Saudi Journal of Biological Sciences 26 (2019) 1995–1999

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Relationship between HIF-1α and apoptosis in rats with traumatic brain injury and the influence of traditional Chinese medicine Sanqi

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ARTICLE INFO

Article history:
Received 10 July 2019
Revised 2 August 2019
Accepted 4 August 2019
Available online 5 August 2019

Keywords:
Traumatic brain injury
HIF-1α
Apoptosis
Traditional Chinese medicine Sanqi
Regulatory network

ABSTRACT

Objective To explore the expression of HIF-1α, neuronal apoptosis and the influence of traditional Chinese medicine Sanqi on hematoma after brain injury in rats. **Methods** Ninety SD rats were divided into 3 groups randomly: blank control group, traumatic brain injury (TBI) group and Sanqi intervention group, and they were decapitated after brain injury at different time points: 6 h, 1 d, 2 d, 3 d, 5 d, 7 d. The model of cerebral hemorrhage was made by autologous non-coagulation in stereotactic locator, the expression of HIF-1α and TUNEL-positive cells (apoptotic cells) in the perihematomal area was detected by immunohistochemistry. **Results** In blank control group, a small amount of HIF-1α was expressed and apoptotic cells were observed. The expression of HIF-1α was up-regulated in the brain injury group from 6 h, and the apoptotic cells increased in abundance. The peak of HIF-1α was reached at 3 d, then decreased, and remained at the high level on the 7 d. Compared with blank control group, the TBI group was statistically significant ($P < 0.05$). The Chinese medicine Sanqi intervention group significantly up-regulated HIF-1α'expression and decreased neuronal apoptosis, which was statistically significant ($P < 0.05$). **Conclusion** HIF-1α's expression was up-regulated around the hematoma after brain injury, and the apoptosis of nerve cells was obviously increased. The traditional Chinese medicine Sanqi can significantly increase the expression of HIF-1α, reduce the apoptosis around the hematoma, and thus play a neuroprotective role.

1. Introduction

Traumatic brain injury (TBI) is a common disease in neurosurgery, and its incidence is increasing year by year. It has become one of the main causes of disability and death among children and young adults (Puccio et al., 2019). Patients with craniocerebral injury mainly manifest as discomfort such as unconsciousness, weakened deglutition reflex function, cough, and increased secretion of respiratory tract. With the rapid development of China's social economy and transportation industry, the incidence of TBI in China has increased year by year, and has become the most important factor of deaths caused by trauma in the rural area of China (Hux, 2019). Rehabilitation treatment for patients with TBI is costly, suffering pains for patients while causing great economic losses for patients and their families, and it is also a heavy burden on society.

In recent years, studies have shown that inducible factor-1 of hypoxia (HIF-1α) is of great importance in curing and repairing brain trauma. After brain injury, it usually induces ischemia and hypoxia in surrounding tissues. HIF-1α has features such as specificity and sensitivity to hypoxia signal response, as well as its presence in tissues and the extensive regulation of target genes. When tissues or cells is under the hypoxia state, HIF-1 can improve hypoxia by regulating oxygen metabolism in tissues or cells (Bogdanovski et al., 2017). HIF-1 is the transcriptional regulator consisted of HIF-1α and HIF-1β, and its expression is positively correlated with the oxygen concentration in the surrounding environment (Khan et al., 2017). As a protein regulated by oxygen concentration, HIF-1α is difficult to be detected at normal oxygen

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2019.08.004
1319-562X/

concentration, and is detected due to reduced degradation in hypoxia. In this experiment, HIF-1α was selected as the detection index in the development of TBI. The intervention of traditional Chinese medicine Sanqi was used to explore the relationship between the expression change of HIF-1α and the apoptosis of nerve cells around the parts of brain injury after TBI as well as the protection mechanism of neuronal cells of HIF-1α after TBI, then, the impact of traditional Chinese medicine Sanqi on the above indicators was observed.

2. Materials and reagents

90 healthy and clean male Sprague-Dawley (SD) rats of 300–350 g (from Animal Center of Fourth Military Medical University), HIF-1α antibody (produced by Sigma in the US), TUNEI kit (produced by Roche), traditional Chinese medicine Sanqi (provided by the Department of Pharmacy, the First Affiliated Hospital of Xinxiang Medical University, batch number: 20110208).

3. Methods

3.1. Animal grouping and model design

The experimental animals were divided randomly into: TBI group, blank control group, and Sanqi intervention group. There're 30 rats in each group, which was randomly divided into 6 subgroups.

Modeling: The rats were anesthetized and fixed on stereotaxic apparatus according to the method described in the literature. A bone window with a diameter of 3 mm was opened at 1.5 mm behind the bregma and 8 mm from the right side of the midline, and the equipment of Lateral fluid percussion (LFP) was connected. The blank control group performed the same operation, but was not connected to the equipment of Lateral fluid percussion. The blank control group and the TBI group were fed by the same normal fodder, and the Sanqi intervention group was given the special fodder for Sanqi [0.4 g/(kg·d)], the rest of the environment is the same. The rats were executed by decapitation at different time points after operation: 6 h, 1 d, 2 d, 3 d, 5 d, 7 d.

3.2. Observation indicators

3.2.1. Quantity of HIF-1α positive cells

All the rats were decapitated, fixed in 4% formaldehyde solution, embedded in paraffin, and their brain tissue was taken out for coronal slices in the hematoma area. After conventional dehydration, waxing, paraffin embedding, brain tissues were cut into slices and each slice was 4 mm, the number of HIF-1α positive cells was observed by SP immunohistochemical assay. Slices of immunohistochemical assay were observed under a 40-fold optical microscope, and their color was brownish yellow which proved that they are positive. Five fields of view were randomly selected and the average value of them was selected.

3.2.2. Count of apoptotic nerve cell

Paraffin slides of brain tissue were conducted by TUNEL (Terminal-deoxynucleoitidyl Transferase Mediated Nick End Labeling), and observed under a 40-fold optical microscope. Those cell nucleus with purple-red particles were TUNEL-positive apoptotic neurons. The apoptotic cell counting method was immunohistochemically associated with HIF-1α.

3.3. Statistical analysis

Statistical differences analysis, analysis of variance and correlation analysis were performed using SPSS 23.0. The measurement data were shown by $\bar{x} \pm s$. One-way analysis of variance was used at each time point in the group and compared with the blank control group. After the difference was determined, the least significant difference method was used for comparison.

4. Results

4.1. HIF-1α expression of Sanqi intervention up-regulation in the parts of brain injury

This paper used immunohistochemistry to detect HIF-1α's expression in the injury parts of brain. HIF-1α positive cells around the injury parts pf brain at different time points in TBI group were obviously increased compared with that in the blank control group, and reached the peak at 3 d. After that, the HIF-1α positive cells decreased slowly and remained high. The HIF-1α positive cells around the parts of brain injury at different time points in the Sanqi intervention group were obviously increased compared with that in TBI group ($P < 0.05$), see Table 1. Fig. 1 showed the expression of HIF-1α in the parts of brain injury at 5 d after trauma in the TBI group and the Sanqi intervention group.

4.2. Sanqi intervention to reduce apoptosis in the parts of brain injury

Neuronal apoptosis in the parts of brain injury was observed at each time point after trauma. Apoptosis was remarkably higher in TBI group than that in other groups, and the number of apoptosis at each time point in Sanqi intervention group was less than that in the TBI group ($P < 0.05$), see Table 2. The apoptosis of the parts of brain injury at 5 d after trauma in the TBI group and the Sanqi intervention group was presented in Fig. 2.

4.3. Construction of brain damage regulation network involved by HIF-1α

Studies have confirmed that HIF-1α is enormously expressed in cells around brain injury tissues, and has an effect on cell growth, angiogenesis, metastasis, apoptosis and drug resistance. On the basis of previous studies, our group has analyzed the regulatory pathway of brain injury by HIF-1α and related target genes, and combined with KEGG database and related literature, preliminary regulation network diagram of brain damage involved by HIF-1α and related target genes was drawn (Fig. 3) and it analyzed the

Table 1
Number of HIF-1α positive cells at different time points in the three groups ($\bar{x} \pm s$).

Groups (F)	6h	1d	2d	3d	5d	7d
Blank control	0	0	0	0	0	0
TBI	5.8 ± 1.64	14.6 ± 1.67	30.2 ± 3.42	39.8 ± 1.48	33.4 ± 2.61	24.6 ± 2.30
Sanqi intervention	9.6 ± 1.67	24.2 ± 2.39	38.4 ± 2.70	49.2 ± 3.11	44.4 ± 1.67	38.2 ± 2.86

Compared with blank control group, the difference was of statistical significance at each time point ($P < 0.05$). The difference between Sanqi intervention group and TBI group was of statistical significance ($P < 0.05$).

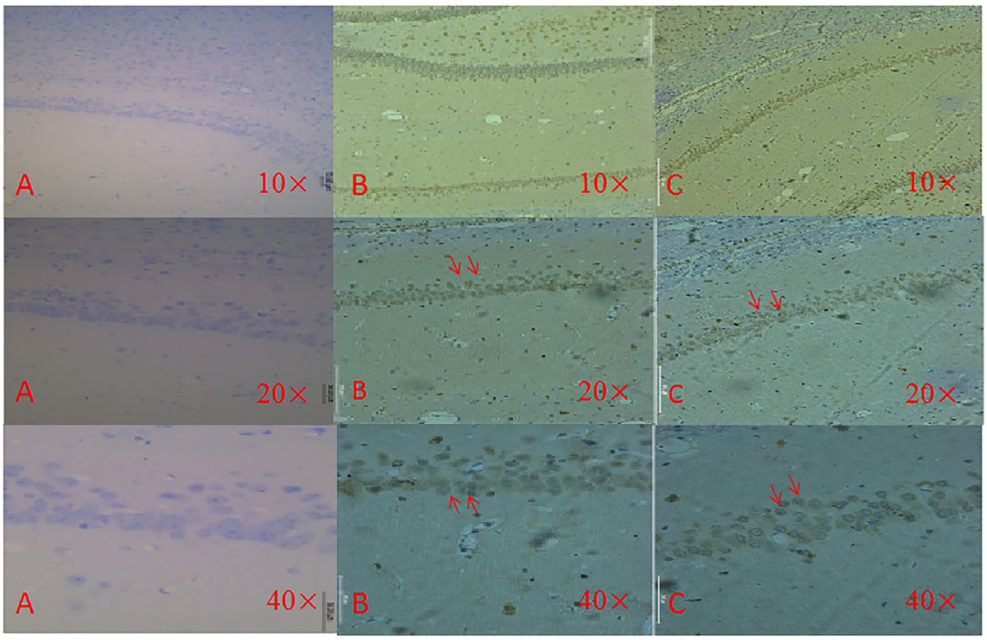

Fig. 1. Expression of HIF-1α in the parts of brain injury of rats at 5 d after brain trauma. The red arrow indicated HIF-1α. (A) Blank control group, (B) TBI group, (C) Sanqi intervention group. Note: Five rats per group at each time point.

Table 2
Number of TUNEL-positive cells at different time points in the three groups ($\bar{x} \pm s$).

Groups (F)	6h	1d	2d	3d	5d	7d
Blank control	1.8 ± 1.3	2.4 ± 0.55	2 ± 1.41	1.6 ± 1.14	2.2 ± 2.05	2.6 ± 1.67
TBI	24.6 ± 1.67	35.4 ± 2.30	49 ± 3.81	60 ± 3.08	51 ± 4	44.4 ± 2.97
Sanqi intervention	19.6 ± 1.67	30.4 ± 3.05	40 ± 2.55	54.4 ± 3.36	40.6 ± 3.36	33.8 ± 3.03

Compared with blank control group, the difference was of statistical significance at each time point ($P < 0.05$). The difference between Sanqi intervention group and TBI group was of statistical significance ($P < 0.05$).

status quo of the therapeutic target of Chinese medicine in the established regulatory network, which could provide some theoretical basis for the treatment of traditional Chinese medicine for brain injury.

5. Discussion

Brain injury may cause cerebral hemorrhage and hematoma oppressing peripheral cerebral tissue, vasospasm, cerebral microcirculation disturbance, cerebral blood flow autoregulation disorder, and no reflow during reperfusion, which may further lead to peripheral decreased cerebral blood flow and formation of peripheral ischemic penumbra of hematoma, these can result in accumulation of HIF-1α when the critical level of cerebral ischemic injury is not reached in degree and time (Khan et al., 2018). Studies have found that HIF-1α was activated through the transcriptional activation of vascular endothelial growth factor (VEGF) by binding it to hypoxia response factors, which increases the stability of VEGF mRNA, up-regulates the expression of VEGF, stimulates the sprouting, migration and proliferation of endothelial cells to form the new blood vessels (Thelin et al., 2016). This is conducive to the reconstruction of the microvascular system in the injured area, which creates a good microenvironment for the repair of damaged neurons and synaptic reconstruction (Wu et al., 2018). In this experiment, HIF-1α expression was up-regulated after brain injury, and the number of apoptotic cells increased. It can be seen that HIF-1α may play a role in neuronal protection during the process of neuronal apoptosis in cerebral hemorrhage by following approaches: (1) It may inhibit the expression of PAR-1, up-regulate anti-apoptotic molecules, and reduce apoptosis; (2) It will promote angiogenesis, etc., mediating cells adapting to hypoxic environment and resisting to apoptosis will promote cell survival (Lu et al., 2015); (3) HIF-1α binds to hypoxia response element and up-regulates VEGF expression. VEGF can stimulate endothelial cell mitosis, participate in endothelial cell differentiation, promote capillary formation, and play an anti-apoptotic cell function (Wu et al., 2014); (4) VEGF can also promote the proliferation of vascular endothelial cells, angiogenesis and blood supply in the ischemic penumbra around the hematoma of the brain injury. The proliferation of vascular endothelial cells forms a new blood vessel extending from the penumbra to the periphery, which can promote the establishment of the collateral circulation of ischemic penumbra and improves the blood supply to the surrounding area, thereby saving the penumbra (Anderson et al., 2009). Therefore, when brain cells are ischemic and hypoxic, HIF-1α is produced and is at the key stage of hypoxia response. It is indicated that the up-regulation of HIF-1α expression after brain injury can play a protective role for neurons.

The traditional Chinese medicine Sanqi has a wide range of pharmacological effects, and it was called “the magical medicine of stopping the blood”, which has the effect of relieving blood stasis, promoting blood circulation and relieving pain. It is used for all kinds of bleeding and bruises in the human body, and it is extremely precious (Wang et al., 2016). Sanqi can relieve blood stasis and gather new blood. All blood stasis is improved and all new

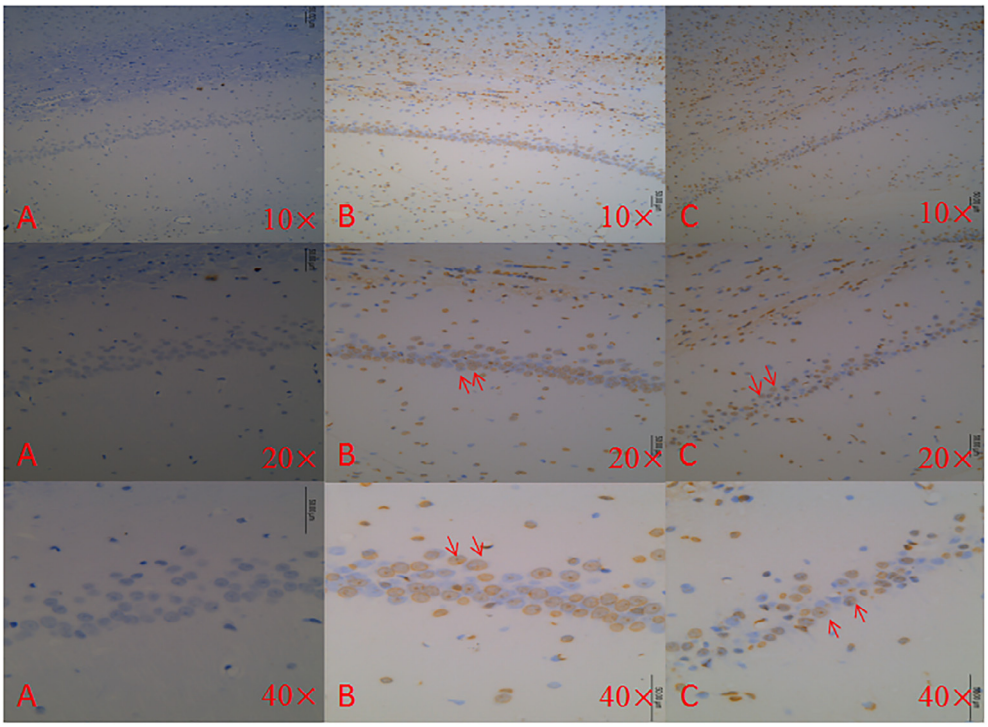

Fig. 2. TUNEL-positive cells in the parts of brain injury of rats at 5 d after brain trauma. Red arrows indicated TUNEL-positive cells. (A) Blank control group, (B) TBI group, (C) Sanqi intervention group. Note: Five rats per group at each time point.

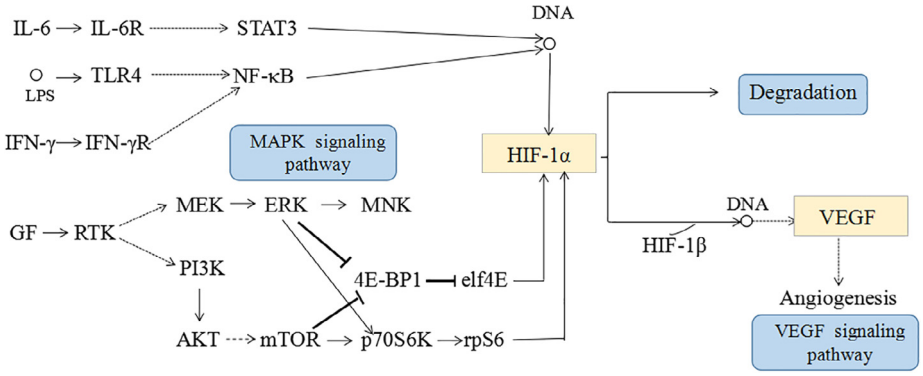

Fig. 3. Regulation network diagram of brain damage involved by HIF-1α and related target genes.

blood is stopped. At the same time, it was confirmed that Sanqi can significantly increase the expression of basic fibroblast growth factor (FGF) and epidermal growth factor (EGF) in the brain of rats with ischemic brain tissue, thus promoting the repair of neurons (Lei et al., 2010). The results of this experiment demonstrated that the intervention of Sanqi can significantly increase HIF-1α's expression and reduce the apoptosis of nerve cells around the brain injury. The expression of HIF-1α was up-regulated after TBI, but HIF-1α's expression was further up-regulated in intervention group, while the apoptosis was significantly decreased, indicating that HVII-1α played a protective role in neurons. Meng et al. (2014) found that in the model of cerebral ischemia-reperfusion injury in vivo and vitro, the Rg1 component in Sanqi can inhibit brain cell apoptosis by counteracting potential damage of mitochondrial membrane and inhibiting caspase-3 activation to decrease the volume of cerebral infarction. Studies have shown that apoptosis inhibitors such as the Bcl-2 family and apoptosis inhibitory protein can counteract apoptosis, reduce infarct area and brain cell edema, and improve neurological function. The Bcl-2 gene is the first gene identified to inhibit apoptosis. Li et al. (2009) have found that Sanqi saponins can inhibit the activation of c-Jun N-terminal kinase (JNK) signal transduction protein by regulating the expression of caspase-1, caspase-3, apoptosis inhibitory protein XIAP, and apoptosis-promoting protein Smac, which reduces brain tissue N-methyl-D-aspartate receptor-1 (NMDAR1-1) and other cells to inhibit apoptosis after ischemic brain injury (Xie et al., 2018). The above studies indicate that Sanqi can protect mitochondria and inhibit apoptosis through a variety of mechanisms of action, thereby playing an important protective role against ischemic brain damage. In addition, studies have argued that Sanqi saponins can inhibit apoptosis, promote neuronal morphology recovery after cerebral ischemia treatment, and increase the number of neurons (Wu et al., 2018) some scholars have proved that Sanqi can inhibit nerves caused by cerebral ischemia. Cell apoptosis has a significant protective effect on cerebral ischemia-reperfusion (Liu et al., 2019), but the mechanism of neu-

ronal apoptosis induced by Sanqi after ischemia inhibition is still unclear. On the basis of previous studies, our group has analyzed the regulatory pathway of brain injury involved by HIF-1α and related target genes, and combined with KEGG database and related literature, preliminary regulation network diagram of brain damage involved by HIF-1α and related target genes was drawn and the status quo of the therapeutic target of Chinese medicine in the established regulatory network was analyzed for the purpose of providing some theoretical basis for the treatment of traditional Chinese medicine for brain injury.

When the brain is injured and ischemic, the blood-brain barrier is damaged, which aggravates brain edema, ischemia and hypoxia, and further causes brain tissue damage (Lasek-Bal et al., 2019). The destruction of the blood-brain barrier is a key link in the aggravation of secondary brain injury. HIF-1α is a nuclear transcription factor that regulates transcription, it is closely related to blood-brain barrier damage after ischemic brain injury. It is abnormally activated under ischemia and hypoxia, regulating a series of related gene expression, including blood-brain barrier that associated with vascular endothelial growth factor and matrix metalloproteinase, etc., they are collectively involved in acute blood-brain barrier injury (Michinaga et al., 2018). Vascular endothelial growth factor is a multi-functional growth factor that specifically acts on vascular endothelial cells, promotes endothelial cell proliferation and angiogenesis in vivo, and enhances vascular permeability. It is a major component involved in the composition of the blood-brain barrier. It has been reported in the literature that Sanqi saponins can also block angiogenesis by inhibiting the expression of VEGF (Qiao et al., 2015), which may be related to the bidirectional regulation of Sanqi saponins with estrogen-like effects.

Acknowledgement

The work was financially supported by The Science and Technology Breakthrough Project of Henan Province (Grant No.: 182102310536).

Declaration of Competing Interest

The authors declared that they have no conflicts of interest to this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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