Shenfu Injection Improves Cerebral Microcirculation and Reduces Brain Injury in a Porcine Model of Hemorrhagic Shock

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Abstract

Background: The aim of this study was to clarify effects of Shenfu infusion (SFI) on cerebral microcirculation and brain injury after hemorrhagic shock (HS).

Methods: Twenty-one domestic male Beijing Landrace pigs were randomly divided into three groups: SFI group (SFI, n=8), saline group (SA, n=8) or sham operation group (SO, n=5). In the SFI group, animals were induced to HS by rapid bleeding to a mean arterial pressure of 40 mmHg within 10 minutes and maintained at 40 ± 3 mmHg for 60 minutes. Volume resuscitation (shed blood and crystalloid) and SFI were given after 1 hour of HS. In the SA group, animals received the same dose of saline instead of SFI. In the SO group, the same surgical procedure was performed but without inducing HS and volume resuscitation. The cerebral microvascular flow index (MFI), nitric oxide synthase (NOS) expression, aquaporin-4 expression, interleukin 6, tumor necrosis factor-α (TNF-α) and ultrastructural of microvascular endothelia were measured.

Results: Compared with the SA group, SFI significantly improved cerebral MFI after HS. SFI up regulated cerebral endothelial NOS expression, but down regulated interleukin 6, TNF-α, inducible NOS and aquaporin-4 expression compared with the SA group. The cerebral microvascular endothelial injury and interstitial edema in the SFI group were lighter than those in the SA group.

Conclusions: Combined application of SFI with volume resuscitation after HS can improve cerebral microcirculation and reduce brain injury.

1. Introduction

Hemorrhagic shock (HS) is a rare but serious complication, which may occur in many emergency diseases and has a high mortality rate [1]. When HS occurs, an acute reduction in blood volume leads to tissue hypoperfusion and sympathetic compensation by peripheral vasoconstriction. With the recovery of systemic perfusion caused by blood transfusion and fluid resuscitation, reperfusion injury occurs with the release of oxygen radicals [2]. Activated inflammatory system caused by ischemia/reperfusion (I/R) injury can lead to vascular endothelial damage, and cause the cerebral microcirculation disturbance [3-4] and brain edema [5].

Shenfu injection (SFI) is a traditional Chinese herbal medicine, and made from the raw materials of ginseng (Panax, family: Araliaceae) and Fuzi (Radix aconiti lateralis preparata, Aconitum carmichaeli Debx, family: Ranunculaceae). Shen-Fu injection (SFI) has been clinically used for treatment of many kinds of diseases associated with syndrome of sudden yang collapse, such as septic shock and cardiac shock. Our previous animal experiments showed that SFI can improve myocardial dysfunction [6-7] and microcirculation [8], reduce inflammation and brain injury [9] after cardiac arrest, which is the most severe state of shock. We also completed a randomized, assessor-blinded, controlled trial and found that SFI can improve clinical outcomes in patients with return of spontaneous circulation after in-hospital cardiac arrest [10]. HS and cardiac arrest have a common pathophysiological feature, which is the severe I/R

injury. We established a porcine model of HS to determine if SFI can improve cerebral microcirculation and reduce brain injury after HS resuscitation. Cerebral microcirculation was assessed with the aid of side-stream dark field (SDF) imaging. NO which is an endogenous vasodilator produced by activated nitric oxide synthase (NOS) plays a dominant role in inflammation and microcirculation after I/R injury [11].The protein expression of NOS (endothelial nitric oxide synthase [eNOS], inducible nitric oxide synthase [iNOS], and neuronal nitric oxide synthase [nNOS]) and inflammatory factors level (interleukin 6 [IL 6] and tumor necrosis factor-α [TNF-α]) in brain tissue were measured. Brain edema biomarker aquaporin-4 (AQP4) was also evaluated.

2. Materials And Methods

Animal preparation

Twenty-one healthy domestic male Beijing Landrace pigs (3 months, 30 ± 2 kg) were fasted overnight with free access to water. The animals were initially sedated with a single intramuscular injection of midazolam (0.5 mg/kg). Anesthesia was induced with a bolus dose of propofol (1 mg/kg) and maintained with pentobarbital (8 mg/kg/h) by ear vein. A cuffed 6.5 mm endotracheal tube was advanced into the trachea. Animals were mechanically ventilated with a volume controlled ventilator (Evita4, Drager Medical, Lubeck, Germany) using a tidal volume of 10 ml/kg and FiO2 at 0.21. End-tidal PCO2 was continuously monitored by in-line infrared capnography to maintain at 35–45 mmHg by adjusting respiratory frequency. A vesical catheter with a thermometric detector was intubated into the bladder to measure body temperature and monitor urine output. The left femoral artery was dissected to insert a 4-F arterial catheter into the descending aorta to measure arterial pressure (MAP). A 6-F arterial sheath was inserted into the right femoral artery for rapid bleeding. A 5-F central venous catheter was inserted via the left femoral vein for volume resuscitation. Room temperature was adjusted to 26 ℃. Body temperature was maintained above 37 ℃ with a heating pad.

Experimental protocol

After operation, the animals were allowed to stabilize for 30 min, and baseline data were obtained. HS was induced by rapid bleeding via the arterial sheath to a MAP of 40 mmHg within 10 minutes and maintained at 40 ± 3 mmHg for 60 minutes [12]. Shed blood was collected in sterile bags (S-400, Sichuang Nightingale Biological Co. Ltd). Additional blood was withdrawn at a MAP ≥ 44 mmHg, or crystalloid solution was infused at a MAP ≤ 36 mmHg. Volume resuscitation started after 1 hour of shock. The animals were resuscitated with the shed blood during the first hour of reperfusion, and then received a basal crystalloid infusion of 10 ml/kg/hr. At 6 hours after the onset of HS, animals were placed in a lateral position. A 10 cm mid-sagittal incision was made over the frontal and parietal portions of the scalp. A 15 mm parietal cranial window was opened by drilling. Then dura mater was incised to observe the cerebral microcirculation as our previous experiment [13]. Animals were euthanized with an intravenous injection of 100 mg/kg pentobarbital after recording microcirculation, and then cortical tissue

samples from parietal lobe were removed. Some samples were frozen in liquid nitrogen and stored at −80 °C for protein analysis. Some samples were stored in 4% paraformaldehyde.

Five pigs were randomly assigned to the sham operation (SO) group, which had no HS or volume resuscitation. Sixteen pigs of HS were randomly divided into two groups: (1) SFI group (SFI, n=8): conventional volume resuscitation and SFI at a dose of 3 ml/kg; (2) saline group (SA, n=8): conventional volume resuscitation and saline at a dose of 3 ml/kg.

Measurements

Microcirculatory imaging

Cerebral microcirculation was recorded by a SDF imaging video microscope (MicroScan; MicroVision Medical Inc, Amsterdam, The Netherlands). Individual videos of 10 seconds were analyzed off-line using a score, in which 0 represents no flow, 1 represents markedly reduced flow, 2 represents reduced flow, and 3 represents normal flow. The microvascular flow index (MFI) score represented the average value of the predominant type of blood flow. Following the guidelines [14], we determined MFI both for large microvessel (diameter > 20 μm) and small microvessel (diameter < 20 μm). To avoid possible bias, the analysis of the videos of the microcirculation was blinded.

Immunohistochemistry (IHC)

IHC was used to evaluate the protein expression of NOS in brain tissues. Cortical tissues fixed with 4% paraformaldehyde were dehydrated with alcohol solutions of gradient concentration (100, 95, 80 and 75%), sliced at 4 μm thickness, and embedded in paraffin. The sections were placed in histosol for the removal of paraffin, rehydrated in graded ethanol, and blocked in 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA, Darmstadt, USA) for 4 hours. Sections were incubated with eNOS antibody (1:1000 dilution; rabbit. no. Ab5589, Abcam Co, China), iNOS antibody (1:1000 dilution; rabbit. no. Ab1956, Abcam Co, China), and nNOS antibody (1:1000 dilution; rabbit. no. Ab3342, Abcam Co, China) at 4 ˚C overnight. The sections were then incubated with biotinylated goat anti-rabbit secondary antiserum. After counterstaining with hematoxylin, Image J software (National Institutes of Health, Bethesda, MD, USA) was used to determine the optical density of the images. Five sections were selected from each group, and the average values were measured.

IL 6 and TNF-α in the brain tissue

Brain homogenates were obtained from the cortical tissue and centrifuged at 2000 x *g* for 15 minutes to remove cellular debris. The levels of IL 6 and TNF-α were measured using specific enzyme-linked immunosorbent assay kits according to the manufacturer's instructions.

Western blotting

AQP 4 protein expression in cortical tissue was measured by western blotting according to manufacturer's instruction. Integrated optical densities of protein bands were digitally quantified using Gel-Pro Analyzer version 3.1 (Media Cybernetics, Silver Spring, MD). Protein levels were normalized to β-actin and presented as a ratio.

Brain ultrastructure

Tissue samples of the cerebral cortex (preserved in 4% paraformaldehyde) were used to assess ultrastructural changes of microvascular endothelia under a transmission electron microscope (H-7650; Hitachi, Tokyo, Japan).

Statistical analysis

Data of normal distribution were expressed as mean ± SD. One-way analysis of variance was used to assess the differences among the three groups followed by Bonferroni's post hoc test to correct for multiple comparisons. Student's t-test was used for the comparisons of shed blood between SFI and SA groups. A *p-v*alue < 0.05 was considered significant. Statistical analysis was performed with SPSS 19.0 software (SPSS Inc, Chicago, IL) and GraphPad Prism version 5 (GraphPad Software, San Diego, CA).

3. Results

No significant differences in body weight, hemoglobin, MAP or shed blood were detected among the three groups (SO, SFI and SA) at baseline (Table 1). There were no differences in 6 hours survival after HS between SFI group (7/8) and SA group (7/8).

Table 1. Baseline characteristics.

	SO (n=5)	SFI (n=8)	SA (n=8)	*F*	*p*
Weight (kg)	30.8±1.8	31.2±1.6	30.7±2.8	.124	.884
Hemoglobin (g/L)	103±5	98±11	104±11	.656	.532
Mean arterial pressure (mm Hg)	97±8	94±14	100±13	.385	.687
Shed blood (mL)		754±84	734±90		.674
Shed blood (mL/kg)		24.3±3.7	24.0±3.1		.902
SO sham operation, *SFI* shenfu injection, *SA* saline.					

From the Fig. 1, the MFI values of both large and small microvessels at 6 hours after HS in the SFI and SA groups were all lower than that in the SO group (p all < 0.05). Compared with SA group, SFI significantly improved the cerebral MFI of both large (p = 0.023) and small (p = 0.034) microvessels.

As shown in the IHC presented in Fig. 2, compared with SO group, the cerebral protein expression levels of NOS (eNOS, iNOS, and nNOS) were significantly increased at 6 hours after HS in the SFI and SA group (p all < 0.05). Compared with the SA group, SFI significantly increased protein expression levels of eNOS (p = 0.043), and reduced levels of iNOS (p = 0.029). However, differences in nNOS were not statistically significant between SFI and SA groups.

The values of IL 6 and TNF-α in cortical tissue were significantly increased at 6 hours after HS in both the SFI and SA groups compared with SO group (p all < 0.05). However, compared with the SA group, SFI significantly reduced the values of IL 6 (p = 0.018) and TNF-α (p = 0.042), as shown in Fig. 3.

As indicated in the western blots shown in Fig.4, the protein expression levels of AQP 4 in the cortical tissue were significantly increased in the SFI and SA groups at 6 hours after HS compared with SO group (p both < 0.05). Compared with the SA group, SFI significantly reduced AQP 4 expression levels (p = 0.005).

The ultrastructure of microvascular endothelia in cortical tissue was investigated by electron microscopy as shown in Fig. 5. Compared with the SO group, obvious endothelium cell damage, including loss of normal cell form, mitochondrial swelling, ridge fracture, cavity changes, tight junction damage and interstitial edema were observed in the SFI and SA groups. Compared with SA group, SFI attenuated damage to endothelial cell and interstitial edema.

4. Discussion

HS is characterized by a severe reduction in circulating blood volume, which can lead to microcirculation disturbance and tissue hypoxia in important organs, especially the brain which can only compensate hypoxemia for short periods [2]. With volume resuscitation, I/R injury can lead to the production of inflammatory mediators and reactive radical species such as nitric oxide, which may cause microvascular endothelial injury. The assessment of microcirculation, which performs the essential functions of delivering oxygen and substrates to cells, should be an end point for resuscitation during the treatment of HS [15]. There is a significant correlation between microcirculatory dysfunction and poorer patient outcomes in HS [16]. In the present study, we demonstrated that the cerebral MFI values of both large and small microvessels after volume resuscitation of HS were significantly decreased compared with the SO group. Combined application of SFI in volume resuscitation can obviously improve microcirculation disturbance after HS.

SFI contains ginsenoside and aconitine, which are made from raw material of ginseng and fuzi by using multistage countercurrent extraction and macroporous resin adsorption technology. SFI as a traditional Chinese herbal formula has the characteristics of multi-components, multi-targets, and multi-effects, and

has been shown to have complex pharmacologic actions [17]. SFI has been widely used for the treatment of sepsis shock in emergency and ICU in China [18], and has been proved to improve the prognosis of patients with cardiac arrest [10]. At present, there are few studies on the effect of SFI on HS. In this study, there were no significant differences in 6 hours survival after HS between SFI and SA groups. This may be related to the short observation time or the small sample size. Therefore, further study is needed on the influence of SFI on the survival of HS. However, in the present study, we demonstrated that SFI can improve the cerebral microcirculation after HS compared with SA group. This is similar to our previous experiment which found SFI can improve the mesenteric microvascular blood flow after resuscitation of cardiac arrest [8].

There are many factors that affect the cerebral microvasculature blood flow during I/R injury after HS. NO secreted by vascular endothelial cells plays an important role in host responses to ischemia [11], and exhibits opposing roles at different stages of I/R injury [19]. At the early stage of I/R injury, NO produced by eNOS mainly relaxes the cerebral vasculature to play a neuroprotective role. But with the prolongation of ischemia, NO produced by iNOS mainly promotes the production of superoxide free radicals leading to neurotoxicity. In this study, SFI significantly up regulated cortical protein expression levels of eNOS and down regulated the iNOS expression after volume resuscitation of HS compared with the SA group. Therefore, this may be one of the reasons why SFI can improve microcirculation and reduce excessive inflammatory response after I/R injury [8-9].

From the results of this study, we found that the inflammatory factors values of IL 6 and TNF-α in cortical tissue were significantly increased after volume resuscitation of HS in both SFI and SA groups compared with the SO group. However, SFI significant reduced the levels of IL 6 and TNF-α compared with the SA group. This is consistent with our previous experimental which found SFI can reduced cerebral inflammatory reaction after resuscitation of cardiac arrest [9]. Excessive production of inflammatory factors of IL 6 and TNF-α in brain can lead to brain tissue edema [20] and the destruction of the blood–brain barrier [21-22].

AQP 4 is a predominant water channel protein expressed in the end-feet of astrocytes, and plays an important role in the formation of brain edema [23]. Our previous experiments found that SFI reduced brain edema after resuscitation of cardiac arrest by inhibiting cerebral AQP 4 expression [9]. In this study, the protein expression levels of AQP4 in the cortical tissue were significantly increased in the SFI and SA groups at 6 hours after HS compared with SO group. However, SFI significant reduced the AQP 4 expression compared with the SA group. The ultrastructure of cortical tissue also showed that SFI can reduce brain edema compared with SA group.

I/R injury in HS can result in endothelial barrier dysfunction, which lead to the increase in permeability through the microvasculature and interstitial edema [24-25]. In the present study, we also found that there were severe microvascular endothelial damages after volume resuscitation of HS, including loss of normal cell form, mitochondrial swelling, ridge fracture, cavity changes, tight junction damage and interstitial edema in cortical tissue. In addition, we found that SFI can alleviate damage of microvascular

endothelia in cortical tissue compared with the SA group. The possible mechanisms include that SFI down regulates iNOS protein expression, and inhibits the production of the inflammatory factor of TNF-α in cortical tissue. Because iNOS induces vascular endothelial cell migration and apoptosis via autophagy in I/R injury [26], and TNF-α is known to disrupt endothelial barrier integrity [22]. Therefore, in the future, SFI can be used as a potential microvascular endothelial cell protector to reduce I/R injury after HS.

From the results of this experiment, we concluded that combined application of SFI with volume resuscitation after HS can reduce the injury of cerebral microvascular endothelium by down regulating iNOS protein expression and inhibiting inflammatory factor of TNF-α, so as to improve the cerebral microcirculation. The effects of SFI on the protection of microvascular endothelium and the down regulation of AQP 4 protein expression help reduce brain edema compared with the SA group.

There were some limitations to the design of this study. There is only a small sample size of animal and a short observation time in this experiment, therefore the results do not reflect the exact changes and complete outcomes that would occur in clinic. HS often occurs in patients with serious bleeding diseases, such as trauma or gastrointestinal bleeding. In this study, the experiment was performed on apparently healthy pigs no persistent bleeding. The optimal volume, ratios, type, and timing of resuscitation fluids following HS remain controversial. In this study, the volume resuscitation procedure of HS is difficult to achieve clinically, and it is not necessarily the best scheme.

5. Conclusions

SFI can improve cerebral microcirculation after volume resuscitation of HS. Compared with the SA group, SFI significantly up regulates eNOS expression and down regulates iNOS expression in cortical tissue. Moreover, SFI also significantly inhibits cerebral inflammatory factors IL 6 and TNF-α, and down regulates cerebral AQP 4 protein expression compared with the SA group. Therefore, SFI significantly reduce cerebral microvascular endothelial injury and brain edema after HS.

List Of Abbreviations

SFI	shenfu infusion
HS	hemorrhagic shock
SA	saline
SO	sham operation
MAP	mean arterial pressure
MFI	microvascular flow index
NOS	nitric oxide synthase
TNF-α	tumor necrosis factor-α
I/R	ischemia/reperfusion
SDF	side-stream dark field
eNOS	endothelial nitric oxide synthase
iNOS	inducible nitric oxide synthase
nNOS	neuronal nitric oxide synthase
IL 6	interleukin 6
AQP4	aquaporin-4
IHC	immunohistochemistry

Declarations

Ethics approval and consent to participate

This study was conducted with the approval of the Animal Care and Use Committee at the Beijing Chaoyang Hospital of the Capital Medical University, and all experiments complied with the principles of laboratory animal use and care formulated by the Administration Office of Laboratory Animals.

Consent for publication

Not applicable.

Availability of data and material

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Competing interests

The authors declare they have no competing interests.

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Authors' contributions

JW and CL designed the experiment protocol and reviewed the final manuscript. JW participated in the animal experiment, analyzed data and drafted manuscript. ZL helped analyze data and draft manuscript. WY, QZ, YL, MZ and HQ took part in the animal experiment and interpreted the results.

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Not applicable

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Figures

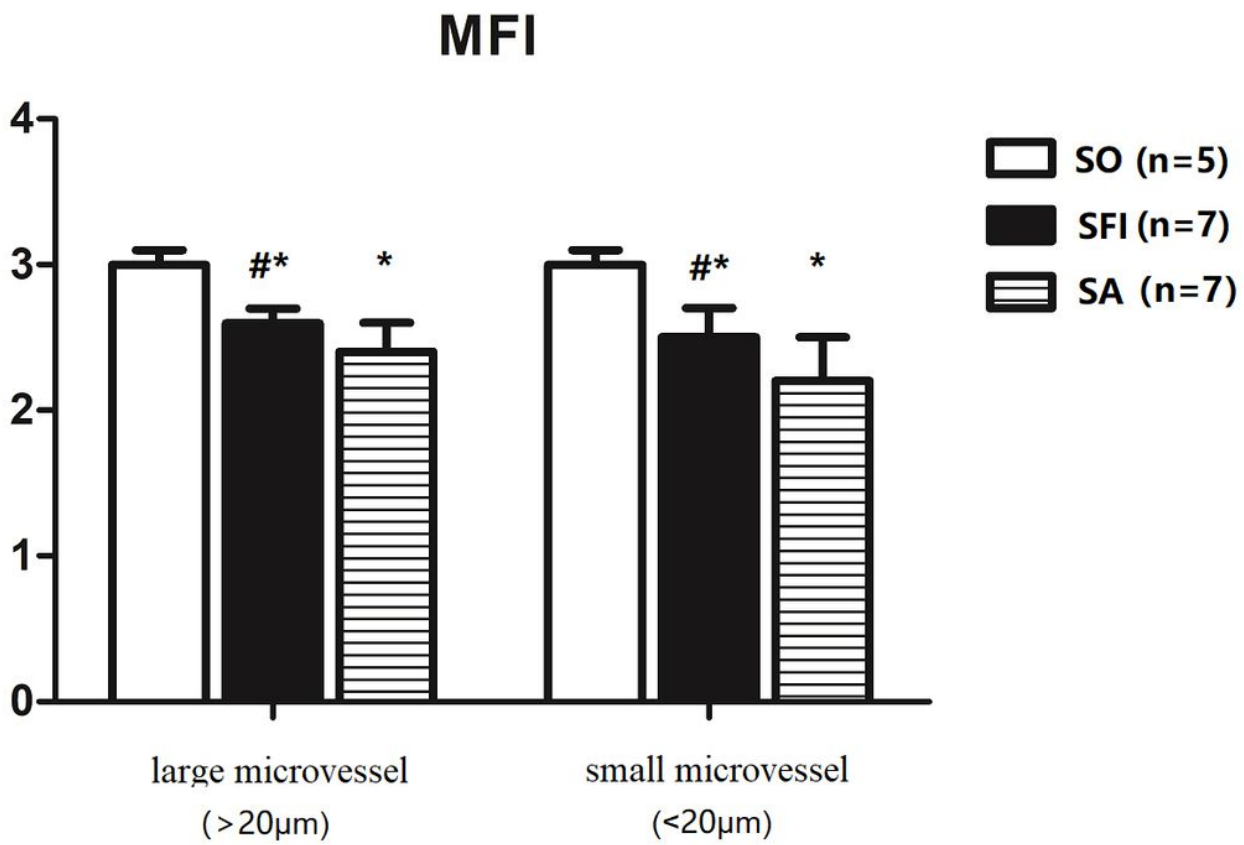

Figure 1

Changes of MFI of both large and small microvessels at 6 hours after hemorrhagic shock. Compared with SA group, SFI significantly improved the MFI of both large and small microvessels after volume resuscitation of hemorrhagic shock. MFI, microvascular flow index; SO, sham operation; SFI, shenfu injection; SA, saline. * p < 0.05 versus SO group; # p < 0.05 versus SA group.

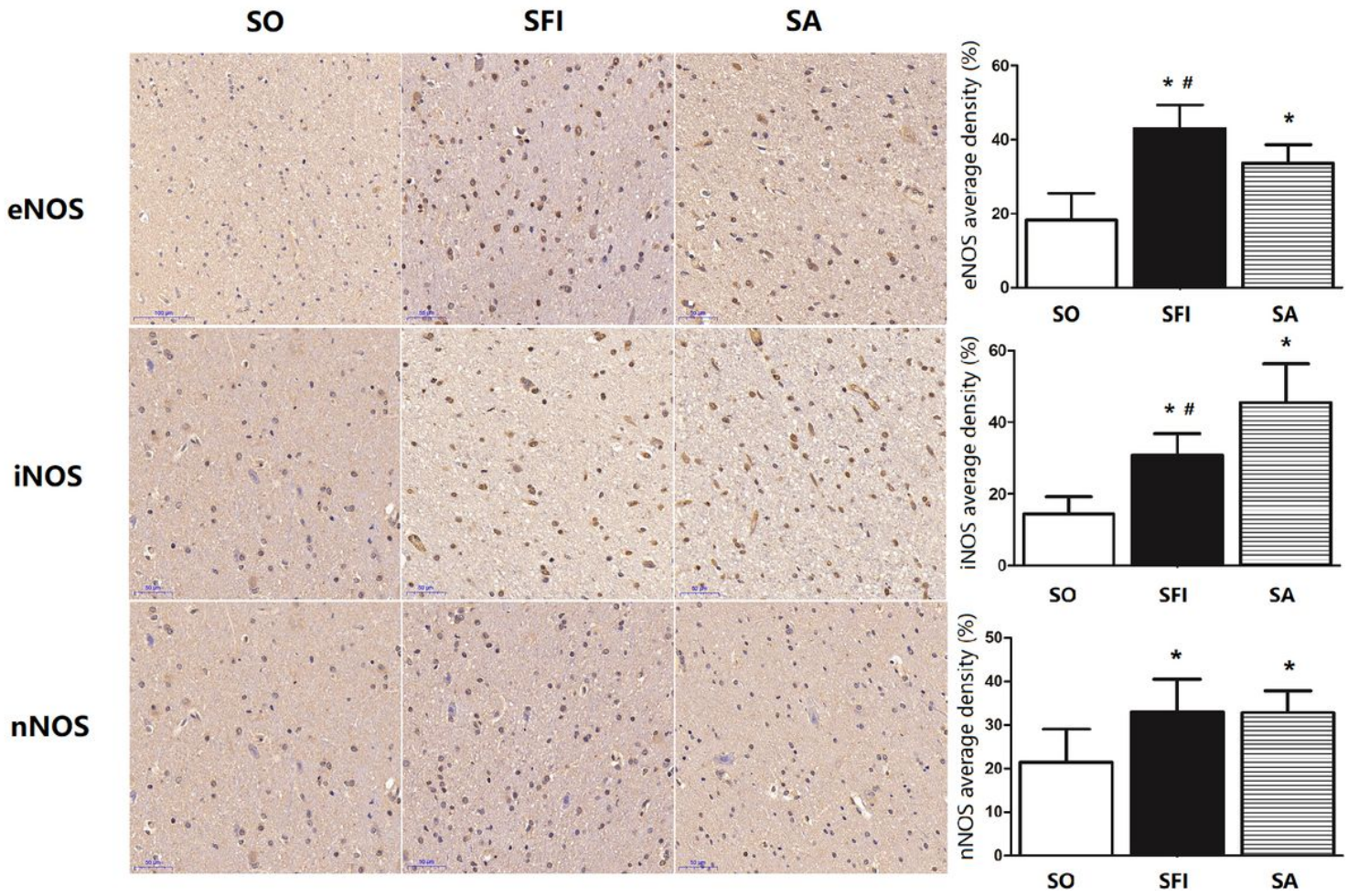

Figure 2

The proteins expression of eNOS, iNOS and nNOS in cortical tissue as determined by immunohistochemistry. Compared with the SA group, SFI significantly increased eNOS expression and reduced iNOS expression at 6 hours after hemorrhagic shock. eNOS, endothelial nitric oxide synthase; iNOS , inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; SO, sham operation; SFI, shenfu injection; SA, saline. * p < 0.05 versus SO group; # p < 0.05 versus SA group.

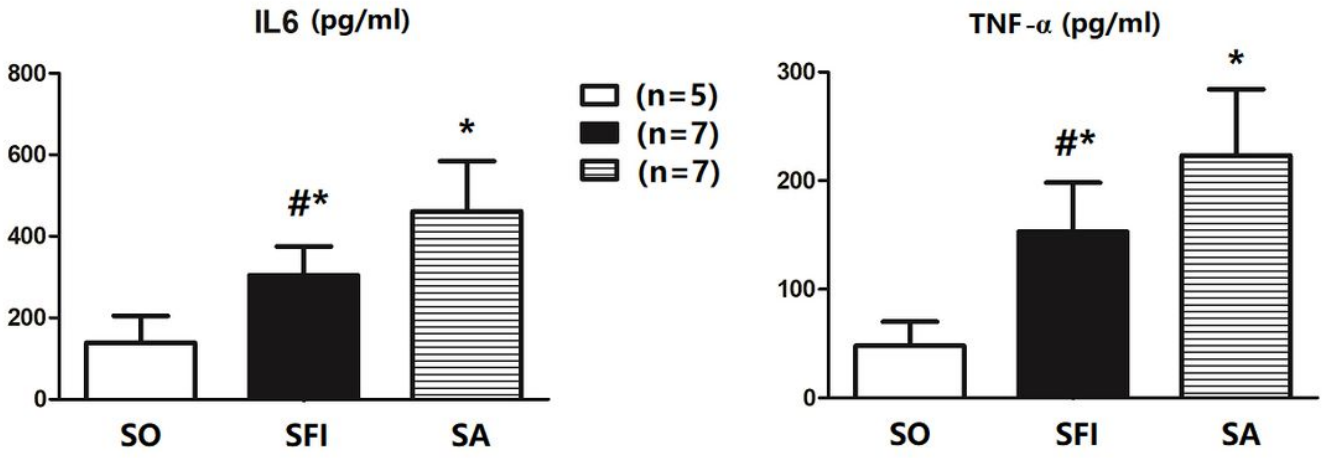

Figure 3

Levels of IL 6 and TNF-α in cortical tissue at 6 hours after hemorrhagic shock. Compared with the SA group, cerebral inflammatory factors levels of IL 6 and TNF-α were reduced in the SFI group. SO, sham operation; SFI, shenfu injection; SA, saline. * p < 0.05 versus SO group; # p < 0.05 versus SA group.

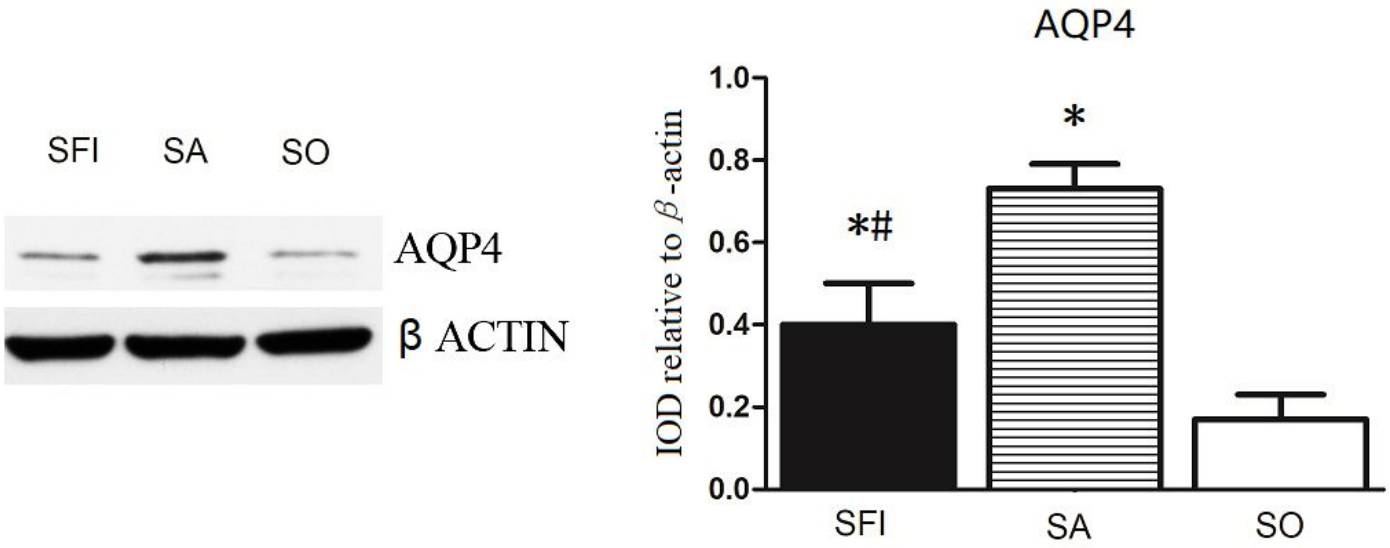

Figure 4

Western blots of the expression of AQP 4 protein in cortical tissue at 6 hours after hemorrhagic shock. Cerebral AQP 4 protein expression was significantly lower in the SFI group than in the SA group. SO, sham operation; SFI, shenfu injection; SA, saline. * p < 0.05 versus SO group; # p < 0.05 versus SA group.

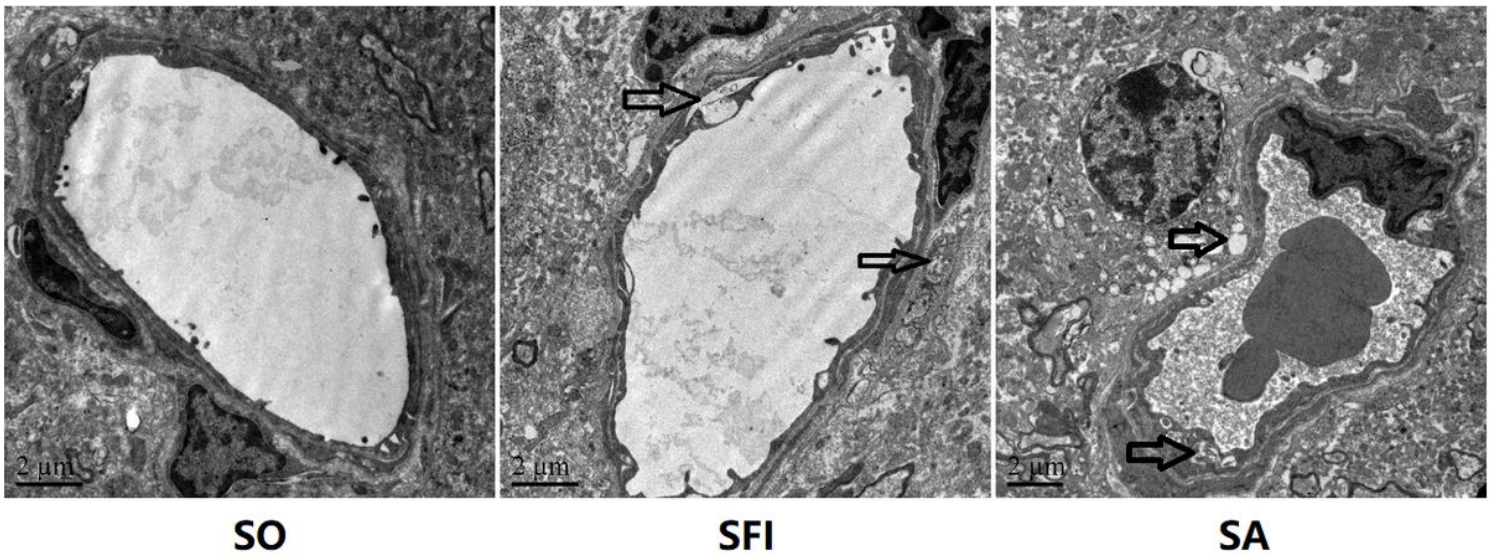

Figure 5

Ultrastructural alterations in cortical tissues at 6 hours after hemorrhagic shock. From the ultrastructure of microvascular endothelium in cortical tissues, endothelial cell injury and interstitial edema after volume resuscitation of hemorrhagic shock were observed in the SFI and SA groups. Arrows show mitochondrial swelling and cavity changes. Compared with the SA group, SFI alleviated the damage of cerebral microvascular endothelia and interstitial edema. SO, sham operation; SFI, shenfu injection; SA, saline.