THYROXINE: A PUTATIVE NEUROPROTECTANT IN CHEMOTHERAPY INDUCED PERIPHERAL NEUROPATHY IN RATS

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ABSTRACT

Objectives: *Anti-neoplastic drugs like Cisplatin, Taxols are associated with the development of peripheral neuropathy (PN). Severe neuropathy can occur in 3% to 7% of treated cases with single agents but can increase to 38% with combined regimens. The treatment options for PN currently include anti-depressants, anti-convulsants and opioid analgesics. These agents are modestly effective for symptomatic relief, but they do not affect the underlying pathology nor do they slow progression of the disease. Therefore, effective treatment for chemotherapy induced neuropathy would be a major advantage for cancer patients. It is well established thyroid hormones lays an important role in regulating the development and regeneration of the nervous system & local administration of triiodothyronine (T3) at the level of transected rat sciatic nerve increased the number and diameter of regenerated axons and SCG10 protein levels about two-fold in the different segments of transected nerve during the regeneration period.SCG10 protein is a regulator of microtubule dynamics in growth cones. The main objective of the study was to evaluate the neuroprotective activity of thyroxine in Cisplatin-induced PN in rats.*

Methods: *PN was induced by Cisplatin - 2mg/kg,i.p. twice weekly for 8 weeks. The degree of protection was determined by measuring electrophysiological properties of sciatic nerve like nerve conduction velocity, motor in-coordination, thermal &cold hyperalgesia, grip strength and histopathological studies.*

Results: *PN was evidenced in Cisplatin control rats and ameliorated with administration of T4 (0.1 mg/kg, s.c.) for 4weeks by augmenting all the above parameters.*

Conclusions: *T4 exhibited neuroprotective activity, which would be attributed to its activity as neurotrophic effect.*

Keywords: *Cisplatin, Neuroprotectant, Peripheral Neuropathy, Thyroxine*

INTRODUCTION

Neuropathic pain is thought to result when sensory neurons generate impulses at abnormal (ectopic) locations, for example at sites of nerve injury or demyelination. In the PNS (Peripheral Nervous System), in addition to firing spontaneously, these ectopic pacemaker sites are often excited by mechanical forces applied to them during movement. The result is spontaneous and movement-evoked pain. Damage to the CNS, such as in stroke or trauma, may cause ectopic firing of central origin or render brain circuits hyper-excitable. The ectopic afferent firing is a primary source of spontaneous pain; it initiates and sustains central sensitization that manifests clinically as neuropathic hypersensitivity. The prevalence of neuropathic pain seems to be increasing, due, in part, to the aging population (as with postherpetic neuropathy), as well as the increasing use of neurotoxic agents in the management of life-threatening illness, such as Cisplatin, Paclitaxel (Taxol), Thalidomide (Thalomid), anti-retrovirals and other agents[1].

Peripheral neuropathy (PN) is derangement in structure and function of peripheral motor, sensory and autonomic neurons. Diabetes mellitus and alcoholism are the most common etiologies of PN but the primary worldwide cause of PN is leprosy. Other common causes of PN include genetic origin, metabolic disorders, infection and traumatic, inflammatory, ischemic, toxic or drug induced (iatrogenic) insults[2].

PNis often considered as having a lancinating or continuous burning pain and is often associated with small-fiber dysfunctions include abnormal sensory signs such as hyperalgesia(an increased response to painful stimulus), allodynia (painful response to innocuous stimulus)[1],

paresthesia (tingling and pricking sensation or numbness of skin) deficits in pain, temperature perception, predisposition to foot ulceration and large-fiber dysfunctions include loss of position and vibration sensation, nerve-conduction abnormalities and distal muscle weakness[3].

Peripheral neurotoxicity is a dose-limiting and disabling side effect of several important chemotherapeutic agents. In particular, Vincristine, Cisplatin, Oxaliplatin, Paclitaxel and Docetaxel are frequently used antineoplastic agents, which are known causes of a peripheral neuropathy, haematological and renal toxicity[4].

The general estimated prevalence of peripheral neurotoxicity in patients treated with chemotherapeutic agents is 30-40%.However, up to 60% incidences have been reported with Cisplatin, Paclitaxel, Docetaxel, Vincristine, Oxaliplatin and Bortezomib[5].

It appears that onset and severity depends on a variety of factors, including concomitant medical conditions such as metabolic disorders like diabetes, alcoholism, malnutrition[6].

Acquired neuropathies, such as diabetes and Cisplatin induced neuropathies are accompanied by positive sensory symptoms like paresthesias (numbness and tingling) dysesthesias (electric shock phenomenon),hyperesthesia (incresed sensitivity to mild painful stimuli), hyperalgesia(increased sensitivity to normally painful stimuli),hyperpathia (pain produced by sub threshold stimuli),allodynia (pain produced by normally non-painful stimuli) and spontaneous painwhereasinherited neuropathies, like Charcot-Marie-Tooth associated with negative sensory symptoms like hypoalgesia and loss of sensation[5]. Cisplatin(cis-diamine-dichloro-platinum)[7] is an effective anti-tumour agent that is currently commonly used for the treatment of various malignancies and particularly ovarian, bladder, lung and testis cancer[8].

Cisplatin predominantly affects the sensory nerve bodies, which are located in the sensory root ganglia. This may be due to the absence of the blood-nerve barrier of this part of the nervous system, resulting in a higher accumulation inside the sensory nerve body[9]. Cisplatin cause early mitochondrial dysfunction with loss of membrane potential. Loss of mitochondrial membrane potential is an early event in models of acquired and genetic neuropathies[10]. Patient compliance to chemotherapeutic regimen is a critical factor in determining the survival of cancer patients. However, chemotherapy-induced peripheral neuropathy (CIPN) is a significant complication in the successful treatment of many cancers. CIPN is also associated with severe and disabling anemia[7].

According to the National Cancer Institute (NCI), CIPN is one of the main reasons that patients prematurely terminate treatment. Early termination of chemotherapy negatively affects patient outcomes, as current oncology practice incorporates dose-dense regimen or combination regimens, which require course completion to decrease the risk of recurrence and increase survival rates[5]. A number of different agents from diverse chemical classes have entered clinical trials for the treatment of CIPN, but only few approved for clinical use while other drugs either ineffective or withdrawn[3]. Current treatment options for symptomatic treatment of CIPN include antidepressants, anticonvulsants. These agents are modestly effective for symptomatic relief, but they neither affect the underlying pathology nor do they slow progression of the disease[11]. Hence a novel approach to bridge the gap in selecting the compound in treatment of CIPN was used .The discovery of use of a drug for a new indication is a arbitrary process, as shown by many past examples like the use of zinc acetate for the treatment of Wilson's disease[12] arsenic for acute promyelocytic leukemia, amphotericin B for leishmaniasis[13] and thalidomide for multiple myeloma[14]. The discovery of these "alternative" uses for drugs different from originally intended drug development process is referred to as drug repurposing or repositioning[15]. Repositioning of drug efforts has many advantages, because the pharmacokinetics and pharmacodynamics of the drug are known, repositioning discoveries are less costly and quicker than traditional discovery efforts, which usually take 10–15 years and cost upward of $1

billion[16]. In this study we have selected Thyroxine to explore for its activity in CIPN.

Thyroid hormones (TH) [T4 (tetraiodothyronine) and T3 (triiodothyronine)], the only iodine-containing compounds with biological activity**.**The cardiac side effect of D isomer of Thyroxine resulted discontinuation of the clinical uses of this hormone. Under normal conditions, about 41% of Thyroxine is converted to T3 and about 21% is converted to metabolically inactive 3, 3,5-triiodothyronine (reverseT3,rT3)[17]**.**T4 stimulates synthesis of Na^+/K^+ ATPase, lipolysis and cholesterol excretion.T4 increases Basal Metabolic Rate, utilization of glucose, and also consumption of oxygen for A.T.P production[18].

MATERIALS AND METHODS

Study design

In-house laboratory bred healthy Wistar rats weighing 200-250g were included for the study. Animals were housed in polypropylene cages on clean paddy husk bedding. Animals were maintained under controlled temperature at $25^0C \pm 2^0C$ with 12hr light/dark cycle with food and water provided *ad libitum*. Animals which did not comply with the above criteria and which were found to be disease were excluded from the study. Before conducting the experiment, ethical clearance was obtained from "Institutional animal ethics committee", Al-Ameen College of Pharmacy, Bangalore.

Cisplatin-induced peripheral neuropathy

Group1: Normal Control
Group2: Cisplatin Control (Cisplatin - 2mg/kg,i.p. twice a week for 8weeks[23])
Group3: Cisplatin Control+ T4 (T4 - 1mg/kg, s.c.[19] twice a week for 8 weeks)
Treatment was given along with the Cisplatin for 8 weeks, after which the following parameters were studied:

- Body weight
- Thermal and cold hyperalgesia by tail immersion test
- Motor in-coordination by rota rod performance test
- Grip strength

Following the above studies the animals were sacrificed and the sciatic nerve was isolated for measurement of nerve conduction velocity (NCV) and histological observations.

Drugs, Chemicals &Instruments

Cisplatin

Cipla Pvt. Ltd.
Thyroxine

ApotexPharmachem India Pvt.Ltd.
Thiopentone
Sodium

Neon Laboratories
AnaestheticEther

Sd Fine-Chem Ltd.
Micro-pipette, Micro-centrifuge tubes

TarsonsProductions Pvt. Ltd.
Bioamplifier,
PowerLab

ADInstrument, Australia

Body weight

Initial and final body weights of the rats weremeasured and the percentage of change in body weight of the experimental groups wascompared with Cisplatin control group.

Measurement ofthermal and cold hyperalgesia using tail immersion test[20]

Thermal and cold hyperalgesia were measured using the tail immersion test in water, maintained at high (46^0C) or low (4^0C) temperature. The duration of tail immersion was recorded, and a cut-off time of 15s was used.

Measurement of motor in-coordination using rota rodperformance test[21]

Rota rod has been used to evaluate motor coordination by testing the ability of rats to remain on a revolving rod. The rate of rotation was adjusted in such a manner that it allowed the normal rats to stay on it for 5min. Each rat was given five trials before the actual reading was taken. The readings were taken at 15 & 25rpm after treatment, in all groups of rat.

Measurement of grip strength[22]

Grip strength meter was used for evaluating grip strength of animals. Before commencement of the experiment, the animals were acclimatized by placing on the instrument for some time to train and then rats were held by the tail above the grid of grip strength meter. The animal was moved until its front legs grasped the grid and it was brought to an almost horizontal position. The base of the tail was then pulled following the axle of the sensor until it released the grid. The force achieved by the animal was then displayed on the screen and was recorded as newtons or kg units.

Measurement of Nerve Conduction Velocity (NCV) [23]

The rats were anesthetized by administration of Thiopentone Sodium - 30mg/kg, i.p. After anesthesia, rat backs were shaved and NCV was recorded. Briefly incision was made at L_4-L_6 spinal segments. The sciatic nerves were surgically exposed from sciatic notch to the gastrocnemius tendon and the left & right sciatic nerves were rapidly removed carefully impregnated on fine filter paper to remove any accompanying blood, then soaked for 10 minutes in Ringer-Locke buffer to prevent spontaneous firing of the nerve[24].

The left sciatic nerves were then placed in a moist nerve chamber (MLT016/B - AD Instruments, Australia) to measure NCV. NCV was measured by stimulating proximally at the sciatic notch by stimulating electrode (MLA270 - AD Instruments, Australia) with 10mV at 1Hz to 5Hz and the action potential was measured using recording electrodes (MLA285 - AD Instruments, Australia) by placing distally to the sciatic notch.NCV was calculated by measuring the distance between stimulating and recording electrodes divided by the latency. Right sciatic nerves were transferred into 2.4% Glutaraldehydesolution for histopathological studies[25].

STATISTICAL ANALYSIS

Statistical evaluations were done by ANOVA, expressed as mean±S.E.M. followed by Bonferroni comparison test using GraphPadInStat (Ver. 3.10) and GraphPad Prism 5 computer programs.

RESULTS

Body weight

Body weight of 8th week Cisplatin-induced neuropathic rats was significantly ($P<0.001$) lower (-40.18%) than the normal rats. T4 treatment significantly ($P<0.001$) reduced percentage of loss of body weight (-22.17%) in Cisplatin treated rats (Fig. 1).

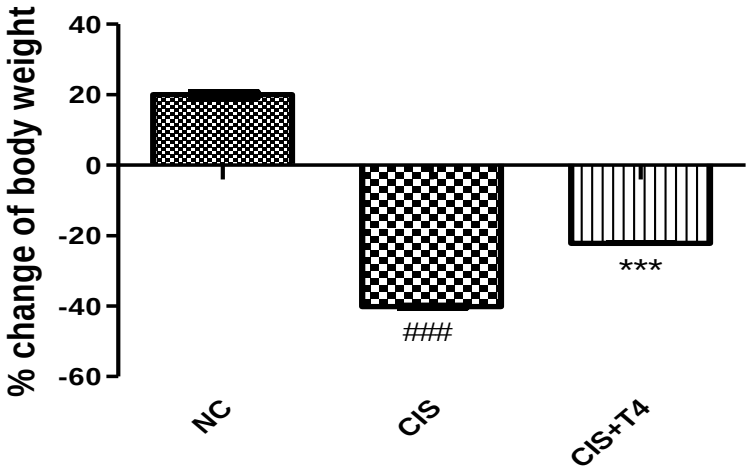

Fig. 1. Effect of treatment of Thyroxineon %body weight change inrats administered with Cisplatin [(2mg/kg, i.p.) twice a week for 8 weeks].NC: Normal control, CIS: Cisplatincontrol, T4: Thyroxine.Values are represented as mean±SEM (n=6). ###P<0.001 Vs Normal control group,

***P<0.001 VsCisplatin control group. One Way ANOVA followed byBonferroni multiple comparisons.

Tail immersion test

In 8 weeks, Cisplatin treated rats showed a significant change (###P<0.001) in tail flick latency in both cold and hot tail immersion test. T4 treatment significantly improved (***P<0.001) cold and hot immersionperformance(Fig.2 and 3)

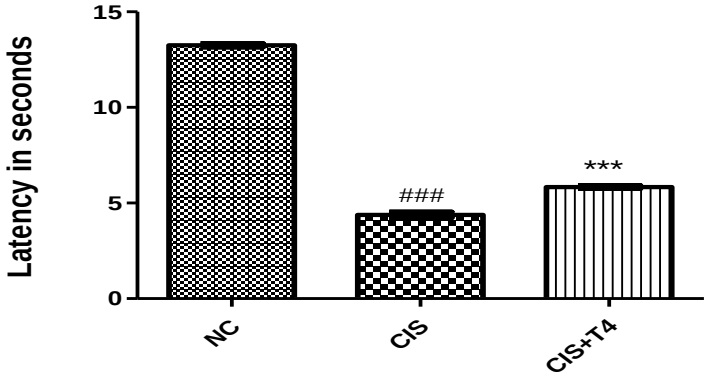

Fig. 2: Effect of treatment of Thyroxine on tail flick latencies (46°C) inrats administered with Cisplatin [(2mg/kg, i.p.) twice a week for 8 weeks]**.**NC: Normal control, CIS: Cisplatincontrol, T4: Thyroxine. Values are represented as mean±SEM (n=6). ###P<0.001 Vs Normal control group, ***P<0.001VsCisplatin control group.

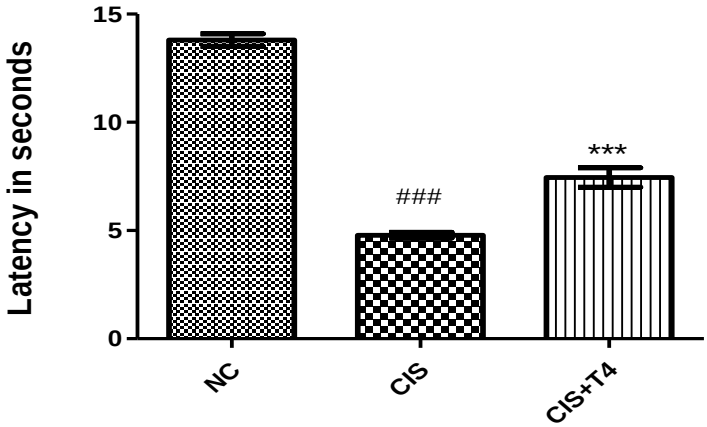

Fig. 3: Effect of treatment of Thyroxine on tail flick latencies (4°C) inrats administered with Cisplatin [(2mg/kg,i.p.) twice a week for 8 weeks]. NC: Normal control, CIS: Cisplatin control, T4: Thyroxine, Values are represented as mean±SEM (n=6).###P<0.001 Vs Normal control group, ***P<0.001VsCisplatin control group.

Rota rod performance test

Rotarod experiment at 15 & 25 revolutions per minute (rpm) showed a significant (###P<0.001) decrease in the retention time on the rotating rod in Cisplatin control group compared to normal control.T4 treatment to Cisplatin control rats significantly reversed(***P<0.001) the retention time to that of normal control at 15 &25 rpm.

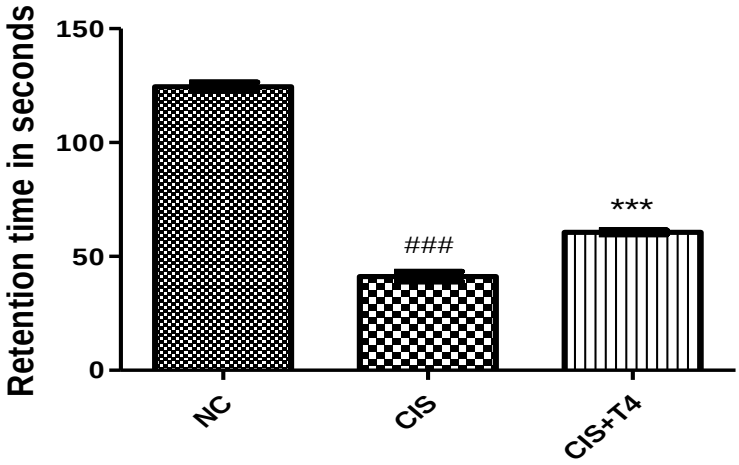

Fig. 4: Effect of treatment of Thyroxineon motor in-coordination by rota rod performance test (15rpm) inrats administered with Cisplatin [(2mg/kg,i.p.) twice a week for 8 weeks].NC: Normal control, CIS: Cisplatincontrol, T4: Thyroxine. Values are represented as mean±SEM (n=6). ###P<0.001 Vs Normal control group, ***P<0.001 VsCisplatin control group. One Way ANOVA followed byBonferroni multiple comparisons.

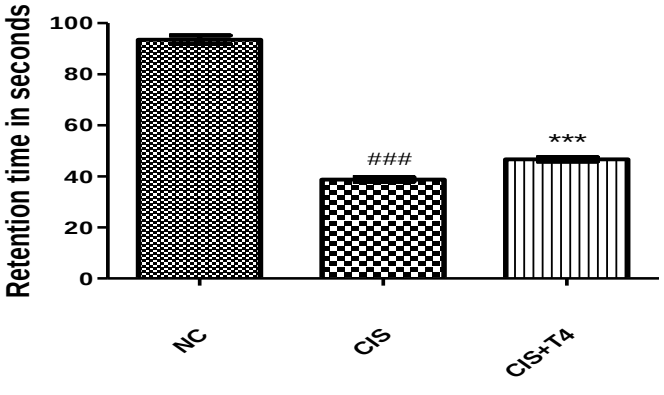

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Fig. 5: Effect of treatment of Thyroxineon motor in-coordination by rota rod performance test (25rpm) inrats administered with Cisplatin [(2mg/kg,i.p.) twice a week for 8 weeks]. NC: Normal control, CIS: Cisplatincontrol, T4: Thyroxine. Values are represented as mean±SEM (n=6). ###P<0.001 Vs Normal control group, ***P<0.001 VsCisplatin control group. One Way ANOVA followed byBonferroni multiple comparisons.

Grip strength

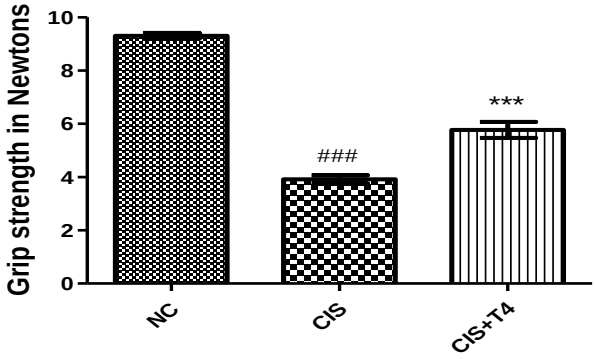


Fig. 6: Effect of treatment of Thyroxine on grip strength in rats administered with Cisplatin [(2mg/kg,i.p.) twice a week for 8 weeks].NC: Normal control, CIS: Cisplatincontrol, T4: Thyroxine. Values are represented as mean±SEM (n=6). ###P<0.001 Vs Normal control group, ***P<0.001 VsCisplatin control group. One Way ANOVA followed byBonferroni multiple comparisons.

Sciatic motor nerve conduction velocity

In 8 weeks, sciatic nerve conduction velocity was significantly (###P<0.001) decreased (31.72%) in Cisplatin treated rats as compared to normal control rats. T4 treatment to Cisplatin control rats significantly (***P<0.001) corrected (20.67%) nerve conduction defects.

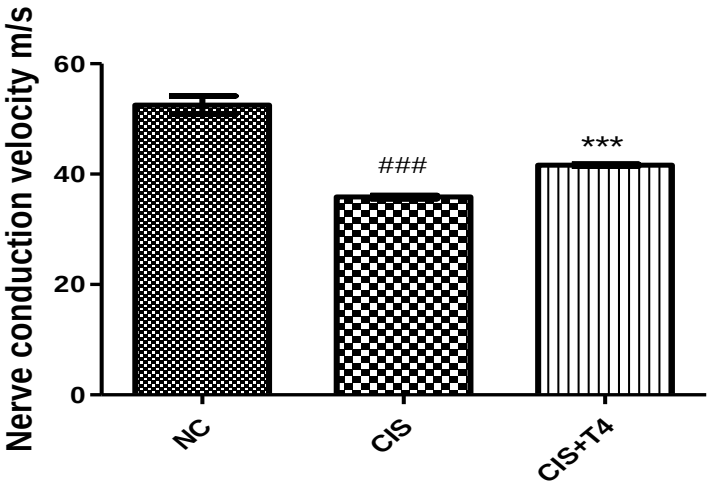
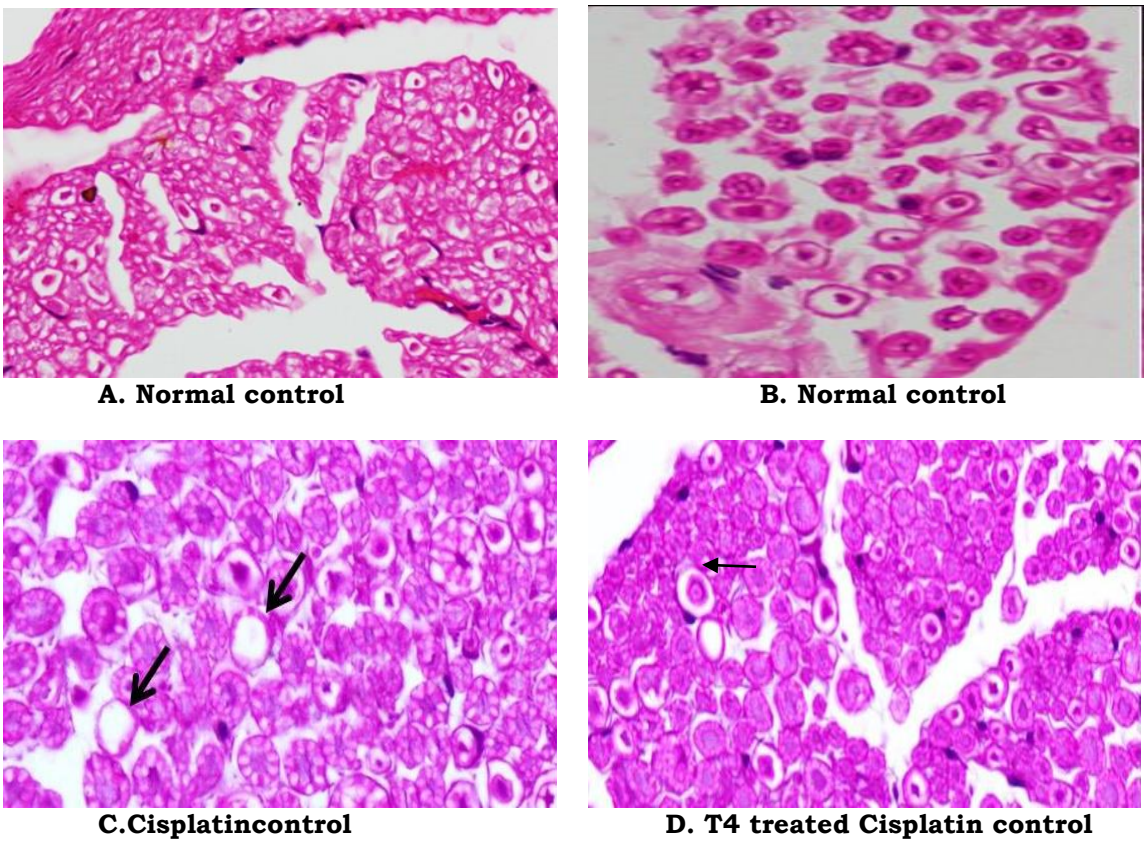


Fig.7: Effect of treatment of Thyroxine on nerve conduction velocity in rats administered with Cisplatin [(2mg/kg,i.p.) twice a week for 8 weeks].NC: Normal control, CIS: Cisplatincontrol, T4: Thyroxine. Values are represented as mean±SEM (n=6). ###P<0.001 Vs Normal control group, ***P<0.001 VsCisplatin control group. One Way ANOVA followed byBonferroni multiple comparisons.

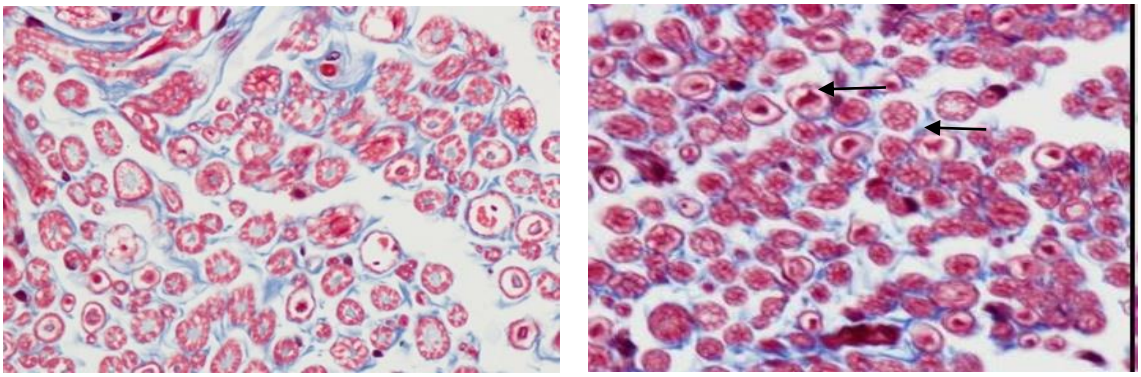
Histology of sciatic nerve



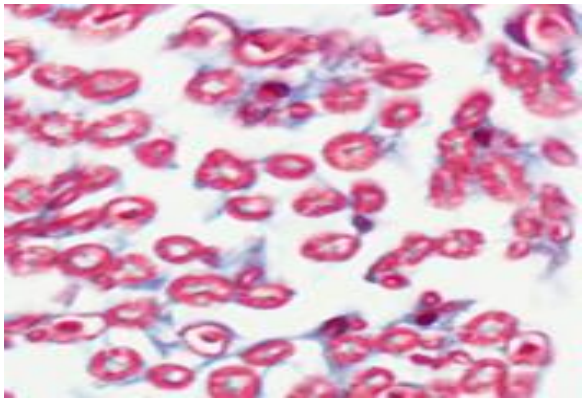
A. Normal control **B. Normal control**

C.Cisplatincontrol **D. T4 treated Cisplatin control**

Fig. 8: Representative microphotographs of the sciatic nerves of control and experimentalgroups of rats. A - Light microscopy transverse section showing closely packed nerve fibers and an occasional endoneurial blood vessel. B - Light microscopy transverse section showing individual nerve fibers and a central axon surrounded by a sheath of myelin. C - Scattered fibers with axonal swelling and degeneration (arrows).D - Light microscopy of transverse section showing scattered fibers with axonal swelling and degeneration. Stain: H&E, Magnification: A-C=X160, D=X80.

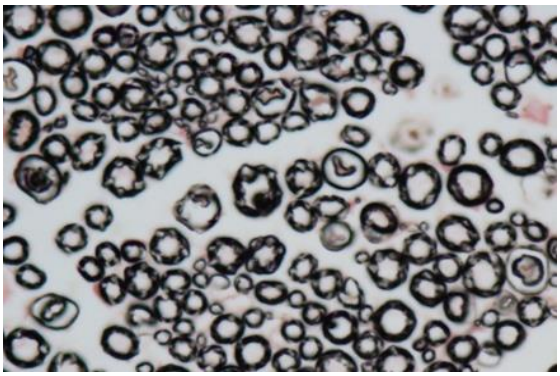


A. Normalcontrol **B.Cisplatincontrol**

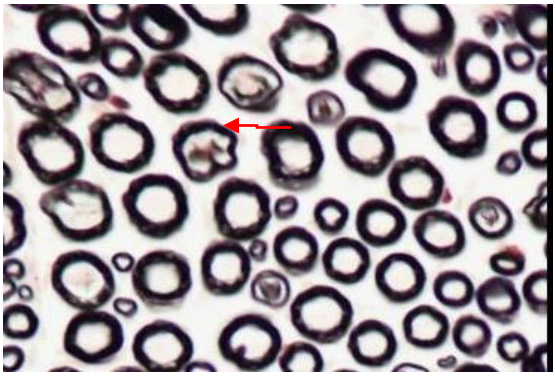


C.T4 treated Cisplatincontrol

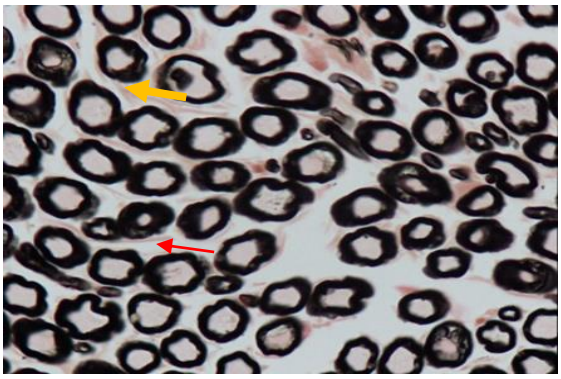
Fig. 9: Representative microphotographs of the sciatic nerves of control and experimentalgroups of rats. A - Transverse section of special stain for collagen highlights the endoneurial matrix separating the nerve fibers and collagenous component is stained blue. B - Transverse section of special stain for collagen highlights axonal degeneration with dilated axons. C - Transverse section showingnormal endoneurial matrix and collagen. Stain: Masson's trichrome, Magnification: A-C=X160.



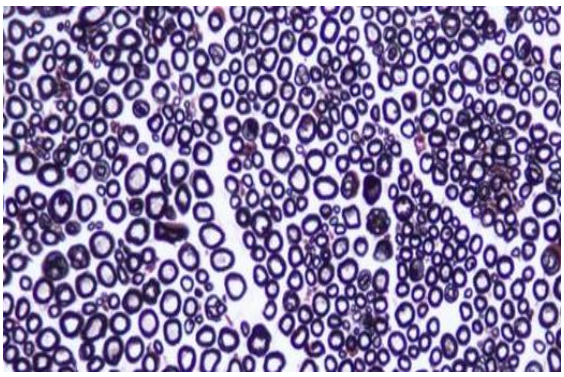
A.Normal control



B. Cisplatin control



C.Cisplatincontrol



D.T4 treated Cisplatin control

Fig.10: Representative microphotographs of the sciatic nerves of control and experimentalgroups of rats. A - Transverse section of special stain for myelin reveals an admixture of large and small diameter myelinated fibers. The thickness of the myelin sheath is proportionate to the width of the axonal diameter. B - Transverse section of special stain for myelin highlights axonal degeneration with dilated axons. C - Highlights the presence of numerous enlarged axonal profiles surrounded by thinner myelin sheaths indicating axonal swelling and axonopathy. D - Transverse section of myelin stain showing near to normal fiber density. Stain: Kulchitsky pal, Magnification: A=X160, B,C=X320& D=X80.

DISCUSSION

The protective effect of T4in Cisplatin-induced peripheral neuropathy was studied. Development of PN in Cisplatin treated rats was evident in cisplatin control rats. Cisplatin treated rats were exhibited decreased body weight;motor in-coordination, decreased grip strength, altered nociception including thermal and cold hyperalgesia and decreased NCV**.** The alteration in these parameters could be due to toxicity of Cisplatin to the peripheral nerves.Cisplatin mainly affects the sensory nerve bodies, which are located in the sensory root ganglia, axonal transport system, the myelin sheath and glial support structures. This may be due to the absence of the blood-nerve barrier of this part of the nervous system[26], resulting in a higher accumulation inside the sensory nerve body leading to early mitochondrial dysfunction with loss of membrane potential.In this study we have selected T4 based on some beneficial effects including T4 provides glial support, synthesizes protein required for myelin sheath[27], The decrease in percentage (-40.18%) of body weight was significantly seen in Cisplatin control rats, T4 treatment significantly reduced the percentage (-22.17%) of loss of body weight in Cisplatin treated rats.Less decrease in percentage of body weight in T4 treated rats indicated that rats do not exhibited the overt hyper-thyroidism.

Pain is most common symptom in CIPN, thus we evaluated nociceptive response in our study. Nociception was observed in Cisplatin control rats. Various mechanisms such as tissue injury, peripheral receptors sensitization, ectopic activity in sprouting fibers, alteration in dorsal root ganglia cells are reported to contribute to pain[28].In the present study we observed significant reduction in nociception with T4 treatment for 8 weeks as T4 significantly improved thermal and cold hyperalgesia, further the effectiveness of the T4 supported by the study wherein a significant improvement in decreased grip strength, motor in-coordination is documented. Cisplatin control rats showed shorter fall of time from rotating rod compared to control rats, suggesting impairment in their ability to integrate sensory inputs with appropriate motor commands to balance their posture.T4 treated rats increased fall of time from rotating rod compared to Cisplatin control rats and enables the rats in lowering the time for spatial recognition and thus helps to maintain their posture during movement on the rod. Several reports have described that CIPN is also associated with decreased nerve conduction velocity (NCV) at the biochemical level, potential etiologic mechanism include decreased insulin-like growth factor-1, neuropeptide expression in the terminal nerve fibers,[29,30] decreased Na^+/K^+ ATPase activity[31] and atrophy of large myelinated fibers. Similarly decreased NCV was found in Cisplatin control rats in our study due to above mentioned mechanisms. Treatment with T4 for 8 weeks improved NCV could be due to increased levels of basal total HSP27 and phospho-HSP27increase in expression of Na^+/K^+ ATPase[30].

Neuropathology is an integral part of the modern multidisciplinary approach to neurotoxicity; hence we have also performed histopathological studies of sciatic nerve in our study.We observed some histopathological alterations in Cisplatin control rats which include presence of scattered fibers with axonal swelling, degeneration and numerous enlarged axonal profiles surrounded by thinner myelin sheaths indicating axonopathy. These histological damagesin our study could result from decreased NCV due to altered sodium cell gradient related to impairment of Na^+/K^+ ATPase activity. T4 treated animals restored all histopathological alterations as evidenced by presence of fiber density to normal, restoration of myelin sheath and normal endoneurial matrix and collagen and amelioration of NCV due to increased expression of Na^+/K^+ ATPase[32].

CONCLUSIONS

Thyroxine treatment effectively prevented many of the behavioral, electrophysiological and histological manifestations of Cisplatin-induced peripheral by decreasing thermal and cold hyperalgesia, improving motor incordination, grip strength, NCV, fiber density and myelin thickness.

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