

Peripheral Nerves

# Citicoline improves functional recovery, promotes nerve regeneration, and reduces postoperative scarring after peripheral nerve surgery in rats

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## Abstract

**Background:** Citicoline has been shown to have beneficial effects in a variety of CNS injury models. The aim of this study was to test the effects of citicoline on nerve regeneration and scarring in a rat model of peripheral nerve surgery.

**Methods:** Seventy adult Sprague-Dawley rats underwent a surgical procedure involving right sciatic nerve section and epineural suturing. Rats were assigned to the control or experiment groups to receive a topical application of 0.4 mL of saline or 0.4 mL (100 μmol/L) of citicoline, respectively. Macroscopic, histological, functional, and electromyographic assessments of nerves were performed 4 to 12 weeks after surgery.

**Results:** In the control versus citicoline-treated rats, SFI was  $-90 \pm 1$  versus  $-84 \pm 1$  ( $P < .001$ ),  $-76 \pm 4$  versus  $-61 \pm 3$  ( $P < .001$ ), and  $-66 \pm 2$  versus  $-46 \pm 3$  ( $P < .001$ ) at 4, 8, and 12 weeks after surgery, respectively. At 12 weeks after surgery, axon count and diameter were  $16400 \pm 600$  number/mm<sup>2</sup> and  $5.47 \pm 0.25$  μm versus  $22250 \pm 660$  number/mm<sup>2</sup> ( $P < .001$ ) and  $6.65 \pm 0.28$  μm ( $P < .01$ ) in the control and citicoline-treated groups, respectively. In citicoline-treated rats, histomorphological axonal organization score at the repair site was  $(3.4 \pm 0.1)$  significantly better than that in controls  $(2.6 \pm 0.3)$  ( $P < .001$ ). Peripheral nerve regeneration evaluated by EMG at 12 weeks after surgery showed significantly better results in the citicoline group ( $P < .05$ ). Nerves treated with citicoline demonstrated reduced scarring at the repair site ( $P < .001$ ).

**Conclusion:** Our results demonstrate that citicoline promotes regeneration of peripheral nerves subjected to immediate section suturing type surgery and reduces postoperative scarring.

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## Keywords:

Citicoline; Peripheral nerve; Scarring

## 1. Introduction

Citicoline administered exogenously provides both choline and cytidine, which serve as substrates for the synthesis of phosphatidylcholine, a primary component of neuronal membrane. It is believed to promote membrane synthesis and repair, which are essential for recovery from neuronal injury [16,30,41,47]. Citicoline has been shown to have

beneficial effects in a variety of CNS injury models and neurodegenerative diseases in humans. In a rat model of cerebral ischemia, citicoline prolongs survival and improves neurological outcome [18,21,46]. Citicoline reduces membrane breakdown and production of free fatty acids, tissue-toxic products of tissue injury secondary to ischemic insult [17,38]. In models of temporary focal ischemia, citicoline decreases the size of cerebral infarcts and prolongs the duration of ischemia required to produce a given behavioral deficit or infarct size [9,33,39]. Citicoline reduces infarct size and decreases mortality in hypotensive rats with subarachnoid hemorrhage and basilar artery occlusion [5]. In a traumatic brain injury model, citicoline reduces brain edema and blood-brain barrier breakdown [10]. In addition,

*Abbreviations:* CNS, central nervous system; Citicoline, Cytidine-5' - diphosphocholine; EMG, electromyography; SFI, sciatic function index.

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combinations of citicoline with the NMDA receptor antagonist MK-801, thrombolytic agents, fibroblast growth factor, or calcium-channel blocker nimodipine show synergistic beneficial effects in experimental neuronal injury models [6,33,39,42,43]. A neuroprotective effect of citicoline has also been shown in clinical studies of citicoline-treated older subjects in whom cognitive and behavioral parameters improved [7,44]. In patients with stroke, citicoline improves neurological outcomes and reduces the volume of the ischemic injury [12–16,23,32,45,46].

Despite the beneficial effects of citicoline in neuronal injury in a variety of CNS injury models and clinical studies, no systematic data are available showing beneficial effects of citicoline in the peripheral nerve injury. We report here that citicoline improves regeneration and functional recovery of sciatic nerves after experimental surgical transection in rats.

## 2. Material and methods

All procedures were performed at the Experimental Animals Breeding and Research Center of the Medical Faculty of Uludağ University, Bursa. Animal care was conducted with the prior approval of the Animal Experimental Ethics Committee of Uludağ University. The functional outcomes and histopathologic and electrophysiologic studies were performed by observers who were blinded to the experimental intervention that the rats had received.

### 2.1. Surgical procedure

Seventy adult female Sprague-Dawley rats weighing 200 to 300 g were used in this study. All animals were housed as 4 rats in one cage and allowed free access to laboratory chow and tap water. Anesthesia was induced with an intraperitoneal injection of thiopental sodium 30 mg/kg. After hair shaving and local skin disinfection with povidone iodine solution, the right sciatic nerve of each rat was exposed through a gluteal muscle-splitting incision. After sharp transection of nerves 10 mm away from the sciatic foramen with a scalpel, an immediate epineural repair of free nerve ends was performed with 8-0 monofilament polypropylene suture (Ethicon/Prolene, Edinbrough, UK). Care was taken not to apply undue tension on the repair site. Seventy rats were randomly assigned to

either the control group (n = 35) or experiment groups (n = 35). Whereas the control group had wrapping of an absorbable gelatin sponge that was impregnated with 0.4 mL of saline at the repair site, the experiment group had wrapping of absorbable gelatin sponges impregnated with 0.4 mL (100  $\mu$ mol/L) of citicoline around the repair site. The wounds were closed in layers, and the rats were allowed free after recovery from anesthesia. All operations were performed by the same surgeon, who used microinstruments and an operating microscope (Zeiss Opmi 6, Oberkochen Germany) under 4 $\times$  magnification.

### 2.2. Functional evaluation

Sciatic function index was measured by walking track analysis preoperatively and at 4, 8, and 12 weeks after the operation [22,27]. Walking tracks were constructed of a 10  $\times$  100-cm wooden corridor, one end opening to a darkened compartment [35]. The hind limbs of the rats were dipped in methylene blue, and the rats were allowed to walk along a sheet of white paper through a crack parallel to the wall. Footprints were analyzed by the following 4 measurements: footprint length, toe spreading, intermediate toe spreading, and distance to opposite foot. The SFI was calculated from these indices [22,27,35].

### 2.3. Electrophysiologic evaluation

Electromyographic evaluation of nerve regeneration was performed at 4 and 12 weeks after the operation. After induction of anesthesia via intraperitoneal injection of thiopental sodium 30 mg/kg, rats were fixed to a table at their extremities using tape and recordings were obtained from the gastrocnemius muscle. Active recording cup electrode was placed on the belly of the muscle, whereas the reference cup electrode was placed on the dorsum of the right foot. Ground electrode was placed on the abdomen. Monopolar pin electrode was placed proximal to the nerve lesion and was used as the stimulator cathode electrode, and a cup electrode was used as anode. Care was taken to keep a minimum distance of 2 cm between the anode and cathode electrodes. To introduce the monopolar pin electrode, an entrance point 2 mm wide was cut on the gluteal skin using an ophthalmic knife and the pin electrode was then placed close to the sciatic nerve. Repetitive stimulations of 0.04-millisecond duration were delivered in increasing intensity,

Table 1  
Results of SFI and electrophysiologic evaluation (EMG)

	4 wk		8 wk		12 wk	
SFI <sup>a</sup>						
Control	-90 $\pm$ 1		-76 $\pm$ 4		-66 $\pm$ 2	
Citicoline	-84 $\pm$ 1	<i>P</i> < .001	-61 $\pm$ 3	<i>P</i> < .001	-46 $\pm$ 3	<i>P</i> < .001
Electrophysiologic evaluation <sup>b</sup>						
Control	2.23 $\pm$ 0.22				3.47 $\pm$ 0.38	
Citicoline	2.08 $\pm$ 0.23	<i>P</i> > .05			4.56 $\pm$ 0.34	<i>P</i> < .05

<sup>a</sup> Rats treated with citicoline showed better results than that of the untreated group at any time point.

<sup>b</sup> The EMG studies at 12 weeks post surgery demonstrated better improvement in nerve function in rats that received choline as evidenced by nerve action potentials (numeric values are expressed as millivolts).

and the best plantar flexion responses were recorded. Care was taken to decrease the artifact and to record a response that starts electronegatively from the isoelectric line.

#### 2.4. Macroscopic evaluation

A gross macroscopic assessment of scar formation was made in 10 rats from each group 4 weeks after surgery. At the end of 4 weeks, the rats were reanesthetized and the surgical area was examined step-by-step by microdissection. Sciatic nerves were exposed and examined to determine the

extent of fibrous tissue surrounding the repair site. Skin closures, muscle fascia closure, nerve adherence to the surrounding muscle cavity tissue, and separability of nerves were assessed by the numerical grading scheme according to Petersen et al [36].

#### 2.5. Histological evaluation

Ten sciatic nerves per group were prepared for histological analysis of perineural scar tissue formation at the end of the first postoperative month. The entire sciatic nerve and

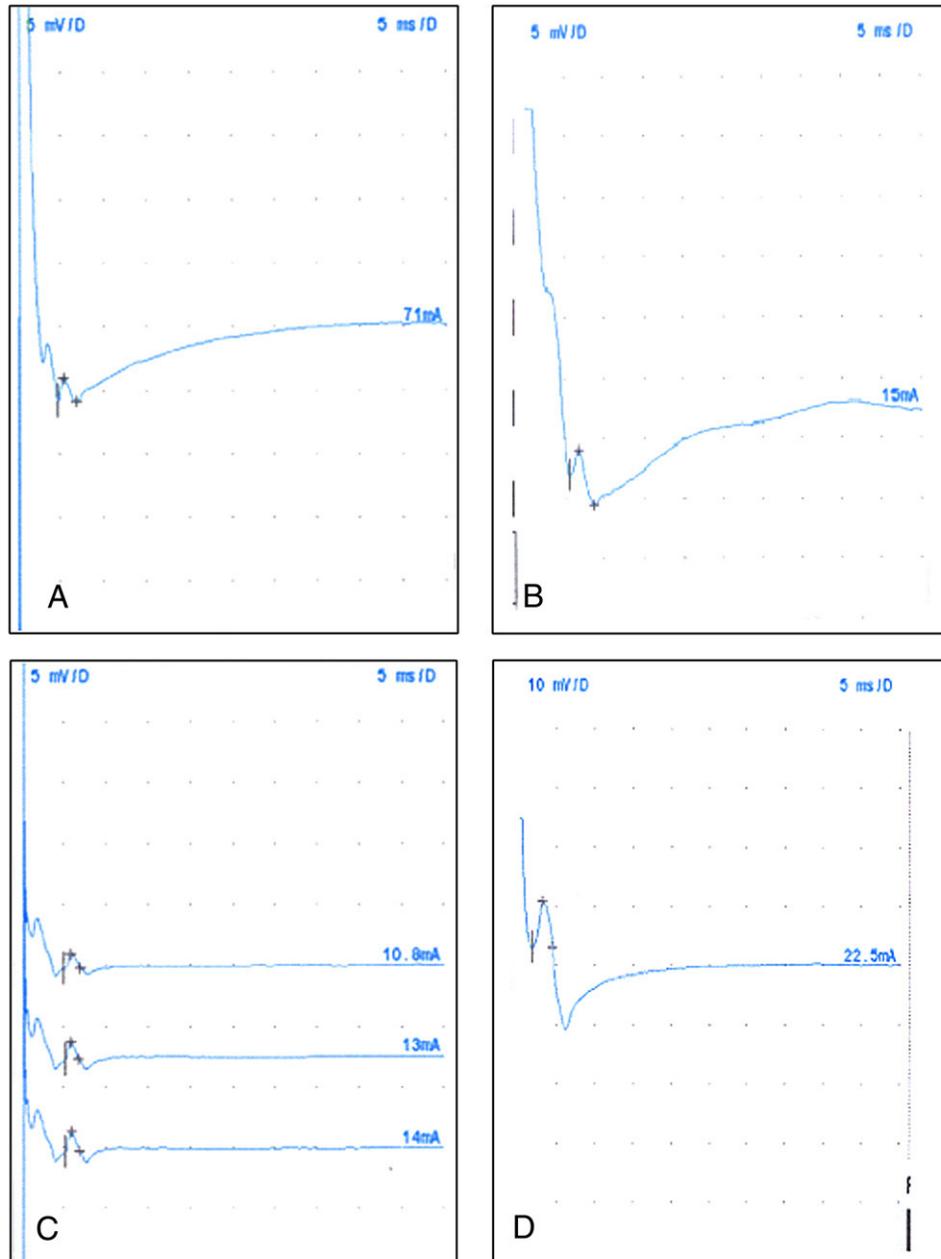


Fig. 1. Upper: Compound muscle action potentials of a rat treated with saline at 4 weeks (A) and 12 weeks (B) post surgery. Distal latency is about the same at 12 weeks compared with that at 4 weeks where there is a mild increase in amplitude that reflects lack of regeneration of axons. Lower: Compound muscle action potentials of a rat treated with citicoline at 4 weeks (C) and 12 weeks (D) post surgery. Shorter distal latency and higher amplitudes were noted at 12 weeks compared with those at 4 weeks, reflecting regeneration of axons (numeric values are expressed as millivolts).

surrounding tissue, including the repaired segment, were removed en bloc and fixed in 10% neutral formalin. After fixation, each specimen was embedded in paraffin. Longitudinal serial sections of 5- $\mu\text{m}$  thickness were obtained from the repair site and stained with Masson trichrome. Epineurial scar formation was examined with the aid of light microscopy. Dense scar tissue surrounding the nerve was discernible as a longitudinal band of dark-staining connective tissue. The thickness of the scar and nerve tissue was measured using an ocular micrometer (Olympus Eyepiece Micrometer, Ax-0026, Hamburg, Germany), and the scar tissue formation index was obtained by dividing the value of the thickness of the scar tissue by the value of the thickness of the nerve tissue.

Twenty-five sciatic nerves per group were prepared for histological evaluation of peripheral nerve regeneration at the end of the 12th postoperative week. Five-micrometer-thick cross sections of tissue originally located 10 mm distal to the repair site were obtained and stained with myelin by using the Weil method [35]. Nerve regeneration was examined with the aid of light microscopy. Transverse sections provided the assessment of axon counts and mean axon and fiber diameters. The diameter of a single axon without the surrounding myelin sheath was evaluated as the

axon diameter, whereas the axon plus the surrounding myelin was evaluated as axon fiber diameter. Axon counts were obtained at  $\times 400$  magnification by using the following sampling technique: Of 100 squares with a surface area of 0.01 mm<sup>2</sup> each, 20 were randomly selected in an ocular grid and used to count myelinated axons. These counts were then normalized regarding surface area. Mean axon and fiber diameters were measured using an ocular micrometer (Olympus Eyepiece Micrometer, Ax-0026). Longitudinal, 5- $\mu\text{m}$ -thick serial sections obtained from the repair site were stained with hematoxylin and eosin; and the longitudinal organization of the regenerating nerve was evaluated according to the following scale described by Brown et al [11]: 1, failure, no continuity of axons from proximal to distal ends; 2, poor organization of the repair site; 3, fair organization of the repair site; 4, good organization of the repair site; 5, excellent organization of the repair site, indistinguishable from normal.

## 2.6. Statistical analysis

The change in SFI and histological measurements except for the evaluation of longitudinal organization at the repair site was analyzed using the Student *t* test. Data from the

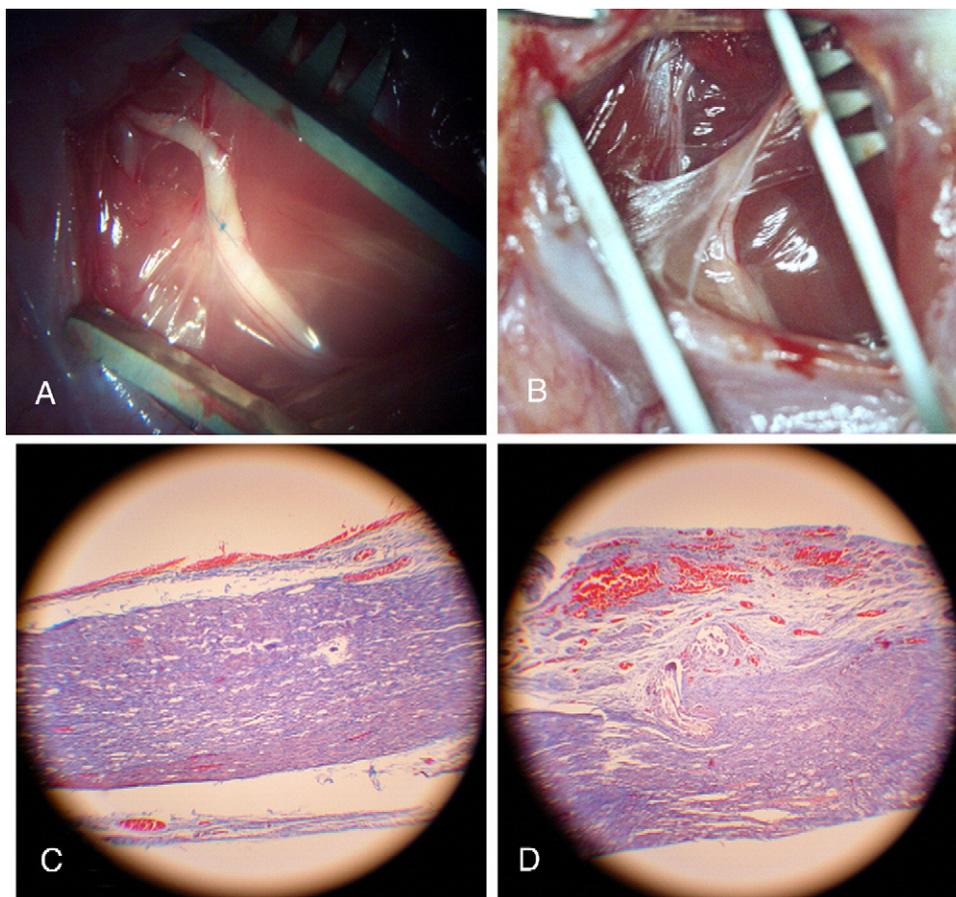


Fig. 2. Photograph demonstrating the macroscopic appearance of sciatic nerves 4 weeks after surgery and the thickness of scar formation. A rat treated with saline shows severe tethering (B) and a very thick scar tissue (D) surrounding the nerve. A rat treated with citicoline shows minimal or no tethering (A) and only a very thin, lucent membrane of scar tissue surrounding the nerve (C). Original magnification  $\times 4$ .

longitudinal organization of the repair site, axon and fiber count, and results of EMG were analyzed with the Mann-Whitney *U* test. A *P* value < .05 was considered statistically significant.

### 3. Results

#### 3.1. Functional evaluation

Preoperatively, the SFI in rats in both groups was approximately zero, indicating normal function. After injury, the SFI decreased to approximately –100, indicating complete loss of function. The SFI values as an indicator of functional recovery were significantly better in rats treated with citicoline than those in rats treated with saline. Statistical difference between the 2 groups was found in every assessment starting from 4 weeks post surgery (Table 1; *P* < .01, Mann-Whitney *U* test).

#### 3.2. Electrophysiologic evaluation

The amplitudes of nerve action potentials that were obtained from electromyographic recordings at 4 and 12 weeks post surgery were analyzed statistically. No statistical difference was found between the 2 groups in the initial recordings at 4 weeks post surgery, but the amplitudes of nerve action potentials that were obtained at 12 weeks post surgery in rats treated with citicoline were significantly higher than those in rats treated with saline (Fig. 1, Table 1; *P* < .05, Mann-Whitney *U* test).

#### 3.3. Macroscopic evaluation

Skin sutures were removed and sciatic nerves were exposed through the original incision. There was no sign of

Table 2  
Results of gross microdissection and scar formation

Gross evaluation <sup>a</sup>	4 wk	12 wk
Skin and muscle fascia closure		
Control	1	1
Citicoline	1	1
Nerve adherence and separability		
Control	2.7 ± 0.2	2.4 ± 0.1
Citicoline	1.7 ± 0.2	1.8 ± 0.2
	<i>P</i> < .01	<i>P</i> < .01
Epineurial scarring <sup>b</sup>		
Control	0.28 ± 0.02	
Citicoline	0.18 ± 0.01	
	<i>P</i> < .001	

<sup>a</sup> The extent of scar tissue surrounding the repair site was numerically graded according to Petersen et al [36] as follows: skin and muscle fascia closure: (1) skin or muscle fascia entirely closed, (2) skin or muscle fascia partially open, (3) skin or muscle fascia completely open, (4); nerve adherence and separability: (1) no dissection or mild blunt dissection, (2) some vigorous blunt dissection required, (3) sharp dissection required. Rats treated with citicoline were noted to have significantly lesser nerve adherence to surrounding tissue and easy separability compared with the untreated group.

<sup>b</sup> Scar formation index was obtained by dividing the value of the thickness of the scar tissue by the value of the thickness of the nerve tissue. Rats treated with citicoline had significantly less scar formation than the untreated group.

Table 3  
Results of histological studies

	4 wk	12 wk	
Axon counts (mm <sup>2</sup> ) <sup>a</sup>			
Control		16400 ± 600	
Citicoline		221250 ± 660	<i>P</i> < .001
Axon diameter (μm) <sup>a</sup>			
Control		5.47 ± 0.25	
Citicoline		6.65 ± 0.28	<i>P</i> < .01
Fiber diameter (μm) <sup>b</sup>			
Control		10.4 ± 0.5	
Citicoline		13.4 ± 2.6	<i>P</i> > .05
Histomorphological axonal organization <sup>a</sup>			
Control	2.3 ± 0.2	3.4 ± 0.1	
Citicoline	2.8 ± 0.1	2.6 ± 0.3	<i>P</i> < .001

<sup>a</sup> Nerves treated with citicoline had significantly higher axon counts and larger diameters compared with the control group and showed better axonal organization.

<sup>b</sup> However, there was no difference between the 2 groups in terms of fiber diameter.

infection or inflammatory reaction. Nerves treated with saline exhibited a dense collagenous scar formation surrounding the repair site, whereas nerves treated with citicoline were surrounded by only a very thin, lucent membrane (Fig. 2).

#### 3.4. Histological evaluation

Nerves treated with saline demonstrated a thick band of dense connective tissue surrounding the nerve. In contrast, nerves treated with citicoline were surrounded by thin dark bands of connective tissue (Fig. 2). Quantification of the dense connective tissue surrounding the nerves revealed a statistically significant reduction in the connective tissue around the nerves treated with citicoline (*P* < .05, Student *t* test; Fig. 2, Table 2).

Nerves treated with citicoline had significantly higher axon counts and mean axon diameters than nerves treated with saline (*P* < .05, Mann-Whitney *U* test; Table 3). No significant statistical difference was found between the 2 groups with respect to the mean fiber diameters (*P* > .05, Mann-Whitney *U* test; Table 3).

On examination of the longitudinal sections at 12 weeks post surgery, the histomorphological organization of axons at the repair site in nerves treated with citicoline was significantly better than that in nerves treated with saline (Fig. 3 and Table 3; *P* < .05, Mann-Whitney *U* test). No inflammatory response against citicoline was observed.

### 4. Discussion

These results show that local application of citicoline improves functional recovery and regeneration of the surgically transected sciatic nerves in rats. Furthermore, citicoline significantly reduces scarring after section suture type of injury.

The observed improvement in the functional recovery is in agreement with the results of the previous experimental studies and shows that citicoline reduces neurological

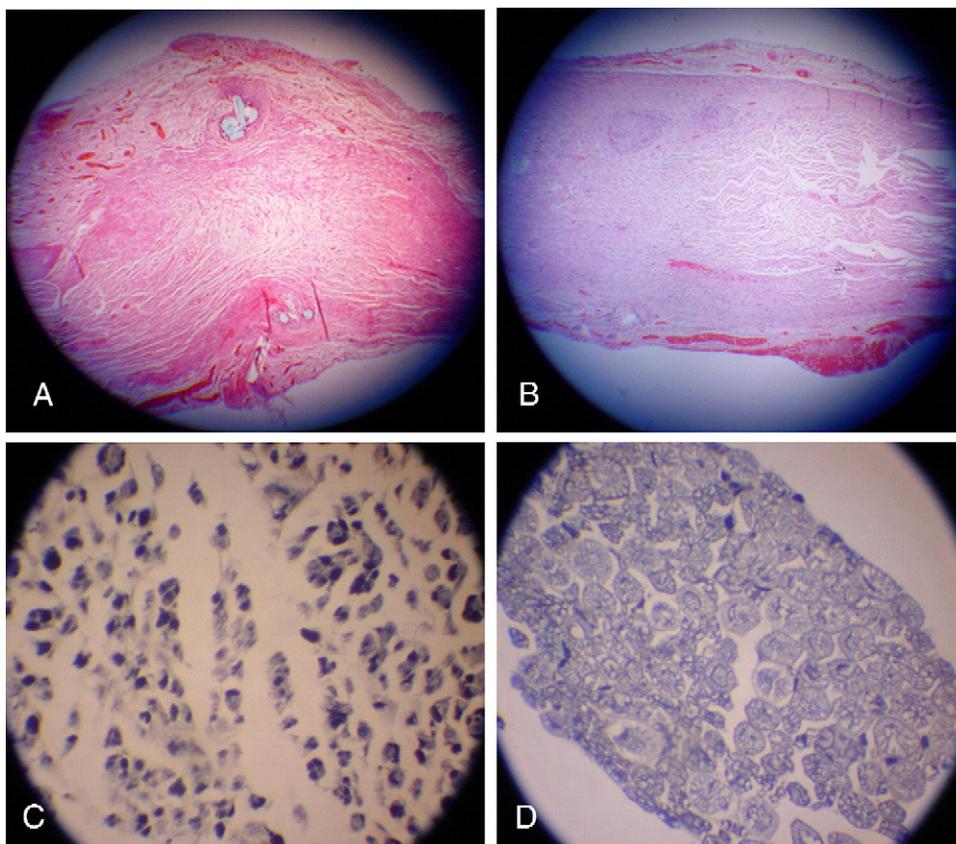


Fig. 3. Photographs showing axonal maturation and organization. Rats treated with citicoline demonstrated better axonal maturation (D) and organization (B), whereas saline-treated rats had poor axonal maturation (C) and organization (A).

deficits in a variety of CNS injury models [9,18,21,33,39]. Previous evidence has demonstrated a neuroprotective effect of citicoline in experimental brain injury, as indicated by an association of the improvement in functional recovery with a reduction in infarct volume after cerebral injury [9,33,39]. In the present study, we observed that the improvement in functional recovery was accompanied by significant increases in axon numbers and thickness (Table 3). The organization at the repair site in the injured nerves treated with citicoline was also significantly better than that in nerves treated with saline (Fig. 2 and Table 3;  $P < .05$ , Mann-Whitney  $U$  test). In addition, electrophysiologic studies revealed higher amplitude values in compound muscle action potentials of rats treated with citicoline, reflecting higher number of normally functioning axons. Taken together, these data indicate that citicoline promotes the survival and/or regeneration of injured neurons that normally die after transection of sciatic nerve fibers.

Despite improvements in operative techniques and adjunctive measures, postoperative scarring still remains to be an unresolved issue that impedes the functional recovery after peripheral nerve trauma [19,20,31,34–36]. Epineural scarring may act as a mechanical barrier impeding axonal outgrowth across the suture site [20,31,35,36]. In addition, extraneuronal scarring may lead to ischemia and irreversible nerve injury by causing tethering of nerves to adjacent

tissue, preventing nerve mobility and in turn leading to traction injury and vasospasm [19,31,36]. Data from the present study clearly show that citicoline significantly reduces scarring after section of sciatic nerves by a suture type of injury. Thus, the reduction in scar volume in citicoline-treated animals may be involved, in part, in the observed improvements in function and regeneration. The mechanism of the reduction in scarring by citicoline is yet to be clarified. However, it is interesting to note that human amniotic fluid, which is very rich in free choline, decreases scarring and promotes regeneration of sciatic nerves in suture type injury models [25,26,35].

The mechanism underlying the observed improvement in function and regeneration in the transected sciatic nerves and reduction in the scarring is presently not investigated in our study. On the other hand, the mechanisms proposed to explain the neuroprotective activity of citicoline in the CNS have been thoroughly reviewed and include (1) stimulation of phosphatidylcholine synthesis and preservation of membrane phospholipids—phosphatidylcholine, sphingomyelin, and cardiolipin—levels [1–4,30,38,40]; (2) preservation of the arachidonic acid content of phosphatidylcholine and phosphatidylethanolamine by inhibiting its release and/or stimulating its incorporation into phospholipids [28]; (3) stimulation of glutathione synthesis and glutathione reductase activity [1,2]; (4) restoration of  $\text{Na}^+/\text{K}^+$ -ATPase

activity [1,37,40]; (5) attenuation of phospholipase A2 activation [1,40]; (6) attenuation of lipid peroxidation [1,17,38]; (7) inhibition of apoptotic enzymes [8,29]; (8) protection of ATP levels [24]; and (9) attenuation of glutamate release [1,16,24]. All or some of these mechanisms may also be involved in the activity of citicoline observed in the present study in the experimental setting of sciatic nerve injury. Clearly, further investigations are needed to understand the mechanism(s) of the protective actions of citicoline in the peripheral nerve injury.

In conclusion, data from the present show, for the first time, that citicoline improves functional recovery and promotes regeneration of injured peripheral nerves, and also reduces postoperative scarring in a rat model of peripheral nerve injury. Further studies are needed to define the mechanism(s) and optimal dose of citicoline in peripheral nerve injuries in experimental animals and humans.

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### Commentary

This is a well-designed study and a well-written article that demonstrates an improvement in nerve regeneration after application of citicoline on the suture site in a rat sciatic nerve transection model. The idea is interesting—the authors extrapolated from the experience in the central nervous system neuroprotection on the rat and applied the treatment to peripheral nerve regeneration. Indeed, peripheral nerve repair may be translated to human use more easily. There are numerous ways to protect a rat from stroke, yet they do not work clinically. As a matter of fact, there were many attempts to improve recovery after nerve repair; yet few advances were made. We should not lose hope though. The effect of citicoline was not very large, but the data provided in this study should lead to further study of citicoline for peripheral nerve repair in other animal models.

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