Int J Clin Exp Med 2017;10(9):14015-14021
www.ijcem.com /ISSN:1940-5901/IJCEM0055280

Original Article

Human tissue kallikrein alleviates microcirculation dysfunction of symptomatic cerebral vasospasm via eNOS upregulation in rabbits

Yunchang Mo*, Yingying Lou*, Chengyu Chen*, Rong Zhang, Anqi Zhang, Meizi Qian, Bo Zheng, Minyuan Zhang, Yelong Ren, Junlu Wang

*Department of Anesthesia, The First Affiliated Hospital of Wenzhou Medical, University, Wenzhou, China. *Equal contributors.*

Received April 12, 2017; Accepted September 10, 2017; Epub September 15, 2017; Published September 30, 2017

Abstract: Cerebral microcirculation dysfunction might play an important role in symptomatic cerebral vasospasm (CVS), and this study was to investigate whether human tissue kallikrein (HTK) can alleviate it. In this study, forty-four rabbits were randomly divided into sham-operated, subarachnoid hemorrhage-operated (SAH), SAH+HTK groups and SAH+HTK+L-NNA groups (n = 11/group). Neurological function and food intake were evaluated on the day before and for 7 days after SAH. Three-dimensional computed tomography angiography was used to determine the diameter of the basilar artery. Western blotting and immunohistochemistry were used to determine the expression of hippocampal endothelial nitric oxide synthase (eNOS) and CD34 respectively. Finally, the microvascular area ratio and field microvessel density (MVD) were quantitated. SAH reduced food intake and caused neurological dysfunction, which were countered by HTK administration. HTK also significantly increased the basilar artery diameter and reduced CVS following SAH. The microvascular area ratio and MVD were reduced in the SAH group, which was countered by HTK treatment. Moreover, HTK treatment increased eNOS expression. Moreover, N-nitro-l-arginine(L-NNA) prevented the protection of HTK; the L-NNA group showed reduced basilar artery diameter, microvascular area ratio, MVD, and eNOS. During post-SAH symptomatic CVS, capillary numbers and the open area of the microcirculation in the hippocampus declined. HTK improved the microcirculation status of symptomatic CVS and upregulated cerebral perfusion pressure by increasing capillary neogenesis and expanding spasmodic microvessels. HTK may exert its action via upregulation of eNOS, which may cause release of more nitric oxide within the hippocampus.

Keywords: CD34, cerebral vasospasm, endothelial nitric oxide synthase, human tissue kallikrein, microcirculation

Introduction

Cerebral vasospasm (CVS) is a common complication of subarachnoid hemorrhage (SAH) caused by aneurysm. CVS can induce brain ischemia and delayed cerebral ischemia (DCI), which are the leading causes of disability and mortality following SAH [1]. SAH comprises only 5% of all strokes, but has a mortality rate of 40% [2]. Cerebral ischemia or infarction post-SAH occurs due to a reduction in cerebral blood flow from the great vessels to the microcirculation [3]. Approximately 62% of post-SAH patients with cerebral infarction experience angiographic vasospasm. However, CVS in angiography is not the exclusive cause of cerebral infarction [4]. CVS can be reduced by drugs, but clinical outcome is not always improved [5]. Cerebral blood flow is reduced after DCI because of microcirculatory constriction [6], microthrombosis [7], blood–brain barrier disruption [8], and endothelial cell apoptosis [9]. Mounting evidence suggests a correlation between the occurrence of DCI and microcirculatory constriction and microthrombosis. Therefore, investigating the changes in cerebral microcirculation post-SAH will provide insight into CVS pathogenesis and may provide an avenue for interventions in post-CVS DCI.

Human tissue kallikrein (HTK) plays multiple roles in physiology, such as dilating cerebral arteries, facilitating brain angiogenesis, and inhibiting cell apoptosis [10]. Previously, we

observed that HTK could expand the spastic basilar artery (BA) after SAH, decreasing the incidence of post-SAH symptomatic CVS [11]. HTK counters ischemia by promoting capillary hyperplasia and hemoperfusion in brain and myocardial ischemic areas [12] and dilates arterioles [13]. Nevertheless, few experiments have examined whether HTK ameliorates microcirculation disturbances by enhancing micrangium hyperplasia in ischemia and dilating spasmodic micrangium in SAH.

In the current study, we established a rabbit model of symptomatic CVS to explore hippocampal microcirculation alterations post-SAH and the effects of HTK on CVS.

Materials and methods

Animal model

The protocol was approved by the special committee on Animal Welfare from the First Affiliated Hospital of Wenzhou Medical University (Approval No. 2015-132), and the rabbits were treated humanely by the guidelines of the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

Animals which died during surgery or developed delayed ischemic neurological deficits post-surgery were excluded from the experiment. The remaining rabbits were randomly assigned to four groups (n = 11/group): sham-operated controls, SAH-operated, SAH+HTK treatment, SAH+HTK+L-NNA treatment.

The model of SCVS after SAH was made as previously reported [11, 14]. The sham-operated group underwent injection of normal saline into the cisterna magna. After 48 hours, the cisterna magna was repunctured, and injection of 1 ml autologous arterial blood was repeated. CVS was confirmed by computed tomography angiography (CTA).

At 30 minutes after the first blood injection, rabbits from the HTK group received 0.01 PNAU/kg in phosphate-buffered saline (PBS; 0.05 mol/L, pH 7.0) intravenously through the ear marginal vein over 5 minutes. This intervention was repeated every 24 hours for 7 days [11]. The L-NNA was intraperitoneally injected at a dosage of 20 mg/kg for 7 days before the intravenously injected HTK. All animals were sacrificed on the seventh day. 1 unit PNAU was defined as the quantity needing to hydrolyze 1 μmol paranitroaniline enzyme in 1 minuteat 37°C and pH 8.0.

Neurological function testing

The rabbits' movements on a flat surface were used to assess neurological function. Deficits included somnolence, reduced activity, weakness in the limbs, circling, and crawling difficulty [14].

BA measurement

BA diameters were measured via 3D-CTA 1 day before and 7 days after SAH induction, as described previously [11]. BA diameters were measured at three individual segments: proximal, middle, and distal. Each segment contained five cross sections [15].

Immunohistochemistry

Immunohistochemical staining was done as described previously [16] (n = 5/group). Formalin-fixed paraffin-embedded tissues were sectioned at 4 μm, deparaffinized, and rehydrated. After incubation with a mouse anti-rabbit CD34 antibody (1:200; Boster-Bio, Wuhan, China) overnight at 4°C, washed by PBS and incubated by a horseradish peroxidase-conjugated secondary antibody (ZSGB-Bio, Beijing, China) for half an hour. The sections were incubated by Liquid DAB Large-Volume Substrate-Chromogen System (DAKO, Glostrup, Denmark) and counterstained with hematoxylin. The immunostaining was evaluated by an Olympus BX-50 light microscope and the Image Pro-Plus 6.0 analysis system. The level of CD34 was measured as the integral optical density.

Western blotting analysis

Hippocampal samples (50 μg protein; n = 6/group) were subjected to gel electrophoresis and transferred to polyvinylidene fluoride membranes using standard methods. Membranes were incubated with rabbit polyclonal anti-eNOS (1:200) and mouse polyclonal anti-tubulin (1:1000) antibodies (Beyotime, Jiangsu, China) followed by incubation with the appropriate horseradish peroxidase-labeled secondary antibodies (1:1000, Beyotime) in 5% non-fat milk in Tris-buffer saline/Tween 20. Bands were

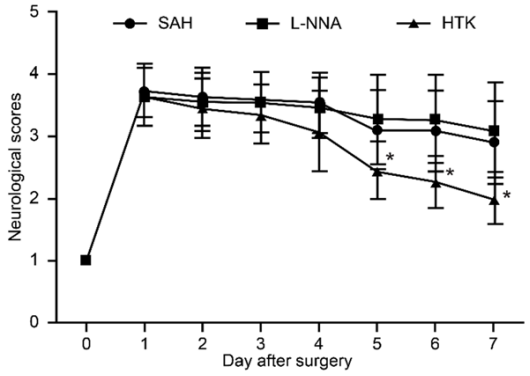

Figure 1. Rabbit neurological scores in three groups. *$p < 0.05$, HTK *vs.* SAH and L-NNA, n = 11/group.

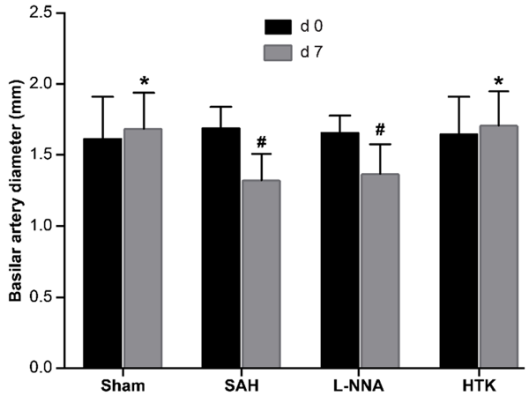

Figure 2. Basilar artery diameter in the four groups. *$p < 0.05$, *vs.* SAH group; #p < 0.05, *vs.* day 0 (d0); n = 11/group.

detected via enhanced chemiluminescence (Beyotime) and autoradiography. The results were quantified by Quantity One Software (Bio-Rad, Hercules, CA).

Field microvessel density (MVD) and microvascular area ratio

Statistical field MVD and microvascular area ratio were analyzed referring to a published protocol [17].

Statistics

Statistical analysis was performed using the SPSS Statistics software package for Windows (version 13.0; Chicago, IL, USA). Differences in neurological scores were analyzed by a Kruskal-Wallis one-way ANOVA followed by multiple comparison procedures with Dunn's method. Other data are expressed as mean ± standard deviation (SD). Statistical differences were detected by *t* tests within groups and one-way analysis of variance (ANOVA) followed by least significant difference tests among groups. Differences in enumeration data among the groups were tested using Fisher's exact test. $p < 0.05$ was considered statistically significant.

Results

No significant differences were found in neurological function in the four groups on the day before SAH induction. After SAH induction, an increase in neurological deficits was observed in the SAH group and L-NNA group. In rabbits that received HTK, these SAH-induced changes were significantly countered, compared with those in the SAH and L-NNA groups at d5 ($p = 0.021$, < 0.05), d6 ($p = 0.001$, < 0.05), and d7 ($p = 0.001$, < 0.05; **Figure 1**).

BA diameters in the sham controls on day 7 were similar to those on pre-injection day. In the SAH and the L-NNA groups, BA diameters were lower than the d0 values ($p = 0.001$, < 0.05). In contrast, in the HTK-treated group, non-arteriospasm was observed on day 7, with a significantly higher BA compared to the SAH group ($p = 0.001$, < 0.05; **Figure 2**).

CD34 was detected in the microvasculature of the hippocampus in all four groups (**Figure 3**). On day 7 post-SAH, the mean MVD values of the SAH and L-NNA groups were lower than that in the sham group ($p = 0.004$, < 0.05). The mean MVD of the HTK group was higher than that in the SAH group ($p = 0.001 < 0.05$; **Figure 3E**). Relative to the controls, the SAH and L-NNA group exhibited a lower microvascular area ratio whereas the HTK group showed a higher ratio ($p = 0.001$, < 0.05; **Figure 3F**). Within the hippocampus, relative to control levels, eNOS levels declined in the SAH and L-NNA groups, and this was countered by HTK treatment ($p = 0.001$, < 0.05; **Figure 4**).

Discussion

Microcirculation changes in CVS post-SAH

A typical CVS is biphasic. The early phase occurs a few hours after SAH, with mild symptoms that quickly remit. In contrast, delayed CVS occurs 3-5 days after hemorrhage, peaks at 7-10 days, and abates after 2-3 weeks [18].

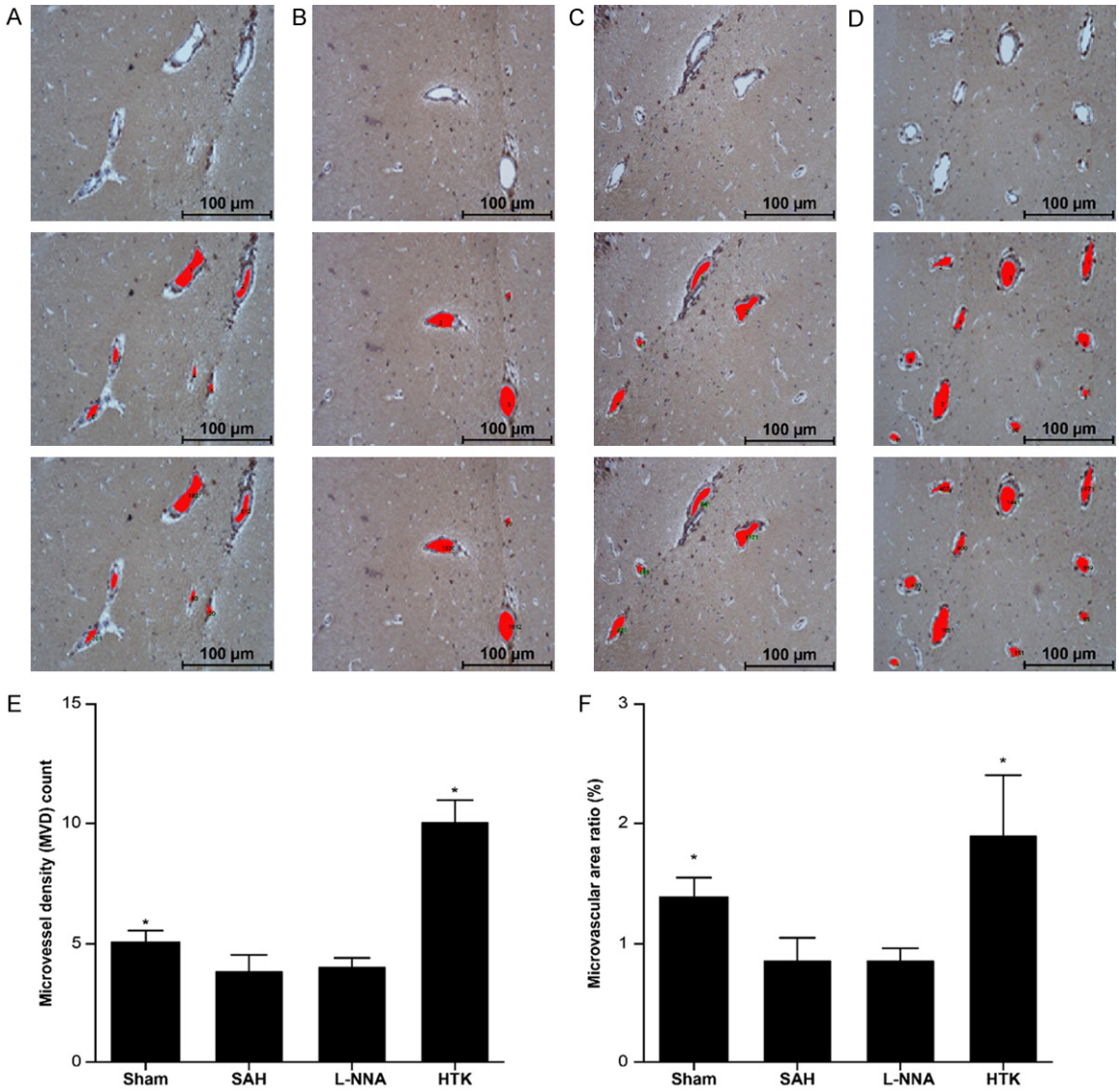

Figure 3. Analysis of microvessels. CD34 immunostaining, MVD, microvascular area ratio shown in four groups. A: Sham group; B: SAH group; C: L-NNA group; D: HTK group. E: Field microvessel density (MVD); F: Microvessel area ratio in the three groups. *$p < 0.05$, *vs.* SAH group; n = 5/group.

Delayed CVS is associated with severe cerebral ischemia and delayed ischemic brain damage and is the main cause of death and severe disability. The incidence of symptomatic CVS is 25%-30% [18].

Although several studies have examined post-SAH CVS-induced brain ischemia and infarction, the pathogenic microcirculatory changes are poorly understood [19]. Mounting evidence shows that microcirculation dysfunction after SAH is the major cause of brain ischemia, with evidence of decreased MVD, twitching or spasm of small cerebral arteries [20], and blocked arteriole circulation, despite the absence of CVS indicators in cerebral angiography [21]. This latter finding is consistent with clinical findings of ischemic neurological deficits without any evidence in cerebral angiography [21].

Cerebral microcirculation is defined as circulation in blood vessels with external diameters within 500 μm (i.e., arterioles, micro-arteries, capillaries, preferential channels, arteriovenous anastomosis, and micro-veins). These vessels exchange nutrients, gases, and hormones between the blood and tissues [22]. A

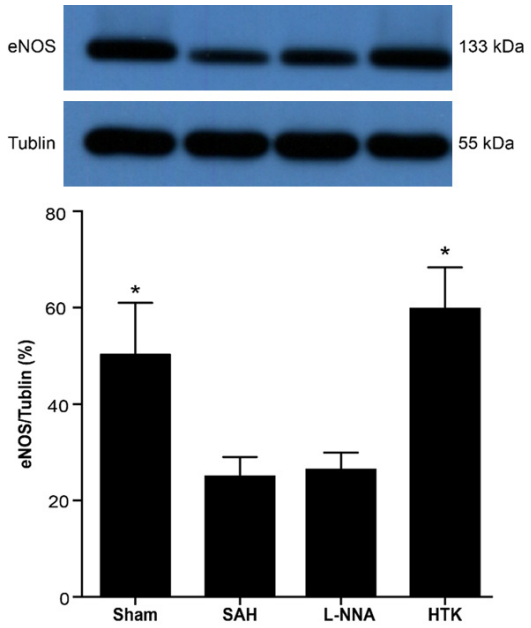

Figure 4. Western blot analysis of eNOS. Blots and densitometric analysis of hippocampal eNOS levels in the four groups. *$p < 0.05$, *vs.* the SAH group; n = 6/group.

second function of the microcirculation is modulation of vascular resistance, which helps to maintain sufficient arterial blood pressure for perfusion. Therefore, microvascular diameter, number, and the degree of opening are critical factors that disrupt cerebral blood flow and cause brain ischemia during CVS.

In the current study, the extent of spasm and neural dysfunction peaked at day 7. This is in accordance with a recent study that reported reductions in hippocampal CD34, microvascular area ratio, and MVD 7 days after SAH. We propose the following underlying mechanism: 1) CVS imbalances diastolic/systolic factors in both the great vessels and the microvessels, leading to vascular occlusion, which minimizes the vessel diameters of the microcirculation [23]. 2) Following CVS, microcirculation perfusion pressure is decreased, resulting in perfusion deficits, and thus post-SAH cerebral ischemia/hypoxia cannot be alleviated [24]. 3) Endothelial cell necrosis, structural rearrangements, endothelial hyperplasia, and the release of vasoactive substances (endothelin, free radicals, inflammatory mediators) after SAH induces direct micrangium injury and an associated hypercoagulable state. This leads to the formation of microthrombus and micrangium obstruction, aggravating the decreased microvascular diameters, thus causing cerebral ischemia/hypoxia [25]. As intact brain microcirculation function is the basis of normal brain function, improvement of the microcirculation is of great significance to the prognosis of SAH.

HTK promotes revascularization

HTK is a new intervention to expand blood vessels in acute cerebral infarction [26] but has not yet been used for the treatment of CVS [11]. We have previously shown that HTK expands the BA after SAH and reduces the incidence of symptomatic CVS. In a model of myocardial and cerebral infarction, HTK promoted capillary hyperplasia and local tissue perfusion [10]. Thus, HTK may counter SAH through promotion of capillary hyperplasia in the ischemic area, expansion of microvascular spasms, and/or enhancement of the microcirculation.

HTK catalyzes the release of bradykinin from kininogens, which acts directly on B1 and B2 kinin receptors to promote vascular proliferation. In our study, HTK increased the post-SAH hippocampal MVD and microvascular area, which indicates that HTK may act via bradykinin to stimulate blood vessel growth and expansion of capillaries. The mechanism may also involve eNOS. Nitric oxide (NO) derived from eNOS has a neuroprotective effect [27]. The kallikrein-kinin system increases NO production via the Akt-B-eNOS pathway, thus promoting the regeneration of blood vessels and expansion of spasmodic capillaries [28]. Previous research showed that eNOS levels decreased after SAH, which led to reduced releaseof NO, one of the leading causes of microcirculation dysfunction and CVS after SAH. We used the eNOS antagonist L-NNA, which can reduce the expression of eNOS. The previous study showed that the L-NNA can also decrease the level of eNOS in the cortex in an SAH rat model [29]. It reduces the expression of eNOS before HTK administration, which may decrease the level of NO. In the current study, HTK countered the SAH-induced decrease in hippocampal eNOS, which may have resulted in the release of more NO to improve microcirculation, and the effect was reduced by L-NNA.

In a rabbit model of symptomatic CVS, intravenous HTK alleviated microcirculation dysfunction, increased nascent capillaries, expanded

spasmodic microvessels, and increased cerebral perfusion via eNOS upregulation. We concluded that HTK is a promising candidate for the prevention and control of CVS.

Acknowledgements

This work was supported by the National Foundation of Natural Science of China, No. 81603685, 81573742 and 81273926; the Natural Science Foundation of Zhejiang Province of China, No. LY15H290006 and LY15-H27001; the Wenzhou Science and Technology Project, No. Y20150229 and Y20140706. The authors thank Dr. Hu Zhangyong for technical assistance with CTA.

Disclosure of conflict of interest

None.

Address correspondence to: Junlu Wang, Department of Anesthesia, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, 325000 Zhejiang, China. Tel: +86-577-55579097; Fax: +86-577-55579097; E-mail: wangjunlu973@163.com

References

[1] Kühn AL, Balami JS and Grunwald IQ. Current management and treatment of cerebral vasospasm complicating SAH. CNS Neurol Disord Drug Targets 2013; 12: 233-241.

[2] Lovelock CE, Rinkel GJ and Rothwell PM. Time trends in outcome of subarachnoid hemorrhage: population-based study and systematic review. Neurology 2010; 74: 1494-1501.

[3] Vatter H, Güresir E, Berkefeld J, Beck J, Raabe A, de Rochemont R dM, Seifert V and Weidauer S. Perfusion-diffusion mismatch in MRI to indicate endovascular treatment of cerebral vasospasm after subarachnoid haemorrhage. J Neurol Neurosurg Psychiatry 2011; 82: 876-883.

[4] Brown RJ, Kumar A, Dhar R, Sampson TR and Diringer MN. The relationship between delayed infarcts and angiographic vasospasm after aneurysmal subarachnoid hemorrhage. Neurosurgery 2013; 72: 702-707; discussion 707-708.

[5] Etminan N, Vergouwen MD, Ilodigwe D and Macdonald RL. Effect of pharmaceutical treatment on vasospasm, delayed cerebral ischemia, and clinical outcome in patients with aneurysmal subarachnoid hemorrhage: a systematic review and meta-analysis. J Cereb Blood Flow Metab 2011; 31: 1443-1451.

[6] Friedrich B, Müller F, Feiler S, Schöller K and Plesnila N. Experimental subarachnoid hemorrhage causes early and long-lasting microarterial constriction and microthrombosis: an in-vivo microscopy study. J Cereb Blood Flow Metab 2012; 32: 447-455.

[7] Sabri M, Ai J, Lakovic K, D'abbondanza J, Ilodigwe D and Macdonald RL. Mechanisms of microthrombi formation after experimental subarachnoid hemorrhage. Neuroscience 2012; 224: 26-37.

[8] Yan J, Li L, Khatibi NH, Yang L, Wang K, Zhang W, Martin RD, Han J, Zhang J and Zhou C. Blood-brain barrier disruption following subarchnoid hemorrhage may be faciliated through PUMA induction of endothelial cell apoptosis from the endoplasmic reticulum. Exp Neurol 2011; 230: 240-247.

[9] Yu Y, Lin Z, Yin Y and Zhao J. The ferric iron chelator 2,2'-dipyridyl attenuates basilar artery vasospasm and improves neurological function after subarachnoid hemorrhage in rabbits. Neurol Sci 2014; 35: 1413-1419.

[10] Hopp S and Albert-Weissenberger C. The kallikrein-kinin system: a promising therapeutic target for traumatic brain injury. Neural Regen Res 2015; 10: 885-886.

[11] Yunchang M, Qinxue D, Binbin J, Xin H, Lili Y, Linbi C, Wujun G, Pengbo Z and Junlu W. Human tissue kallikrein ameliorates cerebral vasospasm in a rabbit model of subarachnoid hemorrhage. Neurol Res 2015; 37: 1082-1089.

[12] Chao J, Shen B, Gao L, Xia CF, Bledsoe G and Chao L. Tissue kallikrein in cardiovascular, cerebrovascular and renal diseases and skin wound healing. Biol Chem 2010; 391: 345-355.

[13] Nagano H, Suzuki T, Hayashi M and Asano M. Effects of a human urinary kininogenase (SK-827) on cerebral microcirculation after glass bead-induced cerebral embolism in rabbits. In Vivo 1992; 6: 497-502.

[14] Endo S, Branson PJ and Alksne JF. Experimental model of symptomatic vasospasm in rabbits. Stroke 1988; 19: 1420-1425.

[15] Chen G, Zhang S, Shi J, Ai J and Hang C. Effects of recombinant human erythropoietin (rhEPO) on JAK2/STAT3 pathway and endothelial apoptosis in the rabbit basilar artery after subarachnoid hemorrhage. Cytokine 2009; 45: 162-168.

[16] Jin X, Wang F, Liu X, Liang B, Chen Z, He J, Zhang H and Zhang J. Negative correlation of CD34+ cells with blood-brain barrier permeability following traumatic brain injury in a rat model. Microcirculation 2014; 21: 696-702.

[17] Maeda K, Chung YS, Ogawa Y, Takatsuka S, Kang SM, Ogawa M, Sawada T and Sowa M.

Prognostic value of vascular endothelial growth factor expression in gastric carcinoma. Cancer 1996; 77: 858-863.
[18] Dorsch NW. Cerebral arterial spasm--a clinical review. Br J Neurosurg 1995; 9: 403-412.
[19] Lucke-Wold BP, Logsdon AF, Manoranjan B, Turner RC, McConnell E, Vates GE, Huber JD, Rosen CL and Simard JM. Aneurysmal subarachnoid hemorrhage and neuroinflammation: a comprehensive review. Int J Mol Sci 2016; 17.
[20] Uhl E, Lehmberg J, Steiger HJ and Messmer K. Intraoperative detection of early microvasospasm in patients with subarachnoid hemorrhage by using orthogonal polarization spectral imaging. Neurosurgery 2003; 52: 1307-1315; disacussion 1315-1317.
[21] Ohkuma H, Manabe H, Tanaka M and Suzuki S. Impact of cerebral microcirculatory changes on cerebral blood flow during cerebral vasospasm after aneurysmal subarachnoid hemorrhage. Stroke 2000; 31: 1621-1627.
[22] Rhoades RA aGA. Medical physiology. 1995. Massachusetts. Little Brown 279-320.
[23] Wan H and Loch MR. Circulatory and vascular changes after aneurysmal subarachnoid hemorrhage. J Neurosurg Sci 2011; 55: 329-341.
[24] Flynn L and Andrews P. Advances in the understanding of delayed cerebral ischaemia after aneurysmal subarachnoid haemorrhage. F1000Res 2015; 4.
[25] Park KW, Metais C, Dai HB, Comunale ME and Sellke FW. Microvascular endothelial dysfunction and its mechanism in a rat model of subarachnoid hemorrhage. Anesth Analg 2001; 92: 990-996.
[26] Wang YD, Lu RY, Huang XX, Yuan F, Hu T, Peng Y and Huang SQ. Human tissue kallikrein promoted activation of the ipsilesional sensorimotor cortex after acute cerebral infarction. Eur Neurol 2011; 65: 208-214.
[27] Santhanam AV, Smith LA, Akiyama M, Rosales AG, Bailey KR and Katusic ZS. Role of endothelial NO synthase phosphorylation in cerebrovascular protective effect of recombinant erythropoietin during subarachnoid hemorrhage-induced cerebral vasospasm. Stroke 2005; 36: 2731-2737.
[28] Emanueli C, Salis MB, Van Linthout S, Meloni M, Desortes E, Silvestre JS, Clergue M, Figueroa CD, Gadau S, Condorelli G and Madeddu P. Akt/protein kinase B and endothelial nitric oxide synthase mediate muscular neovascularization induced by tissue kallikrein gene transfer. Circulation 2004; 110: 1638-1644.
[29] Li Q, Chen Y, Li B, Luo C, Zuo S, Liu X, Zhang JH, Ruan H and Feng H. Hemoglobin induced NO/cGMP suppression deteriorate microcirculation via pericyte phenotype transformation after subarachnoid hemorrhage in rats. Sci Rep 2016; 6: 22070.