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Original Article

Effect of acupuncture on expression levels of NF-κB, IL-1β, IL-6, and TNF-α in rats with cerebral ischemia and reperfusion

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Abstract: Objective: To explore the treatment effects of acupuncture at Baihui point and Zusanli point on the levels of nuclear factor-κB (NF-κB), interleukin -1β (IL-1β), interleukin -6 (IL-6), and tumor necrosis factor-α (TNF-α) in rats with cerebral ischemia and reperfusion. Methods: Sixty SD rats were randomly divided into three groups. The model group (n=20) was selected for cerebral ischemia and reperfusion, another group of rats were selected for acupuncture treatment following cerebral ischemia and reperfusion (n=20), and the control group of rats were fed normally and did not undergo cerebral ischemia (n=20). Results: Expression of IL-1β, IL-6, TNF-α, and NF-κB in the ischemia model group was increased compared to that in the control group. IL-1β expression was decreased in the acupuncture group compared with ischemia model group ($P < 0.05$). In the acupuncture group, IL-6 levels decreased, while in the model group IL-6 levels remained high ($P < 0.05$). TNF-α levels steadily increased in the acupuncture group, yet TNF-α maintained high expression in the model group ($P < 0.05$). NF-κB levels in the acupuncture group decreased gradually during treatment, while NF-kB sustained high expression levels without significant changes in the model group ($P < 0.05$). Conclusions: Acupuncture at Baihui point and Zusanli point effectively reduced the expression levels of inflammatory factors in rats with cerebral ischemia and reperfusion, and demonstrated repairing effects on damaged brain tissue.

Keywords: Acupuncture, cerebral ischemia and reperfusion, NF-κB, IL-1β and IL-6

Introduction

Cerebrovascular disease is a global disease with a high incidence throughout the world. According to statistics from Love et al. [1], the number of patients with cerebrovascular diseases has reached 1.6 million in 2016, most of which were middle aged and elderly people. The findings of Kamat et al. [2] showed that the incidence rate of cerebrovascular disease is increasing in young individuals. It is predicted that young and middle-aged patients will account for 35% of the overall cerebrovascular disease patients in the year 2030. Cerebrovascular diseases often occur suddenly and many patients become disabled and ultimately die due to an untimely response [3]. In the clinic, the most commonly applied method for the treatment of ischemic cerebrovascular disease is thrombolytic therapy with recombinant tissue plasminogen activator (rtPA) [4], but this method is treatment intensive and may cause intracranial hemorrhage and other complications beyond the limited time window [5]. Therefore, improving the prognosis of patients with cerebral ischemia by adopting safe and effective interventions is a difficult problem requiring urgent breakthroughs in modern medicine.

Cerebral ischemia and reperfusion injury is one of the most common pathological mechanisms of cerebral ischemia. However, no exact mechanism has been identified at home or abroad, and the main damage mechanism is through the blood brain barrier [6]. The blood brain barrier preserves and protects the environment of the human brain, and transports substances from the blood into the central nervous system [7]. In cerebral ischemia, oxygen and glucose supplies in the blood are insufficient for the brain, causing damage to the integrity of the blood-brain barrier. This causes harm to neurons of patients, and the inflammatory factors released by the damaged neurons will cause sudden cerebral infarction, cerebral congestion, brain death, etc. [8]. Because of its compli-

cated pathogenesis, the treatment of cerebral ischemia in clinics has gradually begun to advocate the application of traditional Chinese medicine acupuncture [9]. The goal of acupuncture is to improve the prognosis of the patients by reducing the damage caused by cerebral ischemia through multiple channels and multiple targets [10]. Currently, there have been many studies [11-14] proving that acupuncture combined with Western medicine for the treatment of patients with cerebral ischemia has achieved remarkable results. However, due to the complicated structures and points of the human body, studies on the efficacy of acupuncture therapy for the treatment of cerebral ischemia and reperfusion are less reported.

Hwang IK et al. showed that electroacupuncture (EA) at ST36 (Zusanli) and GV20 (Baihui) enhanced cell proliferation and neuroblast differentiation in the rat dentate gyrus [15]. Jin Young Chuang et al. demonstrated that electroacupuncture (EA) at ST36 (Zusanli) and GV20 (Baihui) can ameliorate the reductions in proliferating cells and differentiated neuroblasts in the dentate gyrus induced by type-2 diabetes without significantly reducing blood glucose levels with increasing BDNF levels [16].

Therefore, we established a rat model with cerebral ischemia and reperfusion to study acupuncture at Baihui and Zusanli points to verify whether acupuncture treatment is applicable to patients with cerebral ischemia and reperfusion, and to further provide reliable reference and guidance for clinical practice in the future.

Materials and methods

Animal

Sixty SD rats (30 male and 30 female) with body weights of 250~300 gram were provided by the animal laboratory center of Central South University. Room temperature was 26°C and humidity 75%. 5 rats were fed in one cage.

Modeling method and grouping

All rats were randomly divided into three groups. One group was used as the model group with cerebral ischemia and reperfusion (n=20), one group was treated with acupuncture following cerebral ischemia and reperfusion (n=20), and the final group served as the control group and did not undergo cerebral ischemia or acupuncture, and were maintained on a normal diet (n=20). The rat model with cerebral ischemia and reperfusion was established by Mintorovitch et al. [17]. Briefly, intraperitoneal injection of 10% chloral hydrate (0.3/100 g) was used for anesthesia. After disinfection, the median neck skin was incised 2-3 cm and ligated the external carotid artery and cut it off. A suture was inserted from the cut side of the external carotid artery along the internal carotid artery until resistance was felt. The thread thrombus completely blocked the right middle cerebral artery entrance about 15 mm in. The silk thread above the cutting of the right neck general artery was ligated with about 2 cm of thread indwelled outside the rats' body and the skin was sutured. Animals were anesthetized after 2 hours of cerebral ischemia and the thread was slowly pulled out to the black mark and cut to achieve reperfusion.

Experimental method

At 24 hours after reperfusion, the rats in the acupuncture group received regular daily acupuncture with disposable aseptic acupuncture needles at Baihui and the left Zusanli points. The acupuncture lasted for 7 days. The pulse electrotherapy apparatus was delivered after needle placement so that the local muscle contraction was used to assist the treatment. The parameters of the electrotherapy apparatus were set as a sparse dense wave with the frequencies of 2~100 Hz and intensity of 2 mA for 20 min. Rats in the model group were not given any treatment after cerebral ischemia and reperfusion, and as a control were handled every time the rats in the acupuncture group were given treatment. The rats in the control group did not undergo cerebral ischemia and reperfusion, and were fed normally.

Western blotting

Western blotting was used to detect NF-κB in the ischemic brain tissue of rats. The frozen ischemic brain tissues were placed in homogenate buffer and homogenized and then sonicated three times for 10 seconds at 4°C. The sonicated samples were subjected to centrifugation (10,000× g). Separate cytosol and nuclear protein lysates were prepared by using the Active Motif Nuclear Extract Kit (Active Motif Europe, Rixensart, Belgium). For routine protein quantitation, following the manufacturer's protocol (Thermo Fisher Scientific), equal amounts of protein samples (30 μg) were subjected to

Table 1. IL-1β levels in the control, acupuncture, and model groups of rats

	Control group (n=20)	Acupuncture group (n=19)	Model group (n=17)
3 d	84.71±42.37	71.64±20.84$^{□}$	124.33±35.26$^{□,☆}$
5 d	125.16±34.67	96.27±29.33$^{*,□}$	160.51±21.16$^{*,□,☆}$
7 d	123.59±35.14^{Δ}	85.26±32.92$^{*,Δ,□}$	168.74±8.21$^{*,□,☆}$
F	0.01	3.65	53.06
P	0.99	0.03	< 0.01

Note: *P < 0.05 compared with the IL-1β levels at 3 days. ^{Δ}P < 0.05 compared with the IL-1β levels at 5 days. $^{□}$P < 0.05 compared with the IL-1β levels of control group. $^{☆}$P < 0.05 compared with the IL-1β levels of acupuncture group.

Table 2. IL-6 levels in the control, acupuncture, and model groups of rats

	Control group (n=20)	Acupuncture group (n=19)	Model group (n=17)
3 d	34.27±6.84	54.62±21.52$^{□}$	42.86±8.34$^{□,☆}$
5 d	35.02±5.59	39.26±6.85$^{*,□}$	42.63±7.86$^{□,☆}$
7 d	34.87±5.62	34.77±6.94^{*,Δ}	41.86±8.13$^{□,☆}$
F	0.09	8.30	0.07
P	0.92	< 0.01	0.93

Note: *P < 0.05 compared with the IL-6 levels at 3 days. ^{Δ}P < 0.05 compared with the IL-6 levels at 5 days. $^{□}$P < 0.05 compared with the IL-6 levels of control group. $^{☆}$P < 0.05 compared with the IL-6 levels of acupuncture group.

Table 3. TNF-α levels in the control, acupuncture, and model groups of rats

	Control group (n=20)	Acupuncture group (n=19)	Model group (n=17)
3 d	28.62±4.62	38.76±7.05$^{□}$	36.94±8.07$^{□}$
5 d	29.04±5.04	36.86±7.61$^{*,□}$	37.64±9.24$^{□}$
7 d	28.88±4.73	28.54±4.69^{*,Δ}	37.84±9.01$^{□,☆}$
F	0.04	12.99	0.05
P	0.96	< 0.01	0.95

Note: *P < 0.05 compared with the TNF-α levels at 3 days. ^{Δ}P < 0.05 compared with the TNF-α levels at 5 days. $^{□}$P < 0.05 compared with the TNF-α levels of control group. $^{☆}$P < 0.05 compared with the TNF-α levels of acupuncture group.

sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% Tris-glycine gels. After incubation in blocking buffer and wash three times with Tris-buffered saline and Tween 20 (TBST) buffer (10 mM Tris-base, 100 mM NaCl, and 0.1% Tween 20; pH 7.5), blots were treated with an anti-NF-κB p65 polyclonal antibody (1:1,000), in TBST buffer overnight. Blots were then washed, and the immunoreactive protein was detected using film exposed to enhanced chemiluminescence detection reagents.

ELISA

ELISA was adopted to detect the inflammatory factors of IL-1β, IL-6, and TNF-α. Blood samples were collected at 3, 5, and 7 days following cerebral ischemia and reperfusion in all rats. Whole blood was centrifuged (13000×g for 15 minutes) and supernatants were collected to determine the level of TNF-α, IL-1β, and IL-6 in serum by available quantitative sandwich ELISA kits (R&D, USA). All use of ELISA kits was in strict accordance with the manufacturer's protocols. The concentrations of the samples were calculated according to the standard curve. The serum TNF-α, IL-1β, and IL-6 levels are all expressed as ng/L.

Observation indexes

The expression levels of NF-κB, IL-1β, IL-6, and TNF-α on 3, 5, and 7 days following cerebral ischemia and reperfusion in all rats was examined, and data and variation were plotted.

Statistical method

Statistics software SPSS22.0 was used to analyze the data. All results are expressed by (mean ± standard deviation) and the data among 3 groups was compared by variance analysis. Paired t-tests were used to compare data at two different time points. P < 0.05 suggested that the difference was statistically significant.

Results

Cerebral ischemia and reperfusion results

Of the 40 rats that underwent cerebral ischemia and reperfusion, 36 surgeries were successful (90%). Of all the successful rats, 17 were in the model group and 19 were in the acupuncture group. All rats in the control group were all alive.

IL-1β detection

In the acupuncture group, IL-1β (71.64±20.84) on day 3 was significantly lower than the model

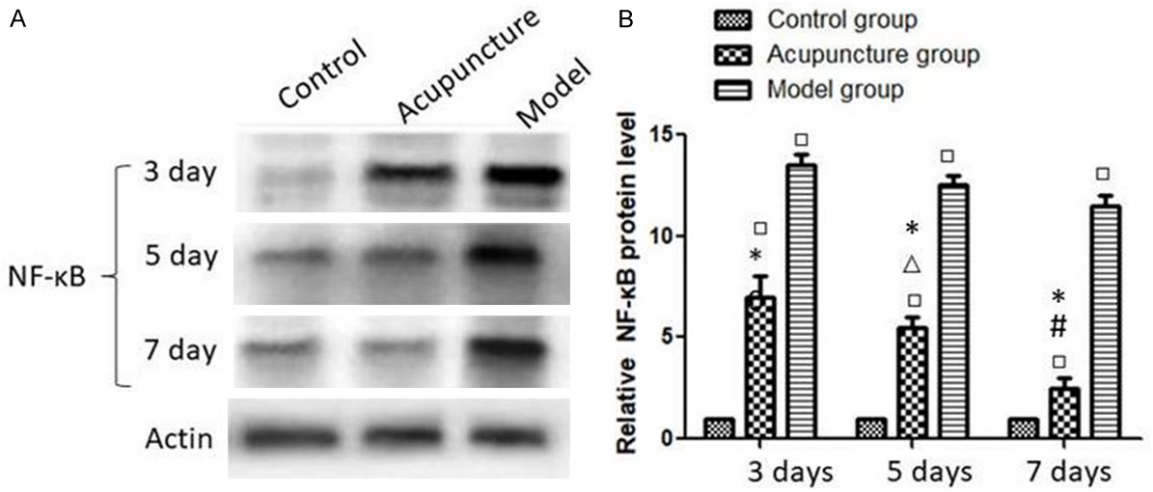

Figure 1. NF-κB protein levels in the control, acupuncture, and model groups of rats. Note: ΔP < 0.05 compared with the NF-κB levels at 3 days. #P < 0.05 compared with the NF-κB levels at 5 days. □P < 0.05 compared with the NF-κB levels of control group. *P < 0.05 compared with the NF-κB levels of model group.

Table 4. NF-κB levels in the control, acupuncture, and model groups of rats

	Control group (n=20)	Acupuncture group (n=19)	Model group (n=17)
3 d	3.14±0.95	14.35±2.06□	16.82±3.27□,☆
5 d	3.18±0.64	9.82±4.69*,□	17.06±3.84□,☆
7 d	3.22±0.73	6.37±2.45*,Δ,□	16.59±4.05□,☆
F	0.05	32.21	0.07
P	0.95	< 0.01	0.94

Note: *P < 0.05 compared with the NF-κB levels at 3 days. ΔP < 0.05 compared with the NF-κB levels at 5 days. □P < 0.05 compared with the NF-κB levels of control group. ☆P < 0.05 compared with the NF-κB levels of acupuncture group.

group (124.33±35.26) and the control group (84.71±42.37), P < 0.05. In the control group on day 3, IL-1β (84.71±42.37) was also significantly lower than that of the model group (124.33±35.26), P < 0.05. On day 5, IL-1β in the acupuncture group was (96.27±29.33), which was significantly lower than that of the control group (125.16±34.67) and model group (160.51±21.16), all P < 0.05. IL-1β in the model group was significantly higher than that of the control group at day 5, P < 0.05. At day 7, the IL-1β in the acupuncture group was (85.26±32.92), which was lower than that of the control group (123.59±35.14), P < 0.05, and lower than that of the model group (168.74±8.21), P < 0.05 (**Table 1**).

IL-6 detection

As shown in **Table 2**, in the acupuncture group, IL-6 was (54.62±21.52) on day 3 which was significantly higher than the control group (34.27±6.84) and the model group (42.86±8.34), all P < 0.05. IL-6 in the model group on day 3 was also significantly higher than that of the control group, P < 0.05. On day 5, IL-6 in the acupuncture group was (39.26±6.85) which was also significantly higher than that of the control group (35.02±5.59) and lower than that of the model group (42.63±7.86), P < 0.05. IL-6 in the model group was significantly higher than that of the control group at day 5, P < 0.05. At day 7, IL-6 in the acupuncture group (34.77±6.94) was lower than that of the model group (41.86±8.13), P < 0.05. On days 3, 5, and 7, the level of IL-6 in the control group and the model group did not change significantly (all P > 0.05), and decreased from relatively high to relatively low, and then decreased much lower in the acupuncture group (P < 0.05).

TNF-α detection

In the acupuncture group, TNF-α was (38.76±7.05) on day 3 which was not significantly different from that in the model group (36.94±8.07), P > 0.05, yet was significantly higher than that of the control group (28.62±4.62), P < 0.05. TNF-α in the model group was significantly higher than that of the control group at day 3, P < 0.05. On day 5, TNF-α in the acupuncture group was (36.86±7.61) which was significantly higher than that of the control group (29.04±5.04), P < 0.05, and lower than

that of the model group (37.64±9.24), P < 0.05. TNF-α in the model group remained significantly higher than that of the control group at day 5, P < 0.05. On day 7, there was no significant difference between the acupuncture group (28.54±4.69) and the control group (28.88±4.73) in TNF-α levels, P > 0.05, and TNF-α in the acupuncture group was significantly lower than that of the model group (37.84±9.01), P < 0.05. On days 3, 5, and 7 the level of TNF-α in the control group and the model group did not change significantly (all P > 0.05) (**Table 3**).

NF-κB detection

The NF-κB levels of the rats in the acupuncture group was (14.35±2.06) which was significantly higher than that of the control group (3.14±0.95), P < 0.05, and lower than that of the model group (16.82±3.27), P < 0.05. NF-κB in the model group on day 3 was significantly higher than that of the control group, P < 0.05. On day 5, NF-κB in the acupuncture group was (9.82±4.69), which was significantly higher than that of the control group (35.02±5.59), P < 0.05, and was lower than that of the model group (17.06±3.84), P < 0.05. NF-κB in the model group remained significantly higher than that of the control group, P < 0.05. At day 7, NF-κB in the acupuncture group was (6.37± 2.45), which was higher than that of the control group (3.22±0.73), P < 0.05, and significantly lower than that of the model group (16.59±4.05), P < 0.05. On day 3, 5, and 7 the levels of NF-κB in the control group and the model group did not change significantly (all P > 0.05), and NF-κB in the acupuncture group decreased from relatively high to relatively low across time points (P < 0.05) (**Figure 1**; **Table 4**).

Discussion

Cerebral ischemia and reperfusion mainly causes neuronal necrosis, vascular endothelial damage, and blood-brain barrier destruction [18]. The expression of inflammatory factors reflects both the injury and repair conditions of cerebral ischemia and reperfusion more accurately. After damage of local neurons and glial cells in patients with cerebral ischemia, various cytokines are released to participate in the local inflammatory response of the injured cells [8, 19]. Relative signals are taken away from the brain by the blood directly to distal effector organs. Therefore, the detection of relative signals in the blood will reflect the local inflammatory damage of brain tissue, and the recovery progress of the inflammatory damage [20] can be measured through monitoring the inflammatory cytokines IL-1β, IL-6, and TNF-α [21]. At the earlier stages of cerebral ischemia and reperfusion injury, the release of inflammatory cytokines in the damaged brain tissue mediates the inflammatory response. Due to the destruction of the blood brain barrier, the inflammatory cells are transferred from the blood to the periphery to activate inflammatory factors in the peripheral immune system. Therefore, a large number of inflammatory factors are seen in rats with cerebral ischemia and reperfusion. NF-κB can be seen in all types of cells in the nervous system [22] and it has been demonstrated by Tabassum et al. [23] that its activation enables an increase in apoptosis of cerebral ischemic neurons. Our work aims to study the feasibility of treating cerebral ischemia and reperfusion with the acupuncture at Baihui and Zusanli points. Additionally, we attempt to provide future guidance and reference for treating patients in the clinic through the establishment of a rat model of cerebral ischemia and reperfusion, and the detection of rat inflammatory cytokines including IL-1β, IL-6, TNF-α, and NF-κB.

In the acupuncture group IL-6 levels decreased, however in the model group IL-6 sustained high expression levels. Regarding TNF-α, it gradually decreased in acupuncture group, yet it sustained a high expression in the model group. The NF-κB in the acupuncture group decreased gradually during the treatment, while in the model group, it maintained high expression without obvious changes. We observed high expression of IL-1β, IL-6, and TNF-α in cerebral ischemia rats, which is consistent with the findings of Li et al. [24]. In the course of treatment, the TNF-α, as the immune system feedback regulation signal, is involved in the repair of damaged neurons, endothelial cells and so on due to cerebral ischemia and reperfusion [25]. In this study, IL-1β reached peak levels in the model group at day 7. IL-1β is an extremely important protein in the immune system and plays a very significant role in adjusting the function of immune response [26]. IL-6 plays a role in promoting inflammatory injury at the early stages of cerebral ischemia, but it turns to neurotrophic effects in the later stages. Therefore, its

expression on day 3 reached a peak due to the severity of the injury. TNF-α is one of the most important effector molecules in the immune system and is directly involved in the inflammatory injury reaction. In this study there was no difference in the expression of TNF-α between the acupuncture group and the model group at the beginning of treatment, and the expression gradually decreased during the treatment and displayed no significant difference on day 7 between the acupuncture group and the control group. This suggests that acupuncture at Baihui and Zusanli points is feasible for the treatment of cerebral ischemia and reperfusion. At the time of ischemia, NF-κB is activated in the nerve cells and the endothelial cells. This causes an interaction between the receptor on the cell surface and the inflammatory factors which amplifies brain tissue damage. In the treatment process of the acupuncture group, activation of NF-κB was reduced as were the infarct areas, which not only maintains immune system function and the normal structure of brain tissue, but also stabilizes the normal metabolic function of the mitochondria and prevents apoptosis of neurons, reducing the damage caused by cerebral ischemia and reperfusion. In this study, NF-κB in the acupuncture group began to decrease gradually, which proves that acupuncture treatment could effectively treat and inhibit brain injury due to cerebral ischemia and reperfusion.

In summary, acupuncture at Baihui and Zusanli points can effectively reduce the expression level of inflammatory factors in rats with cerebral ischemia and reperfusion, and positively impact repair of brain tissue damage. Due to the differences between rats and human, the results we observed in rats may not translate to humans. We will analyze patients with cerebral ischemia and reperfusion to further investigate the results of our experiment.

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Disclosure of conflict of interest

None.

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