Intraperitoneal administration of CDP-choline or a combination of cytidine plus choline improves nerve regeneration and functional recovery in a rat model of sciatic nerve injury

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Objective: Topical cytidine-5′-diphosphocholine (CDP-choline) improves functional recovery and promotes nerve regeneration in sciatic nerve injury in rats. The aims of this study were to test whether systemic treatment with CDP-choline was effective in improving the recovery of injured sciatic nerve, and to determine whether the cytidine and/or choline moieties of CDP-choline contribute to its beneficial actions.
Methods: Seventy Sprague–Dawley rats underwent a surgical procedure that involved transectioning and immediate surgical repairing of the right sciatic nerve. Rats were assigned to one of five groups and administered intraperitoneally 1 ml/kg of saline (control) or saline containing 600 μmol/kg of each of CDP-choline, cytidine, choline, or cytidine + choline.
Results: Recovery in sciatic function index score was greater in rats treated with CDP-choline, choline, or cytidine + choline at 8 and 12 weeks after the interventions. Peripheral nerve regeneration evaluated by electromyography at 12 weeks was also greater in rats receiving CDP-choline (228% of control), choline (168% of control), or cytidine + choline (221% of control). Axon counts and axon density increased significantly following CDP-choline, choline, or cytidine + choline, respectively. Treatment with equivalent dose of cytidine failed to affect sciatic function index, electromyography, and axon counts. Treatment with CDP-choline, but not its metabolites improved nerve adherence and separability score.
Conclusion: These data show that intraperitoneal CDP-choline, as well as the combination of its metabolites, cytidine + choline, improves functional recovery and promotes regeneration of injured sciatic nerves in rats. CDP-choline also improves nerve adherence and separability.

Keywords: Citicoline, Choline, Cytidine, Sciatic nerve, Nerve regeneration

Introduction

Cytidine-5′-diphosphocholine (CDP-choline) is an endogenous compound which is involved in cell membrane synthesis by being formed in the rate limiting step of biosynthesis of phosphatidycholine,[1,2] the most abundant phospholipid in neuronal membranes. Exogenous CDP-choline (also known as citicoline) has been shown to exhibit beneficial effects in various neurodegenerative as well as ischemia-, trauma-, and stroke-related conditions both experimentally and clinically (for recent reviews see Refs. 3–5). Using rat models of cerebral ischemia, previous studies reported that systemic treatment with exogenous CDP-choline reduces membrane breakdown,[6–8] restores reduced phosphatidylcholine levels,[9,10] decreases cerebral infarct size,[11–15] improves neurological outcome,[11] enhances neuronal plasticity,[16] and increases survival.[17] CDP-choline reduces infarct area, and decreases mortality in hypotensive rats with subarachnoid hemorrhage and basilar artery occlusion.[18] CDP-choline restores neural and behavioral deficits[19] and decreases blood–brain barrier breakdown and brain edema[20] in traumatic brain injury models. It provides neuroprotection[21] and improves behavioral and neuroanatomical recovery[22] in experimental spinal cord injury models. In patients with stroke, CDP-choline improves neurological outcomes.[23–26]

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Compared to reports on beneficial effects of CDP-choline in neuronal injury in a variety of central nervous system injury models and clinical studies described above,[6–26] relatively little is known about the effects of CDP-choline in peripheral nerve injury. Recently, we showed[27,28] that topically applied CDP-choline improves functional recovery, promotes nerve regeneration, and decreases perineural scar tissue after immediate[27,28] or delayed[28] surgical repair in a rat model of peripheral (sciatic) nerve injury. We further observed[28] that choline and cytidine, CDP-choline's endogenous metabolites,[29] are both involved in its beneficial effects on the functional recovery and regeneration of injured sciatic nerve in rats. It is not known, however, whether systemic administration of CDP-choline also has beneficial effects in peripheral nerve injury. Thus, in the present study, we first aimed to determine whether systemic administration of CDP-choline has beneficial effects in a rat model of peripheral nerve injury. Secondly, we sought to determine whether choline and cytidine moieties of CDP-choline, alone or in combination at equivalent doses, can mimic CDP-choline's actions on nerve regeneration and functional recovery in the same model. Part of systematically administered CDP-choline is known to be metabolized rapidly to choline in rats and humans[2,29] and cytidine in rats[2] or uridine in humans,[29] which serve as precursors for the synthesis of phosphatidylcholine[2] and in promoting cell membrane synthesis and repair, and neurogenesis.[30–34]

Materials and Methods

Animals and general procedures

A total of 70 adult female Sprague–Dawley rats (Uludag University Medical School, The Experimental Animals Breeding and Research Center, Bursa, Turkey) weighing 200–270 g were used in this study. Rats were housed in a controlled environment and allowed for free access to laboratory chow and tap water.

The experimental protocol was approved by the Animal Care and Use Committee of the Uludag University (Bursa, Turkey). Animal care was conducted with the prior approval of the Animal Experimental Ethics Committee of Uludag University.

Surgical procedures and experiments

The sciatic nerve transection and immediate surgical repair were performed under ketamine (70 mg/kg, i.m.) plus xylazine (10 mg/kg, i.m.) anesthesia as described previously.[27,28] Briefly, after rats had their fur shaved and the skin at the surgical site disinfected with povidone-iodine solution (Glividon™, Bikar Ilac San., Istanbul, Turkey), the right sciatic nerve of each rat was exposed through an incision that split the gluteal muscle. Following sharp transection of the sciatic nerve 10 mm from the sciatic foramen with a scalpel, immediate epineural repair of the free nerve ends was performed with 8-0 monofilament polypropylene suture (Prolene™, Ethicone/Prolene Ltd, Somerville, NJ, USA).

The wounds were closed in layers and rats were treated intraperitoneally either with saline (1 ml/kg), CDP-choline (600 μmol/kg), choline (600 μmol/kg), cytidine (600 μmol/kg), or choline (600 μmol/kg) + cytidine (600 μmol/kg) and returned to their cages after they recovered from anesthesia. The dose of CDP-choline, choline, or cytidine used in the present study was selected from our previous studies in rats.[35–37] This dose of CDP-choline results in several-fold elevations in circulating concentrations of CDP-choline, choline, and cytidine for about 2 hours.[35–37]

All surgical procedures were performed by the same neurosurgeon, who used micro-instruments and a surgical microscope (Zeiss Opmi 6, West Germany), under $\times 4$ magnification.

Functional evaluation

Sciatic function index (SFI) scores were measured by walking track analysis[38,39] that was performed both preoperatively and 4, 8, and 12 weeks after the operation, as described previously.[27,28] The walking track was constructed of a 10×100 cm wooden corridor that was open on one end to a darkened compartment. 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 using the following four measurements: footprint length, toe spreading, intermediate toe spreading, and distance to the opposite foot. The SFI was calculated from these indices.[38,39] The SFI was determined by the observer who was blinded to the intervention in rats.

Electrophysiological evaluation

The electromyographic evaluation of the repaired sciatic nerve was performed at 12 weeks after the operation as described previously.[27] Briefly, the amplitude of compound muscle action potential in response to repetitive stimulation of the sciatic nerve for 0.004 millisecond with increasing intensity was recorded from the gastrocnemius muscle under xylazine (10 mg/kg) + ketamine (70 mg/kg) anesthesia. Care was taken to keep a minimum distance of 2 cm between the electrodes, and to record the response that starts electronegatively from the isoelectric line.

Macroscopic evaluation

At the end of 12 weeks, rats were re-anesthetized and the surgical area was examined in a step-by-step fashion by micro-dissection. The sciatic nerve was exposed and examined to determine the amount of fibrous tissue surrounding the repair site. Adherence and separability of sciatic nerves were assessed using a numerical grading scheme described by Petersen *et al.*[40]

Histological evaluation

Sciatic nerves were prepared for histological evaluation of peripheral nerve regeneration at the end of the twelfth postoperative week. Samples fixed in 4% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) overnight were then post-fixed with 1% osmium tetroxide (OsO_4) in 0.1M phosphate buffer for 2 hours, dehydrated through a graded series of ethanol, and embedded in Spurr's resin (Agar Scientific, Stansted, UK). Semithin 0.5-μm sections perpendicular to the long axis of the nerve fibers that were originally located 3 mm distal to the repair site were then taken and stained with a mixture of 1% toluidine blue and 1% borax in distilled water. A digital camera (Cybershot DSC-F717; Sony, Tokyo, Japan) attached to a light microscope (4S-2 Alphaphot; Nikon, Tokyo, Japan) and Scion Image software (Scion Corp., Frederick, MD, USA) were used to capture images, and the image analysis system was calibrated using a stage micrometer before measurements were obtained. The total surface area of the nerve transverse section was calculated at $\times 4$ magnification. Twenty microscopic fields, selected randomly, were captured for each nerve sample through $\times 40$ objective for accurate recognition and counting of the myelinated nerve fibers. These fields were considered to be representative of the entire surface area. Myelinated axons were counted using the unbiased counting frame method described by Larsen[41] for the peripheral nerve fiber stereology, and by Kaplan *et al.*[42] for the stereological estimation of the number of myelinated axons in the rat sciatic nerve. The square unbiased counting frame was created using Scion Image software macrofeatures and was superimposed on the digital image to be counted. In the unbiased counting frame, the left and bottom lines were used as the exclusion lines, and the upper and right lines were used as inclusion lines.[41,42] Myelinated axons were then quantified in the 20 counting frame, averaged, and normalized with the nerve total surface area. Myelinated axon count was expressed as 'axon count' (axons per nerve) and as 'axon density' (axons per square millimeter). Histomorphologic evaluations were performed by an investigator who was blinded to the intervention in rats.

Statistical analysis

Results are expressed as mean ± SEM. Parametric data (i.e. axon count, axon density, the amplitude of compound action potential, and the change in SFI) were analyzed with one-way ANOVA followed by Tukey's multiple comparisons procedure. Non-parametric data (i.e. nerve adherence and separability scores) were analyzed by using the Kruskal–Wallis one-way ANOVA by ranks test, followed by Dunn's method for multiple comparisons. For all statistical analyses, SigmaStat® statistical software, Version 11, was used (SPSS Science GmbH, Erkrath, Germany). P value <0.05 was considered significant.

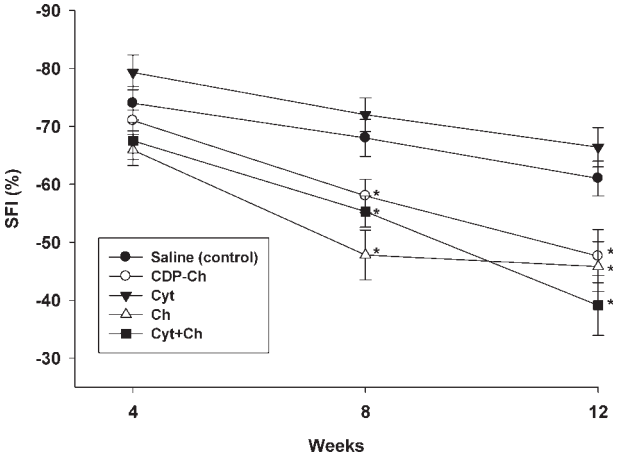

Figure 1 Sciatic function index (SFI) score at 4, 8, and 12 weeks after surgical repair of transected sciatic nerve in rats treated intraperitoneally with CDP-choline, cytidine, choline, or cytidine plus choline. Sciatic nerve injury followed by repair was performed and treatments were administered as indicated in 'Methods' section. SFI scores were determined at 4, 8, and 12 weeks after the treatment. *Significant ($P<0.05$) improvement in SFI score compared to that in saline-treated control rats. CDP-Ch: CDP-choline; Cyt: cytidine; Ch: choline; Cyt+Ch: cytidine+choline.

Results

Functional recovery

The autotomy in the sciatic nerve-repaired leg was observed in 3, 4, 4, 4, and 5 rats treated with saline, CDP-choline, cytidine, choline, and choline+cytidine, respectively. The autotomy was evident particularly at first, second, and third fingers and occurred within 4 weeks after the treatment. In two to three rats from each of all five groups, chronic contracture was developed in the sciatic nerve-repaired leg. Rats with autotomy and/or chronic contracture were excluded from the SFI analyses with the considerations that negative influences of the autotomy and/or chronic contracture on the SFI evaluation are possible.

As seen in Fig. 1, the SFI showed gradual recovery with time in all five groups of rats. In saline-treated control rats, the SFI scores were -74 ± 3, -68 ± 3, and -61 ± 3 at 4, 8, and 12 weeks after surgery, respectively. Statistical analysis using one-way ANOVA revealed significant differences in the SFI at 8 weeks [$F(4,29)=10.11$; $P<0.001$] and 12 weeks [$F(4,29)=8.04$; $P<0.001$] in five different groups of rats (Fig. 1). The SFI scores improved significantly ($P<0.05$) at 8 and 12 weeks in rats treated with CDP-choline compared to that in saline-treated control rats (Fig. 1). Treatment with an equivalent dose of choline or choline+cytidine was also effective in improving SFI scores (Fig. 1).

Electromyographic evaluation

The amplitude of muscle action potential in five groups of rats is shown in Fig. 2. Statistical analysis using one-way ANOVA revealed significant differences [$F(4,30)=8.04$; $P<0.001$] in amplitudes of

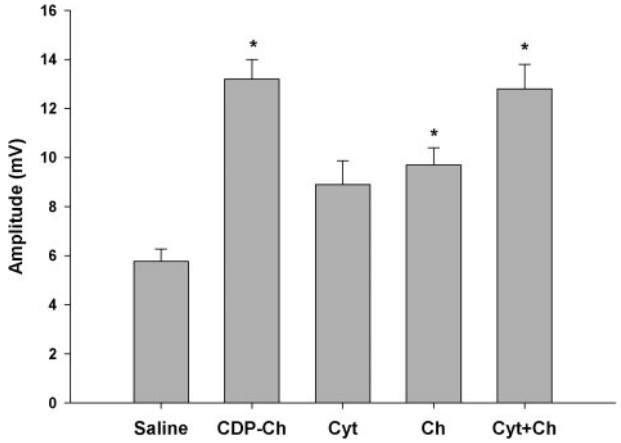

Figure 2 Effects of CDP-choline, cytidine, choline, and cytidine plus choline treatments on the amplitude of muscle action potential at 12 weeks after surgical repair of transected sciatic nerve in rats. Sciatic nerve injury followed by repair was performed and treatments were administered as indicated in 'Methods' section. The amplitude of muscle action potential was recorded at 12 weeks after the treatment from the gastrocnemius muscle. *Significantly ($P<0.05$) greater than the value for saline-treated control rats. CDP-Ch: CDP-choline; Cyt: cytidine; Ch: choline; Cyt+Ch: cytidine+choline.

muscle action potentials obtained from five different groups of rats (Fig. 2). In rats treated either with CDP-choline or the combination of equivalent doses of its endogenous metabolites, choline and cytidine, the amplitude of muscle action potential was higher ($P<0.001$) than that observed in saline-treated control rats (5.8 ± 1.1 mV). Treatment with an equivalent dose of choline increased significantly ($P<0.05$), while cytidine increased slightly, but not significantly, the amplitude of muscle action potentials (Fig. 2).

Axon counts

The results of the axon counts in sciatic nerves from five treatment groups are shown in Fig. 3. Statistical analysis using one-way ANOVA revealed significant differences in the axon counts [$F(4, 37)=5.21$; $P<0.01$], and in the axon density [$F(4,37)=4.25$; $P<0.01$] between different treatment groups (Fig. 3). The mean value of axon counts was 5048 ± 1045, 9618 ± 1017 ($P<0.05$), 7489 ± 498, $10\,050 \pm 969$ ($P<0.01$), or $10\,403 \pm 1010$ ($P<0.01$) in rats treated with saline, CDP-choline, cytidine, choline, or choline+cytidine, respectively. Rats treated with CDP-choline or the combination of choline and cytidine had significantly ($P<0.05$) greater axon density in sciatic nerves than rats treated with saline. Treatment with an equivalent dose of choline alone also increased axon density slightly, but significantly ($P<0.05$), while cytidine alone failed to alter axon density (Fig. 3).

Nerve adherence and separability

At the end of the twelfth postoperative week, nerve adherence and separability were evaluated in all rats. A representative image depicting the significant difference in improvement in nerve adherence has been given in Fig. 4. Nerve adherence and separability scores were improved significantly in rats treated with CDP-choline compared to saline-treated control rats (Table 1). Treatment with an equivalent dose of choline, cytidine, or choline+cytidine failed to alter the nerve adherence and separability scores (Table 1).

Discussion

These data show that intraperitoneal administration of CDP-choline improves neuronal regeneration and functional recovery while decreasing nerve adherence to surrounding tissues and enhancing separability of the tibial and peroneal branches in experimental sciatic nerve injury in rats. Treatment with a combination of choline and cytidine, the endogenous metabolites of CDP-choline, at equivalent dose, was as effective as their parent compound, CDP-choline, in improving functional recovery and neuronal regeneration. Treatment with the equivalent dose of choline alone, but not cytidine alone, was also beneficial in neuronal regeneration and functional recovery. Choline, cytidine, or choline+cytidine combination was ineffective in improving nerve adherence and separability scores, suggesting that the improvement in nerve adhesion and separability score could be an effect of CDP-choline itself and/or by its other intermediate metabolites.

In our recent studies,[27,28] we showed that topical application of CDP-choline increases axon counts and improves functional recovery of the sciatic nerve after sharp transection and surgical repair in rats. Data from the present study confirms and extends these previous observations by demonstrating that systemic, intraperitoneal, administration of 600 μmol/kg of CDP-choline is also effective in increasing axon counts and improving functional recovery of the surgically transected and repaired sciatic nerve. In other studies, it is well established that systemic administration of CDP-choline, at similar dose ranges, reduces neurological deficits and improves functional recoveries in a variety of neuronal injury models.[6–22] Furthermore, when used in combination, CDP-choline enhances neuroprotective effects of glutamate receptor antagonists,[12] calcium channel blockers,[14] thrombolytic agents,[15,43] fibroblast growth factor,[13] or propofol.[44] Taken together, it is highly possible that systemic administration of CDP-choline has the ability to improve functional recoveries in not only brain and spinal cord injuries, but also in injury at the peripheral nerves.

It is well known that, when administered to rats, part of CDP-choline is rapidly metabolized to cytidine and choline[2,29,35–37] resulting in dose-related increases in circulating levels of cytidine and choline.[35–37] These metabolites involve most, if not

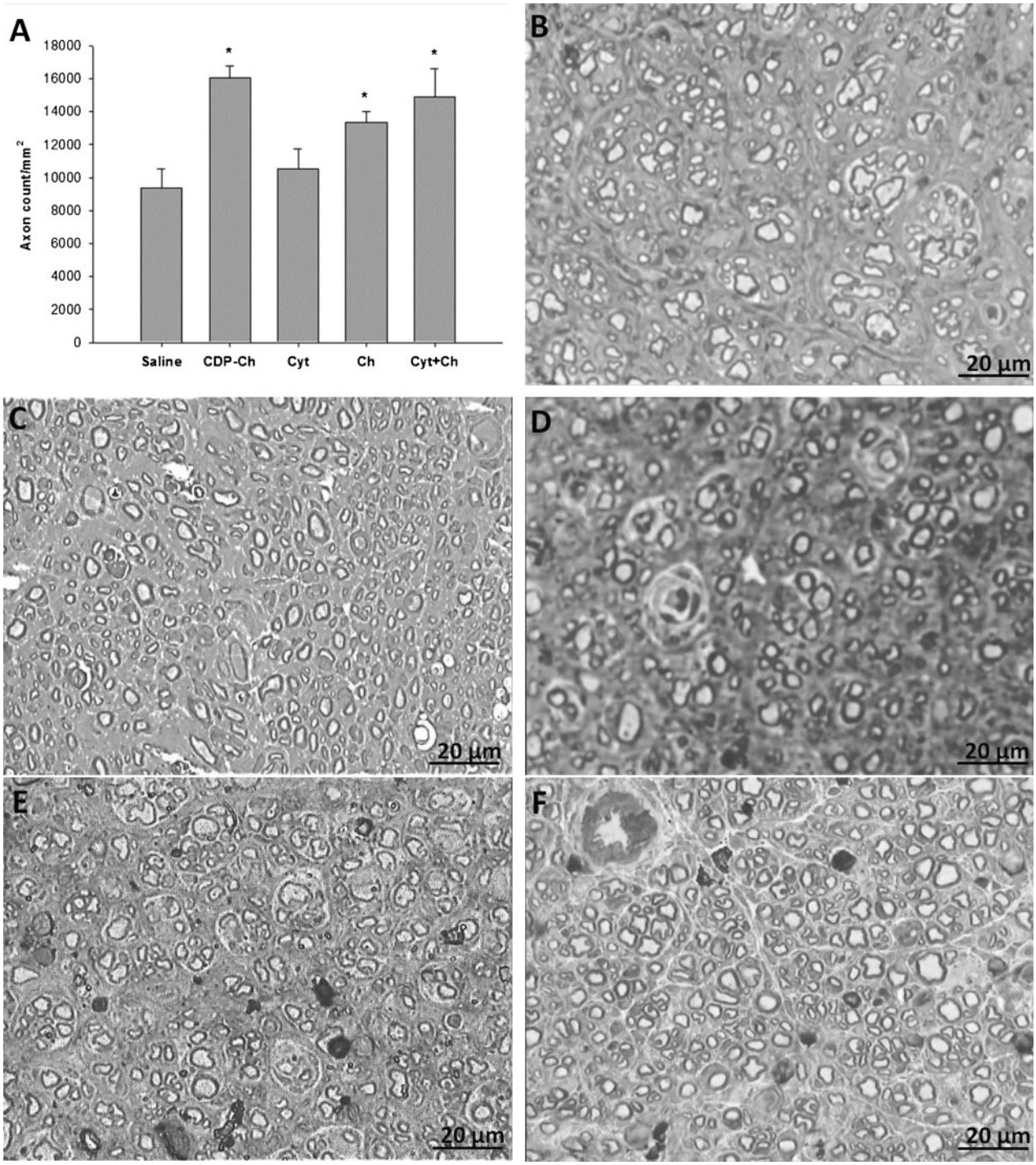

Figure 3 Effects of CDP-choline, cytidine, choline, and cytidine plus choline treatments on axon counts at 12 weeks after surgical repair of transected sciatic nerve in rats. Sciatic nerve injury followed by repair was performed and treatments were administered as indicated in 'Methods' section. Axon count was performed at 12 weeks after the treatment. Bar graph shows axon density (the number of myelinated axons per square millimeter) in each treatment group (A). Photomicrographs of the toluidine blue-stained sciatic nerve sections (×40) indicate myelinated axons of rats receiving saline (B), CDP-choline (C), cytidine (D), choline (E), and cytidine+choline (F). Scale bar=20 µm. *Significantly ($P<0.05$) greater than the value for saline-treated control rats. CDP-Ch: CDP-choline; Cyt: cytidine; Ch: choline; Cyt+Ch: cytidine+choline.

all, biological actions of CDP-choline. Indeed, in the present study, we observed that intraperitoneal combined administration of equivalent dose of choline and cytidine was as effective as an equivalent dose of the parent compound, CDP-choline, in improving functional recovery [i.e. SFI (Fig. 1) and the amplitude of muscle action potential (Fig. 2)] and axon counts (Fig. 3) of the surgically transected/repaired sciatic nerves. These data indicate that such beneficial effects of CDP-choline are likely to be mediated by its endogenous metabolites choline, cytidine, or both. The ability of choline, at equivalent

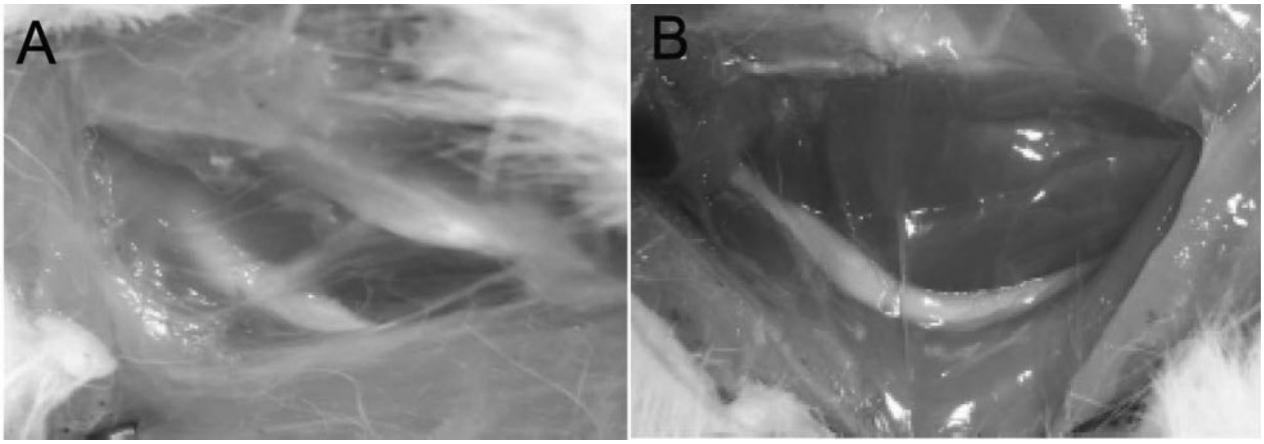

Figure 4 Photomicrographs depicting comparison of sciatic nerves re-exposed 12 weeks after the surgery. Sciatic nerve injury followed by repair was performed and CDP-choline was administered as indicated in 'Methods' section. Sciatic nerves were re-exposed 12 weeks after the surgery and evaluated for nerve adherence and separability. Sciatic nerves of rats treated with saline (panel A) were surrounded by scar and tethered to the surrounding tissue while in rats treated with CDP-choline (panel B), the sciatic nerves were easily dissected from the surrounding tissue. ×4 magnification.

dose, to improve the SFI, elevate the amplitude of muscle action potential, and increase axon counts suggests that choline is the major factor in the observed actions of CDP-choline. Failure of equivalent dose of cytidine alone to improve functional recovery [i.e. SFI (Fig. 1) and the amplitude of muscle action potential (Fig. 2)] and axon counts (Fig. 3) suggests that, at least within the dose range and route used in this study, this metabolite of CDP-choline is not effective. However, given the fact that the magnitude of the elevations in the muscle action potentials and increases in axon counts in response to choline alone were slightly, but significantly, smaller than the observed effect either with CDP-choline or choline+cytidine, suggesting that cytidine may enhance choline's actions. This cytidine-induced enhancement in choline effect could result from stimulation of membrane phospholipid synthesis[31–33] and neurogenesis.[30,34]

Table 1 Effects of CDP-choline, cytidine, choline, and cytidine plus choline treatments on the nerve adherence and seperability score at 12 weeks after surgical repair of transected sciatic nerve in rats

	Nerve adherence and separability score	
Groups (treatments)	Mean±SEM	Median
Saline	2.53±0.33	2.5
CDP-choline	1.33±0.17*	1.0*
Cytidine	2.11±0.26	2.0
Choline	2.25±0.16	2.0
Cytidine+choline	2.38±0.32	3.0

Notes: Sciatic nerve injury followed by repair was performed and treatments were administered as indicated in 'Methods' section. Nerve adherence and separability scores were determined at 12 weeks after the treatment by a numerical grading scheme described by Petersen *et al.*[40] as follows: 1=no dissection or mild blunt dissection; 2=some vigorous blunt dissection required; 3=sharp dissection required. *Significant ($P<0.05$) improvement compared to saline-treated control rats.

The observed improvement in nerve adherence and separability score by i.p. CDP-choline treatment was in good accordance with previous studies[27,28] from our laboratory showing that topical application of CDP-choline improves nerve adherence and separability scores in the repaired experimental sciatic nerve injury.[27,28] Reduced nerve adherence by CDP-choline treatment indicates reduced tethering of the nerve to the surrounding muscle cavity and enhanced nerve separability indicates decreased adherence of the tibial and peroneal nerve components to each other. However, the improvement in nerve adherence and separability scores in CDP-choline-treated rats was not seen in rats treated with equivalent dose of its endogenous metabolites choline, cytidine, or even with cytidine+choline combination. These data indicate that the improvement in nerve adhesion and separability score with CDP-choline is not, apparently, mediated by its circulating metabolites, cytidine and choline. Previous studies from our laboratory showed that i.p. administration of CDP-choline results in elevations not only in circulatory levels of choline and cytidine,[35–37] but also CDP-choline itself and its intermediate metabolites cytidine monophosphate and phosphocholine.[35–37] Thus, the improvement in nerve adhesion and separability score could be an effect of CDP-choline itself and/or by its intermediate metabolites, cytidine monophosphate and phosphocholine. Possible involvement of cytidine monophosphate or phosphocholine in the observed improvement in nerve adhesion and separability score by CDP-choline and the mechanism of action require further investigation.

In conclusion, the present data show for the first time that systemic administration of CDP-choline improves functional recovery, promotes regeneration of the injured sciatic nerves, and reduces nerve adhesion in rats. Treatment with choline+cytidine

combination was as effective as treatment with an equivalent dose of the parent drug, CDP-choline, in increasing axon counts and improving functional recovery of the injured sciatic nerves, but not in improving nerve adhesion and separability. These data suggest that CDP-choline and cytidine + choline combination may be beneficial in surgical treatment of peripheral nerve injuries in humans.

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