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Curcumin reduces blood-nerve barrier abnormalities and cytotoxicity to endothelial cells and pericytes induced by cisplatin

P. Kobutree, A. Tothonglor, A. Roumwong, D. Jindatip, S. Agthong

Department of Anatomy, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

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Background: *Cisplatin is a platinum-based antineoplastic agent used to treat cancers of solid organs. Neuropathy is one of its major side effects, necessitating dose reduction or cessation. Previous studies suggested that cisplatin causes microvascular toxicity, including pericyte detachment. This study aimed to clarify whether these alterations occurred in the blood–nerve barrier (BNB) of capillaries after cisplatin treatment.*

Materials and methods and Results: *Electron microscopic analysis of rat sciatic nerves with cisplatin neuropathy showed increased frequency and severity of pericyte detachment. Moreover, the vascular basement membrane did not tightly encircle around the endothelial cells and pericytes. Cultured human umbilical vein endothelial cells and human brain vascular pericytes showed reduced viability, increased caspase-3 activity and enhanced oxidative stress following cisplatin treatment. In addition, cisplatin decreased transendothelial electrical resistance (TEER) and the expression of the tight junction proteins occludin and zonula occludens-1. Curcumin, a polyphenol found in the root of Curcuma longa, had favourable effects on cisplatin neuropathy in previous work. Therefore, curcumin was tested to determine whether it had any effect on these abnormalities. Curcumin alleviated pericyte detachment, cytotoxicity, oxidative stress, TEER reduction and tight junction protein expression.*

Conclusions: *These data indicate that cisplatin causes BNB disruption in the nerves and might result in neuropathy. Curcumin might improve neuropathy via the restoration of BNB. Whether alterations in the BNB occur and curcumin is effective in patients with cisplatin neuropathy remain to be investigated.* (Folia Morphol 2023; 82, 3: 533–542)

Key words: capillaries, cisplatin, nerve, neuropathy

INTRODUCTION

Cisplatin is a chemotherapeutic agent for treating cancers of several solid organs [10]. One of its major side effects is peripheral neuropathy, often leading to dose reduction or cessation of chemotherapy. Sensory abnormalities were observed in both animals and patients with cisplatin-induced neuropathy [5, 19, 30, 36]. Morphological analysis showed loss of spinal ganglion neurons with nuclear and nucleolar atrophy [2, 35]. In the sciatic nerve, degeneration and demyelination of nerve fibres have also been reported [3, 35]. To date, different agents targeting various

Address for correspondence: D. Jindatip, PhD, Department of Anatomy, Faculty of Medicine, Chulalongkorn University, 1873 Rama 4 Rd., Wangmai, Pathumwan, Bangkok 10330, Thailand, tel: +662-256-4281, fax: +662-252-7028, e-mail: depicha.j@chula.ac.th

underlying mechanisms of cisplatin neuropathy have failed to show clinical efficacy [21, 32]. However, curcumin, a polyphenol found in the root of *Curcuma longa*, has demonstrated beneficial effects on cisplatin neuropathy [2]. This activity warrants further investigation of curcumin as a potential therapy.

Current evidence suggests that vascular dysfunction might play a role in cisplatin neuropathy. Arterial occlusion [20] and endothelial damage [9] were reported in patients receiving cisplatin. In cisplatin-treated rats, reduced nerve blood flow, a decreased number of vasa nervorum and endothelial apoptosis were observed [18]. Our previous study showed reduced density and detachment of pericytes in the nerve capillaries of rats with cisplatin neuropathy [17]. These abnormalities might be associated with an impaired blood–nerve barrier (BNB). This study aimed to confirm whether there were structural defects of BNB in rats treated with cisplatin. In addition, the direct effects of cisplatin on endothelial cells and pericytes were investigated in vitro. Moreover, we examined whether concomitant treatment with curcumin could alleviate the vascular toxicity of cisplatin.

MATERIALS AND METHODS

Tissue collection

The specimens used in this study were from a previous animal experiment [2]. Briefly, female Wistar rats were divided into three groups: control, cisplatin and cisplatin + curcumin (n = 8 each). Cisplatin (Cat. No. NDC 0069-0084-07, Pfizer, New York, NY, USA) 2 mg/kg was injected intraperitoneally twice a week for 5 consecutive weeks (20 mg/kg cumulative dose). During the 5-week administration of cisplatin, 200 mg/kg curcumin (Cat. No. 81025, Cayman Chemical, Ann Arbor, MI, USA) was concomitantly given by gavage to the cisplatin + curcumin group once daily. All animals were left untreated for 3 weeks until they were sacrificed. The rats were sacrificed by anaesthetic overdose and then transcardially perfused with normal saline followed by 4% paraformaldehyde (Cat. no. 818715, Merck Millipore, Darmstadt, Germany). L4 dorsal root ganglia and sciatic nerves were post-fixed in 3% glutaraldehyde (Cat. No. 16220, EMS, Hatfield, PA, USA) and embedded in epoxy resin. The ganglia and left sciatic nerves were used for morphometric analysis, whereas right sciatic nerves were processed for electron microscopic examination.

The presence of neuropathy in the cisplatin group was confirmed by the hot plate test and nerve conduction study during the experiment and before sacrifice. Moreover, ganglion and nerve morphometry showed ultrastructural changes characteristic of experimental cisplatin-induced neuropathy [3, 5, 35, 36]. Curcumin significantly attenuated these abnormalities.

These specimens were from the previous study which showed thermal hypoalgesia in the 5th week and reduced sciatic motor nerve conduction velocity in the 5th and 8th weeks [2]. Moreover, ganglion morphometry showed nuclear and nucleolar atrophy including loss of neurons in the 8th week. Curcumin significantly attenuated these abnormalities.

Transmission electron microscopy

Ultrathin sections (70 nm thickness) of the sciatic nerves divided into the proximal and distal parts were stained with lead citrate and uranyl acetate. The morphology of pericytes and the vascular basement membrane (VBM) shared with endothelial cells was examined with a transmission electron microscope (JEM-1400PLUS; JEOL, Tokyo, Japan). In each rat, 20 capillaries were randomly chosen from serial sections of proximal and distal nerves. Each capillary was evaluated for the presence of pericyte detachment from endothelial cells and VBM, which was classified into two categories. In category 1, the pericyte was completely attached to the VBM and endothelial cell. In category 2, the pericyte had detached from the VBM and endothelial cell at least one point. Subsequently, the lengths of the farthest detachment between the pericytes and VBM were measured in the capillaries of category 2. The thickness of the VBM at the farthest detachment was also measured.

Cell culture

Human umbilical vein endothelial cells (HUVECs; Cat. No. #C-015-5C, Invitrogen, Waltham, MA, USA) and human brain vascular pericytes (HBVPs; Cat. No. 1200, ScienCell, Carlsbad, CA, USA) were grown according to manufacturers' protocols. Each experiment was performed in triplicate and repeated three times. HUVECs and HBVPs were divided into three groups: control, cisplatin, and cisplatin + curcumin. In the cisplatin and cisplatin + curcumin groups, HUVECs and HBVPs were incubated with 3 μg/mL and 1.5 μg/mL cisplatin (Cat. No. 1C 257/51, Korea United Pharm, Seoul, South Korea) for 24 hours, respectively. For curcumin treatment, the cells were co-incubated with 1 μg/mL curcumin (Cat. No. 81025, Cayman Chemical, Ann Arbor, MI, USA) for 24 hours. These doses were

chosen as the lowest concentrations to induce cytotoxicity in the HUVEC and HBVP in the pilot studies.

MTT assay

The MTT assay was used to evaluate the viability of HUVECs and HBVPs. The cells were seeded at 1×10^4 and 5×10^3 cells/well in 96-well plates and allowed to attach for 24 hours. The cells were then treated according to the experimental conditions for 24 hours. Finally, the cells were incubated with 100 μL MTT solution (Cat. No. M6494, Molecular Probes, Eugene, OR, USA) for 2 hours. Subsequently, purple formazan crystals were dissolved in 100 μL dimethyl sulfoxide. The absorbance was measured at 570 nm using a microplate reader (Multiskan GO 1510-02675; Thermo Fisher Scientific, Waltham, MA, USA). The percentage of cell viability was calculated from the absorbance of the sample divided by that of the negative control.

Caspase-3 assay

A caspase-3/CPP32 colorimetric assay kit (Cat. No. #K106-200, BioVision, Milpitas, CA, USA) was used to determine caspase-3 activity. Briefly, the cells were plated at 1×10^6 in the culture vessels. After treatments, the cells were harvested and resuspended in cell lysis buffer. The supernatant of each sample was collected, and the protein concentration was measured. Subsequently, the sample was diluted with cell lysis buffer to obtain a protein concentration of 1 μg/μL and transferred to a 96-well plate. Then, working reaction buffer and DEVD-pNA substrate were added. The plate was incubated at 37°C for 2 hours. Finally, the absorbance was measured at 405 nm using a microplate reader (Multiskan GO 1510-02675; Thermo Fisher Scientific, Waltham, MA, USA). The caspase-3 activity of treated cells was compared with that of controls.

ROS assay

Cells were seeded at 1×10^4 cells/well in 96-well black plates for 24 hours. Then, the cells were incubated with 2',7'-dichlorofluorescein diacetate (DCFH-DA; Cat. No. D688, Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 30 minutes. After the cells were treated according to the experimental conditions, the level of DCF (T0) was measured using a fluorescence microplate reader (Varioskan Flash; Thermo Fisher Scientific, Waltham, MA, USA) at 480 and 535 nm for excitation and emission, respectively. Subsequently, the incubation was continued for 1 hour, and the level of DCF was measured again (T1). The relative production of reactive oxygen species (ROS) was calculated from the ratio of T1 to T0.

GSH/GSSG assay

The levels of oxidized (GSSG) and total glutathione were determined using a glutathione colorimetric detection kit (Cat. No. #9135, Promega, Madison, WI, USA). In brief, cells were plated at 1×10^6 cells/ /mL in a T-25 flask and treated according to the experimental conditions for 24 hours. The pellets were harvested and resuspended in cold 5% 5-sulfosalicylic acid (Cat. No. S2130, Sigma-Aldrich, St. Louis, MO, USA) and then incubated at 4°C for 10 minutes. The supernatant was mixed with the detection substrate and reaction mixture followed by incubation at room temperature for 20 minutes. Total glutathione was determined by measuring at 405 nm using a microplate reader (Multiskan GO 1510-02675, Thermo Fisher Scientific, Waltham, MA, USA). For GSSG detection, the sample and standard were pretreated with 2-vinylpyridine (Cat. No. 132292, Sigma-Aldrich, St. Louis, MO, USA). The level of reduced glutathione (GSH) was derived from subtraction of GSSG from total glutathione. Finally, the GSH/GSSG ratio was calculated.

Transendothelial electrical resistance (TEER) study

HUVECs were cultured in the upper chamber of a transwell insert (Cat. No. MCHT24H48, Merck, Kenilworth, NJ, USA), which was inserted into a 24-well plate at 1×10^4 cells/well. After 24 hours of treatments according to the experimental conditions, cell resistance (R) was measured using a Millicell electrical resistance apparatus (Millicell® ERS-2; Merck, Kenilworth, NJ, USA). The TEER value was calculated using the following formula: TEER value (Ωcm^2) = = $(R_{sample} - R_{blank}) \times$ membrane area (cm^2).

Western blot analysis

HUVECs were seeded at 2×10^6 cells/ml in a culture dish. After being treated according to experimental protocols for 24 hours, the cells were incubated on ice with lysis buffer (Cat. No. #9806, Cell Signaling, Danvers, MA, USA) containing 1 × protease inhibitor cocktail (Cat. No. #5871, Cell Signaling, Danvers, MA, USA) for 5 minutes. Subsequently, the cells were scraped and centrifuged, and the supernatant was collected. The protein concentration of the supernatant was determined using a PierceTM

BCA protein assay (Cat. No. 23227, Thermo Fisher Scientific, Waltham, MA, USA). The sample (1.5 μg/μL) was then mixed with the fluorescent dye (4:1 ratio) and denatured at 95°C for 5 minutes. The marker, sample, antibody diluent, primary antibody (1:200 β-actin [Cat. No. #4970, Cell Signaling, Danvers, MA, USA], 1:200 zonula occludens-1 [ZO-1; Cat. No. #PA5-28858, Invitrogen, Waltham, MA, USA], 1:200 zonula occludens-2 [ZO-2; Cat. No. #PA5-17155, Invitrogen, Waltham, MA, USA], 1:200 claudin-5 [Cat. No. #34-1600, Invitrogen, Waltham, MA, USA], 1:200 occludin [Cat. No. #PA5-20755, Invitrogen, Waltham, MA, USA]), rabbit secondary conjugate, streptavidin-HRP, and luminol peroxide (Cat. No. DM-001, ProteinSimple, Santa Clara, CA, USA) were added to the plate according to the manufacturer's protocol. Subsequently, separation and immunodetection were performed using a WES automated western blotting system (ProteinSimple; Santa Clara, CA, USA). The density of digital images was analysed using Compass software (ProteinSimple, Santa Clara, CA, USA). The expression of each protein was normalized to that of β-actin.

Statistical analysis

One-way ANOVA followed by Tukey's post-hoc test was used to compare the means of the above parameters between groups. The test was performed using SPSS for Windows version 23 (SPSS, Inc., Armonk, NY, USA). Differences were considered statistically significant when $p < 0.05$.

RESULTS

Ultrastructural analysis

Pericyte detachment from endothelial cells appeared to be more prominent in the nerve capillaries of the cisplatin-treated group than in the control group (Fig. 1). In contrast, detachment was less severe in the cisplatin + curcumin group. It was noted that the basement membrane did not tightly wrap around the endothelial cells and pericytes in the cisplatin-treated group (arrows in Fig. 1B) compared to the control and cisplatin + curcumin groups (arrows in Fig. 1A, C). No other pathological findings, such as the accumulation of lysosomes or vacuoles, were detected in the pericytes or endothelial cells in any group.

After morphometric analysis, the number of capillaries with detachment compared with that of total capillaries was significantly higher in the cisplatin group than in the control group (Fig. 2). Curcumin treatment significantly reduced the ratio. In addition, the detachment distance was significantly longer in the cisplatin group than in the control group (Fig. 3). Treatment with curcumin significantly decreased the distance. Regarding the thickness of the basement membrane at the detachment site, there were no significant differences between groups (data not shown).

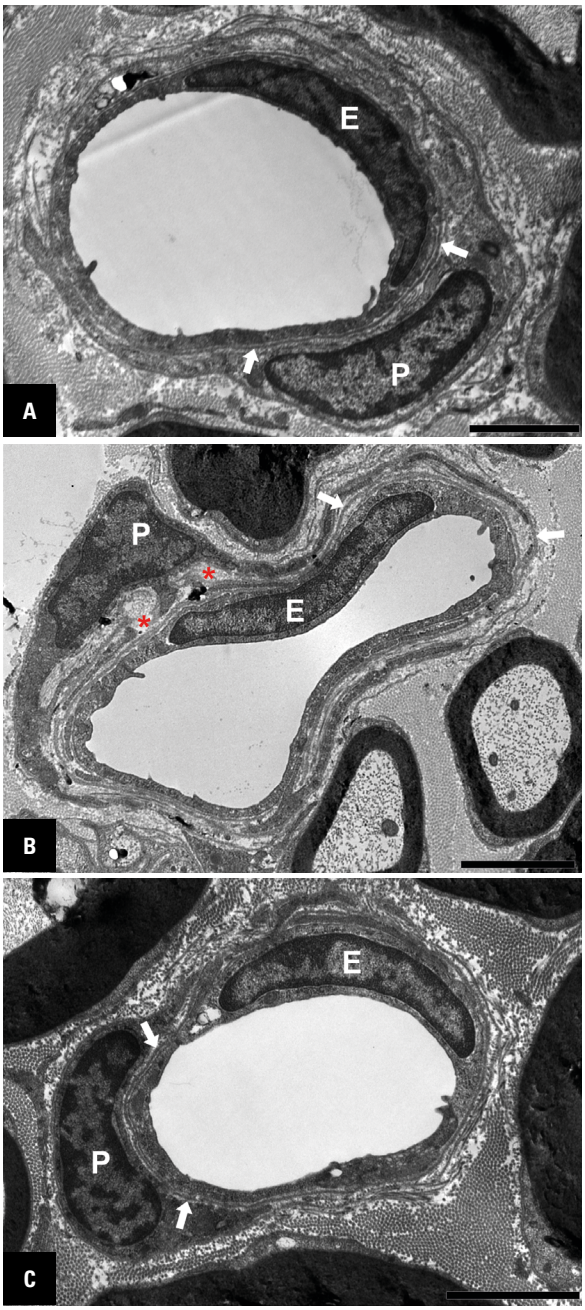

Figure 1. Representative electron microscopic images of capillaries in the sciatic nerves from the control (**A**), cisplatin (**B**), and cisplatin + curcumin (**C**) groups. Arrows indicate the basement membrane shared between the endothelial cell (E) and pericyte (P); *Pericyte detachment from the endothelial cell; scale bars: 1 μm.

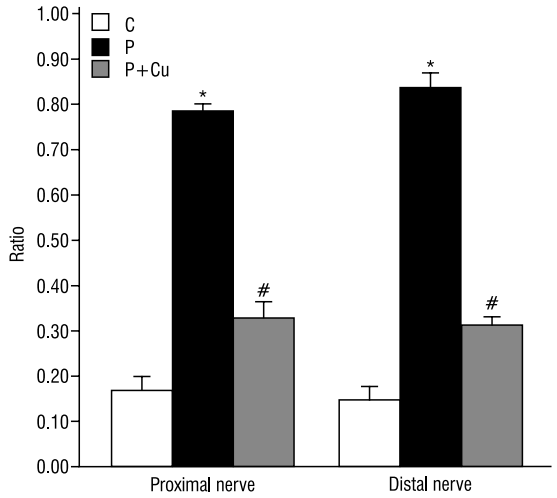

Figure 2. Ratio of the number of capillaries with pericyte detachment to the total number of capillaries in the proximal and distal parts of the sciatic nerve. The graph shows the means and standard error of the mean; C — control; P — cisplatin; P+Cu — cisplatin + curcumin; *p < 0.001 P vs. C; #p < 0.01 P+Cu vs. C and p < 0.001 P+Cu vs. P.

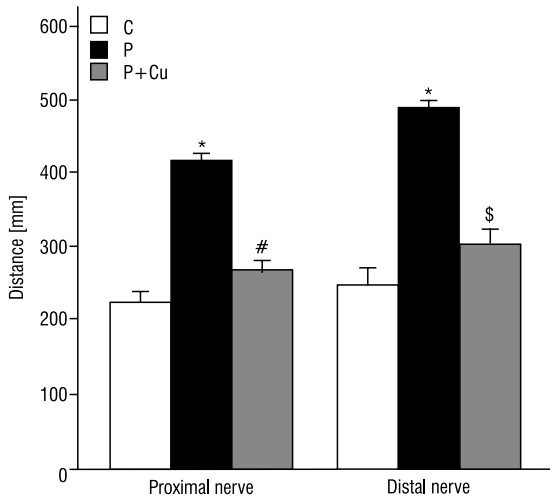

Figure 3. Distance at the widest detachment of pericytes in the proximal and distal parts of the sciatic nerve. The graph shows the means and standard error of the mean; C — control; P — cisplatin; P+Cu — cisplatin + curcumin; *p < 0.001 P vs. C; #p < 0.05 P+Cu vs. C and p < 0.001 P+Cu vs. P; $p < 0.001 P+Cu vs. P.

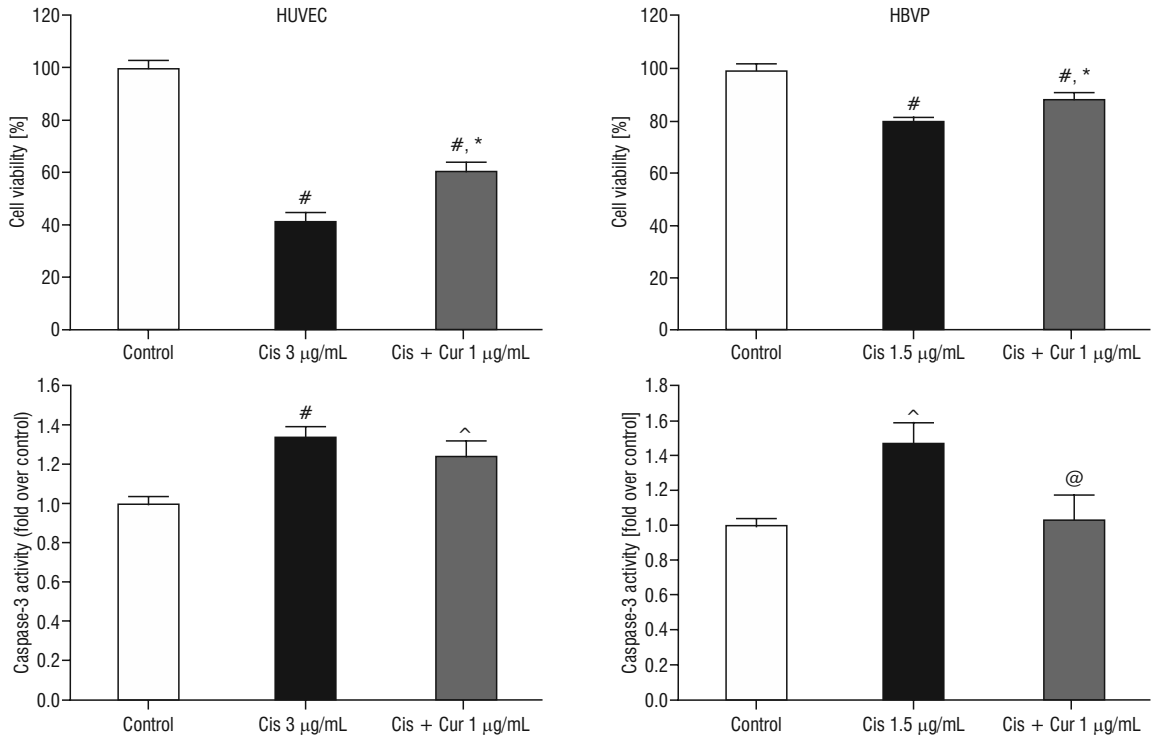

Figure 4. Cell viability and caspase-3 activity of human umbilical vein endothelial cells (HUVECs, left column) and human brain vascular pericytes (HBVPs, right column). The graphs show the means and standard error of the mean; Cis — cisplatin; Cur — curcumin; #p < 0.001 vs. control; *p < 0.01 vs. Cis; ^p < 0.05 vs. control; @p < 0.05 vs. Cis.

Cell viability and caspase-3 activity

The viability of HUVECs and HBVPs was significantly lower in the cisplatin group than in the control group (Fig. 4). In addition, caspase-3 activity was significantly elevated in the cisplatin group compared with the control group in both cell types. Curcumin treatment partially improved the viability of both cell types and caspase-3 activation in HUVECs. In HBVP, curcumin normalised caspase-3 activity.

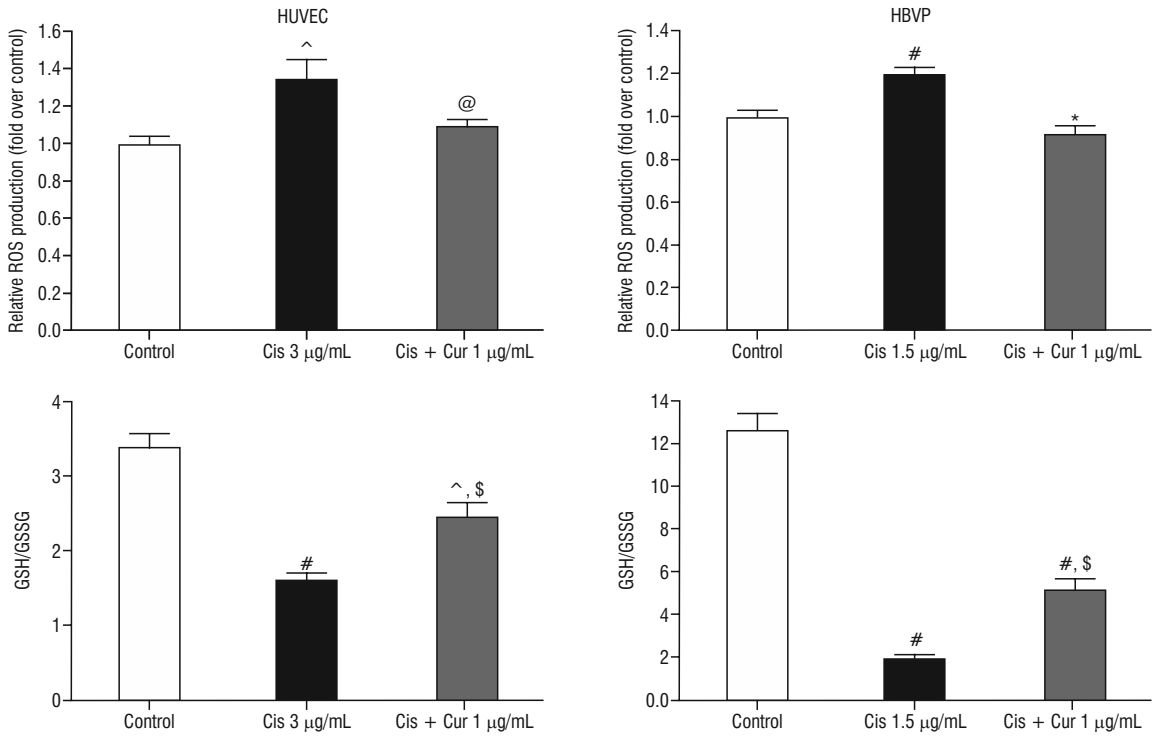

Figure 5. Reactive oxygen species (ROS) production and the ratio of reduced to oxidized glutathione (GSH/GSSG) in human umbilical vein endothelial cells (HUVECs, left column) and human brain vascular pericytes (HBVPs, right column). The graphs show the means and standard error of the mean; Cis — cisplatin; Cur — curcumin; ^ $p < 0.01$ vs. control; #$p < 0.001$ vs. control; @$p < 0.05$ vs. Cis; \$$p < 0.01$ vs. Cis; *$p < 0.001$ vs. Cis.

Oxidative stress parameters

Reactive oxygen species production was significantly increased after cisplatin exposure in HUVECs and HBVPs (Fig. 5). Consistently, the GSH/GSSG ratio was significantly decreased in the cisplatin group relative to the control group in both cell types. Curcumin treatment corrected the higher ROS production and partially elevated the GSH/GSSG ratio in both HUVECs and HBVPs.

Transendothelial electrical resistance

Cisplatin caused a significant reduction in the TEER value of HUVECs, and curcumin partially restored the resistance (Fig. 6).

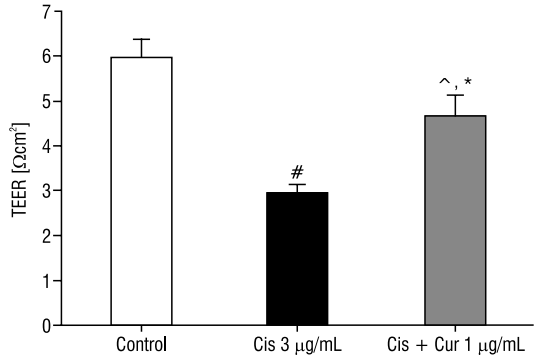

Figure 6. Transendothelial electrical resistance (TEER) of human umbilical vein endothelial cells. The graph shows the means and standard error of the mean; Cis — cisplatin; Cur — curcumin; #$p < 0.001$ vs. control; ^ $p < 0.05$ vs. control; *$p < 0.01$ vs. Cis.

Expression of tight junction proteins

The expression of occludin and ZO-2 in HUVECs was significantly decreased after cisplatin administration (Fig. 7). However, the expression of claudin-5 and ZO-1 was not significantly reduced. Following curcumin treatment, the expression of ZO-1 and ZO-2 was significantly upregulated compared with that in the cisplatin group. In addition, claudin-5 expression showed a tendency toward elevation in the cisplatin + curcumin group. Curcumin had no significant effect on the downregulated expression of occludin caused by cisplatin.

DISCUSSION

A previous study suggested a higher incidence of pericyte detachment in the nerve capillaries of cisplatin-treated rats relative to controls [17]. With quantitative analysis, the present study confirmed

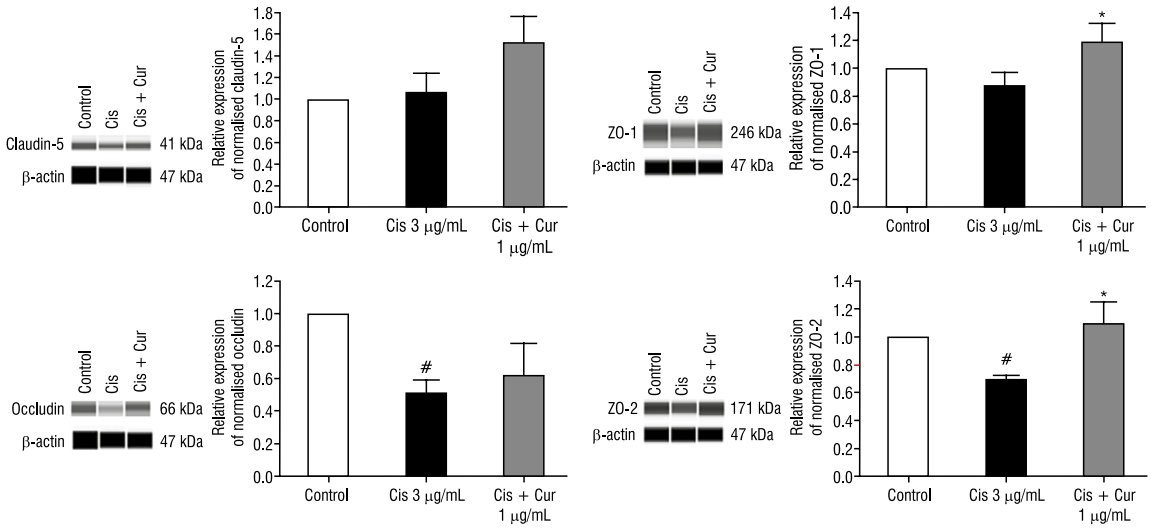

Figure 7. Protein expression of claudin-5, occludin, zonula occludens-1 (ZO-1) and zonula occludens-2 (ZO-2). Representative immunoblots are shown. The density of each protein was normalised to that of β-actin. The graphs show the means and standard error of the mean; Cis — cisplatin; Cur — curcumin; #$p < 0.05$ vs. control; *$p < 0.05$ vs. Cis.

that pericyte detachment occurred with higher frequency and severity in the cisplatin-treated than in the control rats. Pericyte detachment or migration has been observed in various conditions and organs. Increased pericyte migration was found in the brain after traumatic injury [11], in the retina of diabetic rats [29] and in the prolactinoma of the pituitary gland [16]. Although the consequences of pericyte detachment remain unclear, abnormalities in any component of the BNB likely result in barrier dysfunction. Since BNB maintains endoneurial homeostasis, its impairment is likely deleterious to nerve fibres and may lead to neuropathy [28]. This hypothesis is supported by a previous study showing that cisplatin-induced ototoxicity was associated with changes in cochlear endothelial cells and pericytes [37].

Our results showed that curcumin alleviated the pericyte detachment induced by cisplatin in the sciatic nerve. Curcumin, a polyphenol found in the root of *Curcuma longa*, has antioxidant, anti-inflammatory and neuroprotective properties [8, 15]. The mechanisms underlying pericyte detachment induced by cisplatin are still unclear. In vitro experiments on pericytes were performed to clarify this issue.

Using HUVEC and HBVP cultures, this study demonstrated that cisplatin reduced the viability of both cell types with activation of caspase-3. In addition, the production of ROS was increased, and reduced glutathione was decreased in the cells receiving cisplatin. Consistently, endothelial apoptosis and enhanced caspase-3 activity after cisplatin treatment were reported [12, 13, 18, 26]. Oxaliplatin, another platinum-based antineoplastic drug, was also shown to activate caspase-3 and oxidative stress in a rat brain endothelial cell line [7]. All these data indicate that cisplatin induces oxidative stress, leading to caspase-3 activation and apoptosis in endothelial cells and pericytes.

Curcumin improved cell viability, reduced caspase-3 activity and attenuated oxidative stress in both HUVECs and HBVPs. This improvement might be due to the antioxidant property of curcumin [8, 15]. A previous study showed that curcumin ameliorated cisplatin-induced oxidative stress and neuronal death in the mouse optic nerve [27]. Another study demonstrated the favourable effects of curcumin on oxaliplatin-induced neuropathic pain via reduction of oxidative stress and inflammation [38]. Therefore, curcumin was effective against oxidative stress caused by cisplatin, leading to reduced cytotoxicity.

We also showed that the TEER value and expression of the tight junction proteins occludin and ZO-2 in HUVECs were reduced by cisplatin. Lower expression of tight junction proteins was also found in the stria vascularis of the cochlea in cisplatin-treated mice [37]. In oxaliplatin-induced endothelial cytotoxicity, fragmentation of ZO-1 immunostaining in the intercellular junction was observed [7]. The downregulation of endothelial tight junction proteins induced by cisplatin might arise from oxidative stress [31]. These tight junction proteins are crucial for BNB integrity. Perturbation in the expression or localisation

of these proteins is associated with BNB loosening and neuropathies [22]. Pericytes are also important for the normal functions of BNB [28]. Shimizu et al. [33] reported that pericytes controlled the expression of claudin-5 in endothelial cells through secretion of growth factors. In addition to claudin-5, nerve pericytes also express other important components of the BNB, such as fibronectin and collagen type IV [34]. Our results showed that cisplatin causes cytotoxicity and detachment of pericytes. Taken together, the findings show that cisplatin likely causes endothelial and pericyte cytotoxicity, resulting in decreased expression of tight junction proteins and barrier dysfunction. However, it remains to be proven whether these alterations in the BNB occur in patients with cisplatin neuropathy. Moreover, BNB integrity should be examined in neuropathies from other chemotherapeutic drugs or causes.

Curcumin alleviated the decreased TEER value and corrected the expression of ZO-2, including enhancement of ZO-1 expression above the control level. Combined with the above morphometric data, our results indicate that curcumin might attenuate pericyte detachment through the upregulation of endothelial tight junction proteins. Our previous study demonstrated the favourable effects of curcumin on functional and morphological changes associated with cisplatin neuropathy [2]. Curcumin also improved biochemical and histological alterations in the sciatic nerves of cisplatin-treated rats [3]. Therefore, when considering the results of this and previous studies, we propose that curcumin ameliorates cisplatin-induced oxidative stress and cytotoxicity in endothelial cells and pericytes, leading to restoration of tight junction proteins and BNB functions. Apart from chemotherapy-induced neuropathy, increasing evidence supports the beneficial effects of curcumin on neuropathies from various aetiologies [6]. However, whether curcumin can alleviate cisplatin-induced neuropathy in patients remains to be investigated.

Curcumin has several advantages. It is found in the turmeric spice made from the root of *Curcuma longa*, which has long been used in traditional medicine. Hence, it is relatively nontoxic and highly tolerable in humans [14]. In addition, curcumin can be administered concomitantly with chemotherapeutic agents without interfering with antitumor efficacy. On the contrary, several reports have confirmed the enhanced antitumor activity of cisplatin cotreated with curcumin [1, 23, 25]. However, the major limitation is its poor absorption and bioavailability [4]. Novel methods are being developed to overcome this drawback of curcumin, including nanocarriers [24]. Collectively, the evidence shows that curcumin holds promise as a safe and effective therapeutic agent against cisplatin neuropathy.

CONCLUSIONS

This study demonstrated the favourable effects of curcumin on pericyte detachment induced by cisplatin in the capillaries of the sciatic nerve. In addition, in vitro experiments showed a reduction in the viability of endothelial cells and pericytes, TEER, and the expression of some tight junction proteins. These parameters were alleviated by curcumin. These data indicate that BNB disruption is a novel potential mechanism underlying cisplatin-induced neuropathy and that curcumin is effective against this abnormality. In the future, the BNB should be examined in neuropathies from other antineoplastic agents or causes, and drugs with beneficial effects on microvessels should be assessed as potential therapeutic agents.

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