Effect of Urinary Kallidinogenase on Transforming Growth Factor-β_1 and High-Sensitivity C-Reactive Protein Expression in Rat Focal Cerebral Ischemic Injury

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

BE 1 Ting-Fang Dong
BD 2 Hai-Xia Lv
DF 3 Xiao-Lu Niu
CEG 3 Yong-Kun Gui
AEG 3 Ping Zhang
BF 3 Hai-Qing Yan
DF 3 Tong Li

1 Department of Pharmacy, The First Afiliated Hospital of Xinxiang Medical College, Weihui, Henan, P.R. China
2 School of Pharmacy, Xinxiang Medical University, Xinxiang, Henan, P.R. China
3 Department of Neurology, The First Afiliated Hospital of Xinxiang Medical College, Weihui, Henan, P.R. China

Corresponding Author: Ping Zhang, e-mail: pingzhangdoc@126.com
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Background: In this study we investigated the effect of urinary kallidinogenase (UK) on transforming growth factor beta 1 (TGF-β_1) expression in brain tissue. We also explored the neuroprotective mechanism of UK against ischemic injury by measuring serum high-sensitivity C-reactive protein (hs-CRP) level changes after rat cerebral ischemic injury.

Material/Methods: The rat middle cerebral artery ischemia/reperfusion model was established using the suture method. Sprague-Dawley rats were randomly divided into 3 groups: treatment, *Gegen* control, and blank control. Each group was subsequently divided into 5 subgroups according to time (6, 12, 24, 48, and 72 h). Rats in the treatment group were administered UK as treatment. TGF-β_1 expression was observed at each time point using SABC and immunohistochemical staining methods to estimate cerebral infarct volume percentage. Serum hs-CRP levels were also measured.

Results: TGF-β_1 protein expression in ischemic brain tissues of the treatment group significantly increased at each time point ($P<0.01$) compared with both control groups. Treatment group serum hs-CRP levels significantly decreased at each time point ($P<0.05$) compared with both control groups.

Conclusions: UK exerts a neuroprotective effect by upregulating TGF-β_1 expression and inhibiting excessive inflammatory responses.

MeSH Keywords: Activating Transcription Factor 1 • Hypoxia-Ischemia, Brain • Neurology

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Background

The main chemical component of human urinary kallidinogenase (UK) is tissue kininogenase 1 (HKl). In plasma, HK1 catalyzes kininogen hydrolysis into kallidin, which can combine with G protein-coupled receptors (B1 and B2 receptors) in endothelial cells. Only the B2 receptor of kinin is found in normal tissues; B1 receptors are expressed only when damage is induced. In response to ischemic and cellular injuries, such as cerebral infarction, vascular endothelial cells at ischemic sites express B1 receptors. Kallidin then binds to the B1 receptor to promote nitric oxide release, thereby selectively dilating micro-arteries in ischemic tissues [1]. UK is widely used in clinical treatment.

Transforming growth factor beta 1 (TGF-β$_1$), a cytokine that can attenuate inflammation, is considered an effective immunomodulator. The relationship between TGF-β$_1$ and cerebral ischemia has recently gained research attention because the expression and generation of TGF-β$_1$ may provide insights into the protection and repair of ischemic/hypoxic brain tissues [2]. High-sensitivity C-reactive protein (hs-CRP) is a sensitive serum biochemical marker that reflects systemic inflammation and is involved in the occurrence and development of ischemic stroke. The ischemia was closely associated with inflammation. UK can promote angiogenesis and reduce inflammatory cell infiltration, which can protect neurons during ischemia. Increased TGF-β$_1$ expression may be a protective response to ischemic brain damage. C-reactive protein (CRP), a systemic inflammation indicator, may predict ischemic burden. It is important to illuminate the relationship between UK, TGF-β$_1$, and hs-CRP for ischemic therapy.

In this study, a focal cerebral ischemia model of rat middle cerebral artery occlusion via suture was established to observe the effect of UK on TGF-β$_1$ expression and serum hs-CRP level changes in rat brain and to explore the neuroprotective mechanism of UK against focal cerebral ischemia.

Material and Methods

Model preparation

The Henan Provincial Experimental Animal Centre (license number: SCXK (Yu) 2010-0002) provided 108 healthy male Sprague-Dawley rats (weight: 220–260 g). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Xinxiang Medical University. The rats were randomly divided into 3 groups: blank control group (BC, n=36), treatment group (TG,

n=36), and *Gegen* control group (GC, n=36). Rats in each group were further divided into 3 subgroups according to time: 6, 12, 24, 48, and 72 h. For each group, 12 rats were assigned to the 72-h time point, of which 6 were designated for tissue section and 6 were designated for triphenyltetrazolium chloride (TTC) staining to estimate infarct volume percentage. The remaining rats from each group were evenly divided among the remaining time points for tissue section analysis. The TG and GC were intravenously injected caudally with UK (Kailikang, Techpool Bio-Pharma Co., Ltd., 8.75×10^{-3} PNAU/kg) and puerarin (Zhejiang Zhenyuan Pharmaceutical Co., Ltd. Lot: H20020392, 100 mg/kg) 30 min after cerebral ischemia. The same volume of saline was simultaneously applied to the BC group.

The middle cerebral artery ischemia model was established using a modified Zea-Longer suture method. Approximately 3 ml/kg of 10% chloral hydrate was intraperitoneally injected for anesthesia. Rats were fixed in supine position and an incision was made in the neck midline to expose the left common carotid (CCA), internal carotid (ICA), and external carotid (ECA) arteries. The CCA was ligated proximal to the bifurcation. A small hole was cut 5 mm from the CCA bifurcation and a suture was inserted into the ICA. The insertion was stopped when resistance was felt at ~19 mm. At this point, the suture head was at the distal middle cerebral artery (MCA), forming the occlusion. After surgery, the corresponding drugs and saline were immediately intraperitoneally injected. After 2 h, ether was used as rapid anesthesia. The suture was gently pulled out ~10 mm to initiate reperfusion. The following were considered signs of successful establishment of the model: 1) contralateral limb paralysis and contralateral turning symptoms; 2) positive tail-hanging test; and 3) ipsilateral eye fissure decrease. Rats that did not satisfy these criteria were excluded from experimentation.

TTC staining

Seventy-two hours after ischemia/reperfusion, rats were anesthetized and decapitated to collect their brains. Brains were immediately frozen for 20 min. Starting from the anterior frontal pole, five 2-mm coronal slices were made from front to back with a tissue slicer. Slices were placed in TTC staining solution (chlorinated-2.3.5 TTC, Sigma Co.) and incubated at 37°C for 30 min. Tissue sections were then placed in freshly prepared 10% formalin solution for fixation for 24 h. Normal brain tissue turned red, whereas infarcted brain tissue turned white. The slices were removed and arranged in sequence. Sample images were taken using a digital camera and uploaded into a computer.

Preparation of tissue sections

The rats were anesthetized using 10% chloral hydrate and placed on a dissecting table for each time point. Thoracotomy was performed on the left chest, and the left ventricle was
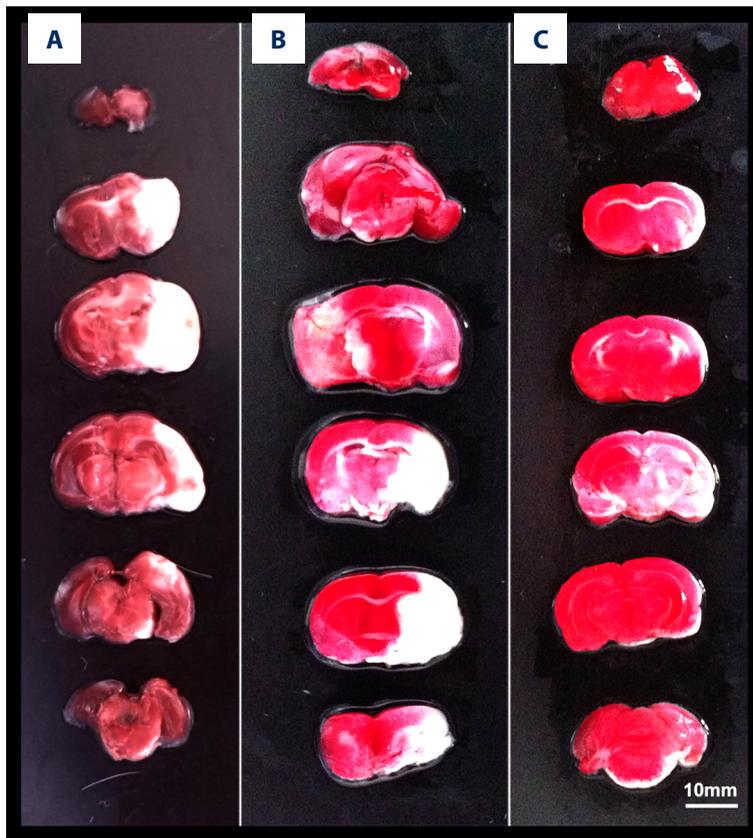
Figure 1. Comparison of cerebral infarct volume in different groups (TTC staining). The blood supply area of left middle cerebral artery presented in white. (**A**) GC; (**B**) BC; (**C**) TG.

catheterized. Blood was first removed using 4°C saline. Post-fixation was performed overnight using phosphate-buffered 4% paraformaldehyde at 4°C, followed by 20% and 30% phosphate-buffered sucrose incubation at 4°C until tissues sank. Consecutive frozen coronal 10-mm slices were made from the temporal lobe. One out of every four slices was used to perform TGF-β_1 immunohistochemical staining as a negative control.

Immunohistochemical staining and cell counting

Slices were washed twice with 0.01 mol/L phosphate-buffered saline and immersed into 3% H_2O_2 for 15 min, followed by droplet addition of non-immune animal serum blocking solution, primary anti-immune TGF-β_1 antibody, and secondary biotinylated goat anti-rabbit IgG, in that order. Reagent SABC was done using a TGF-β_1 protein immunohistochemical SABC kit (Wuhan Boster Biological Engineering Co., Ltd.). Finally, slices were counter-stained with hematoxylin and mounted with neutral gum. TGF-β_1-positive cell morphologies near the infarcted cortex were observed using an optical microscope (400×). The number of positive cells in each expression area field was counted under high-power magnification. Five slices from each rat were selected. Each slice was viewed at 5 randomly selected sites to count the positive cells near the infarcted cortex. At the same time, semi-quantitative image analysis was performed to calculate the final average number of positive cells.

Peripheral serum hs-CRP was determined by immunoturbidimetry using hs-CRP buffer, calibrator solution, and hs-CRP antigen (Finland Orion Diagnostica). Serum was obtained during cardiac catheterization.

Statistical analysis

SPSS 13.0 software was used for statistical analysis. The data are expressed as $\overline{\chi}\pm s$. Levene's test was performed to determine the homogeneity of variance, with a test criteria of a=0.05. If the variance was homogenous, then the LSD test was performed; otherwise, Tamhane's T2 test was performed. $P<0.05$ was considered a significant difference.

Results

Volume change calculation of ischemic lesion by TTC staining

Seventy-two hours after ischemia/reperfusion, infarcted lesions could be observed in the BC, TG, and GC groups. The infarcted zone was mainly located outside the parietal cortex and striatum, and no obvious bleeding was found in infarcted regions (Figure 1). The percentages of infarcted volumes are listed in Table 1.

Table 1. Cerebral infarcted volumes and percentages of each group 72 h after ischemia/reperfusion (mean ±SD).

Group	n	Infarcted volumes (mm³)	Percentage (%)
Blank control	6	187.70±11.04	32.45±1.55
Treatment	6	137.20±8.60	23.15±2.44**
Puerarin control	6	148.30±9.30	28.20±1.50*

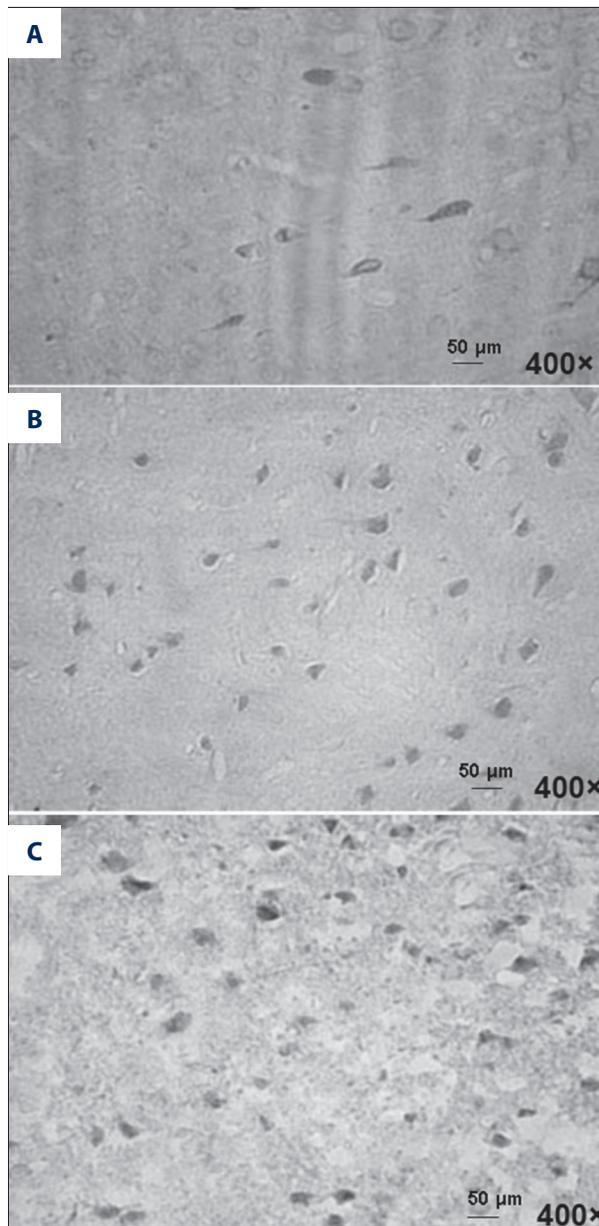** $P<0.01$ compared with blank control group; * $P<0.05$ between compared with puerarin control group.

Figure 2. Immunohistochemical staining 24 h after reperfusion (IHC ×400). The number of TGF-β₁ positive cells increased in the left cortex, with deepened cytoplasmic staining. The deeply stained nuclei were obvious in the TG group. (**A**) BC; (**B**) GC; (**C**) TG.
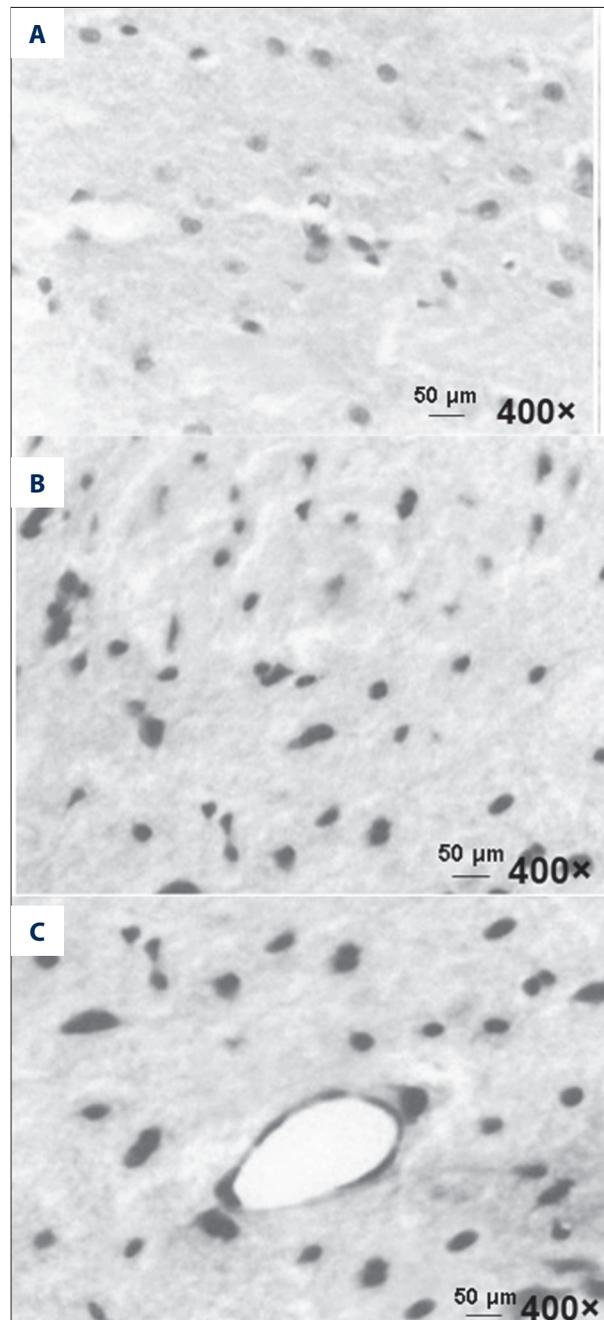

Figure 3. Immunohistochemical staining 48 h after reperfusion (IHC ×400). (**A**) BC; (**B**) GC; (**C**) TG.

Table 2. Changes of TGF-β_1 positive cells number at different time points after cerebral ischemia/ reperfusion (mean ±SD).

Group	6 h	12 h	24 h	48 h	72 h
Blank control	25.35±1.55	31.35±1.30	51.30±1.51	57.45±1.75	54.35±1.15
Treatment	29.55±1.35*	36.45±1.74*	56.25±1.85**	64.35±2.14**	58.25±2.35*
Puerarin control	27.23±2.25	33.10±1.35	54.12±1.76	61.40±2.05	55.35±2.20

* $P<0.05$ and ** $P<0.01$ compared with blank control group and puerarin control group, respectively.

Table 3. Changes of serum hs-CRP level (mg/L) at different time points after cerebral ischemia/reperfusion (mean ±SD).

Group	6 h	12 h	24 h	48 h	72 h
Blank control	4.15±1.45	4.65±1.281	5.00±1.50	6.35±1.75	7.45±1.05
Treatment	2.85±1.25*	3.45±1.75*	3.67±1.87*	4.45±2.04**	5.35±1.35**
Puerarin control	3.55±1.32	4.05±1.68	4.25±2.61	5.05±0.54	6.35±1.23

* $P<0.05$ and ** $P<0.01$ compared with blank control group and puerarin control group, respectively.

TGF-β_1-positive cells by light microscopy observation

Six hours after cerebral ischemia/reperfusion, cerebral cortex TGF-β_1 expression began to increase. Twenty-four hours after reperfusion, TGF-β_1 expression significantly increased. Expression peaked at 48 h and declined at 72 h. The number of positive cells in GC significantly increased over time compared with BC (Figures 2, 3). The number of positive cells in the TG group at each time point significantly increased compared to the BC and GC groups (Table 2).

Serum hs-CRP observation

Six hours after cerebral ischemia/reperfusion, serum hs-CRP started to rise and continued increasing up to 72 h. The serum hs-CRP level in the GC group decreased over time compared to the BC group, and TG serum hs-CRP levels decreased over time compared with the BC and GC groups. The decreases at 48 and 72 h were statistically significant (Table 3, Figure 2).

Discussion

TGF-β_1 is a cytokine with complex cellular functions, including growth regulation, proliferation, differentiation, inflammation response, and tissue repair. Scholars have speculated that TGF-β_1 expression is correlated with blood vessel formation [3]. Several normal tissues or cells synthesize and release TGF-β_1 in vivo. Astrocytes, microglia, and oligodendrocytes can produce TGF-β_1. TGF-β_1 expression is low in normal brain tissue. Increased TGF-β_1 expression can be observed in ischemia/reperfusion under different conditions. Previous studies have shown that TGF-β_1 mRNA increased at the injured sites 1 day

after cerebral ischemia, reached a peak at 2 days, and disappeared by 7 days. Thus, increased neuronal TGF-β_1 expression could be considered a neuronal survival sign [4]. The results of this experiment show that after ischemia/reperfusion, TGF-β_1 increased rapidly and reached a peak 48 h after reperfusion. Therefore, it is clear that TGF-β_1 is involved in the response to ischemia/reperfusion, which is consistent with the literature.

Numerous studies have shown that exogenous TGF-β_1 administration effectively reduces infarct volume after cerebral hypoxic/ischemic injuries in mice [5,6]. We are aware of 5 possible protective mechanisms of TGF-β_1 against cerebral ischemic injury. The first protective mechanism is intracellular Ca^{2+} concentration reduction, which protects nerve cells from glutamate cytotoxicity and increases neurons in the ischemic penumbra for long-term survival [7]. The second protective mechanism is that TGF-β_1 is an important regulating factor in brain injury repair that (a) produces a variety of biological effects in vivo and in vitro on glial cells and neurons, (b) regulates extracellular matrix synthesis and secretion by microglia, astrocytes [8], and (c) promotes tissue repair. In addition, TGF-β_1 regulates reactive oxygen species production through microglia [9]. The third protective mechanism is that TGF-β_1 is a powerful vascular proliferation regulator that induces angiogenesis through basic fibroblast growth factor and macrophages, thereby facilitating necrotic tissue transportation and nerve remodeling [10–12]. The fourth protective mechanism is that TGF-β_1 can antagonize neuronal apoptosis mediated by staurosporine and increased Bcl-2 protein expression. Zhu recently proposed that the primary TGF-β_1 anti-apoptotic mechanism was through activation of nuclear transcription factor NF-κB. Thus, TGF-β_1 inhibits activation of caspase-3 [13], or inhibits BAD expression, thereby increasing BAD phosphorylation to inhibit

apoptosis [14]. The fifth protective mechanism is that TGF-β_1 decreases the chemotaxis of tumor necrosis factor-α and its receptors to inflammatory cells, thereby exhibiting significant regulatory functions in cell migration, inflammation, and immune responses [15–17]. In addition, as an anti-inflammatory factor, TGF-β_1 rapidly induces the anti-hypoxia response shortly after cerebral hypoxia, which effectively prevents the progress of adverse reactions induced by hypoxia. Thus, TGF-β_1 is even stronger than IL-10 [18]. However, TGF-β_1 can also damage central nervous system function. For example, TGF-β_1 is related to disruption of the blood-nerve and blood-brain barrier in diabetic neuropathy [19]. The effects of TGF-β_1 on glial cells and leptomeningeal mesothelial cells are less studied.

CRP is a sensitive acute-phase protein synthesized by the liver. CRP is a sensitive predictor of inflammation and a marker of atherosclerosis. Increased hs-CRP levels can prompt a significant increase in the content and activity of serum pro-inflammatory cytokines. Immunoturbidimetry lowers the detection limit of hs-CRP to the 0.005–0.10 mg/L range. CRP measured using this method is called hs-CRP, which is a valuable cerebral ischemia predictor. This marker fully and sensitively reflects the presence of inflammation. Clinical studies have found increased serum hs-CRP levels in acute cerebral infarction TOAST subtypes [20]. Korean scholars divided 886 cases of stroke caused by middle cerebral artery thrombosis into cortical, lacunar, and deep perforating branch and watershed infarctions. Serum hs-CRP detection revealed that serum hs-CRP levels were increased in cortical infarction compared with the other 3 types [21]. This phenomenon is probably caused by the intense inflammatory response resulting from large-artery atherosclerosis. Another scholar compared the correlation between hs-CRP levels and infarcted volume and found a positive relationship between the 2. The majority of clinical scholars use hs-CRP as an indicator for assessing stroke progress, prognosis, and recurrence [22–24]. A number of scholars have studied guinea pig brain specimens and blood-brain barrier *in vitro* cell culture models, which show that CRP induces blood-brain barrier damage, resulting in cerebral edema through a signal transduction pathway [25]. In the present study, the trend of serum hs-CRP levels shortly after cerebral ischemia was measured. However, further study is required to elucidate the relationship between disease severity and long duration after infarction.

UK is a Class I drug that was developed to treat cerebral infarction. UK, a glycoprotein extracted and purified from human urine, can selectively expand arterioles in ischemic areas, promote vascular regeneration, establish collateral circulation, and improve ischemic area perfusion. Furthermore, UK enhances red blood cell deformability and oxygen dissociation ability, promotes tissue glucose utilization, and inhibits hyperactive platelet aggregation and hematopexis hyperthyroidism. UK also suppresses neuron and glial apoptosis and promotes endogenous nerve regeneration, thereby accelerating the recovery of neurological functions [26–28]. In animal models of cerebral ischemia, UK was confirmed to promote the angiogenesis and opening of collateral circulation, reduce inflammatory cell infiltration, reduce infarct size and cerebral edema, promote the migration of glial cells into the ischemic area, and protect neurons endangered by ischemia [29,30]. In the current study, the cerebral ischemia/reperfusion injury model was established to study the effects of UK intervention. The results showed that the infarct volume in the TG group was significantly reduced compared to the BC and GC groups. The TGF-β_1 expression around infarcted brain tissues was significantly upregulated compared with the BC and GC groups. Serum hs-CRP levels in TG at all time points decreased compared with those in the GC and BC groups. A significant decrease was observed at the 48 h and 72 h time points.

Conclusions

These finding indicate that early UK intervention promotes TGF-β_1 activation, increases TGF-β_1 expression, and inhibits excessive inflammatory response, thereby promoting brain tissue repair and reducing infarct volume. This study showed UK regulates inflammatory factor changes, which may be a neuroprotective mechanism of UK.

Conflict of interest

All authors have no conflict of interest regarding this paper.

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