Int J Clin Exp Med 2017;10(5):7843-7850
www.ijcem.com /ISSN:1940-5901/IJCEM0020573

Original Article

Xanthohumol promotes neuronal and behavioral recovery by suppressing inflammatory response and apoptosis in a rat model of intracerebral hemorrhage

Yang Wang[1], Yidan Zhang[2], Wei Ming[2], Xi Jin[2], Kaihua Zhai[3], Boyu Liu[4], Hongying Bai[3], Xingshan Liu[1]

[1]Colleges of Nursing, Changchun University of Chinese Medicine, Changchun 130117, Jilin, People's Republic of China; [2]The Affiliated Hospital to Changchun University of Chinese Medicine, Changchun 130103, People's Republic of China; [3]Department of Internal Neurology, Second Affiliated Hospital of Zhengzhou University, Zhengzhou 450014, Henan, People's Republic of China; [4]Department of Endocrinology, Third Affiliated Hospital of Xinxiang Medical University, Xinxiang 453000, Henan, People's Republic of China

Received November 26, 2015; Accepted April 13, 2016; Epub May 15, 2017; Published May 30, 2017

Abstract: Exaggerated inflammation and apoptosis are two pivotal pathological processes for secondary brain injury after intracerebral hemorrhage (ICH). Xanthohumol (XN) is a prenylated chalcone derived from hops (Humuluslupulus L.) and has been reported to possess neuroprotective activity. However, the roles of XN in ICH are still unclear. Therefore, the present study was designed to determine the possible mechanisms of action of neuroprotective properties of XN after ICH. In this study, we found that XN significantly increased the neurologic score and reduced the brain water content at 24 h after ICH. XN also attenuated neuronal apoptosis and the expression of pro-inflammatory mediators after ICH in rat brains. Furthermore, XN significantly suppressed p65 phosphorylation in the brain after ICH. In conclusion, our present study demonstrated for the first time that XN promotes neuronal and behavioral recovery by suppressing inflammatory response and apoptosis in a rat model of ICH. Thus, XN may be a promising strategy to inhibit inflammation and neurological impairment in ICH.

Keywords: Intracerebral hemorrhage (ICH), Xanthohumol (XN), inflammation, apoptosis

Introduction

Intracerebral hemorrhage (ICH) accounts for approximately 10% to 15% of all strokes, and is associated with high mortality and morbidity rate throughout the world [1, 2]. It is characterized by the rupture of cerebral blood vessels and subsequent leakage of blood, including blood-intrinsic factors, into the brain parenchyma [3]. Despite its importance, there is a lack of effective treatments for ICH patients in clinics. So, there is an urgent need to develop new treatments for ICH.

Increasing evidences have reported that the inflammatory response after ICH was able to contribute to brain injury [4, 5]. Previous studies demonstrated that nuclear factor κB (NF-κB) is a predominant regulator for inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) and plays an important role after ICH [6]. Furthermore, neuronal apoptosis is observed in experimental ICH and is correlated with neurological function [7, 8]. Therefore, inhibiting inflammatory response and neuronal apoptosis may be important therapeutic approaches for ICH.

Xanthohumol (XN) is a prenylatedchalcone derived from hops (Humuluslupulus L.). Many studies have demonstrated that XN possesses many physiological properties, such as anti-tumor, anti-angiogenic, anti-inflammatory and anti-oxidant potential [9-12]. Most recently, one study showed that XN possesses potent neuroprotective activity, and this activity is mediated, at least in part, by inhibition of inflammatory responses, apoptosis and platelet activation, resulting in a reduction of infarct volume and improvement in neuro-behavior in rats with cerebral ischemia [13]. However, the roles of XN in ICH are still unclear. Therefore, the present study was designed to determine the possible mechanisms of neuroprotective properties of

XN after ICH. Our findings demonstrate that XN could inhibit ICH-induced inflammatory response and apoptosis.

Materials and methods

Animals

Adult male Sprague-Dawley rats (weighing approximately 280-300 g) were obtained from the Animal Breeding Center of Changchun University of Chinese Medicine (China). The animals were housed under conventional conditions at 22°C, 50-60% humidity and a 12 h light/12 h dark cycle. Rats were provided free access to food and water. All of the experiments performed in this study were approved by Changchun University of Chinese Medicine Committee on Animal Care and strictly abided by the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Establishment of the ICH model

ICH model was induced by intrastriatal injection of bacterial collagenase as previously described [14]. In brief, rats were anesthetized with 3.6% chloral hydrate and placed in a stereotaxic frame. An incision was made to expose the bregma, and a burr hole was drilled at the injection site: 3.0 mm left lateral to the midline, 0.2 mm posterior to bregma, 6 mm in depth below the skull. Using a syringe pump, 0.5 U type VII bacterial collagenase in 1 μL sterile saline was infused slowly (0.5 μL/min) into the central striatum. To prevent reflux, collagenase was delivered over 5 min and the needle remained in place for additional 10 min after injection. Rats in the sham group received an equal volume of sterile saline in the same manner.

Experimental design

Rats were randomly assigned to four groups: the sham group (n=20), the ICH + vehicle group (n=20), the ICH + XN 10 mg/kg group (n=20) and the ICH + XN 50 mg/kg group (n=20). Different concentrations of XN (Sigma-Aldrich, St. Louis, MO, USA) were administered i.p. to each animal 1 h after ICH. All efforts were made to minimize suffering. Based on a prior study, all of the animals were sacrificed at 24 h after the operation.

Neurological scoring

Neurological scores were assessed at 24 h after ICH as described previously [15]. The evaluation consists of six tests that can be scored 1-3. These six tests include: spontaneous activity; symmetry in the movement of all four limbs; forepaw outstretching; climbing; body proprioception; and response to whisker stimulation. The maximum score is 18 and the minimum is 3. Higher scores indicate greater function. The neurological evaluation was performed before surgery and at 24 h after ICH.

Hemorrhagic injury analysis

Injury volume was quantified using coronal sections stained with cresyl violet at 20 rostral-caudal levels that were spaced 200 μm apart. Hemorrhagic volumes were calculated using Image Pro Plus 6.0 software (Media Cybernetics, USA).

Measurement of the brain water content

Brain water content was measured at 24 h after ICH as described previously [16]. After euthanasia and decapitation, the brains were quickly removed and immediately weighted as wet weight. Then, samples were placed in an oven for 72 h at 100°C to obtain the dry weight. The brain water content (%) was calculated as a percentage by using the following method: (wet weight-dry weight)/wet weight ×100%.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the brain tissue with using the Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. qRT-PCR was performed with a SuperRT One Step RT-PCR Kit (Biotechnology Jiangsu, China) according to the manufacturer's instructions. The specific primers for TNF-α were sense, 5'-CACGTCGTAGCAAACC-3' and antisense, 5'-GGTGAGGAGCACATAGT-3'; IL-6 were sense, 5'-ATATACCACTTCACAAGTCGG-3' and antisense, 5'-GGCAAATTTCCTGGTTATATCC-3'; and for β-actin were sense, 5'-AAA TCGTGCGTGACATCAAAGA-3' and antisense, 5'-GGCCATCTCCTGCTCGAA-3'. Relative quantification of gene expression was performed using the $2^{-\Delta\Delta Ct}$ method, with β-actin mRNA as an internal control.

Western blot

The total protein samples were extracted from the brain tissue using RIPA lysis buffer (Beyotime, Nantong, China). The protein content was determined by BCA protein assay

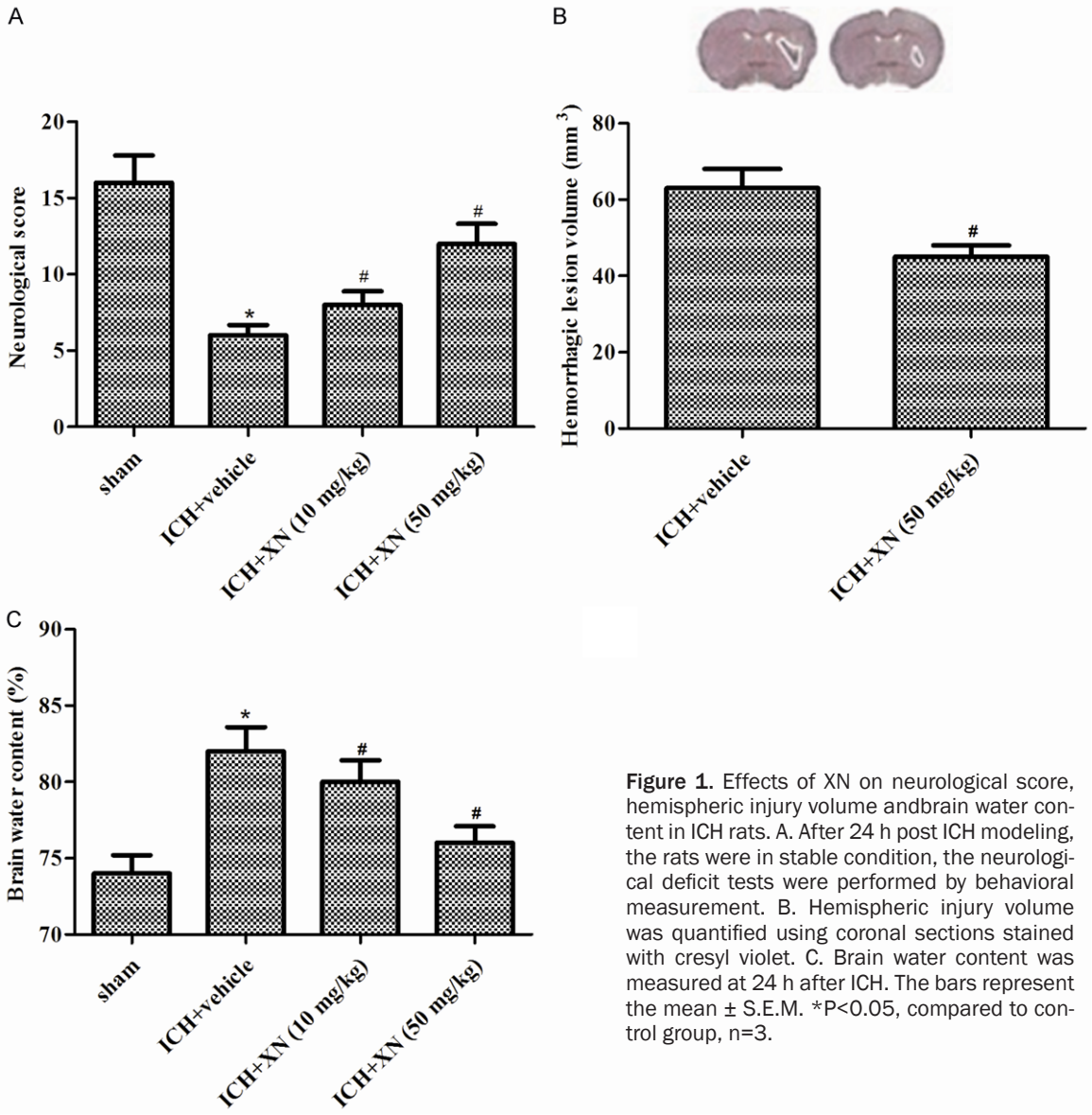

Figure 1. Effects of XN on neurological score, hemispheric injury volume andbrain water content in ICH rats. A. After 24 h post ICH modeling, the rats were in stable condition, the neurological deficit tests were performed by behavioral measurement. B. Hemispheric injury volume was quantified using coronal sections stained with cresyl violet. C. Brain water content was measured at 24 h after ICH. The bars represent the mean ± S.E.M. *P<0.05, compared to control group, n=3.

(Pierce, Rockford, IL). Forty micrograms of protein per lane were separated by 10% SDS-PAGE and electroblotted onto nitrocellulose membranes (Amersham Pharmacia, Munich, Germany). The membrane was then blocked with 5% skim milk and then incubated with primary antibodies overnight at 4°C. The primary antibodies were as follows: anti-TNF-α, anti-IL-6, anti-Bax, anti-Bcl-2, anti-cleaved caspase-3, anti-p-NF-κB p65, anti-NF-κB p65 and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Binding of the primary antibody was detected by peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Bedford, MA, USA) Band pattern was analyzed with Quantity One 4.1.0 software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Results were expressed as mean ± S.E.M. of three independent experiments performed in triplicates. One-way ANOVA followed by Dunnett's t-test was used for statistical analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of XN on neurological score, hemispheric injury volume and brain water content in ICH rats

First, we evaluated the effect of XN on neurological score in ICH rats. At 24 h after ICH, the neurologic score was obviously decreased in

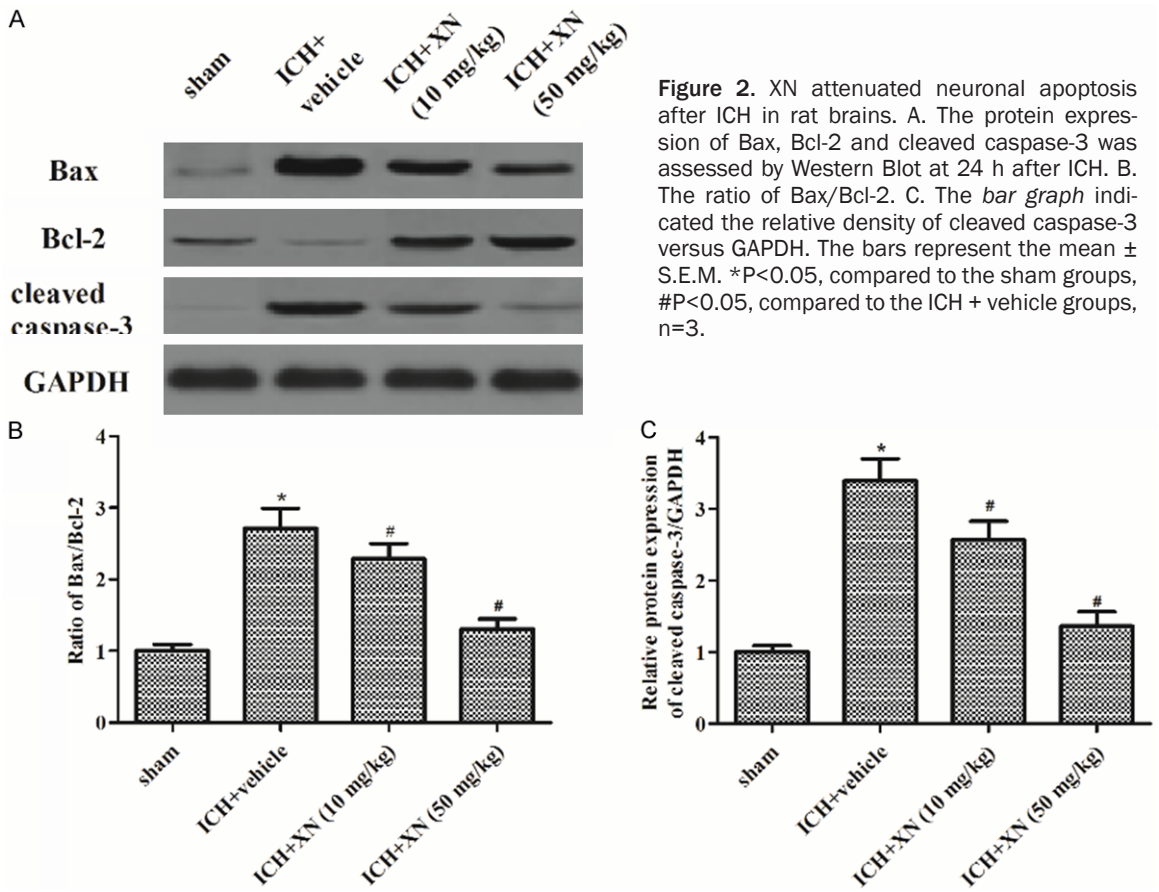

Figure 2. XN attenuated neuronal apoptosis after ICH in rat brains. A. The protein expression of Bax, Bcl-2 and cleaved caspase-3 was assessed by Western Blot at 24 h after ICH. B. The ratio of Bax/Bcl-2. C. The *bar graph* indicated the relative density of cleaved caspase-3 versus GAPDH. The bars represent the mean ± S.E.M. *P<0.05, compared to the sham groups, #P<0.05, compared to the ICH + vehicle groups, n=3.

the ICH + vehicle group, as compared with the sham group. However, XN significantly increased the neurologic score at 24 h after ICH, compared with the ICH + vehicle group (**Figure 1A**). The hemorrhagic lesion volume for the XN group was significantly decreased compared with the ICH + vehicle group at 24 h post-ICH (**Figure 1B**). Then, the effect of XN on brain water content in ICH rats was also determined. The brain water content in the ICH + vehicle group was greatly increased compared with the sham group at 24 h after ICH. XN significantly reduced the increase in brain water content induced by ICH, as compared with the ICH + vehicle group (**Figure 1C**).

XN attenuated neuronal apoptosis after ICH in rat brains

To investigate the effect of XN on neuronal apoptosis, the expression ratio of Bcl-2 to Bax, key factors of the apoptotic signal pathway, was analyzed in rat brains after ICH. As shown in **Figure 2**, in comparison with the sham group, ICH significantly increased the expression of Bax, decreased the expression of Bcl-2. However, after treatment with XN, expression of Bax protein was lower, while expression of Bcl-2 protein was higherin rat brains, as compared with the ICH + vehicle group. Then, we determined the effect of XN on cleaved caspase 3 expression using Western blot. ICH significantly increased the expression of cleaved caspase 3 as compared with sham-treated group. However, XN treatment obviously decreased the expression of cleaved caspase 3, as compared with the ICH + vehicle group.

XN attenuated the release of pro-inflammatory mediators after ICH in rat brains

Accumulating evidences suggested a pivotal role of pro-inflammatory cytokines such as TNF-α and IL-6 in the development and the pathogenesis of ICH. Therefore, we investigated the effect XN on the expression of pro-inflammatory mediators after ICH in rat brains. As shown in **Figure 3A** and **3B**, RT-PCR analysis demonstrated that the mRNA expression of TNF-α and IL-6 was greatly upregulated in the

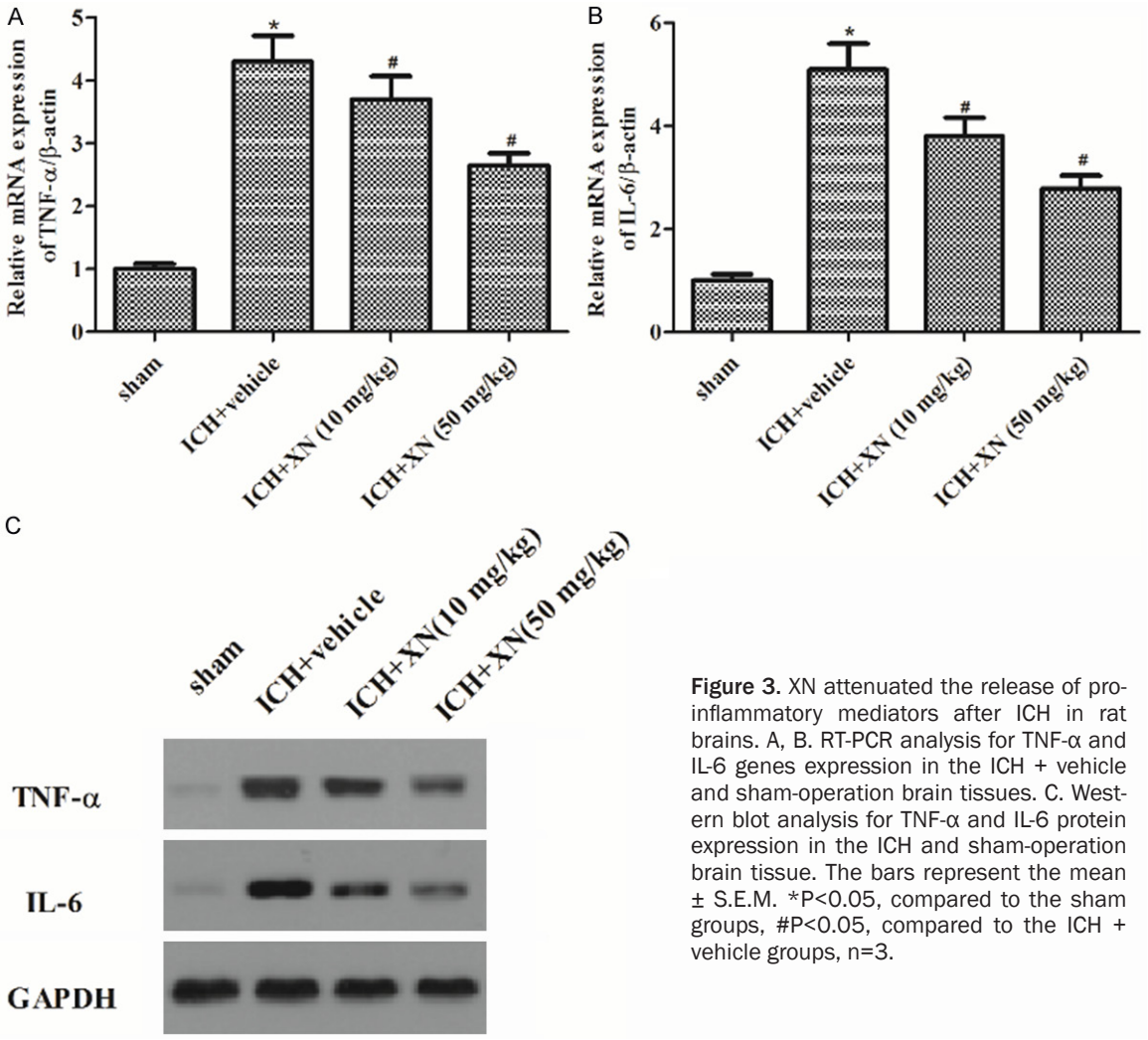

Figure 3. XN attenuated the release of pro-inflammatory mediators after ICH in rat brains. A, B. RT-PCR analysis for TNF-α and IL-6 genes expression in the ICH + vehicle and sham-operation brain tissues. C. Western blot analysis for TNF-α and IL-6 protein expression in the ICH and sham-operation brain tissue. The bars represent the mean ± S.E.M. *P<0.05, compared to the sham groups, #P<0.05, compared to the ICH + vehicle groups, n=3.

ICH + vehicle group compared with the sham group at 24 h after ICH, whereas, XN significantly decreased the upregulation of TNF-α and IL-6 compared with the ICH + vehicle group. Similarly, the results of Western blot showed that XN attenuated the protein expression of TNF-α and IL-6 after ICH in rat brains (**Figure 3C**).

XN inhibited the activation of NF-κB signaling pathway

It is known that NF-κB is involved in the brain injury after ICH. Thus, we examined the effect of XN on NF-κB activation in the brain after ICH. As shown in **Figure 4**, the expression of p65 phosphorylation was enhanced in the ICH + vehicle group compared with the sham group at 24 h after ICH. However, XN significantly suppressed p65 phosphorylation compared with the ICH + vehicle group.

Discussion

In this study, we found that XN significantly increased the neurologic score and reduced the brain water content at 24 h after ICH. XN also attenuated neuronal apoptosis and the expression of pro-inflammatory mediators after ICH in rat brains. Furthermore, XN significantly suppressed p65 phosphorylation in the brain after ICH.

Brain edema is a pathological process for secondary brain injury after ICH and constitutes the main cause of increased intracranial pressure, resulting in brain herniation. Multiple lines of evidence have reported that the degree ofcerebral edema is correlated with poor neurological outcome in patients [17, 18]. XN was found to be a potent neuro-protective agent [19-21]. Yen *et al*. reported that treatment with XN dose-dependently attenuated focal cerebral isch-

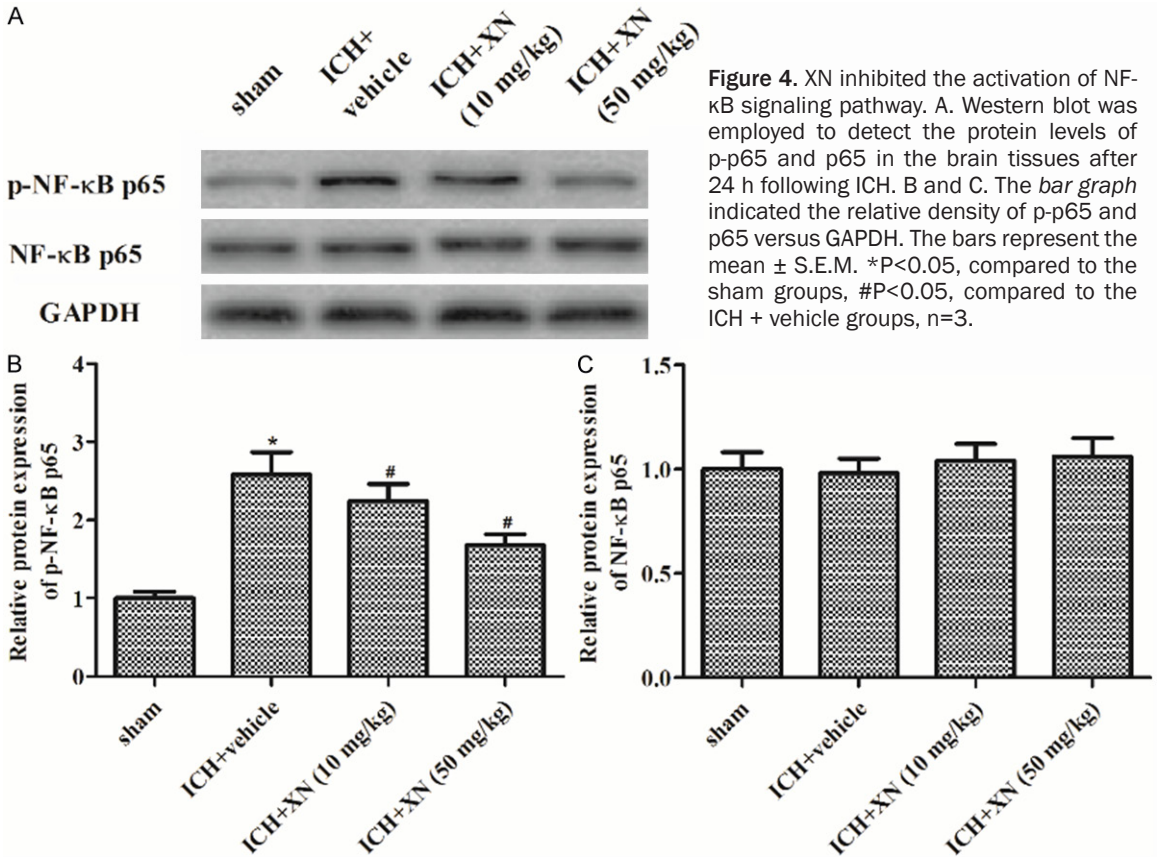

Figure 4. XN inhibited the activation of NF-κB signaling pathway. A. Western blot was employed to detect the protein levels of p-p65 and p65 in the brain tissues after 24 h following ICH. B and C. The *bar graph* indicated the relative density of p-p65 and p65 versus GAPDH. The bars represent the mean ± S.E.M. *P<0.05, compared to the sham groups, #P<0.05, compared to the ICH + vehicle groups, n=3.

emia and improved neurobehavioral deficits in rats with middle cerebral artery occlusion (MCAO) [13]. In line with the previous study, herein, we found that XN significantly increased the neurologic score and reduced the brain water content at 24 h after ICH. These results suggest that XN improved neurological deficits and attenuated brain edema in collagenase induced ICH rats.

Neuronal apoptosis is one of the most severe consequences after ICH [7]. Previous studies reported that up-regulation of pro-apoptotic Bax levels and concurrent down-regulation of anti-apoptotic protein Bcl-2 induced the release of cytochrome c and activation of caspases and eventually resulted in cell apoptosis [22]. Caspase-3, known as the death enzyme, has a critical role in the controlled execution of programmed cell death [23], and its expression is up-regulated in the brain after ICH [24]. In the present study, we found that XN treatment obviously decreased the expression of Bax and increased the expression of Bcl-2, as well as decreased the expression of cleaved caspase 3. These results suggest that XN could promote neural functional recovery in ICH rats by decreasing the number of apoptotic cells in the injured brain tissue.

Increasing evidence suggests that inflammation is a vital factor in the pathological progress of ICH [25, 26]. It has been reported that the expression of pro-inflammatory mediators, such as TNF-α and IL-6, is increased after ICH [27]. This is consistent with our findings that ICH obviously increased the expression of TNF-α and IL-6. However, XN attenuated the expression of pro-inflammatory mediators after ICH in rat brains. These results suggest that XNcould promote neural functional recovery in ICH rats by suppressing the expression of pro-inflammatory mediators.

NF-κB signaling pathway plays a critical role in inflammatory response [28]. NF-κB is activated and sustained elevated for several days after ICH, and it is associated with cell death in perihematomal brain tissue after ICH. Moreover, it was reported that pretreatment with XN almost completely blunted the ischemia/reperfusion (I/R)-induced AKT and NF-κB activation and the

expression of the pro-inflammatory genes, which are known to play an important role in the subacute phase of I/R-induced liver damage [29]. Another study showed that XN also significantly inhibited the excessive production of inflammatory mediators NO, IL-1β, and TNF-α, and the activation of NF-κB signaling in LPS-induced stimulated BV2 cells [30]. Consistent with these results, in the present study, we found that XN significantly suppressed p65 phosphorylation in the brain after ICH. These results suggest that XN inhibits inflammatory response and apoptosis in a rat model of ICH through suppressing the NF-κB signaling pathway.

In conclusion, our present study demonstrated for the first time that XN promotes neuronal and behavioral recovery by suppressing inflammatory response and apoptosis in a rat model of ICH. Thus, XN may be a promising strategy to inhibit inflammation and neurological impairment in ICH.

Disclosure of conflict of interest

None.

Address correspondence to: Xingshan Liu, Colleges of Nursing, Changchun University of Chinese Medicine, Changchun 130117, Jilin, People's Republic of China. Tel: +86-0431-86172319; Fax: +86-0431-86172319; E-mail: bai_hongying@sina.com

References

[1] van Asch CJ, Luitse MJ, Rinkel GJ, van der Tweel I, Algra A, Klijn CJ. Incidence, case fatality, and functional outcome of intracerebral haemorrhage over time, according to age, sex, and ethnic origin: a systematic review and meta-analysis. Lancet Neurol 2010; 9: 167-176.

[2] Ariesen MJ, Claus SP, Rinkel GJ, Algra A. Risk factors for intracerebral hemorrhage in the general population a systematic review. Stroke 2003; 34: 2060-2065.

[3] Ohnishi M, Monda A, Takemoto R, Matsuoka Y, Kitamura C, Ohashi K, Shibuya H, Inoue A. Sesamin suppresses activation of microglia and p44/42 MAPK pathway, which confers neuroprotection in rat intracerebral hemorrhage. Neuroscience 2013; 232: 45-52.

[4] Zhang X, Li H, Hu S, Zhang L, Liu C, Zhu C, Liu R, Li C. Brain edema after intracerebral hemorrhage in rats: the role of inflammation. Neurol India 2006; 54: 402-407.

[5] Gong C, Hoff JT, Keep RF. Acute inflammatory reaction following experimental intracerebral hemorrhage in rat. Brain Res 2000; 871: 57-65.

[6] Fang H, Wang PF, Zhou Y, Wang YC, Yang QW. Toll-like receptor 4 signaling in intracerebral hemorrhage-induced inflammation and injury. J Neuroinflamm 2013; 10: 27-27.

[7] Gong C, Boulis N, Qian J, Turner DE, Hoff JT, Keep RF. Intracerebral hemorrhage-induced neuronal death. Neurosurgery 2001; 48: 875-883.

[8] Qureshi AI, Suri MF, Ostrow PT, Kim SH, Ali Z, Shatla AA, Guterman LR, Hopkins LN. Apoptosis as a form of cell death in intracerebral hemorrhage. Neurosurgery 2003; 52: 1041-1048.

[9] Pan L, Becker H, Gerhäuser C. Xanthohumol induces apoptosis in cultured 40-16 human colon cancer cells by activation of the death receptor-and mitochondrial pathway. Mol NutrFood Res 2005; 49: 837-843.

[10] Albini A, Dell'Eva R, Vené R, Ferrari N, Buhler DR, Noonan DM, Fassina G. Mechanisms of the antiangiogenic activity by the hop flavonoid xanthohumol: NF-κB and Akt as targets. FASEB J 2006; 20: 527-529.

[11] Cho YC, Kim HJ, Kim YJ, Lee KY, Choi HJ, Lee IS, Kang BY. Differential anti-inflammatory pathway by xanthohumol in IFN-γ and LPS-activated macrophages. Intimmunopharmacology 2008; 8: 567-573.

[12] Lee KH. Articels: Inhibition of Matrix Metalloproteinases-12 (MMP-12) and Anti-oxidant Effect of Xanthohumol from Hop (Humulus Lupulus L.). Nat Prod Sci 2012; 18: 261-265.

[13] Yen TL, Hsu CK, Lu WJ, Hsieh CY, Hsiao G, Chou DS, Wu GJ, Sheu JR. Neuroprotective effects of xanthohumol, a prenylated flavonoid from hops (Humulus lupulus), in ischemic stroke of rats. J Agric Food Chem 2012; 60: 1937-1944.

[14] Lee ST, Chu K, Jung KH, Kim SJ, Kim DH, Kang KM, Hong NH, Kim JH, Ban JJ, Park HK, Kim SU, Park CG, Lee SK, Kim M, Roh JK. Anti-inflammatory mechanism of intravascular neural stem cell transplantation in haemorrhagic stroke. Brain 2008; 131: 616-629.

[15] Chen J, Li Y, Wang L, Zhang Z, Lu D, Lu M, Chopp M. Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats. Stroke 2001; 32: 1005-1011.

[16] Chu K, Jeong SW, Jung KH, Han SY, Lee ST, Kim M, Roh JK. Celecoxib induces functional recovery after intracerebral hemorrhage with reduction of brain edema and perihematomal cell death. J Cerebr Blood F Met 2004; 24: 926-933.

[17] Qureshi AI, Palesch YY, Martin R, Novitzke J, Cruz-Flores S, Ezzeddine MA, Goldstein JN, Hussein HM, Suri MF, Tariq N. Effect of systolic blood pressure reduction on hematoma expansion, perihematomal edema, and 3-month outcome among patients with intracerebral hemorrhage: results from the antihypertensive treatment of acute cerebral hemorrhage study. Arch Neurol 2010; 67: 570-576.

[18] Castillo J, Davalos A, Álvarez-Sabín J, Pumar J, Leira R, Silva Y, Montaner J, Kase C. Molecular signatures of brain injury after intracerebral hemorrhage. Neurology 2002; 58: 624-629.

[19] Zamzow DR, Elias V, Legette LL, Choi J, Stevens JF, Magnusson KR. Xanthohumol improved cognitive flexibility in young mice. Behav Brain Res 2014; 275: 1-10.

[20] Panickar KS, Jang S. Dietary and plant polyphenols exert neuroprotective effects and improve cognitive function in cerebral ischemia. Recent Pat Food Nutr Agric 2013; 5: 128-143.

[21] Yao J, Zhang B, Ge C, Peng S, Fang J. Xanthohumol, a Polyphenol Chalcone Present in Hops, Activating Nrf2 Enzymes To Confer Protection against Oxidative Damage in PC12 Cells. J Agr Food Chem 2015; 63: 1521-1531.

[22] Smaili SS, Hsu YT, Sanders KM, Russell JT, Youle RJ. Bax translocation to mitochondria subsequent to a rapid loss of mitochondrial membrane potential. Cell Death Differ 2001; 8: 909-920.

[23] Tawa P, Hell K, Giroux A, Grimm E, Han Y, Nicholson DW, Xanthoudakis S. Catalytic activity of caspase-3 is required for its degradation: stabilization of the active complex by synthetic inhibitors. Cell Death Differ 2004; 11: 439-447.

[24] Simard JM, Geng Z, Woo SK, Ivanova S, Tosun C, Melnichenko L, Gerzanich V. Glibenclamide reduces inflammation, vasogenic edema, and caspase-3 activation after subarachnoid hemorrhage. J Cereb Blood Flow Metab 2009; 29: 317-330.

[25] Wasserman JK, Schlichter LC. Neuron death and inflammation in a rat model of intracerebral hemorrhage: effects of delayed minocycline treatment. Brain Res 2007; 1136: 208-218.

[26] Kleinig TJ, Vink R. Suppression of inflammation in ischemic and hemorrhagic stroke: therapeutic options. Curr Opin Neurol 2009; 22: 294-301.

[27] Wasserman JK, Zhu X, Schlichter LC. Evolution of the inflammatory response in the brain following intracerebral hemorrhage and effects of delayed minocycline treatment. Brain Res 2007; 1180: 140-154.

[28] Tak PP, Firestein GS. NF-κB: a key role in inflammatory diseases. J Clin Invest 2001; 107: 7.

[29] Dorn C, Massinger S, Wuzik A, Heilmann J, Hellerbrand C. Xanthohumol suppresses inflammatory response to warm ischemia-reperfusion induced liver injury. Exp Mol Pathol 2013; 94: 10-16.

[30] Lee IS, Lim J, Gal J, Kang JC, Kim HJ, Kang BY, Choi HJ. Anti-inflammatory activity of xanthohumol involves heme oxygenase-1 induction via NRF2-ARE signaling in microglial BV2 cells. Neurochem Int 2011; 58: 153-160.