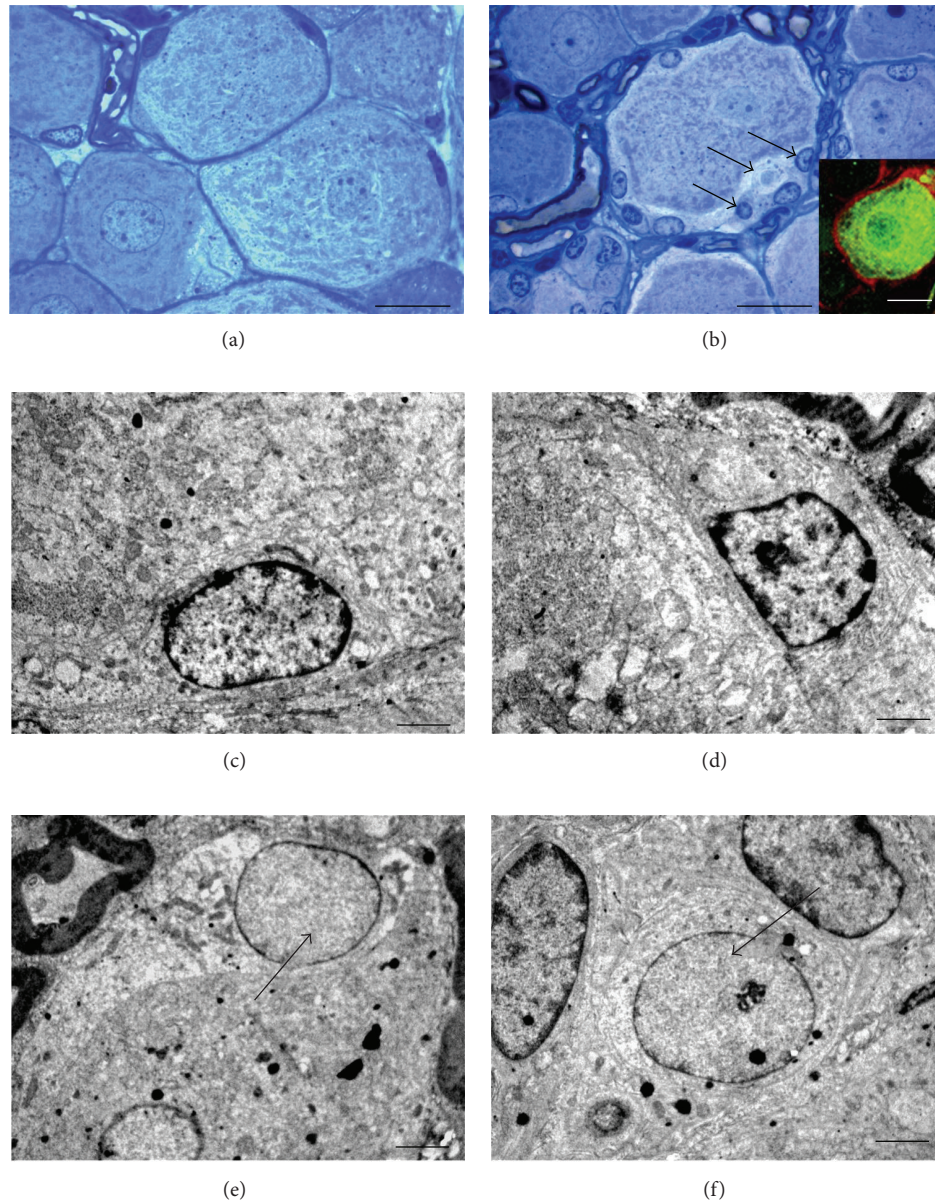
(a)

(b)

(c)

(d)

(e)

(f)

FIGURE 6: High magnification light microscopy pictures of toluidine blue stained semithin sections of controls (a) and DRG harvested 5 days after nerve crush lesion (b) in which many cells are seen surrounding neurons (b, arrows). A double fluorescence staining shows S100-positive cells (red) surrounding a neurofilament 200 KDa-positive (green) neuron (box in b). The ultrastructural analysis in electron microscopy shows at low magnification morphological features of glial cells (c, d) and neuronal-like cells (e, f, arrows). Scale bars: a, b = 20 μm; c–f = 2 μm.

membrane and a dark cytoplasm rich in endoplasmic reticulum (Figures 6(c) and 6(d)). The same morphological characteristics were also seen in the Schwann cells surrounding fibers within the DRG, thus suggesting a glial phenotype.

The other cell profile showed a clear round nucleus with a barely observable chromatin and a nucleolus that was often detectable. The cytoplasm was usually clearer than the other cell type and poorer in cytoplasmic organelles (Figures 6(e) and 6(f)). These clear areas of cytoplasm showed the presence of neurofilaments. These morphological features suggested a less differentiated cell population compared to the one described above.

3.3. In Vivo New Neurons Identification.
The appearance of this new population of cells within DRG after crush lesion led to further investigations in which crush injured animals and control were inoculated *in vivo* with BrdU. For the qualitative evaluation, DRG C5-T1 were harvested and analyzed in immunohistochemistry at different time points after crush lesion (Figures 7 and 8).

After 15 days, many small BrdU-positive cells surrounding neurons were found; a clear BrdU labeling was indeed detected in nuclei belonging to ganglionic neurons (Figure 7(a)). None of the neurons in the control ganglia were found to express BrdU (data not shown). BrdU analysis
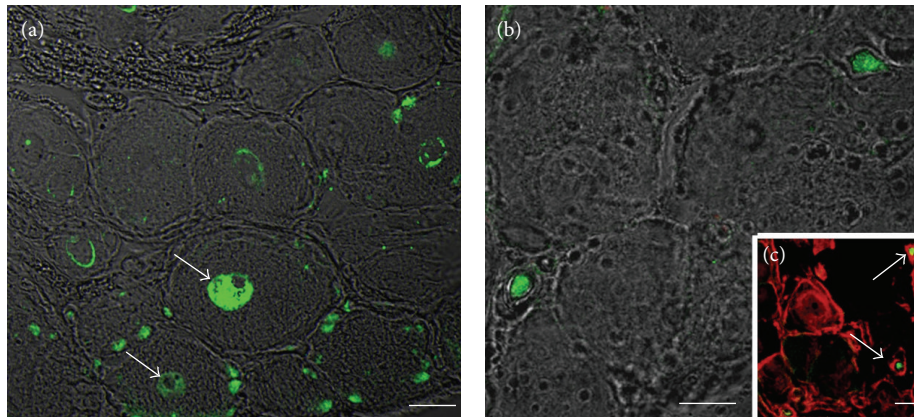
FIGURE 7: Immunofluorescence images of DRG after crush lesion and BrdU treatment for the qualitative evaluation. 15 days after crush lesion, many small cell nuclei surrounding ganglionic neurons as well as some nuclei belonging to sensory neurons are found to be BrdU-positive (a, arrows). Cells localized surrounding the ganglionic neurons are immunolabeled with Ki67, an endogenous marker for proliferation 3 days after crush lesion (b). A double labeling using anti-Ki67 (green) associated with anti-peripherin (red), 7 days after injury, shows a coexpression of the two markers in ganglionic neurons (c). Scale bars: a–c = 20 μm.

was correlated to other markers endogenously expressed in proliferating cells, as, for instance, Ki67, which was found expressed in some cells that would seem to be surrounding large neurons 3 days after crush lesion (Figure 7(b)). As shown in Figure 7(c), after 7 days, we detected a coexpression of Ki67 and peripherin, a neuronal marker specifically expressed by the "small and light" neuronal subpopulation and ubiquitously expressed in young DRG neurons [35]. None of the neurons in the control ganglia were found to express Ki67 (data not shown).

To better characterize the proliferation of the new detected cell population and to define their differentiating pathway, many markers that characterized the earliest steps of the neuronal identity, from stem cell to neuroblast, were applied (Figure 8).

At day 3, small Ki67-positive cells surrounding neuronal profile coexpressed nanog, a marker for pluripotent stem cells (Figure 8(a)). Five days after crush injury, the same cell population surrounding the big neurons, some of which labeled with peripherin, was seen positive for sox-2, a marker for undifferentiated cells (Figure 8(b)). At the same time point, many BrdU-positive cells surrounding the DRG neurons also expressed nestin, a marker specifically expressed in neuronal progenitors (Figure 8(c)). Ten days after crush injury, many of the cells located peripherally to the ganglionic neurons were found to express NeuroD, a marker expressed in cells already committed to the neuronal lineage (Figure 8(d)).

A double labeling for nestin and either GFAP, a marker for immature glia, or S-100, a marker for mature glia, showed that, at day 5, some cells were found coexpressing nestin and GFAP (Figure 8(e)); however, none of the cells expressing nestin were found S-100-positive (Figure 8(f)).

Finally, time course quantification of BrdU-positive nuclei threw light on the progression of DNA synthesis after crush injury. Daily rate of BrdU incorporation in the four time windows (Figure 9) showed that DNA synthesis, for both neuronal and nonneuronal cells, was maximal immediately after crush and decreases progressively along the postlesion time.

4. Discussion

The issue of adult plasticity has always been strongly debated if not denied by the neuroscience community according to Bizzozero's classification of neurons in the perennial cell category [36, 37]. Although the existence of neurogenic areas in the CNS has been commonly accepted [10], only few studies in the past 10 years have begun to come out with evidence of neurogenesis occurring in the peripheral nervous system. Apart from the only site in the PNS where neurogenesis has been undoubtedly documented and accepted, the olfactory neuroepithelium [38–42], the occurrence of adult neurogenesis in other sites of the PNS (dorsal root ganglia and autonomic ganglia) can be only postulated, since experimental data published so far are controversial.

The first evidence for the existence of neurogenesis and, hence, the presence of neural progenitors in DRG was based on counting methods [2, 3, 8, 43]. Although the findings were encouraging, the wide range of results obtained from different groups, due to different methods used to quantify neurons [13], never clarified whether plasticity actually occurs *in vivo* [8, 17]. Recently, new studies added data in support of adult neurogenesis in the PNS, both on nodose ganglion [44] and on DRG [27], after systemic capsaicin treatment. For the first time, here, we present evidence and quantification of a dramatic increase in the neuronal population belonging to the DRG in adult rats as a consequence of peripheral nerve damage and regeneration. Most of the previous studies applied stereology to thick vibratome or paraffin cut sections [23] which do not guarantee that all DRG neurons, especially the smaller ones, are identified. This might explain the disagreement of our results with those published by Degn et al. (1999) [45]. Since the physical disector method is based on
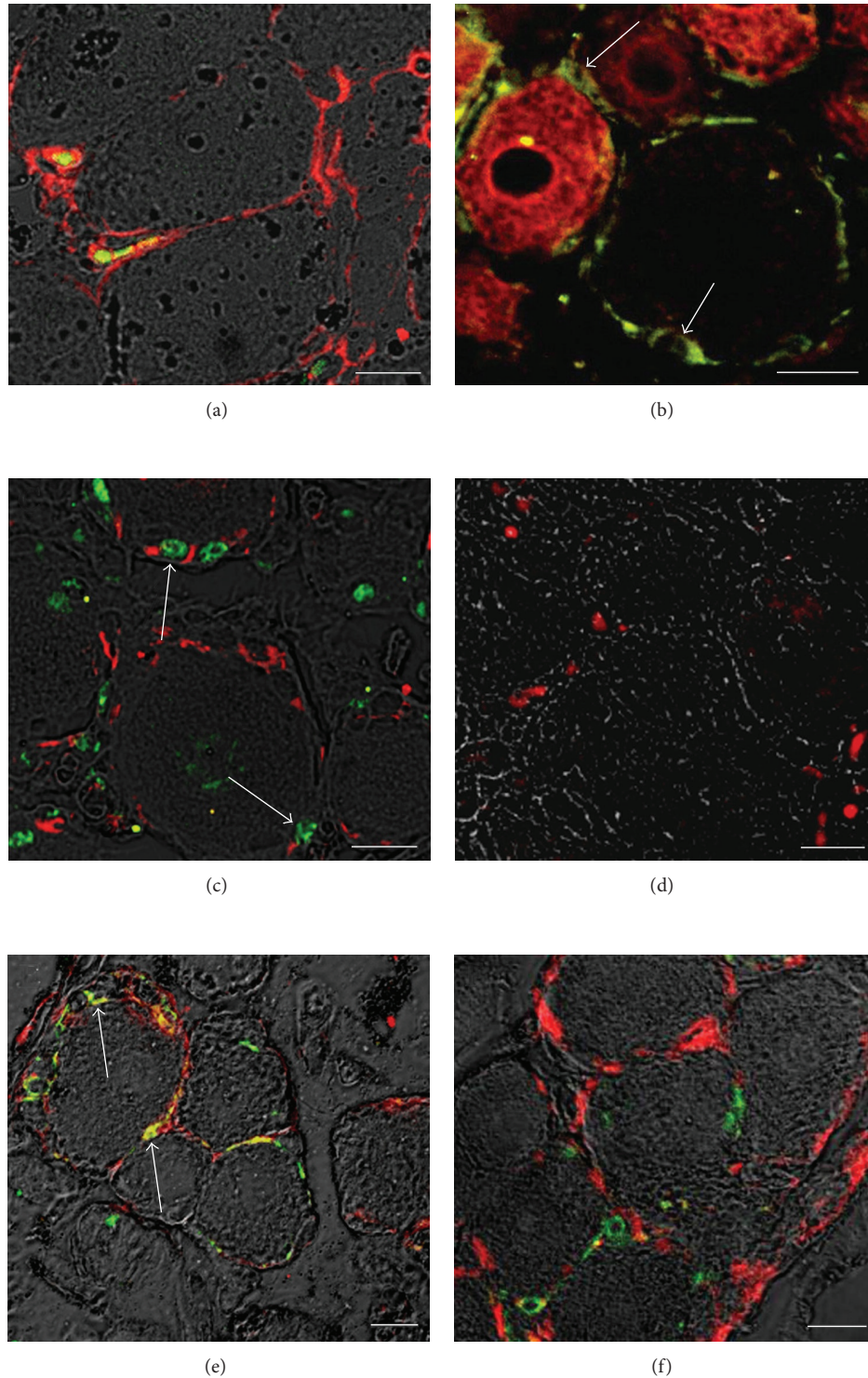
(a)

(b)

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(f)

FIGURE 8: After 3 days from crush lesion, cells coexpressing nanog (red) and Ki67 (green) were found (a). A double immunofluorescence performed at day 5 after injury detects sox-2 positive cells (green) surrounding neurons, some of which are peripherin-positive (red) (b, arrows). At the same time point, the same cells are found coexpressing BrdU (green) and nestin (red) (c, arrows). After 7 days from crush lesion, anti-NeuroD is seen to be expressed in many nuclei surrounding the DRG neurons (d). Further characterization of this cell population was done using markers for both immature and mature glial cells. A double labeling using nestin (green) and the immature glial marker GFAP (red) shows that, at day 5 after injury, the two markers colocalize in some cells (e, arrows). However, no colocalization is observed in double labeling of nestin with S-100 (mature glia, in red) (f). Scale bars: a–f = 20 μm.
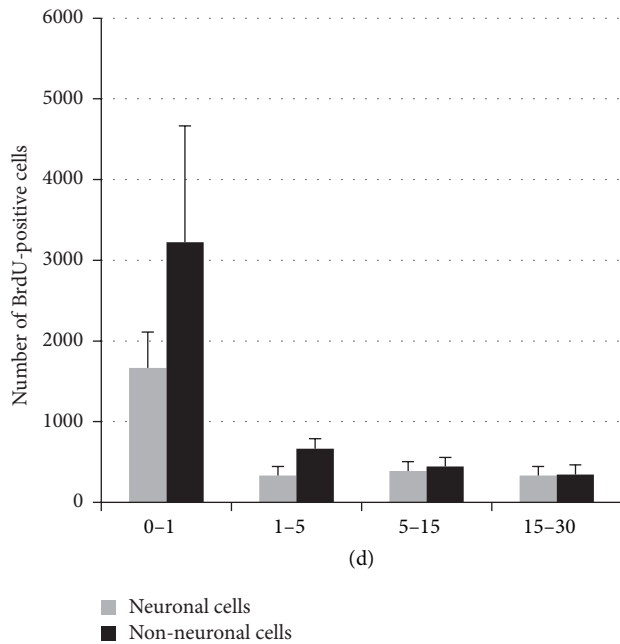
FIGURE 9: Histograms showing stereological analysis of BrdU-positive cells. The number of neuronal and nonneuronal BrdU-positive cells measured in each time window was divided by the days of BrdU treatment in order to predict the daily rate of cells which incorporate BrdU in the different time windows. (See Figure 2 for experimental design of BrdU injection.) All data are expressed as average ± standard error. The data were analyzed using both ANOVA and t-test analyses. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$ versus 0-1 d group.

the adequate recognition of morphological structures, as, for instance, the presence of the nucleus and the volume that the nucleus occupies, good histology and slice thickness are critical for a precise cell count. In this study, semithin sections (2.5 μm) of resin-embedded T1 DRG were used therefore assuring high histological quality, no problems of artifacts, as, for instance, tissue shrinkage [46], and a precise cell count. Moreover, the analysis was done directly under light microscope at a high magnification to better recognize the presence of nuclei, thus discriminating countable from uncountable neurons.

The number of DRG neurons counted in the animals that underwent crush nerve lesion was persistently higher (42%) compared to controls and the data correlate with the volume of the DRG organ, which was also significantly increased. For a more scrupulous validation of our data, we also evaluated the total number of neurons in the contralateral ganglia harvested from the same animals that underwent crush lesion and control, respectively. We thus introduced an internal control to establish the range of variability among the neuronal population belonging to pairs of DRG. Partially in agreement with Ygge and coworkers [47], we found a small and statistically nonsignificant fluctuation in the number of DRG neuronal cells between left and right T1s; therefore, we conclude that a true increase in the number of neurons occurs in the DRG affected by peripheral nerve

crush lesion in comparison to control groups. Moreover, the stereology perfectly correlates with morphological results, which showed *in vivo* the structure and the ultrastructure features of the newborn neurons.

Therefore, the data obtained using different experimental approaches allow us to state that the neuronal population of spinal ganglia after axonotmesis is far from being static; on the contrary, we document here an activation of the DRG cell populations that ends in a neuronal addition. This data are strongly supported by evidence of BrdU-labeled neuronal nuclei and the characterization of the morphological and immunocytochemical steps throughout the differentiating path of these new neuronal cells.

Stereological quantification of BrdU-positive cells showed that, as expected, DNA synthesis peaks at day 1 and progressively decreases after nerve damage for both nonneuronal and neuronal cells. Although it is known that posttraumatic DNA synthesis can increase as a consequence of the damage without necessarily being followed by cell division [48, 49], we believe that part of the BrdU labeling is due to proliferation of new neuronal cells, in accordance with previous literature [50]. Assuming that the BrdU incorporated by neurons at 1 day could reflect DNA repair, we still see an increase in BrdU-positive neurons until 30 days after injury in accordance with the stereological data.

Finally, to further support our hypothesis, we show that Ki67 was found expressed in a subpopulation of neurons. Due to the very restricted window of expression of Ki67, a quantification of Ki67-positive cells would probably lead to a result much less interesting than the qualitative result itself that shows an endogenous marker of proliferation, thus reflecting neurogenesis and not DNA repair, expressed in neuronal nuclei.

Although the possibility that proliferating cells are resident and/or infiltrating macrophages cannot be completely ruled out, the pattern of expression of precocious neuronal and glial markers, as shown by the immunocytochemical characterization, led us to hypothesize that the neuronal progenitors may originate from dedifferentiation of satellite cells. These neuronal progenitors, activated by an exceptional stimulus, for instance, the crush lesion of the peripheral nerves, may proliferate, differentiate into neuroblast cells, and then become new mature neurons. Therefore, we postulate a role for the DRG satellite cells in guaranteeing neuronal cell recruitment as a consequence of damage occurring in the peripheral nervous system.

Conflict of Interests

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

Acknowledgments

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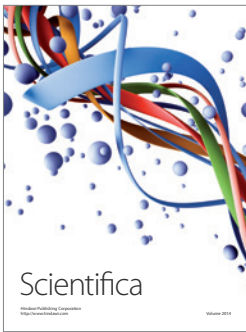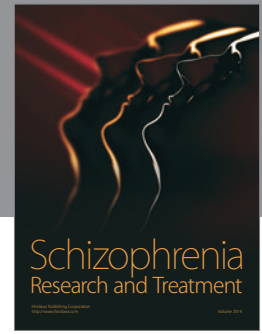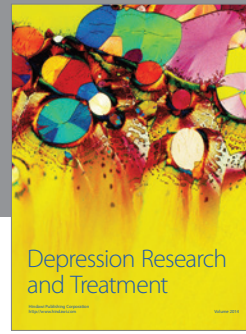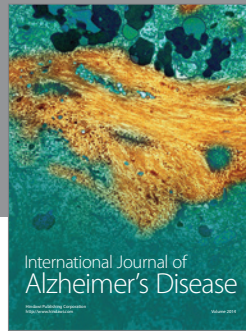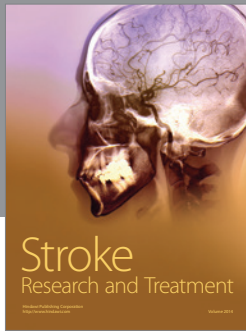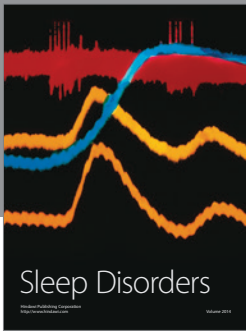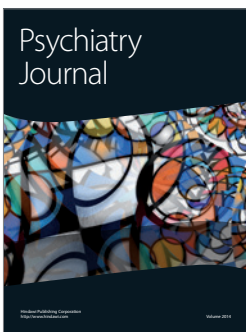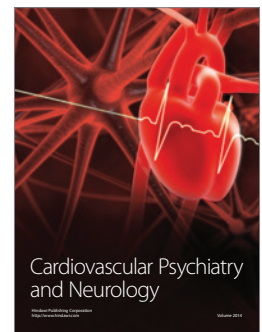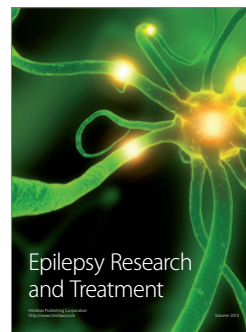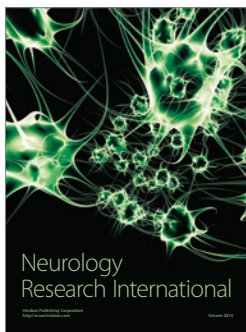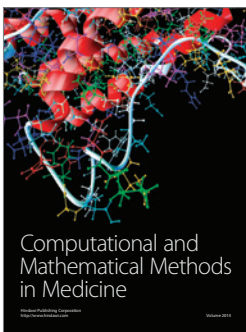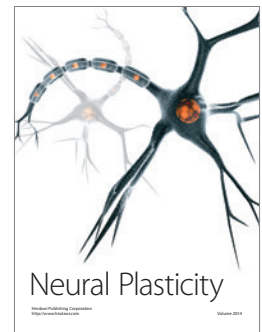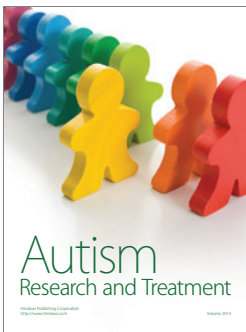
CAN REGENERATED NERVE FIBERS RETURN TO NORMAL SIZE? A LONG-TERM POST-TRAUMATIC STUDY OF THE RAT MEDIAN NERVE CRUSH INJURY MODEL

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Whether post-traumatic regeneration can eventually result in rat peripheral nerve fibers regaining their pretrauma size is still an open question. While it has been shown that, after a sufficient duration in post-traumatic time, the number of regenerated rat peripheral nerve fibers can return to pretrauma numbers and the animal can regain normal prelesion function, no information regarding long-term changes in the size parameters of the regenerated nerve fibers is available. To fill this gap, we have investigated the post-traumatic changes in myelinated axon and nerve fiber diameter, myelin thickness, and g-ratio (the ratio of the inner axonal diameter to the fiber diameter) at three different time points following nerve injury: week-6, week-8, and week-24. A standardized nerve crush injury of the rat median nerve obtained using a nonserrated clamp was used for this study. The results showed that, consistent with previous studies, fiber number returned to normal values at week-24, but both axon and fiber diameter and myelin thickness were still significantly lower at week-24 than prelesion, and the g-ratio, which remained unchanged during the regeneration process, was significantly reduced at week-24 in comparison to the prelesion value. On the basis of these results, the hypothesis that regenerated rat peripheral nerve fibers are able to return spontaneously to their normal pretrauma state, provided there is a sufficiently long recovery time postaxonotmesis, is not supported. © 2012 Wiley Periodicals, Inc. Microsurgery 32:383–387, 2012.

Although peripheral nerves retain a high regeneration potential throughout adulthood,[1,2] it is not known whether regenerated nerve fibers can return to normal levels after a sufficient period of post-trauma recovery time. Mackinnon et al.[3] showed that rat nerve fiber numbers return to normal given a long enough period of time following neurorrhaphy, but as far as we are aware, the possibility that the size parameters of regenerated nerve fibers can return to normal levels has never been investigated. To fill this gap, we have carried out a long-term stereological study on a standardized rat median nerve crush (axonotmesis) model. Since in a previous study[4] we showed that, in these experimental conditions, the morphological size parameters were still significantly different from controls at week-6 postoperatively, we extended the observation up to 8-week and 24-week time-points. We test the hypothesis that the main size parameters of rat peripheral nerve fibers (axon and nerve fiber diameter, myelin thickness and g-ratio) are able to return to prelesion values provided that sufficient recovery time postaxonotmesis is allowed.

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MATERIALS AND METHODS

Animals and Surgery

Ten 8-week-old female Wistar rats (Charles River Laboratories, Milano, Italy), each weighing ~250 g, were used for this study. The animals were housed in large cages in a temperature and humidity controlled room with 12-h light/12-h dark cycles. They were fed with standard food and water ad libitum. Adequate measures were taken to minimize pain and discomfort, taking into account human endpoints for animal suffering and distress. All procedures performed were in accordance with the Local Ethical Committee and the European Communities Council Directive of 24 November 1986 (86/609/EEC).

All surgical procedures were carried out under deep anaesthesia using Tiletamine and Zolazepam (Zoletil) i.m. (3 mg/kg). Each animal was subjected to a median nerve crush injury of the left forelimb by approaching it from the axillary region toward the elbow. The crush lesion was applied at the middle of the arm, using a nonserrated clamp, by compressing the nerve for 30 seconds with a final pressure of 17.02 MPa.[4] Animal well-being was assessed by careful surveillance of passive and active movement, auto-mutilation, skin ulcers, and joint contracture, especially during the early postoperative period. Animals were sacrificed by lethal i.m injection of tiletamine and zoletil at week-8 ($n = 5$) and week-24 ($n = 5$) after crush injury. Data were compared to values obtained from a previous study,[4] where 8-weeks-old female rats were subjected to the same nerve-crush injury under the same experimental conditions, and six crushed left median nerves and six uninjured control left median nerves were harvested at week-6 postoperatively.

Resin Embedding and Design-Based Quantitative Morphology

At the time of euthanasia, a 10-mm-long median nerve segment was harvested immediately distal to the injury site in the axonotmesis groups, and at the corresponding level in uninjured controls. A 4/0 stitch was used to mark the proximal stump of the nerve segment. Nerve samples were fixed by immediate immersion for 6–8 h in 2.5% purified glutaraldehyde/0.5% saccarose in 0.1M Sorensen phosphate buffer. Specimens were then washed in a solution containing 1.5% saccarose in 0.1M Sorensen phosphate buffer, postfixed in 1% osmium tetroxide,[5] dehydrated and embedded in resin. From each resin block, 2.5 μm thick series of semithin transverse sections were cut, starting from the distal stump of each median nerve specimen, using an Ultracut UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany). Sections were stained using Toluidine blue for high-resolution light microscopy examination and design-based stereology.

Design-based stereological analysis was carried out using one randomly selected toluidine blue-stained semithin section. A DM4000B microscope equipped with a DFC320 digital camera and an IM50 image manager system (Leica Microsystems, Wetzlar, Germany) was used for stereology. On the randomly selected section, the total cross-sectional area of the nerve was measured, and 12–16 sampling fields were selected using a systematic random sampling protocol.[6–8] In each sampling field, a two-dimensional disector procedure, which is based on sampling the "tops" of fibers, was adopted to avoid the "edge effect." The total number of myelinated fibers (N), the mean diameter of each fiber (D) and axon (d), as well as mean myelin thickness [$(D - d)/2$] and g-ratio (d/D), were estimated.

Statistical Analysis

Statistical analysis was performed by one-way analysis of variance and tested using the software "Statistica per discipline bio-mediche" (McGraw-Hill, Milano, Italia). Values are expressed as mean ± standard deviation (SD). The level of significance was set at $P \leq 0.05$ (*), $P \leq 0.01$ (**), and $P \leq 0.001$ (***).

RESULTS

For stereological analysis, the results obtained from median nerves harvested at 8 and 24 weeks postlesion were compared to data obtained in a previous study,[4] which included nerves harvested 6 weeks postoperatively, and uninjured control median nerves, all harvested from 8-week-old female rats.

Figure 1 shows high-resolution light photomicrographs of a normal rat median nerve and nerves harvested at 6 weeks, 8 weeks, and 24 weeks postinjury. After 6 weeks from the injury, the regenerated myelinated axons were smaller with a thinner myelin sheath, compared to normal nerves (Fig. 1A), and microfasciculation typical of regenerated nerve fibers was detected; few degeneration signs could still be observed among the regenerated fibers (Fig. 1B). After 8 weeks, the regenerated myelinated fibers were still smaller compared to control nerves, and almost no more degenerating fibers were detectable (Fig. 1C). The time course of nerve fiber maturation was clearly detectable, progressing to a qualitative morphological appearance that at week-24 (Fig. 1D) was similar to controls.

Time course morphological changes were confirmed by the results of the stereological assessment of myelinated nerve fibers (Fig. 2). The total number of myelinated fibers was significantly ($P \leq 0.05$) higher at 6 and 8 weeks postinjury, whereas at week-24, it did not significantly ($P \geq 0.05$) differ from controls. Regarding axon and fiber diameter, both parameters decreased significantly ($P \leq 0.001$) 6 and 8 weeks postlesion. Twenty-four weeks after the injury, the data showed that the fiber size had increased compared to the previous time points analyzed, but it was still significantly ($P \leq 0.01$) smaller than in the control nerves. The myelin thickness results demonstrated that this parameter follows the same trend as axon and fiber diameter. Interestingly, while the mean g-ratio was not significantly ($P \geq 0.05$) different from controls at both week-6 and week-8, statistically significant changes ($P \leq 0.05$) were detectable at week-24, when the g-ratio was lower than prelesion values.

DISCUSSION

Because functional recovery is achieved very quickly after a rat axonotmesis lesion, experimental studies on nerve regeneration after a crush lesion are always based on short-term post-traumatic endpoints: usually 6–8 weeks, and never longer than 12 weeks, as far as we could determine from the relevant literature. However, in spite of the very fast functional recovery, morphological differences persisted even at week-12 post-trauma,[8] leaving open the question of whether post-traumatic regeneration may eventually lead rat peripheral nerve fibers back to normal.

To answer this question, we prolonged the regeneration time in the rat median nerve crush model up to 24 weeks. This is a model in which full functional recovery can already be observed much earlier, namely at week-4 post-trauma,[4] and it allows the following optimal regeneration conditions: (1) pure axonotmesis lesion with no need for surgical neurorrhaphy; (2) no mismatch between regenerating axons and the respective original distal
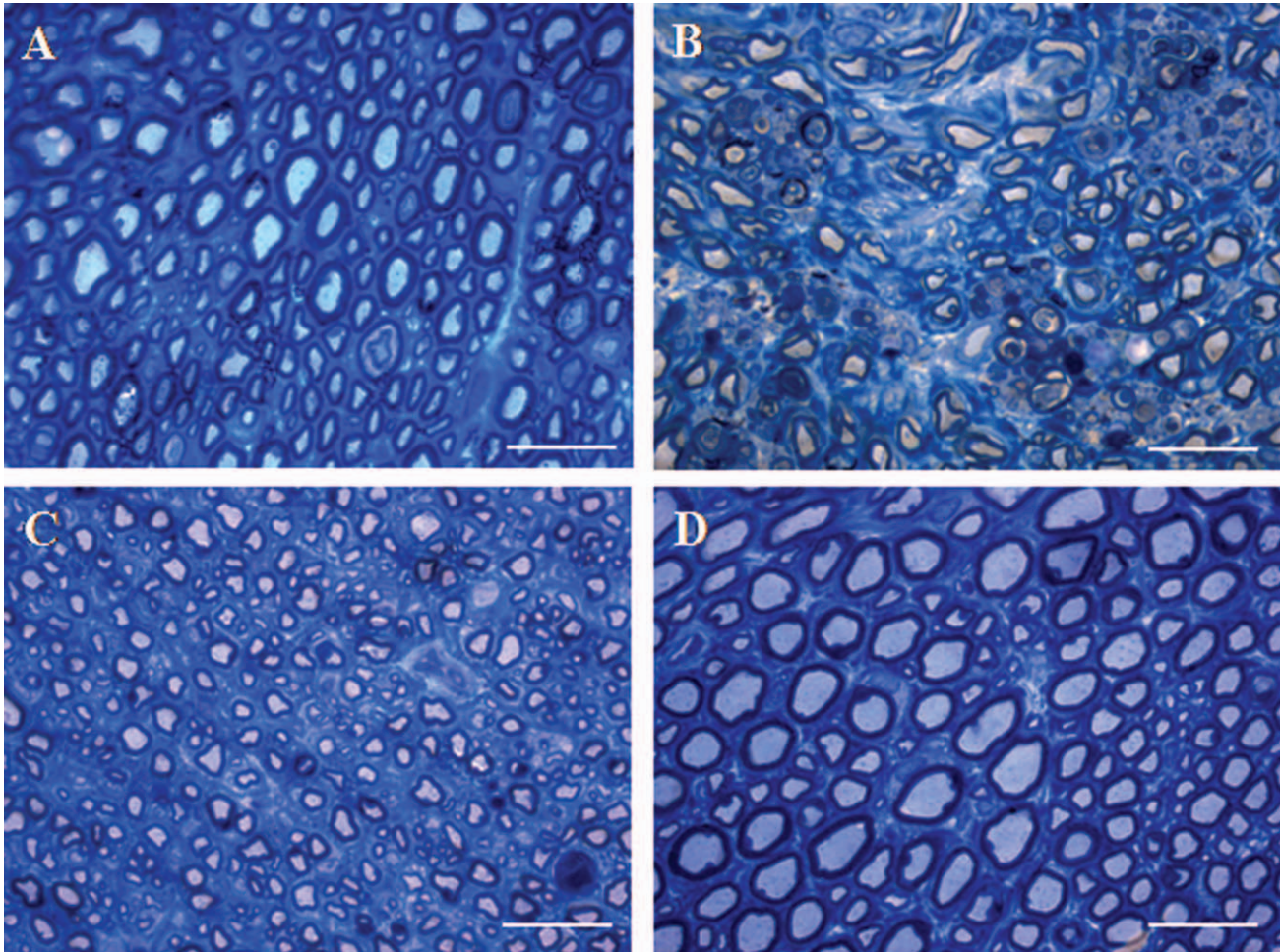
Figure 1. *Photomicrograph of semithin section of normal (**A**), 6 weeks (**B**), 8 weeks (**C**), and 24 weeks (**D**) postinjury median nerves. Magnification bars = 20 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]*

bands of Büngner; and (3) complete functional recovery reached very quickly (already at week-4 post-trauma)

Contrary to our expectations, even extending the observation period for so long post-trauma under optimal regeneration conditions, we did not detect complete recovery or the return to normal preinjury size of the regenerated axons. In fact, we showed that only total fiber number returned to normal. These findings on fiber number are in agreement with the observations of Mackinnon et al.[3] in the neurotmesis model, while all the other morphological predictors of regeneration that we investigated (axon diameter, fiber diameter, myelin thickness, and g-ratio) had still not returned to normal values at the end of our study. Data about changes in the quotient axon diameter/fiber diameter (g-ratio), a measure of the myelination process,[9] deserve particular mention. The observation that the g-ratio remains unchanged in early regeneration stages supports the view that axons and myelin sheaths grow synchronously. On the other hand, at the latest post-traumatic stages, when regeneration is stabilized, the g-ratio is significantly reduced.

This observation indicates that myelin sheath enlargement has overcome axon enlargement and supports the view that the limited post-traumatic recovery of peripheral nerves must mainly be attributable to the neuronal component (axons) rather than the glial one.

Our results are important from both the biological and the clinical perspectives. From a basic science point of view, they provide a better understanding of the nerve regeneration potential by adding an original piece of information, namely that adult peripheral nerve fibers do not retain the capacity for complete regeneration, i.e., a return to prelesion size, even if optimal regeneration conditions and a long-term observation window are guaranteed. It could be argued that, if we had further extended post-traumatic follow-up, thus leaving still more time for the regeneration process, nerve fibers could have returned to normal. However, in view of the long-term stabilization of functional recovery detectable 24 weeks postoperatively in the adopted experimental model, we can reasonably rule out the possibility that the partial recovery of nerve fiber size
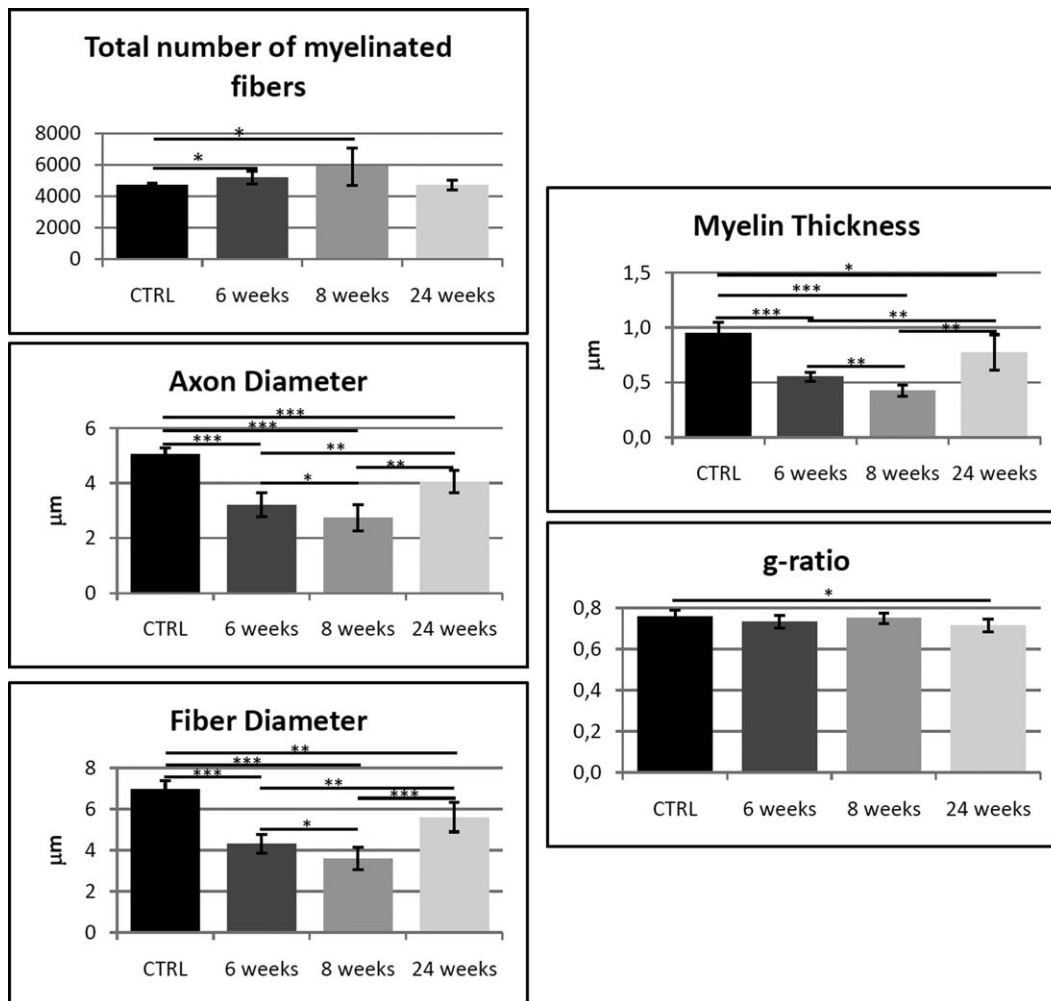
Figure 2. Results of the stereological assessment of peripheral nerve fibres in control rat median nerves (CTRL) and in regenerated rat median nerves, at week 6, 8, and 24 postaxonotmesis. All data reported are presented as means ± standard deviation. * P ≤ 0.05, **P ≤ 0.01, and *** P ≤ 0.001.

reflects too little time still being allowed for regeneration to occur.

In the translational clinical perspective, our results tell us that, without treatment, complete post-traumatic recovery of nerve morphology in a patient should not reasonably be expected, even if surgery is perfectly adequate. This evidence is in line with the observation that although axon regeneration in peripheral nerves is very active,[1,2] electrophysiological parameters do not return to normal levels after nerve lesion and repair,[10,11] and that clinical recovery in patients is usually partial and often frankly unsatisfactory.[12] It can thus be concluded that the persistence of abnormal electrophysiological and/or clinical outcomes after nerve reconstruction does not mean that surgery was inadequate. Our results further support the need to seek effective treatment for improving post-traumatic peripheral nerve fiber regeneration.[13,14]

Our results could influence the selection of regeneration predictors in animal nerve repair studies, supporting the view that fiber size data should always be considered to reduce the risk of a discrepancy between the preclinical stage and the following translation into human subjects.[15,16] Finally, it is interesting to note that the total number of myelinated nerve fibers at week 6 and week 8 postinjury is higher than the number of fibers in the control group. This observation, which could be interpreted as the result of multiple axonal sprouts during the early regeneration phases followed by a late pruning of the branches that did not reach their proper target, supports the need for sufficiently long post-traumatic observation time-points for monitoring the progression of the morphological predictors of recovery.

In conclusion, the hypothesis that a regenerated nerve is able to return spontaneously to a normal (prelesion)

condition, provided that a long enough recovery period is allowed, can be supported on the basis of fiber number and functional recovery data, but not on the basis of axon diameter, fiber diameter, myelin thickness, and *g*-ratio data. Therefore, in the absence of effective treatment, complete post-traumatic recovery, as far as rat peripheral nerve fiber morphology is concerned, is not an achievable goal.

ACKNOWLEDGMENTS

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Decision Letter (AR-TP-PNR-17-0354.R1)

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Date Sent: 05-Feb-2018

Evaluation of Vascular endothelial growth factor (VEGF) and its family member

expression after peripheral nerve regeneration and denervation.

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Key words: VEGF, peripheral nerve regeneration, crush injury, end-to-end repair, median nerve transaction, neuropilin, VEGF receptors.

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Abstract:

Vascular endothelial growth factor (VEGF) represents one of the main factor involved not only in angiogenesis and vasculogenesis but also in neuritogenesis, VEGF plays its function acting via different receptors: VEGF receptor1 (VEGFR-1), VEGF receptor2 (VEGFR-2), VEGF receptor3 (VEGFR-3) and co-receptors Neuropilin-1 (NRP1) and Neuropilin-2 (NRP2).

This study reports on the first *in vivo* analysis of the expression of VEGF and VEGF family molecules in peripheral nerve degeneration and regeneration: for this purpose, different model of nerve lesion in rat were adopted, the median nerve crush injury and the median nerve transaction followed or not by end-to end microsurgical repair.

Results obtained by Real Time polymerase chain reaction showed that VEGF and VEGF family molecules are differentially expressed under regenerating and degenerating condition, furthermore, in order to study the modulation and involvement of these factors in two different regenerative models, crush injury and end-to-end repair, protein expression analysis was evaluated. In addition, immunohistochemical analysis allowed to state a glial localization of VEGF and VEGFR-2 after peripheral nerve crush injury.

Finally *in vitro* assay on Primary Schwann cells culture show that VEGF165 stimulation increases Schwann cells migration, a major process in the promotion of neurite outgrowth.

Introduction

Vascular endothelial growth factor (VEGF) is an endothelial cell mitogen that increases blood vessels permeability and promotes angiogenesis. For this reason VEGF is mainly expressed by endothelial cells but also by activated macrophages and during cancer pathogenesis (Berse, Brown et al. 1992).

VEGF belongs to a family of homodimeric glycoproteins structurally related to the platelet-derived growth factors (PDGF); in mammals, VEGF family consists of five members, VEGF-A, B, C, D and placenta growth factor (PLGF).

Through alternative RNA splicing, different VEGF-A isoforms are generated: VEGF121,145, 165, 183, 189 and 206. VEGF121 is a freely diffusible molecule that lacks the basic amino acid residues and does not bind the extracellular matrix (ECM), VEGF165 contains some basic residues and it is partly diffusible while VEGF189 contains even more basic residues, showing a spatially restricted localization to the matrix around the VEGF-producing cell (Cohen, Gitay-Goren et al. 1995; Ruiz de Almodovar, Lambrechts et al. 2009; Grunewald, Prota et al. 2010).

VEGF binds three tyrosine kinase receptor: VEGF receptor1 (VEGFR-1), VEGF receptor2 (VEGFR-2), VEGF receptor3 (VEGFR-3) and also receptors of the neuropilin family, Neuropilin-1 (NRP1) and Neuropilin-2 (NRP2) considered co-receptor for VEGF (Neufeld, Cohen et al. 1999; Ferrara, Gerber et al. 2003; Takahashi and Shibuya 2005; Ruiz de Almodovar, Lambrechts et al. 2009; Rosenstein, Krum et al. 2010; Carmeliet and Ruiz de Almodovar 2013).

Since the vascular and the nervous system show similar anatomical features and despite the main role of VEGF as a pro-angiogenetic factor, an increasing number of studies focus the attention on VEGF activity on different neural cell types and recent evidence shows a role for VEGF as a neurotrophic and neuroprotective factor for neurons and glial cells. In fact, VEGF stimulates the proliferation of neuronal precursors, increasing the BrdU labeling, in *in vitro* and *in vivo* models of neurogenesis (Jin, Zhu et al. 2002).

Yet, it supports the survival of mesencephalic neurons in explants cultures (Silverman, Krum et al. 1999).

Furthermore it has been reported that VEGF administration enhances axonal outgrowth from dorsal root ganglia adult mice explants promoting the survival of neurons and satellite glial cells (Sondell, Lundborg et al. 1999; Sondell, Lundborg et al. 1999; Hobson, Green et al. 2000; Brockington, Lewis et al. 2004; Pereira Lopes, Lisboa et al. 2011).

Evidence has also been provided that VEGF administration increases the functional recovery after peripheral nerve injury since it was shown that after end-to-end neurorraphy (ETE) and end-to-side neurorraphy (ETS) of transected muscolocutaneous rats nerves, plasmid VEGF transfection in the distal stumps resulted in a better axon regeneration in terms of fibers density, axons diameter and myelin sheath thickness of regenerated axons (Haninec, Kaiser et al. 2012).

The aim of our work is to investigate the expression of VEGF, VEGF receptors and VEGF co-receptors after nerve injury and regeneration. In the present study we carried out a biomolecular and immunohistochemical analysis on rat median nerve experimental models. In particular three different nerve injuries models will be used (crush injury, end-to-end repair and degenerating nerve) in order to analyze mRNA expression and protein expression and localization.

Materials and methods

Animals

All experiments were carried out on adult female Wistar rats (Charles River Laboratories, Milan, Italy) weighing approximately 190-220g.

Surgery

All procedures were performed in accordance with the Ethics Committee and the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Animal well-being assessment was carried out using careful animal surveillance to check for passive and active movement, auto-mutilation, skin ulcers, and joint contracture, especially during early post-operative times.

Adequate measures were taken to minimize pain and discomfort taking into account human endpoints for animal suffering and distress.

For mRNA expression a total of 48 rats were used and three different surgeries were applied: crush injury, end to-end repair and degenerating nerve.

For the crush injury group (n=3), the median nerve of both forelimb were crushed with a non-serrated clamp at mid-humerus level according to the procedure described (Ronchi, Raimondo et al. 2010). For the end-to-end repair (n=3), median nerve was bilaterally transected at the same position described for the crush injury, proximal and distal stumps were immediately sutured using 9/0 epineurial sutures. In the degenerating group, median nerve was bilaterally transected and unrepaired.

For the animals belonging to the control groups (CTRL) nerve was exposed and the skin was closed immediately after (n=3).

For protein analysis, only rats resulting from crush injury and end-to-end repair were used, whereas uninjured rats were used as control (see Total Protein Extraction and Western Blot Analysis section).

For morphological evaluation (see Immunofluorescence section) a total of 6 animals were used, n=3 for crush injury and n=3 for control groups.

All surgical procedures were carried out under deep anaesthesia obtained with Tiletamine + Zolazepam (Zoletil) i.m. (3 mg/kg).

For mRNA and protein analysys, median nerves were harvested 1d, 3d, 7d, 15d and 30d after the different surgeries, for morphological analysis rats were sacrified 7 days after crush injury.

RNA isolation, cDNA preparation.

Samples were frozen at -80° C and processed for RNA extraction. Total RNA was extract with the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. 0.5 µg total RNA were subjected to a reverse transcriptase (RT) reaction in 25 µL reaction volume containing: 1X RT-Buffer (Fermentas); 0.1 µg/µL bovine serum albumin (BSA, Sigma); 0.05% Triton X-100; 1 mM dNTPs; 7.5 µM random exanucleotide primers (Fermentas); 1 U/µl RIBOlock (Fermentas) and 200 U RevertAid™ M-MuLV reverse transcriptase (Fermentas). The reaction was performed for 10 min at 25°C, 90 min at 42°C, 10 min at 90°C. Control reaction "RT⁻" (without the enzyme RT) and "H_2O", without RNA, was also carried out. RNA concentration was quantified using the Nanodrop® ND-1000 spectrophotometer (Celbio, Milano, Italy). cDNA was stocked at -20°C until used as a template for the real-time RT-PCR analysis.

Quantitative real-time polymerase chain reaction (qRT-PCR).

cDNA was diluted 5 times before analyses and 1 µl was analyzed in a total volume of 10 µl using 1X SYBR Green Supermix (Applied Biosystems) and 300 nM forward and reverse primers. Quantitative real-time PCR analysis was performed using chemistry with the StepOne Sequence Detection System (Applied Biosystems), dissociation curves were routinely performed to check the presence of a single peak in agreement to the required amplicon. Reactions were performed in technical and biological triplicate.

Data were analyzed by ΔΔCt relative quantification method normalizing to the housekeeping gene ANKRD (Ankyrin repeat domain 27) and RICTOR (RPTOR Indipendent Companion of MTOR) (Gambarotta, Ronchi et al. 2014).

Primers were designed using ANNHYB software (http://www.bioinformatics.org/annhyb/) and synthesized by Invitrogen (Life Technologies Europe BV, Monza, Italy).

Primers sequences are reported in Table1. Relative expression levels were calculated by (ΔΔCt) method. The normalized relative quantity (NRQ) was determined using the formula: $NRQ = 2^{-(\Delta\Delta Ct)}$. Results were expressed as mean + S.D.

Total Protein Extraction and Western Blot Analysis.

Total proteins were extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Protein pellets were resuspended in boiling Laemli buffer (2.5% SDS, 0.125 M Tris-HCl pH6.8) and followed by 3 min at 100°C. Protein concentration was determined using the Bicinchoninic Acid assay kit (BCA, Sigma), and equal amounts of proteins (40 µg, denaturated at 100°C in 240 mM 2-mercaptoethanol and 18% glycerol) were loaded onto each lane, separated by SDS-PAGE, transferred to iBlot® Transfer Stacks nitrocellulose membrane using the iBlot® Dry blotting Transfer Device (Invitrogen). Primary antibody used were: mouse monoclonal anti-VEGF-A (1:500, ab171828 Abcam), rabbit polyclonal anti-VEGFR-1 (1:1000, #2893 Cell-Signalling Technology), rabbit polyclonal anti-VEGFR-2 (1:1000, #2479 Cell-Signalling Technology), rabbit polyclonal anti-NRP1 (1:1000, #3725 Cell-Signalling Technology), rabbit polyclonal anti-NRP2 (1:1000, #3366 Cell-Signalling Technology), mouse monoclonal anti-β-actin (1:4000 #A5316 Sigma). Secondary used antibodies were horse-radish peroxidase-linked anti-rabbit (#NA934), and anti-mouse (#NA931) both used 1:20000 (GE Healthcare Life Science, Europe).

Immunoflurescence and confocal laser microscopy.

Nerve samples were fixed by immediate immersion in 4% paraformaldehyde for 2 h, washed in a solution of 0.2% glycine in 0.1 M phosphate buffer (pH 7.2), and embedded in OCT. Specimens were cut 10µm thick by a Leica CM3050S cryostat (Leica Microsystems, Wetzlar, Germany). Sections were

permeabilized, blocked [0.1% triton X-100, 10% normal goat serum (NGS)/0.1% NaN$_3$, 1h] and processed for an immunohistochemical protocol. See Table 2 for the list of primary antibodies used. Samples were incubated overnight in primary antibody or in case of double-immunofluorescence experiments, in a mixture of primary antibody and visualized using a solution containing the appropriate secondary antibodies: goat anti-mouse IgG Alexa-Fluor-488-conjugated (1:200, Molecular Probes, Eugene, Oregon), CY3-conjugated anti-rabbit IgG (dilution 1:400, Dako, Milano, Italy). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma) diluted 1:1000 in PBS.

The samples were finally mounted with a Dako fluorescent mounting medium (DAKO) and analyzed by a LSM 510 confocal laser microscopy system (Zeiss, Jena, Germany).

Primary Schwann Cells (SC) Cultures.

Primary Schwann cells (SC) cultures were obtained from fresh adult rat sciatic nerves.

The sciatic nerves were collected and immediately kept in cold DMEM plus glutamax (Invitrogen, UK) containing 100 U/mL penicillin and 100 g/mL streptomycin. Nerves were dissected and the epineurium was stripped off. Nerve fragments were plated in a Petri dish in SC growth medium (DMEM plus glutamax containing 100U/mL penicillin, 100 g/mL streptomycin, 14 M forskolin, and 100 ng/mLNRG11, R&D Systems, UK) and incubated for 2 weeks at 37°C with fresh medium added approximately every 72h.

Nerve fragments were incubated with 0.125% (w/v) collagenase type IV and 117 u/mg dispase for 24 hours and mechanically dissociated using a sterile Pasteur glass pipette in order to obtain a cell suspension. Cell suspension was filtered using a 70µm cell strainer (Falcon; BD Biosciences Discovery Labware, Bedford, MA) and centrifuged at 100×g for 5min to obtain the cell pellet. Finally, the cell pellet was resuspended in SC growth medium, seeded

into a Petri dish pre-coated with poly-D-lysine (Sigma, St Louis, MO, USA), and incubated in the same conditions. SC were purified by an antibody complement method to roll out the remaining fibroblasts (Tohill, Mann et al. 2004; Kaewkhaw, Scutt et al. 2012; Pascal, Giovannelli et al. 2014)

Proliferation Assay.

Primary Schwann cells were seeded at a concentration of 1000 cells/cm^2 on poly-D-lysine coverslips in complete DMEM containing 2%FBS as the control condition, or medium added with VEGF (50 ng/ml) (human VEGF-A165, R&D). After 1, 3, and 7 days, cells were fixed, stained, photographed and counted.

Culture medium was removed, substrates with attached cells were rinsed with PBS with Ca^{2+} and Mg^{2+} and fixed by the addition of 4% paraformaldehyde solution. After 20 min, samples were rinsed with PBS with Ca^{2+} and Mg^{2+} and then stained with 4, 6-diamidino-2-phenylindole (DAPI) diluted 1:1000 in PBS. Cells were photographed using an optical video-confocal microscope (Nikon Eclipse 80i) and the supporting software Image ProPlus (Media Cybernetics USA). For each sample, four images were taken with at low magnification. The number of proliferated cells were counted by using Image J Software. All conditions were performed in triplicate. The counts obtained from assays were analyzed, averaged, and expressed as the number of proliferated cell number/mm^2 ± standard deviation.

Three-dimensional migration: transwell assay.

The Transwell migration assay was used to measure three-dimensional movement. Primary Schwann Cells (10^5) resuspended in 200 μl of DMEM containing 2% FBS were seeded in the upper chamber of a Transwell (cell culture insert, no. 353097, BD Biosciences) on a porous transparent polyethylene terephthalate membrane (8.0-μm pore size, 1×10^5 pores/cm^2). The lower chamber (a 24-well plate well) was filled with DMEM containing 2% FBS with or without VEGF165 (50 ng/ml, R&D). The 24-well plates containing cell culture inserts were incubated at 33°C in a 5% CO$_2$ atmosphere

saturated with H_2O. After 18 h of incubation, cells attached to the upper side of the membrane were mechanically removed using a cotton-tipped applicator. Cells that migrated to the lower side of the membrane were rinsed with PBS, fixed with 2% glutaraldehyde in PBS for 15 min at room temperature, washed five times with water, stained with 0.1% crystal violet and 20% methanol for 20 min at room temperature, washed five times with water, air-dried, and photographed using a Nikon ECLIPSE TS100 inverted microscope equipped with a Nikon Digital Sight DSL1 camera; the images were analysed with ImageJ software. The experiments were repeated three times independently (biological triplicate). Each set included three control condition transwells and three transwells stimulated with VEGF165. Cell counts were expressed as percentage of migrated cells/total number of cells ± standard deviation. All conditions were performed three times independently (technical triplicate). Five images were analyzed for each transwell using the ImageJ software. Cell counts were expressed as percentage of migrated cells/total number of cells ± standard deviation.

Statistical analysis.

For *in vivo* experiments, statistical analysis was performed using one-way analysis of variance (ANOVA) and *post hoc* LSD. Two-way analysis of variance (ANOVA) and *post-hoc* Bonferroni was performed using the Prism Software Package (GraphPad, La Jolla, CA, USA) and SPSS. For *in vitro* proliferation experiments, statistical analysis was performed using one-way analysis of variance (ANOVA) and *post-hoc* Bonferroni using the Prism Software. For *in vitro* migration assay, statistical analysis was performed using Two-Sample t-Test. The level of significance was set at $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***). Values are expressed as mean ± standard deviation (SD).

Results

The expression of VEGF, VEGFR-1, VEGFR-2, VEGFR-3, NRP1 and NRP2 mRNAs were assessed in crush injury, end-to-end repair and degenerating conditions.

The relative quantification (RQ) was determined using the control sample that better represents the mean of the control samples ΔCt; therefore, the relative (and not absolute) gene expression shown in the graphs cannot be compared among different genes.

VEGF mRNA expression is differentially regulated in regenerating and degenerating condition.

VEGF mRNA expression significantly increases in crush group at day 1 ($p \leq$ 0.01 (**)), day 3 ($p \leq 0.05$ (*)) and day 7 ($p \leq 0.01$ (**)) after injury. Whereas VEGF mRNA expression is significantly down regulated in degenerating condition at day 3($p \leq 0.05$ (*)), day 7 ($p \leq 0.01$ (**)) and day 30 ($p \leq 0.05$ (*)). In end-to-end repair group no significant differences are observed (Figure1).

VEGFR-1 is strongly upregulated after crush injury.

In crush injury group the mRNA level of VEGFR-1 is significantly upregulated peaking at day 1 after injury ($p \leq 0.001$ (***)). The expression significantly decreases starting from day 3 ($p \leq 0.05$ (*)) and a significant downregulation is also detectable at day 30. In the end-to-end repair and degenerating condition, mRNA expression of VEGFR-1 does not show significant variations (Figure 2).

VEGFR-2 mRNA expression is strongly down regulated in regenerating and degenerating condition.

Results on mRNA expression of VEGFR-2 in crush injury group showed that expression is significantly decreased 30 days after crush injury ($p \leq 0.05$ (*)).

In the end-to-end repair group a significant down regulation of VEGFR-2 is detectable at day 1 ($p \leq 0.05$ (*)) and 30 ($p \leq 0.05$ (*)) after surgery. VEGFR-2 mRNA expression goes back to control condition level from day 3 up to day 7. Under degenerating condition mRNA VEGFR-2 expression levels did not change compared to control condition (Figure 3).

VEGFR-3 mRNA expression decreases in regenerating and degenerating condition.

mRNA expression of VEGFR-3 significantly decreases starting from day 1 ($p \leq 0.01$ (**)) in crush injury group. Decrement is also detectable at day 3 ($p \leq 0.01$ (**)), day 7 ($p \leq 0.01$ (**)) day 15 ($p \leq 0.001$ (***)) lasting until day 30 ($p \leq 0.001$ (***)) after injury. In degenerating nerve and end-to-end repair groups no regulation occurs (Figure 4).

VEGF co-receptors NRP1 and NRP2 are significantly upregulated in degenerating condition.

The mRNA expression of VEGF co-receptors NRP1 and NRP2 are both upregulated under degenerating condition. NRP1 is significantly upregulated ($p \leq 0.05$ (*)) at day 3 and 15 ($p \leq 0.05$ (*)) and a higher upregulation is detectable at day 30 ($p \leq 0.01$ (**)) in degenerating nerve. The mRNA expression of NRP2 in degenerating condition is strongly upregulated 30 days after injury ($p \leq 0.05$ (*)) (Figure 5).

Comparison between surgery: two-way ANOVA analysis

Through two-way ANOVA analysis several relevant mRNA expression changes have been detected. The most relevant data to us is the VEGFR-3 expression since it is involved in inflammatory response. Its mRNA expression in degenerating nerve is higher compared to degenerating control condition, end-to-end repair and crush injury p-value ranging from $p \leq 0.05$ (*) to $p \leq 0.01$ (***). (See Figure 6 for details).

VEGF, VEGFR-2, NRP1 and NRP2 proteins are differentially expressed after crush injury.

In order to focus the attention on nerve regeneration process, western blot analysis was performed only on crush injury and end-to-end repair group to confirm protein expression of some of genes analyzed by qRT-PCR.

Western blot analysis showed that VEGF expression is detectable in both crush and end-to-end nerves. The 43-kDa band is strongly detectable in control condition, 1 day, 3 days and day 7 after crush injury while a weak band is detectable at day 15 and 30 after injury. In end-to-end nerves a high VEGF protein expression is observed in all time points post repair.

Interestingly VEGFR-2 protein appears in end-to-end nerves starting from day 3 until 30 days. In crushed nerves, high protein expression is found between day 1 and 15 after injury. No band appears in healthy nerves.

NRP1 antibody detects endogenous levels of total NRP1 protein through a 120-kDa band. This antibody also recognizes an 80-kDa protein of unknown origin. Protein expression of NRP1 results with a very small band detectable in healthy control nerves although protein expression level increases in crushed nerves starting from day 1 until day 7 and then decreases until day 30. However the expression is higher compared to control condition. In end-to-end-nerves NRP1 protein expression is higher between day 3 and day 30.

Finally, NRP2 antibody detects endogenous levels of total NRP2 protein recognizing a 130-kDa band. The protein expression level of NRP2 is barely detectable especially in crushed nerves in which a weak band appears starting from day 7 up to day 30 after injury. In end-to-end repair group protein expression of NRP2 appears between day 1 and day 15 with a stronger band detectable at day 3 after surgery (Figure 7).

VEGF, VEGFR-2 have a glial localization.

Since Real Time PCR and western blot analysis do not allow to obtain a morphological evaluation, different immunohistochemical reactions were carried out using VEGF, VEGFR- 2, NRP1 and NRP2 as markers. In order to study the localization of the different markers, double immunostaining with

glial marker (S-100B) and neuronal marker (β-tubulin) were performed on crushed nerves. The employment of the crush injury, which interrupts nerve fibers without severing the connective tissue of the nerve trunk represents a suitable model for the study of the regenerative process providing an optimal regeneration pathway. Furthermore the crush lesion was applied using a standardized and reproducible method, in terms of force, pressure and duration of the compression represented by the use of the not-serrate clump (Ronchi, Nicolino et al. 2009). For this reason, transversal sections of 10 μm of thickness were used to perform immunofluorescence staining only on crushed nerves harvested 7days after injury.

According to Western blot analysis an immunoreactivity for VEGF was found around axons of healthy control nerves (Figure 8A).

Interesting, the same VEGF immunoreactivity was found in nerves 7 days after crush injury, suggesting a glial expression of this marker (Figure 8A-B).

Immunofluorescence analysis was performed with other markers, VEGFR-2, NRP1, NRP2. As shown in Figure 8C, double immunostaining for VEGFR-2 and β-tubulin that represent a typical neuronal marker showed absence of co-localization of these markers suggesting a glial expression of VEGFR-2.

Finally, a double labeling for NRP1/β-tubulin and NRP2/β-tubulin was performed in order to identify co-receptors localization. A co-localization of NRP1 and β-tubulin was detected at nerve axons level (Figure 8D); absence of co-localization was found in case of double labeling NRP1/S-100b (Figure 8E). Co-localization of NRP2 and β-tubulin suggest the same expression pattern found for NRP1 (Figure 8F) with an axonal expression of NPR2.

Administration of VEGF165 do not increase Primary Schwann cells proliferation.

Proliferation assay was performed on Primary Schwann cells (SC) cultures seeded in complete DMEM containing 2%FBS as control condition, or in complete DMEM containing 2%FBS added with VEGF165 (50 ng/ml) (human VEGF-A165, R&D). The number of proliferating cells was then counted quantifying the number of fluorescence labeled nuclei at different time points

1, 2 and 3 DIV after seeding. Schwann cells seeded with complete DMEM containing 2%FBS added with VEGF165 showed a proliferation rate comparable to control conditions, no significant differences have been observed between the two different conditions(Figure 9). Therefore, primary Schwann cell stimulation with VEGF165 did not affect cell proliferation.

Three-dimensional migration assay on Primary Schwann cells culture showed a higher migration rate after VEGF165 stimulation.

To determine whether VEGF165 stimulation on Primary Schwann cell cultures increases the migration of these cells a three-dimensional migration assay was performed.

Primary Schwann cells (10^5) were seeded in the upper chamber of a transwell. In the lower chamber DMEM containing 2% FBS was supplemented with 50 ng/ml of VEGF165 as treatment (see three-dimensional migration: transwell assay section for details).

After 18 h of incubation Primary Schwann cells added with 50 ng/ml of VEGF165 showed a significant higher migration rate ($p \leq 0.05$ (*)) compared to Primary Schwann cells cultured in DMEM-2% FBS (Figure 10).

Discussion

Peripheral nerve injury represents a very complex process that involves different morphological and molecular changes occurring to both proximal and distal stumps (Geuna, Raimondo et al. 2009; Allodi, Udina et al. 2012; Muratori, Ronchi et al. 2012).

After injury, axons distal to regeneration site are interrupted, myelin sheath is degradated and Wallerian degeneration occurs leading to a series of phenotypic changes that promote axonal regeneration. It is well accepted that during the regenerative process various molecular factors are involved in order to form a favorable microenvironment for axonal outgrowth (Navarro, Vivo et al. 2007).

For this reason, various molecules have been investigated in experimental models of neural repair in order to study promising strategies to improve very

important aspects of the regenerative process such as axonal regrowth and target reinnervation (Raimondo, Fornaro et al. 2011; Kang, Kim et al. 2014; Chang, Quan et al. 2016).

Vascular and nervous systems share common molecular pathways during development and regeneration, furthermore the anatomical parallelism between vessel and nerve patterning is well documented (Ruiz de Almodovar, Lambrechts et al. 2009).

Anatomically, both systems are composed of afferent and efferent networks, arteries and veins, motor and sensory nerves and share similar patterning, with vessels running in parallel alongside nerve fibers as a mutual guidance alignment.

Furthermore evidences show that axon guidance and vessel navigation are regulated by similar classes of molecules (Slits, Semaphorins, Netrins,and Ephrins) (Carmeliet and Tessier-Lavigne 2005; Carmeliet and Ruiz de Almodovar 2013).

Even if it was demonstrated that VEGF plays a role during the development of the Central Nervous System (CNS), little is known about its presence and role in Peripheral Nervous System (PNS) (Rosenstein, Krum et al. 2010). This study focuses the attention on VEGF and its receptors and co-receptors expression in three different surgical models used to study peripheral nerve regeneration: the crush injury and the end-to-end repair represent regenerating conditions, while complete nerve transaction reflects a condition in which no signs of axonal regrowth are found (Ronchi, Haastert-Talini et al. 2016).

mRNA expression level for VEGFR-3 after crush injury showed a very low expression in all time points examined compared to control condition. Concerning end-to-end and degenerating nerve, no mRNA expression difference is detectable in all time points. This is probably due, as well reported, to restricted expression of the VEGFR-3 for lymphatic epithelium (Kaipainen, Korhonen et al. 1995; Jussila and Alitalo 2002; Le Bras,

Chatzopoulou et al. 2005). However if we consider the two-way ANOVA comparison, we observed higher VEGFR-3 expression from day 1 until day 30 in degenerating condition compared to crush and end-to-end repair. Crush and end-to-end represents regenerative models, so the immune response may be slow down from day 1 up to 30. In degenerating condition there is no regeneration thus the inflammatory condition may persists over time. This can explain why the VEGFR-3 expression is maintained at high level.

NRP1 and NRP2 are a single pass transmembrane glycoproteins, originally identified as semaphorin receptors mediating axon growth cone collapse. Although, many study reported that NRP1 is involved in neuronal migration, dendritic guidance and repair of the adult nervous system (He, Wang et al. 2002).

NRP2, can bind VEGF165 thus, in addition to neuronal guidance, plays a role in angiogenesis and cardiovascular functions (Favier, Alam et al. 2006).

Our study showed that mRNA expression levels for NRP1 and 2 display a significant increase in degenerating condition. Since NRP1 and NRP2 are involved in several regenerative mechanisms as described above we suppose that in our degenerative model regeneration will not occur, explaining why their expression is manteined.

mRNA and protein expression levels were evaluated by Real Time and western blot analysis respectively. NRP1 and NRP2 display similar mRNA and protein expression levels in both regenerative models (crush injury and end-to-end repair). Finally, double labeling for β-tubulin/NRP1 and β-tubulin/NRP2 performed only on crush injury nerve, the surgical technique that provides clearest regeneration process, shows a co-localization for β-tubulin/NRP1 and β-tubulin/ NRP2 allowing to state an axonal localization of these co-receptors.

VEGF mRNA expression is significantly upregulated during the early phases after peripheral nerve crush injury whereas a strong down-regulation occurs in degenerating nerve suggesting a possible role during the regenerative process.

Furthermore, mRNA expression levels of VEGFR-2, the most implicated in migration and survival of neural and glial cell types of both CNS and PNS (Sondell, Lundborg et al. 1999; Sondell, Lundborg et al. 1999; Jin, Mao et al.

2000; Jin, Zhu et al. 2002; Ogunshola, Antic et al. 2002), is highly expressed over time in crush injury; a significant decrease is detectable at 30 days after injury allowing to suppose that the involvement of VEGFR-2 is restricted to the early phases of the regenerative process.

In order to better characterize the expression of VEGF and VEGFR-2 under regenerating condition, protein expression levels were investigated after crush injury and end-to-end repair. Data obtained from western blot analysis showed a strong VEGF protein expression in control condition and following time points (1 day, 3 days and 7 days after crush injury). VEGF protein expression is also observed in end-to-end repair suggesting a similar expression pattern in the early phase of the regeneration process. Although further *in vitro* experiments need to be done in order to better characterize the molecular pathway involved in these mechanisms.

Concerning VEGFR-2 protein results show that is expressed starting from day 3 until day 30 in end-to-repair. To better characterize protein expression and localization, morphological analysis on crushed nerves were performed showing an intense immunoreactivity for VEGF around axons suggesting a glial expression of this marker.

Furthermore, a double labelling for VEGFR-2 and β-tubulin show absence of co-localization between these two markers suggesting a glial expression also for VEGFR-2.

Interestingly, data are supported by *in vitro* analysis on Primary Schwann cells cultures that significantly increases their migration after VEG165 stimulation compared to control condition suggesting a positive effect of VEGF on glial cells migration which represents a very important process during the peripheral nerve regeneration.

Conclusion

Findings of the present study showed a modulation for VEGF and its family members including VEGFR-1, VEGFR-2, VEGFR-3, NRP1 and NRP2 under degenerating and regenerating conditions. Furthermore, morphological analysis allowed to understand the localization of the VEGF and the VEGFR-2 to Schwann cells after crush injury, used in this study as a suitable model for the study of the regenerative process (Ronchi, Nicolino et al. 2009; Ronchi, Raimondo et al. 2010).

Immunohistochemical results on VEGFR-2 showed also in this case a glial localization of this marker suggesting a potential autocrine VEGF/ VEGFR-2 pathway on Schwann cells.

Morphological evaluation performed by immunofluorescence allowed to identify the glial localization for both factors. Furthermore *in vitro* experiments on primary Schwann cells culture let evaluate the effect of VEGF on the migratory property of the Schwann cells suggesting that VEGF could influence the migration of Schwann cells that represents an important step during the regeneration process.

Acknowledgement

Conflicts of Interest

The authors declare no conflict of interest.

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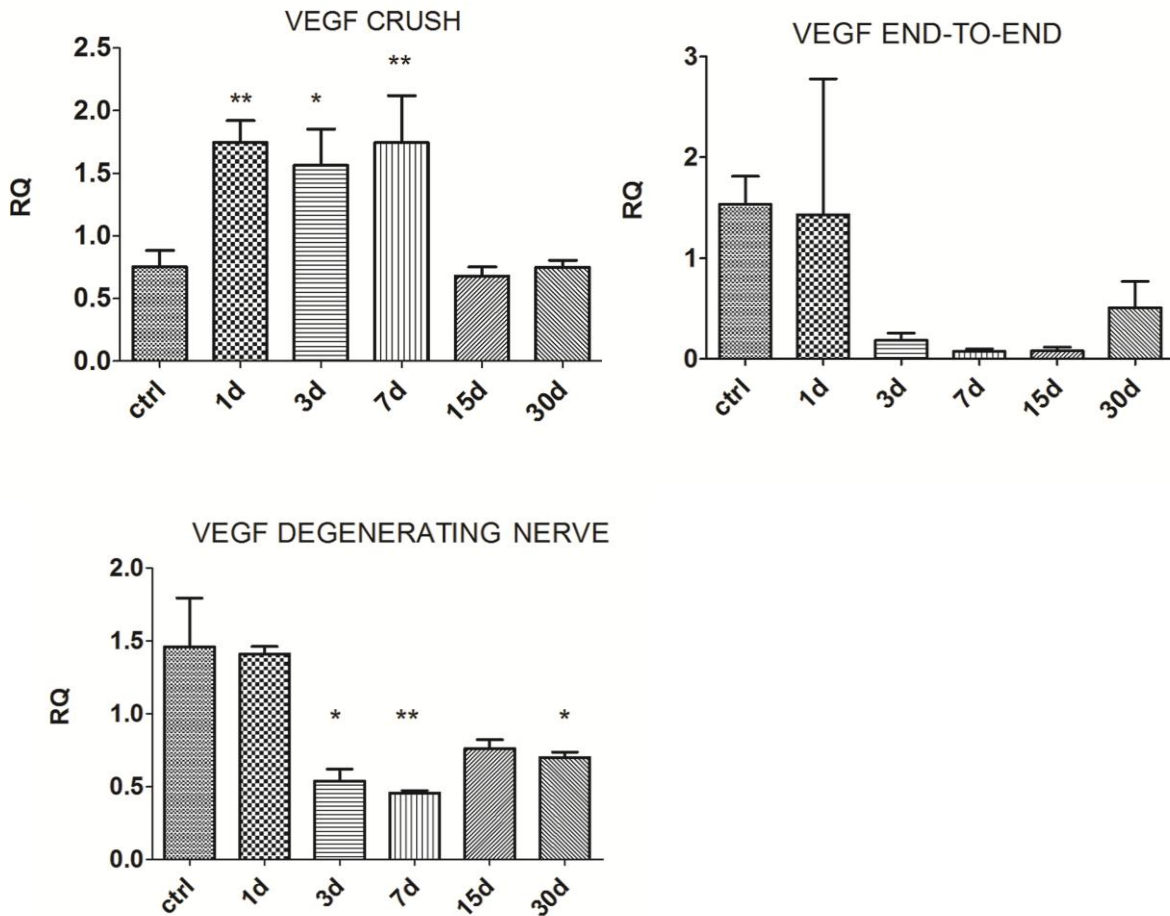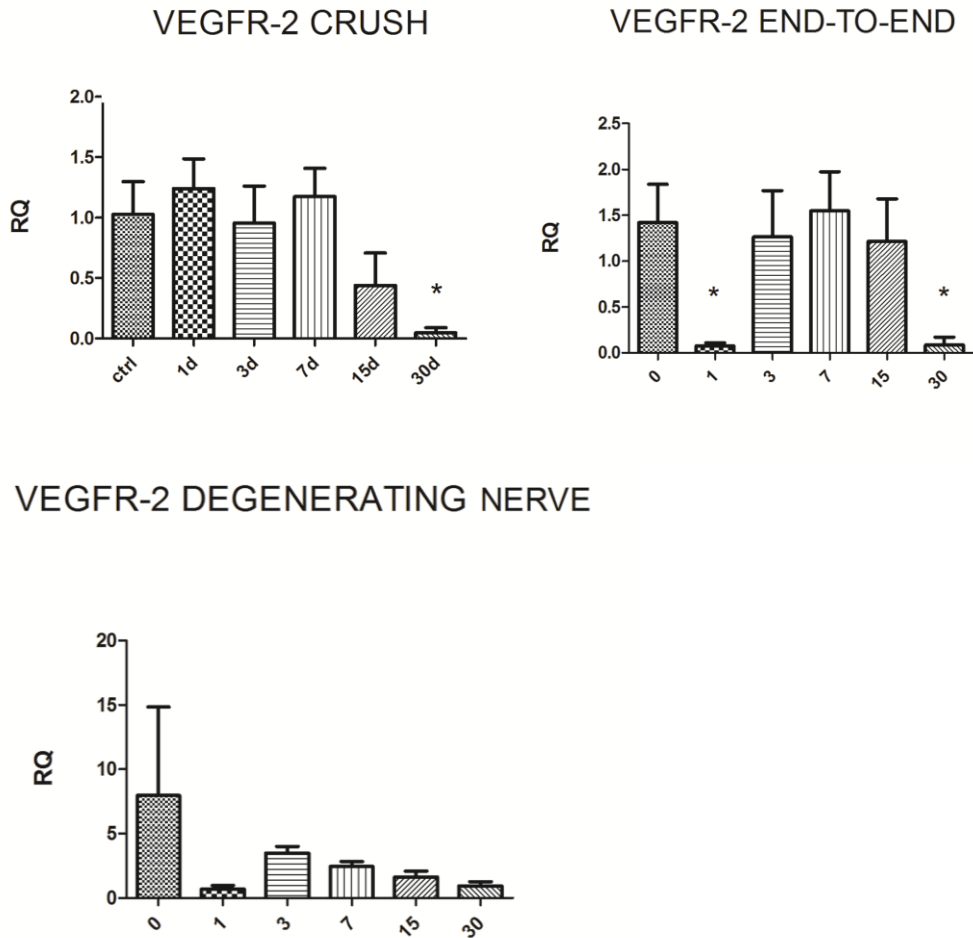
Figure 1: VEGF mRNA expression significantly increases in crush group starting from day 1 ($p \leq 0.01$ (**)), day 3 ($p \leq 0.05$ (*)) and day 7 ($p \leq 0.01$ (**)) after injury. A down-regulation is detectable in degenerating condition at day 3($p \leq 0.05$ (*)), day 7 ($p \leq 0.01$ (**)) and day 30 ($p \leq 0.05$ (*)). In end-to-end repair group a weak increase in VEGF mRNA expression level is detectable at day 30. N=3 in each time point condition.
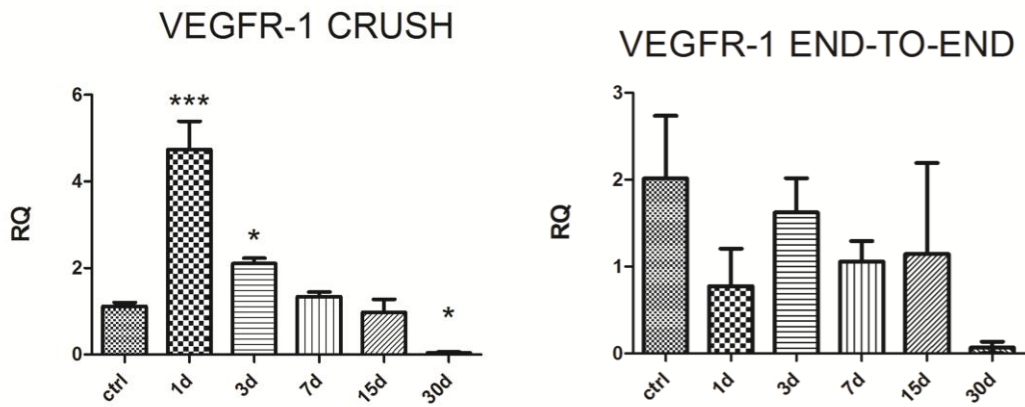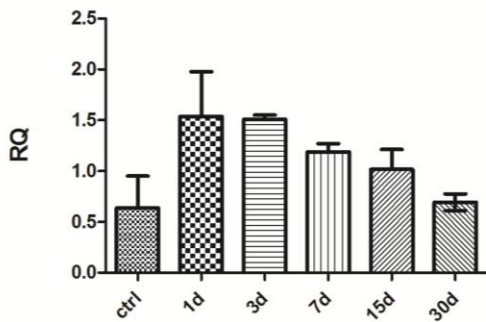
Figure 2: mRNA level of VEGFR1 is significantly upregulated in crush injury group the peaking at day 1 after injury ($p \leq 0.001$ (***)). The expression significantly decreases starting from day 3 ($p \leq 0.05$ (*)) and a significant downregulation is also detectable at day 30. In the end-to-end repair group mRNA expression of VEGFR-1 decrease at day 1, at day 3 expression level increase while a strong reduction is detectable at day 30. VEGFR-1 mRNA expression level increase at day 1 and 3 decreasing gradually in degenerating nerve from day 7. N=3 in each time point condition.

Figure 3: mRNA expression of VEGFR-2 significantly decreased 30 days after crush injury ($p \leq 0.05$ (*)). In the End-to-end repair group a significant down regulation of VEGFR-2 is detectable at day 1 after surgery. mRNA expression of VEGFR-2 remains low from day 1 after injury until day 30 with a very weak increase at day 30 in degenerating nerve. N=3 in each time point condition.
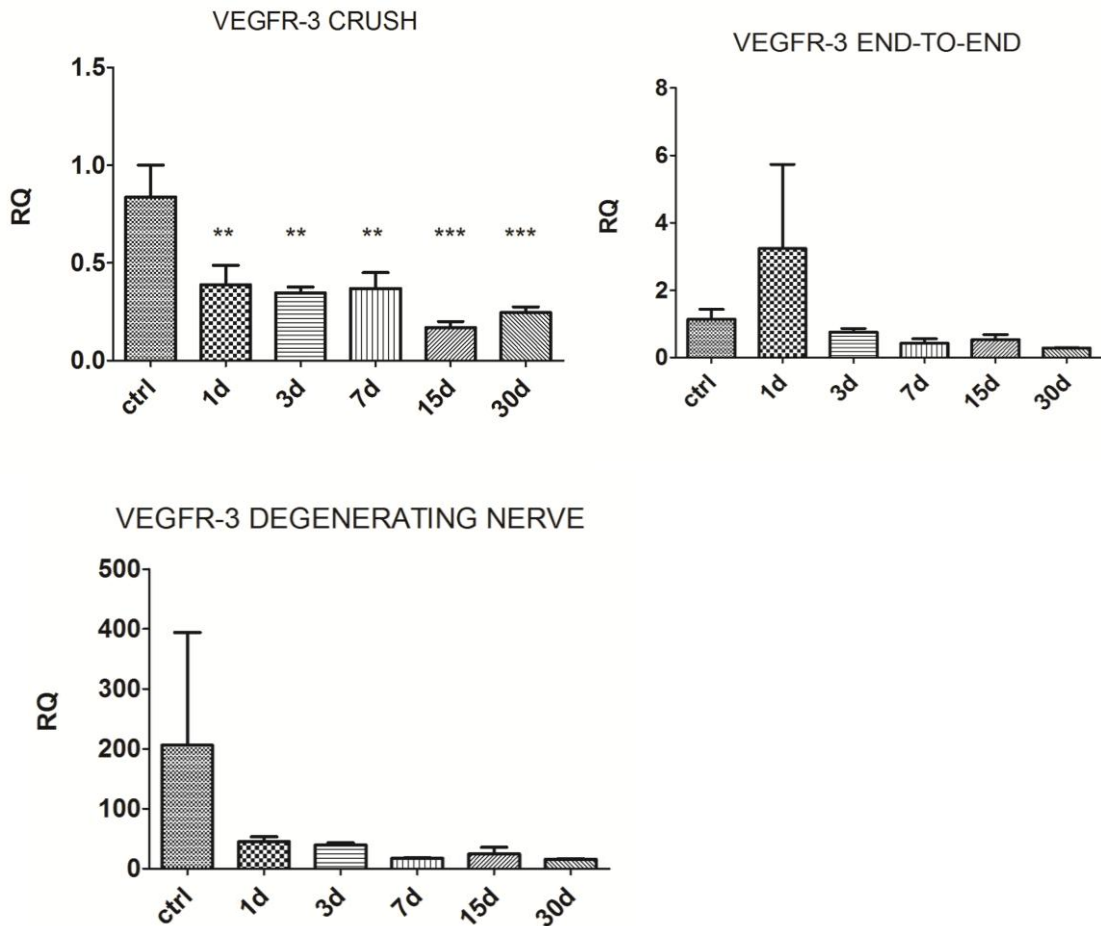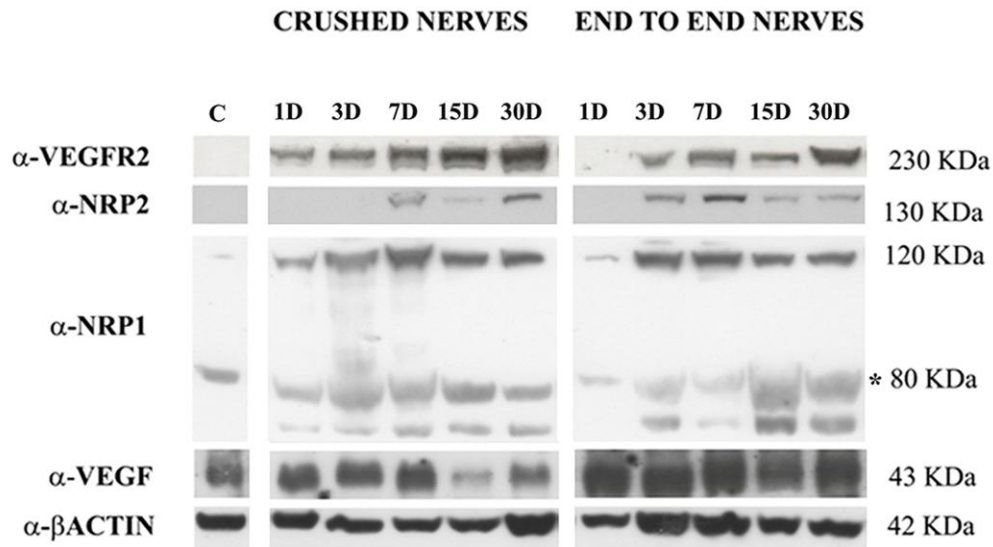
Figure 4: mRNA expression of VEGFR-3 significantly decreases starting from day 1($p \leq 0.01$ (**)) until day 30 ($p \leq 0.001$ (***)) in crush injury group. In degenerating condition a strong down regulation occurs starting from day 1 until day 30. In end-to-end repair group mRNA expression of VEGFR-3 increases at day 1 and decreases from day 3 to day 30 after surgery. N=3 in each time point condition.
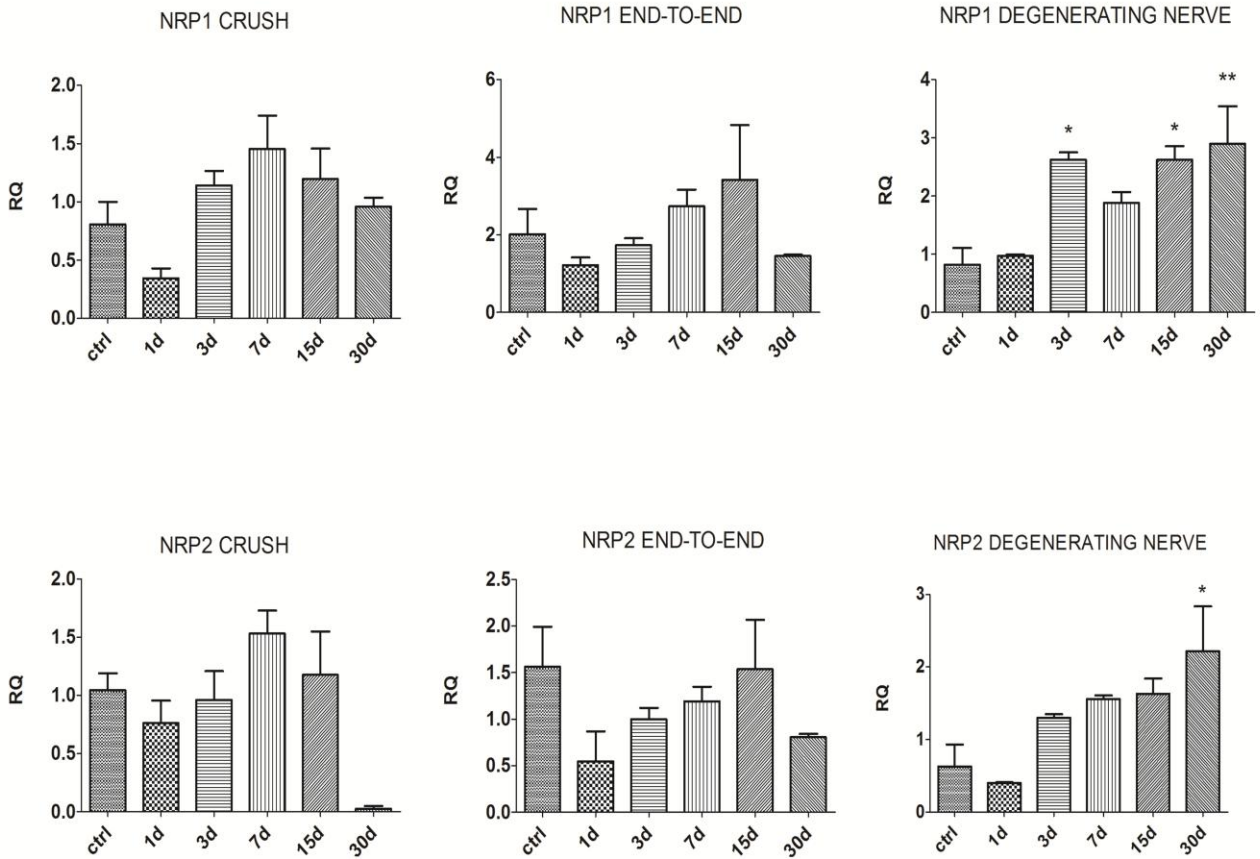
Figure 5: NRP1 and NRP2 are significantly upregulated in degenerating condition. NRP1 is significantly upregulated ($p \leq 0.05$ (*)) at day 3 and 15 ($p \leq 0.05$ (*)) and a higher upregulation is detectable at day 30 ($p \leq 0.01$ (**)) in degenerating nerve. The mRNA expression of NRP2 is strongly upregulated 30 days in degenerating condition ($p \leq 0.05$ (*)).N=3 in each time point condition.
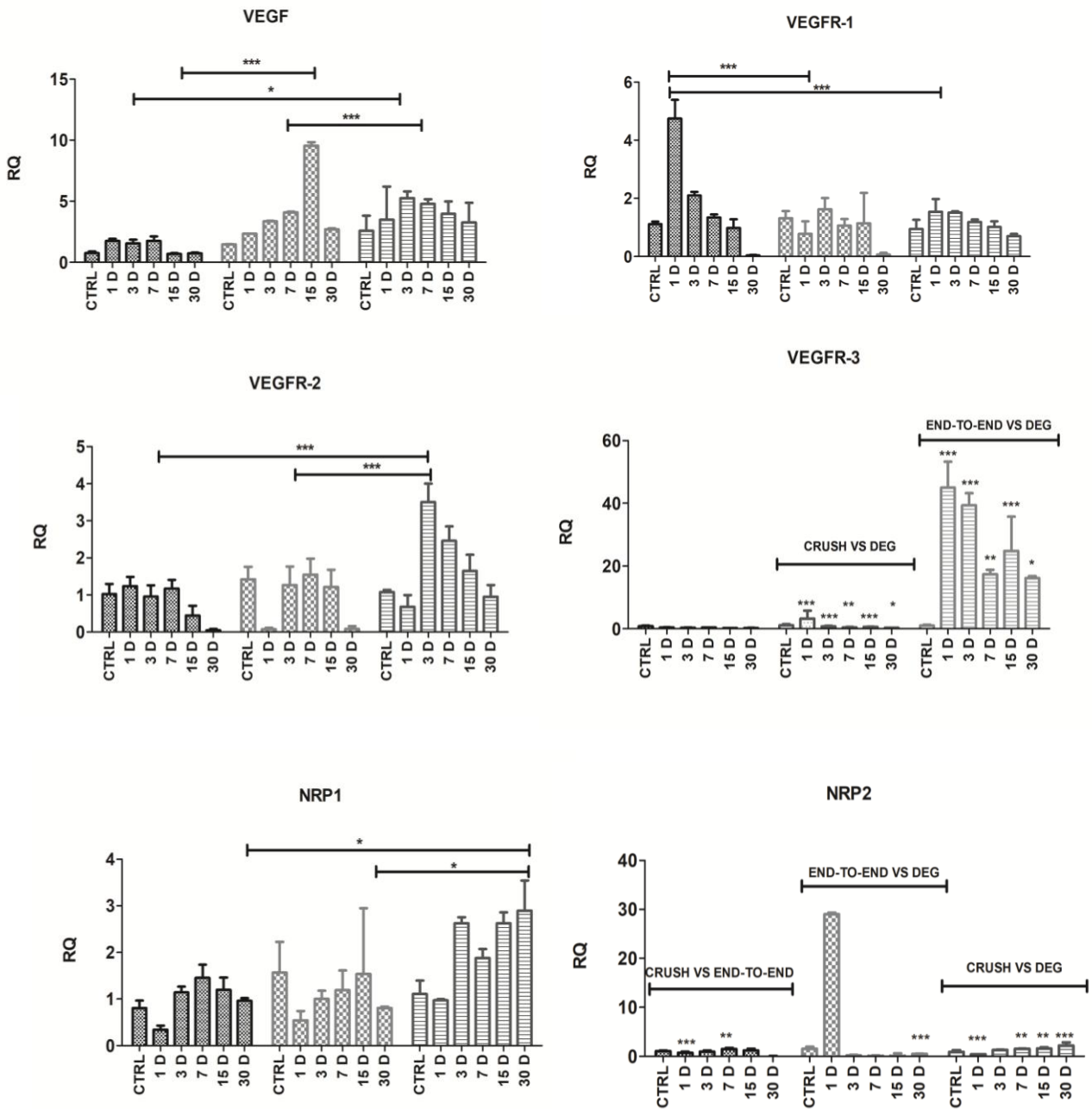
Figure 6: Two-way analysis of variance (ANOVA) and *post-hoc* Bonferroni showed mRNA expression in degenerating nerve is higher compared to degenerating control condition, end-to-end repair and crush injury *p*-value ranging from $p \leq$ 0.05 (*) to $p \leq$ 0.01 (***).

CRUSHED NERVES　　**END TO END NERVES**

|	C	1D	3D	7D	15D	30D	1D	3D	7D	15D	30D	
α-VEGFR2												230 KDa
α-NRP2												130 KDa
α-NRP1												120 KDa
												* 80 KDa
α-VEGF												43 KDa
α-βACTIN												42 KDa

Figure 7: VEGF expression is detectable in both crush and end-to-end nerves. The band is strongly detectable in control condition, 1 day, 3 days, 7 days and 30 days after crush injury. In end-to-end nerves a high VEGF protein expression is observed in all day points post repair. VEGFR-2 band appears in end-to-end nerves starting from day 3 until 30 days. In crushed nerves high protein expression is found between day 1 and day 30 after injury. Protein expression of NRP1 results with a very small band detectable in control nerves although protein expression level increases in crushed nerves starting from day 1 until day 30. In end-to-end-nerves NRP1 protein expression is higher between day 3 and day 30. Protein expression of NRP2 is barely detectable in crushed nerves; in end-to-end repair NRP2 appears between day 3 and day 30 after surgery.
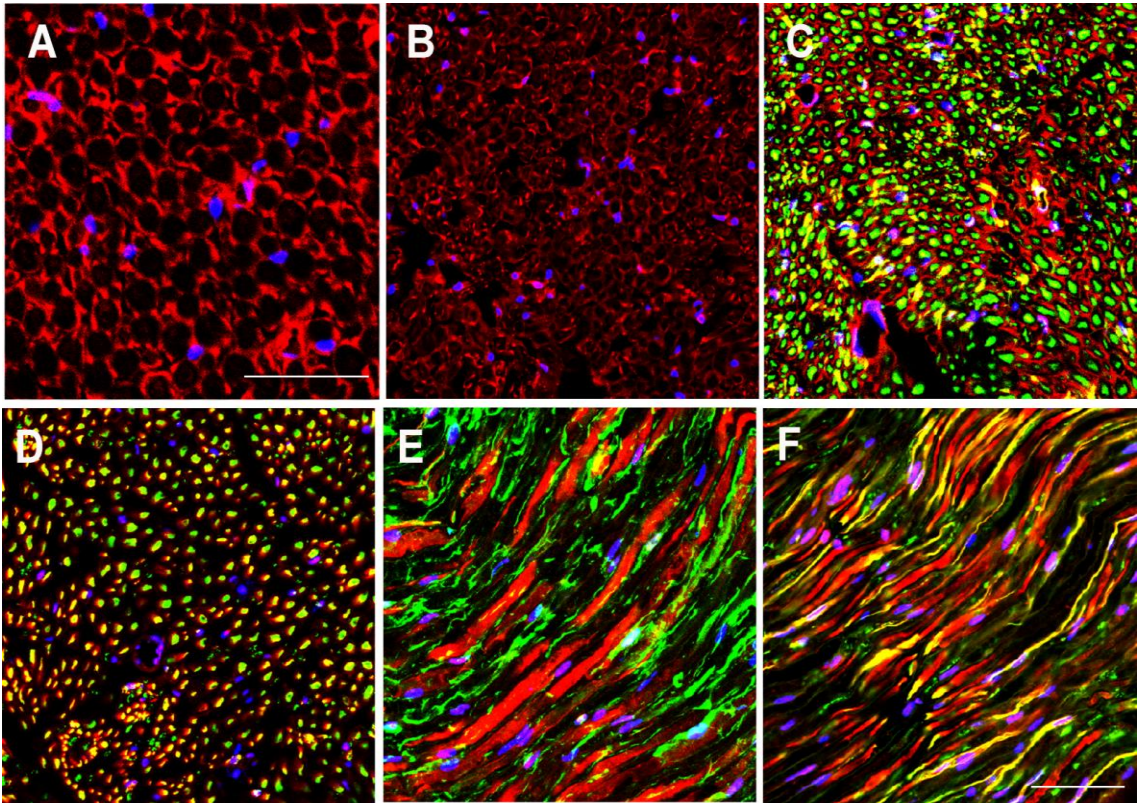
Figure 8: immunofluorescence staining for VEGF (red) in healthy control nerve (A) and crushed nerve 7 days after injury (B); double immunostaining for VEGFR-2 (red) and β-tubulin (green) showed absence of co-localization of these markers (C). Co-localization of NRP1 (red) and β-tubulin (green) is detected at nerve axons level (D); absence of co-localization is found in case of double labeling NRP1 (red) S-100b (green) (E). Co-localization of NRP2 and β-tubulin suggest the same expression pattern of NRP1 (F). Scale bar =100 μm. Nuclei are stained with DAPI (blue). Panel A 63X, panel B-F 40X.
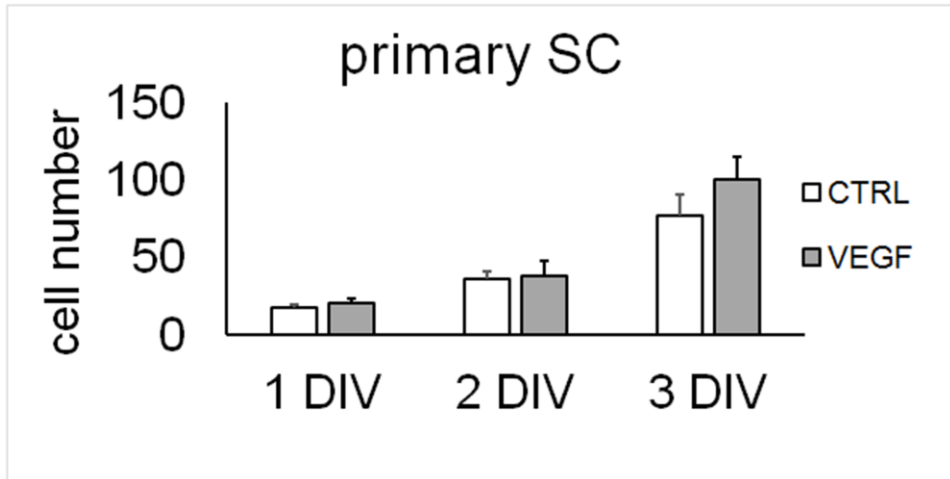
Figure 9: Proliferation assay on Primary Schwann cell culture in control condition and after VEGF165 (50 ng/ml) stimulation.
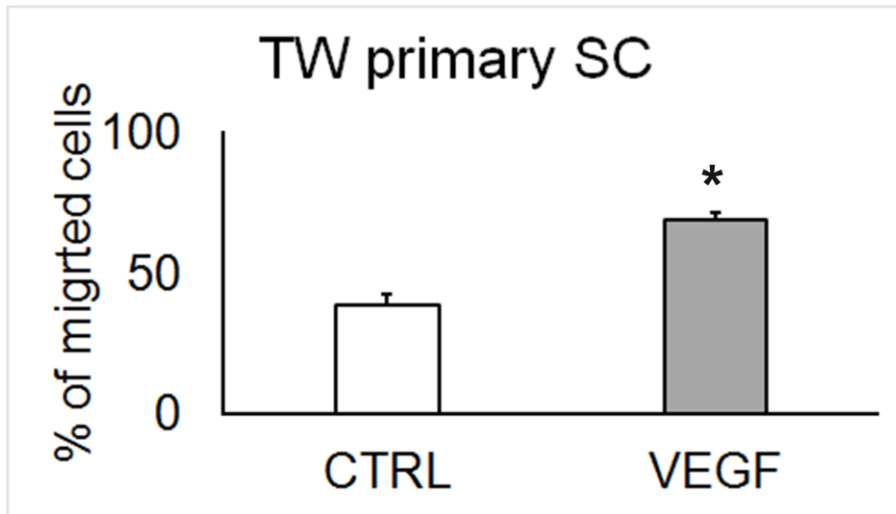
Figure 10: Three-dimensional migration assay on Primary Schwann cells culture in control condition (DMEM/ 2% FBS) and after stimulation with (50 ng/ml) of VEGF165. Data show a higher migration rate after VEGF165 stimulation ($p \leq 0.05$ (*)).

Table 1: Primers used for qRT-PCR reaction.

	Forward primer (5'-3')	Reverse primer (3'-5')	Amplicon size	Accession number
VEGF	CTTCCAGGAGTACCC CGATG	AGGTTTGATCCGCATG ATCTG	151	AY033504.1
VEGFR-1	TTTATCAGCGTGAAG CATCG	CCGAATAGCGAGCA GATTTC	157	NM_019306.2
VEGFR-2	CCAAGCTCAGCA CACAAAAA	CCAACCACTCTGGG AACTGT	190	U93307.1
VEGFR-3	GCTCTGCCTCGGA CTCCT	GATAGGCTGTCCC CGGTG	108	AF402786.1
NRP1	GGCTGTGAAGTAGA AGTGCCTAC	CCTGTGAGCTGGAA GTCATC	122	AF016296.1
NRP2	ACACCCAACGTGG ATTCC	GCTGACTTCCAGCT TGTACG	145	AF016297.1
ANKRD 27	CCAGGATCCGAGA GGTGCTGTC	CAGAGCCATATGGA CTTCAGGGGG	95	NM_001271264
RICTOR	GAGGTGGAGAGG ACACAAGCCC	GGCCACAGAACTC GGAAACAAGG	81	XM_001055633

Table2: Primary antibodies used

Antibody	Manufacturer, Catalog No, species, dilution.
a-β-tubulin	Sigma St. Louis MO, T8758 mouse monoclonal 1:1000
a-S100	Sigma St. Louis MO, S2644 rabbit polyclonal 1:600
a-VEGF-A	Abcam, ab171828 rabbit polyclonal 1:400
a-VEGF Receptor 2	Cell-Signalling Technology, #2479 rabbit polyclonal 1:1000
a-Neuropilin 1	Cell-Signalling Technology, #3725 rabbit polyclonal 1:1000
a-Neuropilin 2	Cell-Signalling Technology, #3366 rabbit polyclonal 1:1000
DAPI	Sigma St. Louis MO, D9542 1:1000

NEW BASIC INSIGHTS ON THE POTENTIAL OF CHITOSAN-BASED MEDICAL DEVICE FOR IMPROVING FUNCTIONAL RECOVERY AFTER RADICAL PROSTATECTOMY.

Abstract:	Objectives: To study the promising effects on potency recovery in patient that undergo nerve sparing radical prostatectomy of the use of chitosan membranes (CS-Me) aimed to protect neurovascular bundles. The aim of this study was two-fold: (1) to evaluate the neuro-regenerative potential of CS-Me on autonomic neurons; (2) to rule out the possibility that a pro-regenerative biomaterial could stimulate the proliferation of prostate cancer cell lines. Material and methods: As regards the neuro-regenerative potential, primary organotypic cultures derived from sympathetic ganglia were cultured on CS-Me over 3 days and neurite extension and axonal sprouting were evaluated. As regards the effects of chitosan (CS) on cancer cells, different human prostate cancer cell lines (PC3, DU-145, LN-Cap) were seeded on CS-coated plates or cultured in the presence of CS-Me dissolution products. Results: Results showed that the CS coating as well as the dissolution products of CS-Me lead to a significantly lower proliferation rate of prostate cancer cell lines. Concerning the pro-regenerative effects on autonomic ganglia, results showed that CS-Me significantly stimulate axon elongation in comparison to control conditions. Conclusion: Altogether, results of this study provide experimental data in support of the safety of CS-Me's clinical employment and of their potential efficacy for improving functional recovery after radical prostatectomy. Yet, our results provide the first experimental evidence that CS-Me stimulate posttraumatic

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axon regrowth of autonomic neurons.

NEW BASIC INSIGHTS ON THE POTENTIAL OF CHITOSAN-BASED MEDICAL DEVICE FOR IMPROVING FUNCTIONAL RECOVERY AFTER RADICAL PROSTATECTOMY.

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ABSTRACT

Objectives:

To study the promising effects on potency recovery in patient that undergo nerve sparing radical prostatectomy of the use of chitosan membranes (CS-Me) aimed to protect neurovascular bundles. The aim of this study was two-fold: (1) to evaluate the neuro-regenerative potential of CS-Me on autonomic neurons; (2) to rule out the possibility that a pro-regenerative biomaterial could stimulate the proliferation of prostate cancer cell lines.

Material and methods:

As regards the neuro-regenerative potential, primary organotypic cultures derived from sympathetic ganglia were cultured on CS-Me over 3 days and neurite extension and axonal sprouting were evaluated.

As regards the effects of chitosan (CS) on cancer cells, different human prostate cancer cell lines (PC3, DU-145, LN-Cap) were seeded on CS-coated plates or cultured in the presence of CS-Me dissolution products.

Results:

Results showed that the CS coating as well as the dissolution products of CS-Me lead to a significantly lower proliferation rate of prostate cancer cell lines. Concerning the pro-regenerative effects on autonomic ganglia, results showed that CS-Me significantly stimulate axon elongation in comparison to control conditions.

Conclusion:

Altogether, results of this study provide experimental data in support of the safety of CS-Me's clinical employment and of their potential efficacy for improving functional recovery after radical prostatectomy. Yet, our results provide the first experimental evidence that CS-Me stimulate posttraumatic axon regrowth of autonomic neurons.

Keywords: *chitosan, autonomic ganglia, prostate cancer, nerve regeneration*

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1. INTRODUCTION

Prostate cancer is the most common cancer among men. An incidence of 214/1000 cases is registered in Europe, surpassing the number of the lung and colorectal cancers (1) and representing the second leading cause of cancer mortality (2).

The current most popular treatment of localized prostate cancer in patients with a life expectancy longer than 10 years is radical prostatectomy (European Association of Urology Guidelines on Prostate Cancer, 2017 update). Unfortunately, in patients who undergo radical prostatectomy, frequently iatrogenic damage to the peri-prostatic neurovascular bundles (NVB) occurs, leading to erectile dysfunction (3).

There have been many efforts with the intent to promote a more rapid and efficient recovery of functions (namely potency and continence) after radical prostatectomy. Patel and colleagues conducted a pivotal study aimed to determine if the use of dehydrated human amnion/chorion membrane (dHACM) allograft wrapped around the NVB during robot-assisted radical prostatectomy is able to accelerate the return to the normal functionality. Interestingly, the authors could demonstrate that the application of dHACM in patients accelerates the recovery of potency and continence, compared to patients who did not receive the membranes (4).

Recently, the application on the neurovascular bundles after nerve-sparing robot-assisted radical prostatectomy of membranes made of another biomaterial of natural origin, namely the chitosan, has been reported to be safe. Authors also appreciated encouraging results regarding potency recovery (5).

Starting from the promising clinical evidences, the present study aims were divided into two different perspectives: 1) to investigate the neuro-regenerative effect of CS-Me on *ex vivo* cultures of autonomic ganglia; 2) to rule out that chitosan employment in oncological surgery might have negative side effects by promoting prostatic cancer cell growth.

2. MATERIALS AND METHODS

2.1. Membranes preparation

CS-Me were produced from highly purified chitosan with a DA of 5% (Altakitin S.A.) as previously described (6). Briefly, CS was dissolved in 0.75% acetic acid to obtain a 1.5% solution, filtered, and poured into Petri dishes, followed by drying at room temperature. The resulting films were treated with a solution of ammonia in methanol/water, followed by intense washing with

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distilled water, and drying. Finally, the films were cut into the required size and sterilized by ethylene oxide.

2.2. Analysis of the regenerative potential of CS-Me on cultures of autonomic ganglia

2.2.1 Autonomic ganglia dissection

For this study adult male Wistar rats (Envigo, Udine, Italy) weighing approximately 190-220g were used. All procedures were performed in accordance with the Ethics Committee and the European Communities Council Directive of 24 November 1986 (86/609/ EEC). Adequate measures were taken to minimize pain and discomfort taking into account human endpoints for animal suffering and distress.

Animals were sacrificed by lethal injection of the anesthetic solution with Tiletamine + Zolazepam (Zoletil) i.m. (3 mg/kg).

Rats were placed under surgical microscope with the ventral side facing up and the caudal end oriented toward you. Using forceps neck skin and muscles were removed allowing to reach the carotid artery that runs alongside trachea. Carotid bifurcation at C2-C3 level, represents an important landmark to identify the superior cervical ganglia that appears as an almond-shaped structure surrounded by connective tissue capsule closely attached to the artery. Once the ganglion is identified, using fine forceps it can be dissected. Following the sympathetic trunk along the toracic level it is possible to identify the stellate ganglion, the cervicothoracic, a large ganglion probably formed by the fusion of the lower two cervicals and the first thoracic ganglia.

2.2.2 Autonomic explants ganglia cultures

The experiment were performed using the organotypic cultures of superior cervical and stellate ganglia (cervicothoracic ganglion) harvested as a model for the sympathetic nervous segment.

The autonomic explants were cultured on two different substrate: chitosan membrane and glass coverslips (control).

After harvesting, the connective tissue capsule around the ganglia was removed and each ganglion was half cut to aid the attachment on the CS-Me , a 50 µl drop of Matrigel in F12 medium (50% v/v) was applied on the substrate before seeding the explants. After 2 hours of incubation at 37°C, SFM (7) culture medium with nerve grow factor (NGF, 50ng/ml) was slowly added to the plate.

2.2.3. Immunofluorescence

After 3 days of culture, autonomic explants were fixed in 4% PFA for 15 min, washed in 0.1 M phosphate buffer (pH 7.2) and processed for immunofluorescence analysis.

Samples were permeabilized, blocked [0.1% triton X-100, 10% normal goat serum (NGS)/0.1% NaN$_3$, 1h] and incubated O/N in anti-βIII-Tubulin (mouse, monoclonal, 1:1000, Sigma-Aldrich) primary antibody; after incubation with secondary antibody goat anti-mouse IgG Alexa-Fluor-488-conjugated (1:200, Molecular Probes, Eugene, Oregon) for 1 hour, autonomic explants were mounted with a Dako fluorescent mounting medium.

2.2.4. Quantification of neurite outgrowth

Autonomic ganglia were scanned using a Zeiss LSM800 confocal laser microscopy system (Zeiss, Jena, Germany). For each sample, confocal Z-stacks were used and different images (10X) were captured in order to reconstruct the entire ganglia.

In order to evaluate the regenerative capability of the autonomic ganglia on the chitosan membranes, Neurite J (ImageJ plugin) was used and two different parameters were evaluated: neurite extension and axonal sprouting (8).

2.3. In vitro cell tests on CS based membranes

In vitro cell tests were performed using LN-Cap, DU-145 and PC3 cell lines. The cytotoxicity test was carried out with the dissolution products of CS-Me, while LN-Cap, DU-145 and PC3 adhesion, proliferation and protein expression were evaluated on a CS coating, as detailed below.

2.3.1. Dissolution products of CS-Me

The effect of the CS-Me-based material extracts was studied on LN-Cap, DU-145 and PC3 cell lines. Material extracts were prepared by incubating CS-Me in Dulbecco's Modified Eagle Medium-F12 (DMEM-F12, Sigma Aldrich) supplemented with 100 U/ml penicillin (Sigma), 0.1 mg/ml streptomycin (Sigma), 1 mM sodiumpyruvate (Sigma), 4 mM L-glutamine (Sigma) and stored at 37°C in a humidified atmosphere of 5% CO$_2$ for 13 days. As control media, samples of culture medium without CS were maintained in the same conditions as CS-Me samples and then collected after 15 days. Then, the proliferation assay was carried out using collected media. In detail, LN-Cap, DU-145 and PC3 cells were seeded and cultivated in the previously prepared extract media, at a density of 5×10^3 cells/cm^2 on Petri dishes. After 1, 3, and 6 days in vitro (DIV), cells were trypsinized and counted in a Burker's hemocytometer chamber. Experiments were

performed as technical triplicates. The counts obtained from assays were analyzed, averaged and expressed as logarithmic scale of viable cells/mm^2 ± SD.

2.3.2. Proliferation assay on CS coating

Culture wells were coated with a solution of 2 mg/ml chitosan in 0.1% acetic acid (EMD Biosciences) or with 0.1% acetic acid alone. Briefly, the solutions were distributed in an excess volume into each well to ensure the entire surface area was covered. Plates were placed at 4°C overnight. The next day, prior to plating prostate cancer cells, remaining chitosan or acetic acid solution was aspirated. Then the proliferation assay was carried out using LN-Cap, DU-145 and PC3 cells. Cell lines were seeded and cultivated in RPMI medium supplemented with 100 U/ml penicillin (Sigma), 0.1 mg/ml streptomycin (Sigma), 1 mM sodiumpyruvate (Sigma), 4 mM L-glutamine (Sigma) and 2% fetal bovine serum (FBS), at a density of 5x10^3 cells/cm^2 on coated Petri dishes. After 2, 5, and 7 days in vitro (DIV), cells were trypsinized and counted in a Burker's hemocytometer chamber. Experiments were performed as technical triplicates.

2.3.3. Total protein extraction, and western blot

Total proteins were extracted by solubilizing cells in boiling Laemmli buffer (2.5% SDS and 0.125 M Tris-HCl pH 6.8), followed by 3 min at 100°C. The protein concentration was determined by the BCA method, and equal amounts of proteins (denaturated at 100°C in 240 mM 2-mercapto ethanol and 18% glycerol) were loaded onto each lane, separated by SDS-PAGE, transferred to a Hybond-C Extra membrane and blocked for 1 h at 37°C in 1× TBST (150 mM NaCl, 10 mM Tris–HCl (pH 7.4), and 0.1% Tween) plus 5% non-fat milk. Membranes were incubated overnight at 4°C in primary antibodies diluted in TBST plus 1% non-fat milk. The day after, they were rinsed four times with TBST for 5 min each at room temperature and incubated for 1 h at room temperature with peroxidase-linked secondary antibody (diluted in TBST plus 1% non-fat milk). Membranes were washed 4 times, 5 min each, with TBST at room temperature, and specific binding was detected by the enhanced chemiluminescence ECL system (Amersham Biosciences) using Hyperfilm™ (Amersham Biosciences).

Primary and secondary antibodies used are: rabbit polyclonal anti-Bcl2 (1:500, sc-492, Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse monoclonal anti-actin (1:4.000, #A5316, Sigma); horseradish peroxidase-linked donkey anti-rabbit secondary antibody (1:10.000, Amersham Biosciences), sheep anti-mouse secondary antibody (1:100.000, Amersham Biosciences).

2.4 Statistical analysis

For *in vitro* experiments, statistical analysis was performed using one-way analysis of variance (ANOVA) and *post hoc* Bonferroni .

For explants ganglia neurites quantification, statistical analysis was performed using Two-Sample t-Test. Statistical analysis were performed using SPSS Software.

The level of significance was set at $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***). Values were expressed as mean ± standard deviation (SD).

3. RESULTS

3.1. CS-Me represent a permissive substrate for neurite regeneration and axonal elongation of autonomic explants ganglia

To evaluate the pro-regenerative capability of CS-Me, we cultured autonomic explant ganglia over 3 days and determined the numbers of extending neurites at certain distance from the explant body according to the Sholl method (8).

Despite the neurites sprouting in both culture conditions is very enhanced (Figure6), it is possible to observe from the graphs (Figure1) that autonomic explant ganglia cultured on CS-Me exhibit an increased neurites length compared to control condition.

Morphological analysis on explants ganglia stained with βIII-Tubulin showed a high neurites outgrowth and a strong neurites arborization supporting the pro-regenerative effect of chitosan membrane (Figure 2).

3.2. Dissolution products of CS-Me negatively affect proliferation of LN-Cap, DU-145 and PC3 cell lines

The proliferation of three different human prostate cancer cell lines: DU-145, LN-Cap and PC3 cultured in medium containing dissolution products of chitosan membranes was evaluated. After 15 days of CS film dissolution at a temperature of 37°C, media collected were used to cultivate three cell lines and proliferation assays were carried out. Cells were plated and cultured in presence of conditioned media and their proliferation was evaluated after 1, 3 and 6 days. As control media, samples of culture medium without dissolution products of chitosan membranes maintained in the same conditions were collected after 15 days. The proliferation assay was performed after 1, 3 and 6 days on DU-145, LN-Cap and PC3 cells cultured with this control condition medium.

Results showed that cancer cells grown in conditioned medium with the dissolution products from CS-Me exibit a significant ($p < 0.05$) lower proliferation compared to control medium. (Figure 3).

3.3. CS coating direct contact with human prostate cancer cell lines: LN-CaP, DU-145 and PC3 substantially alter their morphology and proliferation rate

The anti-proliferative effect of CS was tested on three different human prostate cancer cell lines: DU-145, LN-Cap and PC3. Cells were plated in three different experimental condition: control condition, chitosan coating, acetic acid coating (the latter represents a control of the toxicity, since it is the substance in which CS is dissolved) and the proliferation assay was performed after 2, 5 and 7 days.

All cell lines tested have shown a significant reduction in proliferation when grown on the substrate represented by CS. No interference with proliferation was found by cultivating the cells in the presence of a coating of acetic acid at 0.1%. (Figure 4). Cell morphology reflects the results of the proliferation experiments. Cultured cells on the CS substrate lose their normal morphology and adhesion capacity, they appear rounded and form clusters (Figure 5).

3.4. CS coating affects the expression of BAX and BCL2 proteins suggesting the activation of a pro-apoptotic program

BAX and BCL2 protein expression changes were evaluated on the prostate cancer cell lines: DU-145, LN-Cap and PC3 cells, to study pro-apoptotic and cell survival signaling after 2, 5 and 7 days of culture at three different experimental conditions, control substrate, acetic acid coating, CS coating. In cells cultivated on CS-coated substrates it is possible to observe an increase in the Bax pro-apoptotic protein expression and a simultaneous decrease in anti-apoptotic Bcl2 at the 7 days experimental time point (Figure 6). This pattern of expression suggests an activation of the CS inducted pro-apoptotic program.

4. DISCUSSION

Chitosan (CS) is a derivative of chitin, obtained from the exoskeleton of crustaceans, which is achieving significant interest both in basic research and in clinical settings. Its chemical structure (polysaccharide composed of D-glucosamine and N-acetyl-D-glucosamine linked with β (1-4) bond) gives it a remarkable hypoallergenicity, making it an excellent candidate for the development of innovative applications in the field of medicine and surgery, also thanks to its biocompatibility,

bioavailability and lack of toxicity. For these reasons, CS potential clinical applications range from orthopedic and drug-delivery systems, to scaffolds for regeneration of nerve, skin, bone and cartilage (9-14).

The aim of this of this study was two-fold: (1) addressing the neuro-regenerative potential of chitosan membranes on autonomic ganglia; (2) excluding the possibility that a pro-regenerative biomaterial stimulates cell proliferation in a tumor site.

As regards the first study aim, we have used organotipic cultures that allow to obtain a multicellular *ex vivo* model that preserves both the cytoarchitecture of the tissue and the interactions among cells, providing a closer approximation to *in vivo* conditions (15, 16). Since the prostatic plexus is innervated by autonomic nerve fibers, the regenerative potential of chitosan membrane was assessed through organotipic sympathetic ganglion cultures that represents an innovative and original experimental model for testing autonomic nervous system regenerative properties.

We demonstrated, for the first time in the literature, that CS-Me not only exert a pro-regenerative effect on somatic nervous ganglia (6, 14) but also on autonomic nervous ganglia. This observation is important since it provides a possible mechanistic explanation about the positive effects of CS-Me application on functional recovery after radical prostatectomy (5), namely that the faster recovery of potency is due to the post-surgical stimulation of axonal regrowth in the peri-prostatic neurovascular bundles.

The second aim of this study, regarded the safety of the employment of CS-Me in surgical oncology of prostate cancer. Given the intrinsic cell supportive capacity of CS we wanted to rule out that CS might have any negative side due to a proliferative stimulation of prostate cancer cells. Alongside the pro-regenerative effects, of great interest is the anti-proliferative activity of CS reported on different tumor cell lines. Indeed, the anticancer activity of CS was proved in human breast cancer cell lines (17), in a human gastric carcinoma cell line MGC803 and in a human monocytic leukemia cell line (18, 19). Gibot and colleagues reported a study in which demonstrated the mechanisms underlying the anti-proliferative effect of CS on human melanoma cell lines, suggesting a cell line dependent effect on the apoptosis and proposing it as a future instrument for assessing cancer therapies in the field of melanoma (20). The tumor cells used in this work are cell lines derived from prostate adenocarcinomas: lymph node, brain and bone metastasis, respectively LN-Cap, DU-145 and PC3 cells [9-11].

We wanted to test the effect of CS-Me dissolution products on the proliferation of tumor cells. It is known from the literature that CS products are able to stimulate regeneration and therefore the positive effect on the neuronal population is not only due to direct contact with the material but also

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from its degradation (21). Interestingly, we observed that cancer cells grown in conditioned medium with the dissolution products from CS-Me exhibit a significantly lower proliferation compared to control medium.

The direct contact of the CS with cancer cell lines was tested with the concern of the proliferation, morphology and protein expression of apoptotic markers. This experiments revealed a significant reduction in proliferation of the prostate cancer cell lines grown on the CS substrate. The analysis of the cell morphology also reflected this aspect: cultured cells on the CS substrate lose their normal morphology and adhesion capacity, appearing rounded and forming clusters. From the molecular point of view CS-coated substrates led to an increase in the Bax pro-apoptotic protein expression and a simultaneous decrease in anti-apoptotic Bcl2 at the 7 days experimental time point in all cancer cells tested. This pattern of expression suggests an activation of the CS inducted pro-apoptotic program.

Prostate cancer, whose surgical therapy, radical prostatectomy, remains gold standard therapy today, is the tumor that has an increasing incidence in the male every year, exceeding the number of lung cancer and colorectal cancer [1]. Following the removal of the prostate, the neurovascular bundles may be damaged, leading to functional deficits such as impotence and incontinence. There have been many clinical research efforts designed to minimize side effects and limit residual functional deficiencies [3]. Recently, Porpiglia and colleagues reported the preliminary results of a clinical trial in which they tested chitosan, already known for its effectiveness in promoting nerve regeneration (6), in form of membranes aimed to protect the NVB following radical prostatectomy (5). They showed the feasibility of the application and the safety of the material, meanwhile observing a promising effect on the recovery of the potency in the patients.

Taken together, results of this study provide a strong evidence, from basic science point of view, in favour of the previous promising clinical results and further support the view that application of CS-Me can be a simple, safe and effective strategy to protect the periprostatic plexus following radical prostatectomy. Yet, these results provide the first experimental evidence about the mechanism of action which underlies the effects of CS-Me on autonomic neurons, namely the promotion of posttraumatic axon regrowth, a key factor in neuro-regenerative processes.

Acknowledgments

Conflicts of Interest

Stefano Geuna and Francesco Porpiglia are scientific consultant for Monarch Bioimplants.

Figure legends

Figure 1. Quantification of sprouting and neurites elongation.

Bar Graph and line graph depicting quantification of organotipic autonomic ganglia cultured on CS-Me (red) and control condition (blue).

Figure2. Morphology.

Axonal outgrowth of organotipic autonomic ganglia cultured, stained with anti-β tubulin, on CS-Me (B-D) and control substrate represented by glass slides (A-C).

Figure 3. Effects of dissolution products of CS-Me. Line graph depicting the proliferation of different human prostate cancer cell lines: LN CaP, DU-145 and PC3 cultured in medium collected after 15 days of CS film dissolution.*: $p < 0.05$.

Figure 4. Effects of cell direct contact with CS coating. Proliferation curve experiment: LN-Cap, Du-145 and PC3 cell lines were cultivated on the control, on 0.1% acetic acid coating and on CS coating. Results showed a significant lower proliferation for cell lines cultured on Chitosan. $^{\$/\#}$: $p < 0.05$; $^{\$\$/\#\#}$: $p \leq 0.01$; $^{\$\$\$/\#\#\#}$: $p \leq 0.001$. $ indicates the comparison between CTR and CS coating treatment; # indicates the comparison between acetic acid coating and CS coating treatment.

Figure 5. Effects of cell direct contact with CS coating. Morphology: Representative panel depicting PC3 cell lines cultivated on the control, on chitosan coating and on 0.1% acetic acid coating. Cultured cells on the CS substrate lose their normal morphology and adhesion capacity, they appear rounded and form clusters.

Figure 6. Protein expression analysis. A representative Western blot analysis performed on PC3 cell line cultured in the three experimental conditions showed, at days 5 and 7 of CS coating, an over-expression of the pro-apoptotic protein BAX and a simultaneous down-regulation of BCL2, an anti-apoptotic marker.

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Figure 1. Quantification of sprouting and neurites elongation.
Bar Graph and line graph depicting quantification of organotipic autonomic ganglia cultured on CS-Me (red)
and control condition (blue).

47x27mm (300 x 300 DPI)
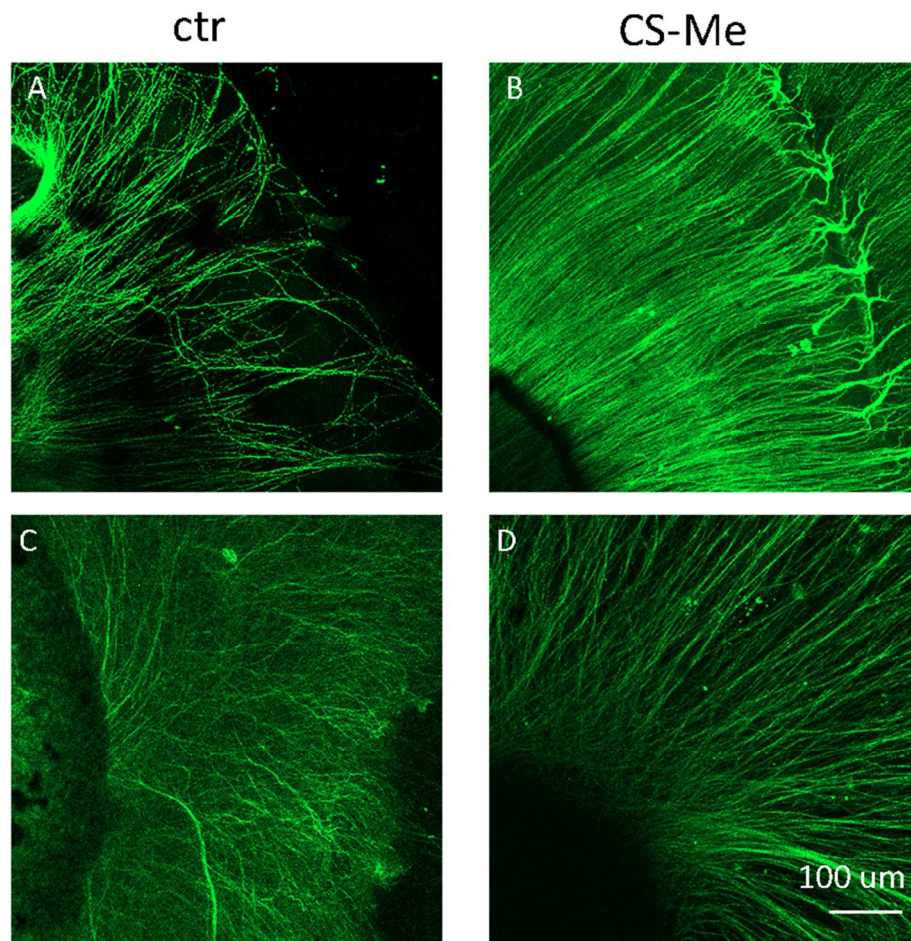
Figure2. Morphology. Axonal outgrowth of organotipic autonomic ganglia cultured, stained with anti-β tubulin, on CS-Me (B-D) and control substrate represented by glass slides (A-C).

80x76mm (300 x 300 DPI)
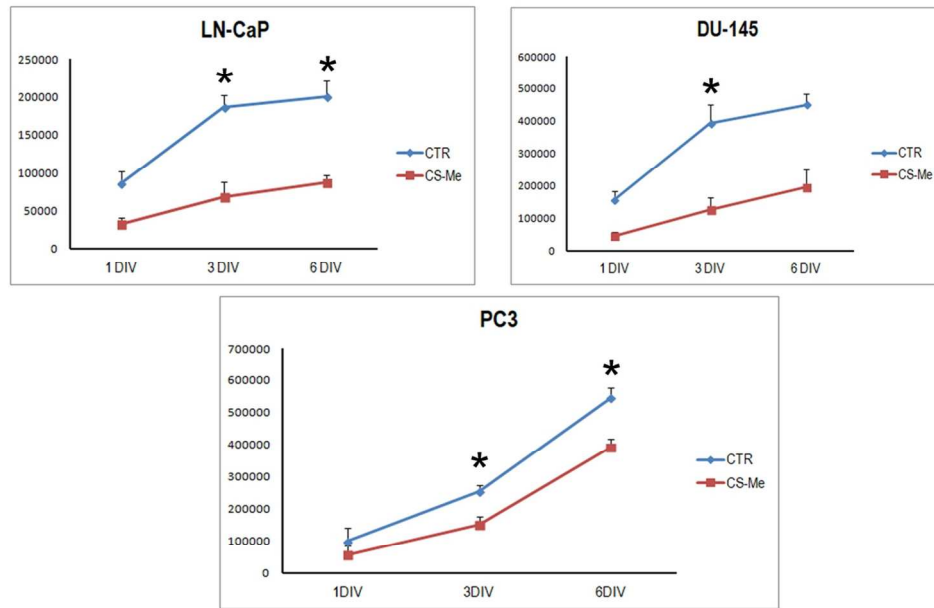
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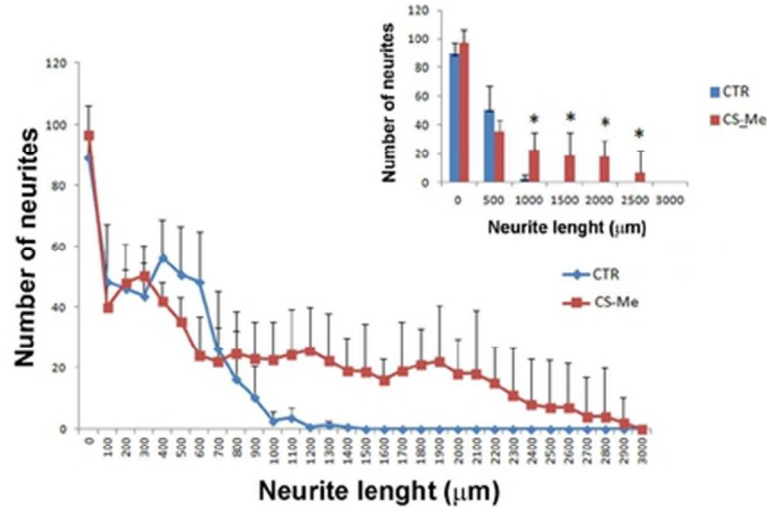Figure 3. Effects of dissolution products of CS-Me. Line graph depicting the proliferation of different human prostate cancer cell lines: LN CaP, DU-145 and PC3 cultured in medium collected after 15 days of CS film dissolution.*: $p < 0.05$.

118x77mm (300 x 300 DPI)
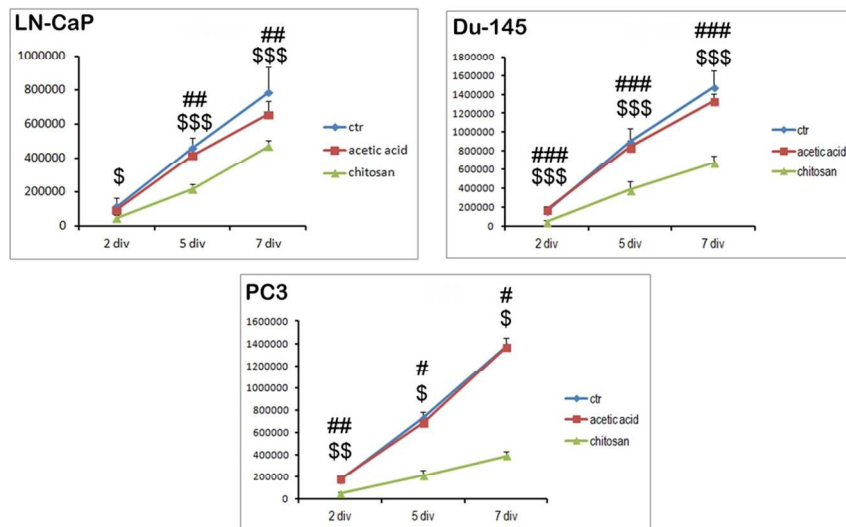
Figure 4. Effects of cell direct contact with CS coating. Proliferation curve experiment: LN-Cap, Du-145 and PC3 cell lines were cultivated on the control, on 0.1% acetic acid coating and on CS coating. Results showed a significant lower proliferation for cell lines cultured on Chitosan. $/#: p < 0.05; $$/##: p ≤ 0.01; $$$/###: p ≤ 0.001. $ indicates the comparison between CTR and CS coating treatment; # indicates the comparison between acetic acid coating and CS coating treatment.

112x70mm (300 x 300 DPI)

Figure 5. Effects of cell direct contact with CS coating. Morphology: Representative panel depicting PC3 cell lines cultivated on the control, on chitosan coating and on 0.1% acetic acid coating. Cultured cells on the CS substrate lose their normal morphology and adhesion capacity, they appear rounded and form clusters.

140x166mm (300 x 300 DPI)
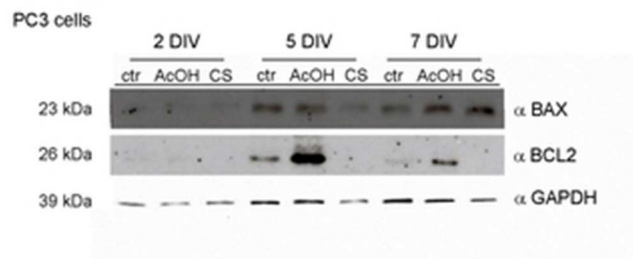
Figure 6. Protein expression analysis. A representative Western blot analysis performed on PC3 cell line cultured in the three experimental conditions showed, at days 5 and 7 of CS coating, an over-expression of the pro-apoptotic protein BAX and a simultaneous down-regulation of BCL2, an anti-apoptotic marker.

30x11mm (300 x 300 DPI)

4. DISCUSSION

4.1 Plasticity and regeneration in PNS after injury

The term of plasticity is referred to the ability of the nervous system to physically change over the time in response to degenerative disease and injuries. Until recently this phenomenon was mainly referred to the CNS with plastic changes in myelination, neuronal proliferation, and synaptic organization (Miller, Gensel et al. 2016).

Instead, in the last decades, this process was studied and described also in PNS in which plastic changes are predominantly based on axonal re-growth and neuron addition (Geuna, Fornaro et al. 2010).

At this purpose during these years of Phd, I focused the attention on different aspects of plasticity and regeneration in the PNS. First of all I studied the neuronal addition and the morphological plastic changes in dorsal root ganglia as a consequence of crush injury of brachial plexus; in a second time I assessed the important issue of long-term regeneration in which, after a long posttraumatic period different morphological predictors of regeneration were investigated; the third phase concerns the possibility that an endothelial growth factor, VEGF could have a role in enhancing the regeneration process.

Finally the last goal of my PhD was the study of the plasticity and regeneration of autonomic ganglia neurons cultured on an innovative chitosan substrate that can improve the regeneration of the peri-prostatic nerves in patients undergo radical prostatectomy.

4.1.1 Investigation of changes in the number of DRG neurons as a consequence of peripheral nerve crush injury

As mentioned above, in the first part of my Phd, I addressed the important issue regarding the generation of new neurons in DRG after peripheral nerve injury. The evidence of neurons generated *ex novo* in sensory ganglia of adult animals is a controversial question still debated (Muratori, Ronchi et al. 2015). For decades several groups have collected data about the progressive increase in DRG neurons number as an age-dependent process (Devor and Govrin-Lippmann 1985; Devor and Govrin-Lippmann 1991; Cecchini, Cuppini et al. 1995; Popken and Farel 1997; Ciaroni, Cecchini et al. 2000; Farel 2002).

Recently new discoveries showed that adult DRG and trigeminal ganglia give rise to neurosphere able to differentiate into neurons and glial cells in different *in vitro* models (Cecchini, Cuppini et al. 1995; Lagares, Li et al. 2007; Li, Say et al. 2007). Furthermore an *in vivo* study demonstrated that as a consequence of nerve injury, DRG neurons undergo plastic changes as a response to injury (Himes and Tessler 1989; Hokfelt, Zhang et al. 1994). For this reason, the issue of adult plasticity has been strongly debated and despite the well known existence of neurogenetic areas in CNS has been commonly accepted, only very few studies have begun to arise the hypothesis that neurogenesis could also occur in a site like the PNS, so different from the olfactory epithelium in which the phenomenon is well described and accepted (Wang and Halpern 1982; Margolis, Verhaagen et al. 1991; Legrier, Ducray et al. 2001; Getchell, Liu et al. 2005; Gould 2007).

The evidence of new neurons in DRG after peripheral nerve injury is based on the results obtained by the quantification of neurons numbers in T1 DRG harvested 30 days after crush injury of the brachial plexus. The quantification was performed using the physical disector method that allowed an adequate recognition of the morphological structures. Results obtained showed an higher increase (42%) in neurons number in T1 DRG that underwent the crush lesion compared to control. The data correlated also with an increase of total volume of those injured DRG. In order to rule out the possibility that the increment in neuron numbers was due to a variability among the neuronal population belonging to pairs of DRG, an internal control was also quantified showing a low and not significant fluctuation in the number of DRG neuronal cells counted between left and right T1 of the same animal. Moreover the stereological results were correlated with the quantification of BrdU positive cells that peaks at day 1 post injury and increase until day 30 post injury in agreement with the sterological data. The evidence of BrdU-immunopositive neurons and neural progenitors labeled with Ki67, nanog, nestin, and sox-2 confirmed the stereological evidence of posttraumatic neurogenesis in DRG. Analysis of morphological changes following axonal damage in addition to immunofluorescence characterization of cell phenotype suggested that the neuronal precursors which give rise to the newly generated neurons could be represented by satellite glial cells that actively proliferate after the lesion and are able to differentiate toward the neuronal lineage.

The crush injury to the peripheral nerves represents an exceptional stimulus that induce the neuronal progenitors to proliferate, differentiate into neuroblast cells and finally become mature neurons.

4.1.2 Study of long-term post-traumatic regeneration of rat median nerve.

As previously described peripheral nerves retain a high regenerative potential throughout the adulthood, but whether post-traumatic regeneration can eventually regain the nerve fibers pre-trauma size is still an open question. At this purpose, it is known that the number of regenerated fiber can return to normal size after a sufficient recovery-time period, but the hypothesis about the possibility that the different nerve fibers parameters can return to normal level has never been deeply investigated due to the fact that the functional recovery is quickly achieved after a rat axonotmesis lesion.

For this reason, the experimental study on nerve regeneration are mainly based on short-term post-traumatic time, for instance 6-8 weeks. In spite of very rapid functional recovery, important morphological parameters are still different to control conditions even at 12 week after injury. This lead to the important question whether the long-term post traumatic regeneration may eventually back to normal different nerve fibers parameters.

To answer this question the post-traumatic time was prolonged until 24 weeks in rat underwent the crush injury that represents a model of injury in which the full recovery is achieved at week 4 post-trauma, moreover allows to obtain regeneration in optimal condition without surgical neurorraphy. Results obtained by the stereological analysis in which total numbers of myelinated fibers, axon diameter, fiber diameter, myelin thickness, and g-ratio were investigated 6, 8 and 24 weeks after injury showed that the total number of regenerated fibers is significantly higher 6 and 8 weeks post injury whereas 24 weeks post injury is not significantly differ from control nerves. Regarding axon and fiber diameter, both parameters significantly decrease 6 and 8 weeks post injury. Interestingly 24 weeks after trauma data showed that fiber size have a small increase compared to the other time points analyzed but still smaller compared with control nerves. The same trend is followed by myelin thickness, while *g-ratio,* that

was not significantly different from control nerves 6 and 8 weeks post lesion is significantly lower 24 weeks after trauma.

Time course stereological analysis were confirmed by the morphological analysis performed by high resolution light microscopy showing that 6 weeks after injury the regenerated myelinated fibers are smaller with a thinner myelin sheath and with the presence of microfasciculation typical of regenerating fibers. 24 weeks after injury the morphology of fibers is similar to control fibers. This results are important from biological and clinical perspective because they provide a better understanding of nerve regeneration potential adding a new information regarding the long-time regeneration; furthermore this findings are also important considering that, although the peripheral nerves are able to regenerate, functional recovery in human is often unsatisfactory.

In the translational clinical perspective this results showed that, without treatment, complete post-traumatic recovery of nerve morphology did not occur. Finally these evidences are in agreement with the consideration that although the regeneration process is very active, the electrophysiological parameters do not return to normal level after repair (Cragg and Thomas 1964; Hoke 2006; Roganovic and Pavlicevic 2006; Luis, Amado et al. 2007) .

4.2 Evaluation of VEGF expression in degenerating and regenerating condition

Peripheral nerve regeneration represents a very complex process that involves different morphological and molecular changes occurring to both proximal and distal stumps (Geuna, Raimondo et al. 2009; Allodi, Udina et al. 2012; Muratori, Ronchi et al. 2012).

After injury, axons distal to regeneration site are interrupted, myelin sheath is degradated and Wallerian degeneration occurs leading to a series of phenotypic changes that promote axonal regeneration. It is well accepted that during the regenerative process many molecular factors are involved in order to create a favorable microenvironment for axonal outgrowth(Navarro, Vivo et al. 2007).

For this reason, various molecules have been investigated in experimental models of neural repair in order to study promising strategies to improve the functional recovery (Raimondo, Fornaro et al. 2011; Chang, Quan et al. 2016).

In the middle part of my PhD, I focused the attention on the study of VEGF expression assuming the important fact that the vascular and nervous systems share common molecular pathways during development and regeneration, furthermore the anatomical parallelism between vessel and nerve is well documented (Ruiz de Almodovar, Lambrechts et al. 2009). Anatomically, both systems are composed of afferent and efferent networks, arteries and veins, motor and sensory nerves and share similar patterning, with vessels running in parallel alongside nerve fibers as a mutual guidance alignment.

Evidences show that axon guidance and vessel navigation are regulated by similar classes of molecules (Slits, Semaphorins, Netrins,and Ephrins) (Carmeliet and Tessier-Lavigne 2005; Carmeliet and Ruiz de Almodovar 2013).

Even if it was demonstrated that VEGF plays a role during the development of the CNS, little is known about its presence and role in PNS (Rosenstein, Krum et al. 2010). For this reason the aim of this project was the study of the expression of VEGF and receptors in three different surgical models: the crush injury and the end-to-end repair ,representing regenerating conditions, and complete nerve transaction, that reflects a condition in which no signs of axonal regrowth are found (Ronchi, Haastert-Talini et al. 2016).

VEGF mRNA expression is significantly up-regulated during the early phases after peripheral nerve crush injury whereas a strong down-regulation occurs in degenerating nerve suggesting a possible role during the regenerative process.

Furthermore, mRNA expression levels of VEGFR-2, the most implicated in migration and survival of neural and glial cell types of both CNS and PNS is highly expressed over time in crush injury; a significant decrease is detectable at 30 days after injury allowing to suppose that the involvement of VEGFR-2 is restricted to the early phases

of the regenerative process (Sondell, Lundborg et al. 1999; Jin, Mao et al. 2000; Jin, Zhu et al. 2002; Ogunshola, Antic et al. 2002).

In order to better characterize the expression of VEGF and VEGFR-2 under regenerating condition, protein expression levels were investigated after crush injury and end-to-end repair. Data obtained from western blot analysis showed a strong VEGF protein expression in control condition and following time points (1 day, 3 days and 7 days after crush injury). VEGF protein expression is also observed in end-to-end repair suggesting a similar expression pattern in the early phase of the regeneration process.

Concerning VEGFR-2 protein results show that is expressed starting from day 3 until day 30 in end-to-repair. To better characterize protein expression and localization, morphological analysis on crushed nerves were performed showing an intense immunoreactivity for VEGF around axons suggesting a glial expression of this marker while a double labelling for VEGFR-2 and β-tubulin show absence of co-localization between these two markers suggesting a glial expression also for VEGFR-2.

Interestingly, data are supported by *in vitro* analysis on Primary Schwann cells cultures that significantly increases their migration after VEG165 stimulation compared to control condition suggesting a positive effect of VEGF on glial cells migration which represents a very important step during the regeneration process.

4.3 Chitosan-based medical device for improving functional recovery after radical prostatectomy

PCa is the most common cancer among men and the surgical treatment is represented by the radical prostatectomy that can induce iatrogenic damage to the peri-prostatic nerve bundles, leading to erectile dysfunction.

Chitosan based nerve grafts are employed to promote neural repair after injury arising more and more interests among basic and clinical research.

Chitosan chemical structure (polysaccharide composed of D-glucosamine and N-acetyl-D-glucosamine linked with β (1-4) bond) gives it a remarkable hypoallergenicity, making it an excellent candidate for the development of innovative applications in the field of medicine and surgery, thanks also to its biocompatibility,

bioavailability and lack of toxicity. For these reasons, chitosan clinical applications range from orthopedic and drug-delivery systems, as scaffolds for regeneration of nerve, skin, bone and cartilage (Brown, Daya et al. 2009; Haastert-Talini, Geuna et al. 2013; Martins, Michelacci et al. 2014; Xu, Jiang et al. 2017).

In vitro and *in vivo* studies have shown that this biomaterial has biocompatible and biomimetic properties to improve the regeneration process in the PNS (Haastert-Talini, Geuna et al. 2013).

Moreover, a recent study has demonstrated the clinical efficacy and safety of the use of chitosan membranes (CS-Me) to protect NVBs following nerve sparing radical prostatectomy (Porpiglia, Bertolo et al. 2017).

Starting from these positive clinical outcome, the aim of this study was two-fold: (1) addressing the regenerative potential of CS-Me on autonomic explants ganglia; (2) rule out the possibility that a pro-regenerative biomaterial could positively influence the proliferation of prostate cancer cell lines. These two steps are discussed in the following sections.

4.3.1 Regenerative potential of CS-Me on autonomic explants ganglia.

The pro-regeneative potential of CS-Me was tested on autonomic explants ganglia cultured on CS-Me over 3 days.

The use of organotipic cultures allowed to obtain a multicellular *ex vivo* model that preserve both the cytoarchitecture and the interaction between cells conforming the tissue, providing a closer approximation to *in vivo* conditions in comparison with dissociated cell cultures (Morano, Wrobel et al. 2014; Geuna, Raimondo et al. 2016).

At this purpose, since the prostatic plexus is innervated by autonomic and somatic fibers, the regenerative potential of CS-Me was assessed through organotipic sympathetic cultures that represents an innovative and first tested model for the regenerative process unlike the widely used DRG of adult rats.

The numbers of extending neurites at certain distance from the explant body were evaluated according to the Sholl method (Torres-Espin, Santos et al. 2014). For the first time, a significant increase in term of neurites elongation of autonomic ganglia

was demonstrated, confirming the biocompatibility and the pro-regenerative capability of this biomaterial and its efficacy for the application in the urological field (Porpiglia, Bertolo et al. 2017).

4.3.2 Assessment of chitosan effect on prostate cancer cell lines: LN-Cap, Du-145 and PC3

The chitosan anti-proliferative activity against different types of cancer cells had been described, for instance, in human breast cancer cell lines (Jiang, Ouyang et al. 2011), in a human gastric carcinoma cell line MGC803 and in a human monocytic leukemia cell line (Qi, Xu et al. 2005; Salah, Michaud et al. 2013).This important effect of is particularly interesting in the view of the clinical application of the CS-Me in a site affected by a prostate cancer.

For this reason, we used different cancer cell derived from prostate adenocarcinomas in order to study the anti-proliferative effect: LN-Cap derived from lymph node metastasis, DU-145 from brain metastasis and PC3 from bone metastasis .

Interestingly, results obtained by the proliferation assay of the different tumor lines cultured both directly on a chitosan surface and on chitosan dissolution products showed a lower proliferation compared with control condition (Brown, Daya et al. 2009; Haastert-Talini, Geuna et al. 2013; Fregnan, Ciglieri et al. 2016).

This study, conducted to better understand the potential of CS devices such as CS-Me, used to protect the peri-prostatic plexus following radical prostatectomy, confirm two important CS effects: the capability to negative influence the proliferation of prostate cancer cell lines and at the same time, exhibits a pro-regenerative effect on autonomic ganglia neuritis elongation, making its application in the urological context safe and effective.

5. CONCLUSIONS

Unlike the CNS, sensory and motor axons of the PNS are characterized by spontaneous regeneration after injury. This is mainly due to the permissive extracellular environment, characterized by the presence of growth factors, extracellular matrix components, and SCs, that influence and promote peripheral axons regeneration (Tucker and Mearow 2008).

During my PhD I addressed several important issues regarding the study of the plasticity of the peripheral nervous system at axonal and neuronal levels. This is particularly interesting because the occurrence of adult plasticity and generation of new neurons in site of PNS is controversial.

In the first chapter of the thesis the study of this phenomenon was referred to the morphological and stereological changes occurring in the sensory neurons of DRG. The choose of a correct stereological methods allowed to state an increment in the neuronal population as a consequence of peripheral nerve injuries while the morphological characterization with neuronal progenitors markers lead the hypothesis about the source of new generating neurons starting from dedifferentiation of satellite glial cells.

In the second part of the thesis the important aspect of long-term regeneration was assessed since the regeneration process and the functional recovery after injury of peripheral nerves is often unsatisfactory. Result obtained are interesting from both the biological and the clinical perspectives showing that the ability of the regenerated peripheral nerve fibers to return spontaneously to their normal pre-trauma state with a sufficient long recovery time post axonotmesis is not supported by the stereological data.

In the third part different experiments concerning the modulation of VEGF and its family members including VEGFR-1, VEGFR-2, VEGFR-3, NRP1 and NRP2 were carried out under degenerating and regenerating conditions showing a modulation of these molecules. VEGF is a endothelial cell mitogen promoting angiogenesis, proliferation, vasculogenesis and increases blood vessel permeability.

The aim of the work presented in this thesis was to carry out biomolecular and immunohistochemical analysis on rat median nerve experimental models in order to analyze mRNA and protein expression and the morphological localization of VEGF and its family members.

Results show a modulation of VEGF during the regenerative process and an increase in SCs migration after *in vitro* stimulation with VEGF displaying its possible involvement during the early phase of the regenerative process.

In the last chapter of this work I would to point out the importance to improve the functional recovery in patient undergo radical prostatectomy. Erectile dysfunction due to damage of peri-prostatic nerves represents an important impairment for these patients, and despite the advancement of the surgical technique such as nerve-sparing robot assisted radical prostatectomy that allow to preserve the peri-prostatic nerves, the percentage of this clinical condition is still considerable. For this reason the development of new strategies such as the application of chitosan membrane as a potential tool to improve the regeneration of peri-prostatic nerves is a available alternative thank to suitable characteristics of this biomaterial.

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