J Orthop Sci (2010) 15:233–239
DOI 10.1007/s00776-009-1438-4

JOURNAL OF
ORTHOPAEDIC SCIENCE
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Original article

Effect of near-infrared light-emitting diodes on nerve regeneration

Motoi Ishiguro[1], Kazuo Ikeda[2], and Katsuro Tomita[1]

[1] Department of Orthopaedic Surgery, Graduate School of Medicine, Kanazawa University, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8641, Japan
[2] Department of Orthopaedic Surgery, Kanazawa Medical Center, Kanazawa, Japan

Abstract

Background. Photobiomodulation by red to near-infrared light-emitting diodes (LEDs) has been reported to accelerate wound healing, attenuate degeneration of an injured optic nerve, and promote tissue growth. The purpose of this study was to investigate the effect of LEDs on nerve regeneration. A histological study as well as a measurement of antioxidation levels in the nerve regeneration chamber fluid was performed.

Methods. For the histological study, the bilateral sciatic nerves were transected, and the left proximal stump and the right distal stump were inserted into the opposite ends of a silicone chamber, leaving a 10-mm gap. Light from an LED device (660 nm, 7.5 mW/cm^2) was irradiated for 1 h per day. At 3 weeks after surgery, regenerated tissue was fixed and examined by light microscopy. For the antioxidation assay of chamber fluid, the left sciatic nerve and a 2-mm piece of nerve from the proximal stump were transected and inserted into opposite sides of a silicone chamber leaving a 10-mm gap. LEDs were irradiated using the same parameters as those described in the histological study. At 1, 3, and 7 days after surgery, antioxidation of the chamber fluid was measured using an OXY absorbent test.

Results. Nerve regeneration was promoted in the LED group. Antioxidation of the chamber fluid significantly decreased from 3 days to 7 days in the control group. In the LED group, antioxidation levels did not decrease until 7 days.

Conclusions. Chamber fluid is produced from nerve stumps after nerve injury. This fluid contains neurotrophic factors that may accelerate axonal growth. Red to near-infrared LEDs have been shown to promote mitochondrial oxidative metabolism. In this study, LED irradiation improved nerve regeneration and increased antioxidation levels in the chamber fluid. Therefore, we propose that antioxidation induced by LEDs may be conducive to nerve regeneration.

Offprint requests to: M. Ishiguro
Received: March 17, 2009 / Accepted December 1, 2009

Introduction

Light-emitting diodes (LEDs), originally used for National Aeronautics and Space Administration (NASA) plant growth experiments in space, have been shown in animal and human studies to have biological effects. LEDs offer an effective alternative to lasers based on safety, superior portability, and low cost. The pinpoint beam of laser light can damage the eye and can limit the irradiation area. Lasers are unsuitable for large or moving targets. The heat production from the laser light can damage tissue and needs to be cooled. Consequently, lasers are not portable devices.[1,2] A variety of studies have determined that light in the red to near-infrared range (630–1000 nm) provides the optimal conditions for biostimulation. Previous reports indicate that low-power laser therapy is effective for wound healing[3] and enhancing peripheral nerve repair.[4] LED therapy can be effective in wound repair in vivo and increased cell growth in vitro.[1,2] Previous reports also indicate that LEDs prevent oral mucositis in pediatric bone marrow transplant patients[5] and radiation-induced dermatitis in breast cancer.[6] In addition to these studies, it has also been shown that LEDs attenuate degeneration of injured optic nerves.[7–10] However, this study is the first, to our knowledge, to investigate the effect of photobiomodulation by LEDs on peripheral nerve regeneration. Using a silicone chamber, we analyzed the histological effects of LEDs on nerve regeneration in rats and measured the antioxidation of the nerve regeneration chamber fluid.

Materials and methods

The study protocol was approved by the institutional review board, and all procedures were performed according to the guidelines outlined by the Institute for

Experimental Animals, Kanazawa University Advanced Science Research Center.

Histological study

Fifteen female Wister rats weighing 150–200 g each (Japan SLC, Hamamatsu, Japan) were anesthetized by intraperitoneal injection of pentobarbital sodium (40 mg/kg body weight). Bilateral sciatic nerves were exposed and mobilized by a back transverse incision. The left nerve was transected in the distal part of the thigh and the right nerve at the level of the distal part of the piriformis muscle. The left proximal stump and the right distal stump were then inserted 2 mm into the opposite ends of a sterile silicone chamber and sutured using double 9-0 monofilament nylon sutures. The length of the silicone chamber was 14 mm, internal diameter was 1.5 mm, and outer diameter was 2.5 mm (Hagitec, Chiba, Japan). The gap between the nerve stumps inside the chamber was 10 mm. The chamber was prefilled with physiological saline (0.9% NaCl) and placed subcutaneously in the back (Fig. 1). The wound was closed by 4-0 nylon sutures.

This is a cross-anastomosed rat nerve regeneration model using a silicone chamber developed by Lundborg et al.[11] We selected this model because a silicone chamber is positioned to the back of a rat, and LEDs may be irradiated from almost the same distance through the skin on the back. The LED device consists of a 30 × 30 cm LED panel and the control device (IS-series; CCS, Kyoto, Japan). LEDs at a wavelength of 660 nm and a power intensity of 7.5 mW/cm^2 were used for this experiment. The LED panel was set approximately 10 cm above the back of the rats (n = 7) in the cage for 1 h per day for 3 weeks.

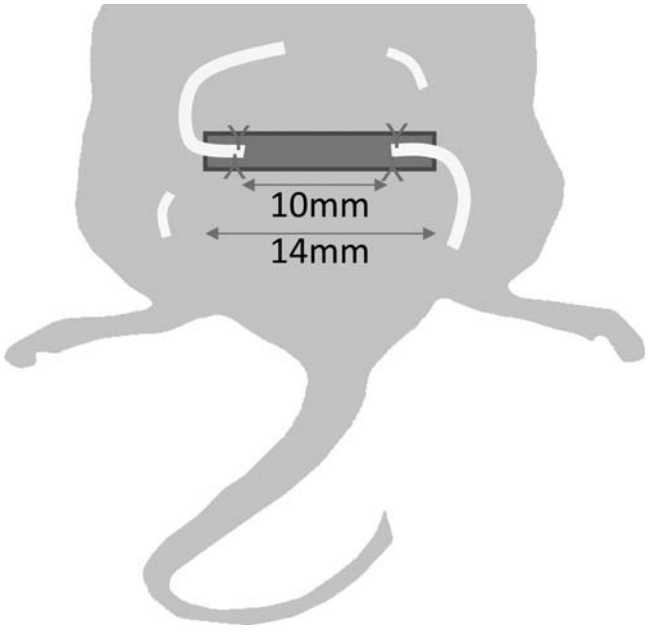

Fig. 1. Procedure for the histological study. The left proximal sciatic nerve stump and the right distal stump were cross-anastomosed to a 14-mm silicone chamber. The gap between the nerve stumps inside the chamber was 10 mm

Three weeks after the surgery, the rats were anesthetized and perfused transcardially with 100 ml of 0.9% saline containing heparin sodium 25 units/ml followed by 100 ml of 0.1 M phosphate buffer containing 2% paraformaldehyde and 2% glutaraldehyde. The silicone chambers were extracted, and the inner regenerated tissue was analyzed. The 10 mm of regenerated tissue was transected into five equal parts and immersed in perfusion liquid. On the following day, these parts were rinsed, postfixed in 1% osmium tetroxide, dehydrated in ethanol, and embedded in epoxy resin. The regenerating nerve was cut at 1-, 3-, 5-, 7-, and 9-mm intervals from the proximal nerve stump. The plastic epoxy-embedded nerves were cross-sectioned at 1 µm, mounted on glass slides, and stained with toluidine blue. Samples were examined by light microscopy (Nikon Instech, Tokyo, Japan).

Assessment of histology

Images were acquired using the COOLPIX micro system. The cross-sectional area was measured by manually tracing the outline of the cross section of ×100 images using the NIH-Image J analysis software. The regenerated tissue consists of an inner endoneurium and an outer perineurial-like cell sheath.[12] The endoneurial area was measured by tracing the area containing Schwann cells, which were identified as cells with a medium dense cytoplasm and pale oval nucleus.[13] The percent endoneurial area is defined as the percentage of the endoneurial area in the cross-sectional area. The presence of myelinated axons was determined in ×1000 images by toluidine blue staining of the myelin sheath. Photoshop-processed images (Adobe Systems, San Jose, CA, USA) were used to determine the total count of myelinated axons. Using NIH-Image J analysis software, the myelinated axons per unit area were quantified, and the total sum of myelinated axons in the endoneurial area was determined.

Antioxidation assay of chamber fluid

A total of 46 rats were anesthetized for the chamber fluid assay (Fig. 2). The left sciatic nerve was transected in the distal part of the thigh, and a 2-mm piece of sciatic nerve was transected from the proximal nerve stump. The proximal nerve stump was sutured on one side of the 14-mm silicone chamber, and a 2-mm nerve fragment was sutured on the opposite side, leaving a 10-mm

gap. The setup for this experiment differed from that of the histological study. The internal diameter of the chamber was 2.0 mm, which facilitated the collection of more than 10 µl of fluid. We plugged the nerve fragment into the distal end of the chamber to prevent the chamber fluid from spilling during fluid collection. It has been previously reported that suturing a 2-mm piece of sciatic nerve into the distal end of the chamber results in fibrin formation, cell migration, and axonal regeneration.[14] LEDs were irradiated in the same way as in the histological study.

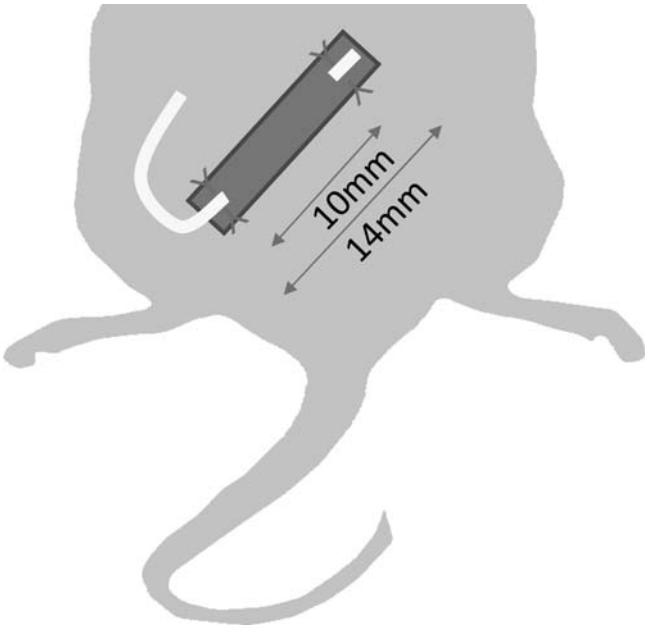

Fig. 2. Procedure for the chamber fluid assay. The left proximal sciatic nerve stump and a 2-mm nerve fragment were anastomosed to a 14-mm silicone chamber. The gap between the proximal nerve stump and the nerve fragment was 10 mm

At 1, 3, and 7 days after the surgery, the chamber was extracted. Fluid from the chamber was collected and centrifuged for 2 min (6000 rpm) to obtain the supernatant, which was stored at −76°C until further analysis. Antioxidation of the chamber fluid was measured using an OXY-absorbent test (Diacron International, Grosseto, Italy). The number of fluid samples ranged from 6 to 10 in each group.

Statistical analysis

Data were analyzed using the Mann-Whitney test and Student's *t*-test. The differences between two groups were considered statistically significant when $P < 0.05$. Statistical analysis was performed using StatView 5.0 statistical software (SAS Institute, Cary, NC, USA).

Results

Histological study

Representative cross sections of the LED group and the control group are shown in Fig. 3. The cross-sectional area of the 1-mm section in the LED group was considerably larger than that observed in the control group ($P < 0.05$) (Table 1). The endoneurial area of the 1- and 7-mm sections in the LED group was also considerably larger than that detected in the control group ($P < 0.05$) (Table 1). The percent of endoneurial area in the 7-mm section in the LED group was significantly higher than that of the control ($P < 0.05$) (Table 1).

Upon examination, we observed myelinated axons in six of seven chambers in the 3-mm section in the LED group. However, this was only observed in three of eight chambers in the control group ($P = 0.0504$, Mann-Whitney's U-test) (Table 2). In the 3-mm section, there

Table 1. Various measurements of the chamber contents

Measurement, by group	1 mm	3 mm	5 mm	7 mm	9 mm
Cross-sectional area of the chamber contents[a]					
Control ($n = 8$)	0.805 ± 0.097	0.394 ± 0.104	0.138 ± 0.051	0.102 ± 0.014	0.674 ± 0.129
LED ($n = 7$)	1.088 ± 0.085	0.376 ± 0.103	0.192 ± 0.034	0.160 ± 0.036	0.586 ± 0.073
P	0.0495*	0.9054	0.4061	0.1352	0.5767
Endoneurial area of the chamber contents[b]					
Control ($n = 8$)	0.497 ± 0.065	0.239 ± 0.071	0.075 ± 0.031	0.039 ± 0.008	0.373 ± 0.073
LED ($n = 7$)	0.071 ± 0.059	0.232 ± 0.069	0.111 ± 0.023	0.095 ± 0.026	0.323 ± 0.041
P	0.0388*	0.9503	0.3797	0.0432*	0.5737
Percentage of the endoneurial area in the total cross-sectional area					
Control ($n = 8$)	61.5 ± 1.6	56.5 ± 4.3	50.4 ± 4.0	37.5 ± 4.9	52.6 ± 4.1
LED ($n = 7$)	64.8 ± 2.9	58.4 ± 4.1	54.1 ± 4.0	55.2 ± 4.7	55.5 ± 2.7
P	0.3186	0.7582	0.5376	0.0223*	0.5824

Results are means ± SE (%)
[a] Measurements were performed by tracing around the cross section
[b] Measurements were performed by tracing the area containing Schwann cells
* Significant difference

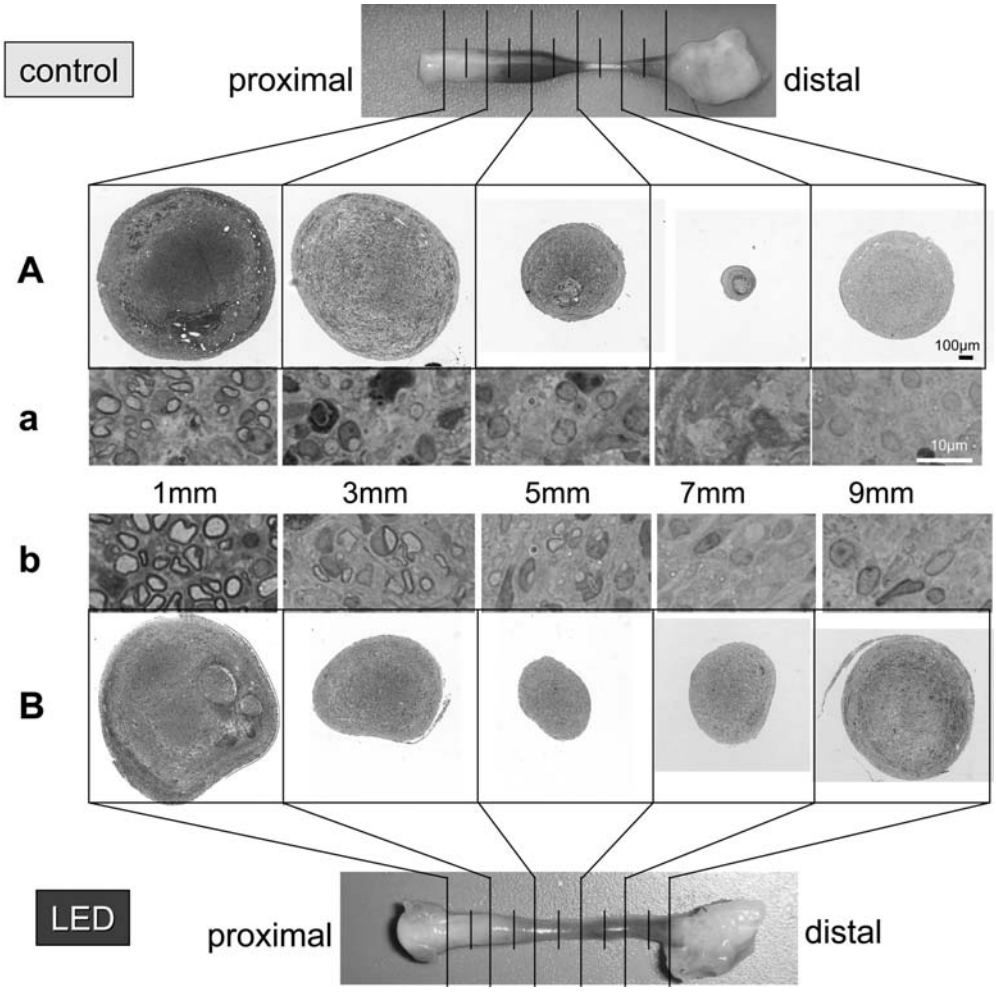

Fig. 3. Macroscopic and microscopic representation of the chamber contents at 3 weeks after implantation. Cross sections at 1, 3, 5, 7, and 9 mm from the end of the proximal nerve stump for the control group (**A**, **a**), and the light-emitting diode (*LED*) group (**B**, **b**). **A**, **B** ×100. *Bar* 100 μm. **a**, **b** ×1000. *Bar* 10 μm. In the 5-mm sections, myelinated axons from the proximal nerve stump are observed in the LED group but not in the control group

Table 2. Number of chambers that contain myelinated axons in each section at 3 weeks

Group	1 mm	3 mm	5 mm	7 mm	9 mm
Control ($n = 8$)	8	3	0	0	0
LED ($n = 7$)	7	6	1	0	0

$P = 0.0504$, Mann-Whitney's U-test

Table 3. Total number of myelinated axons in the 1-mm and 3-mm sections

Group	1 mm	3 mm
Control ($n = 8$)	4747 ± 1343	890 ± 446
LED ($n = 7$)	10922 ± 2640	1957 ± 1076
P	0.0493*	0.3542

Results are means ± SE
*Significant difference

was no significant difference in the total number of myelinated axons between the two groups. However, in the 1-mm section, we observed a significant increase in the LED group compared to that for the controls ($P < 0.05$) (Table 3).

Antioxidation assay of chamber fluid

At day 1, the chamber was filled with fluid. However, there was no bridging tissue inside the chamber. At day 3, the gap was bridged with blood clots that transformed into a fibrin matrix within 7 days (Fig. 4). There was no significant difference in gross observation between the LED and control groups.

Antioxidation measurements of the chamber fluid significantly decreased from 206.4 ± 11.1 μmol at day 3 to 175.9 ± 7.6 μmol at day 7 in the control group ($P < 0.05$). In the LED group, antioxidation measurements did not decrease until day 7. There was no significant difference between the two groups at days 1 and 3. Antioxidation measurements in the LED group at day 7 were 226.9 ± 12.6 μmol. These values were significantly higher than the 175.9 ± 7.6 μmol in the control group ($P = 0.005$) (Table 4).[15]

Table 4. Antioxidation measurements of the chamber fluid at days 1, 3, and 7

Group	Day 1	Day 3	Day 7
Control	195.8 ± 11.4 (n = 6)	206.4 ± 11.1 (n = 8)*	175.9 ± 7.6 (n = 8)*,**
LED	191.3 ± 3.9 (n = 6)	230.4 ± 16.0 (n = 8)	226.9 ± 12.6 (n = 10)**

Results are means ± SE (micromoles)
* Significant differences of results between day 3 and day 7 in the control group ($P < 0.05$)
** Significant differences of results between two groups at day 7 ($P = 0.005$)

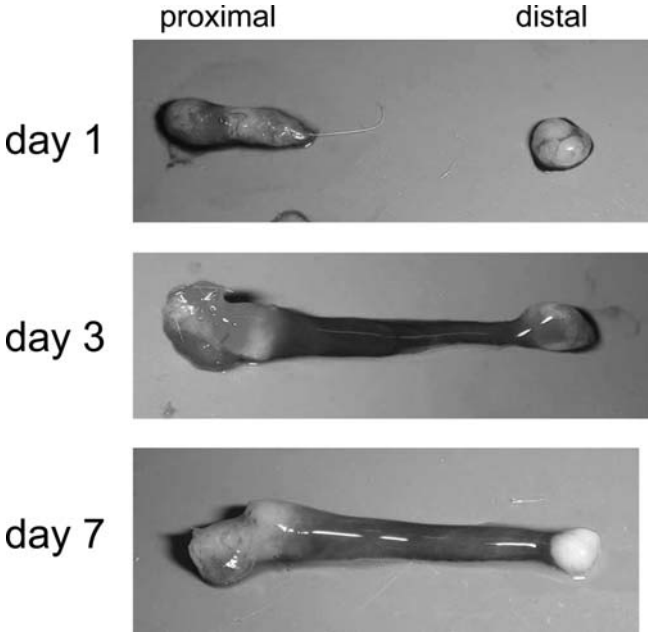

Fig. 4. Chamber contents of the control group at days 1, 3, and 7. At day 1, the fluid-filled chamber contains no bridging tissue between the stumps. By day 3 bridging occurs via blood clots, and by day 7 it has been transformed to a fibrin matrix

Discussion

In this study, LEDs at a wavelength of 660 nm effectively promoted peripheral nerve regeneration in our rat model. We propose that this is due to the antioxidative effect induced by LEDs. Previous studies indicate that red to near-infrared light (630–1000 nm) promotes mitochondrial oxidative metabolism, activates antioxidation, and protects against cell damage.[16] The effect is caused by cytochrome c oxidase, a membrane protein complex present in the mitochondrial inner membrane.[17] Cytochrome c oxidase is a major photoacceptor molecule that is known to absorb light in the red to near-infrared range. Therefore, LED treatment stimulates cytochrome c oxidase activity and activates the basic energy processes in the mitochondria.[16] Light in the 670- to 830-nm range is especially effective owing to the absorption spectrum of oxidized cytochrome c oxidase.[9,18] Therefore, in this study a wavelength in this vicinity, 660 nm, was selected. Previous studies have reported that the optimal condition of LED irradiation is 4 J/cm^2 (50 mW/cm^2) for wound healing.[1,2] In this study, we used a lower power intensity of 7.5 mW/cm^2. Transcutaneous emission attenuates the intensity to 3.0 mW/cm^2. A comparison of the published results indicates that phototherapy at different energy density levels, ranging from <10 J/cm^2 to >150 J/cm^2, can be effective in influencing nerve repair.[4] In this study, we selected to irradiate for 1 h with a total energy density of 27 J/cm^2. Further experimentation may help optimize the irradiation conditions.

In our previous experiment using a rat random pattern skin flap model, we observed that the survival rate increased significantly in LED-treated animals. Moreover, the quantity of free radicals in the flap was significantly suppressed by LEDs. Therefore, we reasoned that LED irradiation decreased the generation of free radicals in the flap and increased cell viability (Hashimoto et al., personal communication). We have continued to study the effects of LEDs on biological tissues. This is the first report suggesting that LEDs affect peripheral nerve regeneration. Particularly, we focused on antioxidation levels in the nerve regeneration chamber fluid.

Based on previous studies of the rat nerve regeneration model using a silicone chamber, clear tissue fluid accumulates up to 12 h in the chamber.[19] This fluid is produced from nerve stumps after nerve injury. The fluid contains neurotrophic factors (NTFs), such as nerve growth factor (NGF) and ciliary neurotrophic factor (CNTF), which ensure in vitro survival and growth of sensory, spinal motor, and sympathetic neurons that contribute axons to the sciatic nerve.[19–23] We did not measure the titer of NTFs in this study. However, we consider the possibility that LEDs may affect NTFs. Based on other studies, at 3 days, the chamber is filled with a clear yellow fluid containing deposits resembling blood clots. At 7 days, the content appears to have condensed, forming a thin, lucent, well-defined matrix along the central axis of the chamber. Fibroblasts and Schwann cells are commonly observed in the most proximal and distal sections.[24–26] In the present study, antioxidation of the chamber fluid decreased at 7 days in the control group. We propose that the reduction in antioxidation of the chamber fluid is due to the generation of free radicals. This occurs as

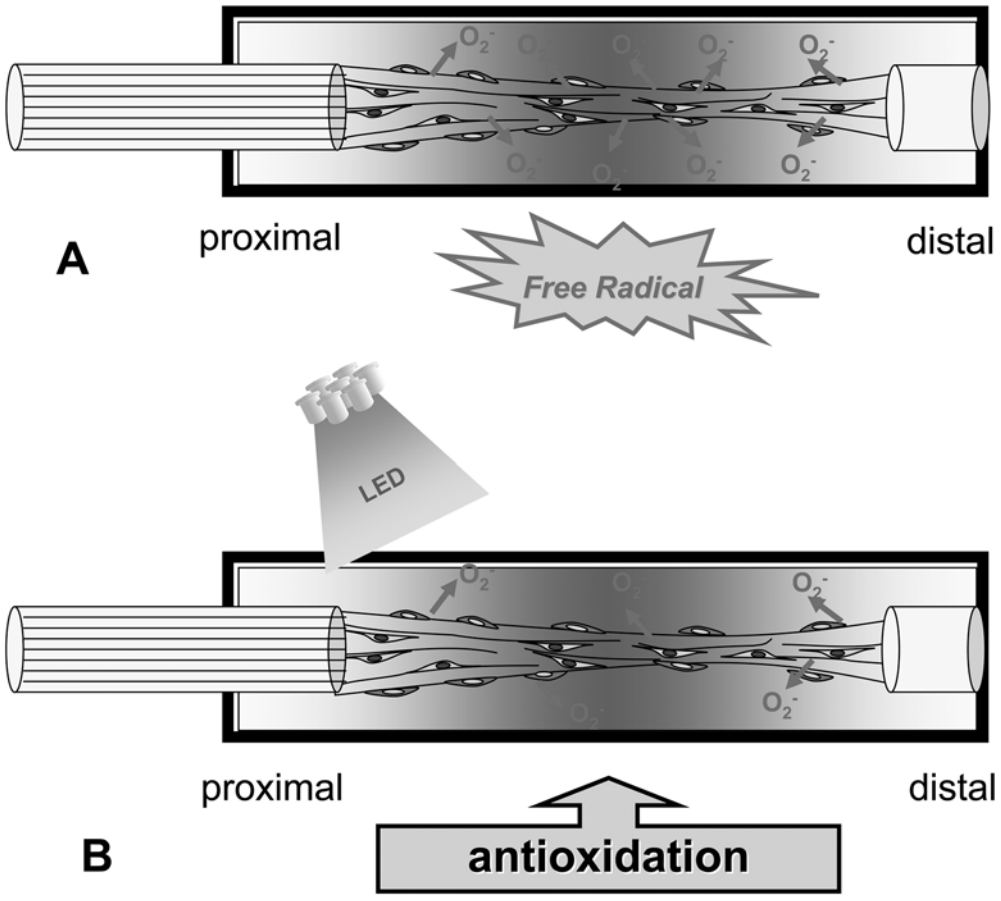

Fig. 5. Nerve regeneration chamber. As cells exit the nerve stumps and axons exit the proximal nerve stump, free radicals are generated, and antioxidation of the chamber fluid decreases (**A**). LED irradiation may affect cells by suppressing a reduction in antioxidation (**B**)

Schwann cells, fibroblasts, endothelial cells, and perineurial cells exit the nerve stumps and axons exit the proximal nerve stump. However, the level of antioxidation was maintained at a high rate at 7 days in the LED group. This suggests that antioxidation induced by LEDs may be conducive to nerve regeneration (Fig. 5). There is some possibility that LEDs may affect angiogenesis of the regenerated tissue, leading to the maintenance of antioxidation in the LED group. Previous studies indicate that LED and low-level laser therapy demonstrate impressive results in the angiogenesis of skin wounds.[27] Blood vessels are not formed in the regenerated tissue at 7 days, but the vasculature of proximal and distal nerve stump may affect the level of antioxidation.

Previous studies of the nerve regeneration model have also shown that circumferential cells are the first cells to enter the chamber and that these cells position themselves at the periphery of the fibrin matrix. Vascular and Schwann cell elements migrate behind the front of circumferential cells and enter the core, rather than the periphery, of the matrix.[25] Previous work also indicates that circumferential cells extend throughout the structure length by 3 weeks in nearly all the chambers, whereas advanced core-migrating elements such as vasculature, fibroblasts, Schwann cells, and axons vary substantially in different chamber systems.[13]

Our histological study suggests that irradiation with LEDs is advantageous to nerve regeneration. The number of myelinated axons in the 1-mm sections was much larger in the LED group than in the controls. In addition, the endoneurial area in the smallest section of the LED group (7-mm section) was significantly larger than that observed in the control group. Previous studies have shown a relation between antioxidants and myelination of dorsal root ganglion (DRG) neurons in vitro.[28] When antioxidants are removed from B27 medium, myelination is lost, suggesting that antioxidants are important for myelination. Our data support this previous work and suggest that LEDs may affect nerve regeneration by maintaining antioxidation and accelerating axonal growth and myelination.

Our study suggests that LEDs may be applied clinically to treat nerve injury. For example, the application of LEDs to neurorrhaphy sites or entrapment points may be effective.

Conclusion

Light-emission diode irradiation of a rat nerve regeneration model promotes nerve regeneration. We propose that this is due to the maintenance of antioxidation in the nerve regeneration chamber fluid.

Acknowledgments. The authors thank Shigenori Tanaka for technical advice in the histological study and Masatake Shiraishi and Yoko Kasai for assistance in presenting and completing this study. This study was supported by Grant-in-Aid no. 18591624 for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

The authors did not receive and will not receive any benefits or funding from any commercial party related directly or indirectly to the subject of this article.

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