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Cisplatin-induced alterations in the blood-nerve barrier: effects of combination of vitamin B1, B6 and B12

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Background: *Cisplatin is a chemotherapeutic agent against solid cancers. However, neuropathy is a major side effect and has no effective treatment so far. Emerging evidence suggests that cisplatin might damage nerve capillaries leading to impaired blood-nerve barrier (BNB). This study aimed to investigate the ultrastructural changes of the BNB in the sciatic nerves and dorsal root ganglia of rats with cisplatin neuropathy and the effects of* $B_{1\text{-}6\text{-}12}$.

Materials and methods and Results: *The results showed that cisplatin 2 mg/kg injected intraperitoneally twice a week for 5 consecutive weeks caused thermal hypoalgesia and structural abnormalities of nerves and ganglia. Co-treatment with oral* $B_{1\text{-}6\text{-}12}$ *(100:100:1) 100, 300 and 600 mg/kg/day for 5 weeks reduced the sensory deficit and structural alterations. Electron microscopic analysis demonstrated the higher frequencies and wider distances of pericyte detachment in the capillaries of cisplatin than control groups. Vitamin B1, B6 and B12 especially the medium dose, reversed these abnormalities. Culture of endothelial cells and pericytes with cisplatin demonstrated reduced cell viability, increased caspase-3 activity, lower transendothelial electrical resistance and decreased expression of tight junction proteins, occludin and zonula occluden-2.*

Conclusions: *Vitamin B1, B6 and B12 could correct these toxic effects of cisplatin. These data confirm that cisplatin causes pathological alterations in the components of BNB which correlate with the severity of neuropathy. Furthermore,* $B_{1\text{-}6\text{-}12}$ *is effective against these abnormalities and deserves further investigations as potential treatment for cisplatin-induced neuropathy.* (Folia Morphol 2023; 82, 1: 53–62)

Key words: pericyte, endothelial cell, cisplatin, nerve, neuropathy

INTRODUCTION

Cisplatin is an antineoplastic agent used to treat cancers of various organs [6]. One of its major side effects is peripheral neuropathy often leading to dose reduction or cessation and thus effectiveness of chemotherapy. Cisplatin-induced neuropathy is mainly characterised by sensory abnormalities in both animal models and patients [2, 16, 22, 28]. Pathological examination showed degeneration and demyelination of nerve fibres [1, 29]. Although various underlying mechanisms have been identified and relevant agents were tested, no clinically effective drugs were achieved so far [18, 24].

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Accumulating evidence suggests vascular dysfunction as another potential mechanism. Patients receiving cisplatin-based chemotherapy had arterial occlusion [17] and endothelial damage [5]. In cisplatin-treated rats, reduced nerve blood flow, decreased number of vasa nervorum and endothelial apoptosis were reported [15]. Recently, the lower density and detachment of nerve pericytes have been found in the rats with cisplatin neuropathy [12]. These abnormalities might impair blood-nerve barrier (BNB). This study aimed to further clarify the association of cisplatin neuropathy with BNB defects. In addition, since beneficial effects of vitamin B1, B6 and B12 in combination ($B_{1\text{-}6\text{-}12}$) were seen in the preliminary study, whether this improvement in neuropathy was associated with ameliorated BNB abnormalities was also investigated.

MATERIALS AND METHODS

Animals

The experiment was approved by the institutional ethics committee (Ref. No. 19/58) and carried out in accordance with the Animals for Scientific Purposes Act 2015, Thailand. Thirty female Wistar rats weighing 250 g were divided into five groups: control (C), cisplatin (P), cisplatin + low-dose (P+LB), medium-dose (P+MB) or high-dose $B_{1\text{-}6\text{-}12}$ (P+HB) (n = 6 for each group).

Drug administration

The cisplatin and cisplatin + $B_{1\text{-}6\text{-}12}$ groups received cisplatin (Pfizer, USA) diluted in normal saline to the final concentration of 0.5 mg/mL for intraperitoneal injection. The dose of cisplatin was 2 mg/kg twice a week for 5 continuous weeks (20 mg/kg cumulative dose). This dose regimen has been shown to induce peripheral neuropathy in rats [2, 28]. The control group received normal saline injection with the volume and schedule equivalent to the cisplatin groups. B1, B6 and B12 (all from Sigma) (100:100:1 by weight) were dissolved in normal saline and given by gavage during the cisplatin treatment once daily for 5 weeks. This ratio of $B_{1\text{-}6\text{-}12}$ was selected based on the previous studies [3, 13]. Low-dose, medium-dose and high-dose $B_{1\text{-}6\text{-}12}$ groups received 100, 300 and 600 mg/kg/day, respectively.

Hind-paw thermal nociception

The details of procedure are described elsewhere [29]. Briefly, the test was done at baseline and the end of 3rd and 5th weeks. Each rat was placed on the hot plate analgesia meter (Harvard Apparatus, UK) maintained at 55°C. When the rat licked its hind paw on either side, elapsed time was recorded as latency. The cut-off duration of 35 s was set to avoid skin burn. The test was repeated at least 3 times with an interval of 15 min and the mean latency was obtained for each rat.

Tissue collection

After the last injection of cisplatin, all the rats were sacrificed by overdose anaesthetics and then transcardially perfused with normal saline. This was followed by 3% glutaraldehyde. L4,5 dorsal root ganglion (DRG) with the proximal and distal parts of sciatic nerves (divided at the trifurcation) were removed, post-fixed in 3% glutaraldehyde and embedded in epoxy resin. These specimens were used for morphometric analysis.

Nerve morphometry

Transverse 1 μm-thick sections of the sciatic nerve were cut, mounted on slides, and stained with paraphenylenediamine. The sections were examined under a light microscope and the cross-sectional areas were chosen using the three-window sampling method [4]. Briefly, under 40× objective lens, three windows of 0.012 mm^2 were randomly placed, one in the middle and the other two in the periphery of fascicle. Images of these windows were imported into a computer via a digital camera. Morphometric analysis was done to obtain the number of myelinated fibres, axon diameter, myelinated fibre density, myelin thickness and g ratio using the Image-Pro Plus software®. The values derived from the three windows were extrapolated to the whole nerve.

DRG morphometry

The L4 DRG were serially cut into 2 μm-thick sections and stained with toluidine blue. The estimation for total number of neurons in each ganglion was done using the physical dissector method. Details of the procedures were described elsewhere [28]. In brief, every 20th section was selected and the number of neurons with prominent nucleus and nucleolus was counted. Then, this number was extrapolated to the total number for the whole DRG. In addition, at least 300 neurons in each DRG were randomly analysed for areas of the nucleus and nucleolus using the Image-Pro Plus software.

Transmission electron microscopy

Ultrathin sections (70 nm thickness) of the L5 DRG and sciatic nerves were stained with lead citrate and uranyl acetate. Morphology of pericytes and the basement membrane shared with endothelial cells was observed with a transmission electron microscope (JEM-1400PLUS, Japan). In each rat, 20 capillaries were randomly chosen from serial sections of each tissue (DRG, proximal and distal sciatic nerves). Each capillary was evaluated for the presence of pericyte detachment from endothelial cell and vascular basement membrane (VBM) which was classified into two categories: Category 1 pericyte completely attached to the VBM and endothelial cell, category 2 pericyte detached from the VBM and endothelial cell at some points. Then, the distances at the widest detachment between the pericytes and VBM were measured in the capillaries of category 2. In addition, the thickness of VBM at the widest separation point was also measured.

Cell culture

Human umbilical vein endothelial cell (HUVEC) (Invitrogen) and human brain vascular pericyte (HBVP) (ScienCell) were grown according to manufacturers' protocols. Each experiment was performed in triplicate and repeated three times. HUVEC and HBVP were divided into three groups: control, cisplatin, and cisplatin + $B_{1\text{-}6\text{-}12}$. $B_{1\text{-}6\text{-}12}$ was prepared in a ratio of 100:100:1 similar to the animal experiment. In cisplatin and cisplatin + $B_{1\text{-}6\text{-}12}$ groups, HUVEC and HBVP were incubated with 3 μg/mL and 1.5 μg/mL of cisplatin for 24 hours, respectively. For $B_{1\text{-}6\text{-}12}$ treatment, the cells were co-incubated with 1 μg/mL of $B_{1\text{-}6\text{-}12}$ for 24 hours. The above doses were selected according to the preliminary data.

MTT assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to evaluate the viability of HUVEC and HBVP. The cells were seeded at 1×10^4 and 5×10^3 cells/well, respectively, 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 (Life technologies, Molecular Probes, 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, Thermo Fisher Scientific, USA). The percentage of cell viability was calculated from the mean absorbance of test samples divided by that of negative control.

Caspase-3 assay

Caspase-3/cpp32 colorimetric assay kit (BioVision, USA) was used to determine the 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. Supernatant of each sample was collected and protein concentration was measured. Then, the sample was diluted with cell lysis buffer to obtain the protein concentration of 1 μg/μL and transferred to a 96-well plate. This was followed by adding working reaction buffer and DEVD-pNA substrate. The plate was incubated at 37°C for 2 hours. The absorbance was measured at 405 nm using a microplate reader (Multiskan GO, Thermo Fisher Scientific, USA). The caspase-3 activity of treated cells was compared with that of controls.

Transendothelial electrical resistance (TEER) study

Human umbilical vein endothelial cell were cultured on the upper chamber of transwell insert (Merck, USA) which was inserted in the 24-well plate at 1×10^4 cells/well. After the cells were grown to confluency, they were treated according to the experimental conditions for 24 hours. Cell resistance (R) was measured using Millicell electrical resistance apparatus (Millicell® ERS-2, Merck, USA). TEER value was calculated using the formula: TEER value (Ωcm^2) = $(R_{sample} - R_{blank}) \times$ membrane area (cm^2).

Western blot analysis

Human umbilical vein endothelial cell were seeded 2×10^6 cells/mL in a cell culture dish. After being treated according to experimental protocols for 24 hours, the cells were incubated on ice with RIPA lysis buffer (Cell Signaling, USA) containing 1× protease inhibitor cocktail (Cell Signaling, USA) for 5 min. Subsequently, the cells were scraped, centrifuged and the supernatant collected. Protein concentration of the supernatant was determined using PierceTM BCA protein assay (Thermo Scientific, USA). Briefly, the sample (1.5 μg/μL) was mixed with the fluorescent dye (4:1 ratio) and denatured at 95°C for 5 min. The marker, samples, antibody diluent, primary antibody (1:200 β-actin [Cell Signaling], 1:200 ZO-1 [Invitrogen], 1:200 ZO-2 [Invitrogen], 1:200 claudin-5 [Invitrogen], and 1:200 occludin [Invitrogen]), rabbit secondary conjugate, streptavidin-HRP, and Luminol-peroxide were

added onto the plate according to the manufacturer's protocol. Subsequently, separation and immunodetection were conducted using WES automated western blotting system (ProteinSimple, USA). Density of digital image was analysed using Compass software (ProteinSimple, USA). Expression of each protein was normalised to that of β-actin.

Statistical analysis

One-way ANOVA followed by Tukey's post hoc test was used for comparing the above parameters between the experimental groups. The test was done using SPSS for Windows version 23. Statistically significant differences were considered when $p < 0.05$.

RESULTS

Body weight

At baseline, the average body weight was similar between groups. However, at the 3rd and 5th weeks, the values of all groups receiving cisplatin were significantly decreased compared with that of the control group (data not shown). Food and water consumption including physical activities were similar between the cisplatin only and cisplatin + $B_{1\text{-}6\text{-}12}$ groups. There was no mortality in any group during the experiment.

Hind-paw thermal nociception

Before the treatment, the latencies were not significantly different between groups (Fig. 1). However, at the 5th week, the latencies of the cisplatin group were significantly longer than that of the control group, indicating thermal hypoalgesia. In addition, the cisplatin + MB and cisplatin + HB groups had significantly shorter latencies than the cisplatin group and not statistically different from the control group. In contrast, the latency of the cisplatin + LB group was close to that of the cisplatin group and significantly longer than that of the control group.

Nerve morphometry

At the fifth week, morphometric analysis of the sciatic nerve showed that the fibre diameters of the cisplatin including cisplatin + LB and cisplatin + HB groups were significantly lower than those of the control and cisplatin + MB groups (Table 1). There were no significant differences between the cisplatin, cisplatin + LB and cisplatin + HB groups. Furthermore, the fibre densities of the cisplatin and cisplatin + HB groups were significantly higher than those of the control and cisplatin + MB groups. The values of

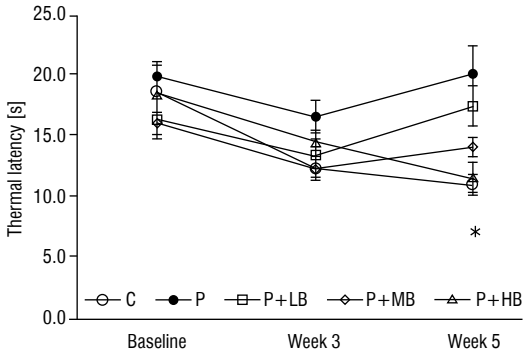

Figure 1. Changes in the thermal latency of hind paw. The graph shows means and standard error of mean; C — control; P — cisplatin; P+LB — cisplatin + low-dose $B_{1\text{-}6\text{-}12}$; P+MB — cisplatin + medium-dose $B_{1\text{-}6\text{-}12}$; P+HB — cisplatin + high-dose $B_{1\text{-}6\text{-}12}$; *$p < 0.01$ P vs. C and P+HB; $p < 0.05$ P vs. P+MB; $p < 0.05$ P+LB vs. C and P+HB.

the cisplatin + MB were not significantly different from those of the control groups. No significant changes were observed between groups in other parameters. However, there were trends toward thinner myelin sheath in all cisplatin-treated groups and higher number of fibres in the cisplatin and cisplatin + HB groups.

DRG morphometry

At the fifth week, the number of DRG neurons and nuclear area were significantly decreased in the cisplatin compared with the control groups (Table 2). However, the nucleolar area was significantly decreased in all cisplatin-treated compared with the control groups. All cisplatin + $B_{1\text{-}6\text{-}12}$ groups had values between those of the control and cisplatin groups.

Transmission electron microscopic analysis

Separation between the endothelial cell and pericyte or pericyte detachment appeared to be wider and more frequent in the nerve and DRG capillaries from the cisplatin compared with the control groups (Fig. 2). The detachment was less prominent in the cisplatin + $B_{1\text{-}6\text{-}12}$ groups. When the number of capillaries with detachment was compared with that of total capillaries included, the ratio was significantly higher in the cisplatin than the control groups (Fig. 3). All doses of $B_{1\text{-}6\text{-}12}$ had the significantly lower ratio than the cisplatin group but remained higher than the control group. However, when considering data of both sciatic nerve and DRG, the ratio of the cisplatin + MB group was the least different from that of the

Table 1. Nerve morphometry

Group	Fibre diameter [μm]	Myelin thickness [μm]	g ratio	Fibre density [/μm^2]	Number of fibre
Control (C)	6.52 ± 0.14	1.29 ± 0.11	0.61 ± 0.03	14,583.3 ± 557.7	8,798 ± 295
Cisplatin (P)	5.57 ± 0.09[a]	1.21 ± 0.02	0.57 ± 0.01	20,305.6 ± 1,073.8[b]	10,026 ± 127
Cisplatin + LB	5.77 ± 0.11[b]	1.21 ± 0.02	0.58 ± 0.01	18,437.5 ± 681.8	8,680 ± 596
Cisplatin + MB	6.62 ± 0.12	1.14 ± 0.11	0.63 ± 0.02	13,555.6 ± 944.6	8,742 ± 266
Cisplatin + HB	5.55 ± 0.23[a]	1.04 ± 0.04	0.62 ± 0.00	22,333.3 ± 1,686.1[a]	9,673 ± 244

Data are means ± standard error of mean; [a]$p < 0.01$ vs. C and MB, [b]$p < 0.05$ vs. C and MB; LB — low-dose $B_{1\text{-}6\text{-}12}$; MB — medium-dose $B_{1\text{-}6\text{-}12}$; HB — high-dose $B_{1\text{-}6\text{-}12}$

Table 2. Dorsal root ganglion morphometry

Group	No. of neuron	Nuclear area [μm^2]	Nucleolar area [μm^2]
Control (C)	21,170 ± 682	150.43 ± 5.64	14.28 ± 0.42
Cisplatin (P)	15,581 ± 328[a]	128.18 ± 4.73[b]	10.29 ± 0.62[c]
Cisplatin + LB	18,113 ± 511	133.01 ± 4.67	11.40 ± 0.46[d]
Cisplatin + MB	19,024 ± 766	137.79 ± 5.46	11.27 ± 0.14[d]
Cisplatin + HB	19,730 ± 1,024	130.26 ± 4.93	11.16 ± 0.53[d]

Data are means ± standard error of mean; [a]$p < 0.001$ vs. C, $p < 0.05$ vs. MB, $p < 0.01$ vs. HB; [b]$p < 0.05$ vs. C, [c]$p < 0.001$ vs. C, [d]$p < 0.01$ vs. C; LB — low-dose $B_{1\text{-}6\text{-}12}$; MB — medium-dose $B_{1\text{-}6\text{-}12}$; HB — high-dose $B_{1\text{-}6\text{-}12}$

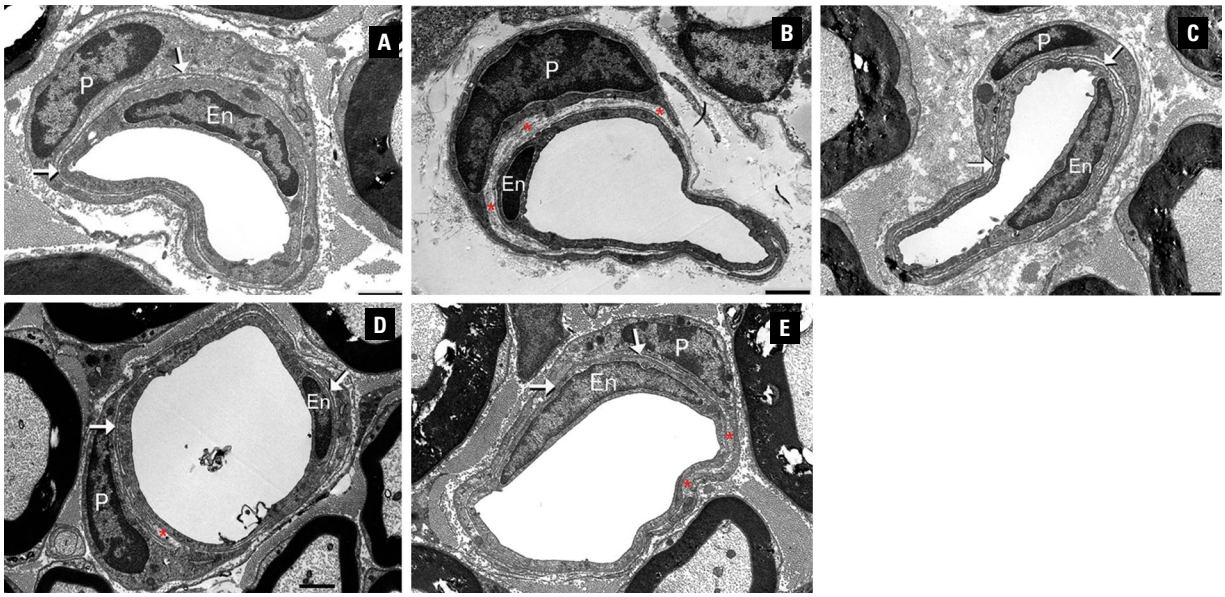

Figure 2. Representative ultrastructural images of capillaries in the sciatic nerves from the control (**A**), cisplatin (**B**), cisplatin + low-dose $B_{1\text{-}6\text{-}12}$ (**C**), cisplatin + medium-dose $B_{1\text{-}6\text{-}12}$ (**D**) and cisplatin + high-dose $B_{1\text{-}6\text{-}12}$ (**E**). Arrows indicate the basement membrane shared between the endothelial cell (En) and pericyte (P); *pericyte detachment or separation between the endothelial cells and pericytes; scale bars = 1 μm.

control group. The changes in the proximal and distal parts of nerve were similar.

The separation distance of the cisplatin group was significantly longer than that of the control group in the sciatic nerves but not the DRG (Fig. 4). All cisplatin + $B_{1\text{-}6\text{-}12}$ groups had shorter distances compared with the cisplatin group. The values of the cisplatin + MB group were the closest to those of the control group. It is worth mentioning that only the cisplatin + HB group had significantly longer distance than the control group in the DRG. As for the thickness of basement membrane at the separation, there were no significant differences between groups (data not shown). Other pathological findings such as accumulation of lysosomes or vacuoles were not detected in the pericytes as well as endothelial cells in any group.

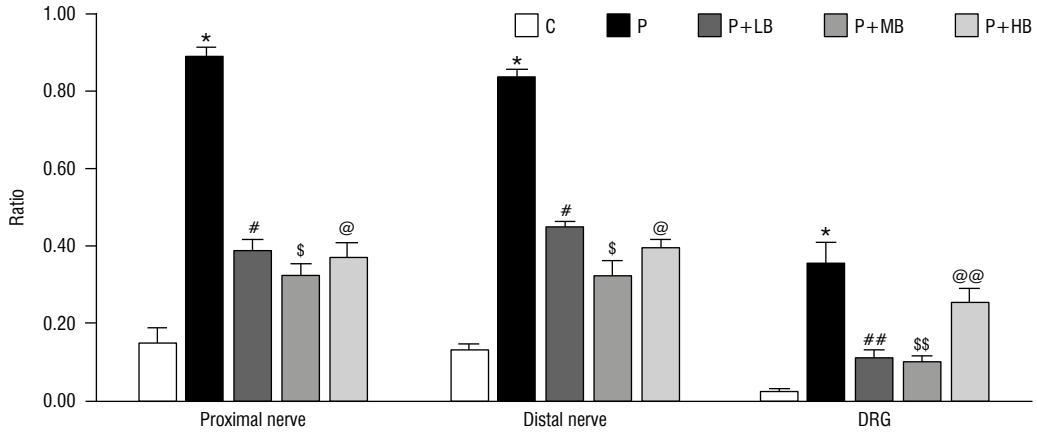

Figure 3. Ratio of the number of capillaries with pericyte detachment from endothelial cells to the total number of capillaries examined in the proximal and distal parts of sciatic nerve including L5 dorsal root ganglion (DRG). The graph shows means and standard error of mean; C — control; P — cisplatin; P+LB — cisplatin + low-dose $B_{1\text{-}6\text{-}12}$; P+MB — cisplatin + medium-dose $B_{1\text{-}6\text{-}12}$; P+HB — cisplatin + high-dose $B_{1\text{-}6\text{-}12}$; *p < 0.001 P vs. C, #p < 0.001 P+LB vs. P and p < 0.001 P+LB vs. C; ##p < 0.001 P+LB vs. P; $p < 0.001 P+MB vs. P and p < 0.01 P+MB vs. C; $$p < 0.001 P+MB vs. P; @p < 0.001 P+HB vs. P and p < 0.01 P+HB vs. C; @@p < 0.01 P+HB vs. C.

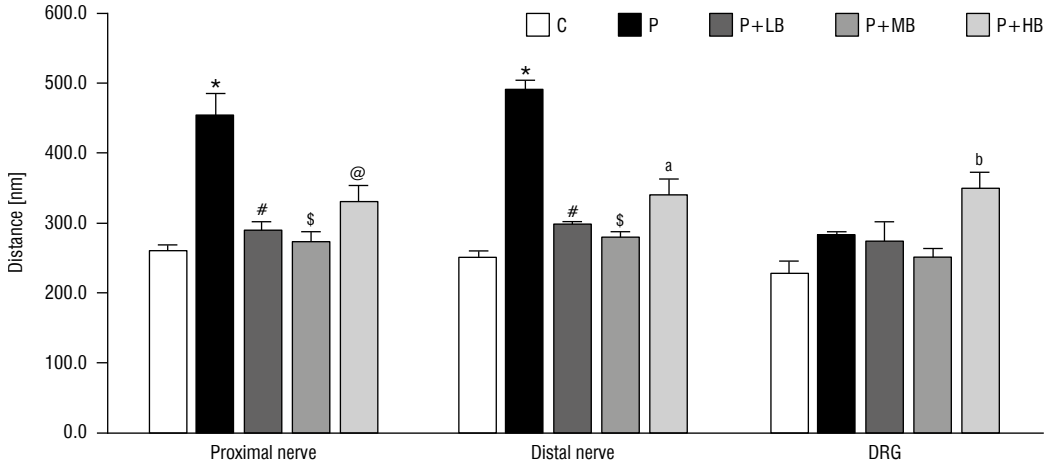

Figure 4. Distance at the widest separation between the endothelial cells and pericytes in the proximal and distal parts of sciatic nerve including L5 dorsal root ganglion (DRG). The graph shows means and standard error of mean; C — control; P — cisplatin; P+LB — cisplatin + low-dose $B_{1\text{-}6\text{-}12}$; P+MB — cisplatin + medium-dose $B_{1\text{-}6\text{-}12}$; P+HB — cisplatin + high-dose $B_{1\text{-}6\text{-}12}$; *p < 0.001 P vs. C; #p < 0.001 P+LB vs. P; $p < 0.001 P+MB vs. P; @p < 0.001 P+HB vs. P; ^{a}p < 0.001 P+HB vs. P and p < 0.01 P+HB vs. C; ^{b}p < 0.05 P+HB vs. C.

Cell viability and caspase-3 activity

Cell viability of the HUVEC was significantly reduced in the cisplatin group compared with the control group (Fig. 5). This was in agreement with the increased caspase-3 activity after cisplatin treatment. Similarly, cisplatin treatment in the HBVP resulted in lower viability and higher caspase-3 activity (Fig. 5). $B_{1\text{-}6\text{-}12}$ was able to significantly enhance the viability of both HUVEC and HBVP but yielded only trends toward less caspase-3 activity in both cell types.

TEER

Transendothelial electrical resistance of the cisplatin group was significantly lower than that of the control group (Fig. 6). Moreover, concomitant addition of $B_{1\text{-}6\text{-}12}$ with cisplatin caused partial restoration of the resistance.

Expression of tight junction proteins

Expression of occludin and zonula occluden-2 (ZO-2) was significantly decreased in the HUVEC exposed to cisplatin compared with the controls (Fig. 7). However, the expression of claudin-5 and zonula occluden-1 (ZO-1) was not significantly different. Following $B_{1\text{-}6\text{-}12}$ treatment, the expression of occludin and ZO-2 was completely reversed to those seen in the control group. It is worth mentioning that the ZO-1 expression was significantly enhanced in the cisplatin + $B_{1\text{-}6\text{-}12}$ group compared with the cisplatin group.

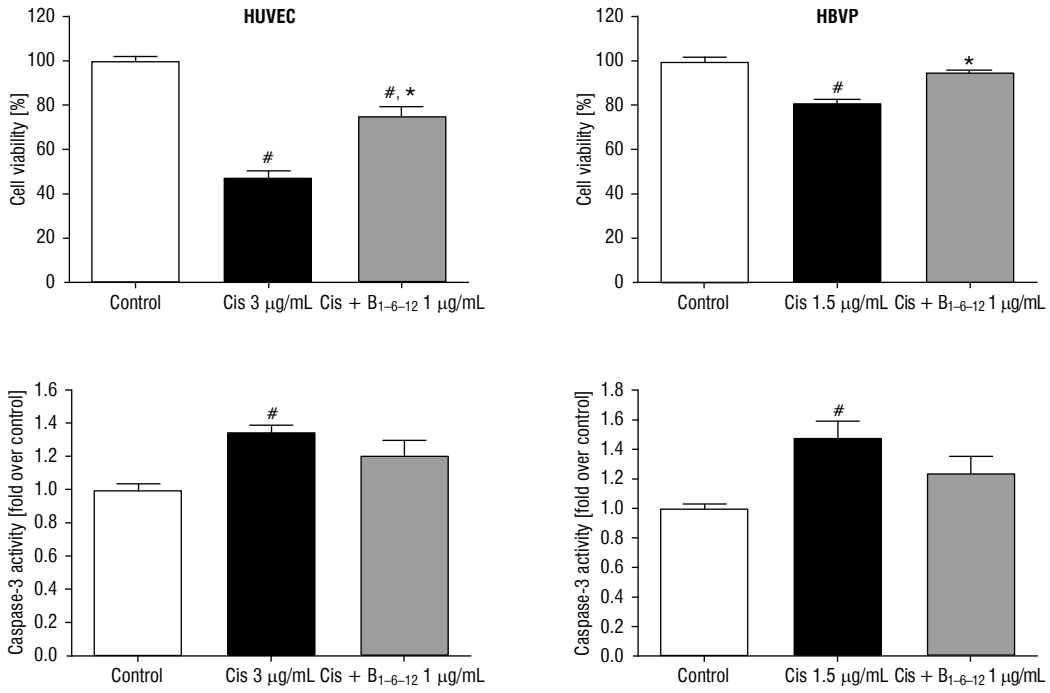

Figure 5. Cell viability and caspase-3 activity of human umbilical vein endothelial cell (HUVEC, left column) and human brain vascular pericyte (HBVP, right column). The graphs show means and standard error of mean; Cis — cisplatin; #p < 0.01 vs. control group; *p < 0.01 vs. cisplatin group.

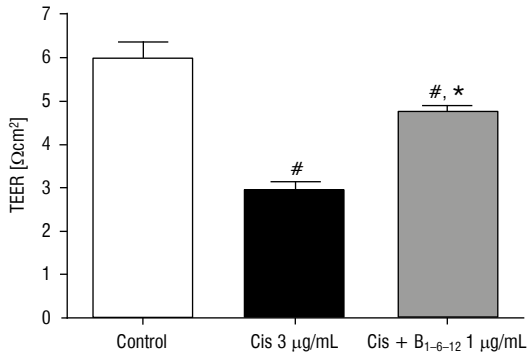

Figure 6. Transendothelial electrical resistance (TEER) of human umbilical vein endothelial cell. The graph shows means and standard error of mean; Cis — cisplatin; #p < 0.01 vs. control group; *p < 0.01 vs. cisplatin group.

DISCUSSION

The rats treated with cisplatin developed neuropathy characterised by thermal hypoalgesia and morphometric changes: reduced fibre diameter, increased fibre density, loss of DRG neurons and shrinkage of nucleus and nucleolus. These features of cisplatin-induced neuropathy were similar to those previously reported [1, 2, 28, 29]. Higher density of nerve fibres was likely due to shrinkage of fibres and slight increase in the number of fibres. Several of these functional and pathological abnormalities were comparable to those observed in cancer patients treated with cisplatin [23, 27].

Vitamin B1, B6 and B12 had beneficial effects on thermal sensation and morphometry. High and medium, but not low doses of $B_{1\text{-}6\text{-}12}$ could significantly reduce the prolonged thermal latency seen in the cisplatin group. Furthermore, medium dose was better than low and high doses in restoring the fibre diameter and fibre density toward controls. As for DRG, all doses of $B_{1\text{-}6\text{-}12}$ appeared to have modest effects on the loss of neurons including shrinkage of nucleus and nucleolus caused by cisplatin. It is worth noting that $B_{1\text{-}6\text{-}12}$ did not have any significant effect on the weight loss used to indicate the general toxicity of cisplatin.

The previous study has demonstrated the pericyte detachment from endothelial cells in the nerves from cisplatin-treated rats which was rarely seen in the controls [12]. This study confirmed with quantitative analysis that pericytes detached with significantly higher frequencies and severity in the cisplatin than in the control rats. It is worth mentioning that despite the higher frequencies of detachment in the DRG from all cisplatin-treated compared with the control groups, the distances were not significantly different. More research is required to clarify why the pericytes detach with shorter distances in the DRG than in the nerves.

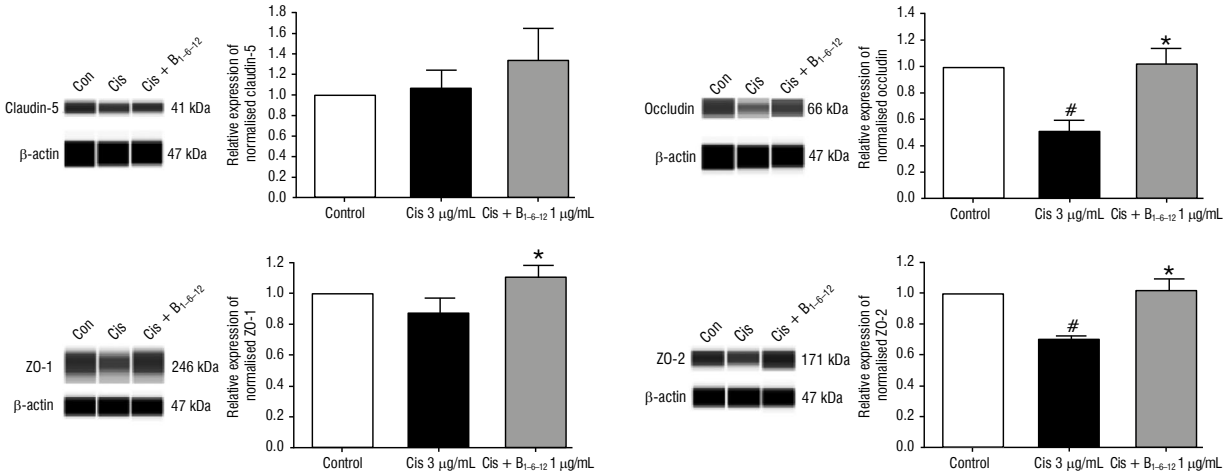

Figure 7. Protein expression of claudin-5, occludin, zonula occluden-1 and -2 (ZO-1 and ZO-2) representative immunoblots are shown. The density of each protein was normalised to that of β-actin. The graphs show means and standard error of mean; Con — control; Cis — cisplatin; #$p < 0.05$ vs. control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

Pericyte detachment or migration has been shown in various conditions and organs. Increased migration of pericytes was observed in the retina of diabetic rats [21]. More pericytes in the anterior pituitary gland detached from the capillary walls of the prolactinoma rats than the normal controls [11]. Pericytes migrated from the vascular wall in response to traumatic brain injury [7]. Implications of the pericyte detachment are still unclear. However, since pericytes, endothelial cells and vascular basement membrane co-operate as the BNB to regulate the microvascular functions [20], the detachment is likely deleterious to the nerve. At least in the cisplatin-induced ototoxicity model, ultrastructural changes in the endothelial cells and pericyte migration in the stria vascularis of cochlea were associated with auditory impairment [30].

All doses of $B_{1\text{-}6\text{-}12}$ could alleviate the elevation in cisplatin-induced pericyte detachment in both sciatic nerves and DRG with the best result seen in the medium-dose group. Moreover, both low and medium doses of $B_{1\text{-}6\text{-}12}$ could equally normalise the distances of detachment. Nevertheless, high dose of $B_{1\text{-}6\text{-}12}$ may be less favourable or even harmful, especially in the DRG. The detachment distance in the high-dose group was significantly longer than those in the other groups. This dose-dependent effect of $B_{1\text{-}6\text{-}12}$ will be discussed later.

Due to the effects of cisplatin and $B_{1\text{-}6\text{-}12}$ on the nerve and DRG capillaries described above, the question which cell component of the BNB was affected by the drugs has emerged. This was clarified using separate cultures of endothelial cells (HUVEC) and pericytes (HBVP). Cisplatin reduced the viability of both cell types at least via activation of caspase-3. This was in consistent with the previous reports of endothelial cell apoptosis and enhanced caspase-3 activity following cisplatin treatment [8, 9, 15, 19]. Furthermore, TEER which represents the barrier function, was also reduced by cisplatin. Moreover, the expression of tight junction proteins in the HUVEC was examined. Expression of occludin and ZO-2, but not claudin-5 and ZO-1, was significantly lower in the cisplatin-treated cells compared with the controls. Reduced expression of tight junction proteins was also found in the stria vascularis of cochlea in cisplatin-treated mice [30].

Pericytes as well as endothelial cells are important for the normal functions of BNB and damage in any of these components might cause neuropathy. The above data from cell culture and ultrastructural analysis indicate that cisplatin causes endothelial and pericyte damage including the BNB disruption. The previous studies have already suggested the importance of tight junction proteins in the BNB integrity and nerve functions. Reduced level of claudin-5 was associated with BNB dysfunction in chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) [14]. Shimizu et al. [25] showed that pericytes controlled the expression of claudin-5 in the endothelial cells through secretion of growth factors. Besides claudin-5, nerve pericytes also express other important components of the BNB, for example, fibronectin, collagen type IV [26].

Vitamin B1, B6 and B12 could partially correct the reduced viability of both HUVEC and HBVP after

cisplatin administration. Furthermore, the decreased TEER was also alleviated by $B_{1\text{-}6\text{-}12}$. Regarding the tight junction proteins, $B_{1\text{-}6\text{-}12}$ was able to correct the reduced expression of occludin and ZO-2 and further enhanced the expression of ZO-1 above the control level. All these data suggest that beneficial effects of $B_{1\text{-}6\text{-}12}$ on functional and morphometric parameters of cisplatin neurotoxicity described earlier might be exerted at least via improvement in the BNB functions. This could be due to less cell toxicity and enhanced expression of some tight junction proteins. Although numerous agents targeting various mechanisms were effective in experimental cisplatin neuropathy, they failed to show significant benefits in clinical trials [24]. Vitamin B1, B6 and B12 are water-soluble vitamins essential for normal functions of the nervous system and frequently prescribed for neuropathies from various causes. However, current evidence of efficacy of B vitamins in chemotherapy-induced neuropathy is still inconclusive [18]. The results of this study support the continued effort to develop $B_{1\text{-}6\text{-}12}$ as the potential treatment for cisplatin-induced neuropathy. It is worth noting that less or unfavourable effects of high-dose $B_{1\text{-}6\text{-}12}$ in the morphometric analysis of DRG and sciatic nerves were found in this study. This might be due to toxicity of all or specific B vitamins. Excessive intake of pyridoxine (B_6) can cause neuropathy [10]. Therefore, optimal dose of these B vitamins must be determined to prevent the overdose side effects. Data in this study suggest the medium dose (300 mg/kg/day per oral) of $B_{1\text{-}6\text{-}12}$ as the most suitable.

The results in this study also suggest the BNB impairment as additional important mechanism underlying cisplatin-induced neuropathy. This is in accordance with the previous study showing the BNB abnormalities in CIDP cases [14]. However, it remains to be proved whether these alterations in the BNB occur in the patients with cisplatin neuropathy. Moreover, the BNB integrity should be examined in neuropathies from other chemotherapeutic drugs or other causes. In the future, drugs with beneficial effects on endothelial cells or pericytes can be assessed for potential treatments against peripheral neuropathy with impaired BNB.

CONCLUSIONS

This study has demonstrated the favourable effects of $B_{1\text{-}6\text{-}12}$ on thermal hypoalgesia and abnormal morphometric parameters of the sciatic nerves and DRG induced by cisplatin. Ultrastructural analysis revealed that cisplatin stimulated pericyte detachment in the capillaries in those tissues. In addition, cell culture experiments showed reduced viability of endothelial cells and pericytes, transendothelial electrical resistance and expression of some tight junction proteins. $B_{1\text{-}6\text{-}12}$, especially the medium dose, could improve the sensory deficit and structural alterations. Moreover, cell viability, barrier function and tight junction proteins were also corrected by $B_{1\text{-}6\text{-}12}$. These data suggest that BNB disruption is one of the pathological mechanisms underlying cisplatin-induced neuropathy and $B_{1\text{-}6\text{-}12}$ are the potential treatment.

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