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Original research

Improvement of peripheral nerve regeneration following nerve repair by silicone tube filled with curcumin: A preliminary study in the rat model

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ABSTRACT

The objective was to assess the effect of locally administered curcumin on peripheral nerve regeneration and functional recovery. Thirty male healthy white Wistar rats were divided into two experimental groups ($n = 15$), randomly: In control group (CG), the left sciatic nerve was exposed and transected proximal to tibio-peroneal bifurcation leaving a 10-mm gap. Proximal and distal stumps were each inserted into a silicone tube and filled with 10 μL sterilized olive oil. In treatment group (TG), the graft was filled with 10 μL curcumin (5 mg/mL) solved in olive oil. Each group was subdivided into three subgroups of five animals each and regenerated nerve fibers were studied 4, 8 and 12 weeks after surgery. Behavioral testing, sciatic nerve functional study, static sciatic index (SSI), gastrocnemius muscle mass and morphometric indices confirmed faster recovery of regenerated axons in TG than CG group ($p < 0.05$). In immunohistochemistry, location of reactions to S-100 in TG was clearly more positive than that in CG group. When loaded in a silicone tube, curcumin improved functional recovery and morphometric indices of sciatic nerve. Curcumin is readily available and its local application is easily performed without limitations of its poor bioavailability in systemic administration.

1. Introduction

A commonly encountered clinical problem which often results in long-term functional deficit is peripheral nerve injury.[1] After complete transection, peripheral nerve regeneration with sufficient recovery is a challenge, and treatment often results in little or no functional recovery.[2] Various techniques and agents have been used to enhance functional recovery of peripharal nerves following transection.[3,4]

Curcumin {1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-hepta diene-3,5-dione} is a traditional spice and a bioactive coloring agent in the rhizome of turmeric (*Curcuma longa*) that has been used for centuries in Asia to treat various illnesses. Curcumin possesses vast pharmacological properties including anti-inflammatory, anti-oxidant, anti-cancer, anti-apoptotic and anti-infectious effects. Curcumin can modulate various types of the inflammatory cytokines and enzymes. It has widely beneficial effects as antioxidant several times more than vitamin E.[5–7] The suggested mechanisms underlying these protective effects are based on inhibitory actions of curcumin on disease-mediated induction of inflammatory transcription factors, protein kinases, adhesion molecules, oxidative stress and inflammation.[8,9] Systemic beneficial effects of curcumin on sciatic nerve crush have already been reported.[10] Bioavailability of curcumin is poor and various approaches have also been used in attempts to improve its bioavailability.[11,12] The present study was designed to evaluate possible local effect of curcumin on peripheral nerve regeneration in a rat sciatic nerve transection model.

2. Materials and methods

Thirty male Wistar rats weighing approximately 300 g were divided into two experimental groups ($n = 15$), randomly: Control group (CG) and curcumin treated group (TG). Each group was further subdivided into three subgroups of five animals each and surveyed 4, 8 and 12 weeks after surgery. Two weeks before and during the experiments, the animals were housed in individual plastic cages with an ambient temperature of (23 ± 3) °C, stable air humidity and a natural day/night cycle. The rats had free access to standard rodent laboratory food and tap water. All measurements were made by two blinded observers unaware of the analyzed groups.

2.1. Surgical procedure

Animals were anesthetized by intraperitoneal administration of ketamine-xylazine (ketamine 5%, 90 mg/kg and xylazine 2%, 5 mg/kg). The procedure was carried out based on the guidelines of the Ethics Committee of the International Association for the Study of Pain.[13] The University Research Council approved all experiments.

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Following surgical preparation in the sham-operation group, the left sciatic nerve was exposed through a gluteal muscle incision and was 7 mm segment was excised proximal to the tibio-peroneal bifurcation where a 10 mm gap was left due to retraction of nerve ends. The gap was bridged using a silicone conduit, entubulating 2 mm of the nerve stump at each end. The inner and outer diameters of the tube were 2 and 6 mm, respectively. In CG the tube was filled with 10 μl sterilized olive oil. In curcumin treated group (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) the tube was filled with 10 μl curcumin (5 mg/mL) solved in olive oil. The Curcumin we used was not solvable in saline. We used olive oil as solvent. Accordingly in control group we used olive oil rather than saline. The animals were anesthetized and euthanized with transcardiac perfusion of a fixative containing 2% paraformaldehyde and 1%glutaraldehyde buffer (pH 7.4) 4, 8 and 12 weeks after surgery.

2.2. Functional assessment of reinnervation

2.2.1. Behavioral testing

Functional recovery of the nerve was assessed using the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale for rat hind limb motor function.[14,15] Scores of 0 and 21 were given when there were no spontaneous movement and normal movement, respectively. A score of 14 shows full weight support and complete limbs coordination. BBB recordings were performed by a trained observer who was blinded to the experimental design. The testing was performed in a serene environment. The animals were observed and assessed within a course of a 4-min exposure to an open area of a mental circular enclosure. BBB scores were recorded once before surgery in order to establish a baseline control and again weekly thereafter to assess functional recovery during 12 weeks. At each time interval five animals from each subgroup underwent Behavioral Testing.

2.2.2. Sciatic functional index (SFI)

Walking track analysis was performed 4, 8 and 12 weeks after surgery based on the method of others.[16] At each time interval five animals from each subgroup underwent SFI measurement. The lengths of the third toe to its heel (PL), the first to the fifth toe (TS), and the second toe to the fourth toe (IT) were measured on the experimental side (E) and the contralateral normal side (N) in each rat. The sciatic function index (SFI) of each animal was calculated by the following formula:

$$\mathrm{SFI} = -38.3 \times (\mathrm{EPL} - \mathrm{NPL})/\mathrm{NPL} + 109.5 \times (\mathrm{ETS} - \mathrm{NTS})/\mathrm{NTS} + 13.3 \times (\mathrm{EIT} - \mathrm{NIT})/\mathrm{NIT} - 8.8$$

In general, SFI oscillates around 0 for normal nerve function, whereas around −100 SFI represents total dysfunction. SFI was assessed in the NC group and the normal level was considered as 0. SFI was a negative value and a higher SFI meant the better function of the sciatic nerve.

2.2.3. Static sciatic index (SSI)

SSI is calculated using the static factors, not considering the print length factor (PL), according to the equation:

$$\mathrm{SSI} = [(108.44 \times \mathrm{TSF}) + (31.85 \times \mathrm{ITSF})] - 5.49$$

where:

$$\mathrm{TSF} = (\mathrm{ETS} - \mathrm{NTS})/\mathrm{NTS}$$
$$\mathrm{ITSF} = (\mathrm{EIT} - \mathrm{NIT})/\mathrm{NIT}$$

At each time interval five animals from each subgroup underwent SSI measurement. Like SFI, an index score of 0 was considered normal and an index of −100 indicated total impairment. When no footprints were measurable, the index score of −100 was given.[17]

2.3. Muscle mass

Recovery assessment was also indexed using the weight ratio of the gastrocnemius muscles 12 weeks after surgery. Immediately after sacrificing of animals, gastrocnemius muscles were dissected and harvested carefully from intact and injured sides and weighed while still wet, using an electronic balance. At each time interval five animals from each subgroup had muscle mass measurement.

2.4. Histological preparation and morphometric studies

Nerve mid-substance in experimental groups were harvested and fixed with glutaraldehyde 2.5%. They were post fixed in OsO4 (2%, 2 h), dehydrated through an ethanol series and embedded in Epon. The nerves were cut in 5 μm in the middle, stained with toluidine blue and examined under light microscopy. Morphometric analysis was carried out using an image analyzing software (Image-Pro Express, version 6.0.0.319, Media Cybernetics, Silver Springs, MD, USA). Equal opportunity, systematic random sampling and two-dimensional dissector rules were followed in order to cope with sampling-related, fiber-location-related and fiber-size related biases.[18] The parameters assessed in histological studies included number of nerve fibers, diameter of nerve fibers, diameter of axons, thickness of myelin sheath and G ratio.

2.5. Immunohistochemical analysis

In this study, anti-S-100 (1:200, DAKO, USA) was used as marker for myelin sheath. Specimens were post fixed with 4% paraformaldehyde for 2 h and embedded in paraffin. Prior to immunohistochemistry nerve sections were dewaxed and rehydrated in PBS (pH 7.4). Then the nerve sections were incubated with 0.6% hydrogen peroxide for 30 min. To block non-specific immunoreactions the sections were incubated with normal swine serum (1:50, DAKO, USA). Sections were then incubated in S-100 protein antibody solution for 1 h at room temperature. They were washed three times with PBS and incubated in biotynilated anti-mouse rabbit IgG solution for 1 h. Horseradish peroxidase-labeled secondary antibody was applied for 1 h. After that all sections were incubated with 3,3′- diaminobenzidine tetrahydrochloride chromogene substrate solution (DAB, DAKO, USA) for 10 min. The results of immunohistochemistry were examined under a light microscope.

2.6. Statistical analysis

The results were expressed as means ± SD. Statistical analyses were performed using PASW 18.0 (SPSS Inc., Chicago, IL, USA). Model assumptions were evaluated by examining the residual plot. Results were analyzed using a factorial ANOVA with two between-subjects factors. Bonferroni test for pairwise comparisons was used to examine the effect of time and treatments. The differences were set at $P < 0.05$.

3. Results

3.1. BBB recovery

Fig. 1 shows BBB scores compared to the baseline. All experimental groups showed the greatest degree of functional deficit one week after surgery. The curcumin treated group showed significant improvement in locomotion of the operated limb compared to the CG group during the study period ($P < 0.05$).

3.2. Recovery of sciatic nerve function and reinnervation

3.2.1. SFI outcome

Fig. 2 shows sciatic function index (SFI) values in experimental groups. Prior to surgery, SFI values in both groups were near zero. After the nerve transection, the mean SFI decreased to −100 due to the complete loss of sciatic nerve function in all animals. At the end of the study period, animals of curcumin group achieved a mean value for SFI of −41.2 ± −2.49 whereas in group CG a mean value of −57.4 ± −5.12 was found. The statistical analyses revealed that the recovery of nerve function was significantly ($P < 0.05$) different

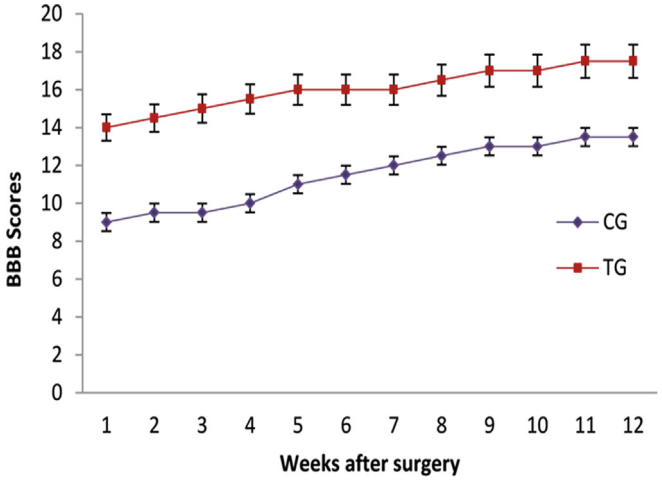

Fig. 1. BBB score for all experimental groups. Local administration of curcumin with silicon tube grafting gave better scores than in CG group. Standard error at each data point is shown with bars.

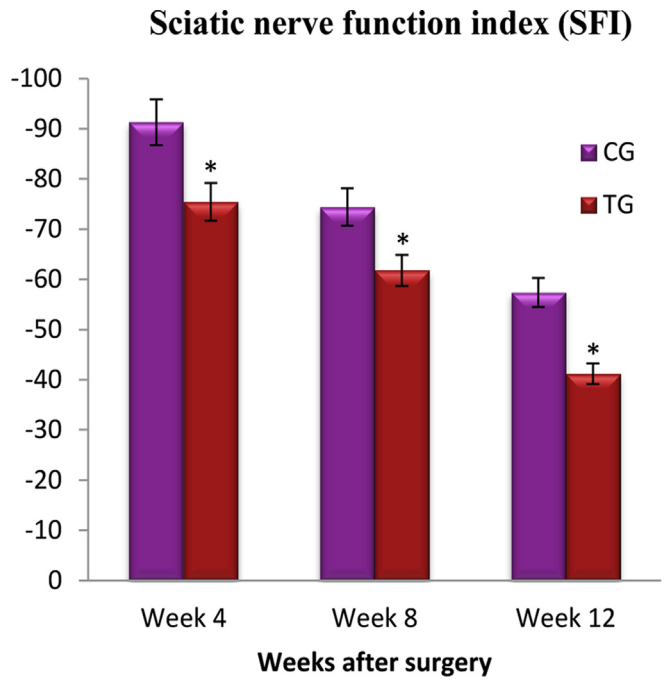

Fig. 2. Bar graph indicating sciatic nerve function index (SFI) values in each experimental group during the study period. Local administration of curcumin with silicone tube gave better results in functional recovery of the sciatic nerve than in CG group. Data are presented as mean ± SD. *$P < 0.05$ vs CG group.

between TG and CG groups and application of the curcumin in silicone tube significantly improved functional recovery in the course of time.

3.2.2. SSI outcome

Changes in SSI were similar to those observed in SFI, indicating significant deficit following the sciatic nerve transection (Fig. 3). Changes in SSI were significant at weeks 4, 8 and 12 weeks of recovery ($P < 0.05$). The contrasts indicated SSI values at week 12 to differ significantly from those obtained from CG, a trend also noticed for SFI ($P < 0.05$).

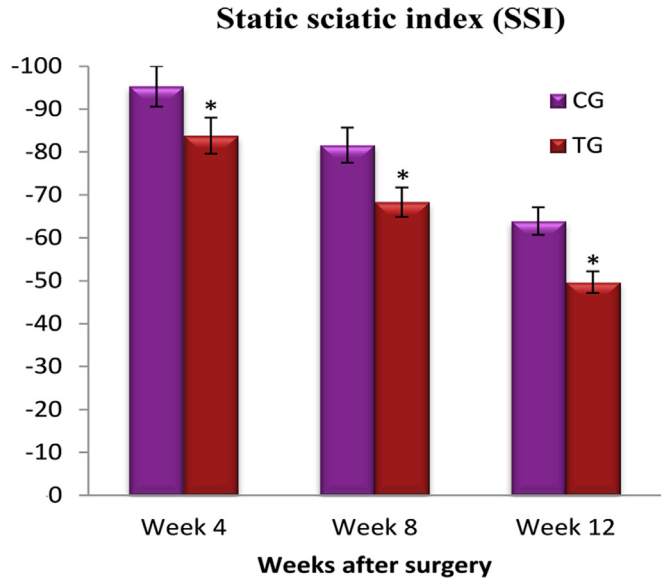

Fig. 3. Bar graph indicating static sciatic index (SSI) values in each experimental group during the study period. Local administration of curcumin with silicone tube gave better results in functional recovery of the sciatic nerve than in CG group. Data are presented as mean ± SD. * $P < 0.05$ vs CG group.

3.3. Muscle mass measurement

The mean ratios of gastrocnemius muscle weight were measured at the end of the study period. Mean weight of the gastrocnemius muscle for animals of the control and treatment groups were 0.7 ± 0.09 and 1.24 ± 0.12, respectively. There was a statistically significant difference between the muscle weight ratios of the TG and CG groups ($P < 0.05$). Because of an increase in muscle mass the TG group, muscle had been reinnervated and fiber diameter has been increased, leading to an increase in cross-sectional area. The results showed that in the curcumin treated group weight loss in the gastrocnemius muscle was ameliorated by curcumin local administration (Fig. 4).

3.4. Histological and morphometric findings

Table 1 shows quantitative morphometric analyses of regenerated nerves for each of the experimental groups. Statistical analysis by means of a one-way ANOVA test showed that the increase in number of fibers, diameter of fibers and diameter of axons, as well as increase in mean myelin thickness in the first 4 weeks of the study period was statistically significant in both groups ($P > 0.05$). On the contrary, 8 and 12 weeks after surgery no significant difference in mean thickness of myelin sheath was observed between groups ($P < 0.05$), however, there was significant difference in number of fibers, diameter of fibers and diameter of axons between two experimental groups.

Using Factorial ANOVA analysis with two between-subjects factors (Group × time); inside the TG group myelin thickness did not show a significant difference between 8 and 12 weeks ($P > 0.05$). The thickness of myelin sheaths showed an interaction across time in both groups. An increase in mean thickness of myelin sheaths was not statistically different between 8 and 12 weeks inside each group ($P > 0.05$).

3.5. Immunohistochemistry

Immunoreactivity to S-100 protein was extensively observed in the cross sections of regenerated nerve segments. The expression of S-100 protein signal was located mainly in the myelin sheath. The

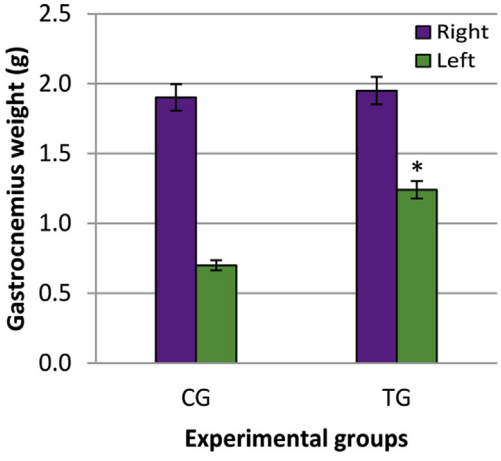

Fig. 4. Gastrocnemius muscle weight measurement. The gastrocnemius muscles of both sides (operated left and unoperated right) were excised and weighed in the experimental groups at 12 weeks after surgery. Data are presented as mean ± SD. * $P < 0.05$ vs CG group.

Table 1
Morphometric analyses of regenerated nerves for each of the experimental groups: values are given as mean ± SD.

Weeks	TG			CG		
	4	8	12	4	8	12
N	3126 ± 259	4037 ± 283	6231 ± 225	1714 ± 289[a]	3145 ± 281[a]	3723 ± 264[a]
D	7.73 ± 0.34	9.69 ± 0.53	10.21 ± 0.78	3.73 ± 0.76[a]	7.95 ± 0.42[a]	8.97 ± 0.53[a]
d	4.49 ± 0.39	5.93 ± 0.71	6.52 ± 0.37	2.88 ± 0.65[a]	4.45 ± 0.73[a]	87.87 ± 0.5[a]
T	1.67 ± 0.06	2.56 ± 0.36	2.61 ± 0.48	0.62 ± 0.11[a]	2.68 ± 0.39	2.32 ± 0.33
G-ratio	0.58	0.61	0.63	0.77	0.55	0.55

N: Number of fibers D: Diameter of fibers (μm) d: Diameter of axon (μm) T: Thickness of myelin sheath (μm).
[a] The mean difference is significant at the 0.05 level.

axon also showed a weak expression indicating that Schwann cell-like phenotype existed around the myelinated axons (Fig. 5). In both TG and CG groups, the expression of S-100 and the findings resembled those of the histological evaluations.

4. Discussion

The design of the present study was a sciatic nerve transection model in rat. In regenerative medicine tissue loss is the most

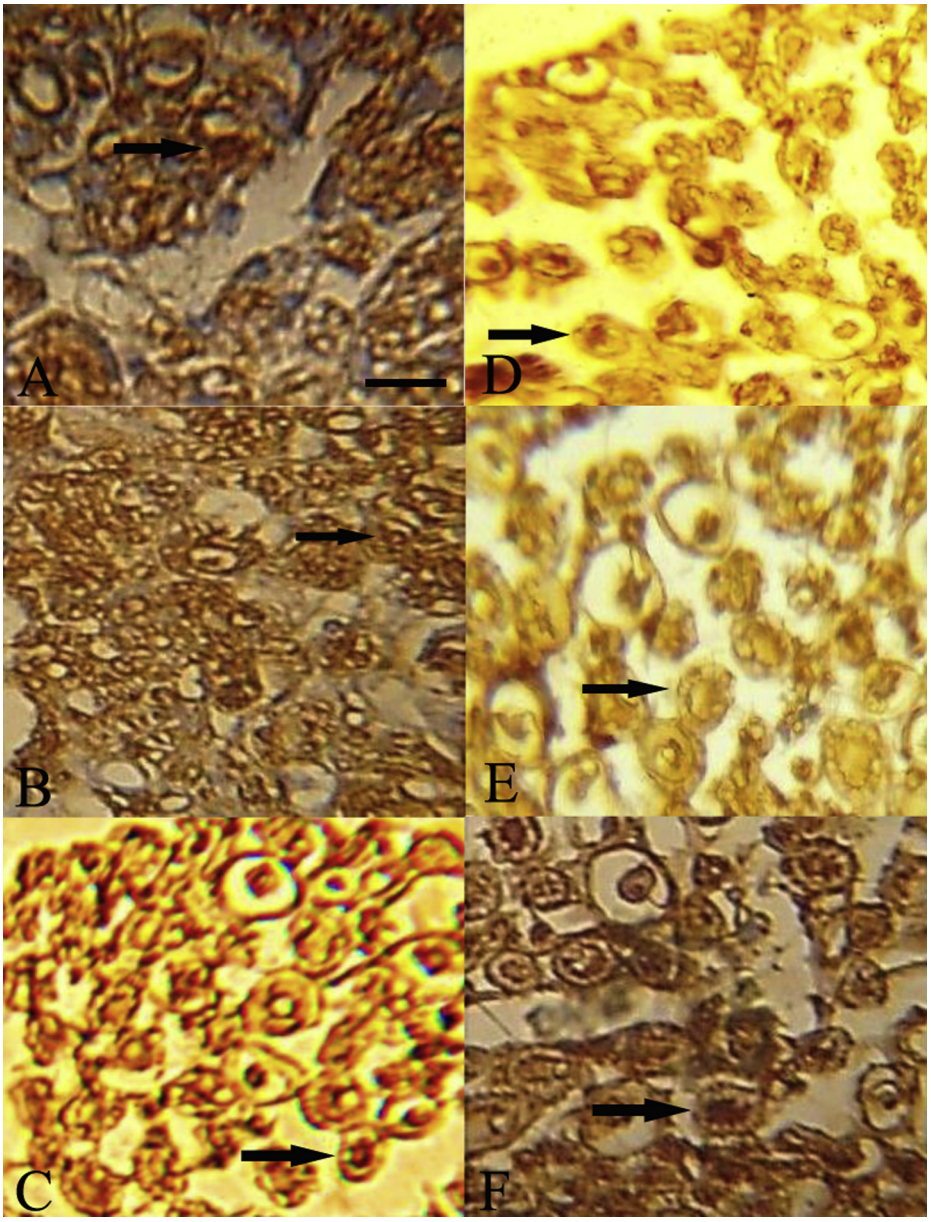

Fig. 5. Immunohistochemical analysis of the regenerated nerves. Representative cross section taken from midpoint of CG (A–C) 4, 8 and 12 weeks after surgery, respectively, and midpoint of TG (D–F) 4, 8 and 12 weeks after surgery, respectively. There was clearly more positive staining of the myelin sheath-associated protein S-100 (arrows) within the periphery of nerve, indicating well organized structural nerve reconstruction in curcumin treated nerve compared to that of the CG during study period. Scale bar: 10 μm.

challenging topic especially where nerve tissue loss is the case.[2] Using autograft as a gold standard is still ongoing in peripheral nerve repair. Since the present study was designed to assess entubulation neurorrhaphy combined with local curcumin administration as an alternative method, no nerve autograft group was considered in experimental design. In the present study, curcumin, a natural phenolic compound, was evaluated for its ability to locally improve regeneration of transected sciatic nerve. The results showed that application of curcumin in a silicone tube resulted in faster improvement functional recovery of the sciatic nerve during the study period. Left gastrocnemius muscle weight was significantly greater in the TG group than in the CG group, indicating indirect evidence of successful end organ reinnervation in the curcumin treated animals. Four weeks after surgery morphometrical indices showed significant improvement in curcumin treated group. At weeks 8 and 12 quantitative morphometrical indices of regenerated nerve fibers showed significant differences between the TG and CG groups, indicating a beneficial effect of local application of curcumin on the nerve regeneration. The Curcumin we used was not solvable in saline. Hence, we used olive oil as a solvent. Accordingly in control group we used olive oil rather than saline. Compared to our other study [19] no comparable difference was observed between saline and olive oil filled groups. Regarding this comparison neither positive nor negative effect of olive oil was observed during the study period.

Both morphological and functional data have been used to assess neural regeneration after induced crush injuries, the correlation between these two types of assessment is usually poor.[20–22] Classical and newly developed methods of assessing nerve recovery, including histomorphometry, retrograde transport of horseradish peroxidase and retrograde fluorescent labeling[23,24] do not necessarily predict the reestablishment of motor and sensory functions.[22,25–27] Although such techniques are useful in studying the nerve regeneration process, they generally fail in assessing functional recovery.[22] Therefore, research on peripheral nerve injury needs to combine both functional and morphological assessment. Castaneda et al.,[28] suggested that arrival of sprouts from the proximal stump at the distal nerve stump does not necessarily imply recovery of nerve function. BBB is widely used to assess functional recovery in spinal cord injured animals, however, it has been demonstrated that it could be most useful in assessment of never repair processes in peripheral nerve injuries[15] and information taken from BBB scale may be invaluable in evaluation of peripheral nerve process. In order to assess hind limb recovery the open field locomotor was used and results of the present study showed that the curcumin treated animals had been improved in locomotion of the operated limb compared to the CG group during the study period. Walking track analysis and static sciatic index has frequently been used to reliably determine functional recovery following nerve repair in rat models.[16,17,26] Static sciatic index is a time-saving digitized static footprint analysis and, a good correlation between the traditional SFI, the newly developed static sciatic index (SSI) has been reported.[29] The Static sciatic index is an easy technique for accurate functional assessment of peripheral nerve regeneration in rats. The present study again showed similar results taken from both SFI and SSI assessments. However, no significant difference was observed in myelin thickness between two groups indicating that selection of a reliable technique for nerve repair assessment is crucial.

Regenerated rat peripheral nerve fibers are not able to return spontaneously to their normal pretrauma state, therefore neuroprotective factors combined with nerve entubulization have been used to accelerate nerve regeneration.[30–32] Growth factors are members of family of neurotrophic factors that support and influence the growth and regenerative capacity of neurons. These substances are produced by a number of tissues during development and direct the formation of the brain and spinal cord and their connections to target organs such as muscle. Nerve growth factor has been demonstrated that promotes nerve regeneration.[32–36]

Curcumin has anti-inflammatory and antioxidants properties. Curcumin is able to modulate the regulation of the inflammatory cytokines such as IL-6, TNF and COX-2.[5] Curcumin is also a good scavenger of the reactive oxygen species and is suggested to be a good neuroprotectant. It can pass through the blood brain barrier, which is an evidence of amplification of its neuroprotectant potential in neurodegenerative disorders such as Parkinson's and Alzheimer's diseases.[6] In addition, it has been reported that curcumin has protective effects after spinal cord injuries. The previous studies have stressed the anti-apoptotic effects of the curcumin on the neurons.[37]

Curcumin has a variety of pharmacologic effects including acts as a neuroprotective agent in several neurologic diseases.[9] For example, curcumin inhibited neuronal cell death from dopaminergic neurotoxicity, apparently through the inhibition of c-Jun N-terminal kinase pathway, and it also protected the brain from I/R-induced neuronal damage by increasing the antioxidant system and decreasing oxidative stress.[38–40]

Despite the multiple pharmacological effects and the Phase I clinical safety even at high doses (12 g/day), curcumin has poor in vivo bioavailability, as demonstrated by its low serum levels and limited tissue distribution.[41–44] Numerous studies have evaluated the level of curcumin after administration and found that either no curcumin or its metabolites, or only low levels, were detected in serum or tissue.[45–48] Curcumin given orally undergoes conjugation, resulting in curcumin glucuronide and sulfates. Curcumin administered intraperitoneally or systemically undergoes reduction to generate tetrahydrocurcumin, hexahydrocurcumin and octahydrocurcumin.[49,50] In addition to the poor bioavailability, curcumin metabolites that are the result of either conjugation or reduction have been found to be biologically inactive by most studies.[51–53] Our other work investigating systemic effects of curcumin on transected nerve regeneration (unpublished data) supports the poor bioavailability of curcumin. It has been confirmed that the most efficient means of delivering curcumin to cells is via incorporation in to liposomes.[54] In a wound healing study Curcumin treatment resulted in enhanced fibronectin and collagen expression.[55] Others demonstrated that the viscous collagen gel containing laminin and fibronectin can provide a potential promoting effect on the regeneration of dissected nerves in the silicone rubber tubes.[56] The mentioned mechanisms could be considered as a rationale for significantly improved and accelerated nerve regeneration in locally used curcumin in the present study.

Even though our preliminary study shows the neuroprotective action of local curcumin in peripheral nerve injuries, data regarding the molecular mechanisms leading to the neuroprotective action in transected models is limited. We have not given the histological and molecular evidence for neuroprotective action of curcumin. This may be considered as a limitation to our study. Therefore, the authors stress that the aim of the current investigation was to evaluate a single local dose and clinical treatment potential of curcumin on nerve regeneration. Mechanism of neuroprotective action remains to be investigated.

Others[10] used dosage of 100 mg/kg for curcumin in intraperitoneal administration in a crush model study. In local application we used a reduced dosage (5 mg/mL) in our study design. Thus, dose response studies should be conducted for curcumin to determine the combination of the graft and the compound that achieve maximal efficacy in nerve transection models.

5. Conclusion

The present study demonstrated that local application of curcumin could accelerate and improve functional recovery after

transection of sciatic nerve. Curcumin is readily available and its local application is easily performed without limitations of its poor bioavailability in systemic and oral administration.

Ethical approval
None declared.

Funding
None declared.

Author contribution
Rahim Mohammadi: Study design, data analysis and writing.
Hadi Mahmoodi: Experimental procedures.

Conflict of interest
None declared.

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