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RESEARCH ARTICLE

Expression of neutrophil gelatinase-associated lipocalin (NGAL) in peripheral nerve repair

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Abstract

Introduction: Trauma is one of the causes of peripheral nerve injuries. Free radicals increase after tissue damage. Free radicals are usually scavenged and detoxified by antioxidants. In this study, we assessed the antioxidative role of the NGAL molecule in sciatic nerve repair in rats. *Materials and methods*: The sciatic nerves of 40 rats were crushed and the total mRNA of samples from day 1 and 3 and week 1, 3, 5 post injury was extracted. The expression of the NGAL gene was confirmed by RT-PCR. For immunohistochemistry analysis, the samples were fixed in paraformaldehyde and cut in 20 micrometer slices by cryostat. *Results*: The expression of NGAL significantly upregulated in day 1, 3 and week 1 following the crushing of sciatic nerves in comparison with the intact nerves. Immunohistochemistry results also confirmed the protein expression of this gene. *Discussion*: The NGAL molecule showed upregulation in the degeneration process after nerve injury, so it may play an important role in nerve repair.

Abbreviations: NGAL: Neutrophil Gelatinase–Associated Lipocalin; **WD**: Wallerian degeneration; **PNS**: peripheral nervous system; **ROS**: reactive oxygen species; **MDA**: Malondialdehyde; **GSH**: Glutathione; **ABC complex**: avidinbiotinylated peroxidase complex; **DAB**: 3, 3′-Diaminobenzidine; **PBS**: phosphate-buffered saline; **LPO**: lipid peroxidation; **HO**: heme oxygenase; **SOD**: superoxide dismutase; **ANOVA**: analysis of variance; **bp**: base pair

Keywords

NGAL, peripheral nerve, repair

History

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Introduction

Peripheral nerve injury, as a main source of morbidity and loss of function in many individuals, is a serious problem in the health system throughout the world. It is estimated to occur once in every thousand (1/1000) in the population per year (1). Several types of trauma can result in nerve injury, which increases with technology in industrialized societies. Neuronal death next to trauma is an important clinical issue. The mechanism of injury includes compression, direct nerve trauma, stretch ischemia, and local anesthetic toxicity (2).

In the distal nerve stump, traumatic injury of a peripheral nerve suggests a procedure called Wallerian degeneration (WD), which offers a suitable cellular and molecular environment for subsequent regenerative axon growth. The degeneration of axons and Schwann cells brings about the activation and recruitment of macrophages, to release cytokines which promote the proliferation and dedifferentiation of Schwann cells (3).

Just one week after injury, degenerated myelin and axons were detected in the lesion location and distal region (4,5), and this procedure took three weeks post trauma (6). Although peripheral nervous system (PNS) axons can be repaired, functional recovery in humans is frequently incomplete. This is due to the fact that the regenerative response of the damaged neuron and of cells adjacent to the injured neuron's axon cannot preserve an effective development - promoting response for long periods. In a suitable situation where injured nerves lie end to end or fill the gap through a nerve graft, the axons are regenerated and remyelinated, and functional recovery is completed in 4–5 weeks (6).

Although the histological features of WD have been well-established, the fundamental cellular and molecular mechanism remains largely unknown and is still under investigation (5). Oxidative stress or free oxygen radicals or reactive oxygen species (ROS) increase and tissues become damaged immediately after the injury. They can react with

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important macromolecules such as DNA, proteins and oxidized lipids (7,8) due to their unpaired electrons (9).

Oxidative stress is defined as an imbalance between reactive oxygen species (ROS) and the antioxidant system. Recently, it has been established that oxidative stress is associated with most of the pathological states and diseases (10) such as epilepsy, ischemia, Alzheimer's disease, multiple sclerosis, spinal cord injury and pain (11–13). Previous studies have revealed that peripheral nerve injury induces oxidative stress in injured neurons (14).

In PNS crush injuries, malondialdehyde (MDA), as a lipid peroxidation indicator in tissue injuries, increases easily due to an excessive amount of lipids in the nerve tissue (7,15–17). The redox homeostasis of the cell is maintained by antioxidant enzymes such as superoxide dismutase, catalase, glutathione S-transferase, NGAL and other elements such as glutathione, vitamins E, C and A, which remove ROS (10).

Neutrophil gelatinase-associated lipocalin (NGAL) is one of the lipocalin family proteins; a 25-kDa protein covalently related with the 92-kDa gelatinase/MMP9 that was identified initially from specific granules of the human neutrophil (18,19), expressed in a variety of normal and pathological human tissues (20,21). when epithelial cells are involved in inflammation, NGAL is also up-regulated. This has mainly been demonstrated for the epithelial cells of the large and small intestine, cells of the upper and lower respiratory tract and the skin (22–24). In addition, it has been revealed that NGAL is up-regulated in tubular epithelial cells of the kidney in response to ischemia (25,26). NGAL was expressed in bone marrow and tissues prone to exposure of micro-organisms (26). Ischemia-reperfusion injuries, heart attacks and strokes are caused by an increase in ROS and, under this condition, the induction of NGAL can be observed (27). NGAL expression was up-regulated after the administration of X-rays or H_2O_2 in HepG2 cells. These results strongly suggest that NGAL expression was induced by ROS. Hence, NGAL could be a valuable biomarker to recognize oxidative stress both *in vitro* and *in vivo* (22). In addition, NGAL is believed to be an acute phase protein whose expression is induced under harmful conditions such as intoxication, renal injury, burn injury, human cancers, inflammatory intestine disease, infection, and other forms of cellular stress (28). Oxidative stress can also induce its expression (29).

In this study, we evaluated the expression of NGAL in different phases of degeneration and regeneration of the sciatic nerves of rats following crush injury.

Materials and methods

Sampling

Forty male Wistar rats weighing 200–250 g were used according to the local guidelines of the Experimental Animal Committee of the animal sciences center of the Baqiyatallah University of Medical Sciences. The animals were housed three per cage and provided with free access to rat food and water. They were kept under constant laboratory conditions of $22 \pm 2\,^{\circ}C$ room temperature, and illumination (12 h each of light and darkness; darkness beginning at 7:00 PM).

All animals were anesthetized with ketamine hydrochloride (90 mg/kg) and xylazine hydrochloride (10 mg/kg). Their right gluteal regions were shaved and cleaned with polyvidon-iodine. The right sciatic nerve was then exposed at the mid-thigh without any damage to the muscle tissue and crushed for 60 s using a jeweler's forceps (no:5). The crush level was marked on the muscle by a 5/0 non-absorbable silk suture, the incision was closed in layers and antibiotic powder sprinkled externally to prevent infection. The rats were then kept in individual cages after surgery.

Subsequently, on the 1st and 3rd days and 1st, 3rd and 5th weeks after injury, the crush level of the sciatic nerve and 5 mm distal to it were harvested. In each group, five samples were transferred in trizol and stored at – 80 °C until RNA extraction, while three samples in formalin were kept in the refrigerator for fixation.

RNA extraction

Total RNA was harvested in conformity with manufacturer's recommendations using trizol reagent (Invitrogen, Carlsbad, CA). Briefly described, samples were homogenized in trizol by mean of an ultrasonic homogenator. After adding 200 µl chloroform (Merck, Darmstadt, Germany) and centrifuging at 12 000 rpm, RNA containing homogenates in the aqueous phase were separated, and the same volume of isopropranol was added. To avoid contamination with proteins, the lowest fraction of the aqueous phase was not incorporated into the total RNA sample. Following centrifugation at 12 000 rpm, precipitated RNA was dissolved in ethanol at 75% and centrifuged again at 7500 rpm. Isolated RNA was eluted in 20 µl RNAase free water, and the quantity and integrity of RNA were measured by Nano Drop (ND-1000 UV-Vis spectrophotometer, USA).

Primer design

The primer sets for NGAL, and β-actin (control gene) are shown in Table 1.

cDNA synthesis and semi quantitative RT-PCR

Aliquots of 500 ng total RNA were reverse-transcribed to create first-strand complementary DNA by superscript III reverse-transcriptase (Invitrogen) according to the manufacturer's protocol. The resulting 1 µl of cDNA was validated with PCR

Table 1. The primer sets for NGAL, and β-actin.

GENES	Primer sequences (5′-3′)	Annealing Temperature (°C)	Size (bp)
NGAL	Forward: 5′TGTACAGCACCATCTATGAGC3′ Reverse: 5′TGCACATCGTAGCTCTGTATC3′	56 °C	175
β-actin	Forward: 5′TCATGAAGATCCTCACCGAG3′ Reverse: 5′TTGCCAATGGTGATGACCTG3′	58 °C	190

in a volume of 25 ml containing 2.5 µl buffer (10× Cinagene, Tehran, Iran), 10 pM deoxynucleoside triphosphate, 0.3 µL rTq polymerase (Cinagene, Tehran, Iran), 10 pm forward primer and 10 pm reverse primer. PCR was carried out in the same solution with heat held at 94°C for 3 min, denaturation at 94 °C for 30 s, and annealing at 56 °C, 58 °C for NGAL and β-actin, respectively, for 30 s, extension at 72 °C for 1 min (35 cycle), terminal extension at 72 °C for 5 min, and a terminal hold at 4 °C. PCR products were separated by1.5% agarose gel electrophoresis, and the quantity of the bands was visually detectable under UV light after dying with ethidium bromide. All results were normalized with β-actin expression to compensate for differences in cDNA amount. Image analysis (using Scion Image software) was done to obtain quantitative data (Scion Corporation, Frederick, MD).

Immunohistochemistry

Details of the immunohistochemistry are already described elsewhere (30). In brief, sciatic nerve samples were placed in 4% buffered paraformaldehyde for fixation.

After overnight immersion in phosphate buffer containing 30% sucrose, 20 µm thick tissue sections were cut on a microtome cryostat (Histo-line Laboratories, Italia) and incubated with NGAL antibodies (1:200 dilution in phosphate buffer) for 12 h at 4 °C. The antibody used in this study were mouse monoclonal IgG1 antibody raised against recombinant NGAL of rat source (Santa Cruz Biotechnology, Inc., USA) at a dilution of 1:200. After incubation with the primary antibody, the sections were washed with PBS and incubated with biotinylated anti-mouse secondary antibody (Santa Cruz Biotechnology, Inc., USA). Antigen-antibody reaction sites were detectable using an ABC complex (avidin-biotin-peroxidase complex) system (Vector Laboratory, Burlingame, CA) with 3,3′-Diaminobenzidine (DAB) as a substrate. For the negative control, phosphate-buffered saline (PBS) was substituted for the primary antibody.

Statistical analysis

Data were analyzed by two-way analysis of variance (ANOVA) followed by a Bonferroni's post-test for multiple comparisons (using SPSS version 13, SPSS, Inc., Chicago, IL). A level of $P<0.05$ was considered statistically significant and all results expressed as means ± SD.

Results

Gene expression evaluation of NGAL

Figure 1 shows the gene expression of NGAL (Figure 1A) and β-actin (Figure 1B) in normal and crushed sciatic nerve samples obtained from semiquantitative RT-PCR.

To obtain quantitative data, image analysis was carried out using Scion Image software. For normalization with β-actin expression to compensate for differences in the cDNA amount, gene expression of NGAL amount/gene expression of β-actin amount in all samples was calculated. Also, the mean ± SEM of five samples in each group was calculated (Table 2).

Figure 2 shows the statistical analysis of groups. The results of statistical analysis show a time variable ($F=36.10$, $p<0.0001$), surgery variable ($F=233.69$, $p<0.0001$) and time*surgery ($F=36.10$, $p<0.0001$). In addition, these results revealed that NGAL gene expression on the 1st and 3rd day and 1st week after injury is significantly high in comparison with the control group (intact nerve) ($p<0.0001$) (Figure 2).

Immunohistochemistry assessment

Immunohistochemistry was carried out to ascertain whether the expression of NGAL in protein level is caused by crush injury (Figures 3 and 4).

A longitudinal section of a normal (intact) nerve was examined and it showed only a lower intensity reaction as background with no reaction to the antibody observed (Figure 3A).

On the 1st day after injury, an intensive reaction to the antibody was seen, revealing an active existence of the NGAL molecule in this stage (Figures 3B and 4A). On the 3rd day after injury, immuno-reactivity to the antibody decreased compared to the 1st day after injury (Figure3C, Figure4B) and, in the 1st week after injury, this reaction decreased

Table2. Mean(NGAL/B-actin)±SEM in groups.

Group	Mean (NGAL/B-actin) ± SEM
Control	0.73 ± 0.069
Day 1	2.14 ± 0.042
Day 3	1.89 ± 0.123
Week 1	1.70 ± 0.097
Week 3	1.00 ± 0.042
Week 5	0.76 ± 0.031

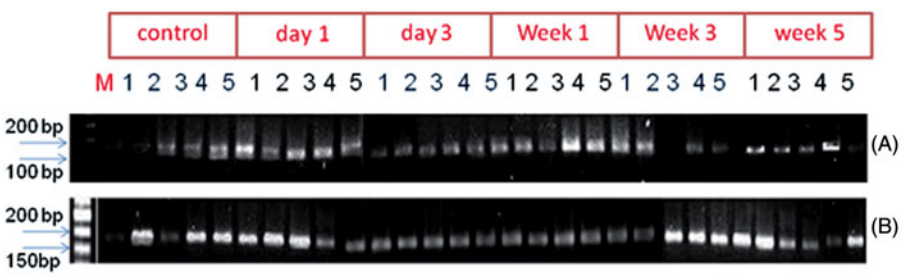

Figure 1. Gene expression of NGAL and β-actin in normal (control) and crushed sciatic nerve samples measured by semi-quantitative RT-PCR. (A) Gene expression of NGAL in normal (control) and crushed sciatic nerve samples: Lane M shows DNA ladder (100 bp). Lanes after M, show five samples of control, day 1, day 3, week 1, week 3 and week 5 after injury, respectively. (B) Gene expression of β-actin in normal (control) and crushed sciatic nerve samples. Lane M shows DNA ladder (50 bp). Lanes after M, show five samples of control, day 1, day 3, week 1, week 3 and week 5 after injury, respectively.

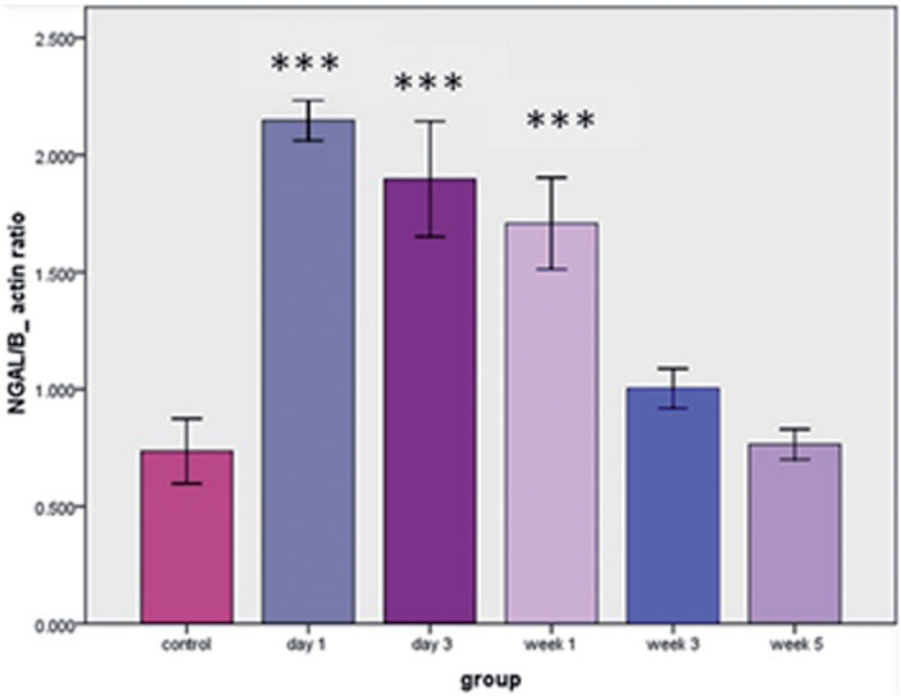

Figure 2. Quantitative data of NGAL gene expression in groups. Data reported as mean ± SEM. *** denotes statistical significance ($P < 0.0001$), when compared to the control.

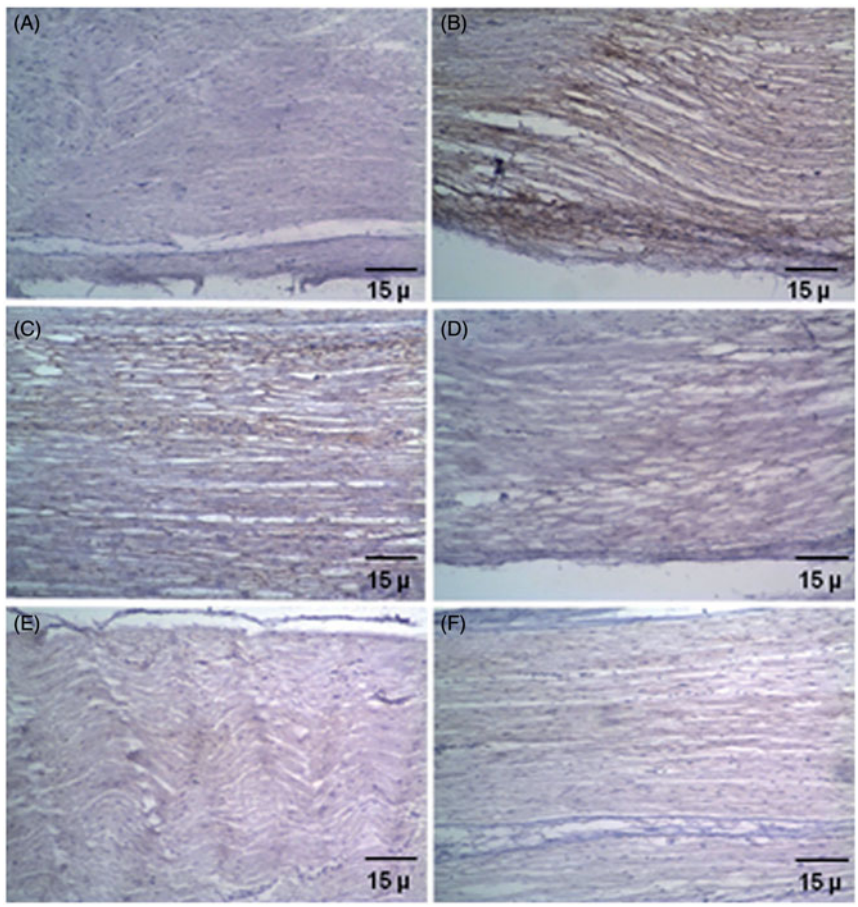

Figure 3. Longitudinal sections of sciatic nerve samples (×10). (A) control (normal), (B) 1st day after injury, (C) 3rd days after injury, (D) 1th week after injury, (E) 3rd weeks after injury, (F) 5th weeks after injury. Brown color indicated NGAL antibody reaction. NGAL protein appears brown.

compared to the 3rd day after injury (Figure3D). In the 3rd week after injury, the intensity of antibody reaction decreased increasingly (Figure3E) and immuno-reactivity for NGAL in the 5th week after injury became negative, revealing the occurrence of regeneration (Figure 3F).

Discussion

A compound-regulated sequence of events was initiated to remove the damaged tissue and begin the healing process of the injured peripheral nerve (31). Subsequent reports in nerve

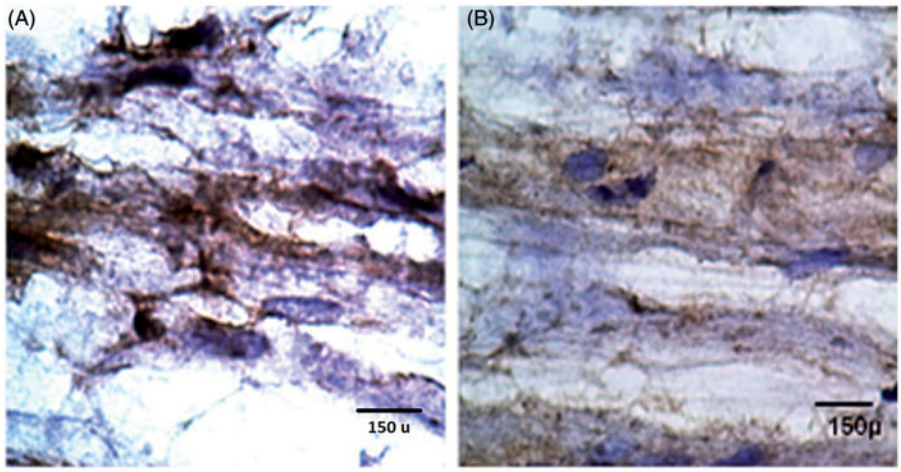

Figure 4. Longitudinal sections of sciatic nerve samples (×100). (A) 1st day after injury, (B) 3rd days after injury. NGAL protein appears brown.

injuries revealed that free oxygen radicals or oxidative stress increased thereby leading to tissue damage (7,16,32). The PNS has a rich source of lipids and may be the major target of oxidative stress facilitated lipid peroxidation (LPO) (13,32). LPO is one of the most important expressions of oxidative stress induced by free oxygen radicals (2,33). LPO has been proposed to be an essential factor in post-traumatic neural tissue degeneration which may modify the permeability of neuronal membranes and decrease cellular functioning, or impairment of membrane-bound receptors and enzymes (1).

In this investigation, our finding through semi-quantitative PCR revealed that the expression of NGAL transcriptome was up-regulated one week after crush injury and gradually decreased until 5 weeks post injury. Immunohistochemisry assessment also revealed that the NGAL protein localized in high intensity in the injured nerve fiber on the 1st day after crush injury, and the immunoreactivity gradually decreased and disappeared in the 5th week after injury.

Malondialdehyde (MDA), as a free radical, is an indicator of lipid peroxidation, and increases in various diseases (33). In crush injury of the rat sciatic nerve, serum levels of MDA, a marker of lipid peroxidation and tissue injury, increased on the 2nd and 7th day post-injury and then gradually decreased on the 14th, 21st and 42nd days. This could be accounted for by the tissue's own protective mechanisms (34).

Roudkenar and colleagues were reported that NGAL has antioxidant capacity and able to prevent or scavenge the free radicals in a concentration-dependent manner (35). There are many experimental studies available showing production of free radicals and the importance of lipid peroxidation on the cell membrane and antioxidant therapy in sciatic nerve crush injury of rats (1,2,33,36–38).

These findings confirmed that oxidative stress is high during the first week after crush injury and gradually decreases until the 5th week. Our results show that high expression of the NGAL molecule in the first week may be due to oxidative stress in the crushed nerve.

The activity of protective antioxidants increases in response to ROS (2). Antioxidants are potential candidates as oxidative stress scavengers for the prevention or treatment of oxidative damage (33). Cells and tissues are supported with a variety of antioxidants to eliminate extra ROS and guard against oxidative injury (14,39).

Glutathione (GSH) is a nucleophilic scavenger of numerous free radicals which protect the cell membrane against oxidative damage. Serarslana et al., in a crushed sciatic nerve study, revealed that the plasma levels of glutathione (GSH) were slightly higher at the initial stage of regeneration compared to the control group (34).

Heme oxygenase-1 (HO-1) is supposed to be involved in protecting cells against oxidative damage. Hirata et al. reported that Schwann cells express HO-1 when they are transformed into myelin-phagocytosing cells from myelinating cells just after crush injury of rat sciatic nerves. At the initial stage of degeneration immediately after nerve injury, HO-1 immunoreactivity was localized in the endoneurium (40).

Heme oxygenase (HO) and superoxide dismutase (SOD) are two strong antioxidants. NGAL is a potent inducer of HO-1 and somewhat SOD-1,2, and it appears that part of the antioxidant property of NGAL could be attributed to the induction of HO-1and SOD-1,2 (28). According to the earlier description, after nerve injury, the nerve fiber undergoes Wallerian degeneration (41).

In the early stage, when free radicals increase, the secretion of antioxidant enzymes also increases, so as to eliminate oxidative stress. From our results, it can be concluded that the NGAL molecule, as an antioxidant agent, was up-regulated to play its role in the degeneration process of the sciatic nerve after injury.

On the other hand, the majority of the cell populations in peripheral nerve fibers are Schwann cells containing a huge amount of lipid. This lipid is a good target for peroxidation by free radicals (13,32) and this procedure is regulated by different antioxidant enzymes. This suggests that NGAL and other antioxidant molecules are up-regulated in the Schwann cells in the process of being transformed into phagocytic cells, so as to engulf the derbies of degenerated nerve fibers.

Conclusion

After crush injury, free oxygen radicals increase and NGAL, as an antioxidant defense, is up-regulated. High expression is seen in the early phase after injury in the degeneration process

of peripheral nerve and NGAL plays an important role in the detoxification of the crush site from free radicals and accelerates nerve fiber healing.

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Declaration of interest

The authors report no declarations of interest.

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