

# Role of GSPE in improving early cerebral vascular damage by inhibition of Profilin-1 expression in a ouabain-induced hypertension model

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**Abstract. – OBJECTIVE:** Grape seed proanthocyanidin extract (GSPE), as one of the most popular natural drug extracted from the grape, has been reported to improve endothelial function and arteriosclerosis. However, little is known about the influence of GSPE on hypertension and vascular remodeling. Profilin-1, an Actin-binding protein, is closely involved in the remodeling of large vessels in ouabain-induced hypertension. To date, there is no effective prevention or treatment in place for the high incidence of ischemic stroke associated with hypertension. In this study, we aimed to determine the role of GSPE via inhibition Profilin-1 in ischemic cerebral cortices of ouabain-hypertension rats and potentially provide a new target to prevent stroke associated with hypertension.

**MATERIALS AND METHODS:** The blood pressure of male Sprague-Dawley (SD) rats was measured during a period of ouabain-induced hypertension. The expression of Profilin-1, vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS) in the cerebral cortex were determined by quantitative Real Time-PCR (qRT-PCR) and Western blot. Histopathological and behavioral tests were also conducted.

**RESULTS:** Blood pressure elevation started at week 5 and reached clinical standards for hypertension at week 8. GSPE was proved to suppress Profilin-1 and VEGF levels through inhibition of Profilin-1-protein kinase B (AKT)-hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) signal pathway and promote eNOS expression. Moreover, the histopathological and ethiological improvement was observed in GSPE over-expression and Profilin-1 inhibition groups.

**CONCLUSIONS:** We detected that GSPE could improve cerebral vascular damage through inhibiting Profilin-1 in an ouabain-induced hypertension model.

*Key Words:*

GSPE, Profilin-1, Gene regulation, Ouabain-hypertension, Cerebral vessels.

## Introduction

Grape seed proanthocyanidin extract (GSPE) is a biological polyphenol compound with a variety of pharmacological activities. It has been reported that GSPE has a powerful superoxide anion scavenging activity that is possibly stronger than vitamin C and other antioxidants. Researches<sup>1,2</sup> have also shown that it can improve cardiac disorders and dyslipidemia. GSPE plays a known role in endothelial protection and reversing arteriosclerosis. However, this compound may also play a role in treating target-organ lesions and local vascular remodeling induced by hypertension. According to the description of the American Heart Association (AHA), stroke is the main complication of hypertension. The key to prevent a stroke is the long-term blood pressure (BP) control<sup>3</sup>. In a latest epidemiological report including over 7 million Chinese stroke patients, 70% suffered an ischemic stroke<sup>4</sup>. Although it has been shown that higher BP does not necessarily lead to a higher incidence of acute ischemic stroke, essential hyper-

tension (EH), continues to remain a major cause of ischemic stroke. Among these patients, the main pathological changes causing stroke have been the remodeling of small vessels (resistance vessel), which reflects the proliferation and hypertrophy of vascular smooth muscle cells (VSMC) and collagen fibers. Vasoconstriction results in tissue and cerebral hypoperfusion and ischemia-hypoxia. On the other hand, ischemia-hypoxia could promote cerebral capillary endothelial cells' lesions and capillary occlusion. But it remains hard to be understood whether there are already changes of molecular biology on cerebral vessels and its effects in the early phase of hypertension. Profilin-1, as an actin-binding protein necessary for maintaining the balance of the cytoskeleton, could be considered as a potent inducer of vascular remodeling<sup>5,6</sup>. Vascular endothelial growth factor (VEGF) is a soluble glycoprotein and powerful mitogen that acts on the endothelial cells of microvessels to stimulate signaling pathways leading to increased capillary density, vascular growth and remodeling in the brain<sup>7</sup>. Hypoxia-inducible factor 1 (HIF-1), a heterodimeric transcription factor, is a critical factor to regulate growth, invasion, and remodeling. HIF is composed of HIF-1 $\alpha$  and HIF-1 $\beta$ . Among them, HIF-1 $\alpha$  is considered as a main regulator by binding to other hypoxia responsive factors to promote VEGF expression<sup>8</sup>. Protein kinase B (AKT), a serine/threonine protein kinase, plays a central role in the signal pathway to regulate cell survival, proliferation. It has been demonstrated that AKT regulates HIF-1 $\alpha$  in the upstream position, which is the major signal pathway to induce proliferation and angiogenesis. And they could regulate VEGF expression<sup>9,10</sup>. Nitric oxide (NO) induces vascular relaxation and is known to be a critical regulator in inflammation. The release of NO can be caused by hypertension-induced impairment of vascular endothelial cell (VEC) function<sup>11</sup>. Impaired VEC can also up-regulate endothelial NOS (eNOS). Nitric oxide synthase (NOS) is the main enzyme that generates NO<sup>12</sup>. We want to investigate the role of GSPE in improving early cerebrovascular damage by inhibiting the expression of profilin-1 in hypertension (especially in pre-hypertension) triggered by ouabain *in vivo*.

## Materials and Methods

### Animals

Five weeks old male Sprague-Dawley (SD) rats (weight 180-200 g) were supplied by the Experi-

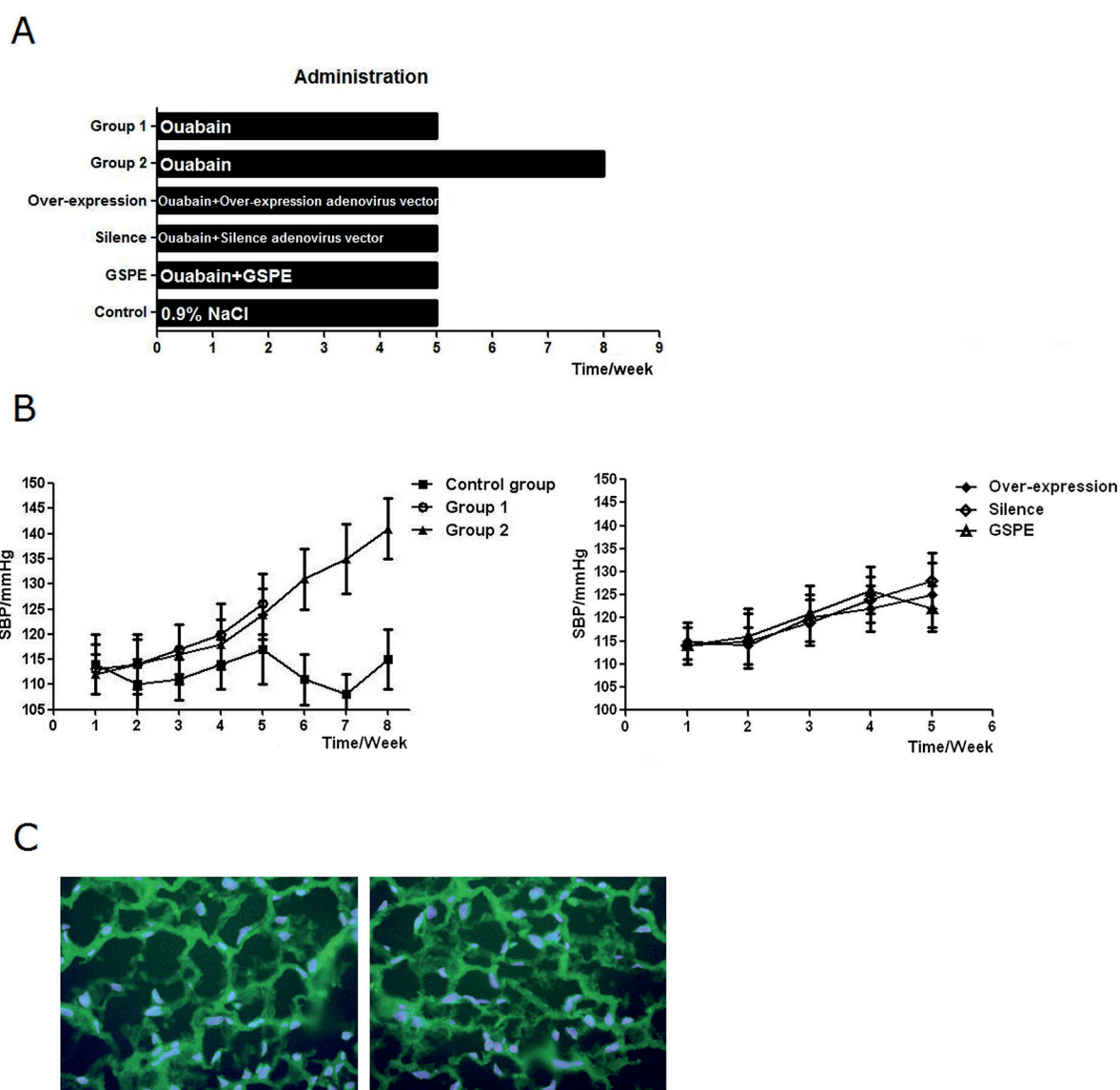
mental Animal Center of Shandong Traditional Chinese Medicine University (Jinan, China) and housed in standard cages under controlled conditions: temperature of 24°C, relative humidity at 40%-70% and a light/darkness cycle of 12/12 h (lights on at 8:00 a.m.) for 2 weeks to adapt to the experimental environment.

### Gene Interference and Recombinant Adenovirus Vector in the Cerebral Cortex of Rat

The rat Profilin-1 gene was inserted into the pIRES2-EGFP plasmid at the XhoI and EcoRI restriction enzyme sites (Invitrogen, Carlsbad, CA, USA) and Profilin-1-IRES-EGFP was amplified to construct the Profilin-1-IRES-EGFP adenovirus vector containing four different Profilin-1 microRNAs (miRNAs). The vectors were co-transfected into HEK293 cells with a negative control miRNA vector to obtain interference efficiency. The most efficient vector, pcDNA6.2-GW/EMGFP-miR-SR-2 (Invitrogen, Carlsbad, CA, USA), was used to construct the adenovirus interference vector pAd-miR-Profilin-1 with Gateway recombination technology. Then, the vector was transfected into HEK293 cells for adenovirus packaging and titrating. Adenovirus vectors were injected into rats through the caudal vein. Verification of the transfection of the vector was conducted by fluorescence detection of adenovirus. Cerebral cortices of the rats were harvested, fixed in 4% neutral formalin (Biyotime Biotech. Shanghai, China) at 4°C for 48 h and subsequently embedded in O.C.T Tissue Freezing Medium. The specimens were sliced into 5-10  $\mu$ m thickness serial coronal sections and stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St. Louis, Missouri, USA) for 5 min. The slides were then sealed with Antifade Mounting Medium Beyotime Biotech. (Shanghai, China) and a cover glass and were observed under a fluorescence microscope (Olympus, Japan).

### Administration

Rats were randomly placed into five groups and injected via intraperitoneal (IP) daily with ouabain (Sigma-Aldrich, St. Louis, MO, USA), at a dose of 27.8 mg/kg/d. The dose of ouabain used in our study was determined from previously published pharmacokinetic data in a rat ouabain-hypertension model with an approximate ouabain plasma concentration of 0.9-1.8 nmol/l<sup>13</sup>. Standard rat chow and water were available throughout the administration phase. Group 1



**Figure 1.** Information about respective administration time and changes in SBP after administration ( $\times 400$ ). (A) Information about respective administration time. (B) Changes in SBP after administration. SBP of six groups were measured all through administration period (Values are means  $\pm$  SD,  $n=10$ ). Ouabain could increase SBP gradually and reach hypertension diagnosis at 8th week. To limit Profilin-1 expression could not impact effective influence on SBP. GSPE may not show extremely significant pharmacological action on SBP-regulation. (C) Fluorescence detection of cerebral cortex (Left, silence; Right, over-expression). Green fluorescence signaled successful transfection and the purple structure is the nucleus.

( $n=10$ ) was injected with ouabain for 5 weeks. Group 2 ( $n=10$ ) was injected with only Ouabain for 8 weeks. GSPE group was administered GSPE orally at a dosage of  $250 \text{ mg/kg}\cdot\text{d}^{14}$  besides ouabain-injection. Over-expression Group ( $n=10$ ) and Silence Group ( $n=10$ ) were respectively injected with ouabain and the adenovirus vector plasmid through the caudal vein to achieve Profilin-1 over-expression/silencing. Control group were only injected with saline in the same dose (Figure

1A). Animals were weighed every week. At the end of weeks 5 and 8, rats were anesthetized with an intraperitoneal (IP) injection of chloral hydrate (Sigma-Aldrich, St. Louis, MO, USA) and sacrificed by decapitation. Brains were fixed in either buffered 4% neutral formalin or 2.5% glutaraldehyde solution for histological examination. All rat protocols were approved by the Institutional Animal Care and Use Committee of the Qilu Hospital, Shandong University, Jinan, China.

### **Blood Pressure Measurement**

The systolic blood pressure of conscious, moderately restrained rats was measured with a tail-cuff sphygmomanometer. The pulse was taken using a rat-tail BP sensor attached to pressure monitors. Signals were amplified and processed by specific software into pressure data. Systolic blood pressures were measured with the transducer (Mode: 1050BP, BioPac System, Goleta, CA, USA) during 30 min and recorded with the specific computer software for data acquisition (Mode: MPI00A, BIOPAC System, Santa Barbara, CA, USA).

### **Behavioral Test of Ouabain-Hypertension Rats**

Rats were subjected to behavioral tests before and after the 5-week ouabain administration as described.

### **Open Field Test**

The test apparatus was an open box divided into 25 squares (20 cm × 20 cm). Rats of each group were dropped into the center of an open field arena (100 cm × 100 cm × 40 cm). There were 16 peripheral squares along the walls, and the rest were in the center zone. The following parameters were measured every 5 min, path length (total number of squares entered by rats, must have at least 3 paws into the neighbored square), duration of center zone; duration of exploratory behavior (mobility), number of unsupported rears (front paws off the floor), and number of fecal boli. Identical procedures and measurements for the open field test were performed by the same researcher blinded to genotype.

### **Scoring System on Provocation Test**

The scores were evaluated according to the reactions of irritated rats, which were divided into four grades. 1. Screaming and jumping when caught by their necks. 2. Outrage and biting when caught by their necks. 3. Screaming, outrage, and biting at testers when caught by their necks, or frequent assault in cages. 0. None of above.

### **Pain Threshold Test**

A physical examination was designed to involve tests for detecting the pain in tails from thermal stimulating temperature. The rat was fixed on the experiment table by a specific net-cage, and the tail was immersed in water heated to 49°C. The amount of time the rat took to pull its tail out of the water was recorded.

### **Hematoxylin-Eosin (HE) and Nissl Staining**

The cerebral cortex was fixed in 0.1 M phosphate buffer (PB, pH 7.0-7.5, Biotime Biotech. Shanghai, China) for 48 h, and dehydrated in graded ethanol solutions. After fixed tissues were embedded in paraffin wax, the specimens were sliced into 5 μm thickness serial coronal sections. For morphometric examination, hematoxylin and eosin (HE) staining and Nissl staining were separately performed and examined by light microscopy to analyze the morphological changes.

### **Ultrastructural Observation**

The cerebral cortices were harvested and cut into 1 mm<sup>3</sup> blocks on ice and immediately placed in a 2.5% glutaraldehyde fixation solution (Biotime Biotech. Shanghai, China). The samples were held in 1% osmium tetroxide at 4°C for 2 h. Subsequently, the 1 mm<sup>3</sup> tissue blocks were dehydrated in a graded ethanol series with acetone, permeated and embedded in epoxide resin. Sections measuring 75 nm in thickness were prepared and stained with uranyl acetate and lead citrate. The stained samples were observed under an H-300 transmission electron microscope (TEM) (Hitachi Electronic Instruments, Tokyo, Japan).

### **Reverse Transcriptase PCR (RT-PCR)**

Discard excessive cerebral tissue to separate microvessels through Glass beads (425-600 μm, Sigma-Aldrich, St. Louis, MO, USA). Expression levels of Profilin-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured by real-time quantitative PCR (RT-PCR) following total RNA extraction and cDNA synthesis (Fermentas Inc, Toronto, Ontario, Canada). The data were expressed as a ratio to GAPDH (Tiangen, Beijing, China). The total reaction volume was 20 μl, including 1.5 μl of cDNA template, 8 μl 2.5 × Real Master Mix, 1 μl 20 × SYBR solution (TaKaRa, Dalian, China), 9 μl double distilled water and 0.25 μl each of the forward and reverse primers (5 mM) (Table I). PCR products were run on a 2 % agarose gel and counted by scintillation fluid. Base pairs (bp) confirmed the specificity of each product. All RT-PCR were carried out with negative controls.

### **Western Blot Analysis**

Protein was extracted from homogenized microvessels, and the concentrations were measured using a BCA assay Biotime Biotech. (Shanghai,



**Table I.** Primers used for Real-Time PCR assay.

Gene	Sense (5'-3')	Anti-sense (5'-3')	Length (bp)
Profilin-1	TTCGTTAGCATTACGCCAGC	GTGCAAAAGCCAAAGGGAG	349
VEGF	ACGACAGAAGGGGAGCAG	AGATGTCCACCAGGGTCTCA	97
eNOS	TGCACCCTTCCGGGGATTCT	GGATCCCTGGAAAAGGCGGT	189
GAPDH	ATTCTACCCACGGCAAGTT	CGCCAGTAGACTCCACGACATA	153

China). Protein samples (50  $\mu$ g) were resolved by electrophoresis in sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred onto polyvinylidene difluoride (PVDF, Amresco Inc., Solon, OH, USA) membrane. The PVDF membranes were incubated with primary antibodies Abcam Biotech. (Cambridge, MA, USA) and secondary antibodies (ZSGB-Bio, Beijing, China) at room temperature. The bands were developed using enhanced chemiluminescence and visualized on Kodak film. The density of the bands was quantitated using the Image J software program (version: 2.0, Image J, Scion Image, Frederick, MD, USA).

#### **Detection of NO Concentrations**

NO concentrations in the cerebral cortex were measured by a spectrophotometer. Nitrate reductase can deoxidize  $\text{NO}_3^-$  into  $\text{NO}_2^-$ , which react with a chromogenic agent to give a colored substance. Ten percent of the cerebral cortex homogenate was centrifuged at 2000  $\times$ g at 4°C for 5 min, and 500  $\mu$ l of the sample was used to measure NO concentrations according to the manufacturer's instructions (Jiancheng, China). The  $\text{OD}_{550}$  of the samples were measured with a spectrophotometer following coloration, and NO concentrations were obtained using the given calculation formula.

#### **Statistical Analysis**

All analyses were performed blindly. Continuous data are expressed as means  $\pm$  standard deviation (SD). The Student's *t*-test was used to compare the differences between the two groups. The Tukey's post hoc test was used to validate the ANOVA for comparing measurement data between groups.  $p < 0.05$  was considered as statistical significance. For categorical variables, the Mann-Whitney U test was used for comparisons between the two groups. The tests were performed with SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL, USA). Differences were considered significant at  $p < 0.05$ .

## **Results**

### **Body Weight and Blood Pressure**

There were no significant differences in the weight of rats ( $p > 0.05$ ). At the end of the eighth week, the average body weight of group 2 was 320 g  $\pm$  11 g. Systolic blood pressures (SBP) (Figure 1B) were almost identical among groups before injection. After five weeks, SBP of ouabain-injected groups increased, compared with the control group [126  $\pm$  6 mmHg vs. 114  $\pm$  5 mmHg, respectively]. However, this difference in SBP between the four experimental groups was not statistically significant ( $p > 0.05$ ). SBP increased continuously and reached the clinical standard for hypertension diagnosis at week 8 (141  $\pm$  6 mmHg). Similarly, there were no significant differences in the SBP between the GSPE and Profilin-1 gene interference groups, compared with that of ouabain-injected groups ( $p > 0.05$ ).

### **Fluorescence Detection Test**

The adenovirus vector was successfully transfected as shown from the green fluorescence (Figure 1C).

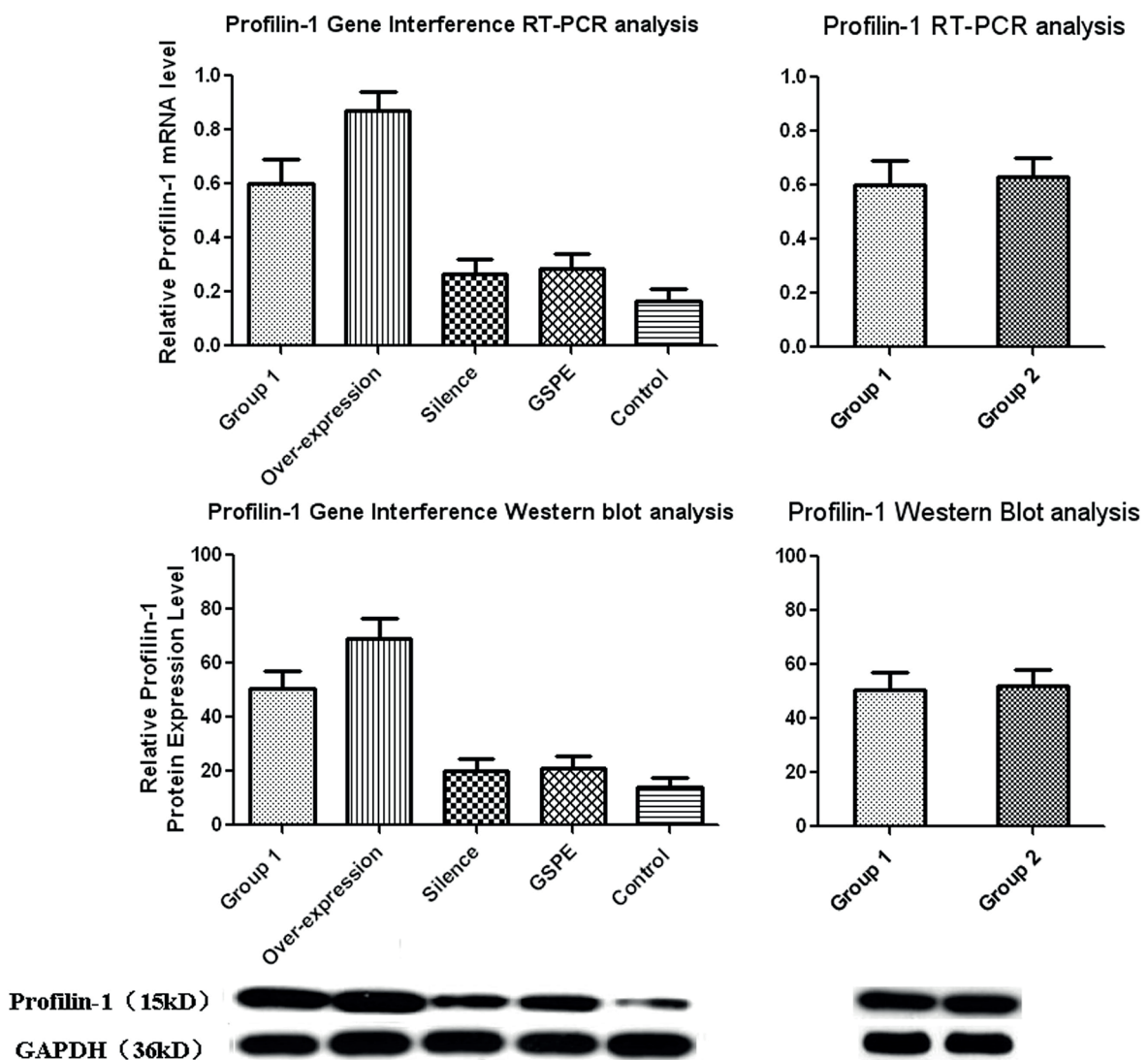
### **Profilin-1 Gene Expression Suppressed by GSPE in the Cerebral Cortex**

We analyzed the gene expression of profilin-1 in the cerebral cortex by RT-PCR (Figure 2). Compared with the vehicle-treated group (0.168  $\pm$  0.025), the group that was given an ouabain injection for five weeks had increased profilin-1 mRNA levels (group 1, 0.603  $\pm$  0.088,  $p < 0.01$ ). We also observed that GSPE decreased the profilin-1 expression (0.284  $\pm$  0.035). Of note, GSPE (0.252  $\pm$  0.037,  $p > 0.05$ ) inhibited Profilin-1 mRNA expression to the same extent as the Profilin-1 silenced group. Profilin-1 levels in the 5-week ouabain injected group (group 1) remained unchanged at the end of week 8 (group 2, 0.630  $\pm$  0.069,  $p > 0.05$ ). We analyzed Profilin-1 protein expression by Western blot. There was a distinctly higher expression in group 1 (50.76  $\pm$

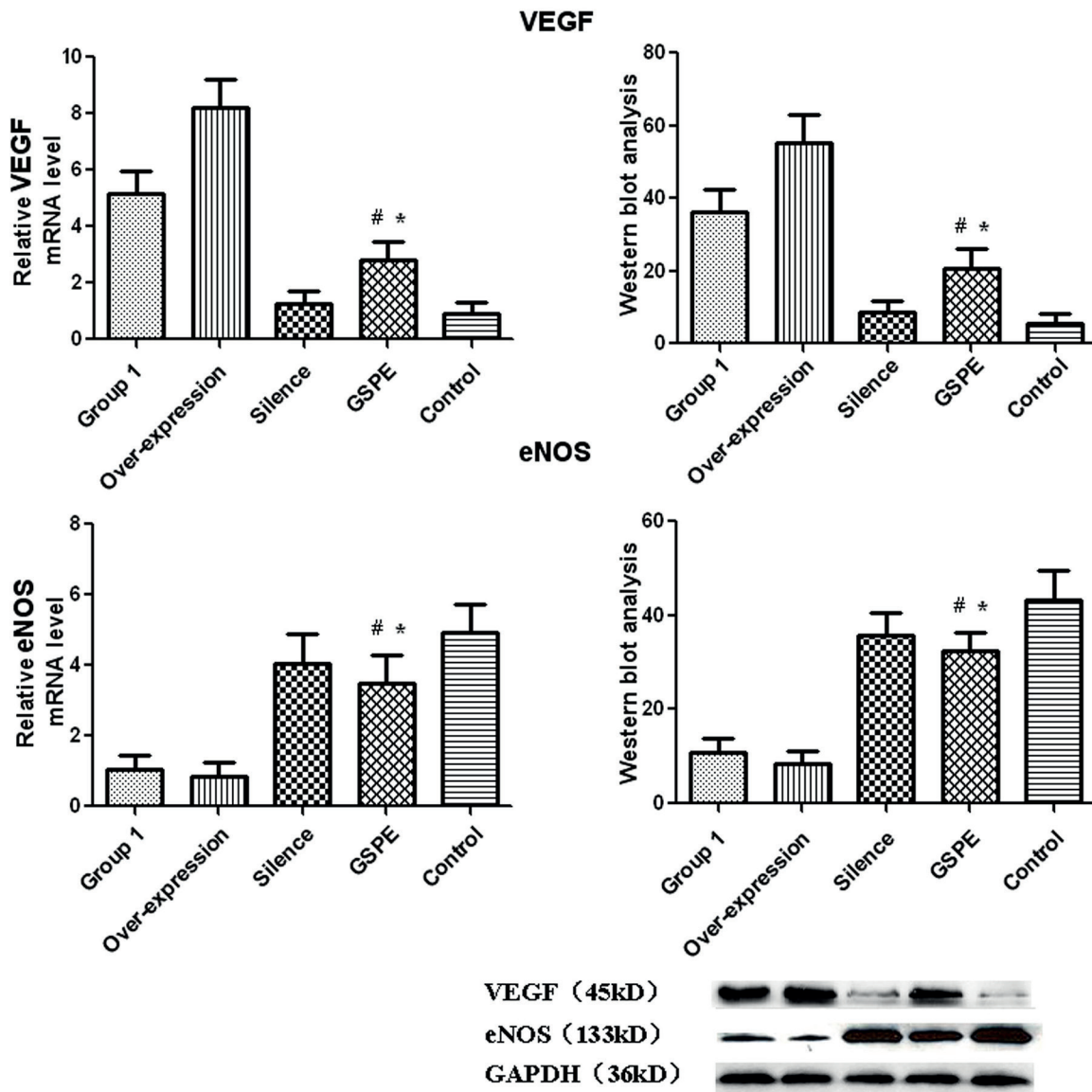
6.19) and group 2 ( $51.85 \pm 6.44$ ) than in the control group ( $13.34 \pm 2.21$ ,  $p < 0.01$ ), but no difference when compared to themselves ( $p > 0.05$ ). GSPE suppressed Profilin-1 expression and Profilin-1 expression in GSPE ( $21.06 \pm 3.27$ ) was  $\frac{1}{2}$  less than Group 1 ( $p < 0.01$ ). Meanwhile, over-expression of Profilin-1 showed higher expression in the over-expressed group ( $69.10 \pm 7.42$ ,  $p < 0.05$ ) and the silenced group ( $18.82 \pm 3.06$ ) displayed a slightly lower expression than that in the GSPE group ( $p > 0.05$ ). There was no statistically significant difference among the GSPE, silence and control groups ( $p > 0.05$ ).

### GSPE Could Increase eNOS Expression

As an indicator of endothelial lesions, the expression of eNOS induced by Profilin-1 declined drastically as shown by RT-PCR and Western blot analysis (Figure 3). eNOS levels were down-regulated markedly after week 5 of ouabain administration [Group 1,  $1.128 \pm 0.276$  vs. the control group,  $4.940 \pm 0.804$ ,  $p < 0.01$ ]. A similar trend was also observed in the over-expressed group [ $0.873 \pm 0.274$ ,  $p < 0.01$ ;  $p > 0.05$ , versus group 1]. GSPE increased eNOS expression normally inhibited by Profilin-1 ( $3.493 \pm 0.789$ ,  $p < 0.01$ ) in accordance with NO Concentration. There were no



**Figure 2.** Profilin-1 mRNA expression levels increased in the ouabain injected groups ( $p < 0.05$ ) and could be up/down-regulated by adenovirus vector. GSPE could prevent Profilin-1 from up-regulated expression ( $p < 0.01$ ), which were all detected by RT-PCR. Protein expression was detected by Western blot. Profilin-1 protein expression levels in the GSPE group was less than  $\frac{1}{2}$  the data of group 1 ( $p > 0.05$ ). There was no discrepancy between group 1 and group 2 ( $p > 0.05$ ). Results are expressed as the mean  $\pm$  SD.

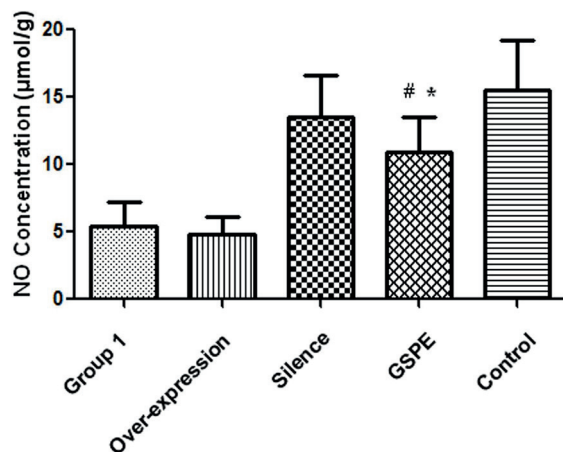


**Figure 3.** RT-PCR and Western blot analysis of eNOS and VEGF. GSPE induces eNOS expression that is inhibited by profilin-1, and prevents Profilin-1 from promoting VEGF expression ( $p < 0.05$ ), which may be also demonstrated by the regulation of the expression of profilin-1 ( $p < 0.05$ ). Results are shown as the mean  $\pm$  S.E.M.

differences between the silenced group ( $4.038 \pm 0.869$ ) and the control group ( $p > 0.05$ ). Protein expression of eNOS, as visualized by Western blot analysis, corresponded to the RT-PCR results. GSPE ( $32.35 \pm 4.13$ ) can prevent Profilin-1 (group 1,  $11.06 \pm 2.09$ ; over-expressed group,  $8.57 \pm 1.99$ ) from decreasing eNOS protein levels ( $p < 0.01$ ), and is approximately three times larger than group 1. The data in the silenced Profilin-1 group appears to be higher than that of the GSPE group ( $p > 0.05$ ), which is similar to the control group ( $43.35 \pm 6.14$ ,  $p > 0.01$ ).

**GSPE Promotes NO Production Usually Inhibited by Profilin-1**

The concentration of NO in tissue homogenates are shown in Figure 4. We observed that the NO concentrations of the GSPE group [ $10.91 \pm 2.62 \mu\text{mol/g}$ ] and the silenced group [ $13.471 \pm 3.13 \mu\text{mol/g}$ ,  $p > 0.05$ ] were much higher than that of group 1 [ $5.464 \pm 1.782 \mu\text{mol/g}$ ,  $p < 0.05$ ]. The over-expressed group [ $4.827 \pm 1.273 \mu\text{mol/g}$ ,  $p < 0.05$ ] followed by the Profilin-1 up-regulated group had the lowest NO concentration. However, these results may correspond to eNOS expression



**Figure 4.** Effects of ouabain-induced hypertension exposures on NO concentration (mean  $\pm$  SD) in the cerebral cortex. NO concentration was markedly decreased in experimental groups ( $p < 0.01$ ). In GSPE and Profilin-1-silenced group, impairment of NO could be relieved with increasing concentration while no clear disparity could be observed between them. Results are expressed as the mean  $\pm$  SD.

mentioned above. The NO concentration in the control group was  $15.52 \pm 3.71$   $\mu\text{mol/g}$  (Figure 4).

#### **GSPE Decreases VEGF Expression that is Promoted by Profilin-1**

The mRNA expression levels of VEGF, a critical indicator of vascular remodeling, was up-regulated in group 1 ( $5.345 \pm 0.775$ ) compared to the control group ( $1.163 \pm 0.252$ ) ( $p < 0.01$ ). Furthermore, the over-expressed group ( $8.382 \pm 0.997$ ) had higher levels of Profilin-1 ( $p < 0.01$ ). There was a decrease in VEGF mRNA and protein expression in the GSPE ( $2.803 \pm 0.421$ ,  $p < 0.05$ ) and Profilin-1-silenced groups ( $1.271 \pm 0.372$ ,  $p > 0.05$ ) ( $p < 0.05$ ). Profilin-1 also increased VEGF protein expression (group 1,  $37.50 \pm 4.82$ ) compared to the control group ( $5.18 \pm 1.23$ ,  $p < 0.01$ ). The over-expressed group had higher VEGF expression levels ( $5.36 \pm 5.24$ ) compared to group 1 ( $p < 0.05$ ), but no further higher expression than Group 1 ( $p > 0.05$ ). Profilin-1 levels were reduced in both the GSPE ( $21.11 \pm 4.23$ ,  $p < 0.05$ ) and silenced groups ( $8.35 \pm 1.97$ ,  $p > 0.05$ ) (Figure 3).

#### **GSPE Decreases VEGF Expression Through Inhibiting AKT Activation and HIF-1 $\beta$ Expression**

AKT (or protein kinase B) is an important signal molecule in regulating cell survival, proliferation, and angiogenesis. All groups of AKT

expression were not different ( $p > 0.05$ ). The levels of phospho-AKT were enhanced by increased Profilin-1 expression or regulation ( $p < 0.05$ ). Over-expressed Profilin-1 did not increase phospho-AKT even further ( $p > 0.05$ ). GSPE completely abolished Profilin-1-promoted AKT activation ( $p < 0.05$ ), and the similar AKT inactivation was observed by down-regulated Profilin-1, demonstrating that GSPE inhibited VEGF expression through the AKT signaling pathway. GSPE could decrease HIF-1 $\alpha$  expression, not HIF-1 $\beta$ , which was increased by Profilin-1 ( $p < 0.05$ ). In Profilin-1 expression's regulation, we also have found the similar change of HIF-1 $\alpha$  expression to follow up-/down- regulated Profilin-1, and these changes could induce VEGF expression accordingly, suggesting that HIF-1 $\alpha$  is a downstream target of Profilin-1 for regulating VEGF expression. But HIF-1 $\alpha$  expression between Group 1 and Over-expression did not show an evident difference ( $p > 0.05$ ) (Figure 5).

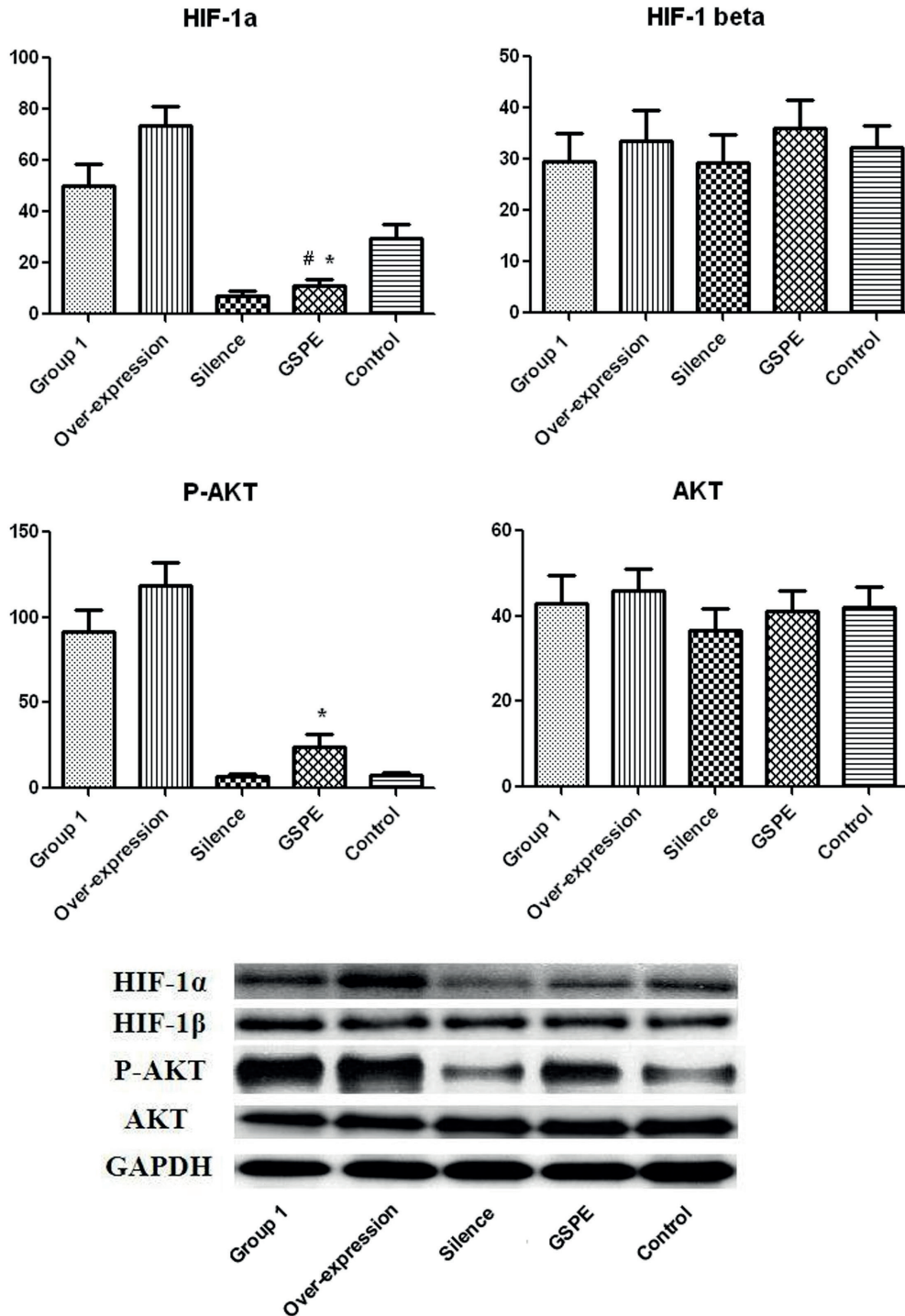
#### **GSPE Improves Cerebral Morphology and Function Impaired by Increased Profilin-1**

The control group appeared normal with fine, granular and dispersed Nissl bodies by HE and Nissl staining. However, the ischemic damage was observed in rats receiving the 5-week ouabain injections (group 1). Loose tissue and edema were present in the ischemic region. Parts of neurons in the cerebral cortices appeared swollen and had sparse, asymmetric cytoplasm in the cell body where there were decreased number of Nissl bodies and an increased number of microglia cells. Apoptosis was also observed in some of the swollen cells. Similar pathological changes were also observed in the over-expressed group. However, atrophy and degeneration of neurons and hyperplasia of microglia cells were significantly absent in the GSPE and Profilin-1-silenced groups (Figure 6A). Ultrastructural changes were observed using a TEM (Figure 6B). The control group had normal cellular and organelle structure with dominant euchromatin in the nucleus of neurons and intact mitochondria. However, group 1 and the overexpressed group displayed early stage pathological impairment induced by cerebral ischemia after 35 days of injection. The neurons had cytolymph edema, reduced organelles, abnormal nuclei with vague nuclear membranes and loss of chromatin. Another sign of an ischemic pathological change may also involve unilamellar vesicles in the nucleus and swollen, vacuolated

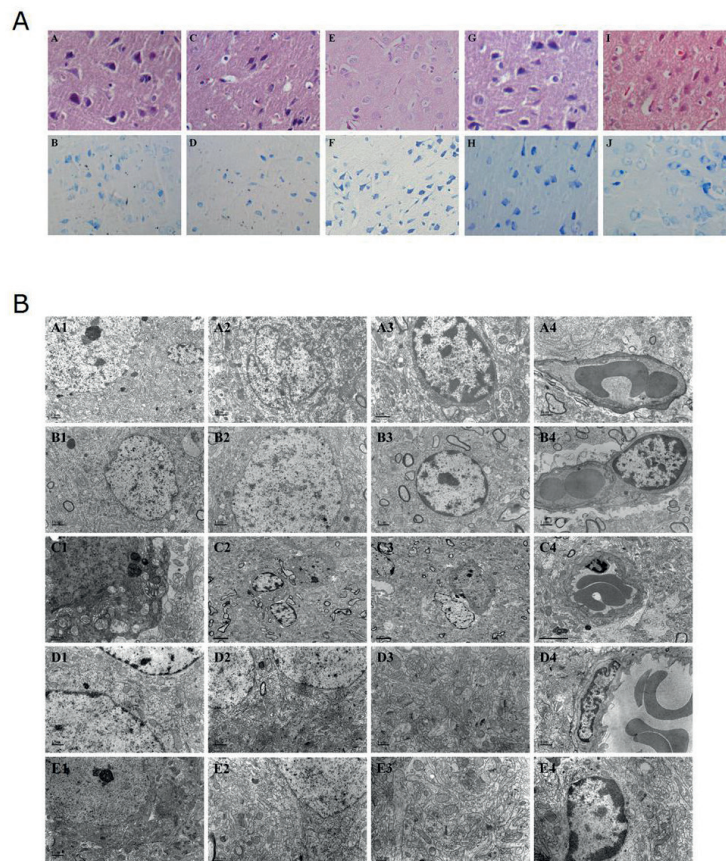


mitochondria with sparse and vague crista. Auto-phagosomes found in neurons indicate apoptosis. The number of injured neurogliaocytes increased around neurons. Also, swollen Glial cell

end plates with large ischemic areas surrounding cerebral capillaries impair the blood-brain barrier. In the GSPE and silenced groups, neuronal lesions were not as profound as the above descri-



**Figure 5.** Immunoblotting analysis for HIF-1 $\alpha$ , HIF-1 $\beta$ , p-AKT, total AKT and GAPDH. GSPE inhibits p-AKT and HIF-1 $\alpha$  expression which could be activated by up-regulated Profilin-1 ( $p < 0.05$ ). # indicates significant difference compared to the control,  $p < 0.05$ . \* indicates significant difference compared to the two increased Profilin-1 groups,  $p < 0.05$ .



**Figure 6.** Morphological changes. (A) Representative light micrographs of the cerebral cortex (hematoxylin-eosin/Nissl,  $\times 200$ ). A. HE staining, control group. B. Nissl staining, control group. C. HE staining, group 1. D. Nissl staining, group 1. E. HE staining, silenced group. F. Nissl staining, silenced group. G. HE staining, overexpressed group. H. Nissl staining, overexpressed group. I. HE staining, GSPE group. J. Nissl staining, GSPE group (magnification  $\times 200$ ). (B) Representative photographs of cerebral cortices show the ultrastructural contrast between the control group and the experimental group by TEM (scale bar: 1  $\mu\text{m}$  or 500 nm). A1-A4, control group. B1-B4, ouabain treatment group (group 1). C1-C4, Profilin-1 over-expressed group. D1-D4, Profilin-1 silenced group. E1-E5, GSPE group. A1. Nucleus of neurons mainly contains euchromatin. A2. Mitochondria with intact structure. A3. Well-structured gliocytes indicate unimpaired nerve tissue. A4. Normal with tight connection of endothelial cells and no-swelling basement membrane in blood-brain barrier. B1. Nucleus could be observed sparse chromatin and the unilamellar vesicles. B2. Hollowed and swollen mitochondria with disappeared crista and increased autophagosome. B3. Typical pathological changes of nucleus and mitochondria in gliocytes with significantly increased autophagosomes (Parts of mitochondria show the ring&chain-shaped structure of the membrane, which is no functional structure and means irreversible damage). B4. Endplate swelling means impaired blood-brain barrier. C1. Apoptosis of neuron-cell body. C2. Higher density of cell nucleus and endoplasmic reticulum swelling means the early change of cell apoptosis. C3 Neuron accompanied by neurogliocytes pathologically. C4 Endplate swelling. D1. Nearly intact karyotheca. D2 Nucleus of neurons mainly contains euchromatin; D3. No pathological structure of mitochondria, Endoplasmic reticulum and Golgi apparatus; D4. Intact blood-brain barrier. E1. Normal structure of nucleus. E2. Nucleus of neurons with main euchromatin. E3. Normal structure of mitochondria. E4. Well-structured gliocytes can be observed.

bed. We observed elevated cytolymph edema and swollen mitochondria, but also fewer autophagosomes and neurogliocytes in or around the neurons. There were no ischemic injuries such as swollen end plates that inhibit the normal structure of the blood-brain barrier.

#### **Effect of GSPE on Improving Cerebral Function**

There were no differences in test results in rats in each of the five groups prior to the behavioral

tests ( $p > 0.05$ ). Table II shows the variables measured and statistical results. In the analysis of the following parameters, the number of squares (path length), duration of exploratory behavior, and the number of unsupported rears, the data in group 1 and the over-expressed group showed that compared to GSPE, the silenced and control groups were statistically significant findings ( $p < 0.05$ ). No significant differences were observed between the three groups ( $p < 0.05$ ). Although the number of center squares in the over-expressed group and

**Table II.** Mean ( $\pm$  SEM) value illustrates the result of each group in the open field test during the 300-s probe trial.

Groups	Squares		Squares of Center		Moving duration		Unsupported Rears		Fecal boli	
	Pre-injection	5 weeks' injection	Pre-injection	5 weeks' injection	Pre-injection	5 weeks' injection	Pre-injection	5 weeks' injection	Pre-injection	5 weeks' injection
Control group	71.1 $\pm$ 4.2	68.6 $\pm$ 5.5	10.1 $\pm$ 1.3	9.6 $\pm$ 1.6	211.7 $\pm$ 6.2	211.6 $\pm$ 8.4	7.7 $\pm$ 1.0	7.2 $\pm$ 1.2	2.50 $\pm$ 0.50	2.30 $\pm$ 0.42
Group 1	73.7 $\pm$ 4.1	97.2 $\pm$ 7.4	10.6 $\pm$ 1.4	15.0 $\pm$ 2.6	206.5 $\pm$ 5.7	241.5 $\pm$ 9.1	8.1 $\pm$ 1.2	14.6 $\pm$ 2.1	2.70 $\pm$ 0.60	4.30 $\pm$ 0.54
Silenced	73.5 $\pm$ 5.1	74.8 $\pm$ 6.0	11.8 $\pm$ 1.8	11.6 $\pm$ 2.0	208.9 $\pm$ 6.1	214.3 $\pm$ 7.6	8.4 $\pm$ 1.1	9.2 $\pm$ 1.9	2.20 $\pm$ 0.44	3.00 $\pm$ 0.52
Overexpressed	75.5 $\pm$ 4.6	98.4 $\pm$ 9.4	11.5 $\pm$ 1.6	16.1 $\pm$ 2.3	206.8 $\pm$ 6.8	244.4 $\pm$ 10.0	9.4 $\pm$ 1.3	15.7 $\pm$ 2.1	2.80 $\pm$ 0.53	4.80 $\pm$ 0.57
GSPE	75.4 $\pm$ 5.5	73.9 $\pm$ 6.8	10.4 $\pm$ 1.5	12.0 $\pm$ 1.9	209.3 $\pm$ 8.4	212.8 $\pm$ 7.7	9.2 $\pm$ 1.3	8.9 $\pm$ 1.7	2.40 $\pm$ 0.58	3.70 $\pm$ 0.54

The measurement was conducted before injection and after 5 weeks of injection, respectively. Compared with the control group, rats in the groups of increased Profilin-1 expression (group 1 and the overexpressed group) were observed to move more frequently including moving longer distances and for a longer duration ( $p < 0.05$ ) with more unsupported rears ( $p < 0.05$ ). To suppress Profilin-1 expression, GSPE may reduce the behavioral indicators effectively with the similar result of silence group ( $p < 0.05$ ). There is no difference among all groups in squares in center zone and number of fecal boli ( $p > 0.05$ ).

group 1 may have increased to some extent, there was no difference within the groups ( $p > 0.05$ ). There were also much lower fecal boli numbers in the GSPE, silenced and control groups compared to the over-expressed group ( $p < 0.05$ ). Table III shows the score of irritation in the rats. The scores of group 1 and the over-expressed group increased after five weeks of ouabain administration and both were much higher than the GSPE and silenced groups ( $p < 0.05$ ) compared to the control group. However, there were no significant differences between group 1 and the over-expressed group ( $p > 0.05$ ). In the pain threshold test, the rats in the over-expressed group and group 1 endured pain for less time compared to other groups after 5 weeks. The endurance of the GSPE and silenced groups were longer in comparison, despite no further improvement caused by GSPE ( $p < 0.05$ ). We did not find any statistical significance between group 1 and the overexpressed group ( $p > 0.05$ ) (Table III).

## Discussion

The link between ouabain and blood pressure has been previously established. As a form of endogenous digitalis (EDLS), ouabain is secreted by the adrenal gland (endogenous ouabain, EO)<sup>13</sup>. Actually, increased levels of EO can be detected in the blood of untreated EH patients and secondary hypertensive patients<sup>15</sup>. EO can induce hypertension through vascular baroreceptor and peripheral vascular contraction by inhibiting the function of Na<sup>+</sup>/K<sup>+</sup>-ATPase (NKA) and being an endogenous regulator of blood pressure<sup>16</sup>. As stated above, we chose ouabain to build the hypertensive model because it has been reported that it plays an essential role in clinic hypertension diagnosis and vascular remodeling<sup>17</sup>. On the basis of the ouabain-hypertension model, we attempted to study GSPE's effect on blood pressure and target-organ protection. GSPEs are a group of polyphenolic bioflavonoids with multiple pharmacological and therapeutic

**Table III.** Mean ( $\pm$  SEM) scores show the irritation levels and endurance on pain of each group.

Groups	Score on Provocation Test		Pain Threshold test	
	Before administration	After administration	Before administration	After administration
Control Group	0.70 $\pm$ 0.30	0.80 $\pm$ 0.33	14.4 $\pm$ 2.2	15.4 $\pm$ 2.4
Group 1	0.60 $\pm$ 0.27	2.20 $\pm$ 0.25	13.1 $\pm$ 2.1	5.3 $\pm$ 1.3
Silenced	0.80 $\pm$ 0.32	1.30 $\pm$ 0.36	12.3 $\pm$ 1.8	10.4 $\pm$ 1.7
Overexpressed	0.60 $\pm$ 0.22	2.40 $\pm$ 0.22	13.4 $\pm$ 2.1	5.1 $\pm$ 1.2
GSPE	0.50 $\pm$ 0.17	1.30 $\pm$ 0.36	12.1 $\pm$ 1.6	11.5 $\pm$ 1.9

The grading was conducted before injection and after five weeks of administration, respectively. In high Profilin-1 expression groups, rats were more irritable and had lower pain tolerance than those of control group ( $p < 0.05$ ). The data showed a decrease for the GSPE and silenced groups which had no discrepancy with the control group ( $p > 0.05$ ).



potentials. It has been widely known that GSPE has the ability to effectively reduce the concentration of Ox-LDL in the blood and protect artery endothelium against oxidative injuries to improve endothelial functionality<sup>18,19</sup>. In our study, we also observed a morphological and functional improvement due to GSPE in week 5 before hypertension was induced. We did not observe GSPE lowering SBP increased by ouabain in experimental animal models, although this has been previously reported in other studies<sup>20</sup>. This may be attributed to the short administration GSPE. And GSPE itself is not a powerful anti-hypertension drug. Furthermore, SBP can be influenced by many other factors. But we did find Profilin-1 expression was suppressed and evident improvement that was caused by Profilin-1's inhibition in molecular biology, morphology, and ethiology of these animals in the Pre-Hypertension Phase. In our study, we have shown that Profilin-1 is involved in molecular biological and morphological changes of the cerebral cortex, which are improved by regulating Profilin-1 through GSPE. To investigate if rats have ethiological changes caused by Profilin-1, we performed three standard tests widely used to study ethiological behaviors in animal models. In the open field test, longer total exploratory travel distance (squares numbering) and duration of movement, as well as an increased in unsupported rears, were found only in the ouabain injected and over-expressed Profilin-1 groups. This test showed the anxiety and increased fear of danger in these groups. The elevated ratio of time spent in the center zone reflected a weakened cognitive ability to explore unfamiliar surroundings and assess potential risk. However, this excess of movement may be attributed to anxiety and not because of impaired cognition. Additionally, higher irrational scores and less time in the pain threshold test also indicated more agitation and hyperactivity in environmental stimulation. These could influence the results of the squares in the center zone. We also found statistically similar figures of defecation among the three groups except for the over-expressed Profilin-1 group. The results from ethiological tests could be explained for gradually elevated blood pressure<sup>21</sup>. But defecation can be also influenced by other factors affecting the gastrointestinal tract and digestive system, but also by feeding patterns. Abnormal ethiological behavior could be observed in the GSPE and silent Profilin-1 groups. This suggests that Profilin-1 may negatively impact the structure and function of the cerebral cortex during the early increasing blood pressure before reaching the diagnosis of hypertension. Profi-

lin-1, with a molecular weight of 12-15 kDa, has a highly conserved structure. Its tertiary structure contains three independent domains in space that are responsible for binding to actin, PIP2, and PLP and for regulating the structure and function of the actin network<sup>22</sup>. Our study has shown the expression of Profilin-1 in the VEC dramatically varied following alterations in cytoplasmic movement and growth status as induced by ouabain. Nevertheless, in spite of the effects of ouabain, we have not observed an identical phenomenon in VEC induced by any other drug. By transmission electron microscopy, cellular ultrastructural changes were observed, including changes in filament structure and reorganization of actin cytoskeleton. These changes were crucial structural changes in VEC during the course of vessel remodeling by hypertension<sup>23</sup>, which agrees with the result of NO measurement and eNOS expression in this study. As an endogenous vasodilator, NO is associated with endothelial physiology and prevents elevation of blood pressure. Surprisingly, NO concentrations have been found significantly decreased in hypertension patients and animal models *in vivo*<sup>24</sup>. The functional mechanism refers to the increased formation of biological activity suppressed by reactive oxygen species (ROS). Elevated eNOS expressions by GSPE are up-regulating Profilin-1 and are regarded as an important mechanism to improve NO production<sup>25</sup>. Our study group has also proved that GSPE could promote eNOS through the AMPK/SIRT1-KLF2 pathway<sup>26</sup>. In the meantime, Profilin-1 accelerated the course of actin polymerization because it has the capacity to catalyze the exchange of actin monomer-coupled ADP for ATP on actin. Therefore, it also has a close relationship with vascular remodeling<sup>27</sup>. In our study, we have found Profilin-1 could also increase VEGF excessive expression to promote vascular remodeling. VEGF results in inappropriate VEC proliferation, motility, and permeability during angiogenesis through the activation of the PIK3/AKT signaling pathway<sup>28</sup>. Thus, escalating VEGF expression is often viewed as an essential index to evaluate and analyze hypertensive vascular growth and remodeling. AKT transmits survival signals from growth factors and regulates cell survival, migration, and proliferation. HIF-1 $\alpha$  has been demonstrated to express constitutively under normoxic conditions, but it could be increased with slower degradation due to ischemia<sup>29</sup>. To identify the relative signaling pathway, we also found that GSPE could suppress AKT activation and HIF-1 $\alpha$  expression. Thus, escalating VEGF expression is often viewed as an



essential index to evaluate and analyze hypertensive vascular growth and remodeling. However, we observed profilin-1 had elevated VEGF expression when SBP had not reached 140 mmHg in the fifth week. This phase is called pre-hypertension in clinical medicine. Actually, except for the function of anti-antioxidant and anti-apoptotic, GSPE could also up-regulate a few proteins, such as C myc, p53. Meanwhile, both of the C myc and p53 were closely associated with the apoptosis and proliferation. Therefore, in the following studies, we would investigate whether the GSPE plays the role of anti-cerebral vascular damage through C myc and p53 pathway in an ouabain-induced hypertension model<sup>30</sup>.

### Conclusions

Increased Profilin-1 expression was found in ischemic lesions in the cerebral cortex induced by ouabain. Ischemic injuries in the early phase and cerebrovascular biological changes were observed in the early phase of hypertension, which was effectively improved by GSPE. Thus, it is reasonable to suggest that GSPE could prevent Profilin-1 from triggering the damage of the cerebral cortex in the early stage of cerebrovascular endothelial impairment and remodeling. Thus, early treatment of hypertension or pre-hypertension with GPSE to inhibit Profilin-1 expression, especially for high-risk patients, might be a fresh pathway for early intervention of hypertension and prevention of cerebral ischemia and degeneration.

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### Conflict of Interest

The Authors declare that they have no conflict of interest.

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