J. of Applied Tissue Engineering
2016; 3(1): 25-33

Metallothionein molecules in sciatic nerve injury and repair

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Received: 8 November 2015/ Accepted: 24 may 2016

Abstract:

Background and purpose: Peripheral nerve injury induces oxidative stress in lesioned neurons. Antioxidants remove excess free radicals and participate in nerve repair. In this study, we assessed the antioxidative role of the metallothionein 1&2 molecules 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 MT-1&2 genes 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 MT-1&2 significantly up-regulated in day 1 and 3 following the crushing of sciatic nerves in comparison with the intact nerves. Immunohistochemistry results also confirmed the protein expression of MT-1.

Conclusion: The metallothionein molecules showed up-regulation in the degeneration process after nerve injury, so it may play an important role in nerve repair.

Keywords: Metallothionein 1 & 2, oxidative stress, Crush injury, peripheral nerve

Introduction

Increased production of reactive oxygen species (ROS) leading to oxidative stress. Oxidative stress contributes to the neuropathophysilogy of several neurodegenerative disorders as well as to trauma, seizures, stroke and neuronal degeneration (1). This condition play an important role in the pathogenesis of peripheral nerve damage (2-6). During the first week after injury to a peripheral nerve, Wallerian degeneration in the distal nerve stump occurs and

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spontaneous regeneration with functional return can be expected (5, 7). The distal nerve stump degenerate rapidly within 1–2 days which is completed between 3 and 7 days after peripheral nerve trauma (8). In distal segment of the crush site, axonal regeneration within 2-5 days after injury leading to good functional recovery at 3-4 weeks and between 1-2 weeks after nerve crush remyelination occures (7). Peripheral nerve trauma leads to a complex series of molecular and cellular events required for neuronal repair (9).

Following tissue injury, ROS and free radicals can damage proteins, lipids, DNA and RNA (10, 11). Neuronal tissue has a rich source of lipids and good candidate for lipid peroxidation (LPO) induced by ROS (12). Malondialdehyde (MDA), an indicator of lipid peroxidation, increases in various diseases and peripheral nerve injuries (4, 6). Antioxidants are the best candidates for prevention of oxidative damage after peripheral nerve injury via intracellular free radical scavenging (6, 11). The metallothioneins (MTs), one of the stress protein induced by oxidative stress, are small, low-molecular weight with zinc-binding capacity that may help protect cells from ROS both *in vivo* and *in vitro* (13-15). In mammals, the metallothionein (MT) family have been subdivided into four members, MT-1 to MT-4. MT1&2 are expressed in most tissues, while MT-3and MT-4 are expressed prominently in the CNS and in stratified squamous epithelia, respectively (1, 16). In most tissues, there is a correlation between MT-1 and MT-2 (MT1 & 2) expression (13, 17); therefore referred to generically as MT-I/-II or MT-1/2 (1).

Metallothioneins are proteins with multiple functions that MT-I/II expression is upregulated in response to injury (18). MT-I/-II demonstrated an important role in protection and regeneration of CNS in response to injury; and in the knockout (KO) mice, oxidative stress and apoptosis are enhanced (13, 17, 19). Following injury, exogenous MT-I/-II strongly promotes regenerative sprouting, when added directly to injured neurons in culture (20). MTs play an important role in protecting against free radicals for treatment of stroke (21) and various human tumors (22). A lot of experiments, have shown the several-fold increasing synthesis of MT during oxidative stress to protect the cells against free radicals (23). However, little is known about the changes in mRNA expression of MT molecules that occur after injury of the peripheral nerve.

So the goal of this study was to evaluate the expression of MT-1 & 2 in different phases of degeneration and regeneration of the sciatic nerves of rats following crush injury.

Material and Methods

Sampling

Forty male Wistar rats weighing 200 to 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±2°C room temperature, and illumination (12 hours 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) (fig. 1)

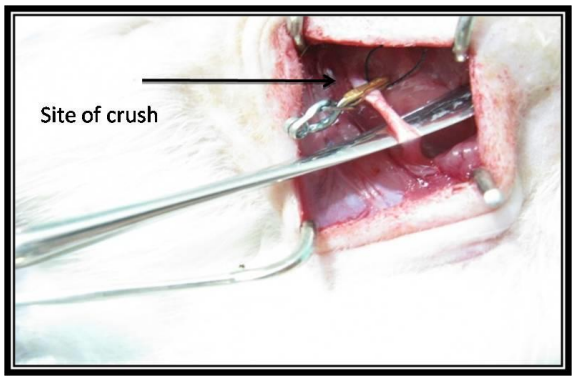

Figure 1. Crush site by jewelry forceps.

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, 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 measuredby Nano Drop (ND-1000 UV - Vis spectrophotometer).

Primer design

The primer sets for β-actin (control gene), MT_1A and MT_2A are shown in Table 1.

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

Product	Sequence(5'-3')	Annealing Tempreture (°C)	Size(bp)
Beta actin forward primer	5'TCATGAAGATCCTCACCGAG 3'	58ºC	190bp
Beta actin reverse primer	5' TTGCCAATGGTGATGACCTG 3'		
MT-1A forward primer	5' TGCCTTCTTGTCGCTTACAC 3'	64ºC	242bp
MT-1A reverse primer	5' TCGTCACTTCAGGCACAGC 3'		
MT-2A forward primer	5' TAGAACTCTGCAGCGATCTC 3'	65 ºC	216bp
MT-2A reverse primer	5' TCCGAAGCCTCTTTGCAGAT 3'		

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 in a volume of 25 mL containing 2.5 µL buffer (10x Cinagene, Tehran, Iran), 10 pM deoxynucleoside triphosphate, 0.3 µL rTq polymerase (Cinagene, Tehran, Iran), 10 pM forward primer and10 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 sec, and annealing at 64°C, 65°C and 58°C for MT-1, MT-2 and β-actin, respectively, for 30 sec, 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 by ١,٥% 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 (24). In brief, sciatic nerve samples were placed in 4% buffered paraformaldehyde for fixation.

After immersion overnight in phosphate buffer containing 30% sucrose, 20 μm thickness sections were cut on a cryostat (Histo line, Italia) and incubated with MT-1 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 MT_1 of rat source (Abcam, UK) 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 (avidinbiotinylated peroxidase complex) system (Vector Laboratory, Burlingame, CA, USA) 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). A level of $P < 0.05$ was considered statistically significant and all results expressed as means ± SD.

Results

Gene expression evaluation of MT-1 & 2

Figure 2 shows the gene expression of MT-1 (Fig.2A), β-actin (Fig2.B) and MT-2 (Fig2. C) in normal and crushed sciatic nerve samples obtained from semiquantitative RT-PCR.

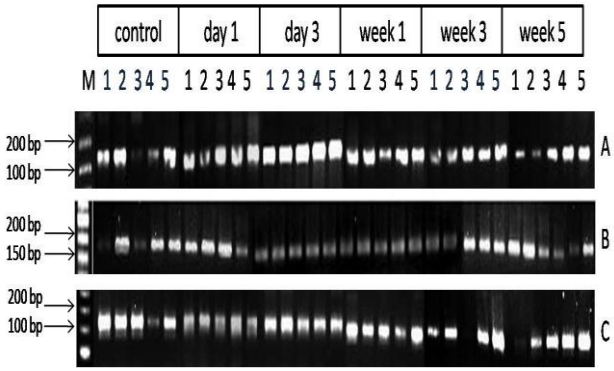

Figure 2. Gene expression of MT-1A, β-actin and MT-2A in normal (control) and crushed sciatic nerve samples measured by semiquantitative RT-PCR. A) Gene expression of MT-1A in normal (control) and crushed sciatic nerve samples: Lane M shows DNA ladder (100 bp). Lanes after M, show 5 samples of control, 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 5 samples of control, day 1, day 3, week 1, week 3 and week 5 after injury, respectively. C) Gene expression of MT-2A in normal (control) and crushed sciatic nerve samples: Lane M shows DNA ladder (100 bp). Lanes after M, show 5 samples of control, day 1, day 3, week 1, week 3 and week 5 after injury, respectively.

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).

Table.**2-** Mean (NGAL/B-actin) ± SEM in groups.

Group	Mean(MT-2/B-actin)±SEM	Mean(MT-1/B-actin)±SEM
Control	2.56±0.11	**2.03±0.04**
Day 1	3.50±0.04	**2.82±0.02**
Day 3	3.71±0.12	**2.89±0.05**
Week 1	2.82±0.05	**2.13±0.03**
Week 3	2.77±0.01	**2.13±0.02**
Week 5	**2.58±0.04**	**2.63±0.01**

Figure 3. shows the statistical analysis of groups for MT-1A gene, respectively. The results of statistical analysis show a time variable (F= 81.24, P<0.0001), surgery variable (F=287.98, P<0.0001) and time*surgery (F=81.24, P<0.0001). In addition, these results revealed that MT-1A gene expression on the 1st and 3rd day after injury is significantly high in comparison with the control group (intact nerve) (P<0.0001) (Fig. 3).

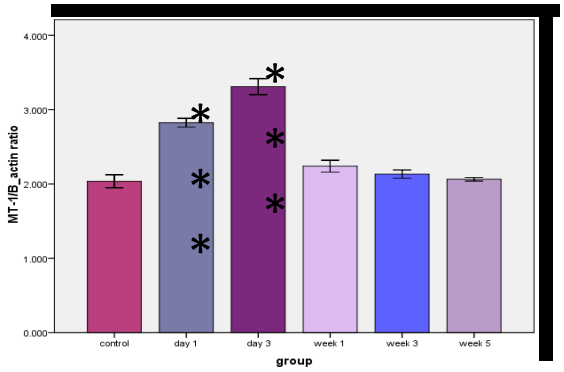

Figure 3. Quantitative data of MT-1 gene expression in groups. Data reported as mean ± SEM.

*** denotes statistical significance (P<0.0001), when compared to the control.

Figure 4 shows the statistical analysis of groups for MT-2A gene, respectively. The results of statistical analysis show a time variable (F= 13.54, P<0.0001), surgery variable (F=62.09, P<0.0001) and time*surgery (F=13.54, P<0.0001). In addition, these results revealed that MT-2A gene expression on the 1st and 3rd day after injury is significantly high in comparison with the control group (intact nerve) (P<0.0001) (Fig. 4).

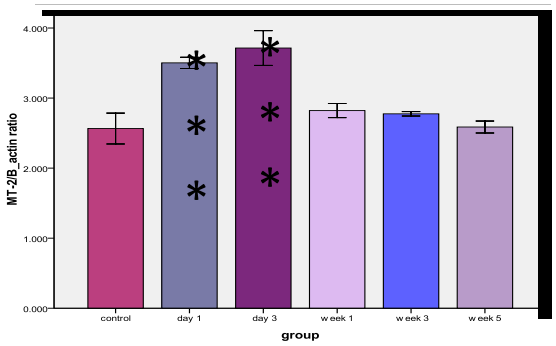

Figure 4. Quantitative data of MT-2 gene expression in groups. Data reported as mean ± SEM.

*** denotes statistical significance (P<0.0001), when compared to the control.

Immunohistochemistry assessment

Immunohistochemistry was carried out to ascertain whether the expression of MT-1A in protein level is caused by crush injury (Fig. 5 and 6).

A longitudinal section of a normal (intact) nerve was examined and it showed only a tint background with no reaction to the antibody observed (Fig.5A).

On the 1st day after injury, an intensive reaction to the antibody was seen (Fig.5B, Fig.6A). On the 3rd day after injury, immunohistochemical reaction to the antibody increased compared to the 1st day after injury revealing an active existence of the MT-1A molecule in this stage (Fig.5C, Fig.6B) and, in the 1st week after injury, this reaction decreased compared to the 1st and 3rd days after injury (Fig.5D). In the 3rd week after injury, the intensity of antibody reaction decreased

increasingly (Fig.5E) and immuno-reactivity for MT-1A in the 5th week after injury became negative, revealing the occurrence of regeneration (Fig. 5F).

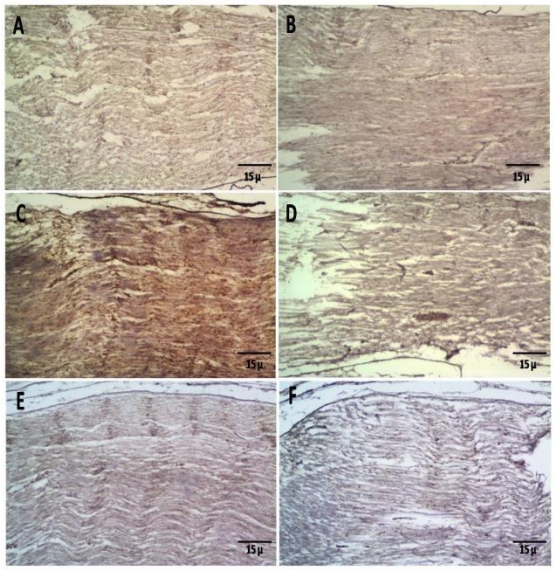

Figure 5. longitudinal sections of sciatic nerve samples (x10). A) control(normal), B) 1st day after injury, C) 3rd days after injury, D) 1th week after injury, E) 3rd weeks after injury, F) 5th weeks after injury. Brown colour indicated MT-1 antibody reaction. MT-1 protein appears brown.

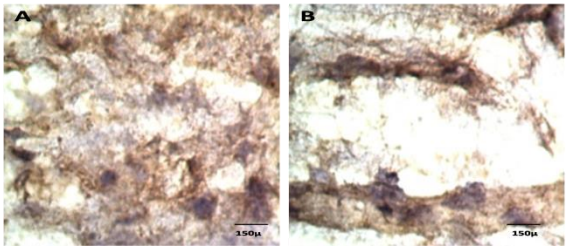

Figure 6. longitudinal sections of sciatic nerve samples (x100). A) 1st day after injury, B) 3rd days after injury. MT-1 protein appears brown.

Discussion

Nerve injuries due to accidents, acute compression, or surgeries are commonly traumatic lesion in peripheral nervous system (25). Tissue injury elicits oxidative stress (1-3). It is generally accepted that peripheral nerve damage induces oxidative stress in lesioned neurons (25).

MDA is used as a lipid peroxidation marker in nerve injuries. In crush injury, serum MDA started to increase on the first day, peaked at 7 days and then gradually decreased to normal levels at 14, 21 and 42 days (26, 27). 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 MT molecules in the first week may be due to oxidative stress in the crushed nerve.

To counteract ROS, biological systems have endogenous antioxidant molecules to protect themselves from oxidative damage (12).

MTs have been suggested to play an important role in protecting against ROS and has a protective role in cerebral ischemia and reperfusion (21). MT-1/2 display neuroregenerative and neuroprotective activity because they have antioxidant properties (15).

The expression of MT-I/II proteins is regulated in response to inflammation and oxidative stress (1). Antioxidant-specific gene induction is mediated by the " antioxidant-response element" (ARE) present in the promoter region of mammalian metallothioneine (1, 28). It is well established that MT-I/-II is a powerful neuroprotective proteins in vitro (20, 29).

The NG2 glial cells express MT-I/-II following CNS injury and this ability of these cells, support axonal regeneration (30).

MT-1 and MT-2 (MT-1&2) are expressed coordinately and have similar effects in most tissues, namely, decreasing oxidative stress (16).

Our results confirm that expression of MT-1 and MT-2 isoforms in crushed sciatic nerve have identical pattern. Extracellular MT-I/-II can promote axon regeneration, both *in vitro* and *in vivo* following an injury to the brain (20), or to a sciatic nerve (31). Axonal regeneration up to 1000 µm past the

transection site has seen by a single intravitreal injection of MT-I/-II, following complete optic nerve transection (17) Previous data showing that in sciatic nerves of MT-I/-II-deficient mice following crush injury, axonal regeneration is impaired (31).

Chung and colleagues have shown that MT expression is up-regulated 4 days after cortical injury (early phase of regenerative sprouting) in CNS (32). Hashimoto et al. used spinal cord compression injury (SCI) models in mice and by double labeling with in situ hybridization and fluorescence immunohistochemistry, MT-1 and MT-2 expression was observed in neurons and glial cells at 1 day post lesion, and MT-2 expression was also detected At 3 days post lesion (33).

In MT-I, II KO mice, reported a reduction and a decreased neurofilament density in the large myelinated axons in the phrenic nerve (34). Also, the expression of MT-I, II is up-regulated in human amyotrophic lateral sclerosis cases (35), it may serve a protective role for MTs (36).

In this investigation, our finding through semi-quantitative RT-PCR revealed that the expression of MT-1 & 2 transcriptome was up-regulated up to three days after crush injury and gradually decreased until 5 weeks post injury. Immuno-histochemisry assessment also revealed that the MT-1 protein localized in high intensity in the injured nerve fiber on the 3st day after crush injury, and the immuno-reactivity gradually decreased and disappeared in the 5th week after injury.

Conclusion

High expression of MT_1 and MT-2 is seen in the degenerative phase of nerve repair after crush injury and they play an important role in the detoxification of the crush site from free radicals.

Our results suggest that MT-I/-II may promote axonal regeneration. The action of MT-1/2 in nerve repair may arise from its antioxidant properties.

Thus, MT-I and MT-II, serve an important antioxidant role elicited in the peripheral nervous system by a crush injury, and they may accelerate neuronal regeneration.

In summary, our results showed that, after crush injury to sciatic nerve, the MT-1 & 2 molecules are upregulated in degeneration phase of repair, when oxidative Stress increasing, and these molecules involved mainly in the detoxify ROS and help to regenerative process.

Acknowledgments

Research was supported by Genomics Division, Systems Biology Institute, Chemical Injuries Research Center of Baqiyatallah University of Medical Sciences, Tehran-Iran.

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