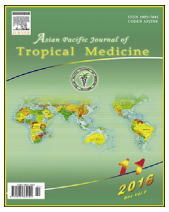
HOSTED BY
ELSEVIER

Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage: http://ees.elsevier.com/apjtm



Original Research http://dx.doi.org/10.1016/j.apjtm.2016.09.004

Mechanism of action of Zhuyu Annao pill in mice with cerebral intrahemorrhage based on TLR4

Wei Chen[1,2,#], Yue-Qiang Hu[1,2,#], Ling-Fei Jiang[3], Lin Wu[2,4✉]

[1]*First Cerebropathy Unit, First Affiliated Hospital of Guangxi University of Chinese Medicine, Nanning 530023, Guangxi, China*

[2]*Key Laboratory of Guangxi Basic Chinese Medicine, Nanning 530000, Guangxi, China*

[3]*Postgraduate Department, Guangxi University of Chinese Medicine, Nanning 530000, Guangxi, China*

[4]*Science Experiment Center, Guangxi University of Chinese Medicine, Nanning 530000, Guangxi, China*

ARTICLE INFO

Article history:
Received 1 Aug 2016
Received in revised form 2 Sep 2016
Accepted 30 Sep 2016
Available online 19 Oct 2016

Keywords:
Zhuyu Annao pill
Intracerebral hemorrhage
TLR4
Gene knockout
Mechanism

ABSTRACT

Objective: To explore the protective effect and possible mechanism of action of Zhuyu Annao pill in mice with intracerebral hemorrhage (ICH).

Methods: Sixty mice were divided into the control group, hemorrhage group, drug-treated group (after hemorrhage), TLR4-knockout hemorrhage group and TLR4-knockout hemorrhage + drug-treated group (after hemorrhage) with 12 in each group. Model of autologous ICH was established in all groups. After drilling and 12 h of fasting, models in the control group hemorrhage group and TLR4-knockout hemorrhage group were all drenched with 10 mL/kg distilled water by intragastric administration. Models in the drug-treated group and TLR4-knockout hemorrhage + drug-treated group were drenched with 6.25 g/kg of Zhuyu Annao pill. All groups were treated for 7 d. Longa scoring method was used to measure the neurological defect scores and determine the brain water contents of all groups; ELISA was employed to detect the inflammatory factor interleukin (IL)-6, tumor necrosis factor-α (TNF-α) and IL-1β in brain tissues; and Western blot was applied to test the expression quantities of apoptotic protein Bax and anti-apoptotic protein Bcl-2 in brain tissues.

Results: At day 3 and 7, compared with the hemorrhage group, the neurological defect scores of the drug-treated group, TLR4-knockout hemorrhage group and TLR4-knockout hemorrhage + drug-treated group decreased significantly ($P < 0.05$). Compared with the hemorrhage group, the brain water contents of the drug-treated group, TLR4-knockout hemorrhage group and TLR4-knockout hemorrhage + drug-treated group reduced significantly ($P < 0.05$). Compared with the hemorrhage group, the inflammatory factor IL-6, TNF-α and IL-1β of the drug-treated group, TLR4-knockout hemorrhage group and TLR4-knockout hemorrhage + drug-treated group decreased significantly ($P < 0.05$). Compared with the hemorrhage group, the expression of apoptotic protein Bax of the drug-treated group, TLR4-knockout hemorrhage group and TLR4-knockout hemorrhage + drug-treated group decreased significantly and the expression of anti-apoptotic protein Bcl-2 increased significantly ($P < 0.05$).

Conclusions: Zhuyu Annao pill can alleviate encephaledema for mice with ICH and reduce inflammatory responses and nerve cell apoptosis. TLR4 can mediate inflammatory injury induced by ICH. Thus, Zhuyu Annao pill can play a protective role for brains by decreasing the expression of TLR4.

1. Introduction

Intracerebral hemorrhage (ICH), also named hemorrhagic stroke, is a kind of strokes and a common gerontological frequently-occurring disease as well with characteristics such as high incidence, fatality and disability rates [1]. It is reported that over 50% of the saved survivors of ICH remain different disabilities, places a heavy burden on their families and on

First author: Wei Chen, First Cerebropathy Unit, First Affiliated Hospital of Guangxi University of Chinese Medicine, Nanning 530023, Guangxi, China.
Tel: +86 13978824543
E-mail: chenwei4926@163.com
✉Corresponding author: Lin Wu, Key Laboratory of Guangxi Basic Chinese Medicine, Nanning 530000, Guangxi, China.
Tel: +86 15177775008
E-mail: Yanwo7073@163.com
Peer review under responsibility of Hainan Medical University.
Foundation Project: This study was supported by the system issue of Key Laboratory of Guangxi Basic Chinese Medicine Research (Grant No. 16-380-58-06).
# These authors contributed equally to this work.

society as well [2]. With the deepening of researches, people found that the secondary nerve injury showing at the 2–3 d after ICH and neurological impairment and cerebral injury exacerbation caused by that contributes to the most serious state of the disease [3]. Researchers have revealed that metabolic disorder around the hematoma, changes of local cerebral blood flow and inflammatory responses caused by the pyrolysis products of red blood cells are important factors for causing the exacerbation of nerve damage [4]. In recent years, the role of inflammatory responses in ICH has arisen enough concern. More and more researches [5,6] have manifested that after ICH takes place, inflammatory cells in tissues surrounding hematoma such as microglial cells are activated and release pro-inflammatory cytokines such as interleukin (IL)-6, tumor necrosis factor-α (TNF-α), which lead to the increase of the expression of intercellular adhesion molecules, while the destruction of the blood–brain barrier would cause the aggregation of peripheral mononuclear macrophages and neutrophil granulocytes in the border zone of hemorrhage, which consequently induces inflammatory cascade, accelerates neuronal death, aggravates encephaledema and finally gives rise to neurological impairment and aggravated cerebral injury.

Toll-like receptor (TLR) is a kind of ancient receptor preserved after experiencing an evolution for hundreds of millions of years. It can mediate innate immune responses, which makes it an important natural defense mechanism [7]. TLR is a member of IL-1 receptor superfamily. By far, 11 kinds of TLR have been discovered and named TLR1–11, respectively. TLR4 is the kind studied widely among TLR, which plays a role in immunological recognition and inflammation regulation mainly by two ways, dependent and independent myeloid differentiation factor 88 (MyD88) [8,9]. As for the transmembrane receptor of endotoxin, the effect of TLR4 in septic shock and Gram-negative bacterial infection has been recognized by many scholars [10,11]. Recent studies have found that heme can act on TLR4 and induce the mass production of inflammation cytokines, causing inflammatory injury. Heme is the lysate of red blood cells, thus TLR4 is considered to play an important role in tissue injuries caused by hemorrhagic diseases.

ICH belongs to the category of "stroke" in Chinese medicine. "Block extravasated blood and water toxin retention" are the basic pathology of edema after hemorrhage [12]. Zhuyu Annao pill is a classic prescription for treating ICH with the therapeutic principle of "activating blood circulation and removing stasis and alleviating water and eliminating toxins". Clinical researches have demonstrated that it obtains a good effect in treating ICH [13]. At present, its mechanism in the treatment of ICH still remains unclear, and the effect of TLR4 in ICH needs further confirmation. This study aimed to explore the protective effect of Zhuyu Annao pill either with effect of TLR4 or without the effect of TLR4 in mice with ICH by establishing models of normal mice and *TLR4* gene knockout ICH mice.

2. Materials and methods

2.1. Animals and groups

Male SPF-level WT C57/BL6 mice (10–12 weeks, 21–25 g) were brought from Guangxi Medical University with a production permit number of SCXK Gui 2014-0002. They were divided into the control group, hemorrhage group and drug-treated group (after hemorrhage). Mice (10–12 weeks, 21–25 g) knockout by TLR4 under the same male SPF-level WT circumstance were introduced form Jackson (USA) and cultivated, and they were grouped into the TLR4-knockout hemorrhage group and TLR4-knockout hemorrhage + drug-treated group (after hemorrhage). The experiment involved 5 groups with 12 mice in each group. Mice died from the exacerbated symptoms in the procedure of the experiment because of were excluded and other mice were supplemented to maintain the original sample size. All experiment procedures involving animals were conducted in accordance to Manipulative Technique for the Care and Use of Laboratory Animals and approved by the ethics committee of the Guangxi University of Chinese Medicine. The experiments were conducted in the Key Laboratory of Guangxi Basic Chinese Medicine, while genes were tested in National Key Laboratory of Diagnosis and Treatment of Infectious Diseases of the First Hospital of Zhejiang Province.

2.2. Models

The autologous ICH models of all WT mice and TLR4-knockout mice were built. The concrete steps were: 4% of chloral hydrate (400 mg/kg) was injected into the abdominal cavity for anesthesia and then the position of those mice were adjusted and fixed. A longitudinal incision was made in the middle of the head and the coronal suture and periosteum-exposed bregma were removed. A hole with a diameter of 1 mm and a depth of 3.5 mm was drilled at 0.8 mm in front of the bregma, 2 mm middle-to-right and 20 μL of blood flow of femoral arteries were collected by a microinjector. The microinjector was located in the hole and injected constantly into the brain striatum of mice at a speed of 2 μL/min for 10 min and then it was placed still for 5 min before pulled out the microinjector. Bone wax was applied to close the wound. Mice showed symptoms such as weakness, spontaneous left-turning, standing still, fore limps could not touch the desktop simultaneously and contralateral limb paralysis indicating the success of model building.

2.3. Drug administration methods

Mice in the control group were not injected with blood flow of femora after drilled. After fasting for 12 h, they were drenched with 10 mL/kg of distilled water by intragastric administration once a day. After the models of the hemorrhage group and TLR4-knockout hemorrhage group were successfully established, mice in the two groups were also drenched with 10 mL/kg distilled water by intragastric administration after 12 h of fasting once a day for 7 d, while after the models of the drug-treated group and TLR4-knockout hemorrhage + drug-treated group were built, they were drenched with 6.25 g/kg of Zhuyu Annao pill after 12 h of fasting once a day for 7 d.

2.4. Evaluation of neurological defect scores

Longa scoring method was used to measure the neurological defect scores at days 1, 3 and 7 after drug administration. 0 score: mice could act freely and symmetrically without any abnormality; 1 score: the left front paws of the mice could not stretched completely when their tails were lifted; 2 scores: the left front paws of the mice could not stretched completely and their left fore limb could not act smoothly; 3 scores: the left fore

limb clung to the chest; 4 scores: activities were left-oriented unconsciously; 5 scores: the left forepaw was pulled back when they turned left; 6 scores: mice rotated to the left around without any other performances; 7 scores: mice could not support themselves but lay down to the left.

2.5. Detection of the brain water content

After treated for 7 d, mice were executed after abdominal anaesthesia by pentobarbital natrium. Their brain tissue was removed. A section, 3 mm thick, in the brain tissue in front-end frontal lobe was obtained. Brain tissues of the two hemispheres were separated. The basal ganglia and free skin of the bilateral semi-section were separated. Brain tissue samples were firstly weighed by electronic analytical balance and the data were expressed with wet weight. Then, they were reweighed after 24 h of bake in an electrothermal oven at constant temperature, and the data calculated this time were expressed as dry weight. The brain water content was calculated as: Brain water content = [(wet weight − Dry weight)/wet weight] × 100%.

2.6. Inflammatory factor detected by ELISA

Ten milligrams of the brain tissue was ground in liquid nitrogen, dissociated on ice and then centrifuged at low temperature to obtain supernatant for further detection (5810Rdesk centrifuge, Eppendorf, Germany). Before the experiments, reagents and samples were mixed adequately and the samples were diluted. Standard hole, black hole and tested sample hole were designed. A total of 100 μL of tested sample was added into the tested sample hole. ELISA was used to detect inflammatory factors IL-6, TNF-α and IL-1β. Kits were purchased from Beijing Zhongshan Company. Microplate reader (Bio Rad, USA) was employed to determine the absorbance at a wavelength of 450 nm to calculate the concentration of the sample.

2.7. Expression of apoptotic proteins tested by Western blot method

Proteins in the brain tissues were extracted by pre-cooling lysate solution. Then, the expression quantities of apoptotic protein Bax and anti-apoptotic protein Bcl-2 in the tested samples were determined by BCA protein assay kit (Beyotime Institute of Biotechnology). Firstly, absorption values were measured and calculated by spectrophotometer. Next, the supernatant was added into loading buffer and closed, boiled and cryopreserved. After loading the sample, polyacrylamide gel electrophoresis was carried out and the color was developed after incubated with antibody. Anti-mouse β-actin antibody, rabbit anti-mouse Bcl-2 antibody and rabbit anti-mouse Bax antibody were bought from Beijing Zhongshan Jinqiao Biotechnology Company Ltd. After films scanning, the gray value of the target strap was analyzed by Adobe Photoshop CS6 with β-actin serving as a reference.

2.8. Statistical management

All data of each group were expressed as mean ± SD. ANOVA was used for comparisons between groups and *SNK-q* was employed for further pairwise comparisons. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Comparison of neurological defect scores of each group

The neurological defect scores of mice in the control group at days 1, 3 and 7 were 0. The neurological defect scores of the hemorrhage group, drug-treated group, TLR4-knockout hemorrhage group and TLR4-knockout hemorrhage + drug-treated group all fell. Comparison of the neurological defect scores of the four groups at the 1st day did not show statistical significance ($P > 0.05$). At day 3 and 7, the neurological defect scores of the drug-treated group, TLR4-knockout hemorrhage group and TLR4-knockout hemorrhage + drug-treated group all decreased significantly as compared with those of the hemorrhage group ($P < 0.05$). Compared with the drug-treated group, the neurological defect scores of the TLR4-knockout hemorrhage group had no significant changes ($P > 0.05$). Compared with the TLR4-knockout hemorrhage group, the neurological defect scores of the TLR4-knockout hemorrhage + drug-treated group also did not showed significant changes ($P > 0.05$) (Table 1).

Table 1
Comparison of neurological defect scores of each group ($n = 12$).

Group	1st day	3rd day	7th day
Control group	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Hemorrhage group	4.43 ± 0.38*	3.24 ± 0.68	2.94 ± 0.77
Drug-treated group	4.40 ± 0.43	2.29 ± 0.47*	1.10 ± 0.62*
TLR4-knockout hemorrhage group	4.37 ± 0.41	2.32 ± 0.45*	1.08 ± 0.61*
TLR4-knockout hemorrhage + drug-treated group	4.41 ± 0.42	2.31 ± 0.46*	1.11 ± 0.59*

Compared with the hemorrhage group at the same time point, *$P < 0.05$.

3.2. Comparison of brain water content of mice in each group

After treated for 7 d, the brain tissues of the mice were taken out and weighed to calculate their brain water contents. The results showed that the brain water content of the hemorrhage group [(80.32 ± 2.68)%] increased significantly as compared with that of the control group [(75.50 ± 2.04)%] ($P < 0.05$). Compared with the hemorrhage group, the brain water contents of the drug-treated group [(76.69 ± 2.15)%], TLR4-knockout hemorrhage group [(76.40 ± 2.04)%] and TLR4-knockout hemorrhage + drug-treated group [(76.24 ± 2.11)%] declined significantly ($P < 0.05$). Compared with the drug-treated group, the brain water content of the TLR4-knockout hemorrhage group did not show significant changes ($P > 0.05$). Then, compared with the TLR4-knockout hemorrhage group, the brain water content of the TLR4-knockout hemorrhage + drug-treated group also had no significant changes ($P > 0.05$).

3.3. Comparison of various inflammatory factors of each group

ELISA was used to determine the levels of inflammatory factors after treated for seven days. The results showed that the

Table 2
Comparison of the inflammatory factors and apoptotic protein of each group (n = 12).

Group	Inflammatory factors (pg/mg)			Apoptotic protein	
	IL-6	TNF-α	IL-1β	Bax	Bcl-2
Control group	25.54 ± 3.94	18.12 ± 3.65	28.21 ± 4.12	0.923 ± 0.039	0.978 ± 0.024
Hemorrhage group	221.23 ± 42.30*	83.32 ± 10.82*	563.19 ± 68.33*	3.146 ± 0.432*	0.124 ± 0.009*
Drug-treated group	134.56 ± 16.69#	58.28 ± 7.70#	147.36 ± 17.60#	1.683 ± 0.384#	0.773 ± 0.018#
TLR4-knockout hemorrhage group	132.61 ± 17.74#	60.32 ± 8.11#	144.98 ± 18.24#	1.669 ± 0.401#	0.779 ± 0.020#
TLR4-knockout hemorrhage + drug-treated group	135.56 ± 15.89#	58.47 ± 7.84#	145.58 ± 17.95#	1.674 ± 0.421#	0.780 ± 0.019#

Compared with the control group, *$P < 0.05$; compared with the hemorrhage group, #$P < 0.05$.

levels of inflammatory factors IL-6, TNF-α and IL-1β in mice of the hemorrhage group increased significantly as compared with those of the control group ($P < 0.05$); compared with the hemorrhage group, the levels of inflammatory factors IL-6, TNF-α and IL-1β in mice of the drug-treated group, TLR4-knockout hemorrhage group and TLR4-knockout hemorrhage + drug-treated group decreased significantly ($P < 0.05$); compared with the drug-treated group, the levels of those inflammatory factors of the TLR4-knockout hemorrhage group did not showed significant changes ($P > 0.05$); finally, compared with the TLR4-knockout hemorrhage group, those levels of the TLR4-knockout hemorrhage + drug-treated group also had no significant changes ($P > 0.05$) (Table 2).

3.4. Comparison of expression of apoptotic proteins of each group

Results of Western blot showed that the expression quantity of apoptotic protein Bax in the hemorrhage group increased significantly as compared with that in the control group, while its anti-apoptotic protein Bcl-2 expression quantity significantly reduced ($P < 0.05$); compared with the hemorrhage group, the expression quantities of apoptotic protein Bax in mice of the drug-treated group, TLR4-knockout hemorrhage group and TLR4-knockout hemorrhage + drug-treated group decreased significantly, but their anti-apoptotic protein Bcl-2 expression quantities increased significantly ($P < 0.05$); compared with the drug-treated group, the expressions of apoptotic proteins in the TLR4-knockout hemorrhage group had no significant changes ($P > 0.05$), and compared with the TLR4-knockout hemorrhage group, the expressions of the apoptotic proteins of the TLR4-knockout hemorrhage + drug-treated group did not show significant changes as well ($P > 0.05$) (Table 2).

4. Discussion

ICH models of normal mice and TLR4-knockout mice were established by autologous ICH. Experiments involved 5 groups. The results of the experiments demonstrated that the brain water content and the levels of inflammatory factors IL-6, TNF-α and IL-1β in mice of the hemorrhage group increased significantly as compared with those of the control group. The neurological function in mice of the control group was not affected. However, the neurological function in mice of the hemorrhage group was damaged significantly from the first day of the experiment. Western blot method was used to further detect the expressions of apoptotic proteins, and the results revealed that the expression of apoptotic protein Bax in the hemorrhage group increased significantly as compared with that in the control group, while its anti-apoptotic protein Bcl-2 expression reduced significantly. Differences in the above results were all statistically significant. It could be told from the results that ICH would cause tissue edema and nerve function impairment, induce the release of inflammatory factors and the expression of apoptotic protein Bax and inhibit the expression of anti-apoptotic protein Bcl-2. While compared with the hemorrhage group, the brain water content and neurological defect score in mice of the drug-treated group decreased significantly, their release of the inflammatory factors also declined significantly and their expressions of apoptotic proteins Bax and Bcl-2 were improved after they were treated with Zhuoyu Annao pill for 7 d. The results indicated that Zhuyu Annao pill can alleviate encephal edema for mice with ICH, improve neurological function, reduce inflammatory response sand inhibit nerve cell apoptosis, which plays a protective role for brains. In Zhuyu Annao pill, peach kernel, *Campsis grandiflora*, leech and ground beetle serve as the major components, which have effects of resolving the hard, removing stasis, breaking blood and expelling blood stasis without harming the healthy qi. The adjuvant drugs could eliminate and dredge qi stagnation and blood stasis in brain vessel and lead the major drugs to the brain directly, which makes *Lumbricus* dart in the cerebral vein so as to eliminate toxins. The combination of those drugs could remove blood stasis, alleviate water retention, promote blood circulation to remove blood stasis, produce saliva and detoxify and recover spiritual mechanism [13].

TLR4 plays its biological role mainly by two ways, dependent and independent MyD88. TRIF-TRAM is the way of independent MyD88 mainly by activating TRAM (the TRIF-related adaptor molecule of TLR4) so as to induce the activation of downstream signal molecules TRIF and transforming growth factor β activated kinase (TBK1) and finally induce the activation of interferon regulation factor 3 (IRF3) and the production of interferon-β [14]. In addition, TRAM can also activate NF-κB and induce the production of inflammatory factors such as IL-10, TNF-α and IL-1β. That is the main mechanism of action of dependent MyD88 pathway [15]. The effect of TLR4 on systemic inflammatory response syndrome is comparatively clear. Gram-negative bacterial infections cause the secretion of endotoxin and activate TLR4. TLR4 could activate NF-κB and produce inflammatory factors by two ways and then lead to inflammatory damage to the bodies. Moreover, inflammatory factors would decline the expression of scavenger receptor-A (SR-A) and reduce the phagocytosing scavenging effect on endotoxin of cells. The long-term preservation of LPS would cause the mass activation of TLR4, which could trigger inflammatory cascade effect, cause overwhelming inflammation, aggravate the damage to body and eventually lead to systemic inflammatory response syndrome [16,17]. Animal studies illustrated that after ICH the activated microglial cells could

increase the release of cytokines to aggravate inflammation damage, while inhibiting the activation of the microglial cells specially could improve neurological function markedly [18,19]. Furthermore, heme, the lysate of red blood cells after ICH, can affect TLR4, mediate the activation of microglial cells, induce the mass production of inflammatory cytokines and, as a result, cause inflammation damage [20–28]. The results of this study demonstrated that the neurological defect scores, brain water content, levels of inflammatory factors IL-10, TNF-α and IL-1β and apoptotic protein Bax in the TLR4-knockout group all decreased significantly as compared with those of the hemorrhage group, while its anti-apoptotic protein Bcl-2 expression significantly increased. Serving as ICH models together, mice without *TLR4* gene showed significantly alleviated encephaledema and nerve function impairment and reduced inflammatory factor release and cell apoptosis after ICH. Wang *et al* [29] found that the infiltration of monocytes and neutrophil granulocytes in mice reduced, peripheral inflammation decreased and the recovery of functions were improved as well after knocking out TLR4. TLR4 itself does not mediate the phagocytosis capacity of cells. It only serves as a recognition receptor instead. So, after knocking out TLR4, the absorption of edema was promoted. The possible mechanism to reduce inflammatory damage was: after ICH occurs, TLR4 expressed by microglial cells and peripheral mononuclear macrophages in central nervous system collaboratively mediated the damage of ICH. There are researches claiming that the activation of the TLR4 signal pathway could regulate the expression of CD36 negatively, which consequently weakens the hematoma clearance ability of the body [30,31]. Its intensive mechanism of action is still needed to be confirmed. Our study manifested that TLR4 could mediate inflammatory damage after ICH and TLR4 may plays an important role in inflammatory responses after brain injury. Besides, targetedly suppressing the expression of TLR4 might help to delay and control the development of ICH.

In order to further explore whether Zhuoyu Annao pill has played its protective role through TLR4, ICH mice were given Zhuoyu Annao pill on the basic of knocking out TLR4 in this study. The results found that the brain water content, neurological defect score, levels of inflammatory factors and apoptotic proteins in mice of the TLR4-knockout hemorrhage + drug-treated group all showed no significant improvement. But before that, we found in researches that Zhuoyu Annao pill could definitely improve ICH and has a protective effect for brains. Therefore, we speculated that the effects of Zhuoyu Annao pill including promoting the hematoma absorption, protecting neurological function and reducing inflammatory response and nerve cell apoptosis might have something to do withTLR4 pathway. Of course, the specific effect of Zhuoyu Annao pill and its exact components playing the effect still need subsequent experiments for intensive study.

Conflict of interest statement

We declare that we have no conflict of interest.

References

[1] Wang X, Arima H, Yang J, Zhang S, Wu G, Woodward M, et al. Mannitol and outcome in intracerebral hemorrhage: propensity score and multivariable intensive blood pressure reduction in acute cerebral hemorrhage trial 2 results. *Stroke* 2015; **46**(10): 2762-2767.

[2] Hong KS, Kim BJ, Lee JY, Kwon SU, PICASSO Investigators. Rationale and design of the PreventIon of CArdiovascular events in iSchemic Stroke patients with high risk of cerebral hemOrrhage (PICASSO) study: a randomized controlled trial. *Int J Stroke* 2015; **10**(7): 1153-1158.

[3] Pan W, Yan Q, Qin M, Jin G, Sun J, Ning X, et al. Detection of cerebral hemorrhage in rabbits by time-difference magnetic inductive phase shift spectroscopy. *PLoS One* 2015; **10**(5): e0128127.

[4] Wang X, Arima H, Heeley E, Delcourt C, Huang Y, Wang J, et al. Magnitude of blood pressure reduction and clinical outcomes in acute intracerebral hemorrhage: intensive blood pressure reduction in acute cerebral hemorrhage trial study. *Hypertension* 2015; **65**(5): 1026-1032.

[5] He L, Ma Q, Wang Y, Liu X, Yuan Y, Zhang Y, et al. Association of variants in *KCNK17* gene with ischemic stroke and cerebral hemorrhage in a Chinese population. *J Stroke Cerebrovasc Dis* 2014; **23**(9): 2322-2327.

[6] Wu J, Chen J, Guo H, Peng F. Effects of high-pressure oxygen therapy on brain tissue water content and AQP4 expression in rabbits with cerebral hemorrhage. *Cell Biochem Biophys* 2014; **70**(3): 1579-1584.

[7] Wang J, Grishin AV, Ford HR. Experimental anti-Inflammatory drug semapimod inhibits TLR signaling by targeting the TLR chaperone gp96. *J Immunol* 2016; **196**(12): 5130-5137.

[8] Ciaramelli C, Calabrese V, Sestito SE, Pérez-Regidor L, Klett J, Oblak A, et al. Glycolipid-based TLR4 modulators and fluorescent probes: rational design, synthesis, and biological properties. *Chem Biol Drug Des* 2016; **88**(2): 217-229.

[9] Hu QP, Mao DA. Histone deacetylase inhibitor SAHA attenuates post-seizure hippocampal microglia TLR4/MYD88 signaling and inhibits *TLR4* gene expression via histone acetylation. *BMC Neurosci* 2016; **17**(1): 22.

[10] Gao HK, Zhou ZG, Li Y, Chen YQ. Toll-like receptor 4 Asp299Gly polymorphism is associated with an increased risk of pancreatic necrotic infection in acute pancreatitis: a study in the Chinese population. *Pancreas* 2007; **34**(3): 295-298.

[11] Chattopadhyay S, Veleeparambil M, Poddar D, Abdulkhalek S, Bandyopadhyay SK, Fensterl V, et al. EGFR kinase activity is required for TLR4 signaling and the septic shock response. *EMBO Rep* 2015; **16**(11): 1535-1547.

[12] Wei L, Xue R, Zhang P, Wu Y, Li X, Pei F. (1)H NMR-based metabolomics and neurotoxicity study of cerebrum and cerebellum in rats treated with cinnabar, a traditional Chinese medicine. *OMICS* 2015; **19**(8): 490-498.

[13] Zheng FK, Wu L, Chen W, Yang JP, Wen HJ. The clinical research of Zhuyu Annao pill in acute cerebral hemorrhage. *J Emerg Trad Chin Med* 2011; **7**(10): 1555-1556.

[14] Chung YH, Kim D. Enhanced TLR4 expression on colon cancer cells after chemotherapy promotes cell survival and epithelial-mesenchymal transition through phosphorylation of GSK3β. *Anticancer Res* 2016; **36**(7): 3383-3394.

[15] Church JS, Kigerl KA, Lerch JK, Popovich PG, McTigue DM. TLR4 deficiency impairs oligodendrocyte formation in the injured spinal cord. *J Neurosci* 2016; **36**(23): 6352-6364.

[16] Lahiri R, Derwa Y, Bashir Z, Giles E, Torrance HD, Owen HC, et al. Systemic inflammatory response syndrome after major abdominal surgery predicted by early upregulation of TLR4 and TLR5. *Ann Surg* 2016; **263**(5): 1028-1037.

[17] Calvano JE, Agnese DM, Um JY, Goshima M, Singhal R, Coyle SM, et al. Modulation of the lipopolysaccharide receptor complex (CD14, TLR4, MD-2) and toll-like receptor 2 in systemic inflammatory response syndrome-positive patients with and without infection: relationship to tolerance. *Shock* 2003; **20**(5): 415-419.

[18] Rodríguez-Yáñeza M, Breaa D, Ariasa S, Blancoa M, Pumar JM, Castillo J, et al. Increased expression of Toll-like receptors 2 and 4 is associated with poor outcome in intracerebral hemorrhage. *J Neuroimmunol* 2012; **247**(1–2): 75-80.

[19] Sansing LH, Harris TH, Welsh FA, Kasner SE, Hunter CA, Kariko K. Toll-like receptor 4 contributes to poor outcome after intracerebral hemorrhage. *Ann Neurol* 2011; **70**(4): 646-656.

[20] Wang YC, Zhou Y, Fang H, Lin S, Wang PF, Xiong RP, et al. Toll-like receptor 2/4 heterodimer mediates inflammatory injury in intracerebral hemorrhage. *Ann Neurol* 2014; **75**(6): 876-889.

[21] Yang Z, Liu B, Zhong L, Shen H, Lin C, Lin L, et al. Toll-like receptor-4-mediated autophagy contributes to microglial activation and inflammatory injury in mouse models of intracerebral haemorrhage. *Neuropathol Appl Neurobiol* 2015; **41**(4): e95-e106.

[22] Cai J, Yuan H, Wang Q, Yang H, Al-Abed Y, Hua Z, et al. HMGB1-driven inflammation and intimal hyperplasia after arterial injury involves cell-specific actions mediated by TLR4. *Arterioscler Thromb Vasc Biol* 2015; **35**(12): 2579-2593.

[23] Ghosh M, Subramani J, Rahman MM, Shapiro LH. CD13 restricts TLR4 endocytic signal transduction in inflammation. *J Immunol* 2015; **194**(9): 4466-4476.

[24] Xu D, Yan S, Wang H, Gu B, Sun K, Yang X, et al. IL-29 enhances LPS/TLR4-mediated inflammation in rheumatoid arthritis. *Cell Physiol Biochem* 2015; **37**(1): 27-34.

[25] Ren W, Wang Z, Hua F, Zhu L. Plasminogen activator inhibitor-1 regulates LPS-induced TLR4/MD-2 pathway activation and inflammation in alveolar macrophages. *Inflammation* 2015; **38**(1): 384-393.

[26] Nair AR, Ebenezer PJ, Saini Y, Francis J. Angiotensin II-induced hypertensive renal inflammation is mediated through HMGB1-TLR4 signaling in rat tubulo-epithelial cells. *Exp Cell Res* 2015; **335**(2): 238-247.

[27] Zhang Z, Gao X, Cao Y, Jiang H, Wang T, Song X, et al. Selenium deficiency facilitates inflammation through the regulation of TLR4 and TLR4-related signaling pathways in the mice uterus. *Inflammation* 2015; **38**(3): 1347-1356.

[28] Chen Z, Liu X, Yu G, Chen H, Wang L, Wang Z, et al. Ozone therapy ameliorates tubulointerstitial inflammation by regulating TLR4 in adenine-induced CKD rats. *Ren Fail* 2016; **38**(5): 822-830.

[29] Wang YC, Wang PF, Fang H, Chen J, Xiong XY, Yang QW. Toll-like receptor 4 antagonist attenuates intracerebral hemorrhage-induced brain injury. *Stroke* 2013; **44**(9): 2545-2552.

[30] Fang H, Chen J, Lin S, Wang P, Wang Y, Xiong X, et al. CD36-mediated hematoma absorption following intracerebral hemorrhage: negative regulation by TLR4 signaling. *J Immunol* 2014; **192**(12): 5984-5992.

[31] Chávez-Sánchez L, Garza-Reyes MG, Espinosa-Luna JE, Chávez-Rueda K, Legorreta-Haquet MV, Blanco-Favela F. The role of TLR2, TLR4 and CD36 in macrophage activation and foam cell formation in response to oxLDL in humans. *Hum Immunol* 2014; **75**(4): 322-329.