Protective effects of nimodipine on cerebrovascular function in chronic alcoholic encephalopathy

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Abstract. In the present study, we used chronic gavage administration of alcohol with gradual increases in alcohol concentration and volume to generate a rat model of chronic alcohol intoxication. We measured the changes in biological, behavioral, pathological and vascular injury-related molecular biological markers, and explored the effects of nimodipine intervention on alcohol intoxication. We found that chronic alcohol consumption induced a variety of behavioral abnormalities, accompanied by severe pathological changes in cerebral arterioles, prefrontal cortex and cerebellar tissue, as well as an upregulation of vascular endothelial growth factor (VEGF), leptin receptor (ob-R) and endothelin-1 (ET-1). Treatment with mimodipine for 15 days significantly improved behavioral abnormalities, alleviated the pathological changes in blood vessels and brain tissues, increased VEGF expression, decreased ob-R expression, reduced plasma ET-1 leakage and protected vascular and neuronal functions.

Introduction

Chronic alcoholic encephalopathy is caused by years of excessive alcohol consumption that results in nutritional and metabolic dysfunction and causes severe damage to the central nervous system. Clinical manifestations of the disease include unresponsiveness, mental disorders, ataxia, eye movement abnormalities and seizures. The pathogenesis of chronic alcoholic encephalopathy has long been recognized as chronic excessive alcohol consumption, which causes vitamin and other nutrient malabsorption, resulting in vitamin B/thiamine deficiency and abnormal glucose metabolism. Moreover, energy deficiency in the central nervous system induces abnormal synthesis and metabolism of phospholipids, resulting in central and peripheral nerve demyelination and axonal degeneration.

Previous studies have found that vascular endothelial growth factor (VEGF), leptin receptor (ob-R) and endothelin-1 (ET-1) interact with each other and participate in the regulation of vascular structure and function (1-4). VEGF is an endothelial cell-specific mitogen and an effective angiogenic and permeability-enhancing factor (5). Leptin is a peptide hormone encoded by the obesity (*ob*) gene and functions by binding to ob-R. As previously demonstrated, leptin regulates lipid and protein metabolism and influences neuroendocrine and endothelial function (6,7). ET-1 is a peptide tissue hormone and is the most potent vasoconstrictive substance (8). ET-1 is also a marker of vascular endothelial injury. Nimodipine is a calcium channel blocker that relaxes blood vessels and improves blood flow. To date, however, the expression and interactions of VEGF, ob-R and ET-1 in chronic alcoholic encephalopathy and the changes in these three factors following nimodipine intervention remain elusive.

Therefore, in the present study, we investigated the changes in rat behavior and brain pathology, as well as the changes in the expression of VEGF, ob-R and plasma ET-1 in brain tissue in a rat model of chronic alcoholic encephalopathy. We also explored the effects of nimodipine intervention on the expression of VEGF, ob-R and ET-1. We confirmed the alcohol-induced cerebrovascular damage in the pathogenesis of alcoholic encephalopathy and shed light on the protective effects of nimodipine on cerebrovascular function in alcoholic encephalopathy.

Materials and methods

Rat model of chronic alcoholic encephalopathy. The experimental animals were provided by the Experimental Animal Center of Shandong University, Jinan, China. All procedures followed international standards (NIH Publication No. 80-23, revised 1996) and were approved by the Ethics Committee of Jinan Fourth People's Hospital (Jinan, China). A total of 120 adult male Wistar rats, after normal feeding for 1 week, were randomly divided into 4 groups: i) the alcoholic group

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(alcohol), where the animals received gavage administration of 40% ethanol (Shanghai Chemical Reagent Co., Shanghai, China), 3 times/day (8 g/kg/day) for 4 weeks, followed by 50% ethanol, 3 times/day (9 g/kg/day) for 4 weeks, and 50% ethanol, 3 times/day (10 g/kg/day) for 8 weeks. Every 4 weeks represented an experimental time point. Animals in this group were further divided into 4 subgroups based on the experimental time points. ii) The control group (control), where the animals received gavage administration of saline (Qilu Pharmaceutical Factory, Jinan, China) for the same time period of 16 weeks. The animals were also divided into 4 subgroups based on the experimental time points. iii) The alcohol + nimodipine group (alcohol + nimodipine), following a 16-week gavage administration of ethanol, the rats were administered nimodipine (Bayer, Leverkusen, Germany), 10 mg/kg, once per day for 15 days. iv) The alcohol + saline group (alcohol + NS), following a 16-week gavage administration of ethanol, the rats were administered equal volumes of saline once per day for 15 days.

Behavioral observations. Rat feeding, nutrition, weight, appearance and personality changes were recorded once a week, on the 7th day of each week.

Hematoxylin and eosin (H&E) staining and electron microscopy (EM). The rats (n=5) were anesthetized with chloral hydrate (Qilu Pharmaceutical Factory) and sacrificed. Rats in the light microscopy group were perfused with formaldehyde (Shanghai Chemical Reagent Co.) through the ascending aorta and the brains were removed after perfusion. The brains were post-fixed, dehydrated, embedded, sectioned, stained with H&E, and then observed under a light microscope. Rats in the EM group (n=2) were perfused with 3% glutaraldehyde (Sigma, St. Louis, MO, USA) through the ascending aorta and the brains were removed. The brains were cut into 1-mm^3 tissue blocks and post-fixed in 3% glutaraldehyde. The tissues were dehydrated in a gradient of ethanol, embedded, sectioned, stained with uranyl acetate and lead citrate (Sigma), and then observed under an electron microscope (Jeol JEM-1200EX; Jeol, Tokyo, Japan).

Western blot analysis. The rats (n=5) were decapitated and the brains were rapidly removed. After washing with cold saline, the brains were homogenized on ice in lysis buffer (Beyotime, Jiangsu, China) and centrifuged at 15,000 rpm for 10 min at 4˚C. Supernatant was collected and protein concentration was determined using a BCA kit (Beyotime). Protein (30 μg) from each sample was loaded onto polyacrylamide gels for electrophoresis and transferred onto nitrocellulose membranes (Milllipore, Billerica, MA, USA). The membranes were blocked with 5% skim milk at room temperature for 2 h and incubated with primary antibodies against [VEGF (1:500; Cell Signaling Technology, Inc., Beverly, MA, USA), ob-R (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β-actin (1:5,000; Bioworld, Atlanta, GA, USA)] at 4˚C overnight. After washing, the membranes were incubated with secondary antibodies (1:10,000; Zhongshan Goldenbridge Biotechnology, Beijing, China) at 37˚C for 1 h. Protein bands were detected using a chemiluminescence detection kit (Millipore) and analyzed using a digital gel imaging system (Alpha Innotech, San Leandro, CA, USA). The expression levels of the target proteins were determined as the ratio of the gray values of the target protein band to that of the internal loading control.

Immunohistochemistry. The rats (n=5) were anesthetized with chloral hydrate and perfused with formaldehyde through the ascending aorta. The brains were removed, dehydrated, embedded and dewaxed. The sections were incubated with 3% hydrogen peroxide for 10 min. After washing, the sections were blocked with goat serum at room temperature for 20 min, and then incubated with primary antibodies against (VEGF, 1:100; and ob-R, 1:100) at 4˚C overnight. The sections were incubated with HRP-conjugated secondary antibodies (1:1,000; Zhongshan Goldenbridge Biotechnology) at 37˚C for 30 min. The sections were incubated with DAB at room temperature for 20 min for color development, and were then stained with hematoxylin, dehydrated, cleared and mounted.

ET-1 radioimmunoassay. Fasting blood samples (2 ml/rat) were obtained. Thirty microliters of 10% EDTA 2 Na and 40 μl aprotinin (400 IU) were added to the blood samples and centrifuged at 1,170 x g for 10 min at 4˚C. The upper plasma layer was collected and stored at -20˚C. The reaction system was prepared using an ET-1 kit (Northern Institute of Biology) and analyzed using an automatic gamma radioimmunoassay analyzer (FJ22008P type; Xi'an Nuclear Instrument Factory, Xi'an, China).

Statistical analysis. Data were analyzed using SPSS10.0 statistical software. All data are expressed as the means ± SEM. The t-test was used if the variances of the two populations were equal. The Wilcoxon Rank-Sum test was used if the variances of the two populations were unequal. An F-test was used to determine whether the variances of two populations were equal. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

General conditions of animals. Compared with the control group, rats in the alcohol group presented mania 5 days following gavage administration of alcohol. Mania became more severe with longer periods of alcohol perfusion. Moreover, rats in the alcohol group presented with dry messy hair, hair loss, fatigue, irritability, loss of appetite, weight loss and loose stool. In addition, 2 rats in the alcohol group had hemiplegia on days 15 and 22 of alcohol perfusion. The difference in body weight between the control and alcohol group was statistically significant at 8 weeks (P<0.05), and the difference was even more significant as the duration of alcohol intake increased. Treatment with nimodipine gradually alleviated mania and improved the appetite, nutrition and hair condition of the rats. Compared with the alcohol + NS group, the average body weight was significantly higher at 12 and 16 weeks following treatment with nimodipine (P<0.05) (Fig. 1).

Histopathological changes

Cerebral arterioles in frontal lobe.

H&E staining. Cerebral cortical arterioles in the control rats were normal (Fig. 2A), while the arterioles in the alcohol and alcohol + NS groups showed mildly thickened arteriolar walls, mild luminal stenosis, partial detachments

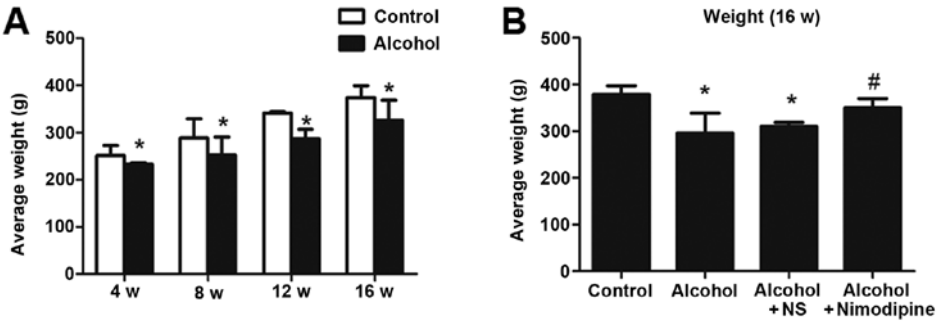

Figure 1. Average body weight of the rats in the different groups at 4, 8, 12 and 16 weeks (w). *P<0.05 vs. control; #P<0.05 vs. alcohol + saline (NS) group.

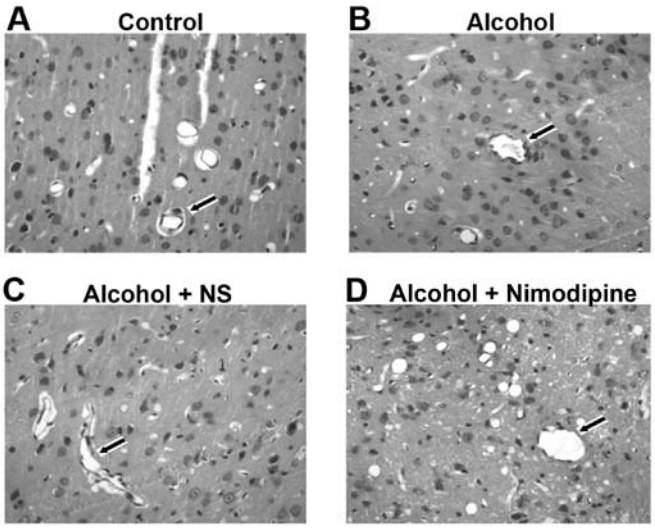

Figure 2. Hematoxylin and eosin (H&E) staining of frontal lobe arterioles in the different groups (magnification, x200). NS, saline. Arrows indicate frontal lobe arterioles.

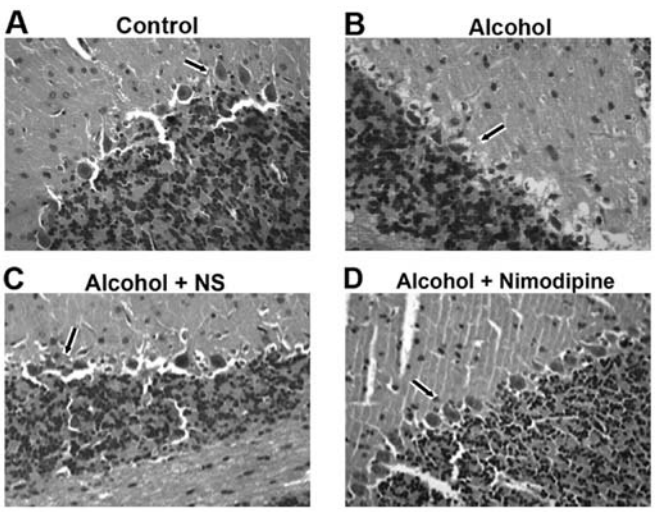

Figure 4. Hematoxylin and eosin (H&E) staining of cerebellar tissues in the different groups (magnification, x200). NS, saline. Arrows indicate cerebellar neurons.

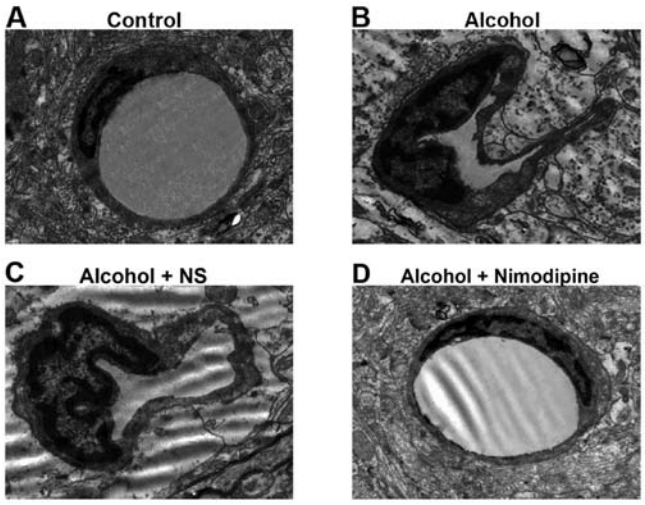

Figure 3. Electron microscopy images of frontal lobe arterioles in the different groups (magnification, x10,000). NS, saline.

of endothelial cells, wrinkled and uneven internal elastic membranes, proliferation of vascular smooth muscle cells, increased intensity of cytoplasmic staining and perivascular interstitial dema (Fig. 2B and C). By contrast, the arterioles in the alcohol + nimodipine group showed intact and smooth endothelial cells,a homogeneous cytoplasm in vascular smooth muscle cells, no significant luminal stenosis and no significant perivascular interstitial edema (Fig. 2D).

EM. The control rats showed structural integrity of vascular endothelial cells and normal nuclear morphology (Fig. 3A). Rats in the alcohol and alcohol + NS groups showed vascular endothelial cell swelling, irregular shaped nuclei, rupture of the nuclear membranes, an uneven distribution of chromatin and narrow vascular cavity (Fig. 3B and C), while the alcohol + nimodipine group showed normal vascular endothelial cells, regular shaped nuclei, an intact nuclear membrane and uniform distribution of chromatin (Fig. 3D).

Cerebellar cells.

H&E staining. The cerebellar Purkinje cells in the control rats had pear-shaped soma, prominent dendrites and regular shaped nuclei; neurons in the granular cell layer were tightly packed (Fig. 4A); the rats in the alcohol and alcohol + NS groups, however, had a significantly decreased number of Purkinje cells and these Purkinje cells had an irregular triangular shape. The cell numbers in the granule cell layer were also decreased (Fig. 4B and C). There was a slight decrease in

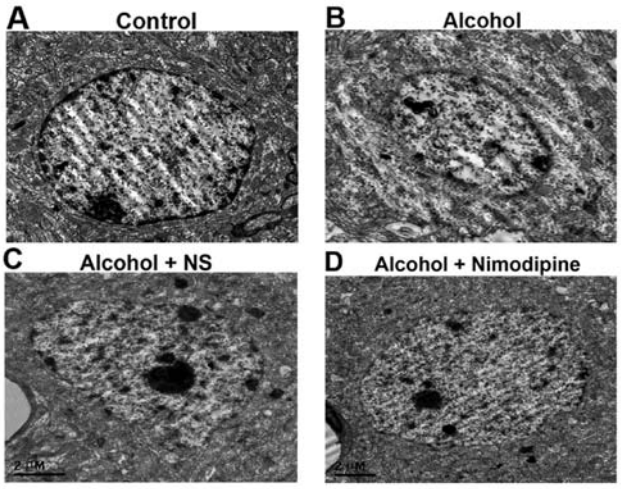

Figure 5. Electron microscopy images of cerebellar neurons in the different groups (magnification, x7,500). NS, saline.

the number of Purkinje cells in the alcohol + nimodipine group and these Purkinje cells had a relatively normal shape. The cell numbers in the granule cell layer were higher compared with those in the alcohol + NS group (Fig. 4D).

EM. The neurons in the control group had an intact and smooth nuclear membrane, evenly distributed chromatin, normal distribution of mitochondria, endoplasmic reticulum, ribosomes and other organelles, and prominent nuclei (Fig. 5A). The neurons in the alcohol and alcohol + NS groups had an incomplete nuclear membrane, reduced and unevenly distributed chromatin, a significant reduction in the number of organelles, a sparse cytoplasm and mitochondrial swelling. Cristae were significantly reduced and showed vacuolar degeneration (Fig. 5B and C), while the neurons in the alcohol + nimodipine group showed a relatively intact membrane, relatively even chromatin distribution, relatively abundant organelles and prominent nuclei (Fig. 5D).

Alcohol-induced upregulation of frontal lobe VEGF and ob-R expression. VEGF and ob-R protein expression gradually increased in the alcohol group with the extended period of alcohol consumption ($P<0.05$) (Figs. 6A and B, 7A and B). Immunohistochemical assay revealed that the number of VEGF- and ob-R-positive cells (yellow-stained cytoplasm) at week 16 was significantly increased in the alcohol group compared with the control group (Figs. 8A and B, 9A and B), which was consistent with the results from western blot analysis.

Treatment with nimodipine upregulates VEGF expression and downregulates ob-R expression. Following a 16-week gavage administration of alcohol, rats in the alcohol + nimodipine group were administered nimodipine for 15 consecutive days. We found that frontal lobe VEGF protein expression was 1.25-fold higher in the nimodipine-treated group than the alcohol + NS group ($P<0.05$, Fig. 6C and D), and ob-R expression decreased to 83.2% of that in the alcohol + NS group ($P<0.05$, Fig. 7C and D). Compared with the alcohol + NS group, the number of VEGF-positive cells in the alcohol + nimodipine group significantly increased (Fig. 8C and D) and that of ob-R-positive cells significantly decreased (Fig. 9C and D). The results from western blot analysis and immunohistochemistry were consistent.

Plasma ET-1 levels increase following gavage administration of alcohol and significantly decrease following treatment with nimodipine. ET-1 levels gradually increased following gavage administration of alcohol and the increase became statistically significant at 4, 8, 12 and 16 weeks after alcohol consumption compared with the control group ($P<0.05$). ET-1 levels at

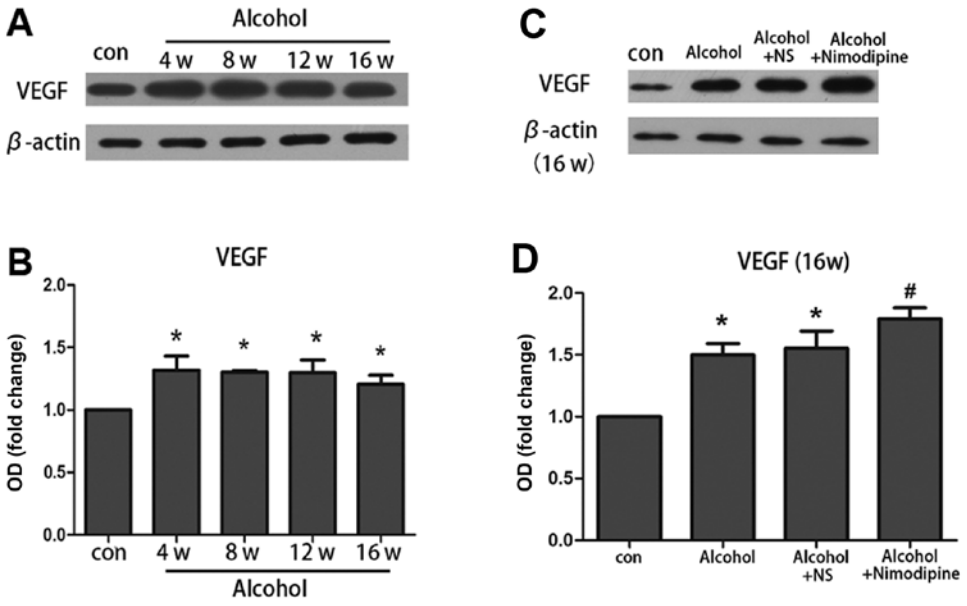

Figure 6. Effects of alcohol and nimodipine on the protein expression of vascular endothelial growth factor (VEGF). (A) Western blot analysis and (B) quantitative representation of the protein expression of VEGF at different time points. (C) Western blot analysis and (D) quantitative representation of the protein expression of VEGF in the different groups. *$P<0.05$ vs. control; #$P<0.05$ vs. alcohol + saline (NS) group. w, weeks.

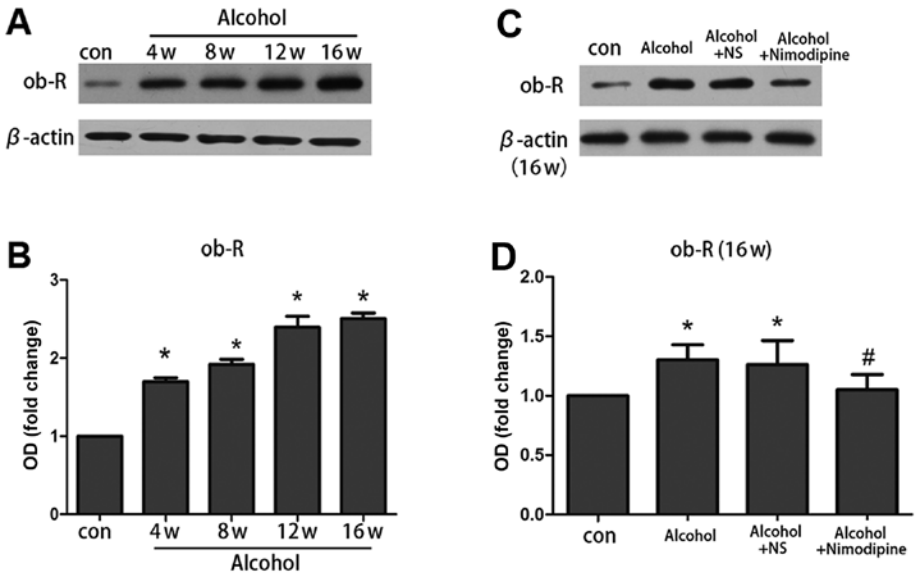

Figure 7. Effects of alcohol and nimodipine on the protein expression of leptin receptor (ob-R). (A) Western blot analysis and (B) quantitative representation of the protein expression of ob-R at different time points. (C) Western blot analysis and (D) quantitative representation of the protein expression of ob-R in the different groups. *P<0.05 vs. control; #P<0.05 vs. alcohol + saline (NS) group. w, weeks.

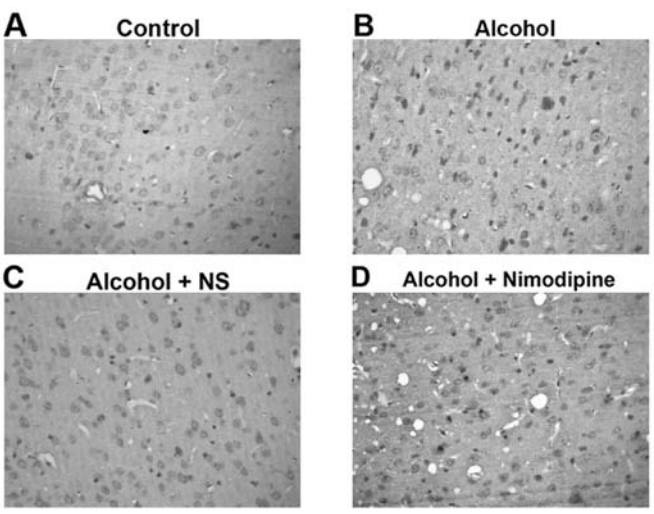

Figure 8. Immunohistochemical staining of vascular endothelial growth factor (VEGF) in frontal lobes in the different groups (magnification, x400).

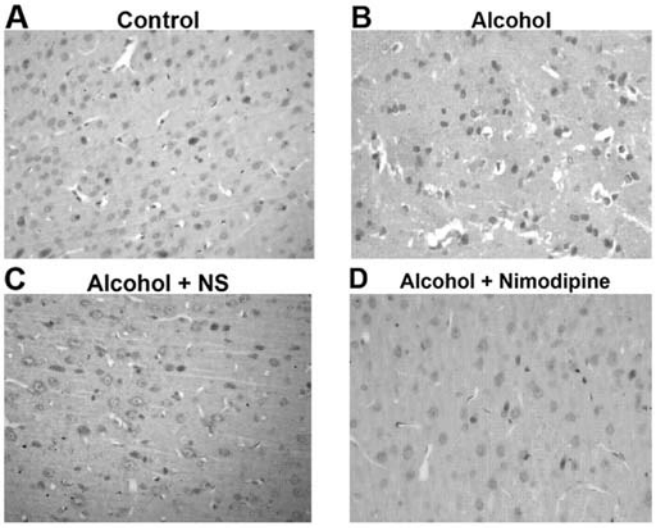

Figure 9. Immunohistochemical staining of leptin receptor (ob-R) in frontal lobes in the different groups (magnification, x400).

week 16 were 1.60-fold higher in the alcohol group compared with the control group. ET-1 levels significantly decreased following treatment with nimodipine. In the nimodipine-treated group, ET-1 levels at week 16 decreased to 84.56% of those in the alcohol + NS group (Fig. 10).

Discussion

Chronic alcoholic encephalopathy is caused by nutritional and metabolic disorders due to long periods of excessive alcohol consumption, which results in severe damage to the central nervous system. The clinical manifestations include unresponsiveness, mental disorders, ataxia, eye movement disorder and seizures. In the present study, we used gavage administration of alcohol with gradual increases in alcohol concentration and volume to generate a rat model of chronic alcoholic encephalopathy. This approach ensures the alcohol consumption and resembles human drinking habits. Our results indicated that 5 days following the gavage administration of alcohol, the rats showed nutritional and behavioral abnormalities; 2 weeks after gavage administration of alcohol, the rats presented with encephalopathy. Light microscopy and EM revealed significant pathological changes in blood vessels and brain tissues.

Chronic alcohol consumption can promote the occurrence and differentiation of melanoma and can enhance the expression of VEGF in mice (9), and enhance angiogenesis in a rat model of choroidal neovascularization (10,11). Cahill and Redmond recently confirmed that long-term alcohol consumption plays a role in atherosclerosis and increases the risk of cardiovascular disease (12). These studies indicate that alcohol

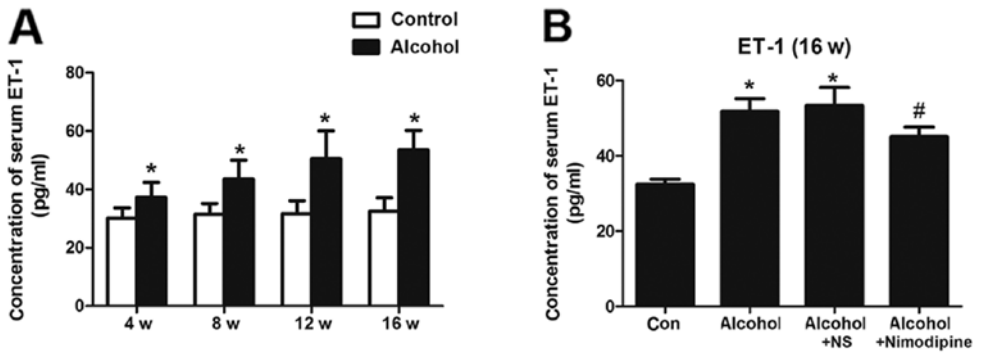

Figure 10. Average concentrations of serum endothelin-1 (ET-1) in the rats in the different groups at 4, 8, 12 and 16 weeks (w). *P<0.05 vs. control; #P<0.05 vs. alcohol + saline (NS) group.

is closely related to vascular diseases. In our study, we investigated the cerebral vascular factors that are related to chronic alcohol intoxication and shed light on the mechanisms behind and treatment for alcoholic encephalopathy.

Alcoholic encephalopathy and VEGF. VEGF is an endothelial-specific mitogen and a potent angiogenic and vascular permeability factor. VEGF can increase microvascular permeability and promote angiogenesis. Normal body fluids contain low levels of VEGF; however, the VEGF expression level is very low and is only involved in the maintenance of normal vascular density and permeability, not in angiogenesis. The expression levels of VEGF are slightly higher in tissues with high metabolism and blood supply. In addition, the overexpression of VEGF can also occur under a number of pathological conditions, such as ischemia and hypoxia (13,14). VEGF is activated by binding to its receptors and induces vascular endothelial cell division, proliferation and migration (15,16).

Numerous studies have found that among the mechanisms involved in regulating VEGF expression, hypoxia is the most important factor. Chronic hydrocephalus-induced hypoxia can promote the expression of VEGFR-2 and increase blood vessel density in brain tissues (17). Hypoxia can also upregulate VEGF and angiopoietin expression and promote prostate cancer (18). Hypoperfusion presents in 3/4 of all chronic alcoholic brain tissues and the decrease in cerebral blood flow is most significant in the frontal lobe (19). Alcohol intoxication can alter red blood cell morphology and reduce red blood cell surface area and oxygen-carrying function (20). In conclusion, alcohol intoxication-induced tissue and organ ischemia and hypoxia play important roles in the regulation of VEGF gene expression.

Our results indicated that, compared with the control group, the alcohol group showed partial detachment of endothelial cells in the frontal lobe arterioles and wrinkled internal elastic membranes. VEGF expression was upregulated with the increase in alcohol concentration and volume, which may be due to alcohol intoxication-induced cerebral ischemia and hypoxia. The increase in VEGF expression may be a compensatory mechanism to promote angiogenesis and improve blood supply.

Alcoholic encephalopathy and ob-R. Leptin is a peptide hormone encoded by the *ob* gene. The ob-R gene maps to human chromosome 1p3, and similar to qpl30, belongs to the class I cytokine receptor family. ob-R has 6 different isoforms (ob-R a-f). ob-Ra is more widely expressed *in vivo* and is highly expressed in the choroid plexus. ob-Rb is highly expressed in the hypothalamic arcuate nucleus, ventromedial nucleus, dorsomedial nucleus, paraventricular nucleus and lateral hypothalamic nuclei, which are involved in the regulation of food intake and body weight. ob-Ra, c and f are selectively expressed in a variety of peripheral organs and are involved in regulating growth, immune function and glucose metabolism (21,22).

ob-Rs and matrix metalloproteinases are highly expressed in atherosclerotic plaques and newly-formed blood vessels, which induce the migration of proliferating smooth muscle cells into the intima, suggesting that leptin and ob-R affect endothelial function and are involved in the process of endothelial damage (2). The binding of leptin and ob-Rs at the atherosclerotic lesion sites can activate the JAK signaling pathway and induce the formation and remodeling of new blood vessels (23). Our study demonstrated that ob-R levels in the alcoholic encephalopathy rat brain tissue were upregulated with the increase in alcohol concentration, volume and duration of alcohol consumption, suggesting that ob-Rs are involved in the pathological process of alcoholic encephalopathy. However, the specific mechanisms involved remain to be further elucidated.

Alcoholic encephalopathy and ET-1. ET-1 is a vasoactive substance produced mainly by the vascular endothelial cells and is a marker of vascular endothelial injury. ET-1 is the strongest and the longest-acting vasoconstrictor peptide. Our study found that plasma ET-1 levels in the alcohol group increased with the increase in alcohol concentration, volume and duration of alcohol consumption. Previous studies have also demonstrated that excessive alcohol consumption causes the body to stay in a high metabolic state, which produces a large amount of oxygen free radicals and results in lipid peroxidation (24); the alcohol metabolite, acetaldehyde, significantly reduced the function of the cellular free-radical scavenger, glutathione (25), which can damage endothelial cells and release large amounts of ET-1. The binding of ET-1 to specific receptors located on vascular smooth muscle cells triggers Ca^{2+} influx, induces smooth muscle contraction, increases body tissue ischemia and hypoxia and creates a vicious cycle (26,27).

Nimodipine intervention. Nimodipine is a second-generation dihydropyridine calcium antagonist and is widely used in the clinical treatment of ischemic cerebrovascular diseases. Nimodipine can prevent platelet intracellular calcium overload, improve cerebral circulation, inhibit free radicals and protect endothelial cells.

Previous studies have demonstrated that calcium overload is associated with vascular endothelial cell apoptosis. Nimodipine inhibits vascular endothelial cell apoptosis by reducing the intracellular calcium concentration (28). Our results revealed pathological destruction and remodeling of cerebral vascular structures in the alcohol group. Nimodipine intervention significantly improved the morphology of cerebral vessels and neurons, which may be due to the reduced calcium overload and free radical production. Low blood sugar levels can increase hepatoblastoma intracellular calcium concentration, activate the protein kinase C (PKC) signaling pathway and upregulate VEGF expression (29). Our study demonstrated that compared with the alcohol group, nimodipine intervention increased VEGF expression. In our study, we defined 15 days after nimodipine treatment as a research time point. By that time point, nimodipine has already improved brain tissue ischemia and hypoxia, it has cleared a lot of free radicals and has changed the neuronal microenvironment; in this case, high levels of VEGF may have anti-apoptotic and neuroprotective effects (30). Angiotensin II can increase Ca^{2+} concentration in vascular smooth muscle cells and increase ob-R expression (31), whereas calcium dobesilate reduces ET-1 expression (32). Our study indicated that, compared with the alcohol + NS group, treatment with nimodipine decreased ob-R and ET-1 expression, which further confirmed that nimodipine can reduce intracellular calcium overload, induce a compensatory reduction in ob-R expression, alleviate vascular endothelial injury, and protect vascular and nerve tissues in chronic alcohol intoxication.

In conclusion, chronic alcohol intoxication induces various behavioral abnormalities accompanied by severe pathological changes in the cerebral arterioles, frontal lobe and cerebellar tissues, as well as an upregulation of VEGF and ob-R expression, and an increase in plasma ET-1 levels. Fifteen days of nimodipine treatment significantly improved the behavioral abnormalities, alleviated the pathological changes in the blood vessels and brain tissues, enhanced VEGF expression and decreased ob-R expression, reduced the plasma ET-1 leakage, and protected neural functions; however, the underlying mechanisms remain to be further elucidated.

Acknowledgements

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