Letter to the Editor

Protective Effects of Tetramethylpyrazine on Cerebrovascular Regulations in Rats with Chronic Alcoholic Encephalopathy

LI Hui[1,#], YANG Xue[2], SHI Wei[3], MA Zhao[4], FENG Guang Kun[1], YIN Yan Ling[1], FAN Yan Xia[1], and JIANG Jie[4]

Recent studies showed that pathology of alcoholic encephalopathy was associated with cerebral vascular damage. TMP (tetramethyl-pyrazine) is widely used in the treatment of cerebrovascular diseases, however, it has not been reported whether TMP can relieve alcohol-induced cerebral vascular damages. The study was performed to investigate the learning and memory, cerebrovascular pathological changes and the expressions of vascular endothelial growth factor (VEGF) and serum levelsofendothelin-1 (ET-1) in the rat model of chronic alcoholic encephalopathy, and explore the effects of TMP intervention on alcoholic encephalopathy. In the present study, the rat model of chronic alcoholic encephalopathy was established by the gavage administration of alcohol; the learning and memory ability was tested by Morris water maze; the expression of VEGF was measured by RT-PCR and Western blot; and the serum levels of ET-1 was measured by radioimmunoassay. We found that alcohol intoxication impaired learning and memory, induced VEGF overexpression and increased ET 1 concentrations. TMP intervention improved learning abilities, increased the VEGF expression and reduced ET-1 level. These results indicate that TMP exhibits therapeutic effects on chronic alcoholic encephalopathy.

Chronic alcoholic encephalopathy can cause serious damages to the central nervous system by long-term heavy drinking. The clinical manifestations include unresponsiveness, mental disorders, ataxia, eye movement abnormalities, seizures and so on. This disease has high incidence and poor prognosis, which often results in irreversible brain damage. The clinical manifestations of chronic alcoholic encephalopathy can be different, such as Wernick encephalopathy, Korsakoff psychosis, and chronic alcoholic dementia[1]. Long-term excessive alcohol intake directly reduces the absorption of B vitamins and leads to impaired glucose metabolism, energy deficiency, abnormal myelin synthesis and demyelination of the central and peripheral nervous system.

Recent studies have found that cerebral vascular damage caused by alcohol is one of the pathological mechanisms of alcoholic encephalopathy. Tan et al. (2007) showed that chronic alcohol intake can stimulate VEGF expression and promote tumor angiogenesis[2]. Yeligar et al. demonstrated that alcohol induces the expressions of ET-1 and endothelin-B receptor (ET-BR) in liver sinusoidal endothelial cells[3]. Therefore, VEGF and ET-1 participate in the regulation of vascular structure and function.

2, 3, 5, 6-tetramethylpyrazine (TMP) is an active alkaloid that was originally isolated from Ligusticum chuanxiong, a traditional Chinese medicine. TMP can pass through blood-brain barrier and is widely used in the treatment of various vascular diseases. In this study, we investigated the effects of TMP on the learning and memory and changes of the expressions of VEGF and serum ET-1 in the model of chronic alcoholic encephalopathy in rats, to provide new means for the treatment of alcoholism encephalopathy.

Experimental animals were provided by the Experimental Animal Center of Shandong University. Experimental procedures were strictly complied with international standards (NIH Publication No. 80-23,

doi: 10.3967/bes2015.098

1. Department of Neurology, Jinan Fourth People's Hospital, Jinan 250031, Shandong, China; 2. Department of Neurology, Shandong Qilu Hospital, Jinan 250012, Shandong, China; 3. Department of gynecology and obstetrics, Jinan Central Hospital Affiliated To Shandong University, Jinan 250013, Shandong, China; 4. Department of Emergency, Jinan Fourth People's Hospital, Jinan 250031, Shandong, China

revised 1996) and approved by Jinan Fourth People's Hospital ethics committee (Approval number: LL-20110002, DATE 2011.5.25). After normal feeding for one week, 80 adult male Wistar rats (10 weeks of age, weight of 230±10 g) were randomly divided into four groups of 20: 1) alcohol group (Alcohol): gavage administration of 40% ethanol (Shanghai Chemical Reagent Company, Shanghai, China) [8 g/(Kg·d)], 3 times/d for 4 weeks, followed by 50% ethanol [9 g/(Kg·d)], 3 times/d for 4 weeks, and then followed by 50% ethanol 10 g/(Kg·d) for 8 weeks. 2) control group (control): gavage administration of equal amount of saline (Qilu Pharmaceutical, Jinan, China) for 16 weeks; 3) alcohol+TMP group (Alcohol+TMP): gavage administration of alcohol for 16 weeks, and then intraperitoneal injection of TMP (Harbin Sanlian Pharmaceutical Company, Harbin, China) 80 mg/kg, once a day for 15 d; 4) alcohol+saline group (Alcohol+NS): gavage administration of alcohol for 16 weeks, and then intraperitoneal injection of equal amount of saline, once a day for 15 d.

Morris Water Maze-place Navigation Morris water maze apparatus was purchased from Shanghai Jiliang Software technology Co. Ltd (Shanghai, China). The rats in each group were released into the water facing the wall from four different quadrants in a pseudo-random order. The time from entering the water to reaching the platform was recorded and defined as the escape latency (EL). If the rats could not find the platform after 60 s, the rats were guided to the platform. The rats received four trainings a day with the inter-training interval of 30 min. The training was given for four times with 30min interval every day for 7 consecutive days.

Spatial Probe It was given in the morning at the day-8. The duration was 60 s. During the probe trial we measured: (1) The time the animals spent in the target quadrant (the platform was located originally) and other quadrants; (2) The numbers of platform crossing (the original platform location).

Electron Microscopy Rats were perfused with 3% glutaraldehyde (Sigma, St. Louis, MO, USA) through the ascending aorta and the brains were harvested. The brains were cut into 1-mm^3 tissue blocks and post-fixed in 3% glutaraldehyde. The tissues were dehydrated through a gradient of ethanol, embedded, sectioned, stained with uranyl acetate and lead citrate (Sigma), and then observed under a transmission electron microscope (Jeol JEM-1200EX; Jeol, Tokyo, Japan).

RT-PCR Rats from each group were decapitated and the brain tissues were rapidly harvested at low temperature. The total RNA was extracted using Trizol (Invitrogen, San Diego, CA, USA). 1 μg total RNA was reverse-transcripted into cDNA using Application Prime Script RT reagent Kit (Takara Biotechnology Co., Ltd., China). 20 μL of SYBR Green system was used and the VEGF levels in each group were detected using Light Cycle 2.0 (Roche, Mannheim, Germany). Real-time PCR for each sample was performed in triplicates and GAPDH was used as an internal reference. Primers were designed as follows: GAPDH: forward 5'-AGGTCGGTGTGAA-CGGATTTG-3'; Reverse 5'-TGTAGACCATGTAGTTGAG-GTCA-3'. VEGF: forward 5'-ACCATGAACTTTCTGCT-C-3', Reverse 5'-GGACGGCTTGAAGAT ATA-3'.

Western Blot Rats were anesthetized and decapitated. Proteins were extracted from hippocampus using lysis buffer (Beyotime, Jiangsu, China), and protein concentration was measured using a BCA kit (Beyotime, Jiangsu, China). 30 μg proteins were used for SDS-PAGE electrophoresis and transferred to a nitrocellulose membrane. After blocking, the membrane was incubated with anti-VEGF primary antibody (1:500, Cell Signal, Technology, Beverly, MA, USA), and then incubated with biotinylated goat anti-rabbit IgG secondary antibody (1:10,000, Zhongshan Golden bridge Biotechnology, Beijing, China). After development and imaging, gray valueswere detected. The relative expression of the target protein was determined as the ratio of the gray values of target protein band to that of the internal loading control. The detailed methods were described previously[1].

ET-1 Radioimmunoassay 2 mL fasting blood samples were taken and mixed with 30 μL 10% $EDTA_2Na_2$ and 40 μL aprotinin (containing 400 IU), centrifuged at 1170×g at 4 °C for 10 min. The upper serum layer was collected and stored at -20 °C. The reaction system was prepared using ET-1 kit (Northern Institute of Biology) and analyzed using automatic γ radioimmunoassay analyzer (FJ22008P type; Xi'an Nuclear Instrument Factory, Xi'an, China).

Statistical Analysis Data were analyzed using SPSS10.0 statistical software. All data were expressed as mean±S.E.M. ($\bar{x}\pm s$). 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. The escape latency in the water maze hidden platform experiments were measured with one-way ANOVA with repeated measurement. The time spent in each quadrant and the numbers of platform crossing in

the probe trial were measured with one-way ANOVA and LSD post-hoc test. Statistical significance was placed at $P<0.05$.

After 7-d training, the EL of animals in each group was rapidly decreased. Compared with control group, the alcohol group had longer EL, spent less time in the target quadrant and had significantly less platform crossing ($P<0.05$). Compared with alcohol+ NS group, the Alcohol+TMP group had shorter EL, spent longer time in the target quadrant and had more platform crossing during the probe trial ($P<0.05$). (Figure 1A, 1C, 1D)

Therefore, alcohol intake impaired the learning and memory abilities and TMP treatment partially rescued the learning and memory impairments. In addition, the rats in the alcohol group had hair loss, fatigue, irritability, loss of appetite, and weight loss (Figure 1B), which had also been seen in our previous study[1]. These physiological changes in the alcohol group might interfere with the Morris water maze performance; however, the underlying mechanisms need to be further studied.

The nuclear morphology and the structural integrity of the vascular endothelial cells in the control group were normal (Figure 2A). In the Alcohol and Alcohol+NS groups, the vascular endothelial cells were swollen with irregular shaped nuclei, ruptured nuclear membrane, uneven distribution of chromatin and narrow vascular cavity (Figure 2B, 2C); whereas, the vascular endothelial cells in the Alcohol+TMP group did not show significant swelling, and had normal shaped nuclei, complete nuclear membrane and evenly distributed chromatin (Figure 2D).

It has been shown that chronic alcohol intake can cause pathological damages to brain blood vessels, which can be ameliorated by TMP treatment. TMP is alkaloid monomers extracted from the rhizome of Chuanxiong. The main component is 2,3,5,6-tetramethyl pyrazine. TMP can dilate blood

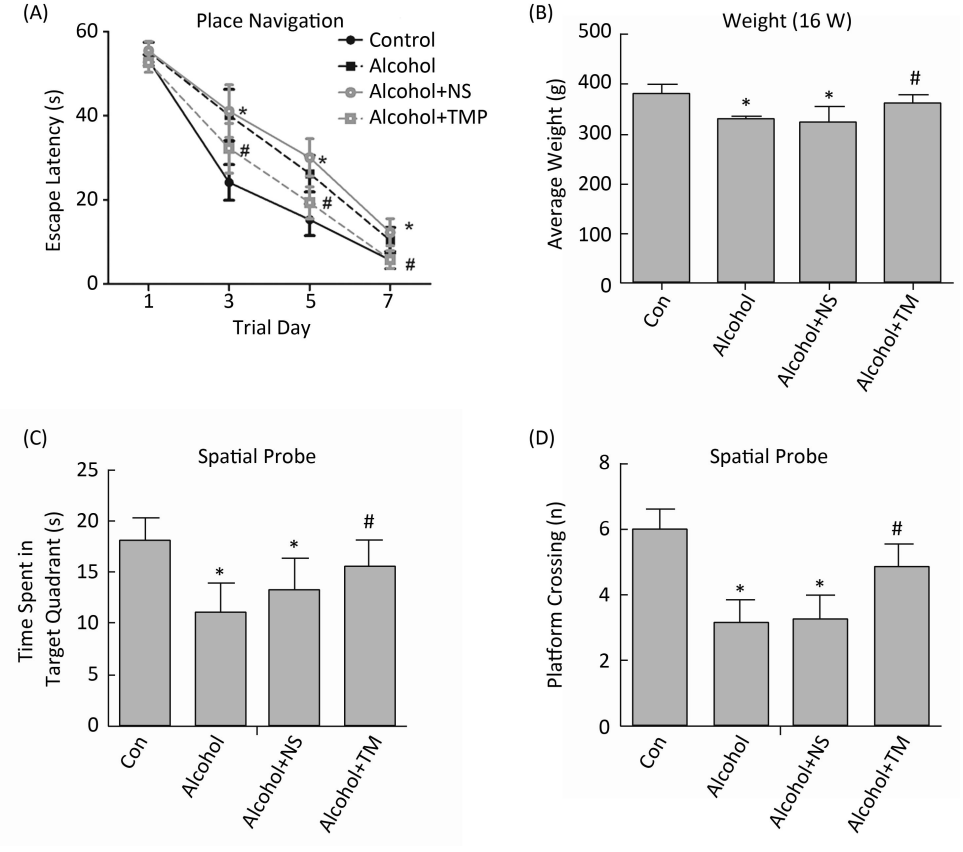

Figure 1. The learning and memory abilities were impaired by alcohol intake and partially rescued by TMP treatment. Mean escape latency(s) of rats in the different groups during navigation training (A). Average weight (g) of rats in different groups (B). Time spent in the target quadrant (C) and platform crossing of rats in the different groups during probe trial (D). $^{*}P<0.05$ *vs*. control; $^{\#}P<0.05$ *vs.* alcohol+saline (NS) group.

vessels, improve micro-circulation and protect vascular endothelial cells[4]. Meanwhile, TMP, as a potassium channel opener, can reduce the calcium influx in a variety of tissues (especially aortic smooth muscle) and play an anti-apoptosis role[5]. Our results showed that alcohol induced pathological changes in the vascular structures, and TMP intervention improved neuronal and cerebral vascular morphology, which may be due to the reduction in calcium overload and free radical production.

Compared with the control group, alcohol administration significantly increased the VEGF mRNA and protein levels in the frontal lobe, which was consistent with our previous findings[1]. Compared with Alcohol+NS group, TMP treatment for 15 d significantly increased the mRNA and protein expression of VEGF by 1.51-fold and 1.28-fold respectively (P<0.05) (Figure 3).

VEGF is an endothelial-specific mitogen and an effective angiogenic factor. Biological characteristics of VEGF are mainly manifested in two aspects: 1) It increases microvascular permeability; 2) It binds to vascular endothelial cell-specific receptors and promotes endothelial cell division and proliferation, which lead to angiogenesis[6]. Hypoxia is the most important factor among the mechanisms that are involved in the regulation of VEGF. Bekhite has confirmed that hypoxia can promote the VEGF expression and induce vascular endothelium differentiation in adipose tissues[7]. TMP can inhibit hypoxia-induced pulmonary vascular leakage through ROS-HIF-VEGF signaling pathway[8]. Our results indicated that compared with the Alcohol group, TMP intervention increased VEGF expression. In this study, we investigated VEGF expression after 15 d of TMP treatment. At this time point, TMP treatment might already have improved the cerebral ischemia and hypoxia, cleared a lot of free radicals and improved neuronal micro-environment. Meanwhile, the consistent high level of VEGF might play neuroprotective and anti-apoptotic roles in our model.

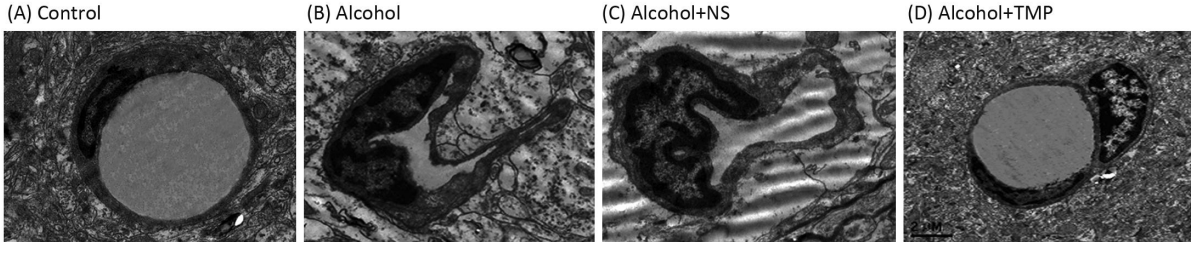

Figure 2. The nuclear morphology and the structural integrity of the vascular endothelial cells were damaged by alcohol, and partially ameliorated by TMP treatment. Electron microscopy images of frontal lobe arterioles in different groups (magnification, x10,000). NS, saline. TMP, Tetramethylpyrazine.

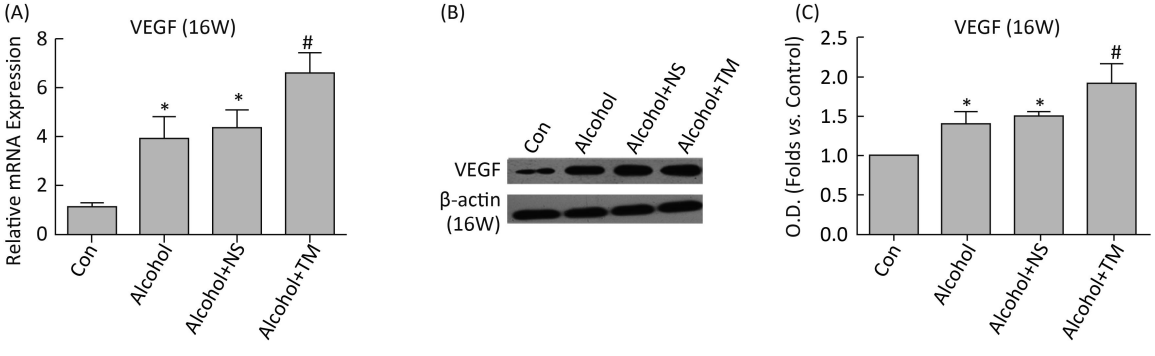

Figure 3. TMP treatment upregulated VEGF levels in the hippocampus. Effects of alcohol and Tetramethylpyrazine on the mRNA and protein expressions of vascular endothelial growth factor (VEGF). (A) PT-PCR analysis, (B) Western blot analysis, and (C) quantitative representation of the protein expression of VEGF in different groups. $^{*}P$<0.05 *vs*. control; $^{\#}P$<0.05 *vs*. alcohol+saline (NS) group.

The ET-1 level in the Alcohol group was 1.65-fold higher than the control group. After TMP treatment, ET-1 levels was significantly decreased to 73.6% of the alcohol +NS group (P<0.05, Figure 4). Lee et al. have shown that TMP can reduce the ET-1 expression in vascular endothelial cells[9]; Cao et al. have also confirmed that TMP could reduce the plasma ET-1 levels in an acute hypoxia dog model[10], which are consistent with our results. However, more studies need to be done to clarify whether TMP plays protective roles through reducing ET-1 expression, or reducing ET-1 leakage to protect the integrity of the vascular endothelial cells.

In summary, alcohol toxicity can impair learning and memory, induce pathological damages in cerebrovascular structures, cause ischemia/hypoxia of brain tissues, induce VEGF overexpression and increase ET-1 concentrations. TMP intervention can improve learning and memory in rats with chronic alcoholic encephalopathy, increase the VEGF expression and reduce ET-1 level, and thus plays a role in protecting blood vessels in the treatment of chronic alcoholic encephalopathy. However, the exact mechanism needs to be further studied.

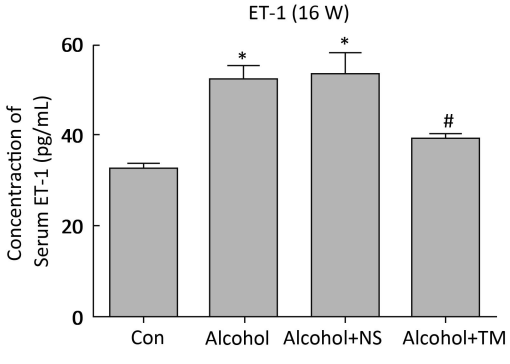

Figure 4. TMP treatment significantly decreased the alcohol-induced increase of ET-1 expression. Average concentrations of serum endothelin-1 (ET-1) in different groups. $^{*}P$<0.05 *vs*. control; $^{\#}P$<0.05 *vs.* alcohol+saline (NS) group.

ACKNOWLEDGMENT

The authors declare that there are no conflicts of interest. We thank the supports from Shandong Academy of Medical Sciences, Electron Microscopy Laboratory of Shandong University Medical School, and Department of Neurology of Qilu Hospital of Shandong University.

$^{\#}$Correspondence should be addressed to LI Hui, Tel: 86-15553172626; Fax: 86-531-81313308; E-mail: lihui19693783@sina.com

Biographical note of the first author: LI Hui, MM, born in 1969, Deputy Chief Physician, major in cerebrovascular disease.

Received: March 20, 2015;
Accepted: September 17, 2015

REFERENCES

1. Li H, Yang X, Shi W, et al. Protective effects of nimodipine on cerebrovascular function in chronic alcoholic encephalopathy. Int J Mol Med, 2014; 33, 201-8.
2. Tan W, Bailey AP, Shparago M, et al. Chronic alcohol consumption stimulates VEGF expression, tumor angiogenesis and progression of melanoma in mice. Cancer Biol Ther, 2007; 6, 1211-7.
3. Yeligar S, Tsukamoto H, Kalra VK. Ethanol-induced expression of ET-1 and ET-BR in liver sinusoidal endothelial cells and human endothelial cells involves hypoxia-inducible factor-1alpha and microrNA-199. J Immunol, 2009; 183, 5232-43.
4. Kao TK, Ou YC, Kuo JS, et al. Neuroprotection by tetramethylpyrazine against ischemic brain injury in rats. Neurochem Int, 2006; 48, 166-76.
5. Tsai CC, Lai TY, Huang WC, et al. Tetramethylpyrazine as potassium channel opener to lower calcium influx into cultured aortic smooth muscle cells. Planta Med, 2003; 69, 557-8.
6. Cariboni A, Davidson K, Dozio E, et al. VEGF signalling controls GnRH neuron survival via NRP1 independently of KDR and blood vessels. Development, 2011; 138, 3723-33.
7. Bekhite MM, Finkensieper A, Rebhan J, et al. Hypoxia, Leptin, and Vascular Endothelial Growth Factor Stimulate Vascular Endothelial Cell Differentiation of Human Adipose Tissue-Derived Stem Cells. Stem Cells Dev, 2013.
8. Zhang L, Deng M, Zhou S. Tetramethylpyrazine inhibits hypoxia-induced pulmonary vascular leakage in rats via the ROS-HIF-VEGF pathway. Pharmacology, 2011; 87, 265-73.
9. Lee WS, Yang HY, Kao PF, et al. Tetramethylpyrazine downregulates angiotensin II-induced endothelin-1 gene expression in vascular endothelial cells. Clin Exp Pharmacol Physiol, 2005; 32, 845-50.
10. Cao W, Zeng Z, Zhu YJ, et al. Effects of tetramethylpyrazine, a Chinese medicine, on plasma endothelin-1 levels during acute pulmonary hypoxia in anesthetized dogs. J Cardiovasc Pharmacol, 1998; 31 Suppl 1, S456-9.