Laser-assisted nerve repair. An experimental study
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Chapter VI

Effects of CO$_2$ Laser Irradiation on Intact Rat Sciatic Nerve

T. Menovsky, M. van den Bergh Weerman, & J.F. Beek

Since several years, the CO$_2$ milliwatt laser has been experimentally and occasionally clinically used for repair of transected peripheral and cranial nerves (KRISHNAMURTHY, 1994; MENOVSKY, 1995c). Although very promising, LANR still needs to be optimised to be effectively and safely used in the clinical practice. Many factors influence the results of LANR, one of the most important being the irradiance applied to the nerve.

Little information could be gained from the literature on the exact action of lasers on peripheral nerve tissue under in vivo conditions. Unlike the brain, in which the action of laser irradiation has been studied extensively (WHAREN, 1984; MARTINIUK, 1989; ROUX, 1990; GAMACHE, 1993), there are only limited data on the extent of damage of peripheral nerves after laser irradiation.

Another issue is the use of protein solders as an adjunct to the welding process. These solders, which provide extra protein for the fusion process, are melted on the outer surface of the repair site to hold the tissue together, resulting in stronger welds (POPPAS, 1988; see also Chapter IV) and theoretically in less thermal damage to the tissue. However, to date there are no comprehensive data on the use of solders for in vivo LANR and how the nerve reacts to the solder.

In a previous study, we have determined the CO$_2$ laser settings which produce the greatest tensile strength (Chapter IV). In the study described in this chapter the functional and thermal damage of rat sciatic nerves irradiated by a CO$_2$ milliwatt laser (with and without solder) at different powers, exposure times was assessed during a 12 week follow up. In the study the nerves were not transected prior to laser irradiation.

Materials and methods

The study was approved by the local Animal Welfare Committee. A total of 88 female rats of an inbred Wistar strain were used in the experiments. The rats (250-300 g) were housed maximal six in a cage and were kept under conventional laboratory conditions. Before surgery, general anaesthesia was accomplished by intraperitoneal injection of mixture of ketamine (90 mg/kg), xylazine (10 mg/kg), and atropine (0.05 mg/kg).

In each rat, the right sciatic nerve was exposed by a modified dorsolateral incision (MENOVSKY, 1995d) and by stomp cleaving the overlying ham-
string muscles. Under the operating microscope, the nerve was prepared free and isolated from the surrounding tissue by a plastic sheet. The diameter of the sciatic nerves ranged between 0.9 and 1.1 mm.

Two experimental groups of laser irradiation were employed. In group I (dose-response study, n=40), circumferential irradiation of the nerve with the CO$_2$ laser was performed at different laser parameters as summarised in table 6.1, using a spot size of 320 µm. For each laser parameter (n=4) the total number of pulses applied to each nerve was between eight to ten. These laser settings have been previously evaluated for their tensile strength in an in vitro study (Chapter IV). For 50 mW power, exposure times were limited to 2.0 s and 3.0 s as below these exposure times no bonding of the nerves could be achieved. The left untreated nerve was sham operated and served as a control. After the procedure, the fascia of the hamstring muscles was closed with 6-0 PGA sutures and the skin was closed with 4-0 PGA sutures. The rats from group I were killed 24 hours after surgery by an overdose fentanyl intraperitoneally and the nerves were carefully dissected. The nerves were fixed in Karnovsky’s fixative, postfixed in osmium tetroxide 1%, stained with uranyl acetate, dehydrated in acidified 2,2-dimethoxypropane, and embedded in epoxy resin. After hardening, semi-thin (1.25 µm) cross sections were cut and stained with toluidine blue and basic fuchsin.

In group II (early and late effects study, n=48), circumferential irradiation of the nerve with the CO$_2$ laser was performed at 100 mW with pulses of 1.0 s and a spot size of 320 µm. In half of the nerves (n=24) a protein solder consisting of bovine albumin powder dissolved in saline was applied to the nerve using a small spatula (soldered nerves). The solder covering the nerve was irradiated using the same laser parameters until the solder was adherent and coagulated on the nerve. The total number of pulses applied to each nerve was between eight to ten. Again, the left untreated nerve was sham operated and served as a control. After the procedure, the fascia of the hamstring muscles was closed with 6-0 PGA sutures and the skin was closed with 4-0 PGA sutures. The CO$_2$ milliwatt laser used for the experiments is described in chapter IV.

The rats from group II were killed 1 day, and 1, 2, 4, 8, and 12 weeks after surgery (n=8 for each survival group) by an overdose fentanyl intraperitoneally and the nerves were carefully dissected. The nerves were processed for light microscopy as described above. Transverse sections were obtained from the proximal and distal nerve segments as well as from the irradiation site. Selected areas were cut for transmission electron microscopy and processed as described in chapter V. In two rats (two weeks survival time) the vena cava inferior was cannulated and the vascular bed was flushed with saline and subsequently with barium. The nerves were dissected and the vascularisation within the nerves was studied by X-rays photographs.

The motor function of the nerves was examined in all rats 24 hours after surgery and thereafter every two days in the first two weeks and weekly in the rest of the survival period using a modified version of the toe-spreading test as originally described by De Medinacelli (1982) and modified by
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Wondergem (1988). In short, the toe-spreading, defined as the distance from the first to the fifth digit, from both hind legs was measured from walking tracts. The relative toe-spreading of the right foot was calculated with the untreated left foot as a control. A 100% motor function loss will result in a relative toe-spreading of 30%, while no motor function loss will result in a relative toe-spreading of 100%. For each measurement, at least four foot steps were recorded.

Results

Group I
Observation of the nerve during irradiation showed gradual changes in appearance of the tissue, which are summarised in table 6.1.

Toe-spreading test
The results of the toe-spreading test are presented in figure 6.1. Irradiations of 50 mW and 100 mW for up to 1.0 s exposure time per pulse resulted in negligible or no deficit in motor function of the nerves (motor function ≥ 90%). Irradiations with 100 mW with prolonged exposure times (≥ 2.0 s pulse duration) and irradiations with 150 mW power resulted in significant decrease in motor function.

Lightmicroscopic changes
Several pathological reactions ranging from total destruction of a part of the nerve to minimal changes were observed, strongly related to the used level of power and exposure duration. The most severe reaction, occurring at 150 mW for 2.0 and 3.0 s exposure time, consisted of vaporisation and perforation of the epi- and perineurium and carbonised and coagulated tissue in which the cells and the tissue structure was not recognisable. More towards the centre of the nerve, massive Wallerian degeneration with endoneurial oedema was observed. In this region, the blood vessels were thrombosed and the injured axons were retracted away from their myelin sheaths and Schwann cells. A few inflammatory cells were seen. Only in the central part of the nerve, a few normal axons and myelin sheaths were observed together with intact blood vessels. The extent of the thermal damage was approximately 500 μm, including the thickness of epi- and perineurium.

At lower powers (50 mW for 3.0 s, and 150 and 100 mW for 0.5 and 1.0 s), the thermal damage was much more confined to the subepineurial axons (Fig. 6.2). The pathological changes consisted of two relatively thin layers. In the outer zone (ca 75 μm), located directly subperineurally, the nerve tissue was oedematous and the nerve fibres had darkly stained cytoplasm. The second zone (ca. 50 μm), located more towards the centre, was also oedematous but the myelin sheaths were thin and separate layers on myelin sheaths were intruding in the axoplasm. Also, enlarged and empty endoneurial tubules were seen. Both the inner and outer zone were undergoing Wallerian degeneration. Very few inflammatory changes were noted. The vessels in the
Table 6.1. Experimental protocol of laser parameters and summary of the macroscopic observations

<table>
<thead>
<tr>
<th>Power (mW)</th>
<th>Exposure time per pulse (s)</th>
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<tbody>
<tr>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>50</td>
<td>x</td>
</tr>
<tr>
<td>100</td>
<td>2-3</td>
</tr>
<tr>
<td>150</td>
<td>3-4</td>
</tr>
</tbody>
</table>

0 no observable effect, 1 drying, 2 shrinkage, 3 whitening, 4 caramélisation (slightly browning), 5 carbonisation, 6 vaporisation

x = not performed

epineurium and vessels located in the injured area were thrombosed and the perivascular space was enlarged. No haemorrhagic lesions could be observed. The epineurium was oedematous and the fibroblasts showed dark picnotic nuclei. The morphological integrity of the epi- and perineurium was not affected. The extent of the total thermal damage was approximately 125 μm. The central part of the nerve was undamaged although the distance between the axons was enlarged, indicating oedema. Around the patent blood vessels in the injured area, a few myelinated axons appeared to be intact.

At 50 mW for 2.0 s and 100 mW for 0.5 s, the thermal damage was slightly less than in the 100 mW group for 0.5 and 1.0 s, despite the absence of macroscopical changes during the laser irradiation. The extent of the thermal damage was approximately 100 μm. The pathological changes were the same as described at 50 mW for 3.0 s and 100 mW for 0.5 and 1.0 s, but less pronounced.

In all groups, morphological changes proximal or distal from the irradiated area were not seen. Sham-operated nerves showed no pathological changes and were identical to historical unoperated nerves.

GROUP II
Observation of the nerve during laser irradiation showed whitening and in some cases slight brown discoloration and some degree of shrinkage of the irradiated area. In the solder group, the solder showed drying, slight brown discoloration, and adherence to the nerve without excessive macroscopic changes of the nerve itself.

All rats did well postoperatively and had no signs of infection or neurological complications except for variable dysfunction of the right sciatic nerve as described below.

Toe-spreading test
The results of the toe-spreading test up to 12 weeks after laser irradiation are presented in figure 6.3. Irradiation of 100 mW for 1.0 s exposure time per pulse resulted in negligible or no deficit motor function of the nerves.
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**Fig. 6.1.** Motor function based on the toe-spreading of the hind legs of rats after irradiation with the CO₂ laser at different laser settings. * not performed

**Fig. 6.2.** Subepineurial thermal damage 24 hours after irradiation at 150 mW and exposure time of 1.0 s (toluidine blue, original magnification x 501). In the outer zone (above), there is swelling of affected fibres with darkly stained axoplasm. In the inner zone (below) some empty endoneurial tubules are seen together with disintegrating myelin sheaths.
Fig. 6.3. Motor function of the hind legs of rats after irradiation with the CO₂ laser without (upper) and with addition of a solder (lower) at different survival times.
Day one
The microscopical changes in the non-soldered nerves are described above. In the soldered nerves, the solder was identified as a homogenous material surrounded and infiltrated by inflammatory cells with a large amount of polymorphonuclear leukocytes (Fig. 6.4). The intraneural changes including the thermal damage were similar to the non-soldered nerves, although the extent of thermal damage was slightly less.

Week one
Some minor adhesions were found in the non-soldered nerves while moderate adhesions of the nerve to the tissue were observed in the soldered nerves. Degeneration of axons and myelin sheaths (Wallerian degeneration), both at the site of irradiation and in the distal nerve segment was noted only in the subperineurial area with a depth of approximately 100 μm. Within this area, cellular reaction consisting of presence of macrophages, lymphocytes, and proliferating Schwann cells was noted. Remnants of myelin were found in Schwann cells and macrophages. The epineurium and perineurium was thickened (due to oedema and collagen production), and the epineurium was characterised by a generalised cell infiltrate and dilated vessels. However, the morphological integrity of the epi- and perineurium was not affected. The blood vessels within the nerve appeared normal. In the epineurium, an increase in normal collagen with active young fibroblasts was noted, containing large amounts of granular endoplasmic reticulum.

In the soldered group, the degenerative changes within the nerve were the same. A lot of inflammatory cells and macrophages could be seen within the epineurium surrounding small remnants of the solder. The extent of the thermal damage was comparable to non-soldered nerves.

Week two
Macroscopically, no adhesions were found in either group of nerves and the solder had macroscopically disappeared. Microscopically, Wallerian degeneration of myelinated nerve fibres was more striking than at one week. Small fragments of lamellar material, possibly remnants of myelin, were enveloped by macrophages and Schwann cells. Clusters of axonal sprouts were seen, indicating regeneration from the proximal nerve segment (Fig. 6.5). A sharp demarcation between the injured and the normal noninjured area existed. The perineurium appeared to be intact and the epineurium contained a markedly decreased number of infiltrating cells with some collagen rich granulation tissue. Clear neovascularization was observed. In the solder group, the degenerative changes were the same as in the non-solder group. The solder has been totally absorbed and a cell rich nonspecific granulation tissue with inflammatory cells, mainly lymphocytes, remained in the epineurium. Angiography revealed an increased vascular pattern at the site of irradiation.

Week four
In the subperineurial area, only a few remnants of the degenerated myelina-
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Fig. 6.4. Solder material (S) upon the epineurium (E) surrounded and infiltrated by inflammatory cells (toluidine blue, original magnification x 400).

Fig. 6.5. Clusters of axonal sprouts were seen at the periphery, indicating regeneration coming from the proximal nerve segment (toluidine blue, original magnification x 400). The epineurium (E) and perineurium (P) is intact.
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ted axons and myelin could be defined. Clusters of small regenerating axons, both myelinated and unmyelinated, were usually present at the Schwann cell surface. A basal lamina was identifiable enveloping the clusters of axons and the morphology of Schwann cells appeared to be normal. The cellular infiltrate in the epineurium of both groups had disappeared and no inflammatory cells or macrophages within the epineurium could be observed. The perineurium had a normal aspect while the epineurium was slightly thickened with clear neovascularization as observed at two weeks.

Week eight
Clear clusters of regenerating axons located subperineurially were observed with both myelinated and unmyelinated nerve fibres. No differences existed between soldered and non-soldered nerves concerning axonal regeneration. At the point of laser irradiation the epineurium in both groups was slightly thicker and minimally disorganised. Nevertheless, no foreign body reaction or other cellular reaction was observed in both groups. Compared to normal nerves, the irradiated nerves had more epineurial blood vessels.

Week twelve
Macroscopically, no adhesions were found during dissection of the nerves. The nerves had a similar appearance as at eight weeks, except for the fact that the subperineurial regenerating axons were larger in size and reached a greater degree of myelination (thicker myelin sheath) (Fig. 6.6). Again, at the point of laser irradiation the epineurium was only slightly thicker and minimally disorganised compared to normal nerves (Fig. 6.7). Besides the subperineurial area, the fibres within the nerve appeared totally normal.

Discussion

In this study, two important issues relating to damage of the nerve following laser irradiation under in vivo conditions were addressed. First, the thermal damage and nerve function of peripheral nerves irradiated with a CO₂ milliwatt at laser settings that were found to give the greatest tensile strength in vitro (Chapter IV) were investigated. A survival time of 24 hours was chosen because thermal damage to biological tissue is then assumed to be complete (THOMSEN, 1991). Second, the wound healing after laser irradiation was studied at different postoperative time intervals whereby in a subgroup of nerves a protein solder was applied to the irradiation site.

As this study focused on thermal damage, the nerves were not transected prior to laser irradiation (transection normally would be performed prior to nerve repair). Reactions resulting from the traumatic division only would have hampered the assessment of the tissue reaction caused by laser irradiation. No reaction resulting from only exploration and mobilisation was found on the sham-operated sides, as was expected (EDSHAGE, 1964).

There are only two previous studies on the effects of CO₂ laser irradiation on intact peripheral nerves. RICHMOND (1986) reports no histological, beha-
Fig. 6.6. Transmission electron micrograph of the subepineurial area showing maturated myelinated and unmyelinated nerve fibres (original magnification x 11,600).

Fig. 6.7. Sciatic nerve 12 weeks after laser irradiation (toluidine blue, original magnification x 200). Note almost normal epineurium (E) and perineurium and normal intra-neural structures.
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vioural, or neurologic deficits following CO$_2$ laser irradiation of intact rat sciatic nerves at a power of 70-80 mW and pulse durations of several microseconds. Myers (1985) did find thermal damage in rat sciatic nerve irradiated with a CO$_2$ laser at relatively high power for tissue welding of 5 W during 0.25 s and 0.5 s, using a relatively large spot size of 2 mm (energy densities of 0.4 J/mm$^2$ and 0.8 J/mm$^2$ at 0.25 and 0.5 s pulse duration respectively). Wallerian degeneration and perivascular and subperineurial oedema marked the nerve injury two days after irradiation. Using a diode laser, Lauto (1997) claimed that no thermal damage occurred after laser welding using protein strips, although some damage to the axons could be observed on the figures provided (Menovsky, 1998b).

In the dose-response study, the pathological changes of the nerve tissue consisted of specific alterations, dependent on the power delivered. At high powers, there was total destruction of the nerve with only a small centre in which healthy nerve fibres could be observed. At lower powers a small zone of subperineurial Wallerian degeneration with oedema, swelling of vascular structures, damaged endothelial cells and vascular occlusion were seen. The central part of the nerve was undamaged. Minimal damage was seen at a power of 100 mW and a pulse duration of 1.0 s (at a spot size of 320 pm resulting in a density of 3.5 J/mm$^2$ per pulse). This result compares favourably to the results of Myers (1985) who found extensive damage at lower energy densities.

Several aspects of the dose-response study are of importance. First, the absence of macroscopic changes during laser irradiation does not necessarily mean that the nerve is not injured. For example, at 50 mW power, where only slight macroscopic changes were observed, there is still an area of damaged nerve fibres notable on histological sections. However, significant visual changes during laser irradiation such as at 100 mW for 1.0 s (Table 6.1) did neither result in extensive histological damage to the nerve nor in severe functional deficit. Second, at short exposure times (≤ 1.0 s) the relative volume of nerve tissue injured is minimal and irradiation at all powers used does hardly lead to any neurological dysfunction. In other words, the subperineurial degeneration does not necessary lead to the development of severe nerve function loss. Finally, at 100 mW for 1.0 s (which is the laser setting which produces the greatest tensile strength in vitro (Chapter IV) only subperineurial damage is present with normal preservation of the nerve structure, including the vascularisation in the centre of the nerve. Consequently, this laser setting seems very suitable for future experiments on LANR and thus this setting was chosen for the second part of this study.

A second important question is whether CO$_2$ milliwatt laser irradiation has any negative effects with respect to axonal regeneration and scar tissue formation. As the CO$_2$ laser has been used to transect peripheral nerves to prevent axonal flow and thus neuroma formation (Fischer, 1983; Hurst, 1984), concern may arise whether the regenerative potential of peripheral nerve is not impaired after irradiation. In the second part of this study, we have shown that the CO$_2$ laser irradiation in the milliwatt range did not affect
the regenerative potential of the nerves. Although some subperineurial damage to the axons was observed followed by classical Wallerian degeneration at the site of injury and distally, vigorous outgrowth of both myelinated and unmyelinated axons across the irradiation site was observed with subsequent maturation of axons with time. The morphological integrity of the epi- and perineurium was not affected by the laser energy nor was regeneration of axons hindered, in great part because intraneural scar formation was absent. This is in agreement with the report of LELE (1963) showing that the perineurium is relatively unaffected by heat-induced injuries to peripheral nerves.

Conventional wound healing proceeds as a continuum, beginning with an initial injury and inflammatory phase, progressing through a granulation or proliferative phase, and is completed by a remodelling phase, possibly resulting in a scar (CLARK, 1985). In peripheral nerves however, extraneural scar formation is undesirable as scar can give adhesions to surrounding structures which can interfere with the physiological longitudinal sliding of the nerves during limb movements. This restriction in nerve mobility can result in nerve injury (HUNTER, 1991), as tethering of the nerves can cause ischaemia and thus further damage. Moreover, painful dysesthesias can occur due to tethering of the nerves to the surrounding tissue.

Almost no information could be found in the literature on the chronic effects of laser irradiation on peripheral nerves. Historically, SCHREIBER (1973) studied the effects of ruby laser on rabbit median nerves at different intervals and observed coagulation necrosis of the epineurium and reactive hyperaemia. In this study, minimal inflammatory reaction was seen around the lased area. New collagen was formed with inward growth from the margins of the lesions. Thereafter, continued healing occurred which resulted in nearly complete repair of the epineurium. It has been shown that laser injury to tissue results in less wound response than other kind of injury (FILMAR, 1989A & 1989B; GREENE, 1994). MIHASHI (1976) noted that lymphatics and blood vessels were sealed in the coagulation zone. As a consequence, local oedema was minimised and haemostasis is achieved. Similar coagulative effects on nerves may account for the minimal pain reported by some patients following laser surgery (KAPLAN, 1973). In laser tissue welding, carbonisation should be avoided as carbonised material causes a prolonged foreign body reaction (FISCHER, 1985) that in turns delays wound healing (FILMAR, 1989A & 1989B). We did not find any signs of a foreign body reaction, probably because of the absence of carbonised tissue. One of the reasons that the wound healing progressed in a normal way is the relative preservation of extracellular matrix components and especially the perineurium which is important for the progression of wound healing. It is also speculated that laser injury induces milder and later inflammatory response and later fibroblastic activity than mechanical damage produced by sutures (CIKRIT, 1990).

Also in the solder group the wound healing proceeded favourably with no adverse effects on peripheral nerve regeneration or intra- or extraneural scar formation. Only small adhesions were found in the first two weeks after surgery, but these were resolved after four weeks.
In conclusion, CO$_2$ milliwatt laser irradiation of in vivo rat sciatic nerves at 100 mW for pulses of 1.0 s and a spot size of 320 μm, i) does not interfere with axonal regeneration, and ii) does not lead to formation of intra- or extraneural scar. Also, iii) the addition of a protein solder does not interfere with normal wound healing. Therefore, the CO$_2$ milliwatt laser at these settings and a protein solder can be safely applied to peripheral nerves for the purpose of tissue welding.
Although information could be found in the literature on the psychic effects of laser irradiation on peripheral nerves. Historically, Schumann (1973) studied the effects of early laser on rabbit median nerve at different intervals and observed anagolic increase of the epineurium and reactive hyperemia. In this study, normal inflammatory reactions vascularity around the cutaneous nerve collagen was intact with smear growth from the injury of the fibers. This reaction continued healing processes which resulted in nearly intact lateral and anterior nerve bundles. It has been shown that laser injury of the skin is subject to inflammatory response with other types of injury (Fleischer, 1974; Wathan, 1973). Similarly, it was noted that mechanical and electrical stimuli were absent. For a non-injury local anesthesia was administered and anesthesia was administered. Similar cumulative effects on foreign body reaction for the minimal pain reported by patients following laser surgery (Fleischer, 1974). In laser tissue welding, carbonation tips should be avoided as carbonated material causes a prolonged foreign body reaction. In other words, that in turn delays normal healing (Fleischer, 1974). We did not find any signs of a foreign-body reaction, probably because of the absence of carbonated tissue. One of the reasons that the wound healing progressed in a normal way is the relative preservation of extraneural anatomycomponents and especially the perineurium which is important for the preservation of normal healing. It is also speculated that laser injury induces solid and granulation tissue response and laser-induced healing tissue may not regenerate damaged produced by laser (Fleischer, 1974).

As in the intact group the wound healing proceeded remarkably with no adverse effects on peripheral nerve regeneration or intra- or extraneural scar formation. Only small adhesions were found in the first two weeks after surgery, but these were removed after four weeks.