



Mediation of Epigenetic Mechanisms in the Regenerative Effect of Uridine in a Rat Model of Sciatic Nerve Injury

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ABSTRACT

AIM: To investigate the possible mediation of epigenetic mechanisms underlying the regenerative effect of uridine in a sciatic nerve transection rat model.

MATERIAL and METHODS: Fifty adult male rats were randomized to sham, control, and uridine groups. After unilateral transection and primary anastomosis of the right sciatic nerve, a single daily dose of saline (1 ml/kg; sham and control groups) or uridine (500 mg/kg; uridine group) was injected intraperitoneally for a week. The sciatic nerves were removed en bloc on the eighth day and levels of histone deacetylase 1 (HDAC1), acetylated histone-H3, and acetylated histone-H4 were analyzed in nerve homogenates. The number of myelinated axons in the sciatic nerve specimens was analyzed histomorphologically.

RESULTS: The HDAC1 levels were significantly greater in the control group than in the sham ($p<0.001$) and uridine ($p<0.01$) groups. Compared to the sham group, the acetylated histone-H3 and histone-H4 levels decreased in the control group (by 81.49% and 79.98%, respectively for both; $p<0.001$) and increased significantly in the uridine group (by 62.54% and 51.68% respectively; $p<0.01$, $p<0.05$). The number of myelinated axons decreased significantly ($p<0.001$) in the control group, which was enhanced significantly by uridine administration.

CONCLUSION: Epigenetic mechanisms may partly mediate the regenerative effect of uridine treatment in a rat model of sciatic nerve injury. Our data provides novel insights in the management of peripheral nerve damage and suggests potential benefit of uridine for degenerative diseases in which epigenetic impairments are involved.

KEYWORDS: Uridine, Sciatic Nerve Injury, Peripheral Nerve Regeneration, Histone Deacetylase, Epigenetic




ABBREVIATIONS: HDAC1: Histone deacetylase 1, PNI: Peripheral nerve injury, CDP-choline: Cytidine diphosphate-choline, HIE: Hypoxic-ischemic encephalopathy, ELISA: Enzyme-linked immunosorbent assay, PBS: Phosphate-buffered saline, SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, PVDF: Polyvinylidene fluoride, TBST: Tris buffer, tween 20, OsO₄: Osmium tetroxide, DNA: Deoxyribonucleic acid, HAT: Histone acetylase

INTRODUCTION

Peripheral nerve regeneration is a challenge in regenerative medicine. Typically, the post-injury regeneration rate of a peripheral nerve is 1 mm/day, which is usually

not sufficient for complete regeneration and functional recovery owing to scar tissue formation (29,45), Schwann cell dysfunction, misdirection of axonal growth cones, or irreversible muscle atrophy after loss of innervation.

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In transection injuries, degenerative changes in the peripheral nerve occur on either side of the injury, resulting in partial or complete sensory/motor loss is observed (8,21,42,46). Following a peripheral nerve injury, the proximal segment undergoes degenerative changes and the distal segment undergoes Wallerian degeneration (29). Pathophysiological changes, such as apoptosis, oxidative stress, inflammation, destruction of the extracellular matrix, and several other events can worsen the extent of damage in a peripheral nerve injury (PNI) (28,47,48,52); however, these complex processes can be disrupted at every stage to prevent post-injury regeneration. Although several surgical and medical approaches targeting these processes have been developed, a treatment that can guarantee full functional recovery in PNI is yet to be discovered (8,29,42,46).

Uridine is the chief pyrimidine nucleoside in circulation (13,72), which is found in breast milk in humans and other mammals (68). It increases the biosynthesis of cytidine diphosphate-choline (CDP-choline) as a rate-limiting compound both in vivo (12), and in vitro (60); it is also a precursor for synthesizing membrane phospholipids via the Kennedy pathway (36). Systemic treatment with uridine reportedly augments the synaptic connections of neurons (58), while exogenous treatment with uridine has been reported to provide neuroprotection and reduce long-term cognitive deficits by preventing apoptotic neuronal loss in experimental hypoxic-ischemic encephalopathy (HIE) (12,22,41), and hyperoxic brain injury models (23). In the HIE model, uridine treatment promoted neuronal recovery by decreasing histone deacetylase (HDAC) activity and increasing levels of acetylated histone-H3 and acetylated histone-H4 (41). A recent study demonstrated the role of epigenetic mechanisms in treating peripheral nerve damage and enhancing functional recovery (27). The authors noted that an HDAC3 enzyme-dependent pathway was responsible for preventing peripheral myelin development and functional regeneration; therefore, inhibiting this pathway can potentially boost peripheral myelination (27).

Therefore, in continuation to our previous observations regarding the effect of uridine in rat sciatic nerve transection and primary anastomosis model (38,39), this study aimed to explore the mediation of epigenetic mechanisms (involving the HDAC1 enzyme, acetylated histone-H3, and acetylated histone-H4 levels) underlying uridine's effects in peripheral nerve regeneration.

■ MATERIAL and METHODS

Study Design and Experimental Animals

This experimental study was approved by the Local Ethics Committee on Animal Experiments, Bursa Uludag University, Bursa, Turkey (approval no: 2021-08/02; 15.06.2021), and carried out in accordance with the ARRIVE Guidelines Version 2.0 (56) and Guide for the Care and Use of Laboratory Animals – 8th edition (49).

Fifty male Sprague Dawley rats (3–6 months old and weighing 300–400 g) were purchased from the Bursa Uludag University Medical Faculty Experimental Animal Research Center, Bursa,

Turkey. All rats were housed in single cages maintained at 22°C with a 12-hour light/dark cycle and free access to food and water.

Rats were randomized to Sham, Control and Uridine groups to further receive intraperitoneal injections of saline, saline and uridine, respectively. Before the treatments, rats in the Control and Uridine groups underwent unilateral sciatic nerve transection surgery and those in the Sham group only received sham surgery as a control for the effects of surgery. Two rats died following sciatic nerve transection surgery; hence, the study finally included 48 rats (n=16 in each group). Following surgery, treatments were initiated and lasted for 7 days. On the 8th day, rats were sacrificed and nerve samples were collected. Half in number (a total of 24 obtained from all three groups) of the nerve samples was used for enzyme and protein analyses while the other half (n=24) was used for histomorphological analyses.

Surgical Procedure

Rats underwent sciatic nerve transection surgery which was followed by immediate primary anastomosis. The rats were anesthetized using 2%-3% sevoflurane (Sevorane Liquid; Aesica Queensborough LLC., Queensborough Kent, UK), followed by the use of 1%-2% sevoflurane for anesthesia maintenance. The rats were placed on a dissection table in a left lateral decubitus position. To approach the sciatic nerve, the femoral head was palpated along the hind leg, and a 2-3 cm long posterolateral skin incision was made from the knee to the upper part of the trochanter major. Next, the plane between the biceps femoris and the gluteus muscle was dissected with the help of microdissection scissors and separated from the underlying fascia. Proceeding with blunt dissection, a retractor was placed between both muscle blocks, and 1-2 cm of the sciatic nerve was exposed extending from the sciatic foramen up to the origin of its tibial and peroneal branches (Figure 1). A full-thickness nerve cut was made in the sciatic nerve 1 cm away from the distal sciatic foramen in all the rats except those in the sham group. In the sham group, no transection was performed to explore normal nerve functions and activity. The distal and proximal nerve stumps were primarily sutured under a microscope with an aperture of 180° (Figure 2). The fascia and skin were closed, and the rats were returned to their cages. All operative procedures were performed by a single neurosurgeon using a surgical microscope (Zeiss Opmi; Carl Zeiss Meditec Inc., Jena, Germany).

Interventions

Before surgical procedures, the rats were randomized into the uridine, control, and sham groups (n=16 in each group). The treatments were initiated on the day of surgery; rats in the sham and control groups were administered daily with 1 ml/kg of saline intraperitoneally for seven days, while the uridine group received 500 mg/kg of uridine (Merck KGaA, Darmstadt, Germany) intraperitoneally daily for seven days. The uridine dose administered was determined as reported in previous studies (38,39).

On the 8th postoperative day, the rats were anesthetized again with sevoflurane (3%), and their sciatic nerves were excised

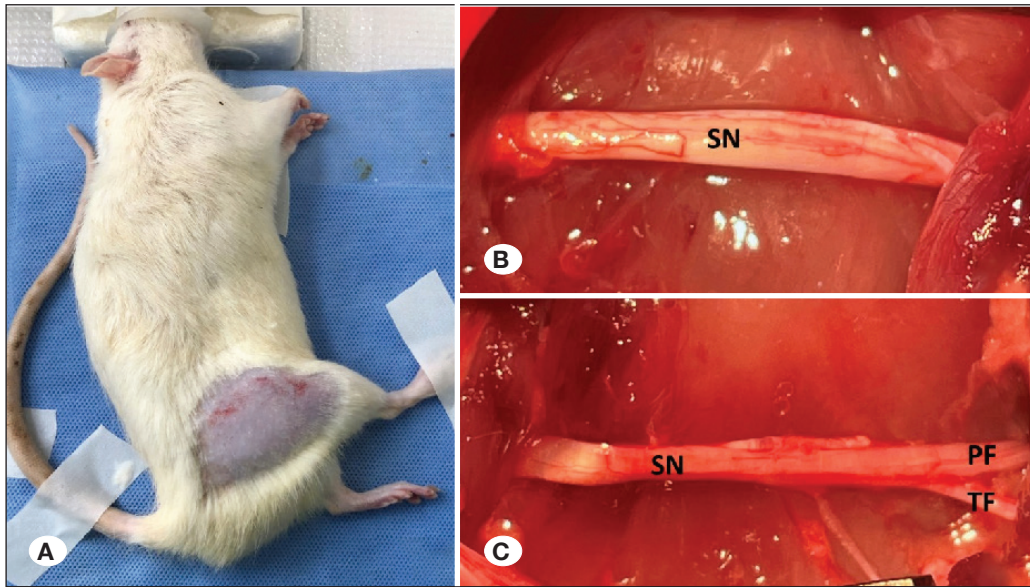


Figure 1: Rat placed in the left lateral decubitus position (A); the right sciatic nerve (B) was exposed with blunt dissection. The sciatic nerve and distal fascicular branch point (C) were exposed. SN: Sciatic nerve, PF: peroneal fascicle, TF: tibial fascicle.

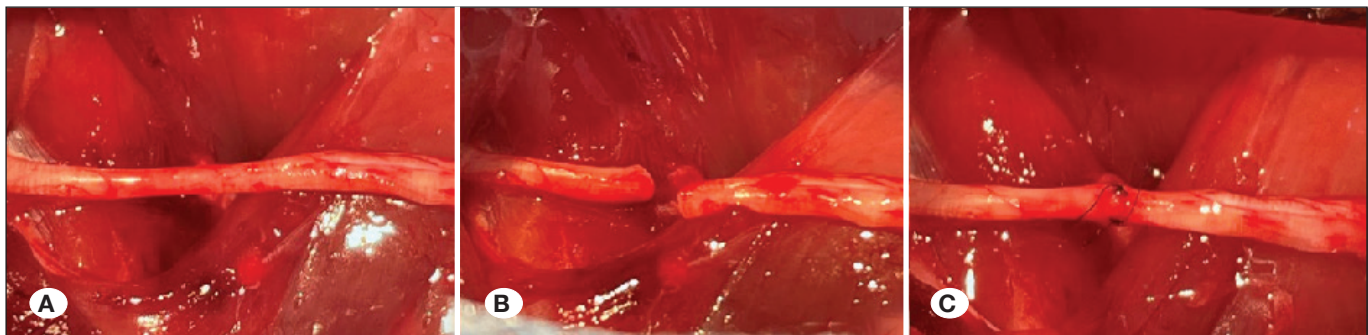


Figure 2: The unifascicular sciatic nerve segment was exposed (A). Subsequently, a full-thickness nerve transection was made with a dermatome knife (B). A primary anastomosis was achieved between the distal and proximal stump using two sutures made 180° apart using the epineurial nerve suturing technique (C).

unilaterally. Sciatic nerve samples from half of the rats in each group were homogenized to perform enzyme and protein analyses; the other half were used for histomorphological analyses. All rats were euthanized with a high dose of sevoflurane after tissue collection.

Macroscopic Evaluation

The operative site and surgical wound were assessed daily until the end of the experiment. The condition of the skin, muscle, and fascia, and the relationship of the sciatic nerve with its surrounding tissue were noted. The Petersen numerical rating score (57) was used to quantify the nerve's adherence to the surrounding soft tissue mass and its dissociation from these structures during macroscopic evaluation. The score uses two parameters: (i) skin and muscle fascia and (ii) nerve adherence and nerve separability. Considering skin and muscle fascia, rats were graded as Grade 1: skin or muscle fascia entirely closed, Grade 2: skin or muscle fascia partially open, or Grade 3: skin or muscle fascia completely open; based on the extent of nerve adherence and nerve separability, rats were graded as Grade 1: no dissection or mild blunt dissection required,

Grade 2: some vigorous blunt dissection required, and Grade 3: sharp dissection required.

Tissue Sampling

On the 8th postoperative day, the surgical wound was re-opened, and the soft tissue was dissected. The sciatic nerves were removed en bloc for enzyme and protein analyses and stored at -80°C. For the enzyme-linked immunosorbent assay (ELISA) and Western blot analyses, the nerve samples were homogenized in 2 ml of ice-cold phosphate-buffered saline (PBS; pH 7.4). For the histomorphological studies, a sufficient amount of Trump's fixative (1.4 g formaldehyde, 1% glutaraldehyde in 1 × PBS, and 1.16 g NaH₂PO₄·H₂O per 100 ml; Sigma-Aldrich, St. Louis, MO, USA) was added to the operative site, incubated for 10 minutes, and subsequently cleared from the surgical field; this step was repeated two more times. Nerve tissues were excised under microscopic guidance and stored at 4 °C in 15 ml tubes containing Trump's fixative.

Enzyme and Protein Analyses

The HDAC1 enzyme levels were determined using a rat-compatible ELISA kit (Bioassay Technology Laboratory, Shanghai, China) as per the manufacturer's instructions. Briefly, the procedure includes incubation of the HDAC colorimetric substrate with a compound containing HDAC activity, followed by treatment with lysine developer to produce a chromophore which was analyzed using spectrophotometry (BioTek Quant; BioTek Instruments Inc., Winooski, VT, USA) at a wavelength of 450 nm.

Levels of acetylated histone-H3 and acetylated histone-H4 were analyzed using Western blot analysis. The total protein levels were measured using the Bicinchoninic acid method; the homogenates were mixed with equal volumes of Laemmli buffer (0.0625 M Tris Base, 0.07 M Sodium Dodecyl Sulfate, 10% glycerol and 5% 2-mercaptoethanol) and boiled for five minutes. Equal amounts of the protein were loaded for all samples, which were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Mini Protean II; Bio-Rad, Hercules, CA, USA). The proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA), which were blocked for half an hour with tris buffer saline-tween 20 (TBST, Sigma-Aldrich, St. Louis, MO, USA), and 5% non-fat powdered milk solution (Carnation, Glendale, CA, USA). Subsequently, the membranes were washed three times with TBST and incubated overnight with anti-acetyl-histone H3 (1:1000; Cell Signaling Technology, Danvers, MA, USA) and anti-acetyl-histone H4 (1:1000; Cell Signaling Technology, Danvers, MA, USA) as primary antibodies, and anti-B-Actin (B-Actin, 1:1000; Cell Signaling Technology, Danvers, MA, USA) as the positive control. The following day, the membranes were washed with TBST again and incubated for 60 minutes with the appropriate secondary antibodies (1:5000; Cell Signaling Technology, Danvers, MA, USA). The intensity of the protein bands was digitized and analyzed using a CDigit scanner (Licor CDigit scanner, Lincoln, NE, USA).

Histomorphological Analyses

Tissue samples in the tubes containing Trump's fixative were fixed for one week by renewing the fixatives every 48 hours. Thereafter, the tissues were subjected to post-fixation procedures (2 h in 1% osmium tetroxide [OsO₄] with a phosphate buffer of 0.1 mol/L) before the histomorphological analysis. Spur's resin (Agar Low Viscosity Resin Kit; Agar Scientific, Essex, UK) was carefully added to the nerve samples (Figure 3A). The mold containing the nerve segments and resin were left overnight to polymerize at 68°C–70°C. The resin-embedded nerve blocks were placed in an ultramicrotome device (LKB Ultratome Main Unit Type 4801 A, Vienna, Austria) set to 1–2 μm, to obtain transverse semi-thin sections. Subsequently, a drop of toluidine blue solution was added to the sections and left for 20–30 seconds; the slides were gently immersed in a jar of distilled water to rinse off the excess solution. This process was repeated several times until the sections were clear. The preparations were dried at 60°C for approximately 15 minutes and covered with a lamella (Objektträger, Isolab, Eschau, Germany). The slides were then analyzed microscopically and stored at room temperature (19).

Using the Zeiss Primo Star light microscope system and Zeiss Labscope software (Carl Zeiss Meditec AG, Jena, Germany), we obtained randomly selected images of the sections (5 MP resolution). The section images magnified to 100x were converted to BMP format using ImageJ 1.53t (Laboratory for Optical and Computational Instrumentation, University of Wisconsin, Madison, WI, USA) which was calibrated using the table micrometer before each examination and count. An Olympus micrometric slide was used for calibration. For all preparations, a count frame (1500 × 1000 pixels; 127.19 × 84.74 μm; 10772 μm²) was created using macros on the images that were obtained with a 100x objective. Additionally, myelinated axons in the field were counted using non-biased counting rules, and the number of axons per square millimeter (axonal density) was determined. For this purpose, axons in contact with the right and lower edges of the frame and all

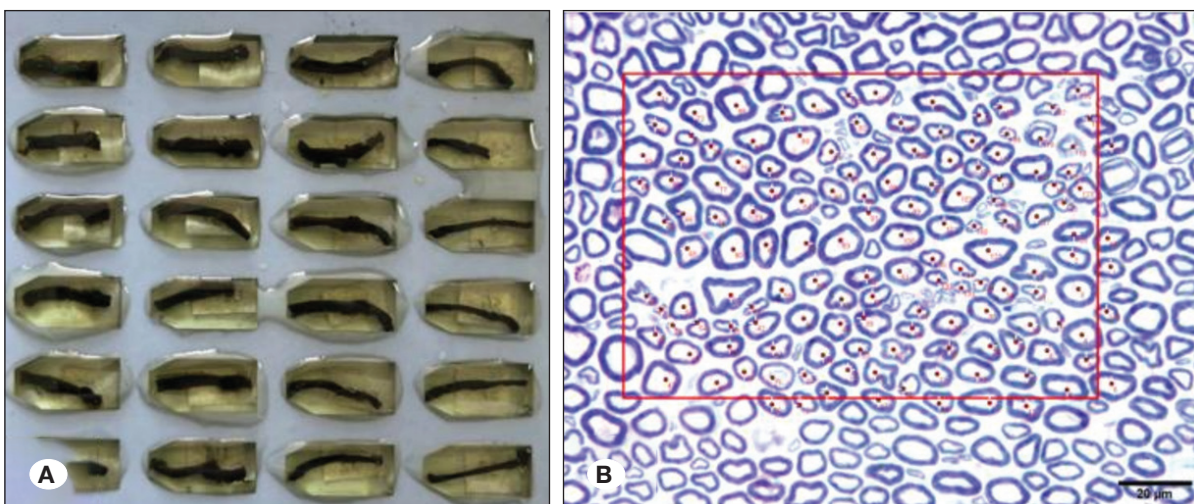


Figure 3: The post-fixation process of the excised sciatic nerve segments for histomorphological analyses was completed and the sections were placed in resin blocks (A). A counting frame was created using ImageJ software before counting the axons (B).

myelinated axons were included in the count, and those axons in contact with the left and upper edges of the counting area were excluded (Figure 3B).

Statistical Analysis

All analyses were performed using SigmaPlot (SYSTAT software, version 12.5, Bayshore Rd, Palo Alto, CA, USA). The variables were described as mean ± standard error of means (SEM). A one-way analysis of variance, followed by Tukey’s post-hoc testing was used to compare multiple groups for enzyme and protein analyses. The histomorphological analysis results were evaluated using the Kruskal–Wallis test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Operative and Macroscopic Evaluations

According to the Petersen numerical rating score (57) for the

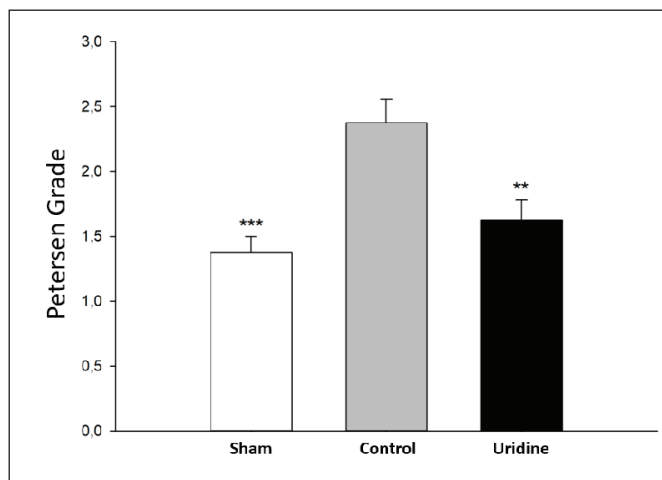


Figure 4: Quantitative results of Petersen’s grading showing nerve adhesion and separability (n = 16 in each group). **p 0.01; ***p<0.001, compared to the control group.

skin and muscle fascia around the wound, two rats had Grade 3 (completely open) wounds, three had Grade 2 (partially healed) wounds, and the remaining rats had Grade 1 (fully recovered) wounds; there was no statistically significant difference between the groups in terms of Petersen grading (Figure 4). In contrast, the groups were significantly different in terms of nerve adhesion and separability; nerve adhesion and fibrotic scar formation were significantly higher in the control group than in the sham group ($p < 0.001$), while these pathologies were significantly improved in the uridine group ($p < 0.01$) (Figure 4). Macroscopic examination of the excised nerve segments revealed that there were more neuromas and misdirection in the control group than in the sham and uridine groups. In contrast, the nerve segments were more intact and had a uniform appearance in the uridine group than in the control group (Figure 5).

Enzyme and Protein Analysis

In comparison to the sham group, the HDAC1 levels were significantly higher in the control group ($p < 0.001$) and significantly lower in the uridine group ($p < 0.01$) (Figure 6). Likewise, as compared to the sham group, the control group showed a significant decrease in the levels of acetylated Histone-H3 and acetylated Histone-H4 by 81.49% ($p < 0.001$) and 79.98% ($p < 0.001$), respectively, and the uridine group showed increased levels of acetylated Histone-H3 and acetylated Histone-H4 to 62.54% ($p < 0.01$) and 51.68% ($p < 0.05$), respectively (Figure 7).

Histomorphological Analyses

The mean ± standard error of measurements (SEM) number of axons counted within the counting frame was 32.52 ± 0.85 , 116.37 ± 3.85 , and 66.75 ± 1.73 for the control, sham, and uridine groups, respectively (Figure 8). The mean axonal density was noted as 3019.09 ± 79.01 in the control group, 10803.04 ± 358.24 in the sham group, and 6196.6 ± 161.48 in the uridine groups (Figure 9). Compared to the sham group, both the number of myelinated axons in the measurement

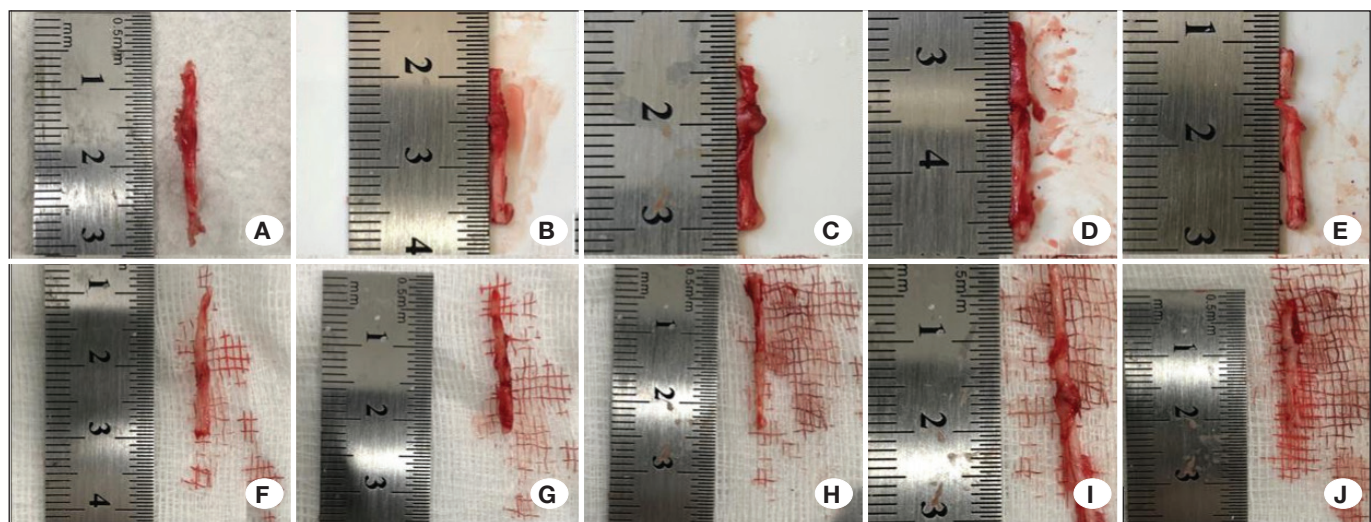


Figure 5: Excised nerve samples of the uridine and control groups. A–E are samples from the control group; F–J show the sciatic nerve segment samples of the uridine group.

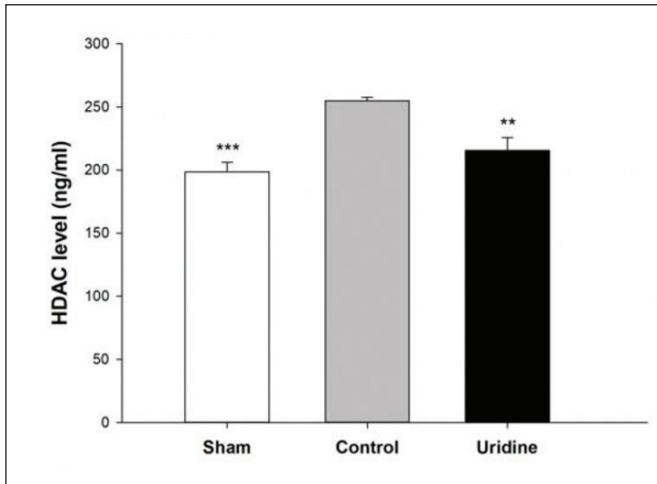


Figure 6: HDAC1 levels in the uridine, control, and sham groups. ** $p < 0.01$; *** $p < 0.001$, compared to the control group ($n=8$). The data are expressed as mean \pm standard error of measurement for each group.

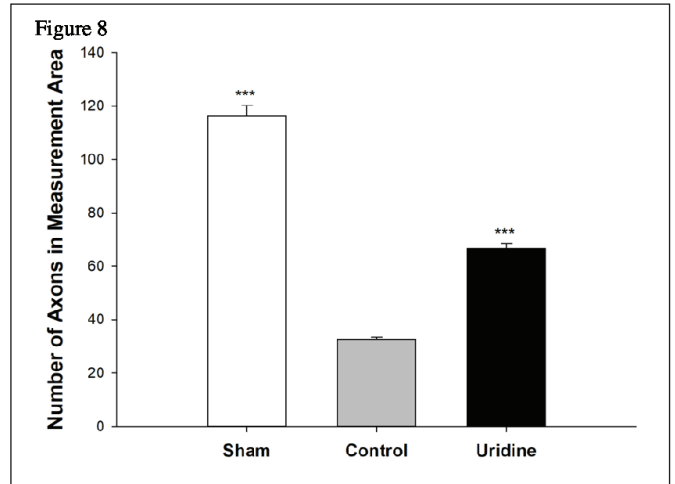


Figure 8: Number of axons in the measurement area. *** $p < 0.001$ compared to the control group ($n=8$ in each group); mean \pm standard error of measurement values are provided for each group.

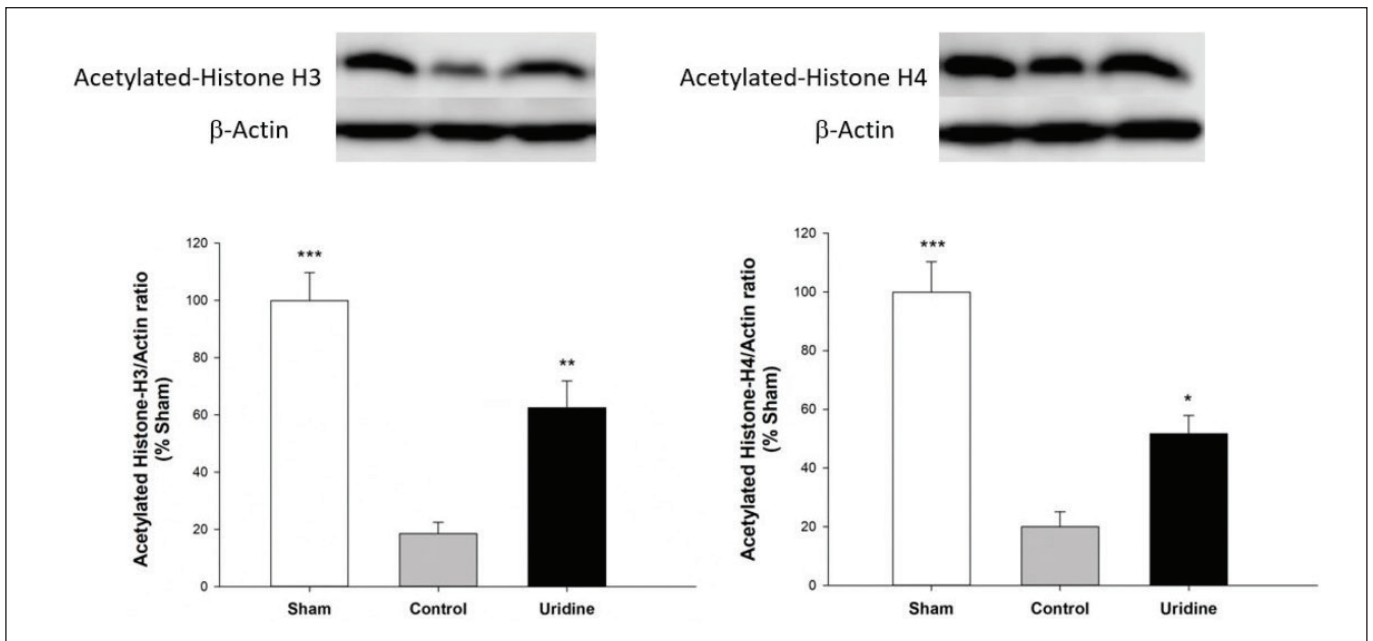


Figure 7: Acetylated histone-H3 (** $p < 0.01$; *** $p < 0.001$) and histone-H4 (* $p < 0.05$; *** $p < 0.001$) protein levels in the uridine, control, and sham groups. p -values compared to the control group ($n=8$ in each group); data are expressed as mean \pm standard error of measurement.

area and axonal densities were significantly lower ($p < 0.001$) in the control group and significantly more in the uridine group ($p < 0.001$) (Figure 10).

DISCUSSION

These results confirm our previous findings regarding the neuroprotective effects of uridine in a rat model of sciatic nerve injury (38,39) and highlight the role of HDAC inhibition as a potential mechanism underlying the action of uridine.

PNI are common causes of severe morbidity with a reported prevalence of 2.8% (50) and an incidence of 13–23/100,000 (5) in developing countries. Surgery remains the gold standard for the treatment of PNI (26); however, despite the introduction of new suturing techniques (44) and other approaches, such as autogenous nerve grafting, vascularized nerve grafting, synthetic tube grafting, and end-to-side anastomosis, full functional recovery is not yet possible and the outcomes are far from being curative. This is because surgery alone cannot assuage the pathophysiological consequences of nerve damage, such as inflammation, oxidation, and apoptosis, which

negatively affect the regeneration process. Therefore, previous studies have targeted one or more of the pathophysiological parameters associated with PNI. So far, a variety of chemical agents, compounds, and other approaches have been tested for the treatment of PNI, which includes chemical agents; coenzyme Q10 and vitamin E (53), ginger and sesame oil (16), telmisartan (76), quercetin (69), hyperbaric oxygen therapy (37), ginkgo (1), pioglitazone (59), levetiracetam (2), lithium (7), surgical approaches; mesenchymal stem cells (34,63,74), the use of synthetic grafts to prevent misdirection of the growth cone (75), local treatment modalities like anti-transforming growth factor beta, hyaluronic acid, human amniotic fluid (55), aprotinin (24), 5-fluorouracil (51), low-dose radiation (20) and electrophysical modalities; electrical stimulation and pulse magnetic field (4,15,33).

Likewise, our previous study showed that treatment with CDP-choline (citicoline), a compound with anti-inflammatory, anti-oxidant, and anti-apoptotic properties (66), improved functional recovery, promoted nerve regeneration, and reduced postoperative scar formation in experimental rat mod-

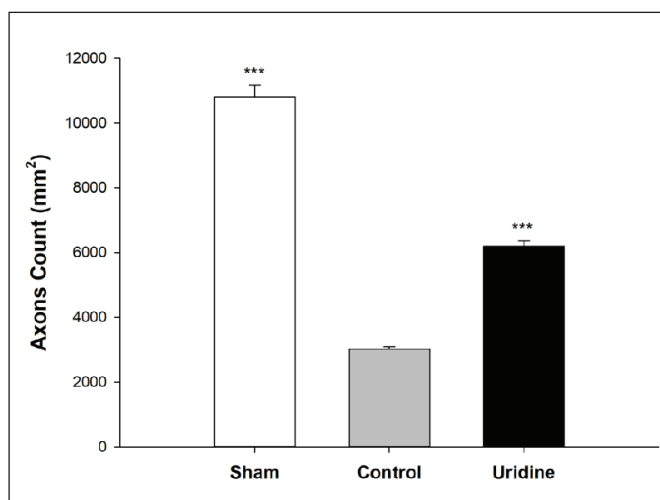


Figure 9: Axon count per square millimeter (axonal density) of the section. *** $p < 0.001$ compared to the control group ($n=8$); mean \pm standard error of measurement values are provided for each group.

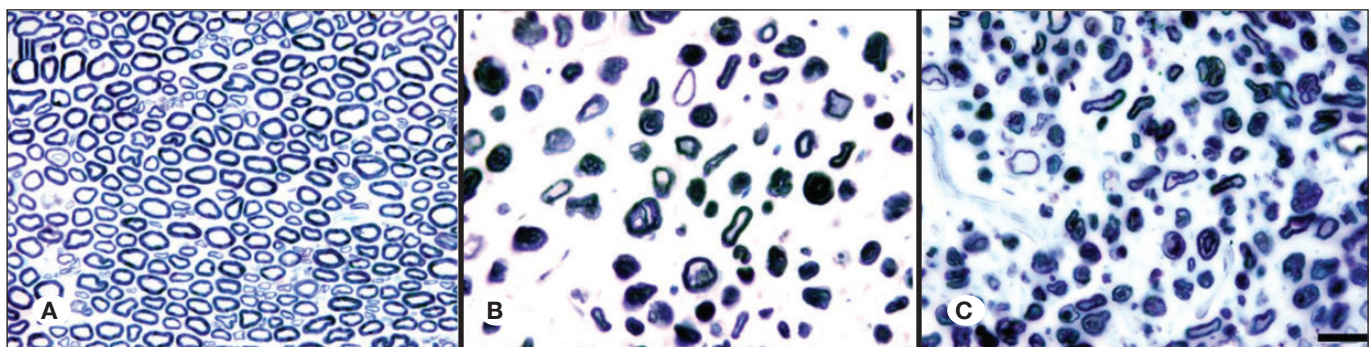


Figure 10: Thin sections of fixed sciatic nerve samples stained with 1% borax-toluidine blue. **A)** sham; **B)** control; and **C)** uridine group. Bar, 20 micrometers.

els (3,9,25,32,54). CDP-choline is an endogenous compound formed in the rate-limiting step of phosphatidylcholine synthesis via the Kennedy pathway (36) which has cardiovascular (14,65), neuroendocrine (10,11,31), and metabolic (30) effects on exogenous administration. However, exogenously administered CDP-choline metabolizes to choline and cytidine while cytidine is rapidly converted to uridine in blood circulation (72). Treatment with uridine exhibits several beneficial effects on nerve tissue, such as increased nerve branching and axonal growth in cultured PC12 cells (58) and protection in HIE (12,22,41) and hyperoxia (24) models. Therefore, our recent studies used experimental models of sciatic nerve injury to test the effects of uridine in major pathophysiological consequences of PNI. We observed that uridine administration improved nerve regeneration and functional recovery (39) by exerting anti-apoptotic and anti-oxidant effects (38).

Abnormal or dysfunctional histone acetylation underlies the pathophysiology of various neurological illnesses, including neurodegeneration (18,62). These acetylation mechanisms are dynamic events and are regulated by histone acetylase (HAT)-HDAC enzyme groups. HATs cause acetylation of lysine residues and relax the intact nucleosome structure between the DNA and histones, allowing transcription factors to reach the target gene and cause its expression. Conversely, HDACs cause deacetylation of the lysine residues; therefore, transcription cannot occur when DNA is firmly bound to the histones (35). Enzymes and proteins involved in histone metabolism are commonly found in the peripheral and central nervous systems. Thus, epigenetic mechanisms involving HDAC inhibition exert neuroprotective action in the pathophysiology of PNI (17,61,64,67).

It is known that inhibition of histone deacetylation is associated with reduced apoptosis (70), and uridine behaves as an anti-apoptotic HDAC inhibitor in a rat model of neonatal HIE (41). Therefore, the present study further explored the potential mechanism of action through which uridine exerts its neuroprotective effects in PNI. As reported previously (38,39), intraperitoneal administration of uridine (500 mg/kg) for seven consecutive days reduced nerve adhesion and fibrotic scar formation in our rat models of PNI. Moreover, the number of myelinated axons in the measurement area and axonal density (per mm²) also enhanced significantly with uridine treatment.

These findings were associated with decreased levels of HDAC1 and increased expressions of acetylated Histone-H3 and acetylated Histone-H4, suggesting mediation of epigenetic mechanisms in sciatic nerve regeneration under the effect of uridine administration. Our findings concur with previous reports showing that HDAC inhibitors, such as trichostatin A, mocetinostat, sodium phenylbutyrate, and valproic acid, reduced axonal degradation (40), improved nerve regeneration (6), enhanced axonal regrowth and remyelination (73), as well as provided functional recovery (71) in models of PNI.

The regenerative effect of uridine was also confirmed by a recent study involving metabolomic analyses in the blastema cells of a vertebrate (axolotl) with high regenerative capacity and in deer antlers, which is the only organ with total regenerative capacity in mammals (43). This study identified uridine as a critical metabolic effector in the regenerative activity seen across species, in addition to other functions such as reactivating the aging stem cells and reducing fibrosis and inflammation (43). The study further stated that oral uridine treatment for two months was associated with increased cognitive function and locomotor activity in aged mice (43). These findings are in line with our previous and current findings in neonatal HIE (12,22,41) and hyperoxia (23) models.

The limitations of our study are that it does not cover all epigenetic mechanisms since it focuses on what has been observed before in the literature, and that it was observed with a small number of subjects. Larger experimental groups and broader enzyme protein analyzes will make a significant contribution in this sense.

CONCLUSION

This is the first study to demonstrate the mechanism underlying the neuroprotective effect of uridine in PNI. Using a rat model of sciatic nerve injury, we observed that uridine functions by modulating the epigenetic pathways associated with pathophysiological consequences of nerve damage to enhance regeneration. Our data provides novel insights into the management of PNI by confirming and extending the effects of uridine.

Declarations

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Availability of data and materials: The datasets generated and/or analyzed during the current study are available from the corresponding author by reasonable request.

Disclosure: The authors declare that they have no conflict of interest.

AUTHORSHIP CONTRIBUTION

Study conception and design: AB, MC

Data collection: AIO, CK, HU

Analysis and interpretation of results: MC, IMK, AB

Draft manuscript preparation: AIO, MC, IMK

Critical revision of the article: AB, MC

Other (study supervision, fundings, materials, etc...): CK, IMK

All authors (AIO, CK, HU, MC, IMK, AB) reviewed the results and approved the final version of the manuscript.

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