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GV20 Penetrating GB7 on the Expression of Wnt1 in Brain Tissue of Rats with Intracerebral Hemorrhage

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Abstract

Background: Intracerebral hemorrhage (ICH) is a life-threatening disease that confuses us for centuries. Acupuncture as a kind of Chinese traditional treatment has been empirically established and widely used in clinics in China, especially for stroke victims. Studies proved that GV20 penetrating GB7, a kind of needling manipulation, with which a needle is inserted into GV20 and then to GB7 under the scalp, is especially effective on the treatment of this disease. Wingless-type1 (Wnt1) is a key factor of Wnt/β-catenin signal pathway, which could promote regeneration and remodeling of neural function after ICH by affecting proliferation and differentiation of the neural stem cells (NSCs). The study on the expression of Wnt1 induced by GV20 penetrating GB7 may give out the theoretical principle of the treatment.

Objective: The goal of this work is to observe the effect of GV20 penetrating GB7 needling on the protein/mRNA expression of Wnt1 in brain tissue of ICH rats.

Methods: In this experiment, we focused on observing the protein/mRNA expression level of Wnt1 by Western blot and real-time fluorescent quantitative PCR (RTFQ PCR) in the rats with ICH, which were treated by Dickkopf-related protein 1 (DKK1), acupuncture or no intervention, comparing with the healthy rats without any treatment at fixed time points.

Results: This study revealed that the expression of Wnt1 had the similar trend in all groups at each time point of corresponding treatment, which began to increase from the third day (3d), reached the peak on the seventh day (7d) then decreased gradually. The acupuncture treatment group showed a higher level of expression.

Conclusion: The results show that GV20 penetrating GB7 has advantage on promoting the protein/mRNA expression of Wnt1, activates the intracellular Wnt pathway, and regulates the transcription of downstream target genes, affecting proliferation and differentiation of the neural stem cells (NSCs), thereby initiates endogenous repair mechanisms of the organism itself. This study provides a powerful scientific basis for acupuncture treatment on ICH.

Keywords: GV20 penetrating GB7; Wnt1; Intracerebral hemorrhage; Neural stem cells

Introduction

Intracerebral hemorrhage (ICH) is a non-traumatic parenchymal hemorrhage [1]. Most of the patients suffer from hemiplegia or cognitive impairment because of the absence of a large number of neurons, which is difficult to be cured by the current treatment. Studies in recent years found that a variety of adult mammals have proliferation ability of neural stem cells (NSCs) in the sub ventricular zone (SVZ) and the sub granular zone (SGZ) [2] of dentate gyrus of hippocampus. Ischemia, hypoxia, injury and other pathological stimuli can induce the proliferation and differentiation of endogenous NSCs in SVZ and SGZ [3,4]. The Wnt/β-catenin signaling pathway plays an important role in the development of the mammals central nervous system and the regulation of proliferation and differentiation of NSCs. ICH stimulates cell proliferation and migration from the SVZ to the injured tissue [5,6]. The use of endogenous regeneration mechanisms in the treatment of stroke patients after ICH seems to be a rational idea and the mechanism of this treatment needs to be explored deeply.

Scalp acupuncture treatment of ICH began to be applied in our hospital from the early 1990s, and a large number of basic studies have been carried out since then. From 2007 to 2009, we employed GV20 penetrating GB7 needling method and treated 144 patients that were randomly divided into control group and acupuncture group. The recovery rate was 58.33%, and the total effective rate was 91.67% [7]. Our previous research on GV20 penetrating GB7 showed that acupuncture can activate endogenous NSCs by regulating external signal, such as neurotrophic factors [8]. Based on this research, we prepared rat model of ICH and detected dynamic changes of the protein/mRNA expression of Wnt1 by real-time fluorescent quantitative PCR (RTFQ PCR) and Western blot, with Dickkopf-related protein 1 (DKK1) as the antagonist of Wnt signaling pathway. The next step is to comparatively observe the influence of GV20 penetrating GB7 on the expression of Wnt1 in brain tissue of ICH rats in order to explore the overall regulating action of the intracellular signal transduction pathway and clarify the mechanism of the acupuncture treatment in promoting

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neural remodeling after ICH. Thus, this study provides a new scientific basis and theoretical principle for the acupuncture treatment of ICH.

Materials and Methods

Chemistry

All the reagent and instruments in this experiment were purchased locally and were of analytical grade. Acupuncture needles of Hwato, 0.35 × 40 mm were used in the acupuncture treatment of GV20 penetrating GB7. The experimental rats were fixed in prone position on the stereotaxic instrument (Chengdu Instrument Factory, STW-1). Engine bit (Shanghai dental Machinery Factory, 307-6) was used to drill a hole in each skull of the rat. Primary antibodies (Rabbit-anti Wnt1, Beijing Boosen), which bind to non-antibody antigen, and secondary antibodies (Goat-anti-Rabbit, Beijing Boosen), which bind to antibody, were both applied in Western blot. GEL imaging system (Alpha Innotech, USA) was employed to detect and analyze different staining through non chemiluminescence imaging of gel electrophoresis (protein/mRNA). Nucleic acid amplification instrument (ABI9700, USA) was supplied for RTFQ PCR, and vertical electrophoresis transfer membrane system (Bio Rad, USA) was used for electrophoresis and transferring membrane.

Animals

Adult male Sprague-Dawley (SD) rats with average weight of 250 g ± 20 g were used in the experiment. The animals had free access to food and water. The experiment was performed in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for the investigation of experimental pain in conscious animals.

Sample collection and processing

ICH molding: 192 healthy male SD rats were chosen randomly, 12 were assigned to blank group and 180 were made to ICH models. Firstly the rats were fixed on the stereotaxic instrument in prone position after being anesthetized and the scalp skin being shaved. Sagittal incision was made in the scalp to expose the anterior fontanel. The micro syringe was fixed with stereotaxic instrument and aimed at anterior fontanel, then was moved 3.5 mm to the right side and 0.2 mm to the back. A sign was marked here, and a hole was drilled in the skull with engine bit on the sign. This is the basal ganglia area of the rat brain. Meanwhile, 60 μl autologous blood was collected from the rat tail with microinjector and slowly injected the blood into caudate nucleus 6 mm beneath the surface of the brain through the hole. The needle was withdrawn after 3-5 min. The hole was closed with dental cement, sterilized with gentamicin, and sutured.

Neurological score: Berdersonscore was used to evaluate the neurological function of the rats. Based on this scoring, rats were held gently by the tail, and suspended one meter above the floor; normal rats extend both forelimbs towards the floor (Table 1) [9]. After the ICH molding, we counted the rats with 1-3 grade as successful ones, which were included in the experiment.

Intervention: The 180 rats to be made into ICH models were first randomized into 3 groups, and 60 rats were in each of them. 10 μL (0.1 μg/μl) GSK-3β inhibitor DKK1 was injected into the rats in DKK1 group 30 min before modeling, GV20 penetrating GB7 needling was applied to the rats in acupuncture group after modeling, and no intervention was employed to the rats in model group either before or after molding. Furthermore, each group was divided into five subgroups by the time point: the third day subgroup (3d), the seventh day subgroup (7d), the fourteenth day subgroup (14d), the twenty-first day subgroup (21d) and the twenty-eighth day subgroup (28d). GV20 penetrating GB7 needling was applied to the rats in acupuncture group for 30 min each time, once a day. 5 min twisting manipulation was given once in each 10 min at the speed of 200 rpm. We sampled the three groups of rats above at corresponding time point. The blank group of rats received no intervention and were sampled directly. All animals were sacrificed after the samples were taken. Out of every twelve samples in each group/subgroup, six were detected for the expression of Wnt1 by Western blot and the other six by RTFQ PCR.

Normal	Grade 0	No observable deficit
Moderate	Grade 1	Forelimb flexion
Severe	Grade 2	Decreased resistance to lateral push (and forelimb flexion) without circling
	Grade 3	Same behaviour as Grade 2, with circling

Table 1: Neurological Examination Grading System.

Western blotting: SDS-PAGE gel was prepared first. The samples were boiled for 5 min, placed on ice and centrifuged for 10 minutes at 4°C under 12000 rpm after the sample tissues were ground, and protein lysate was added. Then the supernatant was dropped into the centrifuge tube. The protein in the sample was measured, and then some sample was taken with injector for electrophoresis, transferring membrane and blocking. Appropriate concentration of primary antibodies was added into the blocking solution. The mixture was kept overnight at 4°C and rewarmed on the following day. Then appropriate concentration of secondary antibodies was added in the sample.

RTFQ PCR: PCR buffer, dNTP, primers and DNA template, etc. were added separately in a centrifugal tube on ice. The mixture was centrifuged, amplified in PCR amplification instrument, and detected through PCR electrophoresis.

Statistical analysis

Data were analyzed with the help of a SPSS version 22, and the results were expressed as Mean ± Standard Deviation (SD). Comparison between groups was performed with one-way analysis of variance (ANOVA). $P<0.05$ was considered to be statistical significance.

Results

Protein expression of Wnt1 in the brain tissues of each group by Western blot

The Western blot results showed that the protein expression is in extremely low level in the blank group. The protein expression of Wnt1 in the model group began to increase from 3d, reached the peak at 7d then decreased gradually in 14d, 21d, and 28d. The protein expression of the model group at each time point were statistically significant compared with the blank group ($P<0.05$). The acupuncture group at each time point expressed the same trend with the model group, but the protein expression in 7d, 14d, 21d, 28d was significantly higher than that of the model group at the same time points. The results were statistically significant ($P<0.05$). DKK1 group had the same expression tendency with the model group at each time point, but the protein expression significantly decreased than that of the same time point of the model group in 7d, 14d, 21d, 28d, and compared with the model group and the acupuncture group at the same time point, the results were statistically significant ($P<0.05$) (Figures 1 and 2).

mRNA expression of Wnt1 in the brain tissues of each group by RTFQ PCR

The RTFQ PCR results showed that mRNA expression was at the

lowest level in the blank group. The level of mRNA expression of Wnt1 in the model group was slightly higher in 3d, reached the peak at 7d then decreased gradually in 14d, 21d and 28. The mRNA expression of the model group at each time point were statistically significant compared with the blank group (P<0.05). The acupuncture group at each time point expressed the same trend with the modle group, but the mRNA expression in 7d, 14d, 21d, 28d was significantly higher than the model group at the same time point. The results were statistically significant (P<0.05). DKK1 group had the same expression tendency with the model group at each time point, but the mRNA expression significantly decreased than that of the same time point of the model group in 7d, 14d, 21d, 28d, and compared with the model group and the acupuncture group at the same time point, the results were statistically significant (P<0.05) (Figure 3).

Discussion

GV20 penetrating GB7 needling

Head acupuncture therapy is a kind of method of treating disease by using needles to stimulate the head acupoints (or stimulation area). The effect of needling the head acupoints on treating ICH has been confirmed and recorded in ancient time. It is said in the ERON song of Compendium of Acupuncture and Moxibustion, booked in the Ming Dynasty and the most important book to study acupuncture, in which the theory of acupuncture and moxibustion is comprehensively

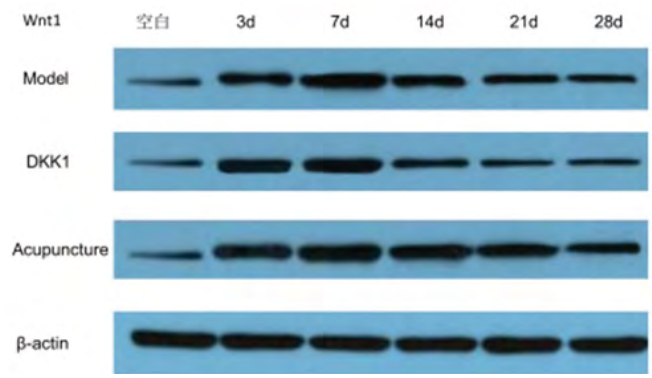

Figure 1: Effect of DKK1, acupuncture or no intervention on the protein expressionof Wnt1 in the normal brain tissues or the ICH brain tissues.

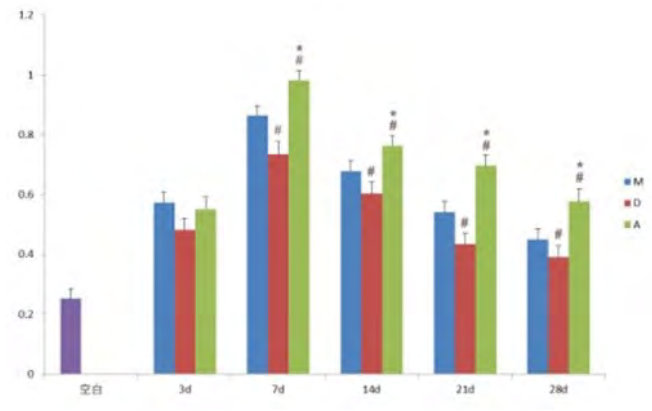

Figure 2: The protein expression of Wnt1 in the blank group, the model group, the DKK1 group and the acupuncture group in 3d, 7d, 14d, 21d, 28d. The results are presented as the mean ± SD. *P<0.01, as compared with the DKK1 group, #P<0.01, as compared with the model group.

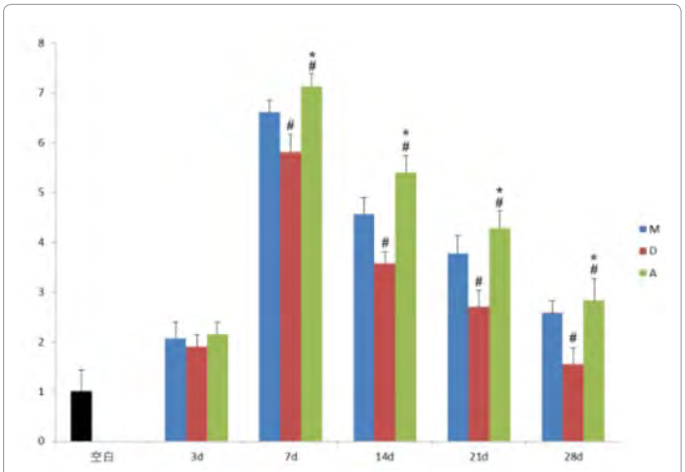

Figure 3: The mRNA expression of Wnt1 in the blank group, the model group, the DKK1 group and the acupuncture group in 3d, 7d, 14d, 21d, 28d. The results are presented as the mean ± SD. *P<0.01, as compared with the DKK1 group, #P<0.01, as compared with the model group.

discussed, that "Aphasia due to stroke is a disease most difficult to be cured. The fontanel (where GV20 is located) and GV 24 should be selected for needling. GV20 is required to be needled with more supplementation and less draining method, and the former first and the latter second. So treated, the patients can be recovered and safe." From the electrophysiology perspective, head is the volume conductor, and needling head acupoints can transmit the bioelectrical effect produced by the acupuncture stimulation to the cerebral cortex by the volume conductor, hence the excitability of neurons in the cerebral cortex is changed. This can reverse the brain neurons excitability inhibited by hemorrhagic stimulation or hematoma compression, awaken the shock or dormancy brain nerve cells, recover the excitability of neural cells in cerebral cortex, and enhance cerebral compensatory function. Therefore, the brain function improves.

GV20 penetrating GB7 is an ideal clinical needling method for the treatment of stroke. Professor Zou Wei has conducted a great deal of research on its mechanism. The parietal lobe, frontal lobe and temporal lobe, where the motor cortex and the sensory cortex locate, are underneath the area of GV20 to GB7. These three lobes are important in processing sensory information, language, and movement coordination. GV20 penetrating GB7 with rapidly twisting manipulation may help restoring consciousness and improving the ability of language and the motor function.

The effect of GV20 penetrating GB7 on the expression of Wnt1 in brain tissue of ICH rats

Wnt was first discovered as a kind of cancer gene in the early 1980s. Until now, there are 19 Wnt genes found in human genome. Among those, Wnt1 is the most important gene in the process of neural development, and has the widest expression in the body. Some experimental results have showed that endogenous NSCs of both rats and humans are activated [10-13] and the active NSCs may take part in the recovery process of nervous system after an ischemic or hypoxic injury [14-19]. Besides, some data display that nerve is enhanced in hippocampus, striatum, and cortex after the brain is injured by ICH, and Wnt signaling pathway is discovered to have the ability of regulating hippocampal neurogenesis of adult SD rats [20]. Some Chinese scholars [21] have found that the time-dependent expression of Wnt1 in the hippocampus of ischemic injury coincides

with the process of proliferation and differentiation of NSCs. They illustrates that Wnt1 plays an important regulatory role in the early process of proliferation and differentiation of NSCs. The research on NSCs of ventral midbrain by Castelo-Branco et al. has discovered that Wnt1 in ventral midbrain mainly promotes the proliferation of NSCs, and is helpful for differentiation of various neurons [22]. In the process of nerve differentiation, blockade of the Wnt signaling pathway can decrease NSCs proliferation, whereas activation of the Wnt signaling pathway may cause the opposite effect [23]. Previous experiments [24,25] showed that the Wnt/β-catenin signaling pathway stimulates self-renewal of NSCs, and by inhibiting gliogenesis, NSCs differentiation towards neuronal phenotype could be activated as well. In addition, down-regulation of Wnt/β-catenin causes degeneration of striatal synapsis, which finally causes impaired motor behaviour [26]. Herein, the expression of Wnt1 is always accompanied by the differentiation of neural cells; when cell differentiation suspends, the expression of Wnt1 stops. This signifies that the expression of Wnt1 is closely related to the differentiation and maturation of nerve cells.

The Wnt signaling pathway controls many events during embryonic development and regulates proliferation, morphology, motility, and cell fate at the cellular level. Activation of the Wnt/β-catenin pathway is characterized by 1) uncoupling β-catenin from degradation complex after receptor engagement by Wnt ligands, 2) β-catenin nucleus translocation, 3) β-catenin interaction with lymphoid enhancer-binding factor-1/T-cell factor-1 (LEF/TCF) transcription factors, and 4) stimulation of target genes (Figure 4) [27-29]. A study also showed that Wnt family gene mRNA expression in NSCs can be isolated from rat SVZ [30]. These results suggest that the Wnt signaling pathway involves deeply in regulating NSCs proliferation and differentiation [31,32] during brain development.

This study utilizes Western-blot and RTFQ PCR methods to observe the changes of the Wnt1 protein/mRNA expression in brain tissue of rats at different time points. These two methods for detecting Wnt1, from protein and gene aspects, reach the same conclusion that GV20 penetrating GB7 can promote the Wnt1 protein/mRNA expression in brain tissue of ICH rats. Although the Wnt1 protein/mRNA expression decreases after 14d, the expression in the acupuncture group is still higher than that of the other groups at the same time point. Along with the increase of acupuncture times, this needling can obviously promote Wnt1 protein/mRNA expression, and urge Wnt1 to combine with the receptors located on the cell membrane through autocrine and paracrine. The stimulation can further activate signaling pathway of intracellular Wnt1, and regulate the expression of downstream target genes, in order to promote the proliferation of endogenous NSCs and the differentiation of neuron. Thereby, endogenous repair mechanism of the organism itself is initiated, and the regeneration and remodeling of neural function after ICH is finally promoted.

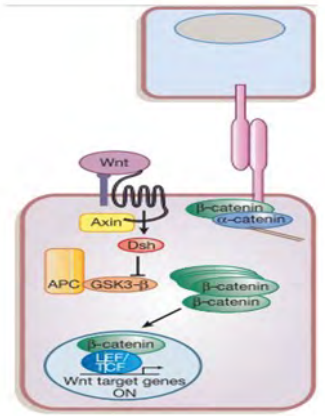

Figure 4: In the presence of Wnt signaling, β-catenin is uncoupled from the degradation complex and transloactes to the nucleus, where its binds LEF/TCF transcription factors, thus activating target genes [27].

Conclusion

This study reveals that GV20 penetrating GB7 needling method can improve the expression of the Wnt signal pathway from protein and mRNA and this result may be one of the important mechanisms of acupuncture on treating ICH.

Conflict of Interest

The authors confirm that this article content has no conflict of interest.

Acknowledgements

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