REVIEW

Peripheral nerve regeneration and intraneural revascularization

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Funding: *This work was supported by a doctoral fellowship from the 'Conseil Régional du Limousin' to MC.*

Abstract
Peripheral nerves are particularly vulnerable to injuries and are involved in numerous pathologies for which specific treatments are lacking. This review summarizes the pathophysiological features of the most common traumatic nerve injury in humans and the different animal models used in nerve regeneration studies. The current knowledge concerning Wallerian degeneration and nerve regrowth is then described. Finally, the involvement of intraneural vascularization in these processes is addressed. As intraneural vascularization has been poorly studied, histological experiments were carried out from rat sciatic nerves damaged by a glycerol injection. The results, taken together with the data from literature, suggest that revascularization plays an important role in peripheral nerve regeneration and must therefore be studied more carefully.

Key Words: *compression; crush; transection; Sunderland's classification; Wallerian degeneration; angiogenesis; traumatic; glycerol*

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doi: *10.4103/1673-5374.243699*

Received: *August 13, 2018*
Accepted: *August 31, 2018*

Introduction
The peripheral nervous system includes motor, sensory and autonomic neurons of spinal and cranial nerves, as well as roots, trunks, ganglia and plexus (Vallat et al., 2009). Peripheral nerves are particularly vulnerable to injuries and are an important source of diseases, playing a role in various pathological processes. Peripheral neuropathies are heterogeneous, including traumatic and non-traumatic injuries (such as genetic, metabolic, deficiency-related, infectious, immune, and drug-induced neuropathies). Moreover, the severity of damage is variable (Martyn and Hughes, 1997). Peripheral neuropathy includes symmetric polyneuropathy, single and multiple mono-neuropathies, and radiculopathies (Martyn and Hughes, 1997). Currently, the pathophysiology of peripheral nerve injuries and the mechanisms involved in spontaneous regeneration are relatively well understood. However, to date, there is no treatment that significantly improves nerve repair after injury and therefore, the use of preclinical models remains essential. Revascularization appears to be a key factor in tissue repair in many other organs (Ferretti et al., 2003). However, little is known about the role of this process in peripheral nerve repair. The aim of the present review is therefore to assess current knowledge on the mechanisms involved in the regeneration of peripheral nerves and on the animal models available, with a focus on the role of vascularization.

We performed a literature search on rodents and humans in Pubmed and google scholar during 1946 to June 2018, which published in English or French. The key words/terms are Seddon's classification, Sunderland's classification, compression, crush, transection, Wallerian degeneration, nerve regeneration, nerve vascularization, intraneural vascular system, revascularization, peripheral nerve angiogenesis and blood-nerve barrier (**Table 1**).

Table 1 Database search strategy

Database	Pubmed, google scholar
Date	1946 – June 2018
Eligibility criteria	Studies conducted on rodents and humans, and published in English and French
Keywords/ keyterms	Seddon's classification, Sunderland's classification, compression, crush, transection, Wallerian degeneration, nerve regeneration, nerve vascularization, intraneural vascular system, revascularization, peripheral nerve angiogenesis, blood-nerve barrier

Pathophysiology of Traumatic Nerve Injuries
The most common peripheral neuropathies are traumatic nerve injuries. In the 1940s, Seddon (1942) published a classification of nerve damage severity based on his observations of trauma cases. In this classification, three levels were defined: neuropraxia, in which the compression injury rapidly repairs; axonotmesis, in which the sheath of the nerve is preserved but a loss of axon continuity is observed; and neurotmesis, in which a complete section of the nerve occurs. Later, Sunderland (1951) refined Seddon's classification into five categories, based on histopathological features rather than on the severity of injury. Furthermore, he added electrodiagnostic and clinical criteria linked to the capacity for regeneration with or without surgical intervention. Finally, Sunderland's classification included 5 degrees of severity (**Figure 1**). Grade I and grade II correspond to the definition

proposed by Seddon (1942), *i.e.*, grade I: neuropraxia and grade II: axonotmesis. In grade III, axons and endoneurium are damaged but not the perineurium. In grade IV, axons, endoneurium and perineurium are damaged but the epineurium is preserved. Finally, grade V correspond to neurotmesis (Seddon, 1942), *i.e.*, complete nerve transection. Later, a grade VI was proposed by Dellon and Mackinnon (1988), which corresponds to the presence of different grades of injury along the same nerve. In the next sections, the different grades of nerve damage described by Sunderland (1951) will be discussed in view of more recent studies.

Grade I

Grade I most often corresponds to peripheral nerve compression. This type of injury is caused by direct pressure on a nerve trunk or root compression (Sunderland, 1951). Moreover, nerve compressive injuries most commonly affect nerves that cross over bony surfaces or pass between rigid structures. Nerve compression syndrome, also named compression neuropathy, can be cited in a number of disorders, for example, cubital tunnel syndrome (ulnar nerve) (Lauretti et al., 2017), carpal tunnel syndrome (median nerve) (Wahab et al., 2017), meralgia paraesthetica (lateral cutaneous nerve) (Khalil et al., 2012), and the tarsal tunnel syndrome (tibial nerve) (Doneddu et al., 2017). Similar lesions are found in sciatica syndrome, which is mostly due to compression of the sciatic nerve by a herniated disc (Markman et al., 2018). Compression neuropathies may also occur in patients who hold a single position for an extended period. These injuries include the "Saturday night palsy", in which the upper arm of a patient is injured, usually as a result of sleeping with the affected arm over the back of a chair. A similar problem can be recognized during the postoperative setting when a limb is held in the same position for a prolonged time (Burnett and Zager, 2004). It is known that mechanical compression may lead to secondary ischemic injury. When the intra-epineural pressure exceeds intra-arterial pressure, nerve ischemia results in axon-loss lesions of variable severity. These two pathological mechanisms, mechanical compression and ischemia, are believed to be involved in grade I injury. Grade I injury is accompanied by morphological alteration of the myelin sheath or segmental demyelination. Electrophysiological examination may reveal demyelinating conduction blocks and sometimes axonal loss. Grade I is clinically associated with a, most often transient, deficiency of motor function, sometimes leading to neuropathic pain. Generally, there is not total loss of both motor and sensory functions, because complete nerve continuity is maintained (Burnett and Zager, 2004).

Different models have been used to study degenerative/regenerative mechanisms involved after grade I and to evaluate potential therapies.

The oldest compression-induced neuropathy model used was direct pressure applied to the sciatic nerve of cats, brought about by use of a bag or by a tourniquet (Denny-Brown and Brenner, 1944a, b). Later this compression-induced neuropathy model was also applied to baboons (Fowler et al., 1972; Ochoa et al., 1972). In this compression model, there is a demyelination of the nerve fibers that then leads to a block of peripheral nerve conduction with a moderate axonal loss. More recently, chemical models have been developed to mimic focal demyelination. For instance, it has been shown that an intraneural injection of phenol into rabbit sciatic nerve induces conduction blocks (Sung et al., 2001). Perineural injection of lysophosphatidylcholine (LPC) was also demonstrated to cause focal demyelination in rodent nerves (Hall and Gregson, 1971). Cold injury models have also been reported in which non-freezing cold injuries caused local failure of nerve conduction at the site of cooling (Kennett and Gilliatt, 1991). However, neuropathy models, induced by chemical products or cold injury, are difficult to compare with mechanically-induced neuropathy since the mechanisms involved are certainly different. Bennett and Xie (1988) developed an animal nerve injury model that appears to mimic many features of neuropathic disorders that occur in humans. The injury is caused, in rodents, by a loose ligation of the sciatic nerve termed chronic constriction injury (CCI). The animals display a block of nerve conduction with a moderate axonal loss, altered spontaneous behavior associated with neuropathic pain and allodynia to thermal and mechanical stimuli.

Grades II, III and IV

Grades II, III and IV are more severe than grade I. As mentioned above, the different grades described in Sunderland's classification depend on the severity of the lesion, with loss of axon continuity and demyelination (grade II), with damage of the endoneurium (grade III) and the perineurium (grade IV) (Sunderland, 1951). Grade II, III and IV types of injuries include different causes of lesions, *i.e.*, crush and stretch. In the following section, we will discuss the mechanisms involved in crush and stretch damage and the animal models that mimic these two types of lesions.

Nerve crush injury is caused by a sudden significant force applied to the nerve, for instance with a blunt object. These lesions can also be induced by a surgical clamp during surgery, by blows resulting from an assault, or by a vehicle impact or accident (Martyn and Hughes, 1997). There are different severities of crush injury, but axon loss is consistently observed. Functional recovery in humans is very heterogeneous and may require microsurgery. However, functional recovery is generally complete, though often requiring considerable time. Pathophysiologically, nerve crush produces an interruption of the continuity of axons leading to Wallerian degeneration. However, there is no interruption of the nerve connective tissue scaffold and, for that reason, regrowth of the nerve fibers is easier and thus allows for better functional recovery (Geuna et al., 2009). However, formation of a traumatic neuroma is frequently observed at injury sites, which can limit or even prevent nerve regrowth.

The nerve crush model is commonly used by researchers studying peripheral nerve regeneration. Generally, experiments are performed on the sciatic nerves of rodents using serrated or non-serrated surgical forceps to inflict the injury. However, various other methods have been described for producing the crush injury. There is therefore a strong heterogeneity in studies, mainly due to the use of different surgical instruments (Chen et al., 1993; Kingery et al., 1994; Savastano et al., 2014). Beer et al. (2001) described the use of a non-serrated clamp aimed at standardizing the pressure exerted to the nerve. This device has provided good reproducibility in different rodent species (Beer et al., 2001; Varejão et al., 2004a, b). In animal models, it is usually accepted that inter-individual variability in tissue regeneration and functional recovery is limited. This feature makes the sciatic nerve crush injury model particularly suitable for the study of the biology of peripheral nerve regeneration as well as for the development of treatment strategies to improve it. However, spontaneous axon regeneration, observed in laboratory animals after crushing the nerve, does not often occur in humans due to frequent extensive fibrosis that is observed at the lesion site. Therefore, in many severe cases, crush lesions in humans require surgery to remove the damaged tissue and replace it with a conduit (Tos et al., 2012). A chemical model has also been developed to mimic grades II and III. For example, chemical neuropathy using an intraneural injection of glycerol into the sciatic nerve of rats showed a complete destruction of nerve fibers and the myelin sheath but maintenance of the connective scaffold, including perineurium and epineurium (Vallat et al., 1988). This model, which was used in the present study to evaluate vascularization during nerve regeneration, will be described in greater detail below.

Peripheral nerves are also vulnerable to excessive stretch. As the stretching force increases, the elastic properties of the nerve are surpassed, and myelin sheaths, axons, and some of the connective tissue may rupture. Experimental nerve stretch studies have shown that an elongation by 8%, of the rat sciatic nerve, increases intraneural pressure and reduces the blood flow by half without reducing nerve conduction velocity (Driscoll et al., 2002). An example of stretch injury in humans is neonatal Erb-Duchenne palsy, a paralysis of the arm that most commonly arises from shoulder avulsion of the brachial plexus during a complicated or difficult birth (Teixeira et al., 2015). In humans, another stretch injury may be produced by a heavy object falling onto a person's shoulder; this results in avulsion of the brachial plexus, and pulling out of the spinal cord rootlets. These avulsion injuries are most often due to vehicle accidents. Avulsion is associated with particularly severe and long-lasting neuropathic pain. In accordance with this feature, experimental models of brachial plexus avulsion show bilateral mechanical and cold allodynia, that last significantly longer than that observed in chronic constriction injury (grade I model) and crush models (Rodrigues-Filho et al., 2003).

Grade V

Grade V, according to Sunderland's classification, is the most severe nerve injury. In this type of injury, there is a total loss of nerve trunk continuity (Sunderland, 1951). The most frequent etiology is transection or laceration of peripheral nerves. Transection injuries are caused by a cutting object (for example, knife wounds, broken glass, metal shards, chainsaw blades, wood splinters, and animal bites) (Martyn and Hughes, 1997). Another example is the inadvertent transection of spinal nerve branches during a surgical intervention. While partial recovery may occur, full spontaneous recovery is impossible. In general, as in the skin and other tissues, the sharper the object that cuts, the neater the injury and the better is recovery. Furthermore, the likelihood of satisfactory functional recovery is greater when the distance between proximal and distal nerve stumps is shorter. In humans, a nerve defect requires the implantation of an autograft using, for example, a segment of a sensory nerve, usually the sural nerve (Berger and Millesi, 1978). Another solution, in the case of tissue loss, is the implantation of tubular reconstruction using either biological materials (biological nerve guides, various autologous tissues, bio-functionalized bio-materials) and/or synthetic materials (silicon, poly ε-caprolactone, chitosan…) (Tos et al., 2012; Haastert-Talini et al., 2013; Reid et al., 2013; Johansson and Dahlin, 2014; Stößel et al., 2018).

In laboratory animals, axonal regeneration occurs spontaneously after complete nerve transection (after suture). Hence, in order to mimic nerve repair in humans, researchers turned to the proximal nerve stump, suturing it to a neighboring stump. In the case of the sciatic nerve, complete transection that is not followed by surgical repair induces the loss of motor function in the ipsilateral paw. Furthermore, sciatic nerve transection also induces loss of sensory function of the paw, explaining the self-mutilation frequently observed in the early post-operative stages. The experimental transection model is a very useful approach to study denervation of distal nerve trunks and the consequences on target skeletal muscles, as well as for studying strategies to prevent muscle atrophy (Karsidag et al., 2012; Moimas et al., 2013; Blom et al., 2014; Gordon and Borschel, 2017). Since alterations in both the distal nerve segment and target muscle occur very early (1 week) after injury, the transection model can be used to study both early and late events (3 to 6 months). In addition, sciatic nerve transection can be used to study direct suture repair (end-to-end neurorrhaphy) (Geuna et al., 2009). If no tissue loss occurs, sciatic nerve transection can be used in rodent models to study tissue glues (Félix et al., 2013) and strategies for reducing post-surgical scar tissue formation (Que et al., 2013). However, even if spontaneous regeneration occurs, a nerve graft may still be necessary to avoid tension or stretch in the nerve, a condition which might limit regeneration and functional recovery (as mentioned above) (Battiston et al., 2009; Siemionow and Brzezicki, 2009).

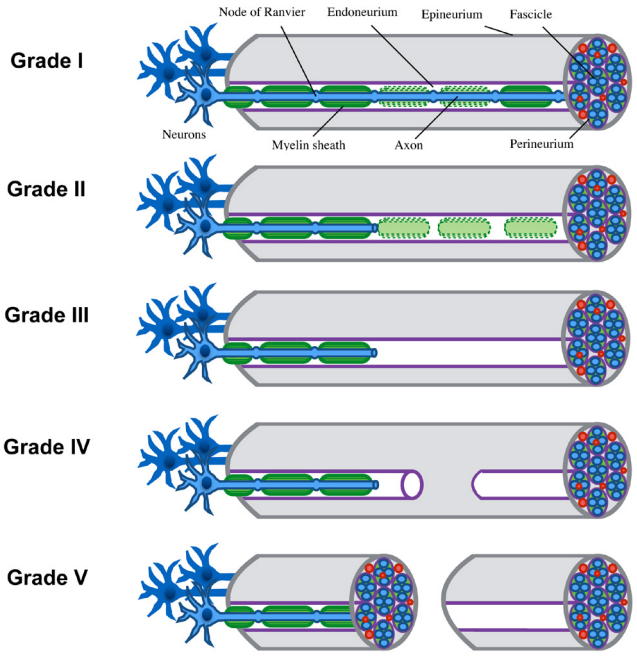

Figure 1 Sunderland's classification of nerve injuries.
Grade I: Temporary interruption of myelin sheath without loss of axonal continuity. Grade II: Loss of continuity of the axon and its myelin sheath, the various connective tissues of the nerve (endoneurium, epineurium and perineurium) are preserved). Grade III, IV and V: Partial (III, IV) or total (V) section of the nerve. A distinction is made between grade III (in which the axon and the endoneurium are damaged but not the perineurium), grade IV (in which the axon, the endoneurium and the perineurium are damaged but the epineurium is preserved) and grade V (in which the nerve is transected). Adapted from Menorca et al. (2013).

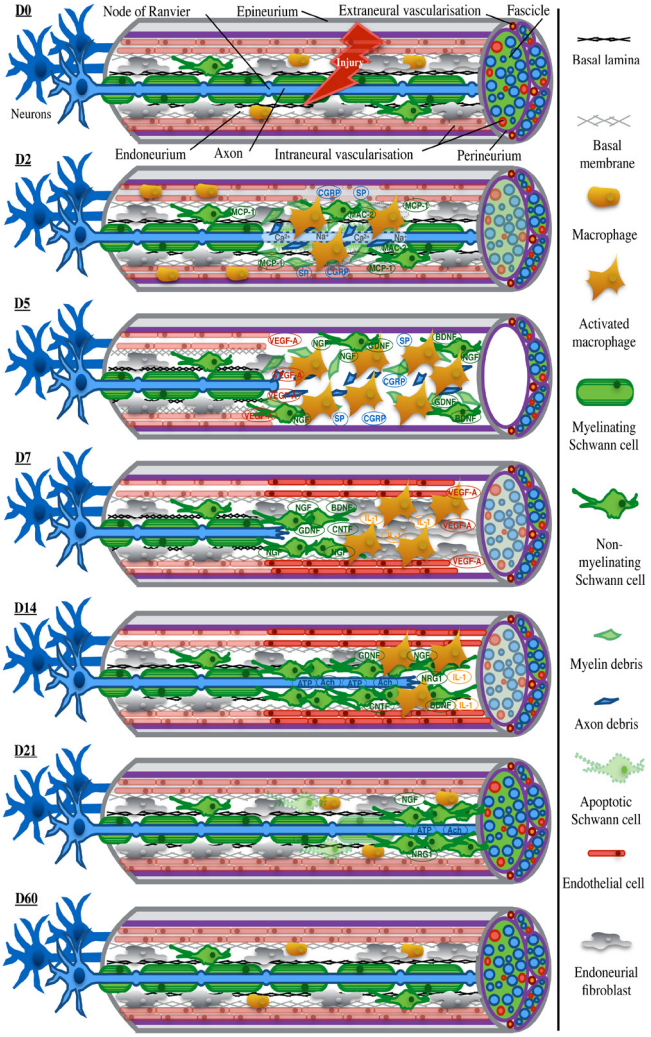

Figure 2 Schematic representation of Wallerian degeneration and nerve regeneration.
At the time of nerve injury (D0), surrounding cells (Schwann cells and endoneurial fibroblasts) are immediately damaged and die by apoptosis. At two days after injury (D2), axons degenerate, notably due to the action of Ca^{2+} and Na^{+} release. Concomitantly, neuropeptides released by damaged axons, such as substance P (SP) and calcitonin gene-related peptide (CGRP), induce the swelling of intraneural blood vessels. This vasodilatation, coupled with the release of monocyte chemoattractant protein-1 (MCP-1) by Schwann cells, stimulates recruitment of migrating and resident macrophages. These macrophages phagocytose axonal and myelin debris and release vascular endothelial growth factor (VEGF)-A, leading to the formation of neovessels (D7). At the same time, intact Schwann cells secrete nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF) and glia-derived neurotrophic factor (GDNF) which stimulate the formation of new Schwann cells. Oxygen and nutrients supplied by neovessels are required for the formation of "bands of Büngner" that then form a physical guide for axonal regrowth (D14). The release of adenosine triphosphate (ATP) and acetylcholine (Ach) by axons allows their self-stimulation (D14–21). Two months after injury, the general appearance of the nerve is almost normal, although some nerve fibers still have a thin sheath of myelin (D60). MAC-2: Galectin-3.

Nerve Degeneration and Regeneration

Wallerian degeneration

Traumatic peripheral neuropathies are usually characterized by section of the axon which is then disconnected from its cell body. Axonal injury produces a complete loss of nerve conduction velocity, which in turn induces muscle weakness and loss of sensation, but also leads to neuropathic pain and adaptive responses (Stoll and Müller, 1999). In addition, nervous tissue damage can be deleterious for patients because recovery is not always complete, with the formation of scar-like tissue impairing optimal limb functionality. If the injury is large or close to the cell body, it can induce neuronal death. However, if the nerve section or the crush injury is distant from the neuron cell body, nerve fibers can regenerate. The degeneration of the distal nerve ending is known as Wallerian degeneration (**Figure 2**). Wallerian degeneration is a unique and structured form of axon degeneration (Stoll et al., 1989). In the first stages of Wallerian degeneration, axonal and myelin debris are produced. Resident macrophages in the nerve tissue then differentiate into activated-macrophages which can phagocytose cellular debris. Moreover, the surrounding Schwann cells (SCs) are a source of monocyte chemoattractant protein-1 which leads to the recruitment of circulating monocytes (Toews et al., 1998). In axons, activation of mRNA translation is observed in the proximal stumps. This increased translation stimulates the formation of the protein complex, importin-phosphorylat-

ed extracellular regulated protein kinase 1/2 vimentin. This complex is transported by the motor protein dynein in a retrograde direction to the cell body and this signal informs the neuron of the axonal damage (Yudin et al., 2008). The neuron then reacts by increasing its volume and by breaking up aggregates of rough endoplasmic reticulum (Nissl bodies), which promotes protein synthesis. In addition, within the first hours after injury, analysis of spinal cord tissue has shown that messenger RNAs for antioxidant proteins (Nrf2, glutathione reductase and heme oxygenase 1) are increased after, in this case, sciatic nerve crush (Renno et al., 2017). The ending of the cut axon rapidly closes and swells because of the accumulation of substances that arrive from the neuron cell body. Only a few hours after the nerve injury, the growing axonal extremity extends filipodia. At first, these are randomly oriented but thereafter, they gain unidirectionality, once the expression of myosin and actin are upregulated within the neuron cell body (Marx, 1995). Concomitantly, regeneration and reorganization of blood vessels accompany the growth of axons. This specific and important aspect of the response to damage will be further discussed below.

From two days after injury (D2), circulating monocytes differentiate into activated-macrophages and contribute to the removal of axonal and myelin debris, a process persisting at least for 2 weeks (Taskinen and Röyttä, 1997; Stoll and Müller, 1999). In addition, phagocytosis is dependent on the galactoside-binding protein of macrophages galectin-3, which supports the elimination of myelin debris by SCs themselves (Reichert et al., 1994; Rotshenker, 2009). In early stages, damaged SCs are involved in protein expression changes at the site of injury. The loss of contact between SCs and damaged axons induces retraction of the myelin sheath and leads to the formation of ovoid structures. These ovoid structures appear two days after a lesion and persist for at least 2–3 weeks (Stoll et al., 1989; Reynolds et al., 1994). In parallel with the loss of axonal contact, SCs progressively acquire a non-myelinating phenotype and become proliferative. Marked down-regulation of the expression of several proteins, including peripheral myelin protein 22 (PMP22), myelin protein zero (MPZ or P0), connexin-32 and transcription factor Krox-20 has been reported (Topilko et al., 1994; Parkinson et al., 2008).

Nerve regeneration

Successful peripheral nerve regeneration after injury relies on both injured axons and non-neuronal cells, including SCs, endoneurial fibroblasts and macrophages. Together, these produce a supportive microenvironment allowing successful regrowth of the proximal nerve fiber ending (**Figure 2**).

Apart from their roles in phagocytosis of debris, macrophages also secrete interleukin (IL)-1 which induces secretion of nerve growth factor (NGF) by SCs, promoting autocrine-stimulated growth and proliferation of SCs (Ronchi et al., 2009). SCs have a peak in mitotic activity at 3–4 days after injury. This event is instrumental in promoting axonal regeneration. Indeed, macrophages are also involved in the production of factors that are mitogenic for SCs and fibroblasts, such as transforming growth factor (TGF)-β and insulin-like growth factor (IGF) (Baichwal et al., 1988; Zochodne, 2000). Finally, macrophages, in damaged nerves, help to rescue valuable cholesterol and especially, produce apolipoprotein E, enabling future myelin reconstruction (Weinberg and Spencer, 1978; Baichwal et al., 1988; Rotshenker, 2003).

In neurons, the production of proteins associated with growth including brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), growth-associated protein (GAP)-43 and c-jun, and of neuropeptides such as calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide (VIP), substance P and somatostatin is increased (Zochodne, 2000). Conversely, neurofilament synthesis and release of neurotransmitters (acetylcholine) are repressed (Fu and Gordon, 1997). Thus, from D3 to D7 post-injury, a growth cone is formed at the ending of the proximal axonal stump. It has been found that calcium plays a major role at the proximal stump in promoting growth cone formation (Geraldo and Gordon-Weeks, 2009). Neurotrophins play a central role in attraction and repulsion of the filipodia. For example, semaphorins, ephrins, and netrins are involved in the guidance of regrowing axons. In contrast, molecules, such as collapsin 1, which promotes growth cone collapse, inhibit the guidance of regrowing axons (Goodman, 1996; Tuttle and O'Leary, 1998). The development of the growth cone may also be inhibited by scar tissue (mainly composed of collagen) and thus, to counteract this, axonal growth cones release proteases and plasminogen activators favoring the degradation of this scar tissue (Geraldo and Gordon-Weeks, 2009). These proteases and plasminogen activators also help to clear any cell-cell or cell-matrix interactions from non-neuronal cells that are hindering progression of the growth cones (Geraldo and Gordon-Weeks, 2009). SCs are the main source of neurotrophic factors which interact with tyrosine kinase receptors to modify the neuronal gene expression profile in order to promote axonal growth. NGF is responsible for promoting axonal growth and SC proliferation (Lunn et al., 1990). At this stage, the SC nuclei become more rounded and their content in heterochromatin is reduced (Ide, 1996). SCs and endoneurial fibroblasts migrate and proliferate at the lesion site to form the neuroma, a tissue bridge that attempts to restore the junction between the proximal and the distal segments. In the distal segment, four days after cutting of the sciatic nerve in the rat, proliferating SCs are aligned in columns forming "bands of Büngner" that are involved in endoneurial regeneration (Burnett and Zager, 2004). Bands of Büngner are linear bands of interdigitating SCs that form a physical guide for axonal regrowth. Neurotrophic factors (*e.g.*, semaphorins, ephrins, netrins...) increase cell survival, and also promote the secretion of regenerating factors (*e.g.*, ciliary neurotrophic factor [CNTF], BDNF, GDNF, and NGF) in neurons and in SCs. Furthermore, SCs produce

neurite-promoting proteins such as fibronectin and laminin, which are incorporated into the extracellular matrix (ECM). Growth cones utilize these proteins for adhesion to the basal lamina (a layer of ECM secreted by SCs, on which the SCs sit) of the endoneurium (Lunn et al., 1990).

Recently, it has been shown that crush injury of the sciatic nerve produces generalized oxidative stress with a significant increase in isoprostanes found in the urine and a decrease in the total antioxidant capacity of the blood from D7 until D14 after injury (Renno et al., 2017).

Three to four weeks after injury, maturation needs to occur before the functional connection is complete. The maturation process includes remyelination, axon enlargement, and finally, functional re-innervation. SC proliferation is then arrested and the production of C-jun promotes differentiation; furthermore, the secretion of neurotrophic factors such as NGF and CNTF occurs (Elfar et al., 2008). The outgrowing axonal endings produce neuregulin-1 (NRG1) type III and I, ATP and acetylcholine, which thus promote the change in the SC phenotype from a non-myelinating phenotype to a myelinating phenotype (Birchmeier and Nave, 2008; Vrbova et al., 2009). In rodents, at five weeks after injury, the general nerve morphology observed using light microscopy appears normal. However, using electron microscopy, a depletion of nerve fiber number (both myelinated and unmyelinated) and the development of small clusters of three or four regenerating axons called "clusters of regeneration" are observed. In addition, macrophage invasion in myelin sheaths has been observed (Vallat et al., 1988). At the molecular level, a proteomic study has shown that five weeks after crush injury in rats, the expression of proteins involved in signaling and metabolism in nerves (aldehyde reductase 1, annexin V, alpha-crystallin B, dimethyl argininase 1, sialic acid synthase, periaxin, ubiquitin C-terminal hydrolase...) are decreased (Jiménez et al., 2005). In contrast, the expression of proteins involved in lipid metabolism (apolipoprotein D, apolipoprotein E, cathepsin B...) and of those involved in cellular processes of maturation and degradation enabling future myelin reconstruction (galectin 3, tropomyosin 4-alpha, tropomyosin 3-gamma...) are up-regulated (Jiménez et al., 2005). These proteomic data indicate that axonal regrowth is complete and that the maturation phase, *i.e.*, remyelination of fibers, is beginning.

Finally, five weeks after sciatic nerve injury in rats, an increase in reactive oxygen species (ROS) and lipoperoxidation has been reported. In nerves, the increase in ROS has been attributed to an increase in myeloperoxidase, a pro-oxidant enzyme secreted particularly by macrophages (Caillaud et al., 2018).

Three months after injury, nerve fibers appear almost normal. However, the endoneurium is still found to be divided into many micro-compartments by a profusion of fibroblasts that gradually differentiate into perineurial cells. Normal perineurium also regenerates within this time. This micro-compartmentalization slowly develops from the periphery. After several months, small newly formed fascicles at the periphery of the original sciatic fascicles are observed (Vallat et al., 1988).

Revascularization During Peripheral Nerve Regeneration

It is well-accepted that angiogenesis and reinnervation, arborization and growth are processes that are intimately connected. For example, Ferretti et al. (2003) have shown, in a model of transplanted human skin equivalents, that vascularization precedes the process of innervation, indicating that the development of nervous tissue is driven by nutritional and trophic factors that are provided by the vascular system. While some authors evidenced that an adequate intraneural vascularization is found concurrently with the regeneration of nerve (Merolli et al., 2009; Goedee et al., 2013), little is known about the specific role of revascularization in nerve regeneration. Thus, the purpose of this review is to revive interest for studying intraneural vascularization after nerve damage.

During embryogenesis, blood vessels and nerves travel together to respectively innervate and supply almost every tissue in the body. Recent genetic insights have shown that they often use common genetic pathways to differentiate, grow and navigate towards their organ targets (Brunet et al., 2014). As an example, netrin-1 has been identified as an essential factor required for axon growth (Madison et al., 2000). In addition, it has been demonstrated that netrin-1 is produced by arterial smooth muscle cells and plays a pro-angiogenic role (Castets and Mehlen, 2010). Furthermore, arterial vessel innervation requires the interaction between netrin-1 and its receptors that are expressed on sympathetic growth cones (Brunet et al., 2014). The vascular and nervous systems thus continuously share molecular tasks in order to function harmoniously. Indeed, recent studies show that, as well as influencing vascular tone, sympathetic nerves may also play a role in arterial maturation and growth (Eichmann and Brunet, 2014). In addition, it has been demonstrated that the repulsive axon guidance molecules, semaphorin-3A, neuropilin-1, and plexin-A1, are required for formation of the lymphatic vasculature (Bouvrée et al., 2012).

Vascularization of peripheral nerves is divided into two longitudinal systems (**Figure 3**). The first one is the extraneural vascular system which is peri-fascicular, connected to the external vascular system by supplying branches (Reinhold and Rittner, 2017). The second one is the intraneural vascular (INV) system, which is intra-fascicular and composed of arterioles, venules and capillaries (Reinhold and Rittner, 2017). The vascular component and the nerve tissue are separated by a blood-nerve barrier (BNB). In nerves, two structures are essential for the formation of this BNB: the perineurium and endoneurium (Mizisin and Weerasuriya, 2011). The perineurium has mainly mechanical functions, whereas the endoneurium controls exchange between both sides. The perineurium wraps around the fascicle in several

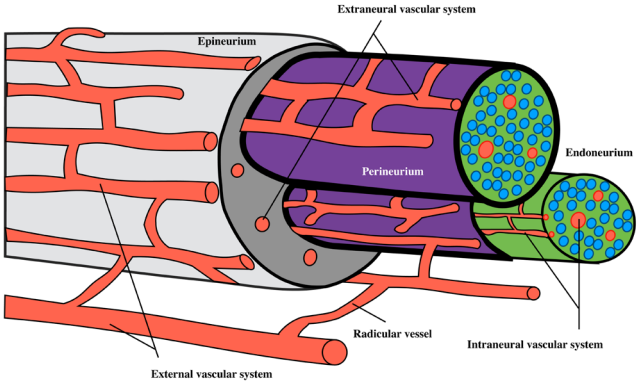

Figure 3 The vascularization of peripheral nerves.
Microcirculation of peripheral nerves derives from external vascular system from which emerge radicular branch vessels supplying the internal vascular system. Internal vascular system consists of longitudinally oriented peri-fascicular vessels that pass through epineurium (extraneural vascular system), reach the perineurium and ultimately join the endoneurium (intraneural vascular system). Adapted from Mizisin and Weerasuriya (2011).

lamellar layers in which each layer involves one fibroblastic cell (and overall there are roughly seven to eight concentric layers). Every layer is covered by a basal lamina, on which myofibroblasts sit, and this organization accounts, in the main, for the stretch compliance properties of the nerve. The endoneurium is composed of endoneurial fibroblasts, glial cells and a dense matrix of collagen tissue around the myelin sheath of each myelinated nerve fiber (Mizisin and Weerasuriya, 2011). The endoneurium also contains endoneurial fluid, similar to the cerebrospinal fluid of the central nervous system. Regulation of endoneurial fluid homeostasis is maintained by endothelial cells of the INV system. At the molecular level, the endothelial cells are maintained by "junctional intercellular complexes" composed notably by claudin, occludin, cadherin, *etc*. The exchanges between blood and endoneurium are carried out by carriers such as gucose transporter-1, monocarboxylate transporter-1, creatine transporter, Na^+-independent L-type amino acid transporter 1, *etc*. (Ubogu, 2013). The last component is the myelin sheath which has both nutritive and protective functions for axons (Reinhold and Rittner, 2017).

Recently, several studies have pointed to the link between the alignment of nerves and blood vessels in the skin, as well as their reciprocal maturation (James and Mukouyama, 2011). However, whereas most of these studies focused on the impact of the nervous system on the vascular network, few studies have investigated how the INV system develops and aids axonal regrowth after peripheral nerve damage (Almgren, 1975; Penkert et al., 1988; Goedee et al., 2013). This is a principal issue since the permanent oxygenation and supply of peripheral nerves play a pivotal role in nerve development, homeostasis and regeneration. For example, it has been demonstrated that there is a close link between peptidergic perivascular unmyelinated nerve fibers and blood vessels (Zochodne, 2000). Indeed, these fibers not only transmit nociceptive information, but they also release their peptides locally in the case of nerve injury. One such peptide, substance P (SP), acts as a local vasodilator, and also induces mast cell degranulation with release of histamine, serotonin, and proteolytic enzymes which promote angiogenesis (Qin et al., 2013). CGRP is a more potent vasodilator than SP and appears to be correlated with angiogenesis (Zochodne, 2000). Vasodilation is essential in the recruitment of macrophages following nerve damage, thus allowing phagocytosis of axonal and myelin debris (Stoll et al., 1989). The peripheral nerve vascularization therefore has an important place in peripheral neuropathies, even more so in the context of the sensitivity of blood vessels to mechanical stress, such as compression and stretching. In addition, impaired vascularization has also been frequently described in peripheral neuropathies of both toxic and diabetic origin (Windebank and Grisold, 2008; O'Brien et al., 2014).

Currently, there is only fragmentary data on the roles of vascularization in nerve regrowth. To better evaluate these roles, we have analyzed vascular regrowth on peripheral nerve sections damaged by a glycerol injection (Vallat et al., 1988). Analyses of sciatic nerves at D2, D7, D14, D21 and D60 after injury were carried out using electron microscopy images of ultrathin sections (**Figure 4**). Results of observations at D2 show that pre-existing blood vessels are ruptured. Erythrocytes and platelets can be observed outside the vessels. However, neo-capillaries are already present, indicating the onset of neo-angiogenesis at two days. Neo-angiogenesis is a multistep process of critical importance both in development as well as in physiological and pathophysiological processes in adults. It involves endothelial cells, sprouting from the parent vessel, followed by their migration, proliferation and alignment to form a tube with anastomoses to other vessels (Nakatsu et al., 2003). At D7 after the injection of glycerol, a profusion of fibroblasts with thin filopodia, associated with an abnormal abundance of collagen fibers, is observed. These fibroblasts are known to gradually differentiate into perineural cells, which are characterized by the presence of numerous pinocytotic vesicles and a discontinuous basal membrane. Indeed, an increase in perineural cells is associated with proliferation of the basal membrane which is observed, in our study, at D14 and D21. In addition, we can note the presence of arterioles surrounded by several neo-capillaries. These observations are in favor of a significant level of angiogenesis and thus, of a regeneration of the INV system. At D60, blood vessels with a normal appearance are observed, thus indicating a complete regeneration of the INV system.

These data are consistent with previous studies on vascular regrowth in animal models of sciatic nerve crush injury and transection (Podhajsky and Myers, 1993, 1994). The vascular response to crush and transection injury is composed of two phases. The early phase comprises the first week after injury and is characterized by an increase in blood vessel size, with the blood vessel number remaining unchanged. Then, during the second phase, from 1 to 6 weeks after injury, an increase

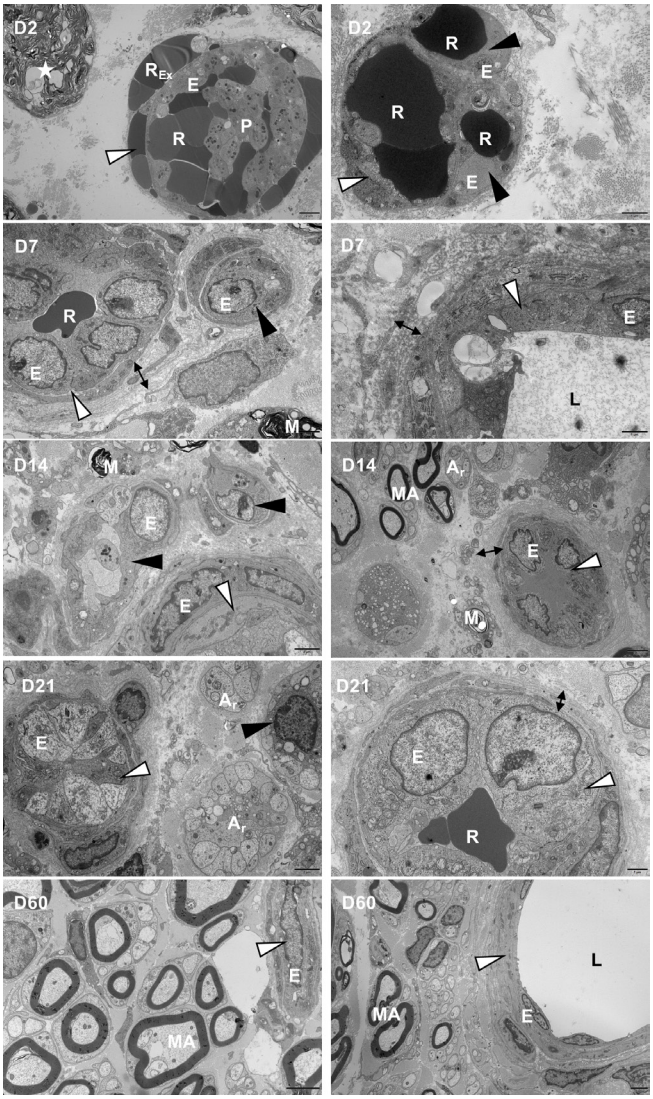

Figure 4 Electron microscopy images of rat sciatic nerve after glycerol injection.
Analyses of sciatic nerve at 2, 7, 14, 21 and 60 days after injury (D2, D7, D14, D21 and D60), were carried out using electron microscopic images of ultrathin transverse sciatic nerve sections. Briefly, nerve samples were collected and fixed in 2.5% glutaraldehyde and post-fixed in 1% osmium tetroxide solution. Samples were then embedded in epoxy resin (Euromedex, Souffelweyersheim, France). Ultrathin sections (60–100 nm thickness) were collected on 200 mesh copper grids and stained with uranyl acetate and lead citrate. Sections were then examined using a JEM-1011 transmission electron microscope at 80 keV (JEOL, Croissy-sur-Seine, France). Two days after glycerol injection, degenerating myelinated fibers are observed and pre-existing blood vessels are ruptured. There are red blood cells and platelets outside the vessels (D2 right image). However, neo-capillaries are already observed indicating the onset of neo-angiogenesis (D2 left image). One and two weeks after injury, observations show the presence of macrophages. One, two and three weeks after injury, observations show a proliferation in the basal membrane of pre-existing vessels and new vessel growth. In addition, we can note the presence of arterioles surrounded by several neo-capillaries, the presence of macrophages and axonal regrowth (D14 and D21). One month after injury, blood vessels with normal and myelinated axons are observed (D60). The white star shows degenerating myelinated fibers (ovoids). The white and black triangles show blood vessels and neo-vessels respectively. Double black arrows show proliferation of the basal membrane; R: red blood cells; R_{Ex}: external red blood cells; P: platelets; Per: pericyte; L: blood vessel lumen; M: macrophages; E: endothelial cells; MA: myelinated axons; A_r: axonal regrowth. Scale bars: 1 µm.

in the number of vessels is observed. The first phase of the vascular response is related to the recruitment of macrophages and the clearance of degenerating axons and myelin sheath debris produced by Wallerian degeneration, while the second phase is associated with nerve regeneration and is comprised of cellular proliferation, axonal elongation and myelination (Podhajsky and Myers, 1993, 1994).

Recently published results suggest that SCs and macrophages are key components in the homeostasis of the INV system. However, the link between vascular guidance of the INV system and SCs after injury is unclear. Thus, improving our understanding of the functions of the INV system, notably by studying the molecular characterization of the BNB that maintains nerve integrity, would seem to be an interesting research approach. In addition, recent studies have emphasized the beneficial effects of vascular endothelial growth factor (VEGF) on neuron survival and SC proliferation. VEGF is a potent angiogenic factor that is required during tissue repair (Pereira Lopes et al., 2011). A study investigating the effects of VEGF on sciatic nerve regeneration demonstrated a positive relationship between increased vascularization and enhanced nerve regeneration (Hillenbrand et al., 2015). Another study showed that VEGF-A, secreted by macrophages, can support and enhance the growth of regenerating nerve fibers, probably through a combination of angiogenic, neurotrophic and neuroprotective effects (Cattin et al., 2015).

Conclusion

Finally, based on the results currently available in the literature, we can propose the following hypothesis on the role of vascularization in nerve regeneration. After peripheral nerve damage, the size of blood vessels of the INV system increases due to the effects of neuropeptides such as SP and CGRP. Vasodilatation, associated with the release of monocyte chemoattractant protein-1 by SCs, allows macrophage recruitment. Migrating and resident macrophages then secrete VEGF. This growth factor then stimulates neo-angiogenesis which supplies the oxygen and nutrients required for the formation of "bands of Büngner" by SCs. However, the molecular interactions between SCs, macrophages and endothelial cells of neo-vessels still remain to be elucidated. In conclusion, this review highlights the importance of revascularization in nerve regeneration. It therefore seems appropriate to intensify research in this area to provide a better understanding of revascularization after nerve injury and additionally to discover new therapeutic avenues.

Author contributions: *Review writing and critical revision: MC, JMV, AD and FB; in vivo study operation: LR and JMV; electron microscopy observation: LR; figures 1 and 2 design and preparation; MC; figure 3 preparation: MC, MC and JMV. All authors approved the final submitted version.*
Conflicts of interest: *None declared.*
Financial support: *Martial CAILLAUD was supported by a doctoral fellowship from the 'Conseil Régional du Limousin'.*

Peer review: *Externally peer reviewed.*

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C-Editors: Zhao M, Yu J; T-Editor: Liu XL