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RESEARCH ARTICLE

The Investigation of Ameliorating Effect of Methylene Blue on Cisplatin-Induced Neurotoxicity in Female Rats

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ABSTRACT

Objective: Cisplatin is indispensable in many solid tumors, and peripheral neurotoxicity is an essential dose-limiting side effect. The study aims to investigate the ameliorating effect of methylene blue, which has anti-oxidant properties, in rats with neurotoxicity due to cisplatin treatment.

Materials and methods: Twenty-four adult female rats were included in the study and divided into 3 groups. The first group (n=8), the control group, did not receive any treatment. The second (n=8) and the third (n=8) group received 2.5 mg/kg/day of cisplatin and 1 ml/kg/day of 0.9% NaCl (saline) twice a week for 4 weeks. Also, the third group received 20 mg/kg/day of methylene blue every day for 4 weeks. Blood samples were collected from rats for malondialdehyde (MDA), glutathione (GSH), tumor necrosis factor- α (TNF-α), and interleukin-6 (IL-6) levels, and electromyography (EMG) and motor function were evaluated.

Results: In non-treated cisplatin injected rats, a significant increase in the lipid peroxidation product MDA and pro-inflammatory cytokines compared to the control group ($p<0.001$). Electromyography measurements also resulted in a significant decrease in cisplatin injected rats' compound muscle action potential (CMAP) amplitude and prolongation in CMAP latency compared to the control group ($p<0.05$). A statistically significant decrease in MDA, TNF-α, and IL-6 levels and a statistically significant increase in GSH levels and CMAP amplitude was observed in the methylene blue-treated group compared to the group injected with cisplatin alone.

Conclusions: We concluded that methylene blue has ameliorating effects against cisplatin-induced neurotoxicity (CIN) by enhancing anti-oxidative capacity, energy metabolism and suppressing inflammatory parameters and oxidative stress.

Keywords: Cisplatin, methylene blue, inflammation, neurotoxicity, tumor necrosis factor-alpha

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INTRODUCTION

Cisplatin was first discovered by the Italian chemist Michele Peyrone in 1844. Its first use in cancer treatment started with testicular cancer by Dr. Rosenberg in 1978. Later, its effectiveness has been proven in many solid tumors such as lung, bladder, endometrium, head and neck tumors [1]. Cisplatin shows its effect by binding to purine bases in DNA in the alkylating chemotherapeutics group. Deoxyribonucleic acid damage and apoptosis occur due to this reaction [2]. Ototoxicity, nephrotoxicity, and neurotoxicity are among the most common side effects of cisplatin [3]. Cisplatin-induced neurotoxicity (CIN) is seen with 30-80% frequency. This side effect is dose-dependent and is in the form of peripheral-sensory neuropathy. Peripheral-sensory neuropathy is characterized by numbness, unpleasant distal paresthesia, Lhermitte's symptoms, significant fiber sensory loss, tremor, and sensory ataxia [4]. The development of this side effect leads to dose reduction, treatment delay, and premature discontinuation of chemotherapy [5].

Neurons in the dorsal root ganglia (DRG) are the main targets of CIN. Chronic damage occurs in DRG neurons by binding cisplatin to DNA [6]. This damage develops oxidative stress, mitochondrial and nuclear

DNA damage, mitochondrial dysfunction, and eventually apoptosis. The mechanism of CIN was demonstrated electro-physiologically and by histopathology examinations in many animal models [7].

Cisplatin-induced neurotoxicity, which is usually irreversible, is the most crucial dose-limiting side effect in cancer treatment. Therefore, the use of effective neuroprotective and anti-oxidant therapies was investigated in various studies [8]. A combination of nicotine and menthol, glutathione (GSH), amifostine, curcumin, vitamin E, melatonin, resveratrol are some of the chemo-protectants used to prevent CIN [9-13].

Methylene blue was used to treat malaria and methemoglobinemia in the 1800s [14]. The high affinity of methylene blue to the nervous system was discovered many years later. The mitochondrial electron transfer chain from NADH to cytochrome c reduces oxidative stress and shows the mitochondrial protective effect [15]. Thanks to this feature, its use in treating many neurodegenerative diseases have been investigated. These include Alzheimer's disease, Parkinson's disease, Bipolar depression, ischemic and traumatic brain injury [16]. In some studies, conducted, the neuroprotective effect of ifosfamide, another chemotherapeutic agent commonly used in hematology and oncology, was revealed using the same method to prevent side effects [17-19].

The primary aim of our study was to determine the ameliorating effect of methylene blue in CIN by the evaluation of inflammatory markers, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and oxidative stress biomarkers including GSH and malondialdehyde (MDA), and to correlate these markers with electromyography (EMG) recordings. To our knowledge, this is the first study in the literature to show the protective effect of methylene blue against CIN.

MATERIALS AND METHOD

Animals

This study used 24 Wistar adult female rats with an average weight of 200 grams. Animals were placed in cages, and they remained on 12-hour light-dark cycle at room temperature. (22.5±2.5 °C). They were fed a standard pellet diet and tap water *ad libitum* throughout the study. All experiments were performed in line with the Guide for the Care and Use of Laboratory Animals rules adopted by the National Institutes of Health (U.S.A). Approval was granted by the Animal Ethics Committee (Ethical number: 03210416). All chemicals were obtained from Sigma-Aldrich Inc. unless otherwise noted.

Experimental Procedure

Twenty-four rats included in the study were assigned into 3 groups. Group 1 (n= 8) served as the control group, and no treatment was administered. As previously described by [20], Group 2 (n=8) was treated with cisplatin intraperitoneally (ip) twice a week at a dose of 2.5 mg/kg for 4 weeks (total dose 20 mg/kg) and 1 ml/kg/day % 0.9 NaCl (saline). According to the recommendations for the conversion of doses between animals and humans published by the Department of Health and Human Services and the FDA, Group 3 (n=8) was exposed to the same dose of cisplatin and treated with methylene blue every day at a dose of 20 mg/kg during 4 weeks [21]. Two rats of group 2 died during the study. There were no deaths in rats receiving cisplatin and methylene blue.

Figure 1. Design and timeline of the study protocol

All rats were tested for EMG and motor function tests at the end of the study. Following EMG recordings, euthanasia was performed with a high dose anesthesia combination (100 mg/kg, Ketasol, Richterpharma/xylazine 50 mg/kg, Rompun, Bayer) and cervical dislocation. After collecting blood samples of animals by cardiac puncture, the biochemical analysis was performed at the end of the study. The study's design was summarized in **Figure 1**.

Electrophysiological Recordings

Electromyography studies were recorded 4 weeks after cisplatin administration. Rats were deeply anesthetized by the mixture of intraperitoneal ketamine (50 mg/kg, Ketasol, Richterpharma)/xylazine (10 mg/kg, Rompun, Bayer). Then, EMG was obtained by stimulating the right sciatic nerve three times supramaximally (duration 0.05 ms, intensity 10 V, frequency 1 Hz, in the range of 0.5-5000 Hz, 40 kHz/s with a sampling rate). In this method, a bipolar needle stimulation electrode placed subcutaneously in the sciatic notch was used (BIOPAC Systems, Inc, Santa Barbara, CA). Unipolar platinum electrodes were used to record compound muscle action potentials (CMAP) from 2-3 interosseous muscles (**Figure 2**) [22].

The Biopac Student Lab Pro software (BIOPAC Systems, Inc. version 3.6.7) evaluated the amplitude of CMAP and the distal latency. During the EMG recordings, the rectal temperatures of the rats were kept at 36.5±0.5 °C by a heating pad. Rectal probes (HP Viridia 24-C; Hewlett-Packard Company, Palo Alto, CA) were used to monitor the rats' temperatures.

Assessment of Motor Function

The motor performances of the rats were evaluated according to the inclined-plate test described by Rivlin and Tator [23]. Rats were placed obliquely on the long axis of the 10-degree inclined plate. The angle of inclination gradually

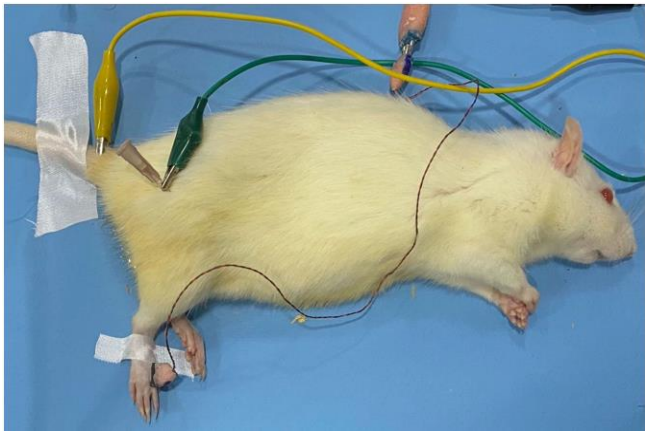

Figure 2. EMG was obtained by stimulating the right sciatic nerve

increased, and the maximum angle of the plate at which the rat kept its position for 5 s without falling was recorded as motor score. Measurement of the inclined plate angle was recorded three times in each rat.

Determination of MDA Level

The thiobarbituric acid reactive substances (TBARS) were used for detecting lipid oxidation by measuring MDA plasma levels as previously described by [24]. The plasma sample was mixed with TBARS reagent and trichloroacetic acid, and the mixture was incubated at 100 °C for 1 hour. This mixture was cooled on ice, and the samples were centrifuged at 3000 rpm for 20 minutes. After centrifugation, the absorbance of the supernatant was read at 535 nm. Calibration was done with tetra ethoxy propane, and MDA levels were stated as nM.

Determination of Tissue GSH Levels

According to the Ellman method, spectrophotometry was used for measuring the GSH content in the plasma samples. In this method, thiols interact with 5, 5'-dithiobis-(2-nitrobenzoic acid) [25]. It converts to an anion form at 412 nm with a maximum peak. GSH levels were expressed as μm and calculated on the standard calibration curve.

Measurement of Inflammatory Markers

An available enzyme-linked immunosorbent assay (ELISA) kit (Biosciences) was used for measuring inflammatory markers, including plasma IL-6 and TNF-a levels.

Statistical Analysis

Statistical package for social sciences (SPSS, version 15.0) was used for all statistical analyses. One-way analysis of variance (ANOVA) and Student *t* test were used for evaluating parametric variables, and post-hoc Bonferroni correction was done for subgroup evaluation. Also, a comparison of the groups of nonparametric variables was determined by the Mann–Whitney U test. Whether the Shapiro-Wilk test determined the normal or non-normal distribution of variables. Variables were presented as mean values±standard error of the mean (SEM), and p≤0.05 was considered statistically significant.

Table 1. The Effects of Methylene Blue on Plasma MDA, TNF- α, IL-6, and GSH Levels in all Groups

	Normal control	Cisplatin + Saline	Cisplatin + 20 mg/kg Methylene blue
MDA (nM)	54.8±6.2	139.5±11.02**	92.2±5.7##
TNF-alfa (pg/ml)	18.6±2.9	86.5±4.4**	41.8±3.3#
IL-6 (pg/ml)	9.2±1.1	651.1±17.9**	485.5±22.4#

*p<0.01, **p<0.001 (different from control group),
#p<0.05, ## p<0.001 (different from cisplatin and saline group)
MDA: Malondialdehyde; TNF: Tumor necrosis factor; IL: Interleukin

RESULTS

Plasma Inflammatory and Oxidative Stress Marker Levels

The effects of methylene blue on plasma MDA, TNF-α and IL-6 levels in all groups are shown in **Table 1**. Note that the results were presented as mean±SEM. Statistical analyses were performed by one-way ANOVA test. The cisplatin injection significantly increased plasma MDA levels compared to the control group (139.5±11.02 vs. 54.8±6.2 nM; p<0.001).

Methylene blue treatment significantly decreased mean MDA levels in 20 mg/kg injection levels compared to the non-treated cisplatin injected rats (92.2±5.7 nM; p<0.001). Cisplatin treatment significantly increased plasma TNF-α and IL-6 levels compared to the control levels (86.5±4.4 vs. 18.6±2.9 pg/ml; p<0.001, 651.1±17.9 versus 9.2±1.1 pg/ml; p<0.001). Methylene blue treatment decreased TNF-α and IL-6 levels compared to the non-treated cisplatin injected rats (41.8±3.3 pg/ml; p<0.05, 485.5±22.4 pg/ml; p<0.05).

The cisplatin injection significantly decreased plasma GSH levels compared to the control group (6.1±0.7 vs. 14.2±1.5 μM; p=0.012). Methylene blue treatment increased GSH levels (10.3±1.8 μM; p=0.023) (**Figure 3**).

EMG and Motor Function Assessment

Table 2 shows all groups' alterations in electrophysiological recordings and inclined plane scores. The amplitude of CMAP was significantly lower, and the latency was significantly prolonged in the non-treated cisplatin injected rats compared to the control group (p<0.05). The amplitude of CMAP was significantly increased when methylene blue treatment was given (p<0.05). Moreover, CMAP latency shortened in the methylene blue treatment group against the non-treated cisplatin-injected rats, but no statistically significant was found. Electromyography recordings in all groups are demonstrated in **Figure 4**.

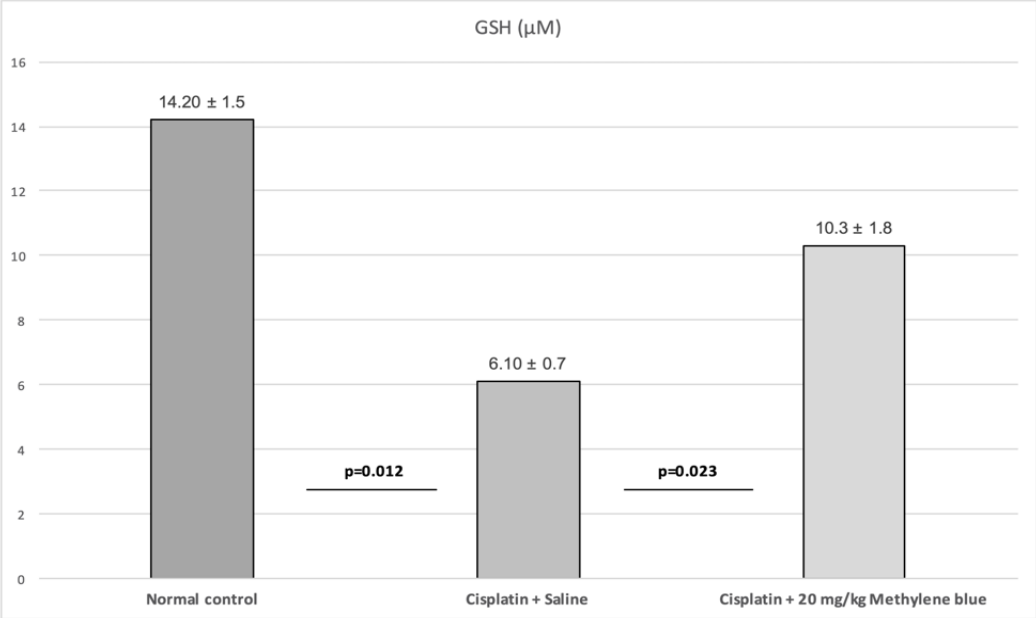

Figure 3. Comparison glutathione (GSH) levels between groups

Table 2. The Alterations in Electrophysiological Recordings and Inclined Plane Scores in All Groups

	Normal control	Cisplatin + Saline	Cisplatin + 20 mg/kg methylene blue
CMAP latency (ms)	2.25±0.03	2.71±0.04*	2.4±0.09
CMAP amplitude (mV)	12.4±0.2	4.6±0.43*	8.7±1.29#
Inclined plane score (°)	89.5±5.1	57.4±6.7**	78.4±4.2#

*p<0.05, **p<0.001 (different from control group),
#p<0.05, ##p<0.001 (different from cisplatin and saline group)
CMAP: Compound muscle action potential

The inclined plane score was significantly lower in the cisplatin group compared to the control group (57.4±6.7 vs. 89.5±5.1 °; p<0.01). Methylene blue treated rats (Group 3) significantly increased the inclined plane scores compared to non-treated cisplatin injected rats (Group 2).

DISCUSSION

This study shows a significant increase in lipid peroxidation products and inflammatory markers in rat models exposed to cisplatin compared to the control group. Still, EMG recordings obtained by stimulating the right sciatic nerve resulted in decreased CMAP amplitude and prolongation in CMAP latency due to chemotherapy. In rats treated with methylene blue, both a significant decrease in oxidative stress and inflammation and a protective effect from CIN were observed.

Several processes cause CIN. Oxidative stress, axonal degeneration, neuroinflammation, ion channel alterations, and immune system activation are among them.

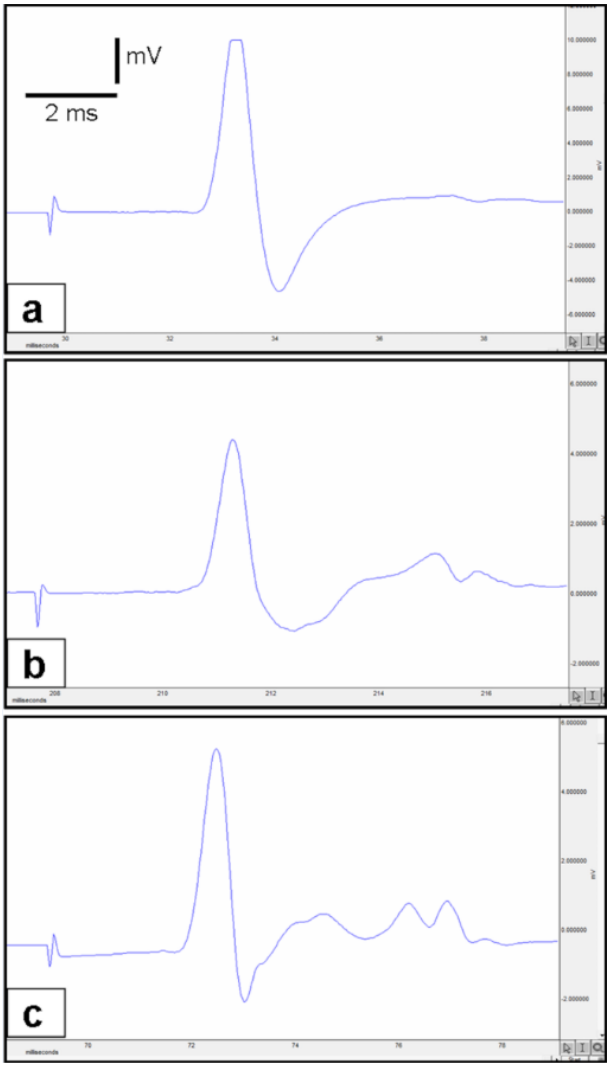

Figure 4. EMG recordings in all groups. a) Normal group, b) Cisplatin+Saline, c) Cisplatin+Methylene blue

The buildup of cisplatin in sensory neurons in the DRG causes nuclear and nucleolar destruction. Apoptosis is induced when platinum compounds bind to nuclear and mitochondrial DNA. Mitochondrial malfunction, oxidative stress, and increased free oxygen radicals have all been linked to this pathway [26]. Lipid peroxidation is also caused by superoxide anions, hydrogen peroxide, and hydroxyl radicals. As a result, MDA levels are rising [27]. Reduced GSH levels and eventual neurotoxicity emerge from damage to multiple anti-oxidant mechanisms such as superoxide dismutase and glutathione peroxidase [28].

Another mechanism in developing CIN is increasing various pro-inflammatory cytokines such as IL-1, IL-6, TNF-α, and INF-γ after treatment [29]. As a consequence of this increase, bradykinin, serotonin, and histamine release are increased. Due to neuro-inflammation, progressive neural sensitization is seen in schwann cells in the DRG, astrocytes, and satellite cells in the spinal canal [30]. In a study conducted in the literature, an increase in IL-6 and IL-6 receptors was observed in patients after breast cancer treatment. The relationship between pain symptoms, chemotherapy-induced neuropathy, and the IL-6 signaling pathway was demonstrated [31].

Long-term usage of platinum compounds has been proven to cause axonal degeneration in human and animal studies published in the literature. The axonal skeleton is disrupted by the loss of myelin and intraepidermal nerve fibers, in particular [32]. Axonal transport loss causes degeneration, especially in the distal axon. The loss of kinesin and dynein motor proteins also contributes to this degeneration [33]. Neuroinflammation occurs as a consequence of oxidative stress. The activated microglia release unregulated reactive oxygen species and reactive nitrogen products. In this case, this results in axonal transport loss and axonal degeneration [34].

There are many studies conducted with anti-oxidants in the literature to prevent CIN [35]. Methylene blue is an antimalarial drug, medical staining agent, and biochemical anti-oxidant. Studies have shown that methylene blue inhibits guanylate cyclase and nitric oxide synthase. It directs electrons from NADH to cytochrome c in the mitochondrial electron transfer chain. This way, it preserves mitochondrial function and prevents oxidative stress [15]. The study conducted by [17] showed that methylene blue resolved the complete resolution of ifosfamide-induced neurotoxicity within 12 hours. In this case, methylene blue was administered as 50 mg IV every 4 hours, and the neurological symptoms associated with ifosfamide completely disappeared after 12 hours. When methylene blue is used in low doses, it prevents lipid β oxidation, oxidative stress, acute inflammatory response, and cytokine release, but when used at high doses, it causes methemoglobinemia [36].

Moreover, methylene blue minimizes free oxygen radicals by increasing global glucose uptake, cerebral blood flow, and oxygen consumption in normal rats [37]. It also improves neurocognitive functions in Parkinson's and Alzheimer's model-generated rats by decreasing the cerebral infarct volume [38]. Another study on rats showed that GSH levels increased and mitochondrial biogenesis was increased in animals treated with methylene blue [39].

Malondialdehyde is the end product of the peroxidation of polyunsaturated fatty acids. It has been used as a biomarker of oxidative stress since the 1960s [40]. In a study conducted by [41], in rat models with ischemia-reperfusion injury in the lung, methylene blue decreased reactive oxygen radicals and MDA levels and increased superoxide dismutase activity. In another rat study with mesenteric ischemia-reperfusion injury, in the group treated with methylene blue, a decrease in superoxide radicals and an increase in GSH levels were observed through xanthine oxidase [42]. In a study conducted by [43], contrary to the literature, in rats with unilateral testicular torsion, treatment with methylene blue showed an increase in opposite testicular damage and oxidative stress compared to the control group.

Consistent with other studies in the literature, a decrease in CMAP amplitude and a prolongation in CMAP latency were observed in rats exposed to platinum in our study. The especially axonal loss was blamed for this change in EMG recordings. In a study [44], the neuroprotective effect of oxytocin was shown in rat models with CIN. Oxytocin administration of 160 μg/kg resulted in a statistically significant prolongation in CMAP amplitude, but the same effect was not observed with 80 μg/kg oxytocin administration. The latency of CMAP was shortened at both doses but was not statistically significant [44]. In our study, in the rat group treated with methylene blue, it was observed that a statistically significant increase in CMAP amplitude and inclined plane score and a shortening of CMAP latency, which did not reach statistical significance.

However, our study has some limitations. First of all, our study is an animal study that results cannot be applied directly to humans. Primarily, more extensive preclinical studies are needed. Secondly, the number of animals used in our study is small due to ethical constraints. The dosage and timing of methylene blue should be determined by forming larger groups.

CONCLUSIONS

We concluded that methylene blue has anti-inflammatory and neuroprotective properties on neuropathy model induced by cisplatin through suppressing neuroinflammation, increasing anti-oxidant capacity and preventing mitochondrial dysfunction. Our results encourage researchers to investigate methylene blue's underlying anti-inflammatory and neuroprotective mechanism, promising in CIN treatment, in-depth.

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