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N-acetylcysteine versus progesterone on the cisplatin-induced peripheral neurotoxicity

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Background: *Cisplatin-induced peripheral nerve neurotoxicity (CIPN) is the main obstacle in cisplatin treatment. The aim of this study was to compare the modulatory effects of N-acetylcysteine (NAC) and progesterone on CIPN, because there are scarce literature data on the protective effect of the progesterone on the CIPN.*

Materials and methods: *Twenty-four rats were divided into four groups: control, cisplatin-treated, concomitant cisplatin-treated and NAC-treated, and concomitant cisplatin-treated and progesterone-treated. Electron microscopic, immunohistochemical, real time polymerase chain reaction and histomorphometric analysis; oxidative/antioxidative markers (MDA/GSH and SOD), neurotoxic/neuroprotective markers (iNOS/nNOS), inflammatory mediators (TNF-α and NF-κB) and BAX were done.*

Results: *The myelin sheath in the cisplatin-treated group elucidated infolding. The myelin was disfigured, degenerated, and extensively split with areas of focal loss. The axoplasm was atrophic. Ballooning and vacuolations of the mitochondria with alterations of Remak bundles structures were observed. Fewer of these changes were noted in the NAC and progesterone-treated groups. Decrease of the antioxidant SOD and GSH (81% and 64%) and increase of the oxidant MDA (9 folds), increment of the neurotoxic iNOS (1.9 folds) and decrement of the neuroprotective nNOS (64%) and elevation of the inflammatory mediators' TNF-α and NF-κB (8.3 and 11 folds) in the cisplatin-treated group. Increase of the antioxidant SOD (1.3 and 2.5 folds) and GSH (120% and 79%) and decrease of the oxidant MDA (69% and 88%), decrement of the neurotoxic iNOS (56% and 68%) and increment of the neuroprotective nNOS (1.6 and one folds) and elevation of the inflammatory mediators' TNF-α and NF-κB were observed in the NAC and progesterone-treated groups, respectively.*

Conclusions: *The toxic effect of CIPN might be attributed to either oxidative or severe inflammatory stress. Progesterone is efficient in ameliorating these effects; however, NAC is better.* (Folia Morphol 2018; 77, 2: 234–245)

Key words: N-acetylcysteine, progesterone, cisplatin-induced peripheral neurotoxicity

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INTRODUCTION

Cisplatin is a platinum-derived chemotherapeutic drug that is now employed in the treatment of head and neck, ovarian, testicular, bladder, colorectal and lung cancers [19]. Peripheral neuropathy is one of the main complications of chemotherapeutics [40]. They range from 10% to 100% depending on the anticancer drug [5]. The peripheral neuropathy occurs in about 20% of patients given standard doses of chemotherapy and in almost 100% of patients treated with high doses [40]. The chemotherapy-induced peripheral neurotoxicity could be considered the main reason for reduction or even stoppage of the treatment [52]. The main roles in the mechanism of cisplatin-induced cytotoxicity are inflammatory cytokines, oxidative stress and DNA damage [10, 30]. The mechanisms of cisplatin-induced neurotoxicity in the nervous system include inflammation, DNA damage, apoptotic cell death, oxidative damage and mitochondrial dysfunction [16, 53].

The efficiency of antioxidative treatments on preventing platinum-induced neurotoxicity has been established in several studies [2, 9, 55]. Antioxidants such as N-acetylcysteine (NAC) [18] have been used to reduce this type of toxicity. NAC is the acetylated form of L-cysteine, which has a peripheral neuroprotective effect through free radical elimination activity; cysteine helps in the synthesis of glutathione which is a very important natural antioxidant [26]. Progesterone is a neuroactive steroid that is considered as an important neuroprotective and remyelinating agent. The probable mechanisms by which progesterone produces its neuroprotective effects are debatable; it could be either through inhibition of oxidative stress and apoptosis or through the decrease of inflammation [50].

Although many researchers studied the protective effect of NAC cisplatin-induced peripheral neurotoxicity (CIPN), its mechanism of action is still debatable. Few types of researchers studied the protective effect of progesterone on the chemotherapy-induced peripheral neurotoxicity.

So, the aim of the present work was to spot the deleterious effects of cisplatin on peripheral nerves, evaluate the modulatory effects of the progesterone on the CIPN and to compare this effect with the effect of the NAC on the CIPN. The comparison included the structural changes of the sciatic nerve and the possible mechanism of function of both drugs. The study was directed on adult male albino rats using electron microscopic, immunohistochemical and oxidative tissue markers histomorphometric analysis and real-time polymerase chain reaction (PCR).

MATERIALS AND METHODS

Chemicals

Cisplatin was purchased from Sigma Chemicals Corp. (Saint Louis, MO, USA). The used vehicle was sterile saline. The rats were injected at a dose of 2 mg/kg/twice weekly/4 weeks intraperitoneally [31, 49]. The neurotoxicity induced by such dose is related to the direct effect of cisplatin and is not secondary to modulation of the drug nephrotoxicity [31]. In addition, such dose doesn't affect the rats' general condition [31].

N-acetylcysteine was purchased from Sigma--Aldrich (Saint Louis, MO, USA). The used vehicle was sterile saline. The rats were injected at a dose of 50 mg/kg/daily/4 weeks intraperitoneally [14]. At this dose, the NAC is not nephrotoxic. In addition, no efficacy occurs to cisplatin at such dose [1].

Progesterone was obtained from Algomhuria Pharm. and Chem. Co. The used vehicle was peanut oil. The rats were injected at a dose of 4 mg/kg/daily/4 weeks intraperitoneally [11]. This dose was selected based on previously published reports showing that this was a relatively low dose that was effective with repeated treatment in other models of nerve injury [22, 44, 45].

Animals

Twenty-four Sprague-Dowley adult male albino rats weighing 200–220 g were used in the present study. The rats were obtained from the animal house, Faculty of Medicine, Cairo University, Egypt. They were acclimatised to standard laboratory conditions; temperature 20°C, allowed free water supply and fed ad libitum. All the animals were treated in accordance with the international guidelines for the care and use of laboratory animals.The study was approved by the Ethics Committee, Faculty of Medicine, Cairo University (2431/2016). The animals were observed twice per day for signs of morbidity and mortality. The general toxicological data were recorded including the motility, food and water consumption, health status and body weight gain. The weights of each individual animal were recorded in the beginning and at weekly intervals until the end of the experiment.

All animal procedures followed the ethical guidelines of experimental animals. Therefore, the study had been performed according to the ethical stand-

ards of the National Institutes of Health guide for the care and use of Laboratory Animals (NIH Publications No. 8023, revised 1978), and we followed these guidelines.

Experimental design

The rats were divided into four groups, 6 rats each:

1 — control group;
2 — cisplatin-treated group;
3 — concomitant cisplatin and NAC-treated group;
4 — concomitant cisplatin and progesterone-treated group.

The animals were sacrificed after 4 weeks. An incision was made on the back of both thighs, and the sciatic nerves were exposed near the greater sciatic foramen. Five small segments, each 5–7 mm in length, from each sciatic nerve were cut and kept in the fixative in the refrigerator overnight.

Ultrastructural preparations. For electron microscopy, small pieces of the sciatic nerve from each rat were fixed in 2% paraformaldehyde and 2% glutaraldehyde solution in 0.1 mol/L phosphate buffered saline (PBS) pH 7.2 and kept in the refrigerator overnight, rinsed in 0.1 mol/L PBS and postfixed in phosphate-buffered 1% osmium tetroxide. To obtain cross-sections, the nerve samples were oriented longitudinally in a flat mold and then embedded in resin. Semithin cross-sections were stained with 1% toluidine blue and examined by light microscope for proper orientation and image analysis of axons number. Ultrathin sections (50–60 nm) were stained with uranyl acetate and lead citrate. These sections were examined and photographed using a Jeom-1400 transmission electron microscope (JEOL Ltd./Japan a Joel), Electron Microscopy Department, Faculty of Agriculture, Cairo University, Egypt.

Immunohistochemistry. Five sciatic nerve paraffin blocks were prepared from each rat. The sections were dewaxed, dehydrated, and incubated with 3% hydrogen peroxide solution for 30 min at room temperature and endogenous peroxidase activity was blocked. Then, the sections were washed with distilled water, a microwave was utilised for tissue antigen retrieval. The buffer solution was cooled and the section was taken. The sections were cleaned with distilled water and put into PBS for 5 min. After PBS was wiped off, the appropriate amount of serum was added into the section at room temperature for 30 min. Then, the serum was removed. All techniques were done according to the manufacturer's instructions.

Apoptosis (BAX) in sciatic nerve. Five sciatic nerve sections from each paraffin block were incubated with anti-rat BAX monoclonal antibody ([E63], (ab32503) 1:100) at 4°C overnight. The sections were maintained at room temperature for 30 min, and rinsed with PBS (pH 7.4) three times, each for 5 min. After removal of PBS, the sections were incubated with anti-rabbit IgG (ab205718) at 37°C for an hour and a half, and rinsed three times with PBS (pH 7.4), each for 5 min after removal of PBS.

Inducible nitric oxide synthase (iNOS). Five sciatic nerve sections from each paraffin block were incubated with a reaction buffer containing primary antibody rabbit polyclonal antibody to iNOS (ab15323 at a dilution of 1:100) for at least 14 h at 4°C. Afterwards the sections were incubated with goat anti-rabbit IgG H&L (HRP) (ab205718) for 20 min at 37°C. Each step was followed by adequately washing with PBS (0.1 mol/L, pH 7.2–7.4).

Tumour necrotic factor alpha (TNF-α). Five sciatic nerve sections from each paraffin block were incubated overnight at 4°C with the following primary antibody rabbit polyclonal to TNF-α (ab66579 diluted 1:500). The primary antibody was diluted in PBS, sections were subsequently incubated for 1 hour at room temperature with secondary with goat anti-rabbit IgG H&L (HRP) (ab205718) in PBS containing 0.3% Triton X-100 and 1% body surface area.

3,3'-diaminobenzidine (DAB) was applied as the chromogen, and all the previous immunohistochemical sections were visualised with DAB (Sigma, Aldrich.) and observed under a microscope to visualise the development of colour. Following rinsing with distilled water and running water successively. The sections were counterstained with haematoxylin, permeabilised with xylene, mounted with resin and treated with a decreasing ethanol series. All antibodies were purchased from Abcam, United Kingdom. Negative control sections were performed using the same previous procedure except that the primary antibody was replaced by non-immune mouse serum immunoreactivity. The iNOS positive control is lung; Raw 264.7 whole cell lysate + PMA + LPS + Brefeldin A. The TNF-α and BAX positive control are the human mammary cancer. The TNF-α antibody gave a positive signal in the following LPS stimulated lysates: THP1, Raw264.7 IF/ICC: PMA/Befreldin/LPS treated RAW246.7 cell line.

Oxidative/antioxidative marker. Five sciatic nerve sections from each rat:

— **Oxidative stress marker:** Malondialdehyde (MDA) was measured according to the manufacturer's instructions. 100 mg of tissue was homogenised in 1 mL PBS, pH 7.0 with micropestle in a microtube. 20% trichloroacetic acid (TCA) was added to sciatic nerve tissue homogenate to precipitate the protein and centrifuged. Supernatants were collected and thiobarbituric acid (TBA) solution was added to the supernatants. After boiling for 10 min in water bath, the absorbance was measured. The concentration of MDA was calculated using the standard curve.

— **Anti-oxidative stress markers:** The techniques were done according to the manufacturer's instructions. The measurement of glutathione (GSH) is based on the reduction of 5, 5 dithiobis (2-nitrobenzoic acid) (DTNB) with reduced glutathione to produce a yellow compound. The reduced chromogen is directly proportional to GSH concentration and its absorbance is measured at 405 nm by using a commercial kit (Biodiagnostic, Egypt) [39]. GSH activity was determined by the procedure of Carlberg and Mannervik 1985. The assay solution contained 10% bovine serum albumin (BSA), 50 mM potassium phosphate buffer (pH 7.6), 2 mM nicotinamide adenine dinucleoticle phosphate (NADPH) and 20 mM oxidised glutathione. Absorbance at 340 nm was recorded at a temperature of 250°C. The activity was calculated using the molar coefficient for NADPH of 6.22 $\mu mol^{-1} \times cm^{-1}$ and expressed in U/g of tissue.

The superoxide dismutase (SOD) activity in tissue homogenate was measured by the inhibition of nitroblue tetrazolium reduction by O_2- generated by the xanthine/xanthine oxidase system. One SOD activity unit was defined as the enzyme amount causing 50% inhibition in 1 mL reaction solution per milligram tissue protein and the result was expressed as U/mg protein.

Image analysis and morphometric measurements. The area per cent of immune expression of BAX, iNOS and TNF-α was done using Leica LAS V3.8 image analyser computer system (Switzerland). Counting of axons was done in the toluidine blue sections. The measurements were obtained by an independent blinded observer, who didn't know the experimental design. In each chosen field, the sciatic nerve was enclosed inside the standard measuring frame. The data was obtained in ten non-overlapping fields per specimen at a magnification of 400. Images were captured live on the screen from sections under a light microscope (Olympus Bx-40, Olympus Optical Co. Ltd., Japan) with an affixed video camera (Panasonic colour CCTV camera, Matsushita Communication Industrial Co. Ltd., Japan).

Gene expression of nuclear factor (NF-κB 65) and neuronal nitric oxide synthase (nNOS) by the real time PCR

Total RNA extraction. Total RNA was extracted from sciatic nerve tissues using the TRIzol method according to the manufacturer's protocol. In brief, RNA was extracted by homogenisation in TRIzol reagent (Invitrogen, Life Technologies, USA). The sciatic nerve tissue homogenate was then incubated for 5 min at room temperature. A 1:5 volume of chloroform was added, and the tube was vortexed and centrifuged at 12,000 g for 15 min. The aqueous phase was isolated, and the total RNA was precipitated with absolute ethanol. After centrifugation and washing, the total RNA was finally eluted in 20 μL of the RNase-free water. The RNA concentrations and purity were measured with an ultraviolet spectrophotometer.

Complementary DNA (cDNA) synthesis. The cDNA was synthesized from 1 μg RNA using Superscript III First-Strand Synthesis System as described in the manufacturer's protocol (Invitrogen, Life Technologies). In brief, 1 μg of total RNA was mixed with 50 μM oligo (DT) 20, 50 ng/μL random primers, and 10 mM dNTP mix in a total volume of 10 μL. The mixture was incubated at 56°C for 5 min and then placed on ice for 3 min. The reverse transcriptase master mix containing 2 μL of 10 × real time buffer, 4 μL of 25 mM $MgCl_2$, 2 μL of 0.1 M DTT, and 1 μL of SuperScript® III real time (200 U/μL) was added to the mixture and was incubated at 25°C for 10 min followed by 50 min at 50°C.

Real-time (RT) quantitative PCR. The relative abundance of mRNA species was assessed using the SYBR Green method on an ABI prism 7500 sequence detector system (Applied Biosystems, Foster City, CA). PCR primers were designed with Gene Runner Software (Hasting Software, Inc., Hasting, NY, USA) from RNA sequences from Gen Bank. All primer sets had a calculated annealing temperature of 60°C. Quantitative RT-PCR was performed in a 25-μL reaction volume consisting of 2X SYBR Green PCR Master Mix (Applied Biosystems), 900 nM of each primer and 2–3 μL of cDNA. Amplification conditions were 2 min at 50°, 10 min at 95° and 40 cycles of denaturation for 15 s and annealing/extension at 60° for 10 min. Data

from real-time assays were calculated using the v1·7 Sequence Detection Software from PE Biosystems (Foster City, CA, USA). Relative expression of studied gene mRNA was calculated using the comparative Ct method. All values were normalised to the beta actin gene and reported as fold change over background levels detected in diseases group (Table 1).

Table 1. The primers sequence for all studied genes

Gene	Primers sequence
NF-κB	Forward primer: 5′-CATTGAGGTGTATTTCACGG-3′ Reverse primer: 5′-GGCAAGTGGCCATTGTGTTC-3′
nNOS	Forward: 5′-CCGTTTCTCCTGGCTCAGTTTA-3′ Reverse: 5′-CCCCAATACC ACATCATCCAT-3′

NF-κB — gene expression of nuclear factor; nNOS — neuronal nitric oxide synthase

Statistical analysis

Statistical analysis was performed using statistical package for the social sciences (SPSS) version 21.0 (IBM Corporation, Somers, NY, USA) statistical software. The data were expressed as means ± standard deviation (SD). Statistical evaluation was done using one-way analysis of variance (ANOVA) followed by Bonferroni pairwise comparisons. Significance was considered when the p-value was less than 0.05.

The percentage of increase or decrease (difference) of all study parameters were calculated per the following formula: Percentage of difference = (Mean difference value between two groups)/(Value of the compared group) × 100.

RESULTS

The general toxicological data (Table 2)

No evidence of general toxicity was observed among the studied groups. The motility, food and water consumption and health status were relatively good.

The mean body weight of the cisplatin-treated group was 8% lower than that of the control group at the end of the 1st week, and the weight was 19% lower at the end of the 4th week.

Compared to that of the control group, the mean body weight of the NAC and progesterone-treated groups were 3% and 5% lower than that of the control group at the end of the 1st week, and the weights were 16% and 18% lower at the end of the 4th week.

Semithin sections

The number of axons of the sciatic nerve and the myelin sheath was affected in the cisplatin-treated group. Although the findings in the NAC-treated group were near to that of the control group, the number in the progesterone-treated group was away from that of the control group (Fig. 1).

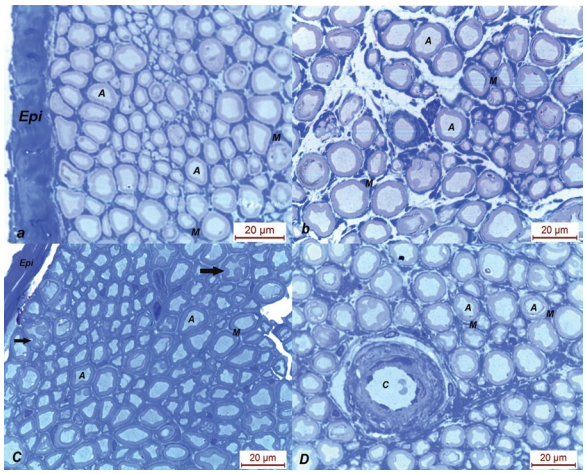

Figure 1. Semithin sections of the sciatic nerve showing the axons (A) and myelin sheath (M) in different groups (Toluidine blue ×1000); **a.** The control group; **b.** Decrease number of axons with degeneration and focal loss of myelin sheath in the cisplatin-treated rat; **c.** Normal axons and myelin in the N-acetylcysteine and progesterone-treated rats; **d.** Decreased the number of axons in the progesterone-treated rat. Note: capillary (C).

Ultrastructural observation

The sciatic nerve of the control group showed myelinated nerve fibres. The axoplasm contains intact mitochondria and the endoneurium interspersed between axons. Schwann cells and regular unmyelinated axons in Remak bundles are also observed (Fig. 2).

Table 2. The mean of the body weight study in the different experimental groups

	Control	Cisplatin-treated	NAC-treated	Progesterone-treated	P
End of the 1st week	210.0 ± 7.1	192.8 ± 5.6	202.3 ± 8.3	198.2 ± 6.8	0.000*
End of the 4th week	232.3 ± 5.6	186.0 ± 2.1	194.0 ± 4.6	189.8 ± 1.8	0.000*

p value > 0.05 is significant; NAC — N-acetylcysteine

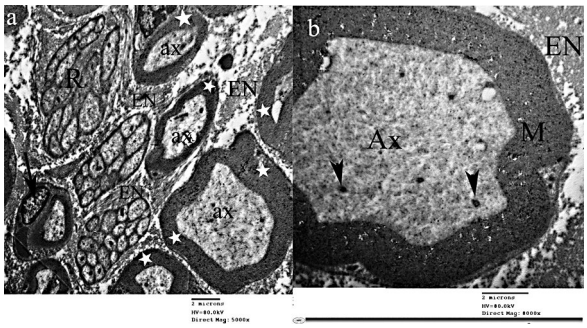

Figure 2. Electron photomicrograph of the sciatic nerve of the control group; **a.** Myelinated nerve fibers (*), axoplasm (ax) and endoneurium (EN) interspersed between axons. Note Schwann cell with its euchromatic nucleus (N) and regular unmyelinated axons in Remak bundles (R) (TEM ×5000); **b.** Myelinated nerve fibers (M), axoplasm (Ax), mitochondria (arrowheads) and endoneurium (EN) (TEM ×8000).

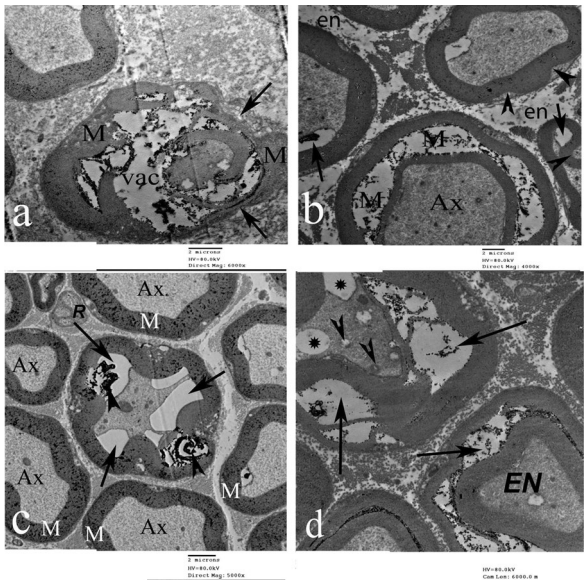

Figure 3. Electron photomicrograph of the sciatic nerve in the cisplatin-treated rats; **a.** Disfigured myelinated nerve fiber (M), degenerated myelin (arrows) and vacuolated (vac) axoplasm (TEM ×6000); **b.** An extensive myelin splitting with focal loss of myelin (M) into an inner part that surrounds the axoplasm (Ax) and outer part. The space between the two parts contains fragmented myelin. Note: excessive infolding of myelin (arrowheads), axonal separation and loss of endoneurium (en) (TEM ×4000); **c.** Extensive myelin (M) splitting with focal thickening of degenerated myelin sheath (arrowheads). Atrophy of the axoplasm (AX) with their vacuolations (arrows). Note alterations of Remak bundle (R) (lack of Schwann cell cytoplasm between axons) (TEM ×5000); **d.** Extensive myelin splitting seeming to entrap the adjacent axon (EN), focal loss of myelin (arrows) with vacuolation of the axoplasm (*). Note abnormal ballooning and vacuolation of the mitochondria (arrowheads) (TEM ×6000).

The myelin sheath in the cisplatin-treated group elucidated infolding. The myelin was disfigured, degenerated, and extensively split with areas of focal loss. The axoplasm was atrophic. Ballooning and vacuolations of the mitochondria with alterations of Remak bundles structures (lack of Schwann cell cytoplasm between axons) were also observed (Fig. 3).

Fewer of the previously mentioned effects were noted in the NAC and progesterone-treated groups suggesting a protective role of both drugs. Thin and loose myelin sheath was observed in both groups suggestive of remyelination. Better findings were observed in the NAC-treated group compared to that of the progesterone-treated group. Myelin infolding and vacuolations of the axoplasm were more extensive in the progesterone-treated group compared to that of the NAC-treated group (Fig. 4).

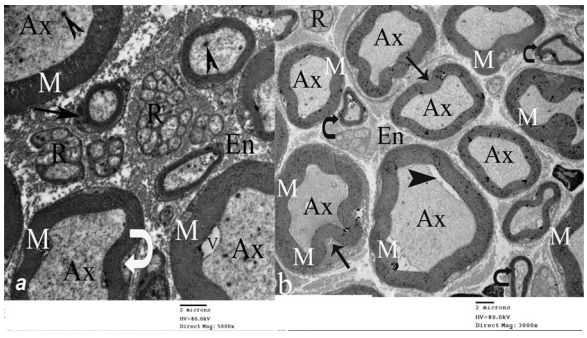

Figure 4. Electron photomicrograph of the sciatic nerve in the NAC and progesterone-treated rats; **a.** Myelinated nerve fibers (M) with slight infolding (curved arrow), unmyelinated axons in Remak bundles (R) with thin and loose myelin sheath, suggestive of remyelination (arrow). Slight vacuolation (V) of the axoplasm (Ax) separated by endoneurium (En). Note intact mitochondria (arrowheads) in the N-acetylcysteine-treated rat (TEM ×5000); **b.** Myelinated nerve fibers (M) with infolding (arrows), unmyelinated axons in Remak bundles (R) with thin and loose myelin sheath, suggestive of remyelination (curved arrows). Vacuolations (arrowhead) of the axoplasm (Ax) in the progesterone-treated rat (TEM ×3000).

Immunohistochemical studies

The immunoreaction of BAX, iNOS, and TNF-α is cytoplasmic. The reaction in the different studied groups was strongly positive in the cisplatin-treated group when compared to that of the control group. The intensity of the reaction decreased in the NAC and progesterone-treated groups (Figs. 5–7).

Histomorphometric study (Tables 3–6)

BAX, iNOS and TNF-α immunoexpression (Table 3). The mean area per cent of BAX immunoexpression of the cisplatin-treated group was 1.3-fold significantly higher than that of the control group.

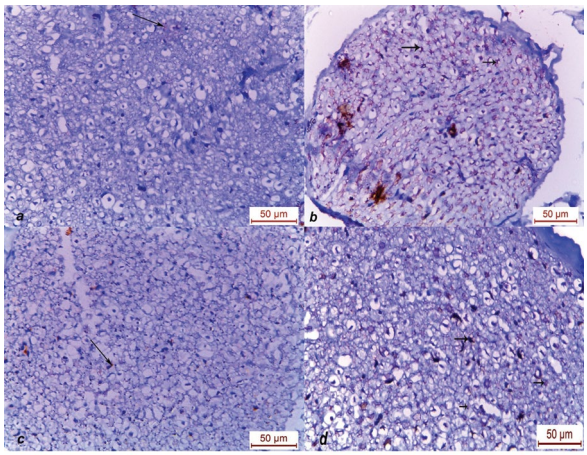

Figure 5. Photomicrograph of the sciatic nerve sections showing the immunoreactivity to bax (arrows) in different groups (BAX ×400); **a.** Weak immunoreaction reaction in the control group; **b.** Strong immunoreaction in the cisplatin-treated rat; **c, d.** Weak immunoreaction reaction in the N-acetylcysteine and progesterone-treated rats.

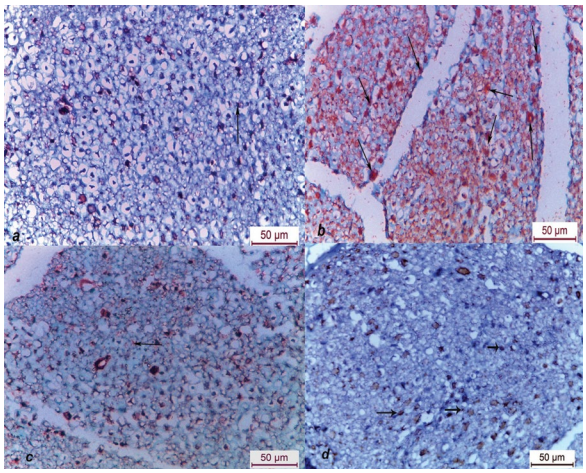

Figure 7. Photomicrograph of the sciatic nerve sections showing the immunoreactivity to tumour necrotic factor alpha (TNF-α, arrows) in different groups (TNF-α ×400); **a.** Weak immunoreaction reaction in the control group; **b.** Strong immunoreaction in the cisplatin-treated rat; **c, d.** Weak immunoreaction reaction in the N-acetylcysteine and progesterone-treated rats.

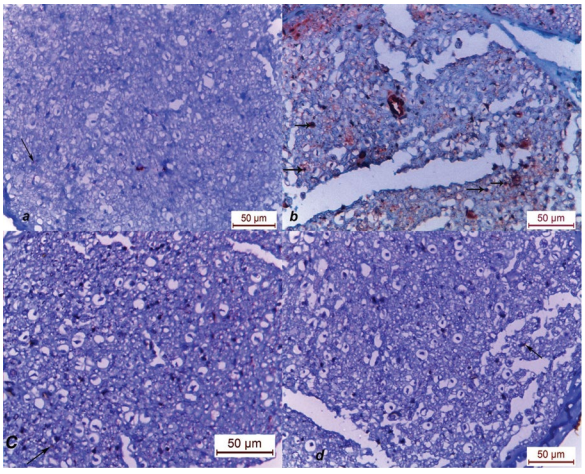

Figure 6. Photomicrograph of the sciatic nerve sections showing the immunoreactivity to inducible nitric oxide synthase (iNOS, arrows) in different groups (iNOS ×400); **a.** Weak immunoreaction reaction in the control group; **b.** Strong immunoreaction in the cisplatin-treated rat; **c, d.** Weak immunoreaction reaction in the N-acetylcysteine and progesterone-treated rats.

The mean area per cent of the immunoreactions of iNOS the cisplatin-treated group was 1.7-fold significantly higher than that of the control group. Contrary, the area per cent in the NAC and progesterone-treated groups were 56% and 68% significantly lower than that of the cisplatin-treated group.

The mean area per cent of the immunoreaction of TNF-α of the cisplatin-treated group was 8.3-folds significantly higher than that of the control group. Although the area per cent's in the NAC and progesterone-treated groups were 55% and 47% significantly lower than that of the cisplatin-treated group, theses per cent didn't return to the control level as these percentages were 31% and 39% higher than that of the control group.

Oxidative/antioxidative markers (Table 4). The mean value of MDA of the cisplatin-treated group was

Table 3. The mean area per cent of BAX, inducible nitric oxide synthase (iNOS), and tumour necrotic factor alpha (TNF-α) immunoexpression in the different experimental groups

Groups	iNOS	TNF-α	BAX
Control	1.13 ± 0.19	0.76 ± 0.18	1.31 ± 0.412
Cisplatin-treated	$3.13 \pm 1.43^{+\#\$}$	$7.09 \pm 0.79^{+\#\$}$	$3.01 \pm 1.02^{+}$
NAC-treated	$1.38 \pm 0.21^{*}$	$3.12 \pm 1.04^{+*}$	1.70 ± 0.34
Progesterone-treated	$0.99 \pm 0.12^{*}$	$3.73 \pm 1.30^{+*}$	1.70 ± 0.34

NAC — N-acetylcysteine; +p < 0.05 — significant difference compared to that of the control group; *p < 0.05 — significant difference compared to that of the cisplatin-treated group; #p < 0.05 — significant difference compared to that of the NAC-treated group; $p < 0.05 — significant difference compared to that of the progesterone-treated group

Table 4. The mean values ± standard deviation of the oxidative markers in the different experimental groups

Groups	MDA [nmol/mg ptn]	SOD [μ/mg ptn]	GSH [mmol/mg ptn]
Control	1.04 ± 0.18	2.90 ± 0.23	58.55 ± 2.02
Cisplatin-treated	$10.33 \pm 2.57^{+\#\$}$	$0.54 \pm 0.24^{+\#\$}$	$20.88 \pm 1.74^{+\#\$}$
NAC-treated	$3.20 \pm 1.31^{*}$	$1.26 \pm 0.24^{+*\$}$	$46.40 \pm 9.34^{+*\$}$
Progesterone-treated	$1.26 \pm 0.24^{*}$	$1.94 \pm 0.16^{+*\#}$	$37.58 \pm 6.91^{+*\#}$

GSH — glutathione; MDA — malondialdehyde; NAC — N-acetylcysteine; SOD — superoxide dismutase; +p < 0.05 — significant difference compared to that of the control group; *p < 0.05 — significant difference compared to that of the cisplatin-treated group; #p < 0.05 — significant difference compared to that of the NAC-treated group; $p < 0.05 — significant difference compared to that of the progesterone-treated group

Table 5. The mean values ± standard deviation of gene expression of nuclear factor (NF-κB) and neuronal nitric oxide synthase (nNOS) in the different experimental groups

Groups	NF-κB	nNOS
Control	1.01 ± 0.01	1.00 ± 0.00
Cisplatin-treated	$12.12 \pm 2.47^{+\#\$}$	$0.36 \pm 0.26^{+\#}$
NAC-treated	$3.86 \pm 0.72^{+*\$}$	$0.94 \pm 0.30^{*}$
Progesterone-treated	$6.25 \pm 0.71^{+*\#}$	0.71 ± 0.26

NAC — N-acetylcysteine; +p < 0.05 — significant difference compared to that of the control group; *p < 0.05 — significant difference compared to that of the cisplatin--treated group; #p < 0.05 — significant difference compared to that of the NAC-treated group; $p < 0.05 — significant difference compared to that of the progesterone-treated group

Table 6. The mean values ± standard deviation (SD) of the number of axons in the different experimental groups

Groups	Mean ± SD
Control	$125.00 \pm 12.91^{*\$}$
Cisplatin-treated	$61.25 \pm 8.54^{+\#}$
NAC-treated	$126.25 \pm 12.50^{*\$}$
Progesterone-treated	$80.00 \pm 8.16^{+}$

NAC — N-acetylcysteine; +p < 0.05 — significant difference compared to that of the control group; *p < 0.05 — significant difference compared to that of the cisplatin--treated group; #p < 0.05 — significant difference compared to that of the NAC-treated group; $p < 0.05 — significant difference compared to that of the progesterone-treated group

significantly nine folds higher than that of the control group. The MDA levels in the NAC and progesterone-treated groups were 69% and 88% significantly lower than that of the cisplatin-treated group.

The mean value of SOD of the cisplatin-treated group was significantly 81% lower than that of the control group. Although the SOD levels in the NAC and progesterone-treated groups were significantly 1.3 and 2.5 folds higher than that of the cisplatin-treated group, these levels didn't return to the control level as these levels were 57% and 33% lower than that of the control group. The level of the SOD in the progesterone-treated group was about 54% higher than that of the NAC-treated group.

The mean value of the GSH of the cisplatin-treated group was significantly 64% lower than that of the control group. Although the GSH levels in the NAC and progesterone-treated groups were 120% and 79% higher than that of the cisplatin-treated group, these levels didn't return to the control level as these levels were 20% and 36% lower than that of the control group. The level of the GSH in the progesterone-treated group was 19% lower than that of the NAC-treated group.

NF-κB and nNOS (Table 5). The mean value of NF-κB of the cisplatin-treated group was significantly 11 folds higher than that of the control group. Although the NF-κB levels in the NAC and progesterone-treated groups were significantly 68% and 48% lower than that of the cisplatin-treated group, these levels didn't return to the control level as these levels were 2.8 and 5.1 folds higher than that of the control group. The level of NF-κB in the progesterone-treated group was 62% higher than that of the NAC-treated group.

The mean value of nNOS of the cisplatin-treated group was significantly 64% lower than that of the control group. The level in the NAC-treated group was 1.6 folds higher than that of the cisplatin-treated group.

Axons number (Table 6). The mean number of axons of the sciatic in the cisplatin-treated group was significantly 50% lower than that of the control group. The mean number of axons in the NAC-treated group was like that the control group. In contrary, the axons number in the progesterone-treated group

was 44% lower than that of the control and NAC--treated groups.

DISCUSSION

Cisplatin-induced sciatic nerve damage in the rat model has been proven in many studies [34]. Cisplatin has a molecular affinity for the peripheral nerves as they lack the vascular and lymphatic barriers, which make them more susceptible to the precipitation of the cisplatin debris [25, 41]. In addition, the mammalian nerves are known to be more susceptible to oxidative stress due to their high content of phospholipids and mitochondria-rich axoplasm [35]. Moreover, their cellular antioxidant defences are weak [35].

Cisplatin administration triggered many pathological changes in the myelin sheath in the form of infolding, extensive splitting, and focal myelin loss. The myelin sheath is the most vulnerable part to neuronal injury by chemotherapy [4].

The mechanisms underlying CIPN are incompletely understood [7]. The first possible studied mechanism was the reduction in the tissue antioxidants (SOD and GSH) and the increase in the tissue oxidants (MDA), which were observed in the cisplatin-treated group. The shift in the balance between oxidants and antioxidants in the favour of oxidants is termed "oxidative stress" [6]. The oxidative stress induced by cisplatin causes deleterious distortion of the nervous tissue which might reach demyelination or even myelin degeneration. Furthermore, during the ongoing process of the release of the nitric oxide (NO) and reactive oxygen species (ROS), the tissue antioxidants are depleted [13], which in turn results in axonal injury [17]. Finally, the total number of axons decreased in this group.

Mitochondrial dysfunction was the second possible studied mechanism of the CIPN [10]. The vacuolated mitochondria detected in the cisplatin-treated group might be due to their damage by the free radicals. Mitochondrial impairment plays a chief role in nervous tissue toxicity [36]. The precipitation of distorted-mitochondria results in more oxidative stress, and more damage to peripheral nerve and neurodegeneration [48].

The third possible studied mechanism of the CIPN was the disturbance of the neurotoxic/neuroprotective mechanism (iNOS/nNOS). An increase in the values of the neurotoxic iNOS and decrease in the values of the neuroprotective nNOS was observed in the cisplatin-treated group. NO could be considered as a double-agent as regard to its neurotoxic and neuroprotective role [8]. The ROS induced by cisplatin resulted in an increase in the values of the neurotoxic iNOS and a decrease in the values of the neuroprotective nNOS [12]. The neurotoxic effect of the iNOS is via caspase-dependent and caspase-independent mechanisms [12], while the neuroprotective effect of the nNOS is via the release of the hypoxia-inducible factor-1, the transcriptional activator of several genes which are involved in the neuroprotection [27].

Inflammation was the fourth studied mechanism of the CIPN. Many proinflammatory mediators such as TNF-α and NF-κB were shown to be involved in cisplatin-induced renal toxicity [47] and in cisplatin-induced vestibular-toxicity [29]. In our study, the proinflammatory mediators NF-κB and TNF-α were highly elevated in the cisplatin-treated group. The proinflammatory cytokines (TNF-α) is upregulated early and transiently at the site of nerve injury and is considered as an initiator of local inflammatory responses [20].

The last possible studied mechanism of the CIPN was the apoptosis. The mean area per cent of BAX gene immunoexpression in the cisplatin-treated group was 1.3-fold higher than that of the control group. The protein encoded by this gene belongs to the BCL2 protein family. This protein functions as an apoptotic activator [24]. Cisplatin-mediated cytotoxicity creates a downstream effect on a variety of molecular factors including activation of p53 and subsequent modulation of Bcl-2 family proteins including the pro-apoptotic protein s BAX [54]. Cisplatin release cytochrome c from mitochondria that translocate BAX [21].

Fewer of the previously mentioned pathological findings were noted in the NAC and progesterone-treated groups suggesting a protective role of both drugs. The structure of the mitochondria in the axoplasm and the myelin sheath were well preserved in both groups. In addition, the myelin sheath in both groups was thin and loose that suggest remyelination. The condition of the sciatic nerve was much better in the NAC-treated group.

Concerning the first possible studied mechanism of the CIPN, both NAC and progesterone ameliorated the oxidative stress-induced by cisplatin. A significant reduction of MDA and a significant elevation of SOD and GSH were observed with both drugs. This pathway allows partial protection against the CIPN as the level of all was away from that of the control group. The main way of action of the NAC was through the

elevation of the GSH as the NAC is a precursor for GSH and aids in its synthesis [23, 32], while the main way of action of progesterone was through the elevation of SOD as progesterone increases the release of SOD [38]. A non-significant difference between NAC and progesterone concerning their way of reduction of MDA was detected. It was postulated that the NAC interferes with the interaction of ROS with proteins, lipids, and DNA by forming complexes with cisplatin; handicapping formation of free radicals and ROS [46].

Concerning the second studied mechanism of the CIPN, the structure of the mitochondria in the axoplasm was well preserved in the use of both NAC and progesterone. The ability of the NAC and progesterone to restore and preserve the normal mitochondrial function was proved in many studies [3, 37, 42]. The normal mitochondrial function is important as the mitochondrial dysfunction is the main cause of redox imbalance and apoptosis in peripheral neurons [21].

Concerning the third studied mechanism of the CIPN, improvement of the disturbance of the neurotoxic/neuroprotective mechanism (iNOS/nNOS) was observed with the use of the NAC and progesterone. The value of neurotoxic iNOS decreased, while the value of the neuroprotective nNOS increased in both groups. The iNOS/nNOS pathway appears to be an excellent way of both drugs in the protection of the nerve against the CIPN. A non-significant difference in both iNOS and nNOS was observed on comparing the two groups with each other, and when comparing each group to that of the control group. Some studies proved the role of progesterone in inhibition of the NO production [15].

Concerning the role of inflammation in the mechanism of the CIPN, the levels of TNF-α and NF-κB in the NAC and progesterone-treated groups were significantly lower. However, the levels both markers were still higher than that of the control group, which reflects the minor role of this pathway. The anti-inflammatory role of the NAC seems to be superior on that of the progesterone as the NF-κB in the progesterone-treated group was 62% higher than that of the NAC-treated group. The anti-inflammatory role of the NAC might be through stabilisation of mitochondria, as the ROS released in mitochondria had been reported to trigger the production of proinflammatory cytokines [28]. This hypothesis is supported by the intact mitochondria in the NAC-treated group. In addition, NAC can suppress NF-κB-activated pathways in which cytokine cascades are induced [33]. On the other hand, progesterone seems to suppress the inflammatory process by inhibiting the release of cytokine and by handicapping immune cell activation and migration [51].

A non-significant difference of the area per cent of BAX was observed in the NAC and progesterone-treated groups. Few types of research reported the anti-apoptotic effect of the NAC through reduction of BAX [43].

CONCLUSIONS

In conclusion, CIPN can be attenuated by concomitant use of the NAC or progesterone with cisplatin. The protective effect of the NAC is superior to that of the progesterone as the main number of axons of the sciatic nerve was lower in the progesterone--treated group. The main way of action of both drugs is through oxidative/antioxidative markers (MDA/GSH and SOD), neurotoxic/neuroprotective mechanism (iNOS/nNOS) and through inhibition of the inflammatory mediators (TNF-α and NF-κB).

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