Article

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Therapeutic Efficacy of Digoxin in Oxaliplatin and Chronic Constriction Injury Model of Neuropathic Pain in Rats

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Background: Neuropathic pain poses a considerable challenge in clinical practice, affecting a significant portion of the global population. Existing treatments often provide only symptomatic relief and are associated with limited efficacy and adverse effects, highlighting the need for novel therapeutic strategies.

Methods: This study investigates the repurposing potential of digoxin for neuropathic pain management. Digoxin works as a soluble epoxide hydrolase (sEH) enzyme inhibitor, traditionally given for cardiac conditions. The neuropathic pain management (anti-nociceptive and anti-inflammatory role) of digoxin at 0.1 & 0.2 mg/kg was studied in a rat model having chronic constriction injury (CCI) and oxaliplatin-induced neuropathy. The reduction in pain was assessed through multiple behavioural assays like the acetone drop test, hot plate test, and pin-prick test. The mitochondrial functions, oxidative stress, & inflammation condition were estimated by biochemical analyses & gene expression studies of relevant markers. The histopathological examination depicts the morphology of tissue damage.

Results: Digoxin administration attenuated neuropathic pain behaviour and reduced neuroinflammation in both CCI and oxaliplatin-induced models. Additionally, digoxin treatment significantly improved mitochondrial function and decreased oxidative stress levels in rat models. Histopathological analysis revealed a reduction in axonal damage in the sciatic nerve. Gene expression analysis indicates downregulation of pro-inflammatory markers such as tumor necrosis factor-α (TNF-α) and nuclear factor-kappa B (NF-κB).

Conclusion: This study concludes that digoxin acts as a sEH inhibitor and holds promise for repurposing for neuropathic pain management.

Keywords: digoxin; neuropathic pain; inflammation; chronic constriction injury; oxaliplatin; repurposing; oxidative stress

Introduction

Neuropathic pain arises from nerve injuries &/or various pathophysiological events that result in persistent pain within nerves, often accompanied by sensory deficits in the somatosensory nervous system [1]. Chronic neuropathic pain affects 3–17% of the general population exhibiting neuropathic characteristics [2]. Various neuropathies stem from conditions such as autoimmune diseases, hereditary neuropathies, inflammatory disorders, human immunodeficiency virus infection, metabolic disorders (e.g., peripheral diabetic neuropathy), stroke, and peripheral nerve damage [3–5]. Clinical manifestations of neuropathic pain encompass hyperalgesia (increase pain perception from a noxious stimulus), causalgia (severe burning pain), paraesthesia (sensations like needle pricks, itching, and loss of sensitivity), and allodynia (pain from non-painful stimuli) [6,7]. Neuropathic pain poses significant challenges to physical well-being and mood, causing anxiety, disrupting sleep patterns, and thereby compromising overall quality of life.

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The primary treatment approach for neuropathic pain involves the use of anticonvulsants (such as pregabalin, and gabapentin) and antidepressants (like duloxetine, and venlafaxine). A second-line options are sustained-release opioids and short-acting opioids like tramadol. Cannabinoids and a select few selective serotonin reuptake inhibitors (SSRIs) like paroxetine and escitalopram are considered as a 3rd and 4th line of treatments, respectively [8]. However, their effectiveness is limited only by providing symptomatic relief without a permanent cure. Furthermore, prolonged usage results in adverse reactions, like addiction potential, increased risk of cardiovascular disorders, and strokes [9–11].

In the quest to alleviate neuropathic pain with fewer side effects, the search for novel therapeutic options is imperative. Repurposing approved drugs presents a promising avenue for developing effective treatments with less time and cost consumption. Recent investigations identified several potential targets, like soluble epoxide hydrolase (sEH), CX3C-chemokine receptor 1, p38 mitogen-activated protein kinases, purinoceptor 4, and toll-like receptor 2, for managing neuropathic pain [12]. Notably, sEH enzyme inhibitors show potential role in the management of neuropathic pain. The sEH enzyme is physiologically widely present in various tissues like the liver, kidney, pancreas, smooth muscle, and lymphoid tissues and converts endogenous epoxides, particularly epoxyeicosatrienoic acids (EETs) to dihydroxyeicosatrienoic acid (DHETs) compounds. Epoxy-fatty acids (EPFAs) show a beneficial role in pain modulation, inflammation, and blood pressure regulation [13]. Inhibiting sEH elevates endogenous EPFAs levels, leading to pain reduction, inflammation alleviation, blood pressure reduction, and potential benefits in cardiovascular diseases and metabolic syndrome [14]. Our research has pinpointed digoxin as an sEH inhibitor with potential repurposing capabilities for pain and inflammation management in rodent models [15]. Digoxin is commonly prescribed by cardiologists in congestive heart failure and specific types of atrial fibrillation to reduce pain, and attract our interest to explore its therapeutic potential in pain management and neuroinflammation models of chronic constriction injury (CCI) and oxaliplatin-induced neuropathic pain. Our study evaluates its impact on various nociceptive pain behaviours, followed by assessments of cell viability, antioxidative activity, mitochondrial functions, and neuro-inflammatory markers.

Materials and Methods

Experimental Animals

We used male Wistar rats (age 7–8 weeks, 200–250 g) housed in polyacrylic cages with controlled environmental conditions, including a 12 h light/dark cycle, temperatures of 25 ± 2 °C, and relative humidity of 60–65%. Study animals procured from the Lala Lajpat Rai Veterinary and Animal Sciences institute, Hisar, Haryana, India (Registration No. 1669/GO/ReBiBt-S/Re-L/12/CPCSEA). Thirty animals were used for CCI model and twenty-four animals were used for the oxaliplatin-induced neuropathic pain model. The experimental work was approved by the Institutional Animal Ethical Committee (IAEC) of Banasthali Vidyapith, Rajasthan, India (BV//2018-19/3776) and in compliance with the Committee for Control and Supervision of Experiments on Animals (CCSEA). Animals in the trial had free access to water and food (dry pellets) during the study.

Reagents and Equipments

Griess reagent (cat. no. G4410), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, cat. no. H3375), oxaliplatin (cat. no. 61825-94-3), dichlorophenolindophenol (DCPIP, cat. no. D1878), collagenase (cat. no. C5138), nicotinamide adenine dinucleotide (NAD) + hydrogen (H) (NADH, cat. no. N8129), ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA, cat. no. E3889), potassium cyanide (KCN, cat. no. 31252), antimycin A (cat. no. 8674), decylubiquinone (cat. no. D7911), rotenone (cat. no. R8875), succinate (cat. no. S3674), cytochrome C (cat. no. C3131), gabapentin (cas no. 60142-96-3), sodium dodecyl sulfate (SDS, cas no. 151-21-3) lauryl maltoside (cat. no. L4641) were purchased from Sigma-Aldrich, (St. Louis, MO, USA). Nitroblue tetrazolium (NBT, cat. no. RM578), thiobarbituric acid (TBA, cat. no. RM1594), digoxin (cat. no. RM1886), trichloroacetic acid (TCA, cat. no. GRM6274), 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB, cat. no. GRM1677), adenosine triphosphate (ATP) (cat. no. RM439), mannitol (cat. no. 147510), tris medium (lot no. 0000164253), sodium nitroprusside (lot no. GRM986) and bovine serum albumin (BSA, cat. no. MB083) were procured from HI-Media (Mumbai, MH, India). Ethylenediaminetetraacetic acid (EDTA, cat. no. 28021), phosphate buffer (cat no. P3619), acetic acid (cat no. 64-19-7), Folin's Ciocalteu's phenol reagent (lot no. A17A50488), potassium dihydrogen phosphate (lot no. QJ3Q632155), phosphate buffer saline (PBS) (MFCD00131855) and sucrose (cat. no. 90701) were purchased from Merck KGaA (Darmstadt, Germany). Alkaline copper reagent (33001-L05) was purchased from s d fine-chem limited (SDFCL), Jodhpur, Rajasthan, India. Micro BSA protein assay kit (cat. no. 23235) was procured from Thermo Fisher Scientific (Waltham, MA, USA). Tumor necrosis factor-α (TNF-α, cat. no. MMC50C) and myeloperoxidase (cat. no. E33856) activity assay kits were procured from Invitrogen (Carlsbad, CA, USA). Prostaglandin E2 (PGE2) was purchased from BIOSS (Woburn, MA, USA). QUTUS hot plate, an Orchid Scientific I R actimeter (Model No. ACT-01, Nashik, Maharashtra, India), a Shimadzu UV spectrophotometer (Model No. UV-1900I, Rajinder Nagar, New Delhi, India), a biotech microplate reader, and a

Biorad RT-PCR machine (Model No. CFX96 optics module, Gurugram, Haryana, India) were utilized in the present study.

Chronic Constriction Injury (CCI) Induced Neuropathic Pain Model

The sciatic nerve is ligated to induce pain, followed by documented protocol [16]. Rats were anesthetized using intraperitoneal administration of ketamine (100 mg/kg, lot no. 2428M1801, Unijules Life Sciences Ltd., Kalmeshwar, Nagpur, India) and xylazine (10 mg/kg, lot no. FHK8006, Indian Immunological Ltd., Hyderabad, India) [17]. A small incision (approximately 1 cm) was made on the centre of the femur skin to expose the sciatic nerve. The nerve injury was induced by tying four ligatures with non-absorbable sutures proximal to trifurcation. The incision was closed with an absorbable suture & povidone-iodine liquid or powder (5% w/w, Win-Medicare Pvt. Ltd., Nehru place, New Delhi, India) was applied to avoid infection. Rats were randomly divided into five different groups (n = 6, in each group). Group I is negative control (sham group) administered only distilled water (5 mg/kg, orally). Group II is a positive control, where the sciatic nerve was ligated and received distilled water (5 mg/kg, orally). Group III is the standard group that received gabapentin (100 mg/kg, oral). Groups IV and V are test groups that received low dose (0.1 mg/kg) and high dose (0.2 mg/kg) of digoxin, respectively. All groups received their respective treatments daily for 24 days. Various behavioural models like the acetone drop test (cold allodynia), hot plate test (heat hyperalgesia), pin-prick test (mechanical hyperalgesia), infrared (IR) actimeter (locomotor activity), and walking track test (De Medinacelli method) examine the anti-nociceptive activity on days 1, 8, 16, and 24. Gabapentin (100 mg/kg) used as a standard drug in the experiment. Animals were sacrificed (ketamine 300 mg/kg along with xylazine 45 mg/kg; i.p. route) on day 25, and biochemical analysis (glutathione (GSH), nitrite, total protein, superoxide dismutase (SOD), thiobarbituric acid reactive substances (TBARS), myeloperoxidase, TNF-α, mitochondrial complexes), and histopathology was performed.

Oxaliplatin-Induced Neuropathic Pain Model

Neuropathic pain was induced by injecting oxaliplatin (dissolved in a 5% glucose (42738, Sisco Research Laboratories Pvt Ltd., Chakala, Maharashtra, India) solution at a concentration of 2 mg/mL and administered at 6 mg/kg) via the intraperitoneal route (i.p.), twice a week for three weeks. The rats were randomly divided into four groups (n = 6 in each group). Group I is a negative control group that received 5% glucose (orally), Group II is a positive control, Group III is the standard group that administered gabapentin (100 mg/kg, orally), and Group IV is the test group that received 0.2 mg/kg of digoxin. All groups received their respective treatment for twenty-one consecutive days. Behavioural studies were executed every week (for 3 weeks) post-drug administration and then sacrificed using high dose of anesthesia (ketamine 300 mg/kg along with xylazine 45 mg/kg; i.p. route) on day 22 [18]. Further, biochemical analysis (GSH, nitrite, total protein, SOD, TBARS, TNF-α, and PGE2), mitochondrial complexes and gene expression using real-time polymerase chain reaction (RT-PCR) were performed.

Behavioral Studies

Cold Allodynia

An acetone drop test was performed by restraining the animal for a few seconds. Then 0.1 mL of acetone was sprayed on the hind paw exterior without any contact with the skin. The responses against acetone were noted for 20 sec and recorded on a 4-point grading scale [19]. The 4-point grading scale describes 0: no response; 1: swift withdrawal, jerking of the paw; 2: extended duration of withdrawal or recurrent flicking; & 3: recurrent licking and tweaking of the paw. This experiment was repeated thrice at five-minute intervals, and a cumulative score of 80 seconds was computed on a 0–9 scale.

Heat Hyperalgesia

The study animals placed on a hot plate analgesiometer with a temperature presented at 55 $\pm$ 2 °C to study hyperalgesia activity. The responses like reaction latency, noting licking, sniffing, and jumping were observed. The experiment was enforced for the 30-second time limit to safeguard the sensitive tissues of the paws [20].

Mechanical Hyperalgesia

A gentle prick on the hind paw sole of each rat with an 18-gauge needle prompted a withdrawal response where the animal withdrew their paw. The pressure was gradually increased on the needle until the rat showed few signs of discomfort, like struggling or attempting to bite. The withdrawal responses were manually noted for 20 seconds for each study animal. This experiment was repeated four times at five-minute intervals [19].

Locomotor Activity

The locomotor activity was determined by the I.R Actimeter instrument having a 65 cm $\times$ 65 cm floor area [21]. The number of horizontal and vertical movements by each rat in each group was recorded for 20 minutes.

De Medinacelli Method

In this experiment, the study animal was trained to walk on a 7.5 cm wide and 65 cm long narrow path with one side closed and a dark end. Before starting the experiment, a white paper was placed on the track base, and the rat's hind paws were coloured with two different inks. During the experiment, each study animal's movement was traced by their paw prints and was manually counted and recorded in centimetres.

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Sciatic Functional Index (SFI)

The footprint measures print length (PL), toe spread (TS), the distance between the first and fifth toes, and intermediate toe spread (ITS), all taken from the third toe to the heel (iTS). A prefix 'N' and 'E' were added for non-operative (N) and experimental (E, with sciatic nerve injury). The non-operated foot print length (NPL), non-operated foot toe spread (NTS), and non-operated foot intermediary toe spread (NIT) of non-operative (normal foot) readings were compared with the experimentally injured foot experimental foot print length (EPL), experimental foot toe spread (ETS), and experimental foot intermediary toe spread (EIT) readings. Three components make up SFI, as determined by the following equations: Print Length Factor (PLF) = [(EPL)-(NPL)/NPL]; Toe Spread Factor (TSF) = [(ETS-NTS)/NTS]; and the Intermediary Toe Spread Fact = [(EIT-NIT)/NIT]. The SFI is the computed variance between the wounded and the normal paw using the following calculations [22].

$$SFI = -38.3\left[\frac{EPL - NPL}{NPL}\right] + 109.5\left[\frac{ETS - NTS}{NTS}\right] + 13.3\left[\frac{EIT - NIT}{NIT}\right] - 8.8$$

SFI suggests normal function and complete impairment of sciatic nerves by computing either zero or –100% respectively. The SFI was calculated on day 1, 8, 16 and 24 post-operation.

Biochemical Analysis

Post behavioural studies, all study animals euthanized by ketamine 300 mg/kg, i.p. and xylazine 45 mg/kg, i.p. [17]. The sciatic nerve was collected from all study groups and washed with 0.9% NaCl. The samples were homogenized with 0.1M phosphate buffer of pH 7.4 and centrifuge. The supernatant was used for further biochemical evaluations.

Superoxide Dismutase (SOD)

The SOD activity was assessed by following standard protocol with specific adjustments [23]. A 60 µL of sciatic nerve tissue lysate mixed with 2.7 mL of sodium carbonate buffer, which includes 0.1 mL of EDTA, 0.2 mL of hydroxylamine hydrochloride, and 2.5 mM of NBT. The absorbance reading was taken at λ 560 nm on a UV spectrophotometer.

Reduced Glutathione (GSH)

A 0.1 mL tissue homogenate was treated with 5% TCA for precipitation. The lysate solution was centrifuged and the supernatant was mixed with 2 mL of DTNB reagent (0.6 mM). The volume was made up of 4 mL using 0.2M phosphate buffer of pH 8.6. The absorbance reading was taken at λ 412 nm on UV spectrophotometer [24].

Thiobarbituric Acid Reactive Substance (TBARS)

The lipid peroxide analyzed by taking 0.2 mL tissue homogenate, 20% acetic acid, 8.1% SDS, and 0.8% TBA and make a final volume of 4 mL [25]. Heat the solution at 95°C for 60 minutes and place at room time (RT) for cooling. Add 5 mL of n-butanol/pyridine mixture and 1 mL of water in the cool solution, thorough mix and centrifuge at 4000 rpm for ten minutes. The absorbance was measured at λ 532 nm on UV spectrophotometer.

Nitrite Content

The radical scavenging activity of nitric oxide was assessed by the Griess-Iliosvoy reaction method. Tissue homogenate was combined with 1 mL of sodium nitroprusside (15 mM) dissolved in PBS, followed by incubation at room temperature (25 °C) for one hour. Subsequently, an equal volume of Griess reagent was added, and the mixture was further incubated in dark environment for thirty minutes. Absorbance readings were then recorded at λ 546 nm [26].

Protein Estimation

The total protein content was determined by mixing tissue homogenate (0.1 mL) with alkaline copper reagent (4.5 mL) and distilled water (0.9 mL), followed by a ten-minute incubation at room temperature. Subsequently, Folin's Ciocalteu's phenol reagent (0.5 mL) was added to the mixture, and the color change was observed after twenty minutes at 640 nm [26].

Myeloperoxidase (MPO)

MPO activity is elucidated as the one unit that alters the absorbance per min at pH 7.0. 1 µM peroxide (used as a substrate) alters the rate of reaction initially and later the optical density was documented at 460 nm [26].

Mitochondria Isolation and Mitochondrial Complex Estimation

The sciatic nerves were collected, dissected, and then digested with collagenase before being incubated for 15 minutes. The resulting tissue homogenate was mixed with 2 mL of a homogenization buffer containing HEPES (0.005 M), EGTA (0.001 M), sucrose (0.075 M), mannitol (0.225 M), and delipidated BSA (1 mg/mL). The tissue samples were homogenized for 2 minutes at 200 rpm using a Teflon glass homogenizer (ABG1709, Pitampura, New Delhi, India) and then centrifuged at 2673 rpm for 15 minutes. The supernatant obtained was centrifuged again at 8452 rpm for 15 minutes to isolate pellets, then resuspended in homogenizing buffer without BSA. Following another centrifugation at 8183 rpm, the resulting pellet was resuspended in 0.25 mL of homogenizing buffer without BSA [27]. The protein concentration of the resuspended mitochondria was determined using the Micro BSA protein assay kit. The activity of mitochondrial complexes I, II, and IV was assessed using 10–45 µg of mitochondrial protein.

Activity of Mitochondrial Complex I

Mitochondrial protein (0.025 mg) was suspended in 0.8 mL of water and incubated for 1–2 min at 37 °C. Further, 0.05 M tris medium (pH 8.0, 200 µL) was added and supplemented with 4 µM antimycin A (AA), 240 µM KCN, 0.8 mM NADH, and 5 mg/mL BSA. Decylubiquinone (50 M) was added to initiate the mitochondrial complex I activity, and the oxidative breakdown of NADH was observed for three minutes to determine the enzyme activity recorded as a decrease in optical density at 340 nm [28].

Activity of Mitochondrial Complex II

Complex II activity includes succinate as a donor and DCPIP as the electron acceptor. 25 µg of mitochondrial protein was suspended in 1 mL of buffer medium containing 0.01 M KH_2PO_4 and 0.002 M EDTA. Succinate (10 mM), rotenone (4 µM), and DCPIP (80 µM) were followed by the inclusion of 0.2 mM ATP, and DCPIP, incubated for 10 min at 30 °C. Further, decylubiquinone (80 µM) was added to initiate the reduction reaction and the absorbance for five minutes was noted at 600 nm [28].

Activity of Mitochondrial Complex IV

Mitochondrial protein (0.01 mg) was added to 1 mL of isosmotic COX medium (0.25 M sucrose, 10 mM KH_2PO_4, and 1 mg/mL BSA). Reduced cytochrome C (0.01 µM), was added along with lauryl maltoside (0.0025 M) to observe the oxidative breakdown of reduced cytochrome C, recorded for three minutes at the optical density at 550 nm [28].

Histopathological Studies

After sacrifice, the sciatic nerve samples were promptly preserved in formalin solution to facilitate tissue fixation. These tissues underwent a dehydration process using various concentrations of ethyl alcohol, followed by immersion in xylene. Subsequently, the tissues were embedded in paraffin to create blocks. Thin sections of tissue, measuring 0.005 mm in width, were then affixed to slides coated with a poly L-Lysine solution to ensure tissue adhesion. The sections were stained with haematoxylin and eosin (H & E) and examined under a light microscope at 20x magnification to observe any axonal damage [13].

Estimation of Inflammatory Markers (TNF-α and PGE2) in Sciatic Nerve Tissue

The sciatic nerve sample lysate was used for the quantification of TNF-α, PGE2, and corticosterone using enzyme-linked immunosorbent assay commercially available kits.

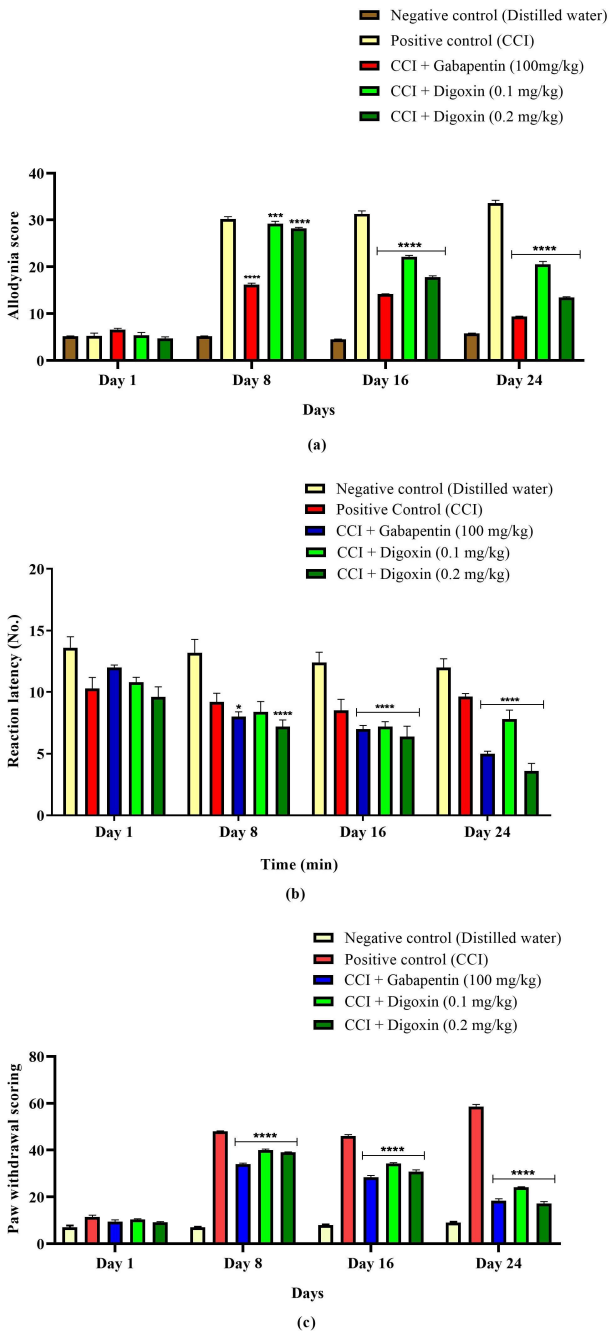

Fig. 1. Effect of digoxin on behavioural models of CCI-instigated neuropathic pain. (a) Consequence of digoxin on cold allodynia analysed by the acetone test. (b) Effects of digoxin on hyperalgesic activity evaluated by the hot plate test. (c) Effect of digoxin in mechanical hyperalgesia estimated by the pin-prick test. Statistics were mentioned as mean ± SD, n = 6 rats per group. *$p < 0.05$ represents when contrasted to the positive control group, ***$p < 0.001$ represents when contrasted to the positive control group, ****$p < 0.0001$ represents when contrasted to the positive control group. CCI, chronic constriction injury. SD, standard deviation.

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Table 1. List of primer and their respective annealing temperatures.

Gene	Primer sequence (5′ to 3′)	Annealing temperature
GAPDH	Forward: CCCACTCCTCCACCTTTGAC Reverse: CCACCACCCTGTTCCTGTAG	58.2 °C
IL-6	Forward: TCTATACCACTTCACAAGTCGGA Reverse: GAATTGCCATTGCACAACTCTTT	61.2 °C
sEH	Forward: AAGCCTGTGGAGCCAGTCTA Reverse: CCAGTTGTTGGTGACAATGC	58.2 °C
NF-κB	Forward: AAGTGATCCAGGCAGCCTTCC Reverse: TTCAGAGATAGCAGTGGGCCATC	62.0 °C

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *IL-6*, interleukin-6; *sEH*, soluble epoxide hydrolase; *NF-κB*, nuclear factor-kappa B.

Gene Expression Analysis by RT-PCR

Isolation of RNA, Synthesis of cDNA and Real-Time PCR Analysis

The RNA was isolated from a sciatic nerve tissue sample using trizol buffer and quantified by NanoDrop™. The RNA was reverse transcribed to cDNA using a verso cDNA synthesis kit following the manufacturer protocol. The RT-PCR was performed by using the SYBR Green PCR master mix (01193104, ThermoFisher Scientific, Waltham, MA, USA) in the Bio-Rad CFX96™ Real-Time PCR System (Bio-Rad, Hercules, CA, USA). PCR conditions included initial denaturation at 98 °C for 5 min, followed by 40 cycles with denaturation at 95 °C for 10s, annealing temperature (variable) at 5s and extension at 72 °C for 30 s. The list of primers and annealing temperatures is included in Table 1. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative amounts of each gene's expression, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal indicator to equalize the mRNA levels [29,30].

Statistical Analyses

Two-way analysis of variance was performed for behavioural (Tukey's multiple comparisons tests), biochemical assessment (Dunnett's multiple comparisons tests), and mitochondrial complexes (Tukey's multiple comparisons test) and represented as mean ± standard deviation (SD). For RT-PCR statistical analyses, an ordinary one-way analysis of variance (Dunnett's multiple comparisons test) was performed and represented as mean ± SD. Additionally, post hoc analysis and graphs were drawn using GraphPad Prism Version 8.0.2 software (Dotmatics, CA, USA).

Results

The anti-neuropathic activity of digoxin was studied on both CCI and oxaliplatin-induced neuropathic pain models. The CCI model study was conducted over 24 days, and the test group was treated with either low (0.1 mg/kg) or high doses (0.2 mg/kg) of digoxin. In contrast, the oxaliplatin-induced neuropathic pain model was conducted for 21 days, and only a high dose (0.2 mg/kg) of digoxin was administered in the test group. Initially, we perform

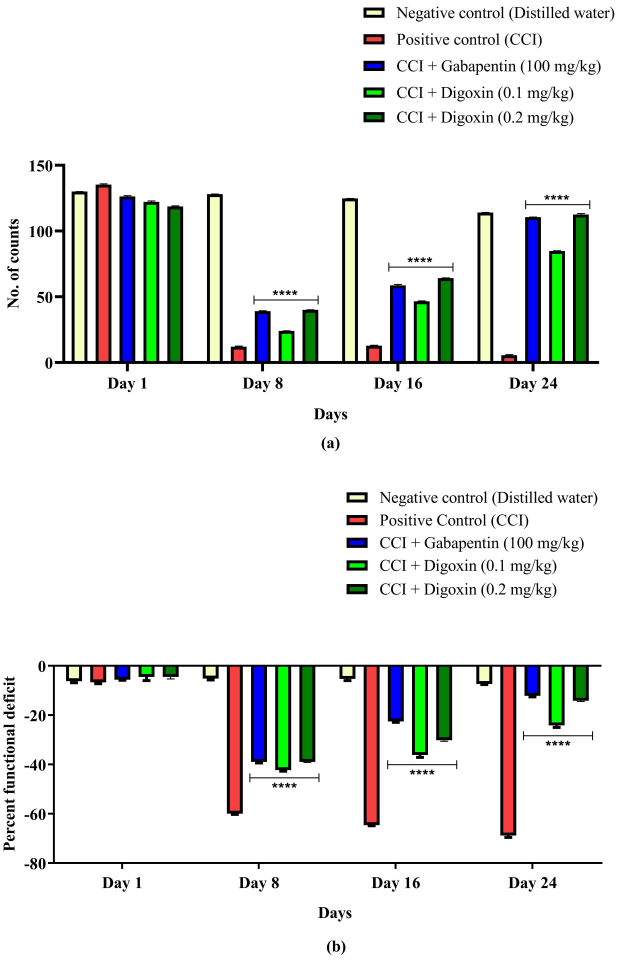

Fig. 2. Effect of digoxin in behavioural models of CCI-prompt neuropathic pain model. (a) Outcome of digoxin on locomotor activity evaluated by the IR Actimeter test. (b) Effects of digoxin on sciatic functional index analyzed by the walking track test. Statistics are mentioned as mean ± SD, n = 6 rats per group. ****$p < 0.0001$ represents when contrasted to the diseased animals (Positive control). CCI, chronic constriction injury; IR, infrared.

a CCI-induced neuropathic pain model that shows promising results with a 0.2 mg/kg dose of digoxin. Notably, the 0.1 mg/kg dosage also shows significant outcomes but

to a lesser extent than the 0.2 mg/kg dose group. Based on the CCI model study outcome, we selected only the high-dose group (0.2 mg/kg) for subsequent experiments subjected to resource optimization. The gabapentin (100 mg/kg) used in the standard group in both models. Behavioral assessments evaluate the pain-relieving effects of digoxin, while biochemical and cellular analyses, including cell viability assays and assessments of mitochondrial complexes, were performed to assess its impact on oxidative stress, mitochondrial function, and inflammatory responses. Histopathological evaluations provided insights into tissue damage and recovery across all study groups.

CCI-Induced Neuropathic Pain Model

Behavioral Studies

Cold Allodynia (Acetone Drop Test). In the CCI-induced neuropathic pain model, there was a notable surge in cold allodynia observed during the acetone drop test (Fig. 1a). However, oral administration of both digoxin and gabapentin led to a substantial reduction in CCI-induced allodynia ($p < 0.0001$). Furthermore, the higher dose of digoxin exhibited a significantly more favorable response compared to the lower dose on day 8 ($p < 0.0001$), 16 ($p < 0.0001$) and 24 ($p < 0.0001$).

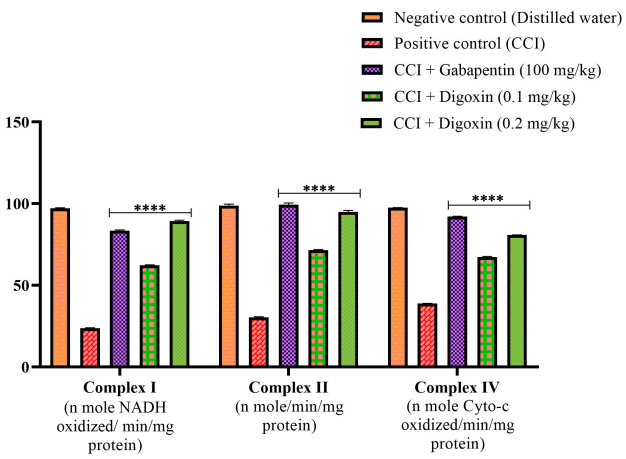

Fig. 3. Mitochondrial complexes assessed in CCI provoked neuropathic pain after the administration of digoxin. Values are mean ± SD, n = 6 rats per group. ****$p < 0.0001$ denotes when contrasted to the diseased animals (positive control). CCI, chronic constriction injury; NADH, nicotinamide adenine dinucleotide (NAD) + hydrogen (H); Cyto-c, cytochrome C.

Heat Hyperalgesia (Hot Plate Test). The hot plate test revealed that neuropathic pain induced by CCI coincided with the onset of heat hyperalgesia (Fig. 1b). Remarkably, oral administration of the digoxin at both low and high doses notably reduced reaction latencies and mitigated CCI-induced heat hyperalgesia on day 8 ($p < 0.0001$), 16 ($p < 0.0001$) and 24 ($p < 0.0001$).

Mechanical Hyperalgesia (Pin-Prick Test). The pin-prick test showed a marked development of mechanical hyperalgesia in CCI-induced neuropathic pain (Fig. 1c). Oral administration of digoxin and gabapentin significantly attenuated CCI-induced mechanical hyperalgesia on day 8 ($p < 0.0001$), 16 ($p < 0.0001$) and 24 ($p < 0.0001$).

Locomotor Activity (IR Actimeter). CCI-induced neuropathic pain showed a marked increase in locomotor disability (Fig. 2a) when equated to the control, evaluated by employing spontaneous locomotor activity in an IR Actimeter. Administration of digoxin and gabapentin orally diminished CCI-provoked locomotor disability and improved locomotor activity remarkably on day 8 ($p < 0.0001$), 16 ($p < 0.0001$) and 24 ($p < 0.0001$).

Walking Track Test. Neuropathic pain induced by CCI demonstrated a negative response in SFI, suggesting a functional deficit in the animals comparable to the negative control group, estimated by walking track tests. Treatment with digoxin and standard drug orally for twenty-four days mitigated the CCI-induced functional deficit substantially ($p < 0.0001$), as shown in Fig. 2b.

Biochemical Analysis

Effect on Antioxidant and Inflammatory Markers. The CCI of sciatic nerve revealed a significant decrease in SOD level (4.98 ± 0.23, $p < 0.0001$), reduced GSH (26.24 ± 0.2, $p < 0.0001$) level, and elevated levels of TBARS (11.2 ± 0.3, $p < 0.0001$), nitrite content (48.34 ± 0.6, $p < 0.0001$), total protein (5.2 ± 0.04, $p < 0.0001$), MPO (92 ± 0.34, $p < 0.0001$), and TNF-α (72 ± 0.76, $p < 0.0001$) in the positive control group as compared to a negative control group in rat sciatic nerve tissue. Administration of digoxin (*Per os*, P.O.) for 24 days elevated the level of CCI-induced decreased level of SOD ($p < 0.0001$), {F (4, 25) = 27.68}; GSH ($p < 0.0001$), {F (4, 25) = 56.41} in a significant manner and markedly reduced the levels of TBARS ($p < 0.0001$), {F (4, 25) = 56.8}; nitrite content ($p < 0.0001$), {F (4, 25) = 29.31}; total protein ($p < 0.0001$), {F (4, 25) = 10.40}; MPO ($p < 0.0001$), {F (4, 25) = 62.95} and TNF-α ($p < 0.0001$), {F (4, 25) = 56.59} as shown in Tables 2,3. Notably, a high dose of digoxin was found to be equally effective as the standard drug gabapentin in ameliorating the antioxidant enzyme level in rat sciatic nerve tissue.

Effect of Digoxin on Mitochondrial Complexes. A remarkable mitochondrial dysfunction in the mitochondrial complexes I, II, and IV activities (23.78 ± 0.2, 30.43 ± 0.3, and 38.83 ± 0.03, respectively, $p < 0.0001$) as compared to the normal control (97.02 ± 0.43, 98.71 ± 0.98, and 97.49 ± 0.08, respectively, $p < 0.0001$), was noted in CCI-induced neuropathic pain. Administration of digoxin and gabapentin *per os* improved mitochondrial enzyme complex I (62.23 ± 0.2, 89.24 ± 0.55, and 83.4 ± 0.47, respectively, $p < 0.0001$) complex II (71.56 ± 0.3, 94.91 ±

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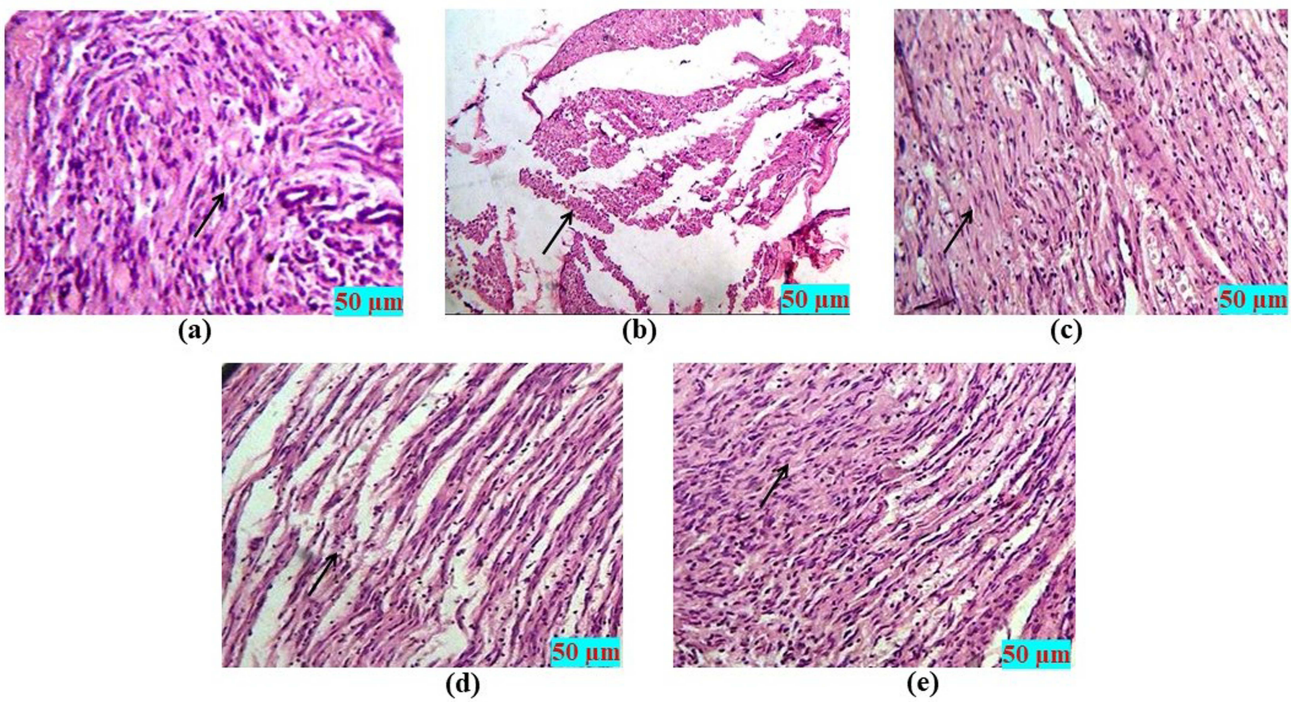

Fig. 4. CCI-induced histopathological changes showing the consequences of digoxin in the sciatic nerve in rats (hematoxylin and eosin stained, scale bar represents 50 µm). (a) Negative control (b) Positive control (c) CCI + Gabapentin (d) CCI + Digoxin (0.1 mg/kg) and (e) CCI + Digoxin (0.2 mg/kg). The black arrows show axonal swelling, fibre derangement and CCI-induced changes in Schwann and satellite cells. (c–e) shows the attenuation of CCI-induced fibre derangement and reversal of Schwann and the satellite cell changes. CCI, chronic constriction injury.

Table 2. Activity of digoxin assessed by antioxidant studies in sciatic nerve tissue samples in CCI-induced neuropathic pain.

Groups	GSH (µg/mg of protein)	Nitrite (µg/mL)	Total protein (mg/mL)	SOD (µg/mg of protein)	TBARS (nmol/mg of protein)
Negative control (distilled water)	55.23 ± 0.4	24.3 ± 0.65	3.7 ± 0.03	26.4 ± 0.62	4.32 ± 0.1
Positive control (CCI)	26.24 ± 0.2$^{\#\#\#\#}$	48.34 ± 0.6$^{\#\#\#\#}$	5.2 ± 0.04$^{\#\#\#\#}$	4.98 ± 0.23$^{\#\#\#\#}$	11.2 ± 0.3$^{\#\#\#\#}$
CCI + gabapentin (100 mg/kg)	52.3 ± 0.53****	26.29 ± 0.4****	4.6 ± 0.03*	26.87 ± 0.2****	5.2 ± 0.03****
CCI + digoxin (0.1 mg/kg)	42.5 ± 0.32****	38.9 ± 0.41****	5 ± 0.07	13 ± 0.64****	6.62 ± 0.5****
CCI + digoxin (0.2 mg/kg)	51.68 ± 0.4****	25.28 ± 0.2****	4.5 ± 0.03**	23 ± 0.32****	5.45 ± 0.2****

Statistics were mentioned as mean ± SD, n = 6 rats per group. *$p < 0.05$ represents when contrasted to the diseased animals. **$p < 0.01$ represents when contrasted to the diseased animals. ****$p < 0.0001$ represents when contrasted to the diseased animals (positive control). $^{\#\#\#\#}p < 0.0001$ represents when compared to the negative control group. GSH, glutathione; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; CCI, chronic constriction injury.

Table 3. Outcome of digoxin on MPO and TNF-α level in different groups of CCI provoked neuropathic pain in rats.

Groups	TNF-α level (pg/mg of protein)	MPO level (U/min/mg of protein)
Negative control (distilled water)	12.03 ± 0.87	11.12 ± 0.2
Positive control (CCI)	72 ± 0.76$^{\#\#\#\#}$	92 ± 0.34$^{\#\#\#\#}$
CCI + gabapentin (100 mg/kg)	14.23 ± 0.65***	12.43 ± 0.3****
CCI + digoxin (0.1 mg/kg)	26.98 ± 0.98****	38.4 ± 0.54****
CCI + digoxin (0.2 mg/kg)	16.67 ± 0.78****	15 ± 0.34****

Statistics were mentioned as mean ± SD, n = 6 rats per group. ***$p < 0.001$, ****$p < 0.0001$ represents when contrasted to the diseased animals (positive control). $^{\#\#\#\#}p < 0.0001$ represents when compared to the negative control group. TNF-α, tumor necrosis factor-α; MPO, myeloperoxidase; CCI, chronic constriction injury.

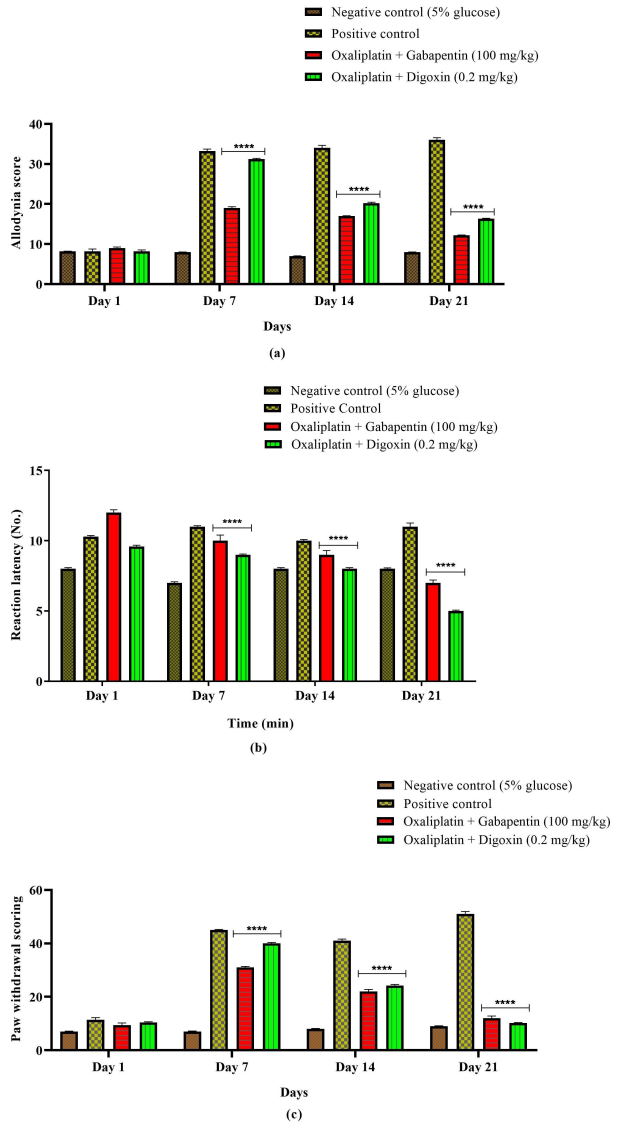

Fig. 5. Effect of digoxin in behavioural models of oxaliplatin-induced neuropathic pain model. (a) Activity of digoxin on oxaliplatin-induced neuropathic pain assessed by the acetone test to analyse cold allodynia. (b) Activity of digoxin on oxaliplatin-induced neuropathic pain assessed by the hot plate test to analyse heat hyperalgesia. (c) Activity of digoxin on oxaliplatin-induced neuropathic pain measured by the pin-prick test to analyse mechanical hyperalgesia. Statistics are mentioned as mean ± SD, n = 6 rats per group. ****$p < 0.0001$ denotes when contrasted to the diseased animals (positive control).

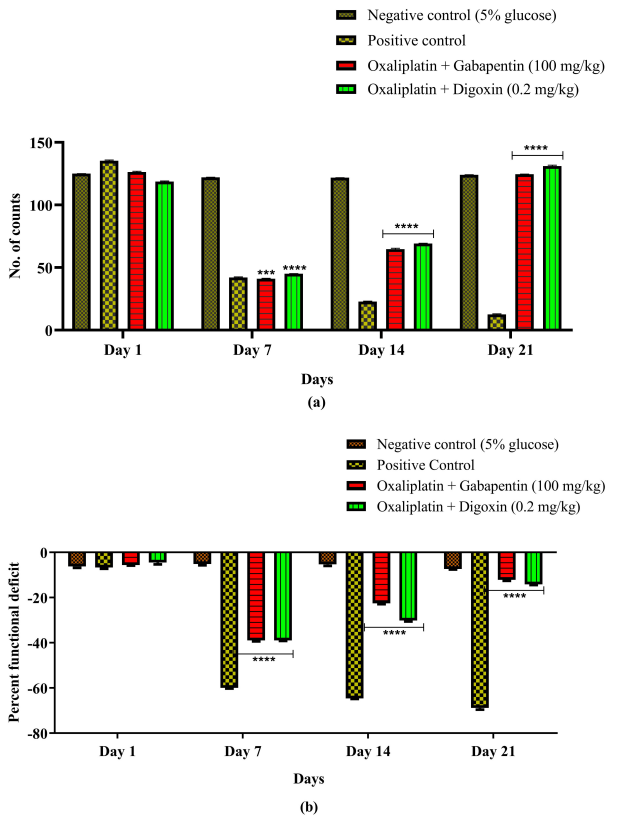

Fig. 6. Effect of digoxin in behavioural models of oxaliplatin-induced neuropathic pain model. (a) Activity of digoxin on oxaliplatin-induced neuropathic pain assessed by IR actimeter to analyze the locomotor activity. (b) Activity of digoxin on oxaliplatin-induced neuropathic pain assessed by walking track test to analyse the functional deficit calculated from Sciatic Functional Index (SFI). Statistics are mentioned as mean ± SD, n = 6 rats per group. ***$p < 0.001$, ****$p < 0.0001$ denotes when contrasted to the diseased animals (positive control).

0.94, and 99.35 ± 0.99, correspondingly, $p < 0.0001$) and complex IV (67.32 ± 0.23, 80.78 ± 0.05, and 92.08 ± 0.09, in that order, $p < 0.0001$) alterations in CCI-induced neuropathic pain in rats (Fig. 3).

Histopathological Analysis

The cross-sectional view of the sciatic nerve demonstrates an increase in the number of Schwann and satellite cells along with axonal swelling. Treatment of digoxin (high dose) and gabapentin via the oral route significantly weakened CCI-induced fibre derangement, decreased swelling, and marked healing (Fig. 4).

Oxaliplatin-Induced Neuropathic Pain Model

Behavioral Studies

Cold Allodynia (Acetone Drop Test). The neuropathic pain induced in rats after oxaliplatin administration demonstrates the development of cold allodynia in the acetone drop test (Fig. 5a) compared with the control group. Post-operative treatment of digoxin at higher doses and gabapentin attenuated oxaliplatin-induced allodynia in a substantial manner on day 7 ($p < 0.0001$), 14 ($p < 0.0001$) and 21 ($p < 0.0001$).

Heat Hyperalgesia (Hot Plate Test). Oxaliplatin-stimulated neuropathic pain triggers a noticeable development of heat-induced hyperalgesia (Fig. 5b) compared to a negative control group that was evaluated by the hot

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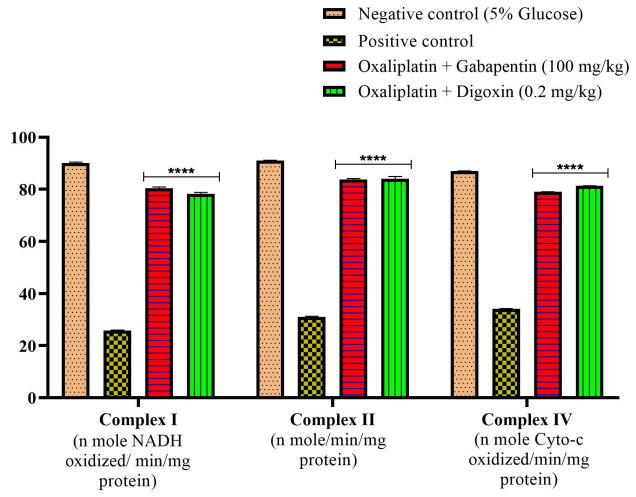

Fig. 7. Outcome of digoxin on mitochondrial complexes considered in oxaliplatin-induced neuropathic pain. Statistics were mentioned as mean ± SD, n = 6 rats per group. ****$p <$ 0.0001 denotes when contrasted to the diseased animals (positive control). NADH, nicotinamide adenine dinucleotide (NAD) + hydrogen (H); Cyto-c, cytochrome C.

plate test. Oral administration of digoxin and gabapentin diminished oxaliplatin-induced heat hyperalgesia by reducing reaction latencies in a significant way on day 7 ($p < 0.0001$), 14 ($p < 0.0001$) and 21 ($p < 0.0001$).

Mechanical Hyperalgesia (Pin-Prick Test). Oxaliplatin-generated neuropathic pain revealed a noticeable development of mechanical hyperalgesia (Fig. 5c) compared to the untreated group that was evaluated by a pin-prick test. Oral administration of digoxin and gabapentin weakened oxaliplatin-induced mechanical hyperalgesia in a substantial manner on day 7 ($p < 0.0001$), 14 ($p < 0.0001$) and 21 ($p < 0.0001$).

Locomotor Activity (IR Actimeter). The neuropathic pain triggered by oxaliplatin resulted in a notable decrease in locomotor activity, as evidenced by reduced line crossings (Fig. 6a) in divergence to the negative control group, as evaluated by spontaneous locomotor activity in the IR Actimeter. Treatment with digoxin and gabapentin orally attenuated oxaliplatin-induced locomotor disability and improved locomotor activity, as evidenced by increased line crossings in a noteworthy way on 7th ($p < 0.0001$), 14th ($p < 0.0001$) and 21st ($p < 0.0001$) day.

Walking Track Test. In oxaliplatin-induced neuropathic pain, there was a notable increase in functional deficit, as indicated by the SFI when compared to healthy animals, as assessed by the walking track test. Oral administration of digoxin and gabapentin significantly attenuated the oxaliplatin-induced functional deficit on 7th ($p < 0.0001$), 14th ($p < 0.0001$), and 21st ($p < 0.0001$) day, as depicted in Fig. 6b.

Biochemical Analysis

Induction of neuropathic pain by oxaliplatin resulted in a significant decrease of SOD (17 ± 0.13, $p < 0.0001$), reduced GSH (12 ± 0.15, $p < 0.0001$) level, and elevated levels of TBARS (38.2 ± 0.31, $p < 0.0001$), nitrite content (42.2 ± 0.51, $p < 0.0001$), and total protein (22 ± 0.16, $p < 0.0001$) in the sciatic nerve tissue of diseased animals compared to the untreated animals. Oral administration of digoxin for 21 days elevated the level of oxaliplatin-induced decreased levels of SOD (44 ± 0.72, $p < 0.0001$), {F (3, 20) = 28.68} and GSH (41.4 ± 0.5, $p < 0.0001$), {F (3, 20) = 79.88} in a significant manner and markedly reduced the levels of TBARS (18 ± 0.2, $p < 0.0001$), {F (3, 20) = 61.74} nitrite content (17.2 ± 0.43, $p < 0.0001$), F (3, 20) = {58.33} and total protein (41.2 ± 0.47, $p < 0.0001$), {F (3, 20) = 83.07} as indicated in Table 4. Markedly, gabapentin showed a similar effect to digoxin in ameliorating the antioxidant enzyme level in rat sciatic nerve tissue.

Estimation of TNF-α and PGE2 in Sciatic Nerve Tissue. Oxaliplatin induced neuropathic pain in rats demonstrated an increased level of pro-inflammatory mediators TNF-α (75.09 ± 0.07, $p < 0.0001$) and PGE2 (79.61 ± 0.03, $p <$ 0.0001) in contrast to the untreated group, 14.63 ± 0.02, and 19.98 ± 0.08, respectively. Oral administration of high doses of digoxin and gabapentin for 21 days resulted in a notable drop in the levels of PGE2 {F (3, 20) = 77.41} and TNF-α {F (3, 20) = 116.44}, ($p < 0.0001$) (Table 5).

Mitochondria Isolation and Mitochondrial Complex Estimation (I, II, and IV). Oxaliplatin-induced neuropathic pain in rats caused significant dysfunction in mitochondrial enzyme complexes and markedly attenuated mitochondrial complexes I, II, and IV (25.78 ± 0.2, 31 ± 0.3, and 34.2 ± 0.03, correspondingly, $p < 0.0001$) as compared to the healthy rats (90.02 ± 0.43, 91 ± 0.18, and 87 ± 0.08, correspondingly, $p < 0.0001$). Treatment of digoxin and gabapentin orally for 21 days improved mitochondrial enzyme complex I (80.4 ± 0.47 and 78.24 ± 0.55, respectively, $p < 0.0001$), complex II (83.8 ± 0.29 and 84 ± 0.94, correspondingly, $p < 0.0001$) and complex IV (79 ± 0.09 and 81.29 ± 0.05, respectively, $p < 0.0001$) alterations in oxaliplatin-induced neuropathic pain in rats (Fig. 7).

Gene Expression Analysis by RT-PCR

Through an extensive literature review, we identified key genes implicated in the inflammatory response during neuropathic pain. We validated our findings through gene expression analysis, calculating the relative fold change of the selected genes. Additionally, we confirmed sEH inhibition by digoxin, showing a significant reduction ($p <$ 0.0001) in the digoxin-treated groups compared to those treated with oxaliplatin in the neuropathic pain model (Fig. 8). Additionally, the expression of inflammatory marker genes interleukin-6 (*IL-6*) ($p < 0.0001$) and nuclear

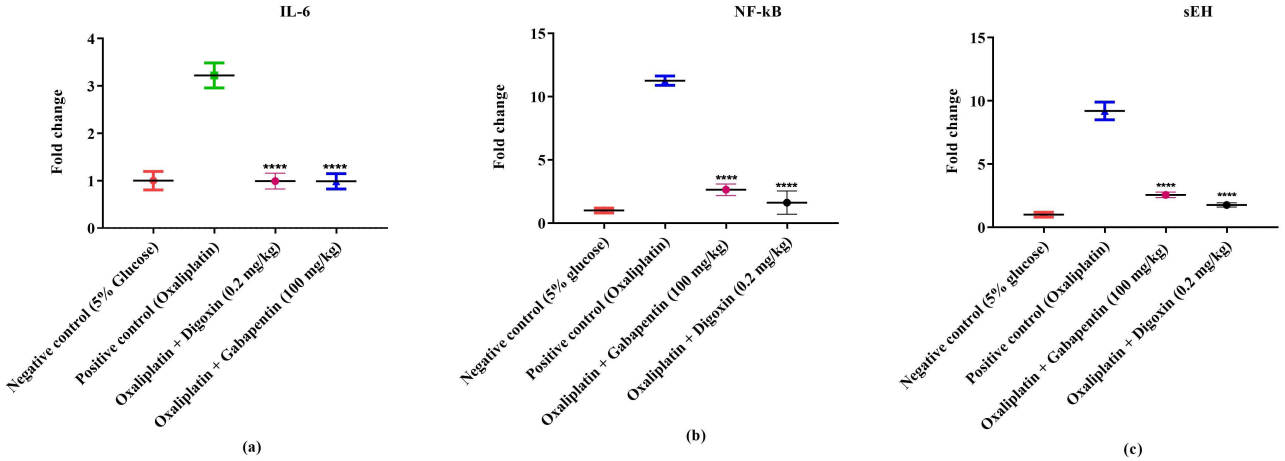

Fig. 8. RT-PCR analysis of genes involved in sciatic nerve tissue of oxaliplatin-induced neuropathic pain model. (a) *IL-6* (b) *NF-κB* (c) *sEH*. Statistics were mentioned as mean ± SD, n = 6 rats per group. ****$p < 0.0001$ denotes when contrasted to the diseased animals (positive control). *IL-6*, interleukin-6; *sEH*, soluble epoxide hydrolase; RT-PCR, real-time polymerase chain reaction.

Table 4. Effect of digoxin in sciatic nerve tissue antioxidants biomarkers in oxaliplatin-induced neuropathic pain model.

Groups	GSH (μg/mg of protein)	Nitrite (μg/mL)	Total protein (mg/mL)	SOD (μg/mg of protein)	TBARS (nmol/mg of protein)
Negative control (5% glucose)	32 ± 0.6	16 ± 0.4	44 ± 0.1	32 ± 0.2	17 ± 0.3
Positive control (oxaliplatin)	12 ± 0.1$^{\#\#\#\#}$	42.2 ± 0.5$^{\#\#\#\#}$	22 ± 0.1$^{\#\#\#\#}$	17 ± 0.1$^{\#\#\#\#}$	38.2 ± 0.3$^{\#\#\#\#}$
Oxaliplatin + gabapentin (100 mg/kg)	39 ± 0.3****	17.4 ± 0.2****	44 ± 0.2****	37 ± 0.4****	19.3 ± 0.5****
Oxaliplatin + digoxin (0.2 mg/kg)	41.4 ± 0.5****	17.2 ± 0.4****	41.2 ± 0.5****	44 ± 0.7****	18 ± 0.2****

Statistics were mentioned as mean ± SD, n = 6 rats per group. ****$p < 0.0001$ represents when contrasted to the diseased animals (positive control). $^{\#\#\#\#}p < 0.0001$ represents when compared to the negative control group. GSH, glutathione; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.

Table 5. Analysis of digoxin on pro-inflammatory mediators in oxaliplatin-induced neuropathic pain model.

Groups	TNF-α (ng/mL)	PGE2 (ng/mL)
Negative control (5% glucose)	14.63 ± 0.02	19.98 ± 0.08
Positive control (oxaliplatin)	75.09 ± 0.07$^{\#\#\#\#}$	79.61 ± 0.03$^{\#\#\#\#}$
Oxaliplatin + gabapentin (100 mg/kg)	19.8 ± 0.07****	24.67 ± 0.1****
Oxaliplatin + digoxin (0.2 mg/kg)	21.46 ± 0.08****	22.83 ± 0.09****

Statistics were mentioned as mean ± SD, n = 6 rats per group. ****$p < 0.0001$ represents when contrasted to the diseased animals (positive control). $^{\#\#\#\#}p < 0.0001$ represents when compared to the negative control group. PGE2, prostaglandin E2.

factor-kappa B (*NF-κB*) ($p < 0.0001$) was also significantly decreased in the digoxin-treated group, which showed the anti-neuropathic pain effect of digoxin.

Discussion

Neuropathic pain poses a significant challenge in clinical management due to its debilitating nature and the limited effectiveness of existing drugs, which offer only symptomatic relief and may cause adverse effects. The complexity of neuropathic pain stems from an insufficient understanding of its underlying mechanisms. There is an urgent need to discover novel therapeutic agents that can offer improved outcomes without the risk of adverse effects and drug interactions exacerbating the condition. This study contributes evidence supporting the therapeutic potential of digoxin in alleviating neuropathic pain induced by both CCI and oxaliplatin in rats. Behavioral studies were conducted on the 8th, 16th, and 24th days for the CCI-induced neuropathic pain model and the 7th, 14th, and 21st days for the oxaliplatin-induced neuropathic pain model. Major behavioural changes were noticed in the second and third weeks of study in both models, aligning with established laboratory findings. The variations in nociceptive threshold induced by sciatic nerve ligation and oxaliplatin were consistent with previously published reports [31]. Neuropathic responses, including cold allodynia, heat hyperalgesia, mechanical hyperalgesia, locomotor activity, and sensory re-

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sponses of the sciatic nerve assessed through various behavioural assays. The experimental findings demonstrated that both low and high doses of digoxin significantly alleviated neuropathic pain and functional deficits in both models during behavioural and functional assessments.

Neuronal inflammation and oxidative stress are a common underlying feature of neuropathic pain resulting from nerve damage and nociception. Recent study has demonstrated that inhibiting the sEH enzyme prevents the conversion of EETs to DHETs, thereby modulating multiple pathways involved in the pro-inflammatory cytokine production and reducing neuronal inflammation [32]. Previous research from our group highlighted the repurposing potential of digoxin as an sEH inhibitor, leading to increased levels of EETs and effective reduction of pain and inflammation [15]. In recent investigations, pretreatment with EETs (100 nM) exhibited protective effects against arsenic trioxide (ATO, 10 μM for 2 h)-induced ROS formation, mitochondrial dysfunction, and apoptosis in Tca-8113 cancer cells. Additionally, EET pretreatment in Tca-8113 cancer cells mitigated ROS generation, caspase activation (caspase-3 and caspase-9), and improved mitochondrial function, ultimately reducing apoptosis in carcinoma cells [33]. Other studies unveiled the cardioprotective properties of sEH inhibitors by reducing ROS formation and enhancing mitochondrial function in sEH-/- mice, H9c2 cells, and diabetic nephropathy [34,35]. However, the efficacy of digoxin in attenuating neuropathic pain models has not been investigated to date.

Chronic neuropathic pain is often accompanied by oxidative stress and dysfunctional mitochondrial complexes [36]. Recent studies have unveiled novel pathways involving mitochondrial regulation in inflammatory signaling and oxidative stress markers [37,38]. In rats with CCI-induced neuropathic pain, treatment with both low (0.1 mg/kg) and high doses (0.2 mg/kg) of digoxin notably reduced levels of MDA, indicating a decrease in oxidative stress. Additionally, in both CCI and oxaliplatin-induced neuropathic pain models, the activity of antioxidant enzymes such as SOD, GSH, and catalase was significantly suppressed, as observed in histological analysis of sciatic nerve sections. These findings align with preclinical study of sEH inhibitors in Drosophila melanogaster, demonstrating efficacy in alleviating inflammation and oxidative stress in Parkinson's disease [39]. MPO, a heme-containing protein released by leukocytes, catalyzes the formation of reactive oxygen species and serves as an indicator of neutrophil accumulation. Study have shown that deficiency of the sEH gene or its inhibition attenuated MPO-labelled neutrophils in the IL-10 (-/-) mouse model of inflammatory bowel disease. Furthermore, the EETs/DHET ratio was increased while TNF-α, interferon-Υ, and NF-κB levels were decreased in the colonic mucosa of IL-10 (-/-) mice [40]. Similarly, the well-known sEH inhibitor AUDA attenuated pulmonary MPO activities and TNF-α levels through NF-κB inhibition in lipopolysaccharide-induced acute lung injury in BALB/c mice [41]. Collectively, these findings suggest that digoxin holds potential to treat inflammation by suppressing oxidative stress and inflammatory cell infiltration through elevation of EETs levels.

Our research uncovered digoxin's protective impact on mitochondrial complexes & attributed it to increased levels of EETs. These compounds help prevent caspase activation and endoplasmic reticulum stress, mitigate ROS generation, and regulate mitochondrial functions to maintain optimal respiratory complexes. In the context of CCI-induced neuropathic pain, our findings indicated elevated levels of TBARS, nitrite content, and MPO, alongside decreased levels of SOD, GSH, and TNF-α, signifying heightened inflammation and oxidative stress, consistent with prior study [42]. Recent evidence suggests that mitochondrial dysfunction contributes to diminished mitochondrial complexes and post-translational modifications, potentially leading to increased lipid peroxidation (malondialdehyde, MDA), culminating in chronic neuropathic pain, cardiovascular issues, and neurodegenerative disorders [27,43]. Our study proposes that digoxin influences the levels of antioxidant enzymes by augmenting their concentration in sciatic nerves while reducing the levels of enzymes associated with oxidative stress in these nerves. Moreover, it significantly enhanced mitochondrial complex activities (complex I, II, and IV) and markedly reduced cell apoptosis based on haematological investigations. The observed increase in cell viability may be attributed to sEH inhibition, regulated by the epoxide hydrolase 2 (*EPHX2*) gene. These results align with previous findings indicating that silencing the sEH enzyme (EPHX2) protected against H_2O_2-induced oxidative damage in intestinal epithelial cells. It further demonstrated the anti-apoptotic effects of EPHX2 inhibition by mitigating ROS, maintaining mitochondrial membrane potential, reducing apoptosis in IEC-6 cells, and enhancing cell viability, as demonstrated in separate studies [44,45]. Additionally, another study suggested that digoxin exhibits nerve healing and neuroprotective effects by its anti-inflammatory action on IL-17, making it a potential adjunctive therapy for peripheral nerve injury [46].

Additionally, significant histopathological improvements and alterations in gene expressions were observed following the administration of digoxin at varying doses for 24 days in both CCI-induced and oxaliplatin-induced rats. Notably, digoxin exhibited notable neuronal healing abilities akin to gabapentin. Our study provides a piece of compelling evidence supporting digoxin's effectiveness as an sEH inhibitor, supported by the reduction in *sEH* gene expression in neuronal tissues, with *GAPDH* serving as the housekeeping gene for control. Furthermore, digoxin demonstrated efficacy in reducing neuronal inflammation, as evidenced by decreased expression levels of pro-inflammatory mediators such as IL-6 and NF-κB in neuropathic pain models. These findings align with previous study where the efficacy of neuropathic drugs has been validated through reductions in inflammatory mark-

ers [40]. In conclusion, our research suggests that digoxin holds promise as a potential alternative to existing drugs, offering fewer side effects and comparable efficacy in managing pain, oxidative stress, restoration of cell viability, improved mitochondrial function, and reduction in key pro-inflammatory cytokines, thereby alleviating neuronal inflammation in neuropathic pain models.

Conclusion

Digoxin administration notably attenuates nociceptive responses in neuropathic pain in CCI and oxaliplatin neuropathic pain models. The study suggests that the attenuation of the sEH enzyme by digoxin plausibly enhanced the levels of EETs that facilitated a reduction in oxidative stress and neuronal inflammation. We observed a remarkable increase in mitochondrial complex activities and a profound decrease in neuronal inflammatory markers post-digoxin treatment. Compared to traditional neuropathic pain medications, digoxin's repurposing as sEH inhibitor introduces a novel mechanism of action. The study demonstrates that digoxin administration attenuates neuropathic pain behaviours and mitigates neuroinflammation, suggesting potential dual benefits in addressing pain perception and inflammatory responses. Additionally, digoxin shows promising outcomes in improving mitochondrial function, which poses an advantageous role in neuropathic pain pathophysiology. Furthermore, the reduction in oxidative stress markers and amelioration of axonal damage in the sciatic nerve highlights the digoxin's potential neuroprotective effects that distinguish it from current clinical drugs.

In contrast to current clinical drugs for neuropathic pain, digoxin presents safety concerns due to its traditional usage in cardiac conditions and associated narrow therapeutic windows. This necessitates careful monitoring to avoid toxicity, particularly with higher doses. Moreover, while encouraging result in animal models, digoxin lacks robust clinical evidence in neuropathic pain management, unlike established medications. Its potential side effects, including gastrointestinal disturbances, cardiac arrhythmias, and visual disturbances, may limit its tolerability and distinguish it from a few existing drugs. Additionally, digoxin's drug interactions, cost, and accessibility issues may pose challenges compared to the readily available neuropathic pain treatments. However, this study provides strong evidence supporting the therapeutic efficacy of digoxin in neuropathic pain attenuation. Thus, pre-clinical evaluations performed in this study encourage the repositioning of digoxin as a promising candidate for the restoration of neuronal functions in neuropathic pain. However, it is pertinent to exhaustively explore the effect of digoxin at molecular levels and investigate its therapeutic potential in clinical scenarios.

Availability of Data and Materials

The authors confirm that the data supporting the findings of this study are available within the article.

Author Contributions

SPat: investigation & writing-original draft. RG: data curation. SJ: preparation of figures. VP: Data analysis and statistical analysis. SwP: RT-PCR and mitochondrial studies. SPal: conceptualization. JD: conceptualization, project administration, editing and reviewing. AS: interpretation of data. SS: conceptualization & supervision. HJL: conceptualization, final approval of the version to be published and supervision. All authors contributed to important editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The experimental work was carried out in accordance with the recommendations of the Committee for Control and Supervision of Experiments on Animals (CCSEA). Ethical approval was granted by the Institutional Animal Ethical Committee (IAEC) of Banasthali Vidyapith, Rajasthan, India (BV//2018-19/3776).

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Conflict of Interest

The authors declare no conflict of interest.

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