Biomedical Research 2016; 27 (2): 322-327 *ISSN 0970-938X*
www.biomedres.info

Curcumin improves sciatic nerve regeneration by up-regulating S100 expression in mice.

Lina Zhao[1], Shusen Cui[2*]

[1]Department of Thyroid Surgery, China-Japan Union Hospital of Jilin University, Changchun, Jilin 130033, China

[2]Department of Hand Surgery, China-Japan Union Hospital of Jilin University, Changchun, Jilin 130033, China

Abstract

Curcumin, a naturally-occurring anti-oxidant ingredient extracted from dried Curcuma plants, has been found to have therapeutic effects in degenerative diseases of central nervous system and mental disorders such as Alzheimer's, Parkinson's disease, depression, seizures and anxiety. However, the Curcumin's neuroprotective effect on peripheral nerve injury has been not reported. This article uses curcumin to treat BALB/c mouse model of sciatic nerve injury, and explores the effects and mechanism of curcumin on sciatic nerve regeneration. Electrophysiological testing finds that 40 mg/kg/day and 20 mg/kg/day intragastric curcumin administration recover sciatic nerve function better than 10 mg/kg/day and saline model control. Luxol Fast Blue (LFB) staining finds that 40 mg/kg/day and 20 mg/kg/day curcumin administrations promote the regeneration of the sciatic nerve myelin structure. Immunohistochemical staining show that curcumin up-regulates S100 expression in L4-6 spinal cord. Real time qPCR and Western-blotting methods are performed to detect S100 expression levels in L4-6 spinal cord. Administrations of 40 mg/kg/day and 20 mg/kg/day dosage result in high S100 levels. These results suggest that curcumin is effective to promote the regeneration of sciatic nerves in a curcumin concentration-dependent manner and the regeneration is associated with up-regulation of S100 expression. The finding of this article is conducive to developing the new use of curcumin to cure peripheral nerve injury in clinic.

Keywords: Curcumin, Peripheral nerve injury, S100, Sciatic nerve, Regeneration.

Accepted January 20, 2016

Introduction

Curcumin is a yellow polyphenol extracts of medicinal Curcuma plants, with anti-oxidant and anti-inflmmatory properties [1,2]. Curcumin has a wide range of targets for central nervous system, and the neuroprotective effects involve a variety of mechanisms, impacting neurotransmitters in the brain, regulating the HPA axis, improving the neural factors and nerve regeneration and inhibiting neuronal apoptosis [3-7]. Study has shown that peripheral nerve injury would inevitably result in corresponding neuron death in spinal cord and dorsal root ganglion, and apoptosis is an important form of impaired neuronal death [8]. However, the effects of curcumin on the functional recovery of peripheral nerves following their injury are lack of adequate understanding. S100 proteins encode by a family of S100 genes [9]. They contain two calcium-binding sites and have highly conservative amino acid sequences in vertebrate animals. S100 proteins have been implicated in a variety of intracellular and extracellular functions [10]. In particular, several S100 proteins are markers in the neural research [11-21]. This article explores the effects and mechanism of curcumin on peripheral neural regeneration in a BALB/c mouse model with sciatic nerve injury.

Materials and Methods

Reagents and animals

Curcumin with a molecular weight 368.39 Da was purchased from Sigma-Aldrich, St. Louis, M. Its structure was shown in Figure 1. Male BALB/c mice (weighing 25 ± 2 g) were fed in in Basic Medical College Laboratory Animal Center of Jilin University (Changchun, Jilin, China) at routine conditions. Animal experiment protocols were approved by Jilin University Animal Ethics Committee.

Modeling and administration

Mouse modeling was performed as referenced to literature [22,23]. Briefly, mice were narcotized and fixed in the prone position. Infrapiriformis was dissected through a 2 cm-longitudinal incision on unilateral rear thigh. Sciatic nerves were separated, then interrupted in the position 0.5 cm below ischial tuberosity, and immediately microsurgically anastomosed, suturing muscles and skins. Then 160 modeled mice were randomized into model control, high-dose (40 mg/[kg·weight]/day), mid-dose (20 mg/kg/day) and low-dose (10

mg/kg/day) groups, respectively, 40 mice each group for 4 different time periods. In experiments, curcumin powder was dissolved in Dimethylsulfoxide (DMSO) and subsequently mixed with 0.9% NaCl saline, forming a concentration series of 1, 0.5, 0.25, 0 mg/ml for high-dose, mid-dose, low-dose and model control groups, respectively. The final volume concentration of DMSO in 0.9% NaCl saline was 0.05%. Curcumin was intragastrically (i.g.) administrated 1 ml daily for continuous 1 week. DMSO-containing 0.9% NaCl saline served as model control.

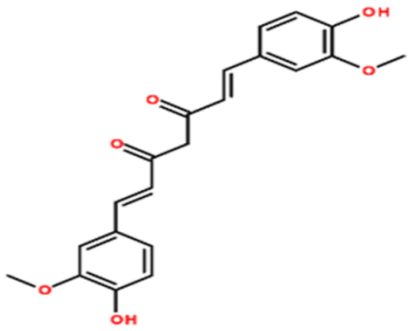

Figure 1. *Structure of curcumin.*

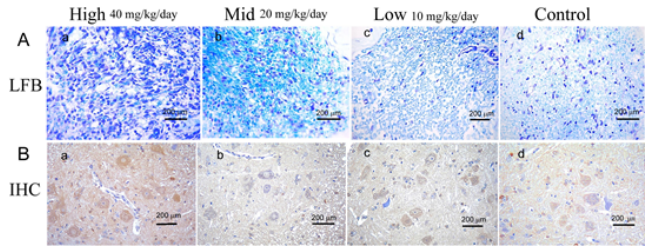

Figure 2. *Immunohistochemical and Luxol Fast Blue (LFB) staining of transverse section of nerve and myelin shealth specimens at 8 weeks (40x magnification).*

Electroneurophysiological testing

Affected sciatic nerves were detected by an electromyograph evoked potential apparatus (Keypoint®, Medtronic A/C Inc., Skovlunde, Denmark) as referenced to literature [23,24].

Briefly, sciatic nerves were exposed at 23°C, then detective electrodes were placed for recording potential amplitude and Motor Nerve Conductive Velocity (MNCV) following a single 10 mA current stimulus.

Spinal cord sampling

Ten mice of each group were narcotized by i.p. injection of 3% ketamine with a dose of 100 mg/kg/day 1 week, 2 weeks, 4 weeks and 8 weeks after curcumin adminnistration, respectively.

After electroneurophysiological test was completed, canalis vertebralis was opened via posterior vertebral column midline incision. L4-6 spinal cords connecting injured sciatic nerves were integrally removed and immediately stored in liquid nitrogen for use.

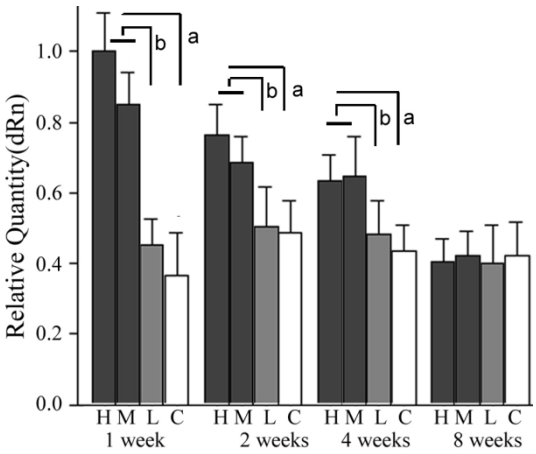

Figure 3. *Real-time qPCR analysis of relative quantity of S100/GAPDH mRNA ratio in the L4-6 spinal cords 1, 2, 4 and 8 weeks following curcumin administration for the high-dose 40 mg/kg/day (H), mid-dose 20 mg/kg/day (M), low-dose 10 mg/kg/day (L) and model control (C) groups . Data are stated as mean ± standard deviation, n=5. ANOVA is used to analyse difference between groups. [a]P<0.05 versus model control. [b]P<0.05 versus low-dose group. GAPDH, glyceraldehyde-phosphate dehydrogenase.*

Immunohistochemical staining and LFB staining

Sciatic nerve between anastomosis (including anastomotic stoma) and distal 1 cm were cut off. Sciatic nerves were fixed 3 days in 10% neutral formaldehyde for immunohistochemical and LFB staining assays. For immunohistochemical assays on S100 proteins, briefly, slices were placed in 3% H_2O_2 for 10 min to block endogenous peroxidase, and boiled 10 min in 0.01 mol/L sodium citrate buffer (pH=6.0).

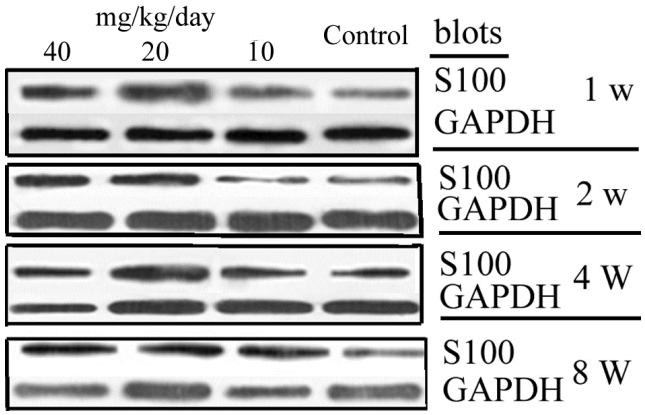

Figure 4. *Western blots of L4-6 spinal cord S100/GAPDH grayscale ratio 1, 2, 4 and 8 weeks following curcumin administration. GAPDH (Glyceraldehyde-Phosphate Dehydrogenase).*

Then, slices were blocked 15 min with anti-species serum following washed by 1 mol/L PBST (pH=7.4). Anti-mouse S100 polyclonal antibodies (Beyotime Institute of Biotechnology, Najing, Jiangsu, China) were added overnight at 4°C. Biotinylated goat anti-rabbit secondary antibodies IgG were added 20 min at 37°C. Streptavidin and biotinylated horseradish peroxidase were added 20 min at 37°C, followed by color development using the DAB (3,3-diaminobenzidine)

kit (Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China). Brown stains indicated positives. Microscopic examination (Olympus PM-10A0, Olympus, Beijing, China) was done by two separately blind pathologists. Each pathologist used image analysis software Image-Pro® Plus 6.0 (Media Cybernetics Inc., Silver Spring, MD) to analyse the interest optical density (IOD) by scanning the stained area. For LFB staining, slices were stained 12 h in LFB solution at 60°C. Then slices were soaked 5 min in 95% ethanol, incubated 15 sec in 0.05% lithium carbonate solution, and washed by 70% ethanol and distilled water, respectively. After dehydration and vitrification, slices were sealed for microscopic observation. In each slice, five microscopic fields of 400X magnification were randomly selected for image acquisition. The diameter and number of myelinated fibers was calculated using image analysis software Image-Pro® Plus 6.0.

Real-time qPCR analysis

Total RNAs were extracted from tissue samples using the TRIzol reagent method. cDNA was cloned by reverse transcriptase using total RNA templates. S100 mRNA were amplified by real-time qPCR using cDNA templates. Primers of S100 and GAPDH were designed by Beacon Designer 7 software (Premier Biosoft Inc., Palo Alto, CA) and synthesized by Sangon Bioengineering, Inc. (Shanghai, China) (see Table 1). A pair of GAPDH primers served as inner control in each reaction system. The reaction conditions included 95°C, 30s; 58°C, 60s; 72°C, 60s; 40 cycles.

Western-blotting

L4-6 spinal cords samples were lysed in icecold Radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Inc., Nanjing, China). Proteins were separated by SDS-PAGE with 12% gels and electroblot onto Polyvinylidine Fluoride (PDVF) films. Film was soaked in rabbit anti-mouse S100 polyclonal antibodies (diluted 5,000 times with PBS containing 1% BSA. Beyotime Institute of Biotechnology, Najing, Jiangsu, China) overnight in 4°C. Horse radish peroxidase-labled goat anti-rabbit secondary antibodies IgG were added 1 h at 37°C. Then color development was performed using Western Blotting DAB Testing Kit (Beyotime Inc., Nanjing, China). X-ray film was exposed, then scanning and analyzing. The protein levels were presented as the relative grayscales of interest protein/GAPDH ratio, analyzed using the software Image-Pro®Plus 6.0.

Statistical analysis

Data were stated as the mean ± standard deviation. SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL) was used for statistical analysis. One-way ANOVA and Dunn' ad hot test were used for comparing differences between groups. $P<0.05$ was statistically significant.

Table 1. *Beacon designer 7 software designs S100 and GAPDH primers.*

Primer Name	Sequence
upper primer 5'	5'-ACTGAAGGAGCTTATCAACAACGA- 3'
lower primer 5'	5'- AGTGTGACTTCCAGGAGTTCATG- 3'
S100 Probe	5'- GTGATGGAGACGCTGGACGAAGATGG-3'
upper primer 5'	5'- CCATTTGCAGTGGCAAAG- 3'
lower primer 5'	5'- CACCCCATTTGATGTTAGT- 3'
GAPDH Probe	5'- CAAGGCCGAGAATGGGAAGCTTGTC- 3'

Results

Neuroelectrophysical testing

The potential amplitude and MNCV were detected for observing the neural function performance (see Table 2). After curcumin administration, the potential amplitude and MNCV of each group went up along from 1 week to 8 weeks. The increment for the high-dose 40 mg/kg/day and mid-dose 20 mg/kg/day curcumin administration was more than the low-dose 10 mg/kg/day and model control in 1, 2, 4 and 8 weeks period, respectively ($P<0.05$ for each comparison).

Table 2. *Neuroelectrophsical potential amplitude (mV) and MNCV(m/sec).*

Curcumin (mg/kg/day)	Potential amplitude, mV				motor conductive nerve velocity (MNCV), m/sec			
	1 week	2weeks	4weeks	8 weeks	1 week	2 weeks	4 weeks	8 weeks
40 High	2.4 ± 0.2[a,b]	5.1 ± 0.2[a,b]	23.2 ± 0.2[a]	33.1 ± 0.6[a,b]	19.8 ± 0.3[a,b]	39.8 ± 0.4[a,b]	62.7 ± 1.7[a]	65.9 ± 1.2[a,b]
20 Mid	2.3 ± 0.2[a,b]	4.9 ± 0.2[a,b]	22.4 ± 0.3[a]	31.3 ± 0.4[a,b]	19.3 ± 0.2[a,b]	36.7 ± 0.7[a,b]	54.8 ± 1.2	62.3 ± 0.6[a,b]
10 Low	1.4 ± 0.1	3.1 ± 0.1	18.7 ± 0.3	20.1 ± 0.4	12.3 ± 0.2	29.9 ± 0.4	50.9 ± 0.4	53.8 ± 0.5
0 Control	1.3 ± 0.1	2.9 ± 0.2	14.3 ± 0.2	17.9 ± 0.5	10.8 ± 0.2	26.7 ± 0.2	49.3 ± 0.5	45.8 ± 0.8

Data are stated as mean ± SD, n=5. ANOVA is used to analyse difference between groups. [a]P<0.05 versus model control. [b]P<0.05 versus low-dose group.

For instance, the potential amplitude and MNCV at 8 weeks for the high-dose 40 mg/kg/day curcumin administration were 1.65-fold and 1.22-fold than the low-dose 10 mg/kg/day, respectively. These electrophysical features were not significantly different between the high-dose and mid-dose groups, or between the low-dose and model control ($P<0.05$ for each comparing). These results suggested that curcumin

administration to sciatic nerve-injured mice can promote the functional recovery of sciatic nerves.

LFB staining

LFB staining was performed to detect nerve myelin status 8 weeks following administration (see Figure 2). Myelin was dyed into blue color but axons with a white background were not dyed. Myelin of the high-dose 40 mg/kg/day and mid-dose 20 mg/kg/day presented regular shapes, uniform thickness, clear boundary and few hyperplasia surrounding myelin.

Table 3. Amount and diameter of myelinated nerve fibers 8 weeks following administration.

Curcumin (mg/kg/day)	40 High	20 Mid	10 Low	0 Control
Myelinated nerve amount (n/mm^2)	79 ± 2^{a,b}	69 ± 2^{a,b}	53 ± 3^{a}	46 ± 1
Diameter of myelin, μm	2.54 ± 0.28^{a,b}	2.28 ± 0.16^{a,b}	1.88 ± 0.30	1.44 ± 0.23

Data are stated as mean ± standard deviation, n=5. ANOVA is used to analyse difference between groups. ^{a}P<0.05 versus model control. ^{b}P<0.05 versus low-dose group.

Myelin of the low-dose 10 mg/kg/day was irregular shapes and thickness, moderately clear border and interfascicular hyperplasia of fibrous connective tissues. The model control myelin presented a worse fiber status.

The amount and diameter of myelinated nerve fibers 8 weeks following administration were measured by image analysis software Image-Pro® Plus. Version 6.0 (see Table 3). The high-dose 40 mg/kg/day and the mid-dose 20 mg/kg/day administration got larger myelinated nerve fibers in amount and diameter than the low-dose 10 mg/kg/day and model control. For instance, the amount and diameter of myelinated nerve fibers in the high-dose 40 mg/kg/day group were 1.49-fold and 1.35-fold than the low-dose 10 mg/kg/day group, indicating markedly effects on recovering sciatic nerve myelin structure.

Immunohistochemical staining

Color density of immunohistochemical positives was semi-quantitatively described. Five microscopic fields were randomly selected from each slice for calculating the Immunohistochemical Optical Density (IOD) value of positive cells. The IOD values and positive expression intensity was positively correlated. The results were shown in Figure 2 and Table 4.

S100 positive expression was clearly seen under the light microscope, with brown fine particles seen in cytoplasm. The nuclei were not colored. Starting from 1 week following curcumin administration, ipsilateral spinal cord S100 positive expression increased. Two weeks after administration, positive staining density got a peak. At this time, the IOD for the high-dose 40 mg/kg/day group was 1.79-fold than the low-dose 10 mg/kg/day. Then from on 4 weeks, positive cells reduced their positive expression color. At every time period, the stain for the high-dose group 40mg/kg/day and the mid-dose group 20 mg/kg/day was significantly stronger than the low-dose 10 mg/kg/day and model control. The results suggest that 40 mg/kg/day and 20 mg/kg/day dose promoted S100 expression.

Table 4. Immunohistochemical optical density value of L4-6 spinal cord S100 protein and S100/GAPDH protein blot grayscale ratio.

Curcumin mg/kg/day	Immunohistochemical optical density(IOD)				S100/GAPDH blot grayscale ratio			
	1 week	2 weeks	4 weeks	8 weeks	1 week	2 weeks	4 weeks	8 weeks
40 high	1.31 ± 0.02^{a,b}	2.01 ± 0.02^{a,b}	1.021 ± 0.02^{a,b}	0.63 ± 0.02^{a,b}	1.12 ± 0.02^{a,b}	1.80 ± 0.02^{a,b}	1.43 ± 0.02^{a,b}	0.23 ± 0.02
20 mid	0.98 ± 0.03^{a,b}	1.98 ± 0.02^{a,b}	1.002 ± 0.02^{a}	0.58 ± 0.03^{a,b}	0.83 ± 0.0^{a,b2}	1.79 ± 0.02^{a,b}	1.11 ± 0.03^{a}	0.27 ± 0.02
10 low	0.62 ± 0.02	1.12 ± 0.02	0.819 ± 0.02	0.33 ± 0.01	0.56 ± 0.02	1.32 ± 0.02	0.83 ± 0.02	0.22 ± 0.02
0 Control	0.58 ± 0.04	1.08 ± 0.02	0.618 ± 0.01	0.42 ± 0.01	0.52 ± 0.03	1.28 ± 0.25	0.72 ± 0.01	0.22 ± 0.01

Data are stated as mean ± standard deviation, n=5. One-way ANOVA is used to analyse difference between groups. ^{a}P<0.05 versus model control. ^{b}P<0.05 versus low-dose group. GAPDH, glyceraldehyde-phosphate dehydrogenase

Real-time qPCR

S100 mRNA was hardly detected in normal sciatic nerves of mice. L4-6 spinal cord S100 mRNA results of real-time PCR analysis were shown Figure 3. After curcumin administration, S100 mRNA level in L4-6 spinal cord connecting with inured sciatic nerve increased in each group. S100 mRNA for the high-dose 40 mg/kg/day and mid-dose 20 mg/kg/day increased to peak at 1 week. S100 mRNA level for the low-dose 10 mg/kg/day and model control groups increased to peak at 2 weeks. The increment in both high and mid-dose groups was significantly more than those in both low-dose and model control groups (P<0.05). At this time, for instance, S100 mRNA levels for the high-dose 40 mg/kg/day group was about 2.2-fold than the low-dose 10 mg/kg/day. After time period of 8 weeks, S100 mRNA levels had no statistically significantly difference between groups. These results demonstrated that the high and mid-dose obviously promoted S100 expression.

Western-blotting

Figure 4 showed the image results of S100 Western blotting. The S100/GAPDH grayscale ratio results were shown in Table 3. S100 protein levels of each group up-regulated after

curcumin administration and got a peak at 2 weeks. The increment in both high and mid-dose groups was significantly more than those in both low-dose and model control groups ($P<0.05$ for each). At this time, for instance, S100 protein levels for the high-dose 40 mg/kg/day group was 1.4-fold than the low-dose 10 mg/kg/day. These results suggest that S100 expression in the high and mid-dose groups was significantly promoted compared to the low-dose and control group. After time period of 8 weeks, S100 protein levels had no statistically significantly difference between groups ($P>0.05$ for each).

Discussion

Curcumin has been reported to have a wide range of effects in central nervous system [25-29]. However, its effects on peripheral nerve regeneration is not understanding. This article explores the effect and mechanism of curcumin on peripheral nerve regeneration. Neuroelectrophysiological experiments were performed to examine the functional recovery of sciatic nerve. We found that curcumin administration at a high-dose 40 mg/kg/day and mid-dose and 20 mg/kg/day can promote the functional recovery of sciatic nerve. This outcome was testified by the LFB staining experiments studying the amount and diameter of myelinated nerve fibers. We found that both amount and diameter of regenerated nerve myelin in the high-dose and mid-dose groups were larger than the low-dose and model control groups. This finding is a direct structural evidence for sciatic nerve recovery at a tissue level.

To explore the molecular mechanism of sciatic nerve regeneration, we tested S100 expression because several S100 expression has been considered as the marker for nerve regeneration [11-21]. Immunohistochemical staining, Real-time qPCR and Western blotting were performed to detect S100 expression at the tissue, mRNA and protein levels, respectively. S100 mRNA levels up-regulate and peak at 1 week period following administration. In immunohistochemical and Western blotting assays, S100 protein levels up-regulate to peak at 2 weeks. S100 mRNA levels peak 1 week earlier than S100 protein levels. This is because mRNA degrades fast and at the same time protein expression accumulate. The accumulated S100 proteins promote the sustained regeneration and functional recovery of sciatic nerves.

Conclusion

curcumin administration can promote the sciatic nerve regeneration in a concentration-dependent manner, in particular 40 and 20 mg/kg/day, and such regeneration is associated with up-regulation of S100 expression. The finding of this article is conducive to developing the new use of curcumin for cure peripheral nerve injury in clinic.

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***Correspondence to:**

Shusen Cui

Department of Hand Surgery

Jilin university

China