Original Article

Targeting Oxidative Stress, Autophagy, and Apoptosis by Quercetin to Ameliorate Cisplatin-induced Peripheral Neuropathy in Rats

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Abstract

Background: Quercetin is a flavonoid, with antioxidant and autophagy-modulating activities. Cisplatin is one of the platinum-based anticancer drugs. Early development of peripheral neuropathy as an adverse effect of cisplatin interferes with the continuation of therapy. Oxidative stress and autophagy impairment may play a role. **Aim:** This study aimed to explore the possible protective effects of quercetin against cisplatin-induced peripheral neuropathy. **Methods:** Twenty-four male Wistar rats were divided into three groups: Group 1 (control group) and Group 2 (cisplatin group) where peripheral neuropathy was induced using single ip injection of cisplatin. Group 3 (cisplatin + quercetin group) received single ip injection of cisplatin and was then treated with quercetin for 14 days. At the end of the experiment, nociception was evaluated by tail immersion test, and then, blood was collected for analysis of nerve growth factor. Sciatic nerve was used to assess histopathological changes and light chain 3-II by immunohistochemical staining. Reduced glutathione, malondialdehyde, mTOR, and caspase-3 were estimated in sciatic nerve tissue homogenate. **Results:** This research work revealed that quercetin significantly improved cisplatin-induced nociceptive impairment, attenuated cisplatin-induced oxidative stress, autophagy, and apoptosis to protect against neuronal death. **Conclusion:** From the current study, quercetin can act as a promising protective agent against cisplatin-induced peripheral neuropathy.

Keywords: Apoptosis, autophagy, cisplatin, neuropathy, quercetin

Introduction

Cisplatin is a platinum-based antineoplastic drug, commonly prescribed in clinical practice for several types of solid tumor treatment such as breast, ovarian, testicular, lung, head and neck, and many other cancer types.[1,2] However, the effectiveness of cisplatin therapy is hampered by the development of severe peripheral neuropathy which necessitates a reduction of dose or early chemotherapy termination which represents a great obstacle interfering with its anticancer effect.[3,4]

Cisplatin neurotoxicity generally occur after cumulative doses more than 350 mg/m^2, the severity of neurotoxicity and the probability of chronicity increases with long duration of administration and higher cumulative doses of cisplatin. The peripheral neuropathy induced by cisplatin is mostly a sensory neuropathy that may be associated with varying degrees of impairment of motor function.[5] It is represented clinically by absent deep tendon reflexes, numbness of fingers and toes, and paresthesia which increase in a glove/stocking distribution, and on continuation of drug treatment, fine motor coordination may be lost and gait disturbance appears.[6]

The exact mechanism of cisplatin neurotoxicity is not completely understood. However, peripheral neuropathy induced by platinum agent is initiated by platinum adduct accumulation in dorsal root ganglia.[5] Generation of reactive oxygen species (ROS) is considered a major player, as cisplatin reduces

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levels of antioxidant enzymes such as glutathione peroxidase, catalase, and superoxide dismutase leading to inhibition of the antioxidant defense against damage of free radicals.[7,8] The increased ROS increases biomolecules damage leading to lipid peroxidation and react with DNA causing DNA damage.[5]

Despite long time of research, protective approaches against side effects induced by cisplatin including neurotoxicity still unavailable.[9,10] Therefore, finding effective medications that have a protective effect against cisplatin-induced peripheral neuropathy remains an urgent medical need.

Autophagy is an intracellular degradative process whereby senescent or damaged organelles or proteins are sequestered in autophagosomes and targeted for destruction in lysosomes for recycling of their products and preserving normal tissue homeostasis.[11] Regarding peripheral nervous system, autophagy plays essential roles for function of Schwann cell during both myelination and re-myelination[12,13] as well as synaptic integrity and receptor turnover.[14,15]

Mounting evidence has revealed that autophagy impairment underlies neuropathic pain and therapeutic intervention-modulating autophagy can be considered a potential strategy in the treatment of pain behavior.[16-18] Moreover, accumulated evidences demonstrated that autophagy may protect against cisplatin-induced renal tubular cell death[19,20] and cisplatin-induced ototoxicity.[21] However, the role of autophagy induction in preventing cisplatin-induced neurotoxicity has received much less attention.

Quercetin, the most abundant dietary polyphenolic flavonoid, exhibits different pharmacological effects such as antioxidant, antitumor, and anti-inflammatory actions. Moreover, its activity as an autophagic inducer has been reported.[22,23]

Quercetin has been described as a neuroprotective agent in various models of neurological disorders.[24-26] Indeed, increasing reports have shown that quercetin produced the inhibition of nociceptive neurotransmission and decreased pathological pain in many experimental models, such as diabetic model, chronic constriction nerve injury model, and cancer pain model.[27-29]

Based on the growing interest in the role of autophagy dysfunction in the pathological process of neuropathic pain, this study aimed to explore the possible protective effects of quercetin against cisplatin-induced peripheral neuropathic pain and the underlying mechanism.

Materials and Methods

Drugs and chemicals

Cisplatin (10 mg/10 ml vial; a product of EIMC United Pharmaceuticals, Cairo, A.R.E). Quercetin (powder; a product of Sigma-Aldrich, St. Louis, Missouri, USA). Ketamine (50 mg/ml solution for injection; a product of Rotexmedica GmbH, Germany). Xylazine (100 mg/ml vial for injection; a product of BIMEDA Company). All chemicals and solvents used in this experiment are of high analytical gradient.

Animals and experimental design

We performed the current study using 24 8-week-old male Wistar rats, with an average weight of 150–200 g, obtained from Tanta University Animal House. Rats were kept at 20°C ± 2°C in wire mesh cages (12-h light/dark cycle) and fed a standard laboratory diet and water *ad libitum*. An adaptation period for 1 week was allowed to all animals before starting the experiment. All experiments were performed according to the guidelines for the care and use of experimental animals, with an approval of the Animal Experiment Ethics Committee in Faculty of Medicine, Tanta University, Egypt (Approval N. 34410/1/21). Approval code 34410/1/21, Tanta university faculty of medicine research ethics committee FWA00022834, IRB0010038, Date 20-1-2021.

The rats were randomly divided into three groups (n = 8 per group): Group 1 (control) received physiological saline by intraperitoneal (ip) injection, Group 2 (Cisplatin) received single injection of cisplatin ip at a dose of 7 mg/kg body weight[30] and served as untreated cisplatin-induced peripheral neuropathy group, and Group 3 (cisplatin + quercetin) received single injection of cisplatin ip at a dose of 7 mg/kg body weight and then was treated with ip injection of quercetin at a dose of 50 mg/kg body weight for 14 consecutive days.[31] Nociception was evaluated by tail immersion test at day 0 and 14th day. On the 14th day, all animals were fasted overnight, and blood was collected by cardiac puncture under ketamine-xylazine anesthesia. Blood was rapidly collected in a well sterile dry centrifugation tube and allowed to clot for 30 min at room temperature, and then, 20-min centrifugation (1000 × g at 4°C) was done. Sera were collected and kept at −80°C for further biochemical estimation of serum nerve growth factor (NGF).

Tail immersion test

For assessment of nociceptive reaction after drug treatment, tail immersion method was used, where tail of each rat was immersed in a warm water (47° ± 1°C) bath until tail withdrawal (flicking response) or signs of struggle were observed. The time between the onset of painful stimulus and the animal's response was recorded as reaction time.

Tissue sampling

Both sciatic nerves from each rat were rapidly excised and washed with ice-cold saline.

For histopathological examination

One of the two nerves was fixed in 10% formalin embedded in paraffin and sectioned and stained with hematoxylin and eosin.

For detection of autophagy marker light chain 3-II

After deparaffinization and rehydration, the sciatic nerve section was placed in a 10 mM citrate buffer solution (pH 6.0) for antigen retrieval. Endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 in methanol for 15 min. Additional washing in phosphate-buffered saline (PBS) was performed before 30 min of incubation at 37°C in 10% normal goat serum. Then, the sections were incubated overnight with monoclonal light chain 3 (LC3) antibody at 4°C. The

sections were then treated with an avidin–biotin affinity system for 30 min at room temperature and stained with 3,3'-diaminobenzidine and hematoxylin. It was expressed as negative if no dots or barely visible dots in <5% of the cells, mild if detectable dots are present in 5%–25% of cells, moderate if readily detectable dots in 25%–75% of cells, and high if dots are detectable in >75% of cells.

Processing for transmission electron microscopic examination

A very small piece (2 mm) of the sciatic nerve was cut and fixed in a mixture of 2.5% glutaraldehyde and 0.1 M cacodylate buffer for 24 h, postfixed for 2 h in 1% buffered osmium tetroxide, and dehydrated by ascending grades of alcohol and cleared in propylene oxide. Then, the small samples were embedded in absolute resin, sectioned with ultramicrotome, placed on grids, and stained with 4% uranyl acetate and 2% lead citrate. The sections were examined using Transmission Electron microscope (in Transmission Electron Microscope Unit, Tanta University) to detect histopathological changes in the axon and myelin sheath of sciatic nerve fibers.

Preparation of tissue homogenate

The other sciatic nerve from each rat was subjected to homogenization where a piece of sciatic nerve was weighed and homogenized in 10 volumes of 50 mM, 7.4 pH ice-cold PBS. Tissue homogenate was centrifuged (7700 × *g* for 30 min at 4°C). The supernatant was collected and stored frozen at −80°C for biochemical assay.

Biochemical analysis

Serum NGF was estimated by an enzyme-linked immunosorbent assay (ELISA) (NGF Assay Kit, Abcam, USA, Cat N: ab207223) according to the manufacturer's instructions.

Sciatic nerve homogenate was used for determination of the following parameters.

Sciatic nerve mammalian target of rapamycin (mTOR) was measured using ELISA kit obtained from CLOUD-CLONE CORP. (CCC) USA Cat N: E-31046Ra and caspase-3 using ELISA kit obtained from Sun Red Bio. China Cat. N: 201-11-0281.

The total protein content was determined according to Lowry *et al*. method.[32]

Colorimetric assay for reduced glutathione (GSH) and malondialdehyde (MDA) using commercially available kits (Biodiagnostic, Egypt).

Statistical analysis

Values of all measured parameters were expressed as mean ± standard error of the mean. Independent sample *t*-test was used to detect the significance between the two groups. The difference was considered significant at $P < 0.05$. The statistical analysis was processed using the Statistical Program of Social Sciences (SPSS) for Windows, version 14 (SPSS Inc.,Chicago, IL, USA).

Results

Evaluation of the nociceptive reaction in rats

Sensory function in rats was assessed using tail immersion test. At day 0, there were no significant differences between the three groups (data not shown). On day 14 examining rats in the cisplatin group, they exhibited a significant decrease in withdrawal latency when compared to the control group ($P < 0.001$) signifying thermal hyperalgesia, while withdrawal latency increased in the group receiving cisplatin + quercetin to reach a significant level versus the cisplatin group ($P < 0.01$) [Figure 1].

Effect of quercetin on oxidative stress and lipid peroxidation

As shown in Figure 2, cisplatin-treated rats exhibited a marked decrease in the level of GSH content in sciatic nerve tissue, and upon quercetin treatment, it increased significantly ($P < 0.01$) [Figure 2a]. However, administration of cisplatin led to a significant elevation in the lipid peroxidation product, malondialdehyde (MDA) ($P < 0.05$), and this elevation was reversed on coadministration of cisplatin + quercetin ($P < 0.05$) [Figure 2b].

Effect of quercetin on nerve growth factor

Cisplatin-treated rats exhibited a marked decrease in serum NGF versus the control group ($P > 0.05$), while the cisplatin + quercetin treatment group showed a significant elevation in relation to the cisplatin group ($P < 0.05$) [Figure 3].

Effect of quercetin on mammalian target of rapamycin (mTOR) and caspase-3

Cisplatin administration led to a nonsignificant reduction in sciatic nerve content of mTOR versus the control group ($P > 0.05$) with a further reduction upon cisplatin + quercetin treatment to reach a significance versus the cisplatin group [Figure 4]. On the other hand, sciatic nerve content of caspase-3 elevated significantly on cisplatin administration while cisplatin + quercetin treatment was capable of decreasing its level in relation to the cisplatin group [Figure 5].

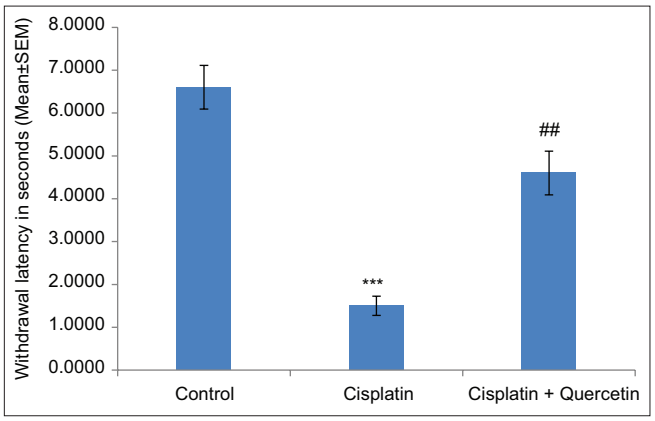

Figure 1: The effect of cisplatin and quercetin on tail immersion test. Data were presented as mean ± SEM ($n = 8$). * and # indicate a significant change from the control and cisplatin groups, respectively, ***$P < 0.001$, ##$P < 0.01$. SEM: Standard error of the mean

Histopathological and immunohistochemical results

Histological examination of the control group revealed normal histological appearance of sciatic nerve with normal distributed nerve axons within its myelin sheath. On the other hand, hydropic degeneration occurred in sciatic nerve fibers after cisplatin administration with edema and fragmentation of axons, these abnormalities were alleviated on treatment with cisplatin + quercetin and the nerves became more organized with decreased areas of degenerated fibers [Figure 6]. Immunohistochemical (IHC) analysis of the control and cisplatin + quercetin groups showed negative immunostaining with LC3-II antibody. On the other hand, moderate immunostaining in cisplatin-treatment groups were noticed with numbers of brown punctate staining were obviously augmented [Figure 7].

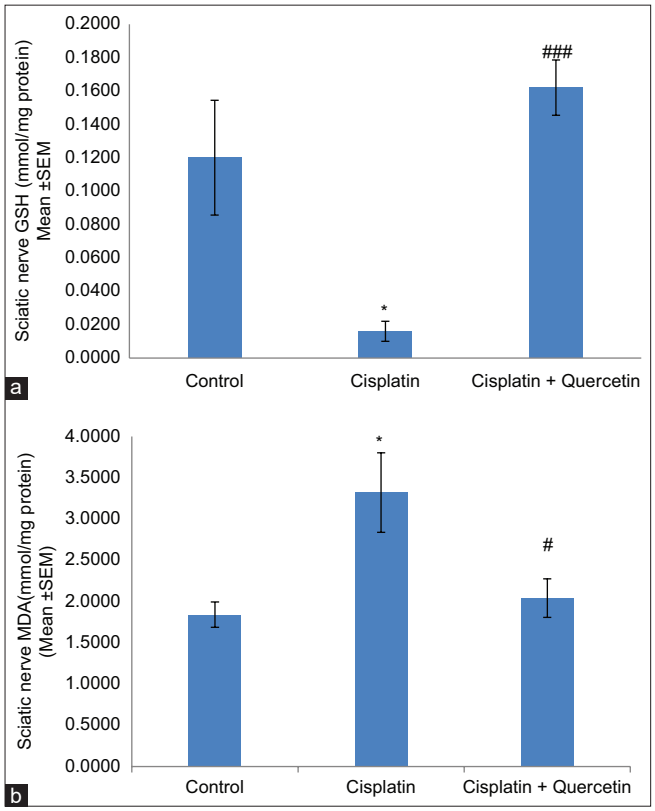

Figure 2: Quercetin reversed oxidative stress and lipid peroxidation induced by cisplatin. Effect of cisplatin and quercetin on reduced glutathione (GSH) (a). Effect of cisplatin and quercetin on malondialdehyde MDA in sciatic nerve (b). Data were presented as mean ± SEM (n = 8). * and # indicate a significant change from the control and cisplatin groups, respectively, at $P < 0.05$, $^{###}P < 0.001$. SEM: Standard error of the mean. MDA: Malondialdehyde

Transmission electron microscopic results

The control group revealed normal ultrastructural appearance of sciatic nerve. The myelinated axons showed identical axoplasm with normal mitochondria and were surrounded by regular myelin sheaths. Cisplatin-treatment groups showed that some myelinated axons were disorganized with extensive splitting of their myelin sheaths. Concomitant administration of cisplatin with quercetin showed approximately typical appearance of the myelinated axons [Figure 8].

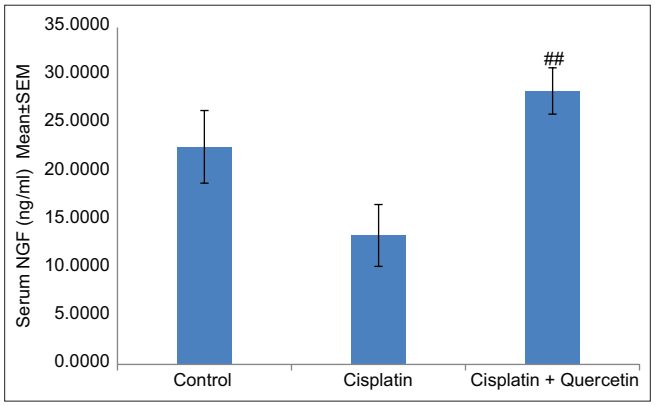

Figure 3: The effect of cisplatin and quercetin on serum NGF level. Data were presented as mean ± SEM (n = 8). ## indicates a significant change from cisplatin group at $P < 0.01$. SEM: Standard error of the mean, NGF: Nerve growth factor

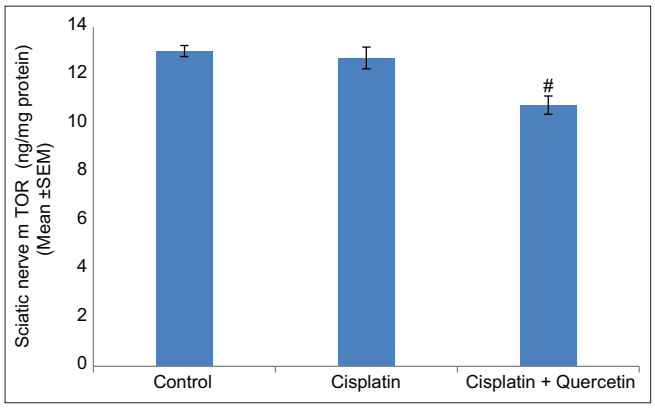

Figure 4: The effect of cisplatin and quercetin on mTOR. Data were presented as mean ± SEM (n = 8). # indicates a significant change from the cisplatin group at $P < 0.05$. SEM: Standard error of the mean

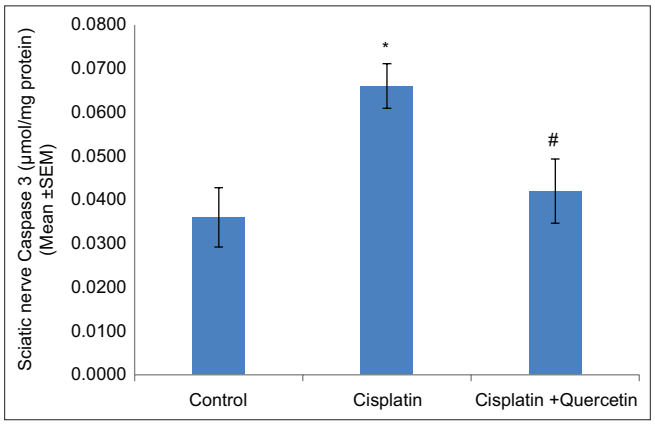

Figure 5: The effect of cisplatin and quercetin on caspase-3. Data were presented as mean ± SEM (n = 8). * and # indicate significant change from the control and cisplatin groups, respectively, at $P < 0.05$. SEM: Standard error of the mean

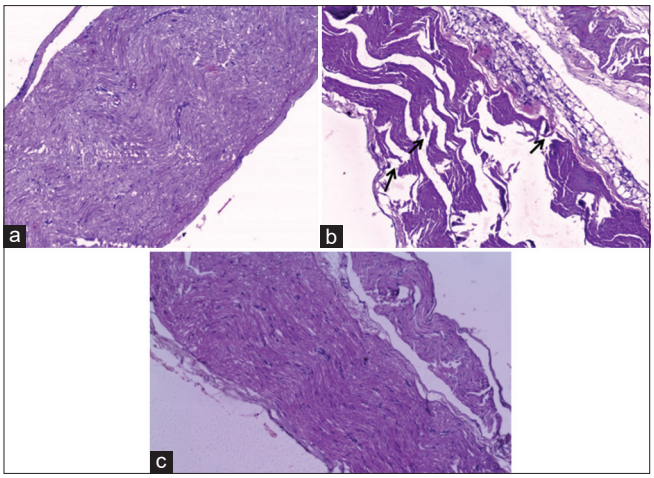

Figure 6: Histopathological findings in all studied groups. longitudinal sections of sciatic nerve from: (a) Control group showing normal nerve architecture with normal nerve fibers, (b) Cisplatin group showed disarranged nerve fibers, fragmentation, and degeneration of axons (→), (c) Cisplatin + quercetin group showed improvement in sciatic nerve architecture (H and E, ×200)

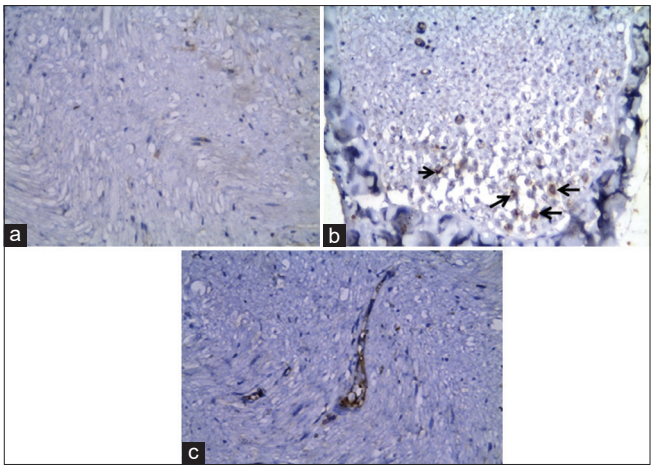

Figure 7: Immunohistochemical staining for LC3-II in sciatic nerve sections. (a) Control group showed negative immunostaining. (b) Multiple brown punctate staining were observed in cisplatin-treatment groups (→). (c) Cisplatin + quercetin group showed negative immunostaining. (×400). LC#: Light chain 3

Discussion

Regarding the pathological role of perturbations of autophagy in peripheral neuropathy, studies that explore pharmacological agents that may promote autophagy have garnered much interest.[33] The present study investigated the protective role of quercetin in the context of cisplatin-induced painful neuropathy via controlling autophagy. Herein, our study reveals the neuroprotective potential of quercetin against cisplatin-induced painful neuropathy through ameliorating oxidative stress, reducing NGF levels and the modulation of autophagy. To the best of our knowledge, our study is the first to describe the neuroprotective effects of quercetin against cisplatin-induced painful neuropathy by promoting autophagy.

The peripheral neurotoxic effect of cisplatin is the major dose-limiting side effect; our results showed great affection of the sensory function in the form of significant reduction of nociceptive threshold during performing tail immersion test in the cisplatin group as previously shown in other studies.[34,35]

It is well documented that cisplatin increases the production of oxygen-free radicals and reduces antioxidants with subsequent lipid peroxidation.[34,35] In the current study, administration of cisplatin led to a decrease in GSH and at the same time an increase in the lipid peroxidation marker, MDA. The previous findings matched with the histopathological examination of sciatic nerve sections that revealed axonal degeneration after cisplatin administration.

Quercetin is a flavonoid known for its antioxidant and anti-inflammatory effects, quercetin treatment, remarkably increased the withdrawal latency; pointing out the potential effect of quercetin in modulating pain. Moreover, the antioxidant profile improved significantly. In consistence, the present study revealed marked improvement in the histopathological findings in light and electron microscope upon quercetin cotreatment.

NGF is a crucial neurotrophin involved in regulating the development and survival of neurons in the central and peripheral nervous systems and the neuroprotective properties of NGF has been reported in various experimental models of the peripheral neuropathy.[36] It is well-established that NGF has a crucial role in survival and maintenance of both sympathetic and sensory nerves, resulting in neuroprotective and axonal growth effects.[37] In the current study, we reported decreased NGF in serum of rats treated with cisplatin alone as compared with the control group, coming in line with Cheng *et al*.,[38] who found that serum level of NGF decreased on treatment with oxaliplatin when compared to controls. Meanwhile, serum NGF significantly increased in rats supplemented simultaneously with quercetin and cisplatin, the present observation is consistent with prior reports suggesting that quercetin may induce synthesis and secretion of NGF in glial cells, brain, and retina.[39-41]

Autophagy, a cellular housekeeping process, is mandatory in eukaryotic cells for removing damaged organelles and denatured proteins, allowing cells to update their organelles.[42] Mammalian target of rapamycin (mTOR), one of the downstream kinases of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt), acts as a crucial regulator of autophagy by modulating multiple aspects of the autophagic process, such as initiation, propagation, and termination. In stress conditions, mTOR is inhibited to enhance the autophagic process.[43] In the context of nervous system, the PI3K/Akt/mTOR signal pathway is activated in the pain related to the central nervous system[44] and has been proven to participate in chronic neuropathic pain and spinal

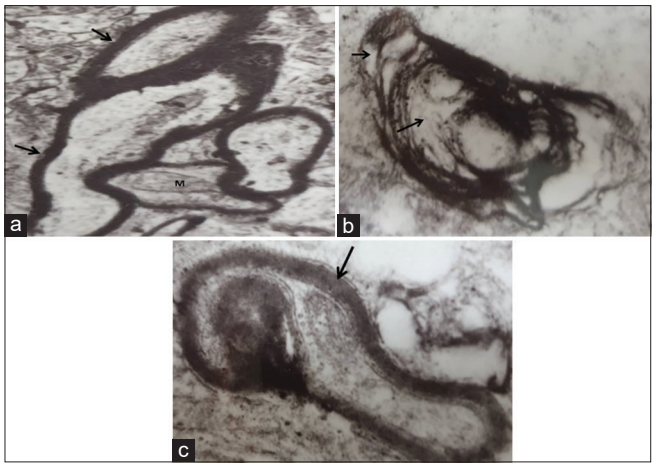

Figure 8: An electron micrograph of the myelinated axons of sciatic nerve. (a) The control group, showing myelinated nerve fibers of different sizes with compact regular myelin sheaths (→) and their axoplasm containing normal mitochondria (m). (b) Cisplatin group showing extensive splitting of their myelin sheaths (→). (c) Cisplatin + quercetin group showing nearly normal appearance of the myelinated nerve fibers (→) (TEM × 25000)

microglia and its inhibition alleviated chronic neuropathic pain and reduce microglia in chronic constriction injury model and sciatic nerve endometriosis in rats.[45,46] In the current study, mTOR content in sciatic nerve decreased in both drug treatment groups, whether the group received cisplatin alone as well as the group received cisplatin and quercetin. In the cisplatin group, sciatic nerve content of mTOR was lower than that in the control group, indicating that autophagy was initiated perhaps as a protective mechanism against cisplatin-induced cellular stress, while on quercetin treatment, mTOR content showed a much more decrease pointing out its autophagic-inducing activity which came in accordance with Cao *et al*.[47]

Microtubule-associated protein LC3 is mandatory for expansion and closure of autophagosomes during the autophagic process and the processing of LC3 to LC3-II is considered a reliable biochemical indicator for autophagic activity, as long as the autophagy pathway is fully functioning.[48] Increased LC3-II levels can be an indicator of either increased autophagosome formation as well as reduced autophagosome breakdown, in cases of delayed trafficking to the lysosomes, reduced fusion between autophagosome and lysosome, or impaired lysosomal proteolytic activity leading to accumulation of autophagosomes.[49] Herein, we examined the IHC staining for LC3-II in sciatic nerve samples and confirmed their upregulation in cisplatin-induced neuropathic rats.

Interestingly, in a study by Zhang *et al*.,[50] cisplatin was found to initiate the early stages of autophagy but suppresses its terminal stages in pheochromocytoma-derived cell line. They observed that the LC3-II–LC3-I ratio and expression of beclin-1 were elevated in cisplatin-treated cells by Western blot analysis with a higher accumulation of autophagosomes than autolysosomes, indicating that cisplatin activates early stages of autophagy but blocks autophagic flux.

In rat astrocytes, cisplatin low dose suppressed the autophagy expression-related molecules including LC3-II. Moreover, analysis of autophagic flux revealed decreased numbers of autophagosome and autolysosome.[51] Prior studies demonstrated autophagy abrogation in chemotherapy-induced neuropathic pain.[38,42]

In parallel, it has been reported that the downregulation of Schwann cell autophagic activity is an early process in the origin of neuropathic pain. Indeed, following peripheral nerve injury, the autophagic activity was disrupted in spinal GABAergic interneurons and glial cells, suggesting that the disruption of autophagy might take a part in neuropathic pain induction and maintenance. On the other hand, enhancement of autophagy in spinal microglia and Schwann cells can attenuate neuropathic pain by providing molecular proteins and energy for essential cell functions, as well as suppressing the neuroinflammatory response.[52] Considering the widely accepted nation that quercetin exerts inhibiting effect on mTOR signaling pathway,[53,54] in the current study quercetin administration significantly reduced mTOR content in sciatic nerve and markedly offset cisplatin-induced autophagy impairment as displayed by reduction of LC3-II immunostaining, signifying quercetin potential in promoting autophagy and maintaining autophagic flux which might be hampered by cisplatin administration. In line, quercetin was demonstrated to alleviate high glucose-induced damage to Schwann cells by upregulating autophagy.[55] Consistently, it was reported that quercetin could attenuate renal ischemia/reperfusion injury via inhibiting mammalian target of rapamycin (mTOR) and stimulating AMP-activated protein kinase-regulated autophagy pathway.[56]

To determine the fate of neurons in case of cisplatin and cisplatin + quercetin treatment and whether the sciatic nerve cells will undergo apoptosis or not, we measured the apoptotic marker, caspase-3, and we found that caspase-3 elevated significantly on cisplatin administration. This finding came in harmony with previous studies.[57,58]

On the other hand, sciatic nerve content of caspase-3 reduced significantly with quercetin treatment. The antiapoptotic properties of quercetin were reported in other studies, manifested by significant reduction of caspase-3.[59,60]

Induction of apoptosis by cisplatin supports our previous result of hampering autophagy in sciatic nerves of cisplatin-treated animals. Autophagy is considered a cell survival mechanism, while apoptosis is a cell death mechanism. Both processes are connected together through beclin-1, which is a key component in autophagosome formation during the process of autophagy. Beclin-1 can be cleaved by several members of caspase family such as caspase-3 to inhibit autophagy and change fate of the cell from autophagy to apoptosis.[61]

The present study verifies the neuroprotective effect of quercetin. This effect could be, in part, attributed to the

dramatic change in oxidative stress, enhanced autophagy, suppressed apoptosis, and elevated levels of NGF which may impart the neuroprotective potential of quercetin against cisplatin-induced painful neuropathy.

Conclusion

Quercetin significantly attenuates cisplatin-induced peripheral neuropathy in rats, and this effect could be indebted to the modulation of autophagy, elevated levels of NGF, and ameliorating oxidative stress, together with attenuating cisplatin-induced apoptosis. Therefore, it can be concluded that quercetin may be a potential protective agent against cisplatin-induced peripheral neuropathy, and this application will require further investigation.

Recommendation

Further studies are definitely needed for more understanding of the hampering effect of cisplatin on neuronal survival and disruption of autophagy-related proteins, which may provide therapeutic targets for the neuronal protection against cisplatin-induced neuropathy.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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