Original Article Identification of molecular mechanism underlying therapeutic effect of tanshinone IIA in the treatment of hypoxic vestibular vertigo via the NO/cGMP/BKCa signaling pathway

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Abstract: This study aimed to investigate the molecular mechanisms underlying the effect of Tashinone IIA (Tan) on the treatment of ischemic vertigo. Sprague-Dawley (SD) male rats were divided into a SHAM group, a MODEL group, a MODEL+Tan (10 mg/kg) group, a MODEL+Tan (20 mg/kg) group, a MODEL+Tan (40 mg/kg) group and a MODEL+Tan (80 mg/kg) group. The escape latency was observed among different groups of rats, while the production of NO/cGMP and the expression of BKCa were measured in vivo and in vitro by H&E staining, Western Blot and IHC assays. While the rats with ischemic vertigo showed prolonged escape latency, the treatment by Tan (40 mg/kg and up) shortened the escape latency in rats with ischemic vertigo. Moreover, the reduced production of NO/cGMP and expression of BKCa protein in the MODEL group were increased by a certain extent upon the treatment of 40 mg/kg or 80 mg/kg Tan. H&E staining of MVN neuron cells collected from different rat groups also validated in vitro, as the cells treated with 5 ug/ml and 10 ug/ml Tan increased the levels of NO/cGMP production and BKCa protein expression. At a certain dose, Tan could increase the production of NO and cGMP as well as the expression of BKCa, which would subsequently aid the treatment of ischemic vertigo.

Keywords: Vestibular vertigo, Tanshinone (TAN IIA), nitric oxide (NO), cGMP, BKCa

Introduction

As a frequently diagnosed clinical symptom, vertigo represents an illusion of movement triggered by the asymmetric balance of the vestibular system. It was reported that the annual prevalence of vertigo is 4.9%, while the prevalence of unbalanced movement in the older population is > 30% [1-3]. Neurotologic survey has shown that vestibular vertigo (VV) accounts for 25% of dizziness. Therefore, with an annual prevalence of 5.2% and incidence of 1.5%, VV is not only very frequent at the population level, but also exerts a considerable impact on individuals, since 80% of individuals affected by VV have been absent from work or have interrupted their daily activities in order to seek medical consultation. Although VV seriously impacts the quality of life of its patients, the pathological mechanisms underlying the symptoms of VV are yet to be fully clarified. In fact, many studies have shown that various chemical processes contribute to the symptoms of VV by playing important functions in the vestibular nuclei [4]. Previous studies have also suggested the role of glutamate as the main neurotransmitter traveling from the vestibular nerve to the neurons in the medial vestibular nuclei (MVN) [5, 6]. In addition, the levels of calcium ions may be closely related to the occurrence of vertigo and hence are considered as an effective substance in the treatment of VV [7].

As a derivative of phenanthrenequinone, Tashinone IIA (Tan) is a key component in Danshen. Tan shows strong in vitro cytotoxicity against various types of tumor cells, including leukemia cells, breast cancer cells, and NCI-H460 cells [8-10]. Tan also shows a protective effect under certain conditions. It was shown that Tan leads to a reduced brain infarct volume and significantly alleviates the neurological deficits in rats undergoing temporary focal cerebral ischemia [11]. As a key water soluble extract from Danshen, salvianolic acid B (Sal B) shows a broad range of pharmacological properties. In addition, as an active diterpenoid quinone pigment in Danshen. Tan exerts a cardioprotective effect by inducing vasodilatation via the phosphorylation of endothelial nitric oxide synthase (eNOS) through the Akt/PI3K/AMPK pathway, which in turn induces the production of NO. In addition, Tan and Sal B can stimulate the uptake of L-arginine by endothelial cells via increasing the mRNA expression of CATs, such asCAT-2B and CAT-1.

The regulation of arterial tone controls the adequate blood flow in the entire body. Generally, vascular endothelium produces multiple substances involved in the regulation of arterial tone. Among these substances, nitric oxide (NO) acts as a second messenger to directly activate big conductance Ca2+-activated K+ channels (BKCa) [12]. Alternatively, NO can induce the hyperpolarization of smooth muscle cells (SMCs) by generating cGMP [13, 14].

Disorder and abnormal firing of MVN neurons can be triggered by hypoxic inhalation [15]. In addition, BKCa channels, small conductance Ca2+-activated K+ channels (SK channels), Ca2+ channels, and sodium channels are present in MVN neurons [16-21]. In a previous study, the effect of BKCa channels in MVN neurons on hypoxia-induced neuronal disorder was investigated. The results suggested that the activity and expression of BKCa channels in MVNs are decreased by hypoxia. Subsequently, the decreased activity of BKCa channels in MVNs aggravates the severity of hypoxia-induced neuronal disorder.

It has been reported that Tan up-regulated the production of NO by promoting the expression of NOS [22, 23]. Furthermore, NO functions as a vasodilator via activating its downstream signaling pathways, including cGMP, PKA and BKCa [24, 25]. Moreover, as a well-known vasodilator, the deregulation of BKCa has been found in patients with VV [26]. In this study, we investigated the therapeutic effect of Tan in an animal model of VV as well as the effect of Tan on the BKCa signaling pathway.

Materials and methods

Animals and treatment

In this study, 21 purchased Sprague-Dawley (SD) male rats (age: 6 weeks, weight: 200 ± 20 g) were studied. All rats were housed in an environment of 30%-70% humidity, under a temperature of 20~25°C and a 12 h light-dark cycle. In addition, all rats had free access to water and food. After 3 days of adaptation, the rats were divided into a SHAM group (sham-operated rats), a MODEL group (rats suffering from ischemic vertigo), a MODEL+PBS group (ischemic vertigo rats treated with PBS) and various MODEL+TAN II A groups (ischemic vertigo rats treated with 10 mg/kg, 20 mg/kg, 40 mg/kg and 80 mg/kg of TSA, respectively). And TSA was dispersed in 0.5% sodium carboxymethyl cellulose, and intraperitoneally administered upon the model rats accordingly. The rats were anaesthetized with chloral hydrate (0.35 ml/ 100 g body weight) and fixed in supine position. The neck skin was cut to be separated completely from the muscles. Right CCA and SCA were exposed and separated, followed by penetrating ligation. To observe the effect of TSA on the treatment of ischemic vertigo, we measured the escape latency, which is defined as the time required for the model rats to regain balance on a platform (no less than 30 s) after a combined stimulation by rotation and electricity. The animal experiments in this study were all approved by the Animal Ethics Committee of our institution and all animal procedures were performed in accordance with the guide for the care and use of laboratory animals.

Cell culture

U251 and SH-SY5Y cells were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences and maintained in a DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT). During this study, the cells were cultured in a 5% CO₂ incubator (Model, Thermo Scientific 8000, Thermo Fisher Scientific, Waltham, MA) at 37°C and 95% humidity. During cell passage, the original medium was removed by centrifugation at 1000 rpm and the cells were rinsed twice with PBS before being seeded in a new DMEM medium containing 10% FBS. The culture cells were treated with 1 ug/ml, 5 ug/ml and 10 ug/ml Tan as well as the blank control and functional analysis was performed to study the effect of Tan on the expression of BKCa signaling pathway.

Western blot analysis

The samples of MVN tissues (30 mg in each group) were grinded to a uniform powder at low temperature, washed 3 times by PBS, lysed with a protein lysis buffer, incubated on ice for 20 min, and subsequently centrifuged at 13,000 r/min and 4°C for 15 min to collect the supernatant. The supernatant was then collected and the protein concentration in the supernatant was determined using a bicinchoninic acid (BCA) protein assay kit. The protein samples (50 µg in each group) were then dissolved in a 2 × sodium dodecyl sulphate (SDS) sample buffer, boiled at 100°C for 5 min, separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto a nitrocellulose membrane. The membrane was then blocked in 5% skimmed milk overnight at 4°C, washed twice by PBS. Subsequently, anti-BKCa and anti-β-actin (internal control) primary antibodies (Cell Signaling Technology, Danvers, MA, US) were added onto the membrane and incubated overnight. After being washed three times in Tris-buffered saline tween-20 (TBST), the membrane was incubated with HRP-labeled goat anti-rabbit IgG secondary antibodies (1:5000, Cell Signaling Technology, Danvers, MA, US) for 1 h, and washed three times with TBST (5 min each time). Enhanced chemiluminescence (ECL) kit (Millipore, Burlington, MA) was then used for coloration. After liquid removal, the membrane was exposed to X-ray and the absorbance of protein bands was analyzed using a Gel imaging analysis system. During the analysis, β -actin was used as the internal reference to calculate the relative protein expression of BKCa.

H&E staining

The samples of MVN tissues were collected from the rats in each group and fixed using 10% neutral buffered formalin (NBF) for at least 24

h, and then dehydrated using conventional gradient alcohol of 75%, 85%, 95%, 95%, and 100%, respectively (5 min for each dehydration step). Subsequently, the tissues were cleared twice with dimethylbenzene (5 min/each time), immersed in the wax and embedded with paraffin. Afterwards, the tissue blocks were cut into 3 µm sections, which were then placed in an incubator at 60°C for 1 h. After cooling, the sections were dehydrated using conventional gradient alcohol, cleared with dimethylbenzene, and taken out from the incubator for rinsing. Subsequently, the sections were stained by hematoxylin for 7 min, and washed, followed by differentiation with hydrochloric alcohol for 5 s, and 1 min of soaking in eosin. In the next step, the sections were dehydrated with conventional gradient alcohol of 75%, 85%, 95%, and 100%, respectively (5 min for each dehydration step), cleared twice with dimethylbenzene (5 min/each time), and mounted in neutral balsam on a draught cupboard. Subsequently, the sections were placed under an optical microscope to observe the pathological changes in MVN neuron cells.

IHC assay

The samples of MVN tissues were fixed using formaldehyde, embedded with paraffin, and cut into 4 µm serial sections. The sections were then incubated in an incubator at 60°C for 30 min, dewaxed with conventional xylene, dehydrated using gradient alcohol, and soaked in 3% H₂O₂ for 10 min. After being washed three times (3 min/each) with distilled water and subsequent high-pressure antigen retrieval, the sections were placed in a cold bath to be cooled down to room temperature, followed by two rinsing (3 min/each) with phosphate buffered saline (PBS) (0.01 M pH 7.4). Subsequently, the sections were incubated in a 5% BSA sealing solution for 20 min, followed by removal of the excessive liquid. In the next step, the sections were incubated overnight at 4°C with anti-BKCa primary antibody (1:100, Abcam, Cambridge, MA), and then washed three times (3 min/each) with PBS. Subsequently, the sections were incubated for 30 min with IgG secondary antibody (1:1000, Abcam, Cambridge, MA) and washed three times (3 min/each) with PBS, followed by 5 min of staining using a diaminobenzidine (DAB) kit (Abcam, Cambridge, MA). After the sections were counter stained with hematoxylin, the sections were observed



Figure 1. The escape latency of in rats from the SH-AM group, MODEL group, MODEL+PBS group, MO-DEL+Tan (10 mg/kg) group, MODEL+Tan (20 mg/ kg) group, MODEL+Tan (40 mg/kg) group and MO-DEL+Tan (80 mg/kg) group was measured and the results indicated that the treatment by Tan at a higher dose (40 mg/kg and up) could reduce the escape latency (*P < 0.01 as compared with SHAM group, **P < 0.05 as compared with MODEL group).

under a microscope. During the observation, five different fields were randomly selected for each section under the microscope. The number of BKCa positive cells was then counted and the positive expression rate of BKCa in each group was calculated.

ELISA

The levels of NO production in MVN tissues and cultured SH-SY5Y cells were detected by an ELISA kit. In brief, an ELISA kit for NO detection (A012-1, Jiancheng, Nanjing, China) was used according to the instruction of the kit, and the optical density (OD) of each sample was measured at a wavelength of 550 nm on a MULTISKAN MK3 plate reader (Thermo Fisher Scientific, Waltham, MA).

Statistical analysis

Statistical analysis was performed using the SPSS 19.0 software (IBM Armonk, NY), and the measurement data were expressed as mean \pm standard deviation (SD). Comparisons between two groups were carried out using *t*-tests, while one-way analysis of variance (AN-

OVA) was conducted for comparison among multiple groups. A p value of < 0.05 was considered as statistically significant.

Results

Tan at a higher dose (40 mg/kg and up) reduced the escape latency

As shown in **Figure 1** and compared with that in the SHAM group, the escape latency in the MODEL group was greatly prolonged, while the treatment by 10 mg/kg and 20 mg/kg of Tan showed little effect on the escape latency in model rats. However, once the dose of Tan was increased to 40 mg/kg and 80 mg/kg, the escape latency was significantly reduced in model rats, indicating that a higher dose of Tan (40 mg/kg and up) exerts a positive effect during the treatment of ischemic vertigo.

Tan at a higher dose (40 mg/kg and up) increased the production of NO and cyclic guanosine monophosphate (cGMP) to a certain extent

We also measured the dynamic changes in the production of NO and cGMP after the Tan treatment of model rats. As displayed in **Figure 2**, the production of NO (**Figure 2A**) and cGMP (**Figure 2B**) was evidently reduced in the MO-DEL group compared with that in the SHAM group, while the treatment by PBS, Tan (10 mg/kg) and Tan (20 mg/kg) showed insignificant effects on the production of NO and cGMP. However, as the dose of Tan was increased to 40 mg/kg, the production of NO (**Figure 2A**) and cGMP (**Figure 2B**) in model rats started to increase, while the treatment by 80 mg/kg Tan exhibited a more significant efficacy.

Tan at a higher dose (40 mg/kg and up) exhibited a therapeutic effect on the repair of damaged MVN neurons

Furthermore, we carried out H&E staining on MVN neuron samples collected from the model rats. As shown in **Figure 3**, large multi-polar neurons and bulky Nissl bodies were clearly seen in the MVN neurons of the SHAM group (**Figure 3A**), which also showed small and medium-sized cells scattered around the large cells. Meanwhile, the rats in the MODEL group (**Figure 3B**) exhibited deformed large cells featured by evident enlargement, pyknosis and



Figure 2. Tan at a higher dose (40 mg/kg and up) increased the production of NO and cGMP in model rats to a certain extent. A. NO production in the rats of the SHAM group, MODEL group, MODEL+PBS group, MODEL+Tan (10 mg/kg) group, MODEL+Tan (20 mg/kg) group, MODEL+Tan (40 mg/kg) group and MODE+Tan (80 mg/kg) group (*P < 0.01 as compared with SHAM group, **P < 0.05 as compared with MODEL group); B. cGMP production in rats of the SHAM group, MODEL group, MODEL+PBS group, MODEL+Tan (10 mg/kg) group, MODEL+Tan (20 mg/kg) group, MODEL+Tan (40 mg/kg) group, MODEL+Tan (20 mg/kg) group, MODEL+Tan (40 mg/kg) group and MODE+Tan (80 mg/kg) group (*P < 0.01 as compared with SHAM group, **P < 0.05 as compared with MODEL group).

apoptosis. In addition, the treatment by 10 mg/kg Tan (Figure 3C) and 20 mg/kg Tan (Figure 3D) resulted in slight changes in cell morphology, although some large cells were still surrounded by similarly denatured MVN neuron cells. Moreover, in model rats treated by 40 mg/kg of Tan (Figure 3E), we observed an apparently lower number of denatured MVN neuron cells, while the number of normal large cells surrounded by colloid cells was significantly increased. Similarly, a better therapeutic efficacy was observed in the MODEL+Tan (80 mg/ kg) group (Figure 3F).

Tan at a higher dose (40 mg/kg and up) upregulated the expression of BKCa

To investigate whether Tan promotes the activation of BKCa and subsequent vasodilation in the treatment of ischemic vertigo, we also carried out Western Blot analysis (**Figure 4**) and IHC assay (**Figure 5**) to measure the expression of BKCa in different groups. Consistent with the effect of Tan shown above, the treatment of model rats by 10 mg/kg and 20 mg/kg Tan A showed no effects on the BKCa expression in model rats, while the treatment by 40 mg/kg or 80 mg/kg Tan obviously up-regulated BKCa expression. Thus, the above results collectively validated the positive effect of Tan in the treatment of ischemic vertigo in vivo.

Tan at a higher dose (5 ug/ml and up) increased the production of NO, cGMP and BKCa proteins in vitro

We also studied the effect of Tan on in vitro production of NO, cGMP and BKCa proteins. As shown in Figure 6, U251 cells were treated with different concentrations of Tan (1 ug/ml, 5 ug/ml and 10 ug/ml, respectively) before the levels of NO (Fig.6A), cGMP (Figure 6B) and BKCa (Figure 6C and 6D) were measured. The results showed that the treatment by 1 ug/ml Tan did not significantly change the levels of NO, cGMP and BK-Ca in U251 cells, while the treatment by 5 ug/ml and 10 ug/ml Tan obviously increased the levels of NO, cGMP and BKCa.

Since similar results were obtained in SH-SY5Y cells (**Figure 7**), the effects of Tan on promoting the production of NO, cGMP and BKCa proteins were confirmed in vitro.

Discussion

In this study, the escape latency in the MODEL group was greatly prolonged, while the treatment by 10 mg/kg and 20 mg/kg Tan showed little effect on the escape latency in model rats. However, the treatment by 40 mg/kg and 80 mg/kg Tan apparently reduced the escape latency of model rats, indicating that a higher dose of Tan (40 mg/kg and up) can exhibit a therapeutic effect in the treatment of ischemic vertigo.

As a chemical discovered in Salvia miltiorrhiza, Tan plays a role in the anti-inflammatory activity, apoptosis, activation of methyl guanine transferase, and lipid peroxidation in cells [27-29]. A previous study demonstrated that Tan can prevent brain injury and cardiovascular diseases, including cerebrovascular diseases, hyperlipidemia, and coronary heart diseases [30]. In addition, as a key ingredient in Danshen anshinone, Tan can prevent the development of temporary ischemia injury in the brain [11]. Another study also showed that the vasodilation effect of Tan is dependent on the function of endothelial cells. In fact, endothelial cells



Figrue 3. Tan at a higher dose (40 mg/kg and up) could exert a positive effect on repairing the damaged MVN neuron cells. A. H&E staining of MVN neuron cells collected from the SHAM group; B. H&E staining of MVN neuron cells collected from the MODEL group; C. H&E staining of MVN neuron cells collected from the MODEL+PBS group; D. H&E staining of MVN neuron cells collected from the MODEL+Tan (10 mg/kg) group; E. H&E staining of MVN neuron cells collected from the MODEL+Tan (20 mg/kg) group; F. H&E staining of MVN neuron cells collected from the MODEL+Tan (20 mg/kg) group; F. H&E staining of MVN neuron cells collected from the MODEL+Tan (20 mg/kg) group; F. H&E staining of MVN neuron cells collected from the MODEL+Tan (40 mg/kg) group; G. H&E staining of MVN neuron cells collected from the MODEL+Tan (80 mg/kg) group.



Figure 4. Western Blot analysis showed that Tan at a higher dose (40 mg/kg and up) could increase the expression of BKCa. A. Western Blot analysis of BKCa expression in the SHAM group, MODEL group,

MODEL+PBS group, MODEL+Tan (10 mg/kg) group, MODEL+Tan (20 mg/kg) group, MODEL+Tan (40 mg/ kg) group and MODE+Tan (80 mg/kg) group; B. Relative expression of BKCa protein in the SHAM group, MODEL group, MODEL+PBS group, MODEL+Tan (10 mg/kg) group, MODEL+Tan (20 mg/kg) group, MODEL+Tan (40 mg/kg) group and MODE+Tan (80 mg/kg) group (*P < 0.01 as compared with SHAM group, **P < 0.05 as compared with MODEL group).

can control vascular functions by releasing endothelium-derived hyperpolarizing factor (ED-HF), prostaglandin I2 (PGI2), and NO [31]. Another study also reported that Tan can reduce the blood pressure in patients with hypertension, possibly by enhancing the synthesis of eNOS and the production of NO [32]. Similarly, it was shown that L-NMMA can inhibit the production of NOS and reduce the extent of vasodilation [33]. These results indicate that NO plays an important role to maintain Tan-triggered vasodilation in the coronary arterioles of rats. Therefore, by inducing the release of EETs and NO, and by activating BKca channels, Tan exerts a strong effect to dilate coronary arterioles. In this study, the production of NO and cGMP was evidently reduced in the MODEL group, while the treatment by PBS, 10 mg/kg and 20 mg/kg did not significantly increase the levels of NO and cGMP in model rats. However, the treatment by 40 mg/kg and 80 mg/kg Tan



Figure 5. Tan at a higher dose (40 mg/kg and up) can up-regulate the expression of BKCa. A. IHC assay of BKCA expression in the SHAM group; B. IHC assay of BKCA expression in the MODEL group; C. IHC assay of BKCA expression in the MODEL+PBS group; D. IHC assay of BKCA expression in the MODEL+Tan (10 mg/kg) group; E. IHC assay of BKCA expression in the MODEL+Tan (20 mg/kg) group; F. IHC assay of BKCA expression in the MODEL+Tan (40 mg/kg) group; G. IHC assay of BKCA expression in the MODEL+Tan (80 mg/kg) group.

increased the production of NO and cGMP to a certain extent. As the only receptor of NO found so far, soluble guanylate1 cyclase (sGC) is produced from L-arginine by NOS. Since NO can bind to the sGC heme, it subsequently activates sGC and increases the concentration cGMP, which in turn allows sGC to send an NO signal downstream to cGMP-regulated phosphodiesterase, cGMP-gated cation channels, and signaling cascade-cGMP-dependent protein kinase. Finally, cGMP leads to the relaxation of blood vessels via various mechanisms, such as the hyperpolarization of cavernosal and arterial SMCs by cGMP-dependent protein kinase (PKG), which then phosphorylates and activates BKCa to induce the effects of vasodilation [34, 35].

In a previous study, it was shown that NO can significantly elevate the spontaneous rate of firing in MVNs by activating the cGMP signaling. In addition, such effect of NO is not dependent on the pathways of cAMP, PKA or PKG. Moreover, NO not only can activate the CNG channels in MVNs, but may also affect the ion conductance responsible for inducing the spontaneous firing in these neurons. In fact, the firing by basal MVNs is primarily dependant on persistent influx of Na+ ions and the inactivation of K+ current in type-A and type-B cells [17, 36, 37]. Furthermore, extensive studies have shown that cGMP and NO are involved in the regu

lation of both Na+ influx and K+ current [38]. Moreover, as a key regulator of calcium signaling and neuronal excitability, BKCa channels are present in various excitable cells and can be activated by either membrane potential depolarization or an increased level of intracellular calcium [20, 39-42]. In addition, a reduced activity of BKCa channel may induce the hyperexcitability of neurons, thus implicating BKCa channels in the control of neuron activity during ischemic/hypoxic injury [40, 42-47]. Finally, BKCa channels may act as an "emergency brake" to inhibit membrane depolarization, thus preventing the occurrence of neuron injury caused by a large current of calcium influx [44]. In this study, the expression of BKCa was evidently suppressed in the MODEL group, while the treatment by 10 mg/kg and 20 mg/kg Tan did not evidently increase the expression of BKCa in rats with ischemic vertigo. In contrary, the treatment by 40 mg/kg and 80 mg/kg Tan evidently increase the expression of BKCa in the model rats.

The reduced activity of BKCa channels may be caused by the increase of firing frequency upon hypoxia, indicating that the reduced activity of BKCa channels is responsible for hypoxia-induced hyperactivity in the MVN. Furthermore, the activation of BKCa channels by NS1619 can reduce the level of hypoxia-triggered depolarization in MVN neurons, suggesting that the



Figure 6. Tan at a higher dose (5 ug/ml and up) increased the production of NO, cGMP and BKCa protein in U251 cells A. NO production in U251 cells treated with 1 ug/ml, 5 ug/ml and 10 ug/ml Tan (*P < 0.01 as compared with untreated cells); B. cGMP production in U251 cells treated with 1 ug/ml, 5 ug/ml and 10 ug/ml Tan (*P < 0.01 as compared with untreated cells); C. Western Blot analysis of BKCa protein expression in U251 cells treated with 1 ug/ml, 5 ug/ml, 5 ug/ml and 10 ug/ml Tan. D. Relative expression of BKCa protein in U251 cells treated with 1 ug/ml, 5 ug/ml and 10 ug/ml Tan.

activation of BKCa channels in transient hypoxia can protect neurons against ischemic/hypoxic injury by reducing the magnitude of hypoxia-induced neuronal hyperactivity. In this study, the MVN neuron cells collected from the SHAM group showed large multipolar neurons and bulky Nissl bodies surrounded by small and medium-sized cells. In contrary, the MO-DEL group showed evidently enlarged and deformed cells along with signs of pyknosis and apoptosis. The treatment with 10 mg/kg or 20 mg/kg Tan failed to change the deformed morphology of the cells, while the treatment with 40 mg/kg or 80 mg/kg Tan significantly reduced the number of denatured MVN neuron cells.

Our study featured the effect of Tan in the treatment of hypoxic vertigo via modulating the NO/cGMP/BKCa signaling pathway, which explained the molecular mechanism underlying the therapeutic effect of Tan. Accordingly, this study could provide supportive materials for the clinical treatment of hypoxic vertigo. The limitation of this study was that only animal models were utilized, with no according clinical trials. Therefore, further clinical studies are needed to confirm the conclusion of this study.

Conclusion

It was hypothesized in this study that Tan could alleviate the symptoms of vertigo by activating eNOS and by increasing the expression of BKCa in MVN neuron cells. To confirm this hypothesis, we established a rat model of ischemic vertigo, and used molecular biology tools to study the effect of Tan on vasodilatation and potential signaling pathways implicated in the neuroelectrophysiology of MVN. Furthermore, the effects of Tan on BKCa current and the expression of BKCa in ischemic VV were further elucidated. The results from this study will help to



Figure 7. Tan at a higher dose (5 ug/ml and up) increased the production of NO, cGMP and BKCa protein in SH-SY5Y cells A. NO production in SH-SY5Y

cells treated with 1 ug/ml, 5 ug/ml and 10 ug/ml Tan (*P < 0.01 as compared with untreated cells); B. cGMP production in SH-SY5Y cells treated with 1 ug/ ml, 5 ug/ml and 10 ug/ml Tan (*P < 0.01 as compared with untreated cells); C. Western Blot analysis of BKCa protein expression in SH-SY5Y cells treated with 1 ug/ml, 5 ug/ml and 10 ug/ml Tan. D. Relative expression of BKCa protein in SH-SY5Y cells treated with 1 ug/ml, 5 ug/ml and 10 ug/ml Tan.

further understand the mechanisms underlying the effects of Tan in the treatment of hypoxic vertigo.

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Disclosure of conflict of interest

None.

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References

- [1] Neuhauser HK. Epidemiology of vertigo. Curr Opin Neurol 2007; 20: 40-46.
- [2] Jonsson R, Sixt E, Landahl S, Rosenhall U. Prevalence of dizziness and vertigo in an urban elderly population. J Vestib Res 2004; 14: 47-52.
- [3] Sloane PD, Coeytaux RR, Beck RS, Dallara J. Dizziness: state of the science. Ann Intern Med 2001; 134: 823-832.
- [4] Matsuoka I, Ito J, Takahashi H, Sasa M, Takaori S. Experimental vestibular pharmacology: a minireview with special reference to neuroactive substances and antivertigo drugs. Acta Otolaryngol Suppl 1984; 419: 62-70.
- [5] Inoue S, Yamanaka T, Kita T, Nakashima T, Hosoi H. Glutamate release in the rat medial vestibular nucleus following unilateral labyrinthectomy using in vivo microdialysis. Brain Res 2003; 991: 78-83.
- [6] Yamanaka T, Sasa M, Matsunaga T. Release of glutamate from the vestibular nerve in the medial vestibular nucleus as a neurotransmitter: in vivo microdialysis study. Acta Otolaryngol Suppl 1995; 520 Pt 1: 92-93.

- [7] Olesen J. Calcium entry blockers in the treatment of vertigo. Ann N Y Acad Sci 1988; 522: 690-697.
- [8] Song Y, Yuan SL, Yang YM, Wang XJ, Huang GQ. Alteration of activities of telomerase in tanshinone IIA inducing apoptosis of the leukemia cells. Zhongguo Zhong Yao Za Zhi 2005; 30: 207-211.
- [9] Wang X, Wei Y, Yuan S, Liu G, Lu Y, Zhang J, Wang W. Potential anticancer activity of tanshinone IIA against human breast cancer. Int J Cancer 2005; 116: 799-807.
- [10] Hu H, Zhang Y, Huang F, Deng H. Inhibition of proliferation and induction of apoptosis by tanshinone II A in NCI-H460 cell. Zhong Yao Cai 2005; 28: 301-304.
- [11] Lam BY, Lo AC, Sun X, Luo HW, Chung SK, Sucher NJ. Neuroprotective effects of tanshinones in transient focal cerebral ischemia in mice. Phytomedicine 2003; 10: 286-291.
- [12] Van Hove CE, Van der Donckt C, Herman AG, Bult H, Fransen P. Vasodilator efficacy of nitric oxide depends on mechanisms of intracellular calcium mobilization in mouse aortic smooth muscle cells. Br J Pharmacol 2009; 158: 920-930.
- [13] Jackson-Weaver O, Osmond JM, Riddle MA, Naik JS, Gonzalez Bosc LV, Walker BR, Kanagy NL. Hydrogen sulfide dilates rat mesenteric arteries by activating endothelial large-conductance Ca(2)(+)-activated K(+) channels and smooth muscle Ca(2)(+) sparks. Am J Physiol Heart Circ Physiol 2013; 304: H1446-1454.
- [14] Eichhorn B, Dobrev D. Vascular large conductance calcium-activated potassium channels: functional role and therapeutic potential. Naunyn Schmiedebergs Arch Pharmacol 2007; 376: 145-155.
- [15] Inoue S, Yamanaka T, Okamoto H, Hosoi H. Effect of a glutamate blocker, ipenoxazone hydrochloride on the hypoxia-induced firing in the medial vestibular nucleus. Acta Otolaryngol Suppl 2004; 58-60.
- [16] Vassias I, Patko T, Vidal PP, de Waele C. Modulation of the beta1-3 voltage-gated sodium channels in rat vestibular and facial nuclei after unilateral labyrinthectomy and facial nerve section: an in situ hybridization study. Brain Res Mol Brain Res 2003; 120: 73-78.
- [17] Serafin M, de Waele C, Khateb A, Vidal PP, Muhlethaler M. Medial vestibular nucleus in the guinea-pig. II. Ionic basis of the intrinsic membrane properties in brainstem slices. Exp Brain Res 1991; 84: 426-433.
- [18] Ris L, Capron B, Nonclercq D, Alexandre H, Sindic C, Toubeau G, Godaux E. Labyrinthectomy changes T-type calcium channels in vestibular neurones of the guinea pig. Neuroreport 2003; 14: 1585-1589.

- [19] de Waele C, Serafin M, Khateb A, Yabe T, Vidal PP, Muhlethaler M. Medial vestibular nucleus in the guinea-pig: apamin-induced rhythmic burst firing--an in vitro and in vivo study. Exp Brain Res 1993; 95: 213-222.
- [20] Saito Y, Takazawa T, Ozawa S. Relationship between afterhyperpolarization profiles and the regularity of spontaneous firings in rat medial vestibular nucleus neurons. Eur J Neurosci 2008; 28: 288-298.
- [21] van Welie I, du Lac S. Bidirectional control of BK channel open probability by CAMKII and PKC in medial vestibular nucleus neurons. J Neurophysiol 2011; 105: 1651-1659.
- [22] Jin HJ, Li CG. Tanshinone IIA and cryptotanshinone prevent mitochondrial dysfunction in hypoxia-induced H9c2 cells: association to mitochondrial ROS, intracellular nitric oxide, and calcium levels. Evid Based Complement Alternat Med 2013; 2013: 610694.
- [23] Zhou ZW, Xie XL, Zhou SF, Li CG. Mechanism of reversal of high glucose-induced endothelial nitric oxide synthase uncoupling by tanshinone IIA in human endothelial cell line EA.hy926. Eur J Pharmacol 2012; 697: 97-105.
- [24] Sinha S, Sinharoy P, Bratz IN, Damron DS. Propofol causes vasodilation in vivo via TRPA1 ion channels: role of nitric oxide and BKCa channels. PLoS One 2015; 10: e0122189.
- [25] Jing F, Liu M, Yang N, Liu Y, Li X, Li J. Relaxant effect of chloroquine in rat ileum: possible involvement of nitric oxide and BKCa. J Pharm Pharmacol 2013; 65: 847-854.
- [26] Lopez-Dyck E, Andrade-Urzua F, Elizalde A, Ferrer-Villada T, Dagnino-Acosta A, Huerta M, Osuna-Calleros Z, Rangel-Sandoval C, Sanchez-Pastor E. ACPA and JWH-133 modulate the vascular tone of superior mesenteric arteries through cannabinoid receptors, BKCa channels, and nitric oxide dependent mechanisms. Pharmacol Rep 2017; 69: 1131-1139.
- [27] Xia WJ, Yang M, Fok TF, Li K, Chan WY, Ng PC, Ng HK, Chik KW, Wang CC, Gu GJ, Woo KS, Fung KP. Partial neuroprotective effect of pretreatment with tanshinone IIA on neonatal hypoxia-ischemia brain damage. Pediatr Res 2005; 58: 784-790.
- [28] Gao J, Yang G, Pi R, Li R, Wang P, Zhang H, Le K, Chen S, Liu P. Tanshinone IIA protects neonatal rat cardiomyocytes from adriamycin-induced apoptosis. Transl Res 2008; 151: 79-87.
- [29] Kang BY, Chung SW, Kim SH, Ryu SY, Kim TS. Inhibition of interleukin-12 and interferongamma production in immune cells by tanshinones from Salvia miltiorrhiza. Immunopharmacology 2000; 49: 355-361.
- [30] Zhou L, Zuo Z, Chow MS. Danshen: an overview of its chemistry, pharmacology, pharma-

cokinetics, and clinical use. J Clin Pharmacol 2005; 45: 1345-1359.

- [31] Campbell WB, Falck JR. Arachidonic acid metabolites as endothelium-derived hyperpolarizing factors. Hypertension 2007; 49: 590-596.
- [32] Kim DD, Sanchez FA, Duran RG, Kanetaka T, Duran WN. Endothelial nitric oxide synthase is a molecular vascular target for the Chinese herb Danshen in hypertension. Am J Physiol Heart Circ Physiol 2007; 292: H2131-2137.
- [33] Rosenblum WI. Re: role of endothelial nitric oxide and smooth muscle potassium channels in cerebral arteriolar dilation in response to acidosis. Stroke 2002; 33: 1742-1743; author reply 1742-1743.
- [34] Robertson BE, Schubert R, Hescheler J, Nelson MT. cGMP-dependent protein kinase activates Ca-activated K channels in cerebral artery smooth muscle cells. Am J Physiol 1993; 265: C299-303.
- [35] Archer SL, Huang JM, Hampl V, Nelson DP, Shultz PJ, Weir EK. Nitric oxide and cGMP cause vasorelaxation by activation of a charybdotoxin-sensitive K channel by cGMP-dependent protein kinase. Proc Natl Acad Sci U S A 1994; 91: 7583-7587.
- [36] Serafin M, de Waele C, Khateb A, Vidal PP, Muhlethaler M. Medial vestibular nucleus in the guinea-pig. I. Intrinsic membrane properties in brainstem slices. Exp Brain Res 1991; 84: 417-425.
- [37] Johnston AR, MacLeod NK, Dutia MB. Ionic conductances contributing to spike repolarization and after-potentials in rat medial vestibular nucleus neurones. J Physiol 1994; 481: 61-77.
- [38] Ahern GP, Klyachko VA, Jackson MB. cGMP and S-nitrosylation: two routes for modulation of neuronal excitability by NO. Trends Neurosci 2002; 25: 510-517.
- [39] Ghatta S, Nimmagadda D, Xu X, O'Rourke ST. Large-conductance, calcium-activated potassium channels: structural and functional implications. Pharmacol Ther 2006; 110: 103-116.

- [40] Smith MR, Nelson AB, Du Lac S. Regulation of firing response gain by calcium-dependent mechanisms in vestibular nucleus neurons. J Neurophysiol 2002; 87: 2031-2042.
- [41] Faber ES, Sah P. Physiological role of calciumactivated potassium currents in the rat lateral amygdala. J Neurosci 2002; 22: 1618-1628.
- [42] Hendrich J, Alvarez P, Chen X, Levine JD. GDNF induces mechanical hyperalgesia in muscle by reducing I(BK) in isolectin B4-positive nociceptors. Neuroscience 2012; 219: 204-213.
- [43] Nelson AB, Gittis AH, du Lac S. Decreases in CaMKII activity trigger persistent potentiation of intrinsic excitability in spontaneously firing vestibular nucleus neurons. Neuron 2005; 46: 623-631.
- [44] Womack MD, Hoang C, Khodakhah K. Large conductance calcium-activated potassium channels affect both spontaneous firing and intracellular calcium concentration in cerebellar Purkinje neurons. Neuroscience 2009; 162: 989-1000.
- [45] Zhang R, Sun H, Liao C, Yang H, Zhao B, Tian J, Dong S, Zhang Z, Jiao J. Chronic hypoxia in cultured human podocytes inhibits BKCa channels by upregulating its beta4-subunit. Biochem Biophys Res Commun 2012; 420: 505-510.
- [46] Veltkamp R, Domoki F, Bari F, Busija DW. Potassium channel activators protect the N-methyl-D-aspartate-induced cerebral vascular dilation after combined hypoxia and ischemia in piglets. Stroke 1998; 29: 837-842; discussion 842-833.
- [47] Hughes JM, Riddle MA, Paffett ML, Gonzalez Bosc LV, Walker BR. Novel role of endothelial BKCa channels in altered vasoreactivity following hypoxia. Am J Physiol Heart Circ Physiol 2010; 299: H1439-1450.