Vincristine-Induced Neuropathy in Rats is Mediated Via NMDA Excitotoxicity and Impairment of CGRP: Possible Neuroprotective Effect of Erythropoietin

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Abstract

Background/Aims: Vincristine (VCR) is a potent anticancer drug and neurotoxicity is one of its most important dose-limiting toxicities. In this study, we investigated the effect of VCR by neurophysiological recordings and the tail flick test. To elucidate the underlying mechanism of action of VCR, expression of both N-methyl-D-aspartate (NMDA) receptor, an index of glutamate excitotoxicity and calcitonin gene-related peptide (CGRP), an important regulator of vascular tone, were measured in both spinal cord and sciatic nerves. The role of erythropoietin (EPO) in the protection against VCR-induced neurotoxicity was also examined.

Methods: Rats were divided into control group, VCR-treated group and two groups given EPO in two different doses concomitant with VCR administration.

Results: VCR significantly decreased the amplitude of maximum compound action potential (MCAP) and prolonged the duration of action potential (AP) and relative refractory period (RRP), decreased chronaxie and the latency of tail flick test, but it had no effect on conduction velocity. VCR increased NMDA receptor expression and it decreased CGRP expression. The smaller dose of EPO improved all VCR-induced changes, except chronaxie, while its higher dose reversed all parameters and its effect was more prominent on tail flick test latency and NMDA receptor expression.

Conclusion: VCR resulted in axonal degeneration. It caused increased neuronal excitability and induced a state of glutamate excitotoxicity. Finally, VCR caused a decrease in blood flow in the nervous tissue resulting in vascular neurotoxicity. EPO had an obvious neuroprotective effect probably through decreasing NMDA receptor expression and increasing CGRP expression both centrally and peripherally.


Introduction

VCR is a chemotherapeutic agent that can be used in the treatment of many types of human cancer. It is a purified alkaloid extracted from the periwinkle plant Vinca rosea Linn. of the family Apocynaceae [2,3]. However, like many chemotherapeutic drugs it is toxic to peripheral nerves and the development of VCR-induced neuropathy seems to be dose-related and to occur in the early stage of treatment [3]. Thus, the clinical use of VCR is limited by the predictable development of the neuropathy [4]. If this effect could be prevented, it might be possible to use VCR more effectively in the treatment of malignant tumors at higher doses and for longer durations. Thus far, the mechanisms of VCR-induced neuropathy are poorly understood, which has hindered the development of protective measures against this toxic effect.

Erythropoietin (EPO), a well-established hematopoietic factor responsible for the production of red blood corpuscles, was discovered to have multiple functions outside the bone marrow. As it was found that recombinant human EPO crosses the blood-brain barrier [5], interest has focused on its function in the nervous system. Studies have shown that it reduces injury both centrally in acute ischemic stroke patients [6], as well as peripherally in sciatic nerve compression [7]. Recently EPO was also demonstrated to protect and reverse experimental diabetic neuropathy [8].

Thus the aim of the present study was to clarify the alterations in VCR-induced peripheral neuropathy using a behavioral assay, the tail flick test and neurophysiologic studies performed on the rat sciatic nerve. In addition, some possible underlying mechanisms of action of VCR were investigated by measuring NMDA receptor and CGRP expression in both the spinal cord and sciatic nerves. Furthermore, we studied the potential beneficial role of EPO (in 2 different doses) in preventing...
VCR-induced peripheral neuropathy and whether it reversed the underlying pathology.

**Material and Methods**

I- Animals:

40 male Sprague Dawley rats, weighing from 150-200 grams, were purchased from the animal house of the National Research Center, Cairo University. They were given free access to water and food and maintained on 12 hr light/dark cycle. All experimental procedures were carried out in compliance with guide for care and use of laboratory animals published by the US National Institute of Health (NIH publication 85-23 revised 1985).

II- Experimental protocol:

Rats were assigned into four equal groups:

- **Group I**: Control group; received no treatment.
- **Group II**: Vincristine-treated group (VCR Group); Vincristine sulfate (VCR) (Korea United Pharmaceuticals) was injected at a dose of 150 \( \mu \text{g/kg} \), intraperitoneally, three times weekly for five consecutive weeks [9,10].
- **Group III**: Erythropoietin-treated group 1 (EPO1 Group); This group of rats was treated with vincristine at the same dose and route of administration as in group II & received concomitantly human recombinant erythropoeitin (EPO) (Egyptian International Pharmaceutical Industries Co., 10th of Ramadan City, Egypt) at a dose of 40 \( \mu \text{g/kg} \), intraperitoneally, three times weekly for five consecutive weeks [8].
- **Group IV**: Erythropoietin-treated group 2 (EPO2 Group); This group of rats was subjected to same treatment protocol as in group III, but the EPO dose used was doubled (80 \( \mu \text{g/kg} \)).

At the end of the experimental protocol, all groups were subjected to Tail Flick test as a behavioral nociceptive reaction. Then rats were sacrificed by cervical dislocation followed by decapitation. Sciatic nerves of both limbs were exposed by a longitudinal skin incision in the hind legs. Each nerve was dissected free from the surrounding connective tissue and completely excised with the epineuria intact: One nerve was used for electrophysiological studies and the other one for assessment of gene expression of calcitonin gene-related peptide (CGRP) and N-methyl-D-aspartate (NMDA) receptor.

III- Electrophysiological recordings:

The sciatic nerve was mounted in a nerve chamber (MLT012/B. AD Instruments) designed for the recording of action potentials from isolated nerves. It contains 15 stainless steel wire electrodes of 0.8mm diameter spaced at intervals of 5mm and 10mm. Each end of the nerve is tied with short lengths of thread that are pushed into split silicone tubing at either end of the bath to help position the nerve without stretching it. The nerve was positioned over the electrodes and embedded in paraffin oil to maximize signal amplitude and prevent drying.

The proximal part of the nerve was stimulated by 2 platinum stimulating hook electrodes. The recording electrodes were placed 1 to 2cm apart from the stimulating ones.

The electrophysiological measurements were performed using an AD Instruments (Greenwich, CT) Power Lab 4/25 stimulator and BioAMP amplifier followed by computer assisted data analysis (Chart 5.0 and SCOPE 3.7; AD Instruments) and displayed at a sampling rate of 40K/sec. Sciatic nerves were stimulated with square wave pulses of 200 \( \mu \text{sec} \) duration at 1-10 volts for action potentials, conduction velocities and refractory periods measurements and of 10-1000 \( \mu \text{sec} \) at 50 \( \mu \text{v-5V} \) for chronaxie. The following parameters were assessed:

1- **Evoked maximal compound action potential amplitude (MCAP):**

The stimulating voltage was set to produce a maximal compound action potential using square wave pulses of supra-maximal strength and 0.2ms in duration. The maximum amplitude was measured from the baseline to the peak of action potential [11].

2- **Duration of action potential (AP):**

The time elapsed in milliseconds between the onset of depolarization to return to the baseline was recorded [12].

3- **Relative refractory period (RRP):**

Twin maximum pulse stimuli with stimulus interval 2msec were used to record 2 action potentials. Then, the stimulus interval was decreased gradually by 10 \( \mu \text{sec} \) increment. The interval where the amplitude of the second CAP decreases and reaches 75% of the first maximum CAP was recorded and taken as a standardized measure of refractoriness for comparison between groups [12].

4- **Chronaxie:**

The rheobase was measured as the threshold stimulus voltage for an active response with a long duration pulse. Chronaxie is the pulse width corresponding to twice the rheobase [13].
5- Conduction velocity:

This was measured by dividing the distance between the stimulating and the recording electrodes by the time elapsed between the application of the stimulus until the peak of the MCAP [13].

IV- Behavioral assay:

Tail flick (immersion) test:

This behavioral test is used to assess the nociceptive response to acute thermal pain stimulus. This measure was chosen because of the role small fiber dorsal root ganglia sensory neurons play in pain transmission [14]. The animals were restrained into restrainer cage with their tail hanging free and allowed to adapt for 30 minutes before testing. The lower 5cm portion of the tail is marked. This part of the tail was immersed in a cup of freshly filled water of exactly 55ºC. Within a few seconds, the rat reacted by withdrawing the tail. The latency of this tail withdrawal reflex was recorded by a stop watch in all animal groups [15].

V- Detection of NMDA Receptor and CGRP Expression by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR):

About 30mg of nerve and spinal cord tissues were stored at -80ºC in lysis buffer that contain guanidium thiocyanate and ß-mercaptoethanol for RNA extraction.

RNA Extraction:

Total RNA was extracted from both nerve and spinal cord after homogenization according to manufacturer’s instructions. The concentration of extracted RNA was measured by spectrophotometer at 260nm.

Reverse transcription and polymerase chain reaction (RT-PCR):

For amplification of the targets, reverse transcription and PCR were run in two separate steps. Briefly, equal amounts of total RNA were heat denatured and reverse transcribed by incubation at 42°C for 90min with 12.5U avian myeloblastosis virus reverse transcriptase (AMV) (Promega Corp., Madison, WI), 20U ribonuclease inhibitor RNAsin (Promega Corp.), 200N deoxy–nucleoside 5’-triphosphate mixture and 1nM oligo-dT primer in a final volume of 30 µl of 1x avian myeloblastosis virus reverse transcriptase buffer. The reactions were terminated by heating at 97°C for 5min and cooling on ice. The cDNA samples were amplified in 50 µl of 10 x PCR buffer in the presence of 2.5U Taq DNA polymerase (Promega Corp.), 200mM deoxy–nucleoside 5’-triphosphate mixture, and the appropriate primer pairs (1nM of each primer These sets of primers of CGRP and NMDA were designed from GenBank (accession no. G35510 and 691379, respectively).

Forward primer: 5’- GAGATCAGGAGTTCAAGACC -3’ and
5’-TCCAAACTGGTCACACCTCTACT-3’

Reverse primer: 5’- TTGGCTCACTGCAACCTCC -3’ and
5’- CAGCTTTGGTGACAGCATCTCT-3’ respectively.

PCR consisted in a first denaturing cycle at 97°C for 5min, followed by a variable number of cycles of amplification defined by denaturation at 96°C for 1.5min, annealing for 1.5min and extension at 72°C for 3min. A final extension cycle of 72°C for 15min was included. Annealing temperature was adjusted at 55°C [16].

Agarose gel electrophoresis:

All PCR products were electrophoresed on 2% agarose stained with ethidium bromide and visualized by UV transilluminator.

Semi-quantitative determination of PCR products:

Semi-quantitation was performed using the gel documentation system (BioDO, Analyser) supplied by Biometra.

According to the following amplification procedure, relative expression of each gene [16] was calculated following the formula: R = Densitometrical Units of each gene/Densitometrical Units of β-actin.

PCR Detection of β-Actin:

The "house-keeping" gene β-actin was assessed by PCR for the presence of RNA in all samples and for semi-quantitation of PCR products. cDNA was generated from 1 µg of total RNA extracted with AMV reverse transcriptase for 60min at 37 °C. For PCR, 4 µl cDNA was incubated with 30.5 µl water, 4 µl 25 mM MgCl2, 1 µl dNTPs (10mM), 5 µl 10 x PCR buffer, 0.5 µl (2.5U) Taq polymerase and 2.5 µl of each primer containing 10pmol. β-actin primers (forward 5-TGTTGTCCCTGTATG CTTCT-3, reverse 5-TAATGTCACGCTCC-3) were designed from GenBank (accession no. J00691). The reaction mixture was subjected to 40 cycles of PCR amplification as follows: Denaturation at 95 °C for 1min, annealing at 57°C for 1 min and extension at 72°C for 2min. The PCR product yielded 206bp fragment.
Statistics:
All data are expressed as means ± SE. Statistical analysis was performed using one way ANOVA (Microcal origin software, Inc. Version 5). Student’s t-test served as Post hoc test. p value <0.05 was considered statistically significant.

Results
Electrophysiological recordings:
The results of the electrophysiologic recordings performed in sciatic nerves of the various study groups are shown in Table (1).

1- Sciatic nerve MCAP amplitude:
Following VCR injection for 5 weeks, the mean MCAP was significantly reduced compared to the control group (p<0.05). Concomitant administration of EPO at the smaller dose together with VCR in the EPO1 group completely reversed the effect of VCR and no significant difference in the amplitude of MCAP was measured compared to the control group. In the EPO2 group, however, the mean MCAP was significantly lower than in the control group, but still significantly higher than in the VCR group. There was a significantly lower mean MCAP in the EPO2 group compared to the EPO1 group (p<0.05).

2- Duration of AP:
The duration of AP was significantly prolonged in the VCR-treated group (p<0.05). In the EPO1 and EPO2 groups this prolongation was completely abolished and the duration of AP showed no significant change compared to the control group.

3- RRP:
With regard to the RRP, it was found to be significantly prolonged in the VCR group compared to the control group (p<0.05). Again this effect of VCR was completely abolished by the concomitant administration of the two doses of EPO, so that the RRP in the EPO1 and EPO2 groups was not significantly different from the values of the control group.

4- Chronaxie:
In the VCR group chronaxie was significantly shortened (p<0.05) compared to the control group. The smaller dose of EPO in the EPO1 group was not able to reverse this effect. The EPO2 group however showed a significantly prolonged chronaxie compared to the VCR group (p<0.05) and it was not significantly different from the control.

5- Conduction velocity:
The conduction velocity was not significantly altered in any of the tested groups compared to the control group (p>0.05).

Behavioral assay: Tail flick test:
The latency of tail withdrawal reflex in response to immersion of the tail in warm water was significantly shortened in the VCR group compared to the control group (p<0.05). In the EPO1 group the latency was significantly lower than that of the control group, but significantly higher than in the VCR group (p<0.05). In the EPO2 group, the higher dose of EPO completely reversed the effect of VCR in the tail flick test, so that there was no significant difference between the EPO2 group and control, while the latency was significantly longer than in the VCR and EPO1 groups (p<0.05).

Detection of NMDA receptor and CGRP Expression:
The results of CGRP and NMDA receptor expression are shown in Table (2) and Figs. (1,2).

NMDA receptor expression was significantly increased in the VCR group (p<0.05) in both the spinal cord and sciatic nerves. In the EPO1 group, concomitant administration of EPO at the smaller dose with VCR, resulted in a significant decrease in the expression of NMDA receptors compared to the VCR group (p<0.05), but it was still significantly higher than in the control group (p<0.05) in the sciatic nerves. With the higher dose of EPO, the EPO2 group showed a complete reversal of the effect of VCR in the sciatic nerves, as the NMDA receptor expression was not significantly different than that in the control group (p>0.05). In the spinal cord, however, the expression of NMDA receptors was significantly lower in the EPO2 group, but it remained higher than in the control group (p<0.05).

With regard to the expression of CGRP, the VCR group showed significantly reduced expression in the spinal cord and sciatic nerves compared to the control group (p<0.05). In the EPO1 and EPO2 groups there was a significant increase of the expression of CGRP in sciatic nerves compared to the VCR group, but it was still significantly lower than in the control group (p<0.05). In the spinal cord, however, CGRP expression in both EPO1 and EPO2 groups was significantly increased compared to the VCR and the control groups (p<0.05).
Table (1): Results of electrophysiologic recordings and behavioral assay (tail flick test) in the different experimental groups expressed as mean ± SE (n=10 rats/group).

<table>
<thead>
<tr>
<th></th>
<th>Control Group</th>
<th>VCR Group</th>
<th>EPO1 Group</th>
<th>EPO2 Group</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MCAP (mV)</strong></td>
<td>44.83±2.1</td>
<td>22.49±2.3*</td>
<td>43.22±2.44∞</td>
<td>33.65±2.58*∞#</td>
</tr>
<tr>
<td><strong>Duration of AP (msec)</strong></td>
<td>0.67±0.12</td>
<td>0.80±0.03*</td>
<td>0.64±0.01∞</td>
<td>0.64±0.02∞</td>
</tr>
<tr>
<td><strong>RRP (msec)</strong></td>
<td>0.12±0.01</td>
<td>0.14±0.005*</td>
<td>0.11±0.003∞</td>
<td>0.10±0.01∞</td>
</tr>
<tr>
<td><strong>Chronaxie (msec)</strong></td>
<td>87.5±2.5</td>
<td>77.5±3.78*</td>
<td>79.16±3.27</td>
<td>89.28±2.02∞#</td>
</tr>
<tr>
<td><strong>Conduction Velocity (m/sec)</strong></td>
<td>43.09±1.79</td>
<td>87.21±2.11</td>
<td>38.46±1.36</td>
<td>40.46±1.35</td>
</tr>
<tr>
<td><strong>Tail Flick Test (sec)</strong></td>
<td>14.87±0.55</td>
<td>7.81±0.64*</td>
<td>10.58±1.08*∞</td>
<td>14.64±0.39∞#</td>
</tr>
</tbody>
</table>

* Significant compared to control group (p<0.05).
∞ Significant compared to VCR group (p<0.05).
# Significant compared to EPO1 group (p<0.05).

Table (2): NMDA receptor and CGRP expression (in sciatic nerves and spinal cord) in the different experimental groups. Results are expressed as mean ± SE (n=10 rats/group).

<table>
<thead>
<tr>
<th></th>
<th>Control Group</th>
<th>VCR Group</th>
<th>EPO1 Group</th>
<th>EPO2 Group</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NMDA receptor Expression:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In sciatic nerves</td>
<td>1.21±0.09</td>
<td>3.55±0.27*</td>
<td>2.19±1.00∞</td>
<td>1.51±1.04∞#</td>
</tr>
<tr>
<td>In spinal cord</td>
<td>0.02±0.06*</td>
<td>1.67±0.21*</td>
<td>0.56±0.12*∞</td>
<td>0.51±0.03*∞</td>
</tr>
<tr>
<td><strong>CGRP expression:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In sciatic nerves</td>
<td>4.99±0.29</td>
<td>2.38±0.16*</td>
<td>3.91±0.21*∞</td>
<td>4.15±0.12*∞</td>
</tr>
<tr>
<td>In spinal cord</td>
<td>-6.63±0.18</td>
<td>1.46±0.19*</td>
<td>6.16±0.15*∞</td>
<td>6.78±0.26*∞</td>
</tr>
</tbody>
</table>

* Significant compared to control group (p<0.05).
∞ Significant compared to VCR group (p<0.05).
# Significant compared to EPO1 group (p<0.05).

Discussion

Neuropathy accompanies peripheral nerve injury after a wide variety of insults, including metabolic disorders, traumatic nerve injury and neurotoxic drugs. Chemotherapy-induced neuropathy, caused by drugs, such as VCR, occurs in cancer patients who receive these drugs as antineoplastic agents [17].

In the present study, the amplitude of the maximum compound action potential (MCAP) was significantly decreased, while the duration of action potential was significantly prolonged in the VCR group compared to the control group. A similar reduced action potential amplitude was recorded by Ja’afar et al. [10]. On the other hand, the study of Adelsberger et al. [18] showed that another chemotherapeutic agent, Oxaliplatin, which is also neurotoxic like VCR, increased the action potential amplitude in contrast to our study, but similar to our findings prolonged the duration of action potential. The significant decrease in the mean am-

Lane M: PCR marker with 100 bp ladder.
Lane 1: PCR products of NMDA gene in Control Group.
Lane 2: PCR products of NMDA gene in VCR Group.
Lane 3: PCR products of NMDA gene in EPO1 Group.
Lane 4: PCR products of NMDA gene in EPO2 Group.

Fig. (1): An agarose gel electrophoresis showing PCR product of NMDA gene.

Lane M: PCR marker with 100 bp ladder.
Lane 1: PCR products of CGRP gene in Control Group.
Lane 2: PCR products of CGRP gene in VCR Group.
Lane 3: PCR products of CGRP gene in EPO1 Group.
Lane 4: PCR products of CGRP gene in EPO2 Group.

Fig. (2): An agarose gel electrophoresis showing PCR product of CGRP gene.
plitude of action potential suggests that a significant number of sensory and motor fibers are affected by the drug. This was confirmed by several histologic studies, which found VCR-induced axonal degeneration [10,19] as well as disorganization of the axonal microtubule cytoskeleton and increase in the caliber of the unmyelinated sensory axons [20]. It could also be demonstrated that the VCR-induced toxicity is localized in the axons not in the cell bodies [19,21], a pattern that is known as the "dying-back neuropathy".

In the VCR group of the present study, no significant change was observed in the conduction velocity compared to the control group. Similar results were recorded by other investigators [3,10,22]. The preservation of nerve conduction velocity, despite a reduced amplitude and prolonged duration of action potential during VCR neuropathy, may reflect that in the present study the axonal degeneration may have been too mild and too early to show affection of the nerve conduction. This is consistent with the findings of Ja'afar et al. [10] that showed no significant change in conduction velocities of sciatic nerves between the control and VCR group, despite a decrease in amplitude of MCAP. In their histological examinations they detected minor changes in Schwann cells and myelin sheath vacuolation, which represent an early stage of myelin disruption [23,24]. Another study observed a decreased nerve conduction velocity in hyperglycemia that rapidly improved by insulin therapy, suggesting that the axonal dysfunction was induced by impaired Na\(^+\)-K\(^+\) ATPase rather than by mechanical demyelination of the nerves [28]. On the other hand, other studies observed decreased nerve conduction velocity in VCR-induced neuropathy in rats [9,26] and rabbits [27,28]. This may be explained by the higher dose of VCR used, which may cause early and direct toxic effects on the Schwann cells with consequent early myelin changes and/or the species difference in these studies from our own.

In the present study, the relative refractory period (RRP) was found to be significantly prolonged in the VCR group. It was shown that RRP measurement is more sensitive than routine measures of nerve conduction in detection of axonal disorders [29,30] and early neurotoxicity [31]. To our knowledge, no other study has investigated the effect of VCR on the relative refractory period. But another study, using Oxaliplatin, also observed a prolonged RRP [32]. It proposed that the acute form of this drug-induced neurotoxicity and specifically the observed prolonged RRP is mediated through an effect on axonal voltage-gated transient Na\(^+\) channels, which is involved in the subsequent process of long-term axonal degeneration. This is further confirmed by another study, which showed that Oxaliplatin altered voltage-gated Na\(^+\) channel kinetics in rat sensory neurons [18]. Inactivation of transient Na\(^+\) channels was also reported as the cause of prolonged RRP in human diabetics [33]. In our study, the prolonged RRP with normal conduction velocity suggests the presence of early and mild axonal degeneration and confirms that RRP is a more sensitive marker for the detection of early axonal affection.

Chronaxie can be used to define the excitability of nerves and muscles. It is the duration of a pulse of current of twice rheobasic strength. VCR-induced shortening of chronaxie in the present study, suggests that VCR increased nerve excitability.

Another evidence that VCR increased nerve excitability in the present study is seen in the results of the tail-flick test. In this test there was significant shortening of the reaction time in the VCR-treated group compared to control, indicating a decreased nociceptive threshold to thermal stimuli. A similar result was observed by Kamei et al. [34] in VCR-treated mice. They found that the degree of thermal hyperalgesia depended on the duration of treatment. Hyperresponsiveness of VCR-treated nociceptors was also reported in response to heat [26] and mechanical stimulation [35] in rats. Thermal hyperalgesia could not be observed in other animal models [36,37]. This discrepancy might be due to differences between the dose and treatment schedule of VCR.

The exact mechanisms underlying VCR-induced neuropathy are poorly understood. Recently, a report demonstrated that inflammatory mediators such as macrophage-derived interleukin-6 plays a critical role in VCR-induced mechanical allodynia in mice [38], while others found that protein kinase A, protein kinase C and nitric oxide second messenger pathways in the periphery of nerves [39] contribute to the VCR-induced neuropathy. Axonal dysfunction was also shown to be induced by impaired function of the Na\(^+\)-K\(^+\) ATPase in hyperglycemic neuropathy [28], while other investigators showed that the Na\(^+\)-K\(^+\) pump plays no role in the development of neuropathy in end-stage kidney disease [40]. Another study reported that chemotherapy resulted in nerve injury-evoked increase in calcium channel alpha(2) delta-1 subunit expression [41].

The observed increased excitability in the present study suggests the presence of an excito-
toxicity. Glutamate mediates excitatory synaptic transmission through the activation of ionotropic glutamate receptors that are sensitive to N-methyl- D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or kainate. Excess and sustained activation of the ionotropic glutamate receptors causes fulminating neuronal death, namely, glutamate neurotoxicity or excitotoxicity [42]. The Ca\(^{2+}\) influx through NMDA receptors mediates the rapidly-triggered NMDA neurotoxicity, while Na\(^{+}\) influx contributes to the swelling of the neuronal cell body [43]. Therefore, we studied the expression of NMDA receptors in the different groups of this study both in the sciatic nerves and spinal cord. A significant increase in NMDA receptor expression was detected in the VCR-treated group compared to the control. To our knowledge, no other studies measured the expression of NMDA receptors in association with VCR neurotoxicity. However, our results are consistent with studies that showed that NMDA receptor antagonists decreased VCR-induced hyperalgesia [44,45]. Another study suggested that activation of peripheral NMDA receptors by glutamate injection contributes to human pain and afferent discharge in rats [46].

In contrast to our results, other studies reported that NMDA receptor-mediated signaling pathways were not involved in VCR-induced neuropathic pain [39,47] and using an NMDA receptor antagonist did not reduce VCR-induced cell death in an in vitro study on primary cerebellar granule neurons [48].

In the present study, VCR resulted in a significant reduction of CGRP expression in the sciatic nerves and spinal cord. It was reported that epineurial peptidergic terminals mediate a vasodilatory response through CGRP that increases blood flow in the downstream endoneurial compartment [49]. Thus, it may be suggested that VCR by decreasing CGRP decreases the blood flow to the nerve producing ischemia. This is the first time that CGRP expression has been measured in conjunction with VCR or any other chemotherapeutic agents. Other investigators reported that cisplatin, ifosfamide and etoposide produced vascular neurotoxicity [50]. Won et al. [42] showed that ischemia induces excitotoxicity. Therefore, the VCR-induced excitotoxicity in our study may not only be due to increase in the expression of NMDA receptors, but may also be due to ischemia. It also appears that VCR mediated its effects at the level of both the spinal cord and peripheral nerves.

EPO, a 165-amino acid sialoglycoprotein, is essential in the regulation of erythropoiesis. Among its various clinical applications, EPO is a very effective and widely used treatment for anemia in cancer patients undergoing chemotherapy. However, the bone marrow is not the only target tissue of EPO and the wide expression of functional receptors for EPO explains its nonerythropoietic functions [51]. EPO receptors were found to be expressed in both dorsal root ganglia [52] and peripheral nerves [51]. Several studies have shown that administration of EPO affords significant neuroprotection in peripheral and central nervous system injury models [8,47,54-57], thus raising the possibility that it has a nonspecific neuroprotective property. On the other hand some investigators could not detect any neuroprotective effect of EPO in a rabbit model of meningitis [58].

In the present study, EPO1 reversed most of the VCR-induced changes in the electrophysiological parameters measured, namely the decreased amplitude of MCAP, prolonged duration of AP and prolonged RRP, but had no significant effect on chronaxie. It also shortened the latency of the tail flick test. EPO2 on the other hand, had a similar effect to EPO1, but was also able to reverse the decreased chronaxie induced by VCR. It was also more potent than EPO1 with regard to tail flick test, but it was less effective on MCAP. These results indicate that EPO had an obvious neuroprotective effect in VCR-induced neuropathy in rats of the present study.

Bianchi et al. [8] found that EPO was able to partially reverse diabetes-induced loss in nerve functions (MCAP and conduction velocity) and impairment in nociceptive thresholds. Similar improvement of these parameters by EPO was observed in Cisplatin-induced peripheral neurotoxicity [59,60]. To our knowledge, our study is the first to use EPO as a neuroprotective agent in VCR-induced neuropathy.

Several mechanisms of action were proposed for the protective effect of EPO. It was suggested that EPO ameliorates or prevents neuronal injury by antiapoptotic [61], antioxidant [62], anti–inflammatory [63] effects in cell culture and animal models of neurological diseases.

In the present study, the increase in NMDA receptor expression by VCR was significantly reduced by both doses of EPO in the spinal cord and sciatic nerves. Yet, the higher dose of EPO in the EPO2 group produced a complete reversal of the VCR-induced changes, while the smaller dose
only caused a partial reversal at the peripheral nerve level. This clearly indicates that one of the mechanisms of action of EPO as a neuroprotective agent is mediated through decreasing excitotoxicity. It also seems that the higher dose was better capable of preventing excitotoxicity as it had a more prominent effect not only on NMDA receptor expression, but also on improving the latency of the tail flick test and chronaxie, which are both indicators of excitability. An inhibitory effect of EPO on excitotoxicity was reported in several neurotoxic models. EPO was shown to reduce excitotoxicity in mouse hippocampal slice cultures [64] and in newborn mouse brain injury [65]. Another study showed that the neuroprotective effect of EPO was abolished via a NMDA receptor antagonist [66]. Won et al. [67] also found that EPO is a potent protector against kainate excitotoxicity. On the other hand, another study could not show that EPO reduced NMDA receptor increase in intracellular Ca²⁺ [62].

The reduction in CGRP expression observed with VCR administration in the spinal cord and sciatic nerves was reversed by EPO in our study. On the level of the spinal cord both doses of EPO even increased CGRP expression to higher levels than normal. Similarly Toth et al. [68] reported that EPO increased the density and intensity of CGRP expression within outgrowing axons after crush injury. Thus it seems that another mechanism for the improvement of VCR-induced neurotoxicity by EPO may be via the restoration of blood flow in central and peripheral nervous tissue by increasing CGRP. This is consistent with the finding of Paschos et al., who reported that EPO plays a crucial role as an inhibitor of tissue ischemia [69]. Multiple studies have shown that whereas normal brain expresses both EPO and EPO receptors in a highly restricted and limited manner, a marked induction of both proteins occurs following ischemic and other stressors in animal models [70] as well as in human disease [71,72].

It can be concluded that VCR decreased the amplitude of MCAP and prolonged the duration of AP, indicating axonal degeneration in sciatic nerves. This degeneration apparently was early and mild, as it did not affect the conduction velocity, but prolonged the RRP, which is an early marker of axonal degeneration. VCR also increased the excitability, as evidenced by the decreased chronaxie and latency time of the tail flick test, suggesting the presence of a state of excitotoxicity. The measurement of NMDA receptor expression showed that it was increased by VCR in the sciatic nerves and spinal cord, indicating the clear presence of a glutamate excitotoxicity both centrally and peripherally. VCR also decreased the expression of CGRP, the neuropeptide that mediates vasodilation and increased blood flow in the nervous system. Thus, it can be concluded that VCR produced excitotoxicity both directly by increasing the expression of NMDA receptors and indirectly by causing ischemia, another factor that increases NMDA receptor expression. EPO improved VCR-induced neurotoxicity as evidenced by the electrophysiologic recordings and the tail flick test. There may exist several mechanisms of action of EPO, but we were able to detect that EPO decreased NMDA receptor expression and increased CGRP expression, thus decreasing the excitotoxicity caused by VCR. The two doses of EPO used in the present study both had similar effect in ameliorating excitotoxicity, but the higher dose was more effective.

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