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Uridine Treatment Improves Nerve Regeneration and Functional Recovery in a Rat Model of Sciatic Nerve Injury

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ABSTRACT

AIM: To investigate the regenerative potential and long-term functional effects of uridine treatment in a rat model of sciatic nerve injury.

MATERIAL and METHODS: Male Sprague-Dawley rats were randomized to receive sham surgery plus saline (Sham group), right sciatic nerve transection and primary repair plus saline (Control group), right sciatic nerve transection, and primary repair plus 500 mg/kg uridine (Uridine group). Saline or uridine was injected intraperitoneally (i.p.) for seven days, and the rats were monitored for 12 weeks after surgery. We evaluated electrophysiological and functional recovery using electromyography (EMG) and sciatic functional index (SFI) at six and 12 weeks, respectively. At 12 weeks, rats were decapitated and their right sciatic nerves were examined in macroscopic and histomorphologic manners.

RESULTS: Functional evaluation by SFI and sciatic nerve conduction velocity analyzed by EMG both decreased in the Control group but recovered in the Uridine group 12 weeks after surgery. Additionally, upon experiment completion, Uridine treatment was observed to enhance nerve adherence, separability scores, and the number of myelinated axons.

CONCLUSION: These results reveal that short-term Uridine treatment provides morphological and electrophysiological benefits, which are represented by long-term functional improvement in a rat model of sciatic nerve injury. These findings validate and extend our knowledge on Uridine's regenerative effects in peripheral nerve injuries.

KEYWORDS: Electrophysiology, Peripheral nerve regeneration, Sciatic functional index, Sciatic nerve injury, Uridine

ABBREVIATIONS: ANOVA: Analysis of variance, **CDP-choline:** Cytidine-5'-diphosphocholine, **CNS:** Central Nervous System, **EMG:** Electromyography, **E:** Examed in Control and Uridine groups, **I.P:** Intraperitoneally, **ITS:** Intermediate Toe Spread; distance from the second to the fourth toe, **N:** Normal in the Sham group, **OsO4:** Osmium Tetroxide, **PC12:** Pheochromocytoma-12, **PL:** Print Length; distance from the heel to the third toe, **SEM:** Standard error of means, **SFT:** Sciatic Functional Index, **TS:** Toe Spread; distance from the first to the fifth toe, **UDP:** uridine-5'-diphosphate, **UMP:** Uridine-5'-monophosphate, **UTP:** uridine-5'-triphosphate

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INTRODUCTION

Peripheral nerve regeneration is one of the unresolved issues in regenerative medicine. The regeneration of an injured peripheral nerve is affected by various factors, including emerging pathophysiological events and significant nerve tissue loss. Despite the proposal of several therapeutic approaches for achieving satisfactory recovery in sensory/motor function, no cure for peripheral nerve injury has been discovered. The most common treatment for peripheral nerve injury includes direct surgical treatment or nerve grafting, even though the reported clinical results are far from satisfactory (33).

Therefore, novel approaches that provide pathophysiological benefits at both the cellular and molecular levels are necessary, which is reflected by electrophysiological and functional recovery. Oxidative stress (50), apoptosis (51), inflammation (49), extracellular matrix degradation (32), and several other pathophysiological events compromise peripheral nerve injury. Thus, therapeutic strategies targeting such pathological processes potentially have long-term effects and confer benefits in re-gaining function.

Uridine is a ubiquitous pyrimidine nucleoside that, when administered exogenously increases neuronal levels of cytidine-5'-diphosphocholine (CDP-choline) (14,61), an endogenous intermediate formed at the rate-limiting phase of phospholipid synthesis via the Kennedy pathway (40). The contribution of Uridine in membrane synthesis may underlie the promotion of axonal sprouting in cultured pheochromocytoma-12 (PC12) cells (54). Neuroprotective effects of Uridine have been studied in experimental models of various central nervous system (CNS) pathologies. Uridine provides neuroprotection in neonatal rats subjected to hypoxic-ischemic (12,27,43) or hyperoxic (2,28) brain injuries, and the results were associated with anti-apoptotic (12), anti-oxidant (2), and epigenetic (43) effects. Furthermore, short-term treatment with Uridine against either hypoxic-ischemic or hyperoxic brain injury was associated with significant recovery in cognitive function in young adult rats over time (27,28).

Consistent with previous findings in CNS disease models, we recently demonstrated that Uridine treatment exhibited anti-apoptotic and anti-oxidant effects in a rat sciatic nerve injury model with unilateral transection followed by primary repair (41). This study primarily investigated the effects of Uridine treatment on significant pathological events that occurred in this model in the short term (seven days). Hence, this study was conducted to investigate whether the alleviation at the cellular and molecular levels in pathological consequences of sciatic nerve injury by Uridine treatment for the same period is reflected by a functional recovery in the longer-term by follow-up for 12 weeks.

MATERIAL and METHODS

Animals and the Operative Procedure

Twenty-eight adult male Sprague-Dawley rats weighing 270–350 grams were purchased from Bursa Uludag University Faculty of Medicine Experimental Animal Breeding, Application and Research Center and housed in a controlled environment at the Department of Pharmacology by allowing free access to laboratory chow and tap water. We conducted experiments after receiving ethical approval from the Local Ethics Committee for Animal Experiments of Bursa Uludag University (Approval ID: 2017-15/04). The experiments and animal care followed the Guide for the Care and Use of Laboratory Animals (22) and ARRIVE Guidelines 2.0 (52).

The rats were fixed to the operating table in the left lateral position under sevoflurane (Sevorane Liquid; Aesica Queen borough Ltd., Queen borough, UK) anesthesia at 1%–5% titration. The surgical region was prepared by shaving the area of interest and was cleaned using 70% alcohol followed by a 10% povidone-iodine solution (Glividon; Bikar Inc., Istanbul, Turkey). Surgical interventions were performed using microsurgical methods under an operating microscope (Zeiss Opmi-6; Carl Zeiss Meditec Inc. USA) by the same neurosurgeon. All rats underwent a 3 cm-long posterolateral skin incision extending from the right gluteal region to the posterior of the thigh. The sciatic nerve, along with M. biceps femoris and m. gluteus superficialis junction lines were exposed and isolated from surrounding tissues from the sciatic foramen to the point of the tibial and peroneal branches by opening the surrounding fascia with blunt dissection. Except for the rats in the Sham group, a full-thickness incision with a dermatome blade was made 1 cm away from the sciatic foramen using a metal dissector placed under the sciatic nerve. Proximal and distal nerve stumps were sutured with 8-0 polypropylene sutures (Ethicon Inc, Somerville, NJ, USA) using a microsurgical method at 180° aperture, and primary repair was achieved. During the anastomosis procedures, every precaution was taken to avoid producing tension. Then, the skin and fascia were sutured together with non-absorbable suture material (Ethicon Inc, Somerville, NJ, USA). In the Sham group, the sciatic nerve was exposed using the same surgical procedures but was left intact. Rats were returned to their cages following recovery from anesthesia.

Treatments

After undergoing full-thickness sciatic nerve transection and primary repair, rats were randomized to Control and Uridine groups and received saline (1 ml/kg; i.p.) and Uridine (500 mg/kg; dissolved in saline; i.p.) injections, respectively, once a day for seven days. For seven days, rats in the Sham group received daily saline injections (1 ml/kg; i.p.). The dose of Uridine (500 mg/kg) administered in this study was selected from our recent report (41) as the effective dose that provided anti-apoptotic and anti-oxidant effects in the same experimental model of sciatic nerve injury and from our previous studies in hypoxic-ischemic (27,28,43) or hyperoxic (2,28) brain injury models.

Within 12 weeks after surgery, two rats in the Control group and one rat in the Uridine group were found dead in their cages. Furthermore, one rat in the Control group developed sores on his right toes, resulting in self-amputation. Therefore, these four rats were excluded from the study, which was conducted on 24 rats (eight rats in each group).

Functional Evaluation

Functional assessment was made by Sciatic Functional Index (SFI), which was calculated using data derived from walking track analyses at six and 12 weeks after the surgical operations, as previously described (7). Briefly, all rats were subjected to a gait test on a walking track made of a 144 × 10 × 10 cm (length × width × height) wooden corridor. We painted the plantar surfaces of the hind limbs of the rats with methylene blue and the rats were allowed to walk through the open end of the corridor on a sheet of white paper. To entice the rats to wander, they were fasted overnight and a food pellet was placed at the open end of the corridor. A blinded researcher analyzed the measurements of normal (N; in Sham group) and experimental (E; in Control and Uridine groups) print length (PL; distance from the heel to the third toe), toe spread (TS; distance from the first to the fifth toe) and intermediate toe spread (ITS; distance from the second to the fourth toe) obtained from the footprints and we calculated the SFI using the Bain-Mackinnon-Hunter formula (5), as described (31,56).

Electrophysiological Evaluation

The electrophysiological assessment was made by electromyography (EMG) recordings through neural conduction velocity measurement using a data acquisition system (MP36, BIOPAC Systems, CA, USA). EMG recordings were taken at six and 12 weeks after surgery, immediately following the completion of SFI tests. Briefly, rats were fixed on the operating table in a prone position under sevoflurane (Sevorane Liquid; Aesica Queen borough Ltd., Queen borough, UK) anesthesia and their fur was shaved. Two lines were drawn on the backs of rats, one connecting the iliac crests and the other 2 cm distally to it, and nerve impulses were induced on these lines using a surface stimulator placed on the shaved skin. The anodes and cathodes of the stimulating cup were placed on the same side during each application by centering the spine. The recordings were taken from the gastrocnemius muscle using a recording electrode placed on this muscle, while the reference or ground electrode was placed on the tendon of the gastrocnemius muscle or the abdomen, respectively. Stimulation responses were delivered for 1 ms at a constant intensity of 30 mV. Nerve conduction velocities were calculated by recording the amplitude time and latency of compound muscle action potentials obtained from the recording electrode due to electrical impulses from two different locations with a fixed interval of 2 cm. The best three responses, accompanied by a clinically observed plantar flexion, in EMG were recorded for 10 ms and the data were presented as the average of these three responses.

Macroscopic Evaluation

Rats in each group were sacrificed under sevoflurane anesthesia immediately after completing the electrophysiological evaluation at 12 weeks, and the surgical area was evaluated macroscopically by a blinded researcher. For this purpose, the incision was opened and the adhesion and separability of the sciatic nerve from surrounding tissue were evaluated. During a macroscopic evaluation, the criteria that determine the adhesion of the nerve to surrounding muscle mass and separability from these structures were converted into quantitative scores using the previously described numerical grading scheme (53).

Histomorphological Evaluation

A 1-cm portion of the sciatic nerve, including the anastomosis area, was resected 12 weeks immediately after macroscopic evaluation to perform a histomorphological evaluation. Nerve tissues were fixed overnight in 0.1 mol/L phosphate buffer containing 4% glutaraldehyde (pH: 7.4) and post-fixed for 2 h in 0.1 mol/L phosphate buffer containing 1% osmium tetroxide (OsO4). Subsequently, they were treated with propylene oxide, dehydrated in a graded series of alcohol, and embedded in Spur's resin (Agar Scientific, Stansted, UK) for polymerization. Transverse semi-thin sections, 0.5 µm thick, were taken from resin blocks using an ultramicrotome. The slide was stained with 1% toluidine blue-1% borax mixture on each slide with three cross-section samples. Five-megapixel images were obtained using Zeiss Primo Star light microscope system (Carl Zeiss AG, Ober kochen, Germany) and Zeiss Lab scope software with random sampling throughout the sections. Images captured at 100x magnification were converted to BMP image format, and we performed visual analyses using Scion Image (version 4.0.3.2) software. Myelinated axons were counted using the unbiased counting frame method (44) for peripheral nerve fiber stereology and estimated using a previously-reported method (38) for the stereological estimation of the number of myelinated axons in the rat sciatic nerve.

Statistical Analyses

We performed statistical analyses using Sigma Plot software Version 12.5 (SPSS Science GmbH, Erkrath, Germany). Data were expressed as mean± standard error of means (SEM). The normality of the data was determined by the Shapiro-Wilk test. The difference between groups was analyzed using the parametric one-way Analysis of Variance (ANOVA) followed by the post-hoc Tukey test for data showing normal distribution. The difference between groups was analyzed by using the non-parametric Kruskal–Wallis H test, followed by Dunn's method for multiple comparisons with Bonferroni correction for data that failed the normality test. The statistical significance level was set at $p<.05$.

RESULTS

Functional Recovery

Sciatic nerve transection injury significantly (p=.001) reduced SFI in Control or Uridine rats compared to Sham rats. Uridine treatment provided a significant (p=.001) functional recovery compared to the Control group at six and 12 weeks after surgery (Figure 1A). We calculated the SFI scores in Control or Uridine group as −75.4 ± 3.3 and −77.9 ± 5.6 or −45.9 ± 2.7 and −37.8 ± 1.8 at six and 12 weeks after surgery, respectively. One-way ANOVA revealed significant differences in SFI between groups at six weeks [$F(2,21) = 274.29$; p=.001] and 12 weeks [$F(2,21) = 270.68$; p=.001].

Electrophysiological Improvement

Sciatic nerve conduction velocity, measured by EMG recordings, was significantly reduced (p=.001) in Control or Uridine rats compared to Sham rats at six weeks, but Uridine treatment provided a significant (p=.001) improvement 12 weeks after surgery (Figure 2). We measured the sciatic nerve conduction velocities in Control or Uridine group as 36.4 ± 3 and 45.9 ± 2.3 or 36.7 ± 1.6 and 64.9 ± 2.8 at six and 12 weeks after surgery, respectively. One-way ANOVA revealed significant differences in sciatic nerve conductivity between groups at 6 weeks [F(2,21) = 13.88; p=.001] and 12 weeks [F(2,21) = 19.31; p=.001].

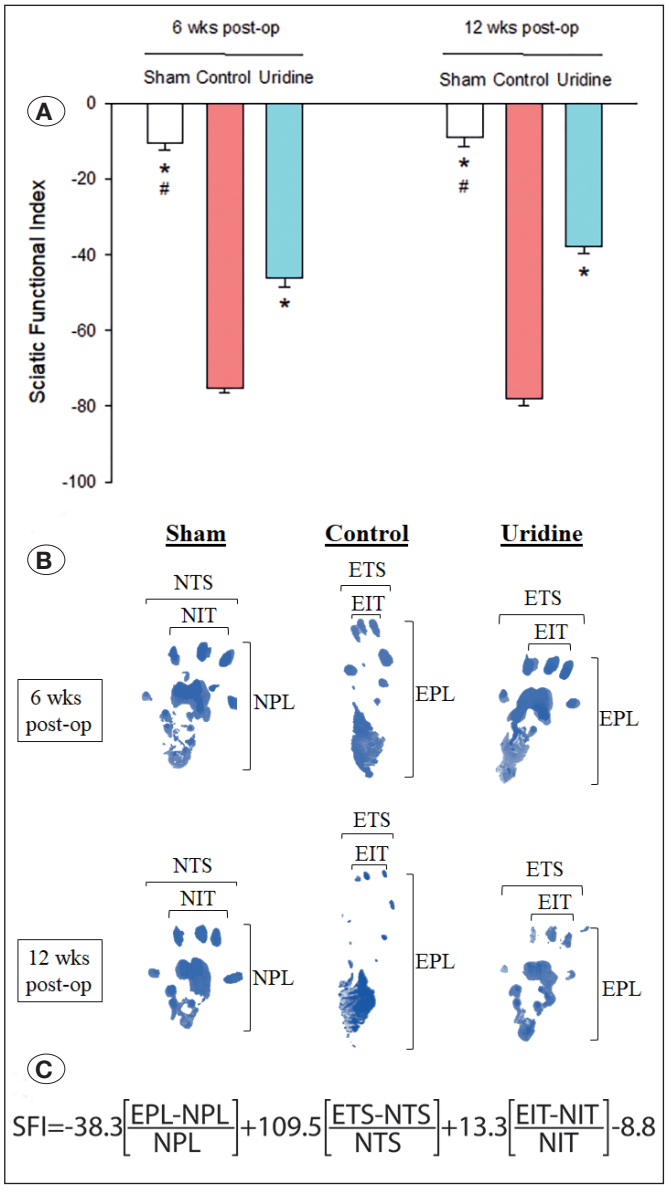

Figure 1: Results of the walking track analysis. **A)** Graph depicting the Sciatic Functional Index (SFI) in rats in Sham, Control, and Uridine groups at six weeks post-operatively (6 weeks post-op) and 12 weeks post-operatively (12 weeks post-op); *p=.001 compared to Control group and #p=.001 compared to Uridine group. **B)** Representative images of footprints (obtained by painting the plantar surfaces of right hind limbs with methylene blue) of rats in Sham, Control, and Uridine groups at six weeks post-operatively (6 weeks post-op; upper panel) and 12 weeks post-operatively (12 weeks post-op; lower panel). **C)** The Bain-Mackinnon-Hunter formula[20]used for calculating SFI data. **NPL,** normal print length, **NTS,** normal toe spread, **NIT,** normal intermediate toe spread, **EPL,** experimental print length, **ETS,** experimental toe spread, **EIT,** experimental intermediate toe spread.

Nerve Adherence and Separability

On completion of functional and electrophysiological evaluations at 12 weeks after surgery, rats in all groups were sacrificed and sciatic nerves were evaluated macroscopically. We discovered that nerve adherence and separability scores were impaired (p=.001) in the Control group (2.3 ± 0.2) and significantly improved (p=.002) in the Uridine group (1.5 ± 0.2) (Figure 3A). One-way ANOVA revealed significant differences in nerve adherence and separability scores [F(2,21) = 21; p=.001] between groups. Figure 3B-D depict representative images showing macroscopic appearances of sciatic nerves in different groups. All rats in the Control group had thick connective tissue covering the right sciatic nerve (Figure 3C), whereas rats in the Uridine group had a delicate membranous tissue surrounding the right sciatic nerve (Figure 3D).

Axon Count

The number of myelinated axons per square millimeter (mm^2) significantly reduced (p=.025) in the Control group (10234 ± 1161/mm^2) compared to the Sham group (14072 ± 825/mm^2) and significantly increased (p=.039) in the Uridine group (13923 ± 479/mm^2) (Figure 4A). One-way ANOVA revealed significant differences in myelinated axon counts [F(2,21) = 6.055; p=.019] between groups. Figure 4B-D depict representative images of the stained transverse sections.

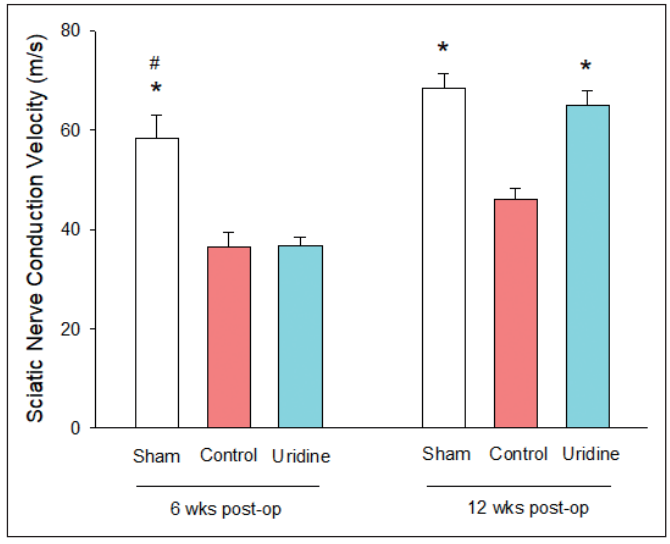

Figure 2: Graph depicting sciatic nerve conduction velocity in meters per second (m/s) in rats in the Sham, Control, and Uridine groups at six weeks post-operatively (6 wks post-op) and 12 weeks post-operatively (12 weeks post-op); *p=.001 compared to the Control group and #p=.001 compared to the Uridine group.

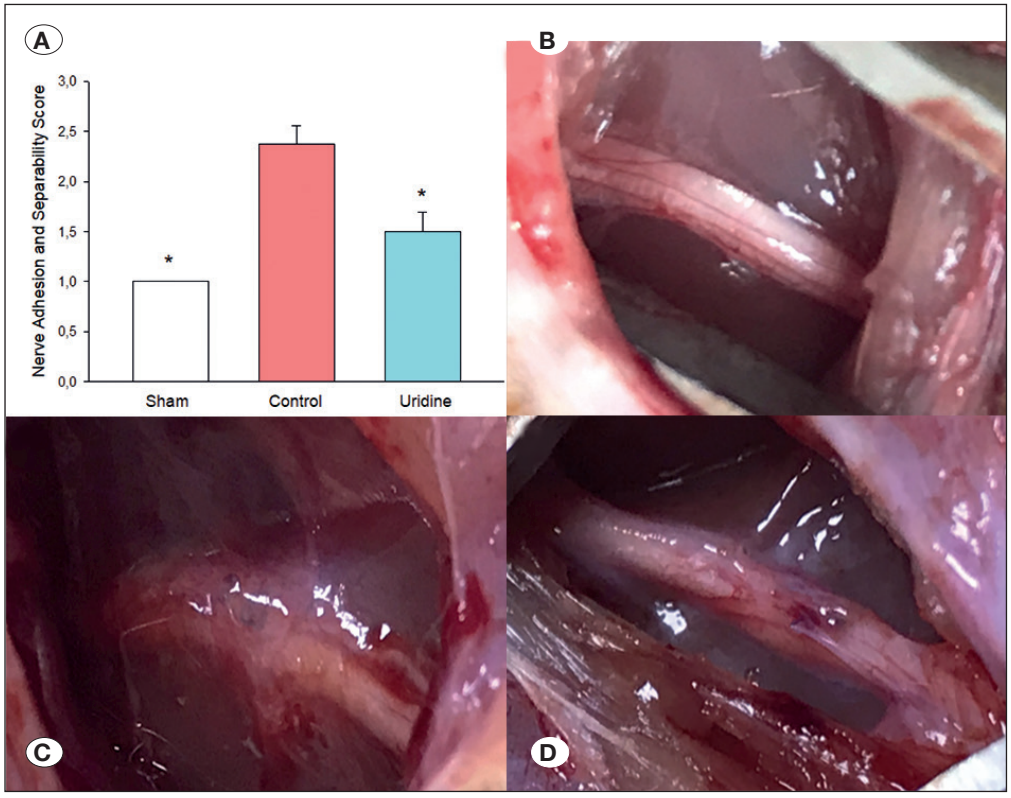

Figure 3: Results of macroscopic evaluation. **A)** Graph depicting nerve adhesion and separability score in rats in the Sham, Control, and Uridine groups at 12 weeks post-operatively; *p=.001 compared to the Control group. **B)** Representative image of the right sciatic nerve of a Sham-operated rat at 12 weeks post-operatively. **C)** Representative image of the thick connective tissue surrounding the right sciatic nerve of a rat in the Control group at 12 weeks post-operatively. **D)** Representative image of the fine membranous tissue surrounding the right sciatic nerve of a rat in the Uridine group at 12 weeks post-operatively.

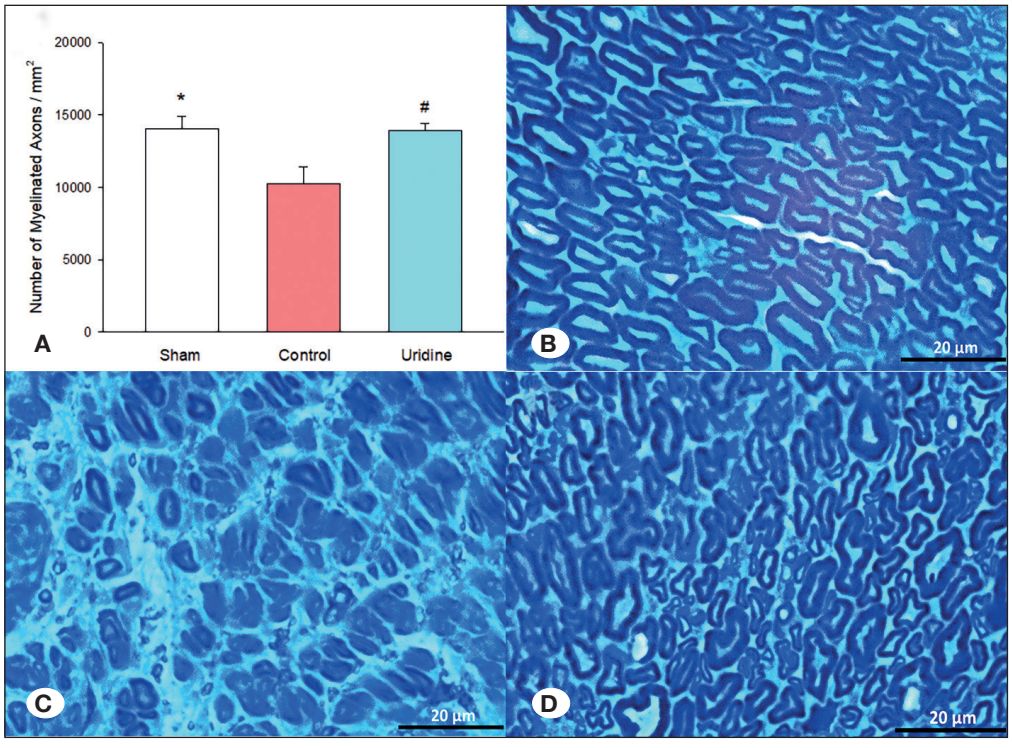

Figure 4: Results of histomorphological evaluation. **A)** Number of myelinated axons per square millimeter (mm^2) in rats in the Sham, Control, and Uridine groups at 12 weeks post-operatively; *p= .025 and #p=.039 compared to the Control group. **B-D)** Representative micrographs of toluidine blue-stained sections of right sciatic nerves in rats in the Sham, Control, and Uridine groups.

DISCUSSION

For the first time, these findings reveal that systemic Uridine treatment for seven days provides a long-term functional and electrophysiological improvement in a rat model of sciatic nerve injury. The effect of Uridine is associated with increased axon count, as well as decreased nerve adhesion and separability. These findings validate and further explain the results from our recent study (41) that Uridine exhibits anti-apoptotic and

anti-oxidant effects in the same model, indicating a putative mechanism for Uridine's long-term effects.

Peripheral nerve injury causes various physiopathological alterations in both the proximal and distal regions of the nerve. During transection injury, the proximal segment of the neuron suffers degenerative alterations, whereas the distal segment undergoes Wallerian degeneration (21). The proximal part of the surviving nerve is often degenerated by calcium flow followed by the activation of calcium-associated proteases up to the first Ranvier node (26). Portions of the preexisting elements of the cytoskeleton in axons start elongating from the initial node of Ranvier at the lesion's proximal site (60). Axon regeneration reportedly requires the contribution of neurotrophic factors, cell adhesion molecules, extracellular matrix proteins, and several other molecules (24,60).

Treatment of transected peripheral nerve injury with a full functional recovery remains a challenge in regenerative medicine. The gold standard treatment for such injury is axon-to-axon anastomosis (29), and functional recovery is affected by several factors, including the number of viable neurons, the quality of axonal growth, the orientation of the regenerated axon, and the condition of the target tissue to influence an ideal nerve repair (37,46,58). Other surgical approaches, such as direct nerve repair, nerve grafting, nerve transfer, fibrin glue, nerve conduits, and cell-based therapy do not provide 100% recovery, and are generally time-consuming and expensive, and carry the risk of donor unavailability and immunosuppression (33).

However, non-surgical therapies, such as analgesics, opioids, and corticosteroids, can only provide pain relief but cannot promote functional recovery (33). Alternative therapeutic approaches with phytochemicals, including quercetin, ursocholic acid, curcumin, and several others have been proposed to confer benefit with experimental evidence, even though most of them induce side effects at human doses (33). Furthermore, an ideal medication must target at least some pathophysiological events, including oxidative stress (50), apoptosis (51), inflammation (49), degradation of the extracellular matrix (32), and several others that compromise recovery in peripheral nerve injury. Although several therapeutic approaches have been proposed for the anatomical and functional recovery of the injured peripheral nerve (33), newer approaches for treating peripheral nerve injury, which target pathophysiological events and govern optimum properties, such as having ignorable side effects, are required.

Uridine is one such possibility when combined with axon-to-axon anastomosis. Uridine is an endogenous pyrimidine nucleoside (9,59) and a precursor for membrane phospholipid synthesis (14) via the Kennedy pathway (40). Uridine might confer benefit in peripheral nerve injury for the following reasons: (i) Uridine is a precursor of CDP-choline (14,55), which enhances nerve regeneration and functional recovery in sciatic nerve injury models (7,30,39), (ii) Uridine promotes axonal growth and sprouting in cultured PC12 cells (54), (iii) Uridine inhibits inflammation and fibrosis in bleomycin-induced lung injury (18), and (iv) Uridine provides neuroprotection in hypoxic-ischemic (12,27,43) and hyperoxic brain injury models (2,28) by inhibiting apoptotic cell death (12) and oxidative damage (2). Considering these findings, we recently examined Uridine's acute effects and discovered (41) that systemic administration of Uridine for seven days exhibited anti-apoptotic and anti-oxidant effects in a rat model of peripheral nerve injury performed by full-thickness sciatic nerve transection followed by immediate primary repair. Hence, this study was designed to investigate the longer-term benefits of Uridine treatment in the same model.

In this study, we discovered that intraperitoneal Uridine injection for seven consecutive days at a dose (500 mg/kg) proven to exhibit immediate anti-apoptotic and anti-oxidant effects provided sciatic nerve regeneration 12 weeks after transection injury, as demonstrated by functional and electrophysiological recovery. Compared to the Control group, Uridine treatment significantly enhanced SFI and accelerated nerve conduction velocity. Furthermore, Uridine increased the number of myelinated axons, while decreasing nerve adhesion and separability. These findings could be associated with the recent observation of Uridine modulating cellular and molecular mechanisms (41).

Direct action of Uridine on sciatic nerve injury in all its short- and long-term effects may be speculated, but it may not be coherent because Uridine has no known receptors (8). Depending on our current understanding, the mechanism(s) by which Uridine promotes peripheral nerve regeneration may involve the effect(s) of compounds synthesized from Uridine endogenously. Uridine can be phosphorylated in the body to form Uridine-5'-monophosphate (UMP), uridine-5'-diphosphate (UDP), and uridine-5'-triphosphate (UTP) (9,13,45). UTP and UDP are ligands to several P2Y receptors; UTP activates P2Y2 (47) and P2Y4 (20) receptors, while UDP activates P2Y6 (19) and P2Y14 (16) receptors. Activation of these receptors was shown to enhance nerve growth (16), proliferation (48) and differentiation (3), inhibit apoptosis (17), counteract oxidative stress (23), and suppress the matrix metalloproteinase production (42) to promote neuroprotection and neuroregeneration (6).

Uridine is also a precursor for the synthesis of CDP-choline (14,45,55,61), an endogenous intermediate formed in the rate-limiting phase of phosphatidylcholine synthesis via the Kennedy pathway (40). Exogenously-administered CDP-choline was shown to exhibit cardiovascular (15,57), metabolic (34), and neuroendocrine (10,11,35) effects, as well as counteract endotoxemia (36,62). Furthermore, CDP-choline was reported to exert neuroprotective effects in hemorrhagic/hypoxic/ischemic brain injury models (1), spinal cord trauma (63), and glaucoma (25). Concerning peripheral nerve regeneration, we previously showed that CDP-choline administration dose-dependently (39) improves nerve regeneration and functional recovery in a rat model of sciatic nerve injury (4,7) by modulating the levels and activities of matrix metalloproteinases (30). Therefore, Uridine might have exhibited its effects in this model through various mechanisms, including in vivo conversion to CDP-choline.

CONCLUSION

The treatment of peripheral nerve injuries remains an issue in regenerative medicine. For the first time, the current findings reveal that systemic Uridine administration for seven days provides nerve regeneration and functional recovery in a rat model of sciatic nerve injury over time. The effect of Uridine is associated with increased axon count, as well as decreased nerve adhesion and separability. The findings validate and extend our knowledge on Uridine's longer-term effects in sciatic nerve injury by adding to its immediate anti-apoptotic and anti-oxidant actions in this model, indicating that Uridine is effective in treating peripheral nerve injuries.

DISCLOSURE and CONFLICTS of INTEREST

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The authors report no proprietary or commercial interest in any product or concept discussed in this article.

AUTHORSHIP CONTRIBUTION

Study conception and design: AB, MC

Data collection: MKK, CK, BS

Analysis and interpretation of results: MC, MKK, AT

Draft manuscript preparation: MC, IMK, MKK

Critical revision of the article: AB, MC, IMK

Other (study supervision, fundings, materials, etc...): PL, AC

All authors (MKK, AT, CK, BS, PL, AC, IMK, MC, AB) reviewed the results and approved the final version of the manuscript.

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