



SYNERGISTIC NEUROPROTECTIVE ACTIVITY OF BENFOTIAMINE CO-ADMINISTERED WITH ERYTHROPOIETIN IN CHEMOTHERAPY INDUCED PERIPHERAL NEUROPATHY IN RATS

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Abstract: Chemotherapeutic drugs like Cisplatin, Taxols used in cancer are associated with the development of peripheral neuropathy (PN). Severe neuropathy can occur in 3% to 7% of treated cases with single agents but the severity 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 neither 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. Benfotiamine (BT) a lipid soluble form of thiamine plays important role in various biological pathways reported beneficial effect in diabetic retinopathy. Erythropoietin (EPO) has recently been considered as a tissue protective cytokine. EPO is reported to protect neurons from ischemia reperfusion-induced injury, metabolic stress, HIV-induced damage or even mechanical injury such as nerve compression or trauma. Therefore the present study was undertaken to evaluate the individual and combination effects of BT and EPO in cisplatin induced peripheral neuropathy in rats. 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, behavioral parameters like motor in-coordination, thermal & cold hyperalgesia, grip strength, biochemical parameters like measurement of endogenous antioxidants and histopathological studies. PN was evidenced in Cisplatin control rats and ameliorated with administration of BT (100 mg/kg p.o daily) and EPO (500U/kg i.p. thrice weekly) for 8 weeks by augmenting all the above parameters. BT exhibited neuroprotective activity, which would be attributed to its activity as neurotrophic effect.

Key words: Thyroxine; Neuroprotectant; Cisplatin; Peripheral Neuropathy

INTRODUCTION

Neuropathic pain is arising as a result of generation of sensory impulses at abnormal (ectopic) locations, for example at sites of nerve injury or demyelization. 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 appears 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 agents¹.

Derangement in structure and function of peripheral motor, sensory and autonomic neurons causes' condition called Peripheral Neuropathy (PN). Metabolic disorders such as 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) insults².

PN is 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 weakness³.

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 PN, haematological and renal toxicity⁴.

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 Bortezomib⁵.

It appears that onset and severity depends on a variety of factors, including concomitant medical conditions such as metabolic disorders like diabetes, alcoholism, malnutrition⁶. 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 (increased 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 pain whereas inherited neuropathies, like

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Charcot-Marie-Tooth associated with negative sensory symptoms like hypoalgesia and loss of sensation⁵.

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 cancer⁸.

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 body⁹.

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 neuropathies¹⁰.

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 anaemia.

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 rates⁵.

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 withdrawn³. 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 disease¹¹.

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 disease¹² arsenic for acute promyelocytic leukemia, amphotericin B for leishmaniasis¹³ and thalidomide for multiple myeloma¹⁴.

The discovery of these "alternative" uses for drugs different from originally intended drug development process is referred to as drug repurposing or repositioning¹⁵. 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 billion¹⁶. In this study we have selected BT,

EPO and studied individual and combination effect in CIPN.

EPO has also protected neurons from apoptosis in cell culture studies and in animal models of central nervous system injury, EPO has shown to increase expression of antioxidant enzymes, reduces nitric oxide mediated formation of radicals, normalize cerebral blood flow and promote neuroangiogenesis. EPO has shown to regulate bone marrow erythroid cell proliferation, differentiation, and survival through its binding to an erythroid progenitor cell surface EPO receptor (EPOR). The EPOR also is expressed in myelin sheaths of radicular nerves in human peripheral nerves, suggesting both a developmental and potential protective role for EPO not only in the central nervous system, but also in disease entities that involve the peripheral nervous system.¹⁷ BT is a transketolase (TK) activator. BT was shown to prevent experimental diabetic retinopathy and *in vitro* hyperglycemia-induced endothelial dysfunction. The effects of benfotiamine on *in vivo* endothelial function remained unknown. BT has shown to inhibit hexosamine pathway, advanced glycation end product (AGE) formation pathway and the diacylglycerol (DAG) protein kinase C (PKC) pathways. Therefore the present study was designed evaluate whether PN can be reversed by treatment with BT, EPO and their combination¹⁸.

MATERIALS AND METHODS

Experimental animals

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°C±2°C 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 diseased were excluded from the study. Before conducting the experiment, ethical clearance was obtained from "Institutional animal ethics committee", Al-Ameen College of Pharmacy, IAEC Number: AAC/P-48, India.

Experimental Design

Twenty four rats were randomly divided into four groups (Group I-Group IV). Group I served as normal control group. Group II, Group III, Group IV, and Group V were induced PN and included in the study as experimental rats. Group II served as cisplatin control received only cisplatin 2mg/kg, i.p. twice a week for 8 weeks¹⁹ whereas Group III, Group IV and Group V received cisplatin + EPO (500U/kg i.p.²⁰ thrice weekly for 8 weeks), BT (100 mg/kg p.o.²¹ daily for 8 weeks) and combination of EPO + BT respectively. After 8 weeks various behavioral biochemical, electrophysiological parameters were determined to evaluate the severity of PN in treated group as compare to normal and cisplatin control group. The researchers carrying out the behavioral studies were blinded with respect to the treatment administered.

Drugs, Chemicals and Instruments

Cisplatin	Cipla Pvt. Ltd.
Erythropoietin	Biocon Limited.
Benfotiamine	Strides Arco Lab Pvt.Ltd
Thiopentone Sodium	Neon Laboratories
Anaesthetic Ether	Sd Fine-Chem Ltd.
Micro-pipette and	
Micro-centrifuge tubes	Tarsons Pvt. Ltd.
Bioamplifier, PowerLab	ADInstrument, Australia

Assessment of General Toxicity

To assess the general condition of animals, they were examined daily for clinical signs such as alopecia, piloerection or hind limb weakness and mortality. Body weight was measured using digital balance (Essae® DS-252). Loss of body weight was compared between body weight measured at the beginning and at the end of the study.

Behavioral parameters**Assessment of Thermal and cold Hyperalgesia²²**

Tail immersion test (warm water): Rat's tail was marked at lower 5 cm portion and immersed in warm water bath (46°C). The withdrawal response of tail or sign of struggle was observed and the reaction time was recorded using stop watch (cut-off time 12 sec). The tail was dried carefully after recording every response. The reaction time was recorded before and after treatment in normal, diabetic and treated groups. Reductions in tail withdrawn time specify hyperalgesia.

Tail immersion test (cold water): The lower half portion of the tail was immersed in a beaker of cold water (4°C). Tail withdrawal or flicking response from cold water was recorded as the reaction time (cut-off time 10 sec) in seconds. Shortenings in tail withdrawn time stipulate cold hyperalgesia.

Rota-Rod Performance test²³: Motor incoordination was evaluated by a Rota-Rod apparatus. The Rota-Rod unit consists of a rotating rod, 75mm in diameter, which was divided into four parts by compartmentalization to permit the testing of four rats at a time. Briefly, in a training session, the rats were placed on the rod that was set to 15 rpm and the performance time that each rat was able to remain on the rota-rod was recorded. The rats were subjected to three training trials from 3h – 4h intervals on two separate days for acclimatization purposes. In the test session, the rats were placed on the rota-rod and their performance times were recorded. All the readings were taken in triplicate.

Grip strength measurement²⁴: Grip strength meter was used for evaluating grip strength of animals. Before commencement of experiment the animals were acclimatized by placing them on the instrument for few minutes. Rats were held by the tail above the grid of grip strength meter 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 Newton.

Isolation of sciatic nerve

The rats were anesthetized by administration of thiopentone sodium, 30mg/kg, and i.p.²⁵ after anesthesia, rat backs were shaved and NCV was recorded. Briefly incision is made at L₄-L₆ spinal segments. The sciatic nerves were surgically exposed from sciatic notch to the gastronomies tendon and the left and right sciatic nerves were rapidly removed, left sciatic nerve carefully impregnated on fine filter paper to remove any accompanying blood soaked for 10 minutes in Ringer-Locke buffer to prevent spontaneous firing of the nerve²⁶ and used for measuring NCV, a segment of left sciatic nerve transferred in to 0.05mol/L phosphate buffered (30g/L) glutaraldehyde solution for histopathological studies and right sciatic nerves were rinsed with ice cold saline homogenized in chilled phosphate buffer (pH 7.4) and used to assay lipid peroxidation, reduced glutathione super oxide dismutase and catalase .

Preparation of nerve homogenate

A segment of right sciatic nerve, approximately 1.5 cm in length, 5mm proximal and 5 mm distal was used for preparing the 10% w/v homogenates for biochemical estimation. Tissue homogenates were prepared in 0.1M phosphate buffer (pH 7.4). The homogenate was centrifuged at 1000 rpm 4°C for 3 min and the supernatant divided into two portions, one of which was used for measurement of lipid peroxidation (LPO) and the remaining supernatant was again centrifuged at 12,000 rpm at 4°C for 15 min and used for the measurement of endogenous antioxidants such as superoxide dismutase (SOD), catalase (CAT) glutathione (GSH).

Measurement of lipid peroxidation

The extent of lipid peroxidation in terms of thiobarbituric acid reactive substances (TBARS) formation was measured according to the method of Esterbauer and Cheeseman. Tissue extracts were mixed separately with 1 ml TCA (20%), 2 ml TBA (0.67%) and heated for 1 h at 100°C. After cooling, the precipitate was removed by centrifugation. The absorbance of each sample was measured at 535 nm using a blank containing all the reagents except the sample. As 99% TBARS are malondialdehyde (MDA), so TBARS concentrations of the samples were calculated using the extinction coefficient of MDA, which is $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ and were expressed as μM of malondialdehyde per mg protein ²⁷.

Measurement of superoxide dismutase (SOD) and catalase activity

Sciatic nerve homogenate was centrifuged at 4°C, 17,500 ×g for 10 min. Supernatant was used for the measurement of SOD activity by pyrogallol autooxidation method ²⁸ and catalase activity by H₂O₂ degradation method, which is a quantitative spectroscopic method developed for following the breakdown of H₂O₂ at 240 nm in unit time. The sample readings were taken by placing 1 ml of phosphate buffer and 100 μl of tissue homogenate in the reference cuvette and 1 ml of H₂O₂ and 100 μl of homogenate in the test cuvette in the spectrophotometer. For each measurement, the reading was taken at 240 nm 1 min after placing the cuvettes in Shimadzu spectrophotometer ²⁹.

Measurement of reduced glutathione (GSH) activity

Reduced glutathione was assayed by the method described by Tietzl³⁰. Briefly 1.0ml of sciatic nerve homogenate (10%w/v) was precipitated with 1.0 ml of sulphosalicylic acid (4%). The samples were kept at 4°C for at least 1 h and then subjected to centrifugation at 1200g for 15min at 4°C. The assay mixture contained 0.1 ml supernatant, 2.7 ml phosphate buffer (0.1M, pH 7.4) and 0.2 ml 5,5, dithiobis (2-nitro benzoic acid) (Ellman’s reagent, 0.1 mM, pH 8.0) in a total volume of 3.0 ml. The yellow colour developed was read immediately at 412 nm and the reduced GSH levels were expressed as µg/mg protein.

Electrophysiological parameters

Measurement of Nerve Conduction Velocity (NCV):

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 10 mV at 1Hz to 5 Hz and the action potential was measured using recording electrodes (MLA 285) by placing distally to the sciatic notch. NCV was calculated by using the following formula:

$$\text{Nerve Conduction velocity} \left(\frac{\text{m}}{\text{sec}} \right) = \frac{\text{distance}}{\text{latency}}$$

Histopathological studies

The segment of left sciatic nerves were isolated and transferred in to 0.05mol/L phosphate buffered (30g/L) glutaraldehyde solution for histopathological studies (H&E, Kulchitsky Pal staining and Massion’s trichome staining).

Statistical analysis

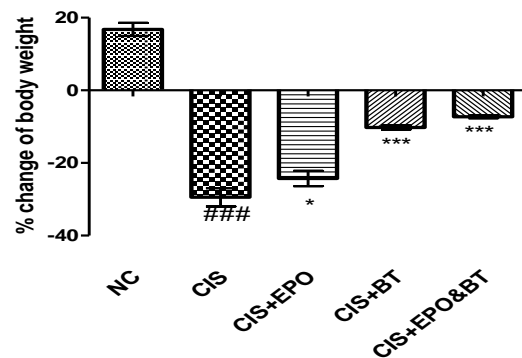
Statistical evaluations were done by ANOVA, expressed as mean± S.E.M. followed by Bonferroni comparison test using Graph Pad In Stat (Ver.3.10) and Graph Pad Prism 5 computer programme. P<0.05 was considered statistically significant.

RESULTS

Assessment of General Toxicity

The percentage body weight of normal and cisplatin control rats treated with EPO, BT and combination at 8th week was found to be 16.79±1.792g, -29.43± 2.524g, -24.27±2.112-10.20±0.5908g,-7.253± 0.4246. The percentage of change of body weight of cisplatin control rats significantly high (P<0.001) as compared to normal control, similarly the percentage change of body weight of cisplatin control treated rats with EPO, BT and combination was significantly less (P<0.05, P<0.001, P<0.001) respectively as compared to cisplatin control rats (Fig.1).

Figure 1: Effect of treatment of EPO, BT and combination on %body weight change in rats administered with Cisplatin [(2mg/kg, i.p.) twice a week for 8 weeks].



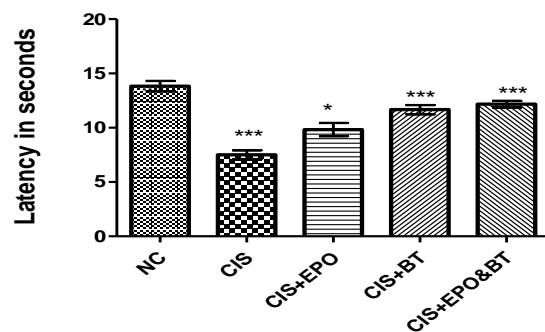
NC: normal control, CIS: cisplatin control, EPO: erythropoietin BT: benfotiamine. Values are represented as mean ±SEM. Experimental groups statistically compared with cisplatin ***P<0.001, *P<0.05and ### P<0.001 when cisplatin control group compared with normal control group. One Way ANOVA followed by Bonferroni multiple comparisons.

Behavioral studies

Thermal hyperalgesia:

The tail flick latencies in hot immersion test of normal, cisplatin control and treated rats with EPO, BT and combination were found to be 13.83±0.4773, 7.500±0.4282, 9.833±0.6009, 11.67±0.4216, 12.17±0.3073. The tail flick latency of cisplatin control rats significantly less (P<0.001) at 8th week as compared to normal rats. The tail flick latency of cisplatin control rats treated with EPO, BT and combination was significantly less (P<0.05, P<0.001, P<0.001) respectively as compared to cisplatin control rats. (Fig.2).

Figure 2: Effect of treatment of EPO, BT and combination on tail withdrawal latencies in rats administered with cisplatin [(2 mg, /kg i.p.) twice a week for 8 weeks] after immersion in a hot (46°C) water bath].



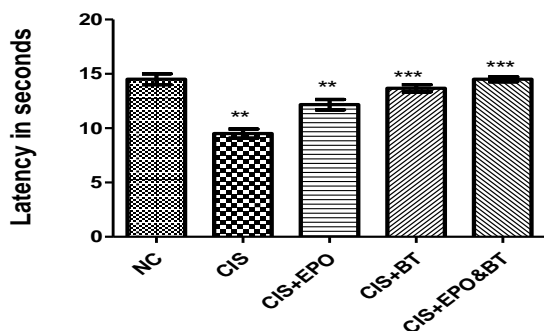
NC: normal control, CIS: cisplatin control, EPO: erythropoietin BT: benfotiamine. Values are expressed in mean ± SEM (n=6), Experimental groups statistically compared with cisplatin control *** P<0.001, * P<0.05 and *** P<0.001 when cisplatin control group compared with normal control group. One Way ANOVA followed by Bonferroni multiple comparisons.

Cold hyperalgesia:

The tail flick latencies in cold immersion test of normal, cisplatin control and treated rats with EPO, BT and combination were found to be 14.50±0.5000, 9.500±0.4282, 12.17±0.4773, 11.67±0.3333, 12.17±0.2236. The tail flick latency of cisplatin control rats significantly less (P<0.001) at 8th week as compared to

normal rats. The tail flick latency of cisplatin control rats treated with EPO, BT and combination was significantly high ($P < 0.05$, $P < 0.001$, $P < 0.001$) respectively as compared to cisplatin control rats. (Fig.3).

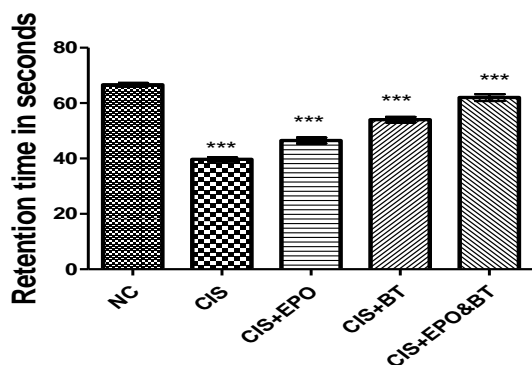
Figure 3: Effect of treatment of EPO, BT and combination on tail withdrawal latencies in rats administered with cisplatin [2 mg, /kg i.p.) twice a week for 8 weeks] after immersion in a cold (4°C) water bath].



NC: normal control, CIS: cisplatin control, EPO: erythropoietin BT: Benfotiamine. Values are expressed in mean ± SEM (n=6), Experimental groups statistically compared with cisplatin control *** $P < 0.001$, ** $P < 0.01$ and ** $P < 0.01$ when cisplatin control group compared with normal control group. One Way ANOVA followed by Bonferroni multiple comparisons.

Measurement of muscle in coordination: Time latencies at 15 rpm of normal, cisplatin control and treated rats with EPO, BT and combination were found to be 66.67 ± 0.6667 , 39.67 ± 0.7149 , 54.00 ± 1.000 , 62.001 ± 2.38 respectively. Time latency at 15 rpm of cisplatin control rats significantly less ($P < 0.001$) at 8th week as compared to normal rats. Time latency of cisplatin control rats treated with EPO, BT and combination was significantly high ($P < 0.01$) respectively as compared to cisplatin control rats. (Fig.4).

Figure 4: Effect of treatment of EPO, BT and combination on muscle in coordination in rats administered with cisplatin [2 mg, /kg i.p.) twice a week for 8 weeks at 15 rpm].

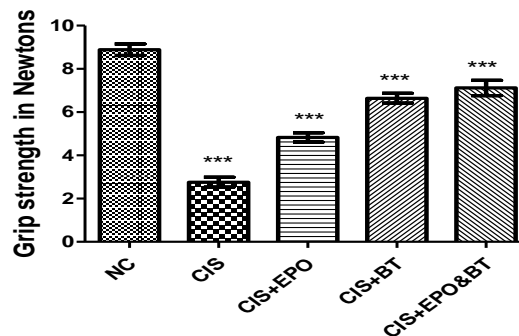


NC: normal control, CIS: cisplatin control, EPO: erythropoietin BT: benfotiamine. Values are expressed in mean ± SEM (n=6), Experimental groups statistically compared with cisplatin control *** $P < 0.001$ and *** $P < 0.001$ when cisplatin control group compared with normal control group. One Way ANOVA followed by Bonferroni multiple comparisons.

Measurement of grip strength: The grip strength of normal, cisplatin control and treated rats with EPO, BT and combination were found to be 8.887 ± 0.2650 ,

2.757 ± 0.2295 , 4.830 ± 0.2161 , 6.642 ± 0.2333 , 7.120 ± 0.3549 respectively, the grip strength of cisplatin control rats significantly less ($P < 0.001$) at 8th week as compared to normal rats.. The grip strength of cisplatin control rats treated with EPO, BT and combination was significantly high ($P < 0.001$) as compared to cisplatin control rats. (Fig.5).

Figure 5: Effect of treatment of EPO, BT and combination on grip strength in rats administered with cisplatin [2 mg, /kg i.p.) twice a week for 8 weeks].

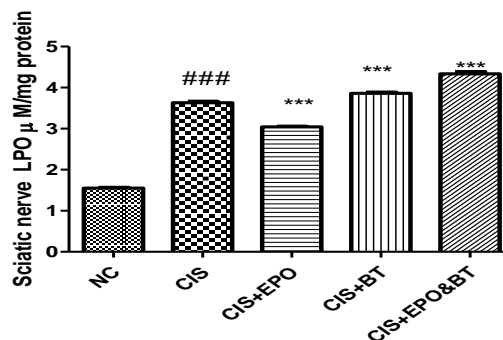


NC: normal control, CIS: cisplatin control, EPO: erythropoietin BT: benfotiamine. Values are expressed in mean ± SEM (n=6), Experimental groups statistically compared with cisplatin control *** $P < 0.001$ and *** $P < 0.001$ when cisplatin control group compared with normal control group. One Way ANOVA followed by Bonferroni multiple comparisons.

Biochemical studies

Measurement of lipid peroxidation: The sciatic nerve MDA levels of normal, cisplatin control and treated rats with EPO, BT and combination were found to be 1.552 ± 0.01558 , 3.633 ± 0.03252 , 3.045 ± 0.01204 , 3.862 ± 0.02242 , 4.333 ± 0.05064 respectively, the MDA levels of cisplatin control rats significantly more ($P < 0.001$) at 8th week as compared to normal rats.. The MDA levels of cisplatin control rats treated with EPO, BT and combination was significantly less ($P < 0.001$) as compared to cisplatin control rats. (Fig. 6).

Figure 6: Effect of treatment of EPO, BT and combination on sciatic nerve LPO in rats administered with cisplatin [2 mg, /kg i.p.) twice a week for 8 weeks].

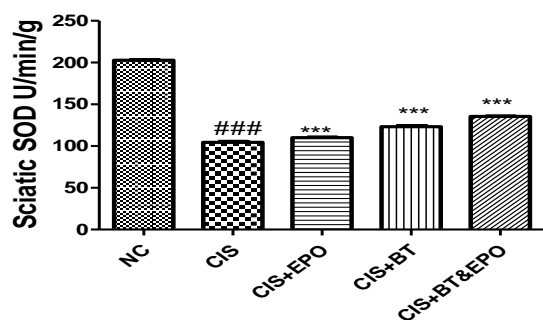


NC: normal control, CIS: cisplatin control, EPO: erythropoietin BT: benfotiamine. Values are expressed in mean ± SEM (n=6), Experimental groups statistically compared with cisplatin control *** $P < 0.001$ and ### $P < 0.001$ when cisplatin control group compared with normal control group. One Way ANOVA followed by Bonferroni multiple comparisons.

Estimation of superoxide dismutase (SOD)

The sciatic nerve SOD activity of normal, cisplatin control and treated rats with EPO, BT and combination were found to be 202.7±0.7472, 104.5±0.9500, 110.2±0.3141, 123.2±1.096, 135.6±0.1517 respectively, the SOD activity of cisplatin control rats significantly less (P<0.001) at 8th week as compared to normal rats. The SOD activity of cisplatin control rats treated with EPO, BT and combination was significantly high (P<0.001) as compared to cisplatin control rats. (Fig. 7).

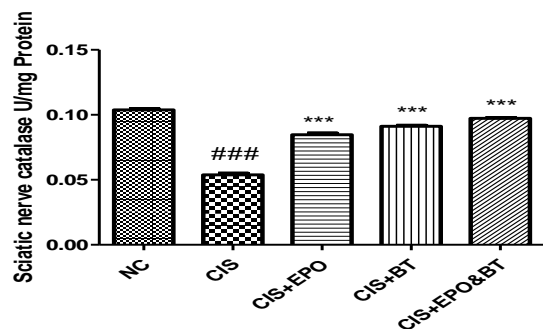
Figure 7: Effect of treatment of EPO, BT and combination on sciatic nerve SOD activity in rats administered with cisplatin [(2 mg, /kg i.p.) twice a week for 8 weeks].



NC: normal control, CIS: cisplatin control, EPO: erythropoietin BT: benfotiamine. Values are expressed in mean ± SEM (n=6). Experimental groups statistically compared with cisplatin control ***P<0.001 and ###P<0.001 when cisplatin control group compared with normal control group. One Way ANOVA followed by Bonferroni multiple comparisons.

Estimation of catalase: The sciatic nerve catalase activity of normal, cisplatin control and treated rats with EPO, BT and combination were found to be 0.1038±0.0007923, 0.05383±0.001195, 0.08467±0.001229, 0.09117±0.0007923, 0.09733±0.0004944 respectively, the catalase activity of cisplatin control rats significantly less (P<0.001) at 8th week as compared to normal rats. The catalase activity of cisplatin control rats treated with EPO, BT and combination was significantly high (P<0.001) as compared to cisplatin control rats. (Fig.8).

Figure 8: Effect of treatment of EPO, BT and combination on sciatic nerve catalase activity in rats administered with cisplatin [(2 mg, /kg i.p.) twice a week for 8 weeks].

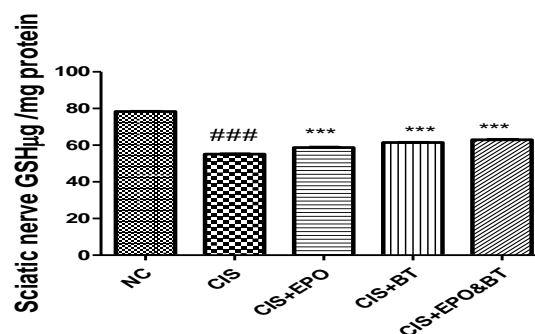


NC: normal control, CIS: cisplatin control, EPO: erythropoietin BT: benfotiamine. Values are expressed in mean ± SEM (n=6). Experimental groups statistically compared with cisplatin control ***P<0.001 and ###P<0.001 when cisplatin control group compared with normal control group. One Way ANOVA followed by Bonferroni multiple comparisons.

Measurement of reduced glutathione content:

The sciatic nerve glutathione content of normal, cisplatin control and treated rats with EPO, BT and combination were found to be 78.34±0.08613, 55.10±0.05596, 58.72±0.1420, 61.44±0.1231, 62.94±0.06627 respectively, the glutathione content of cisplatin control rats significantly less (P<0.001) at 8th week as compared to normal rats. The glutathione content of cisplatin control rats treated with EPO, BT and combination was significantly high (P<0.001) as compared to cisplatin control rats. (Fig.9).

Figure 9: Effect of treatment of EPO, BT and combination on sciatic nerve GSH content in rats administered with cisplatin [(2 mg, /kg i.p.) twice a week for 8 weeks].

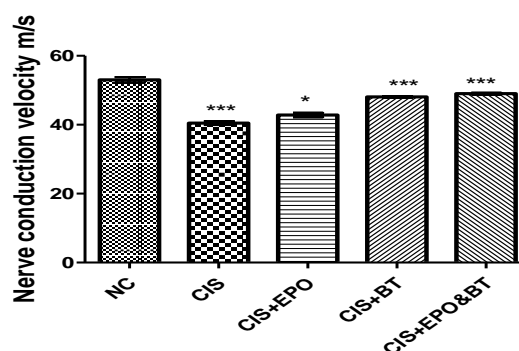


NC: normal control, CIS: cisplatin control, EPO: erythropoietin BT: benfotiamine. Values are expressed in mean ± SEM (n=6). Experimental groups statistically compared with cisplatin control ***P<0.001 and ###P<0.001 when cisplatin control group compared with normal control group. One Way ANOVA followed by Bonferroni multiple comparisons.

Measurement of Nerve Conduction Velocity (NCV)

The sciatic NCV of normal, cisplatin control and treated rats with EPO, BT and combination were found to be 53.03±0.8136, 40.48±0.5559, 42.82±0.6464, 46.31±0.2766, 49.01±0.2477 respectively, the sciatic NCV of cisplatin control rats significantly less (P<0.001) at 8th week as compared to normal rats. The sciatic NCV of cisplatin control rats treated with EPO, BT and combination was significantly high (P<0.05, P<0.001, P<0.001) respectively as compared to cisplatin control rats. (Fig.10).

Figure 10: Effect of treatment of thyroxine, erythropoietin, benfotiamine and their combination on nerve conduction velocity in rats administered cisplatin [(2 mg, /kg i.p.) twice a week for 8 weeks].



Values are expressed in mean ± SEM (n=6). Experimental groups statistically compared with cisplatin control *** P<0.001, * P<0.05 and *** P<0.001 when cisplatin control group compared with normal control group.

DISCUSSION

The curative effect of, EPO, BT and their combination in cisplatin induced peripheral neuropathy was studied. The Decreased NCV, muscle in coordination, grip strength, pain sensitivity and body weight is seen in cisplatin treated rats. 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. This may be due to the absence of the blood-nerve barrier of this part of the nervous system¹², resulting in a higher accumulation inside the sensory nerve body leading to early mitochondrial dysfunction with loss of membrane potential³¹Particularly the decrease in NCV is due to decrease in the activity of Na⁺/K⁺ ATPase.³²

Our results indicated that cisplatin control rats showed body weight reduction during the experimental period.EPO, BT and combination improved body weight from the initial value. Rats treated with combination exhibited less percentage of loss of body weight compared to EPO and BT alone thus the combination improved general health of rats by improving the body weight. Reactive oxygen species (ROS) such as superoxides and hydroxyl radicals cause vascular endothelial damage and reduced nitric oxide mediated vasodilatation. Studies have also showed evidence that superoxides and peroxyntirite impairs endothelium dependent vascular relaxation of epineural arterioles of the sciatic nerve in rats ^{33,34}EPO has recently been considered as a tissue protective cytokine especially within nervous tissue, kidney and cardiac muscle. EPO is reported to protect neurons from ischemia reperfusion-induced injury, metabolic stress, HIV-induced damage or even mechanical injury such as nerve compression or trauma ¹⁷

EPO has also protected neurons from apoptosis in cell culture studies and in animal models of central nervous system injury, EPO has shown to increase expression of antioxidant enzymes, reduces nitric oxide mediated formation of radicals, normalize cerebral blood flow and promote neuroangiogenesis. EPO has shown to regulate bone marrow erythroid cell proliferation, differentiation, and survival through its binding to an erythroid progenitor cell surface EPO receptor (EPOR). The EPOR also is expressed in myelin sheaths of radicular nerves in human peripheral nerves, suggesting both a developmental and potential protective role for EPO not only in the central nervous system, but also in disease entities that involve the peripheral nervous system.³⁵

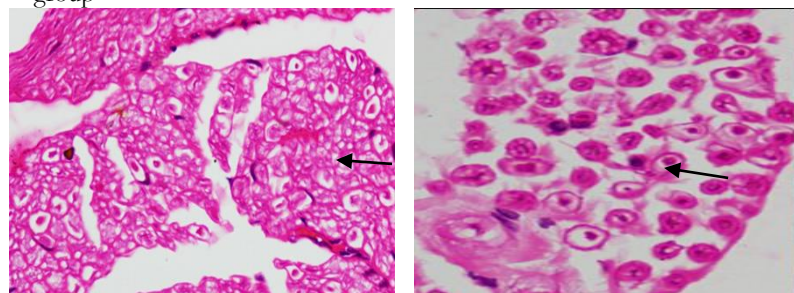
The decrease in the NCV, muscle in coordination, grip strength, pain sensitivity is significantly increased in EPO treated rats compared to cisplatin control. The protective role of EPO probably due to increase in expression of antioxidant enzymes and its ability to inhibit apoptosis. BT is a transketolase (TK) activator.³⁶ BT was shown to prevent experimental diabetic retinopathy and in vitro hyperglycemia-induced endothelial dysfunction. The effects of benfotiamine on in vivo endothelial function remained unknown.BT has shown to inhibit hexosamine pathway, advanced glycation end product (AGE) formation

pathway and the diacylglycerol (DAG) protein kinase C (PKC) pathways. BT was significantly augmented the decrease in the NCV, muscle in coordination, grip strength, pain sensitivity compared EPO treated cisplatin control rats. The protective role of BT probably due to its activity as co enzyme in various biological pathways³⁷and inhibiting the superoxides and hydroxyl radicals induced epineural arterioles vascular endothelial damage and reduced vasodilatation.

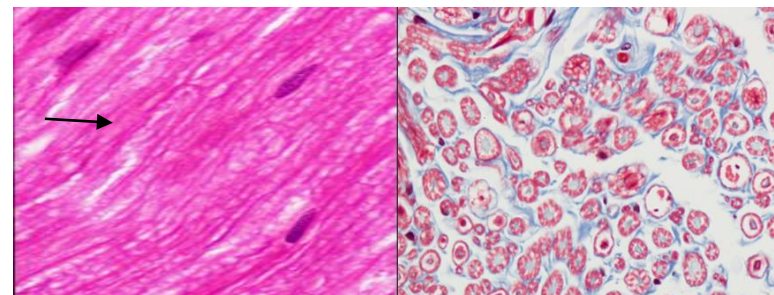
Morphological study of siatic nerve of normal rats showed closely packed nerve fibers normal endoneurial matrix separating the nerve fibers(Fig 11A, Fig 11D), admixture of large and small diameter myelinated fibers and the thickness of the myelin sheath is proportionate the width of the axonal diametr(Fig 11 E).

Conversly, cisplatin control rats displayed the histological damages like reduced nerve fiber density in the endoneurium (Fig 12A), scattered fibers with axonal swelling and degeneration (arrows). (Fig 12 B), presence of thick axons (Fig 12 C), presence of numerous enlarged axonal profiles surrounded by thinner myelin sheaths indicating axonal swelling and axonopathy. (Fig 12 F)

Figure 11: Histology of rat sciatic nerve of normal control group

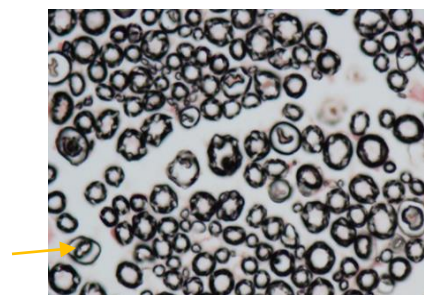


A. Hematoxylin and Eosin 160XB.Hematoxylin and Eosin 160X



C.Hematoxylin and Eosin 160X

D.Masson's trichrome 160 X

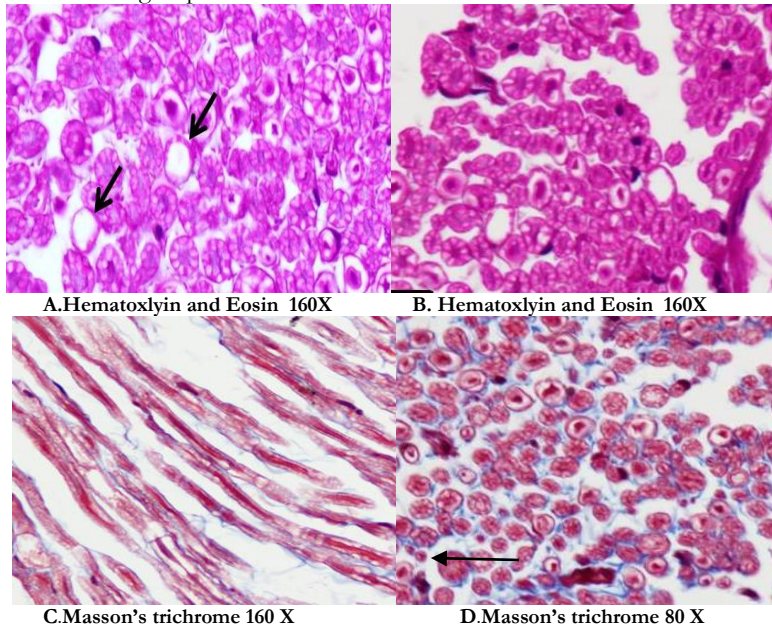


E.Kulchitsky pal stain 160X

(A) light microscopy transverse section showing closely packed nerve fibers and an occasional endoneurial blood vessel. (B) light microscopy transverse

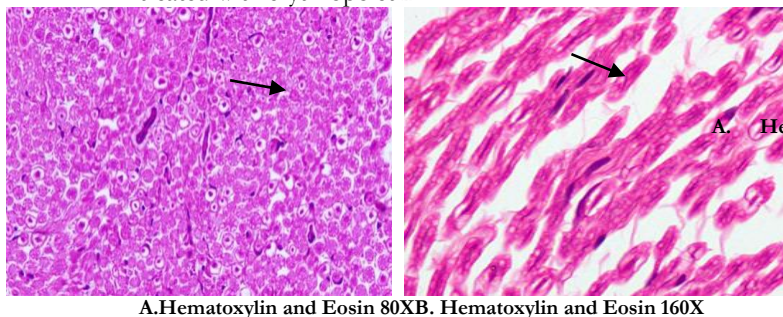
section showing individual nerve fibers and a central axon surrounded by a sheath of myelin.(C) longitudinal section showing the elongated schwann cell nuclei and longitudinally oriented axons with myelin sheaths.(D)transverse section of special stain for collagen highlights the endoneurial matrix separating the nerve fibers and collagenous component is stained blue. (E) 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 the width of the axonal diameter.

Figure 12: Histology of rat sciatic nerve of cisplatin control group

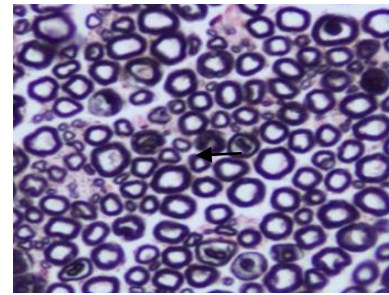


(A) light microscopy of transverse section showing small nerve funicles with reduced fiber density,(B) scattered fibers with axonal swelling and degeneration (arrows). (C) light microscopy of longitudinal section showing thick axons. (D)transverse section of special stain for collagen highlights axonal degeneration with dilated axons. (E) transverse section of special stain for myelin reveals the same. (F)higher magnifications highlight the presence of numerous enlarged axonal profiles surrounded by thinner myelin sheaths indicating axonal swelling and axonopathy.

Figure 13: Histology of rat sciatic nerve of cisplatin control treated with erythropoietin.



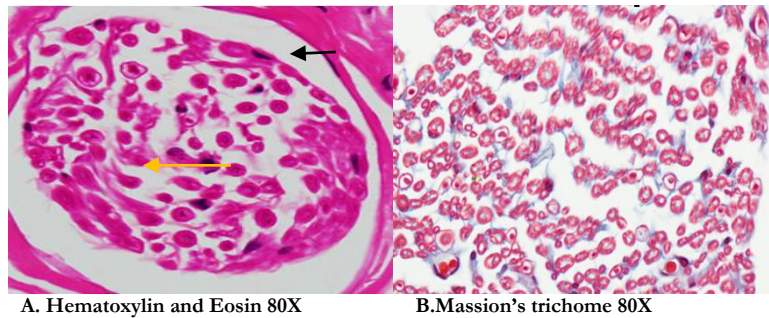
A.Hematoxylin and Eosin 80XB. Hematoxylin and Eosin 160X



C. Kulchitsky pal stain 160X

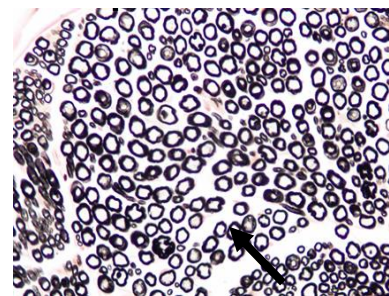
(A) light microscopy of transverse section showing normal density of myelinated fibers. (B) light microscopy of longitudinal section reveal normal longitudinally oriented nerve fibers. (C) transverse section of myelin stain confirms the same.

Figure 14: Histology of rat sciatic nerve of cisplatin control treated with benfotomiane



A. Hematoxylin and Eosin 80X

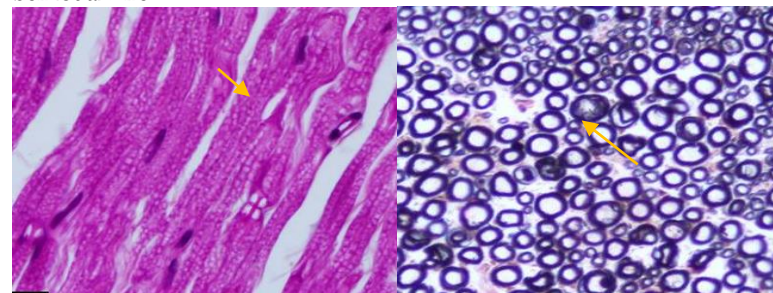
B.Massion's trichome 80X



C.Kulchitsky pal stain 80X

(A) light microscopy of transverse section of a small nerve funicle with preserved fiber density.(B) transverse section showing normal endoneurial matrix and collagen.(C) transverse section of special stain for myelin confirms mild to moderate reduction of density of myelinated fibers.

Fig.15: Histology of rat sciatic nerve of cisplatin control treated with combination of erythropoietin and benfotiamine.



A. Hematoxylin and Eosin 80X

B. Kulchitsky pal stain 80X

(A) light microscopy of longitudinal section showing normal fiber density.(B) transverse section of special stain for myelin confirms the same.

The altered sodium cell gradient due to impairment of Na^+/K^+ ATPase activity leading to altered membrane environment which in turn causes histological

damages, and decrease myelin protein expression. Cisplatin control rats treated with EPO exhibited normal density of myelinated fibers (Fig.13.A), normal longitudinally oriented nerve fibers. (Fig.13. B) and was further confirmed by the transverse section of myelin stain (Fig.13. C). Similarly cisplatin control rats treated with BT exhibited a small nerve funicle with preserved fiber density (Fig.14.A), showing normal endoneurial matrix and collagen. (Fig.14.B), however special stain for myelin confirms mild to moderate reduction of density of myelinated fibers. (Fig.14. C)

Cisplatin control treated with combination of EPO and BT revealed normal fiber density (A) in longitudinal sections (Fig.15.A) and further the transverse section of special stain for myelin confirms the same Showing (Fig.15.B). Treatment with EPO, BT and combination almost completely prevented the histological damages induced by diabetes. EPO and BT could probably prevent the histological damages induced by chemotherapy due to prevention of chemotherapy induced oxidative stress.

CONCLUSIONS

- EPO, BT and their combination has shown significant curative effect in cisplatin induced peripheral neuropathy in rats.
- Combination of EPO and BT exhibited synergistic effect

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