

Original Article

Radial extracorporeal shock wave therapy improves cerebral blood flow and neurological function in a rat model of cerebral ischemia

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Abstract: We performed middle cerebral artery occlusion (MCAO) in rats to investigate the effect and some of the underlying mechanisms of radial extracorporeal shock wave therapy (rESWT) in cerebral ischemia rats. We measured neurological function and cerebral blood flow (CBF) using a full-field laser perfusion imager and brain infarct volume on days 3, 12, and 30. Immunofluorescence, western blot, and real-time polymerase chain reaction (PCR) techniques were used to detect the expression of vascular endothelial growth factor (VEGF), neuron-specific enolase (NSE), nestin, Wnt3a, and β -catenin in the ischemic hemisphere. The dose of rESWT used on the head revealed remarkable advantages over sham rESWT, as demonstrated by improved neurological function scores, increased CBF, and reduced brain infarct volume. Furthermore, applying rESWT to the head and limbs enhanced short-term neurological function. Our results confirmed that rESWT can induce VEGF expression over an extended period with a profound effect, which may be the primary reason for CBF recovery. High NSE and nestin expression levels suggest that rESWT enhanced the number of neurons and neural stem cells (NSCs). Wnt3a and β -catenin expression were up-regulated in the ischemic hemisphere, indicating that rESWT promoted NSC proliferation and differentiation via the Wnt/ β -catenin pathway. Overall, our findings suggest that an appropriate rESWT dose delivered to the head of rats helps restore neurological function and CBF, and additional application of rESWT to the limbs is more effective than treating the head alone.

Keywords: Radial extracorporeal shock wave, cerebral ischemia, neurological function, CBF, neural stem cells, Wnt/ β -catenin pathway

Introduction

Cerebral ischemia is the most common cerebrovascular condition. Thrombolytic therapy and functional rehabilitation training help prevent neurological damage after cerebral ischemia [1, 2]; however, many patients are unable to receive thrombolytic treatment because of its limited therapeutic window [1] and must use drugs that have limited abilities to improve circulation and neurological function. Kinesiotherapy is also suggested, but it is tedious and therefore difficult to maintain over an extended period. Other treatments have therefore been suggested as supplementary methods to recover neurological function after cerebral ischemia. In clinical settings, repetitive transcranial magnetic stimulation seems to promote motor

function in the affected limb [3]. Transcranial low-level light therapy and pulsed transcranial ultrasound stimulation lead to significant functional benefits in a mouse model of cerebral ischemic [4].

Extracorporeal shock wave therapy (ESWT) was first successfully used for treating urinary stones [5]. Since then, it has been widely applied in the medical field as a noninvasive therapy and has been used to treat muscle, bone, and joint diseases [6-8], and chronic soft tissue injuries [9-11]. Recent studies have shown that ESWT can treat sciatic nerve injury by enhancing nerve function and preventing denervation atrophy [12]; it can induce nerve fiber regeneration after 2 weeks [13]. Furthermore, ESWT improves cardiac function in the setting

Table 1. The behavior evaluation of rats

Test	0	1	2	3
Spontaneous activity	No movement	Did not rise at all and barely moved in the cage	Moved around and reached at least one upper rim of the cage	Moved around and approached at least three walls of the cage
Forelimbs outstretching	Left forelimb did not move	Left forelimb moved minimally	Leftside outstretched less than the right, and walking with the forelimbs was impaired	Both forelimbs were outstretched, and the rat walked symmetrically on forelimbs
Response to vibrissae touch	-	Did not respond to a stimulus on the left side	Reacted slowly to a stimulus on the left side	Reacting by turning the head or was equally startled by the stimulus on both sides
Epistaxis	Epistaxis	No epistaxis	-	-
Dietary intake	Ate nothing	Barely ate	Normal	

Note: The behavior of rats was evaluated through spontaneous activity, forelimb outstretching, response to vibrissae touch, epistaxis and dietary intake. The behavior scores were the total of the respective scores.

of ischemic heart failure and promotes re-vascularization in the ischemic myocardium by increasing vascular endothelial growth factor (VEGF) and interleukin (IL)-8 secretion [14, 15]. Additionally, transcranial ESWT is a safe treatment for unresponsive wakefulness syndrome, resulting in improvements on the Glasgow Coma and German Coma Remission Scales [16]. Because of the remarkable efficacy of ESWT in treating various diseases, we hypothesized that it may increase local brain blood flow after cerebral ischemia and restore neurological function by inducing VEGF expression to stimulate vasculogenesis and Wnt/ β -catenin signaling, which is involved in neural stem cell (NSC) proliferation and differentiation.

Materials and methods

Ethics

All animal experiments were approved by the China Medical University Laboratory Animal Center and followed the guidelines for the care and use of laboratory animals.

Animals and grouping

Male Sprague Dawley (SD) rats (Liao Ning Chang Sheng Biotechnology, China, 240-260 g) were used in this study. The rats were randomly assigned to group I (n=45), group II (n=15), or the control group (n=45). Fifteen rats from group I and the control group and 5 rats from group II were sacrificed on days 3, 12, and 30 following the middle cerebral artery occlusion (MCAO) operation.

Cerebral ischemia model

Cerebral ischemia model was induced using a modified Zea Longa [17] intraluminal filament

technique in all the three groups. After anesthesia, the right common carotid artery was exposed, and the external carotid artery was closed and cut with microscissors. A filament (Guang Zhou Jia Ling Biotechnology, China, 3400AAA) was inserted into the stump of the external carotid artery and gently advanced into the internal carotid artery, approximately 18 mm from the carotid bifurcation, until mild resistance was felt; the filament was withdrawn 2 h after MCAO. Cerebral ischemia induction was considered successful based on the following signs: failure to extend the left forepaw, circling to the contralateral side, or leaning to the contralateral side. The model that we chose scored 2 or 3 on the Longa test [17].

Treatment protocol

The rESWT (STORZ MEDICAL AG, Switzerland) dosage was tested in healthy and cerebral ischemic rats. The criterion was the maximum dosage that did not damage brain tissue. We employed a ceramic transmitter that could be applied to superficial tissue at a depth of 20 mm [18]. To test whether rESWT was safe, we evaluated rat behavior according to **Table 1**. After behavioral testing, the rats were sacrificed to observe whether there was evidence of hemorrhage on the brain surface.

Coupling was used between the head and transmitter, and rESWT was applied directly to the right side of the head of healthy rats. We modified the dosage from 3.5 to 3.0 to 2.0 to 1.0 bar using 200 impulses and 10 Hz. Three rats were used to test each dosage. rESWT at a dosage below 2.0 bar, 200 impulses, and 10 Hz did not cause hemorrhages on brain surface, and the behavior scores remained high. Ultimately, we chose 2.0 bar, 200 impulses, and 10 Hz as a suitable dosage.

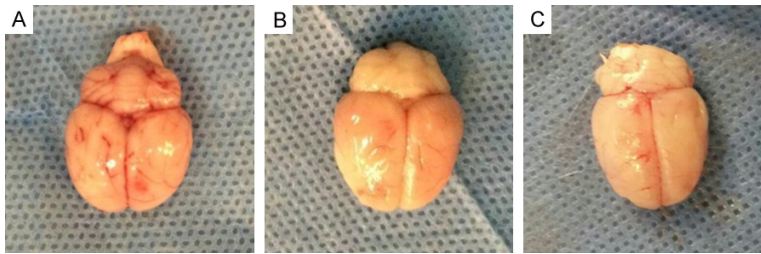


Figure 1. Safe use of rESWT in the brains of control rats and 72 h after MCAO. rESWT at dosages of 2.0 or 1.0 bar, 200 impulses, and 10 Hz (A and B, respectively) was safely administered to control rats. There was no brain surface hemorrhaging 72 h after MCAO when using rESWT at a dosage of 1.0 bar, 200 impulses, and 10 Hz (C).

After testing, rESWT was administered at a dosage of 2.0 bar, 200 impulses, and 10 Hz in a three rats 72 h after MCAO. Their behavior scores decreased, and the brain surface hemorrhaged, so we reduced the dosage to 1.5 or 1.0 bar with 200 impulses and 10 Hz on six additional rats 72 h after MCAO. rESWT at a dosage below 1.0 bar, 200 impulses, and 10 Hz was safe after MCAO and was used in the experiments.

The rats in groups I and II received rESWT (1.0 bar, 200 impulses, 10 Hz directly on the right side of the head) 72 h after MCAO every 3 days. Also, the rats in group II received rESWT (2.0 bar, 200 impulses, 10 Hz on the left side of the limbs) every 3 days beginning 6 days after MCAO. The rats in the control group did not receive rESWT; the coupling was used, but the transmitter did not directly touch the rats' head.

Behavioral tests of neurological function

All rats were tested using modified neurological severity scores (mNSS) [19] on days 3, 12, and 30 after MCAO by three evaluators who were blind to groups and time points. Motor tests were performed by lifting the rats by the tail and observing either flexion of a forelimb (1 point), flexion of a hindlimb (1 point), or head movements $>10^\circ$ to the vertical axis within 30 s (1 point). Tests were performed by placing the rat on the floor and observing ambulation of either normal walking (0 points), an inability to walk straight (1 point), circling toward the paretic side (2 points), or falling to the paretic side (3 points). The placing test (1 point) and proprioceptive test (1 point) were performed to assess sensory function. Beam balance tests were conducted to observe balance with a

steady posture (0 point), grasping a side of the beam (1 point), hugging the beam while one limb falls from the beam (2 points), hugging the beam while two limbs fall from the beam, spinning on the beam (>60 s) (3 points), attempting to balance on the beam but falling off (>40 s) (4 points), attempts to balance on the beam but falling off (>20 s) (5 points), or falling off the beam with no attempt to

balance or hang onto the beam (<20 s) (6 points). Reflexes were also observed for absent and abnormal movements, including pinna reflex (1 point); corneal reflex (1 point); startle reflex (1 point); or seizures, myoclonus, or myodystrophy (1 point). The final neurological severity score was the mean of the three results.

Brain infarction assessment

Rats in the control group and group I were deeply anesthetized, and their brains were rapidly removed. After the brains had been frozen in liquid nitrogen, six serial sections from each brain were cut at 2.5 mm each. To measure changes due to ischemia, brain slices were stained with a solution containing 2% 2,3,5-triphenyltetrazolium chloride (TTC) dissolved in saline at 37°C for 20 min. Next, the slices were transferred to 5% neutral buffered formalin and stored at 4°C before analysis. Infarct volume (%) was measured using Image-Pro Plus (IPP) software (Media Cybernetics, USA). Percentage of infarct volume = (contralateral hemispheric volume-ipsilateral non-infarcted volume)/total hemispheric volume [20].

Cerebral blood flow

Cerebral blood flow (CBF) was measured after evaluating neurological function. Imaging was performed using a full-field laser perfusion imager (FLPI2, Moor Instruments Ltd., UK). Rats were anesthetized with 1% isoflurane inhalation in air via a facemask and placed in the prone position, and the skin was incised to access to the skull. A physiologic solution was applied to keep the surface moist [21]. Rats from the control group and group I were evaluated on 3, 12, and 30 days after MCAO. Blood

Table 2. The scores of behavior evaluation before and after rESWT

	Healthy rats				Rats 72 h after MCAO		
	3.5 Bar (n=3)	3.0 Bar (n=3)	2.0 Bar (n=3)	1.0 Bar (n=3)	2.0 Bar (n=3)	1.5 Bar (n=3)	1.0 Bar (n=3)
Before rESWT	12.00	12.00	12.00	12.00	9.33	8.67	9.00
Immediately after rESWT	3.33	10.67	12.00	12.00	8.00	8.67	9.00
10 min after rESWT	9.67	12.00	12.00	12.00	9.33	8.67	9.00
30 min after rESWT	11.67	12.00	12.00	12.00	9.33	8.67	9.00
Hemorrhage on brain surface (in at least 1 rat)	Yes	Yes	No	No	Yes	Yes	No

The behavior of rats was evaluated before rESWT and immediately, 10 min and 30 min after rESWT. There were no obvious differences before and after rESWT at a dosage below 2.0 bar, 200 impulses, and 10 Hz in healthy rats and below 1.5 bar, 200 impulses, and 10 Hz in rats 72 h after MCAO.

Table 3. Neurological function of the experimental and control groups

Group	3 days (n=45)	12 days (n=30)	30 days (n=15)
GI	6.71±0.55	2.83±0.65	0.47±0.64
Control group	6.62±0.53	4.40±0.85***	3.40±0.73***
GII	6.83±0.40	1.90±0.23*	0.33±0.14

Note: A significant improvement in mNSS was found in GI compared with the control group (***P<0.001) at both 12 and 30 days after MCAO. Neurological function significantly improved in GII compared with that in GI on day 12 (*P<0.05).

fluxes were measured in infarcted regions of interest and corresponding contralateral cortical areas to calculate the percentage of CBF reduction.

Immunofluorescence analysis

After behavioral testing, animals in the control group and group I were sacrificed. The brains were then collected, and the infarct area and surrounding tissues were prepared for immunofluorescence studies. Rats were transcardially perfused with 4% paraformaldehyde in 1× phosphate-buffered saline (PBS, pH 7.4). The brains were rapidly removed, embedded in paraffin wax, and sectioned at 5 µm. Paraffin sections were dewaxed and pre-treated using heat-mediated antigen retrieval. Next, the sections were washed with PBS and blocked in blocking buffer (1% bovine serum albumin PBS) for 30 min, followed by incubation with antibodies against VEGF (1:200, Abcam, UK), NSE (1:200, Abcam), or nestin (1:100, Abcam) at 4°C overnight. The sections were then washed in PBS, followed by further incubation at room temperature for 1 h with an FITC-conjugated anti-rabbit IgG (1:200, Beyotime, China) and rinsing with PBS. Next, the sections

were treated with DAPI for 5 min at room temperature to stain nuclei and washed again in PBS. The sections were mounted in 90% glycerol with an anti-fading agent. All sections were analyzed using IPP software.

Western blot analysis

Rats were sacrificed after performing behavioral tests, and the brains were rapidly removed. Proteins were extracted from the ischemic cerebral hemisphere using cold radio immunoprecipitation assay buffer. Equal amounts of protein extracts were loaded and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12% polyacrylamide gels. After electrophoresis, the proteins were transferred onto polyvinylidene fluoride membranes. The membranes were then immersed in blocking solution for 1 h at room temperature, followed by incubation with antibodies against VEGF (1:1000, Abcam), NSE (1:5000, Abcam), nestin (1:3000, Abcam), Wnt3a (1:20,000, Abcam), and β-catenin (1:10000, Abcam) overnight at 4°C. Subsequently, membranes were rinsed and incubated with a secondary antibody (1:5000, Sigma, USA), followed by ECL detection. The density of each band was quantified with ImageJ software (National Institutes of Health, USA).

Real-time PCR analysis

Real-time PCR was used to measure VEGF, NSE, nestin, Wnt3a, and β-catenin mRNA levels in the affected cerebral hemisphere. After sacrifice, the brain specimens were homogenized, and total RNA was extracted with TRIzol. RNA purity and concentration were analyzed before

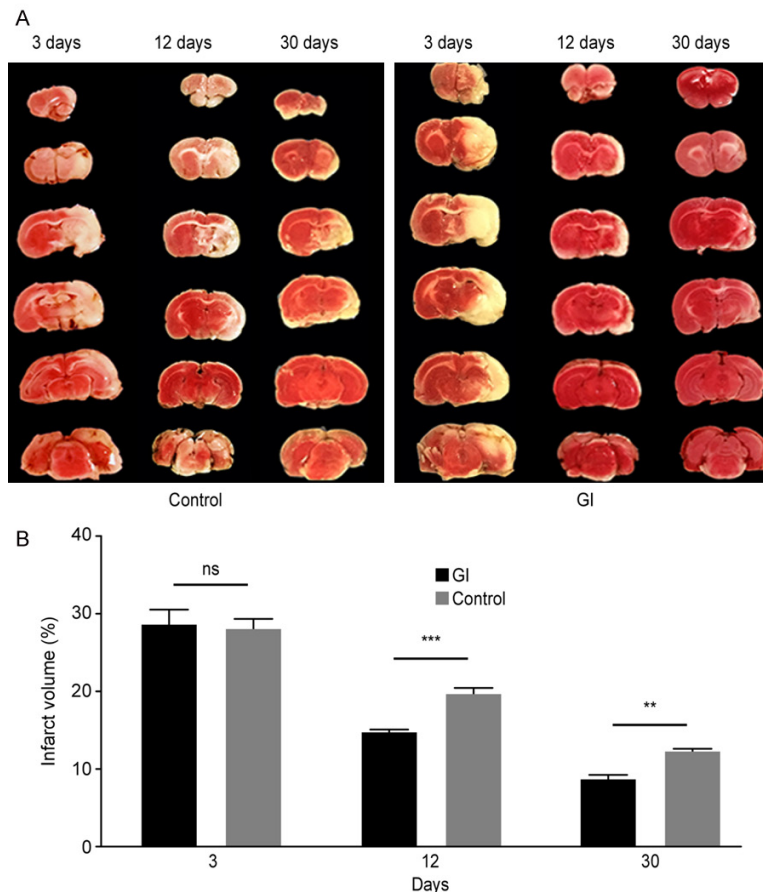


Figure 2. TTC staining to measure brain infarction volume. TTC staining showing infarction volume (A) and infarction volume divided by total brain volume (B). The white area indicates the brain infarction size. rESWT significantly reduced the brain infarction volume to total brain volume ratio (%) on days 12 (*** $P < 0.001$) and 30 (** $P < 0.01$).

RNA was reverse transcribed to cDNA. Next, a 20- μ L real-time PCR reaction was conducted with the following cycling conditions: 95°C for 30 s, 95°C for 5 s, 60°C for 34 s, 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. The primers used in this study were as follows: VEGF: 5'-CCGTCCTGTGTGCCCTAAT-3' and 5'-AAACAAATGCTTTCTCCGCT-3'; NSE: 5'-GATGCTGGAGTTTCGATGG-3' and 5'-AGGATGAGGTCTGGAGTTC-3'; Nestin: 5'-CAACCACAGGAGTGGGAAC-3' and 5'-TCTGGCATTGACTGAGCAAC-3'; Wnt3a: 5'-TCTGCCATGAACCGTCACAACAAT-3' and 5'-C-CAGCAGGTCTTCACTTCGCAACT-3'; and β -catenin: 5'-TGCAGCGACTAAGCAGGA-3' and 5'-TC-ACCAGCACGAAGGACA-3' (Beijing Ding Guo Chang Sheng Biotechnology, China). The housekeeping gene was glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Quantification was performed by analyzing the cycle thresh-

old (Ct) values according to the $2^{-\Delta\Delta C_t}$ method.

Statistical analyses

Quantitative data are expressed as mean $\pm$ SD. All analyses were conducted using SPSS statistical software. Statistical comparisons were performed by one-way analysis of variance (ANOVA) followed by the Least Significant Difference test to compare differences at each time point. A probability value < 0.05 was considered significant.

Results

rESWT dosage

rESWT was safely used directly on the head of healthy rats at a dosage below 2.0 bar, 200 impulses, and 10 Hz; in model rats, the safe dosage was 1.0 bar, 200 impulses, and 10 Hz. These protocols did not induce hemorrhage (Figure 1) or behavior changes (Table 2). We found that rESWT at a dosage of 2.0 bar, 200 impulses, and 10 Hz had different effects on heal-

thy and model rats. This dosage only induced behavioral score changes in rats that had undergone MCAO 72 h earlier (Table 2), presumably because the brain tissues were weakened and could not tolerate the same rESWT dosage.

Neurobehavioral recovery

We quantified mNSS to evaluate the neurological function. No differences were observed between the three groups at 3 days after MCAO ($P > 0.05$). However, by days 12 and 30 after MCAO, there was a remarkable improvement in mNSS between group I and the control group ($P < 0.001$). Neurobehavior was significantly more impaired in group II than in group I on day 12 ($P = 0.016$), and mNSS in group II were lower than those in group I on day 30, alth-

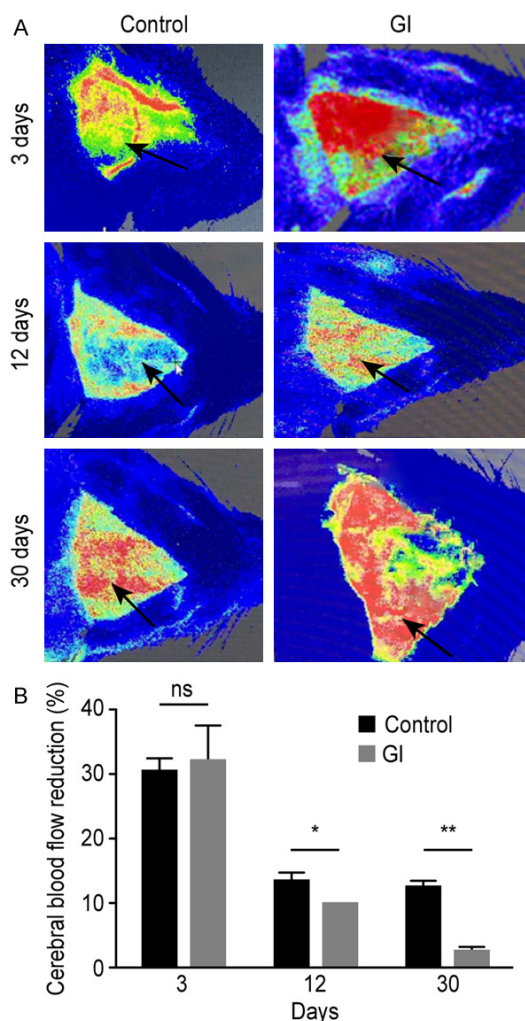


Figure 3. CBF redistribution after MCAO in SD rats using laser speckle imaging. Representative color-coded laser speckle images taken 3, 12, and 30 days after MCAO. Brighter colors are associated with greater blood flow (A). In group I, the mean CBF value in the left cerebral hemisphere was remarkably reduced compared with that in the control group on days 12 (* $P<0.05$) and 30 (** $P<0.01$) (B).

ough there was no difference between groups I and II (Table 3).

Brain infarction volume and CBF

TTC staining did not show differences in brain infarction volume on day 3 ($P>0.05$) between group I and the control group. However, group I differed significantly from the control group on days 12 ($14.36\pm1.03\%$ vs. $19.56\pm1.47\%$, respectively; $P<0.001$) and 30 ($8.34\pm1.04\%$ vs. $12.10\pm0.75\%$; $P=0.003$) (Figure 2). CBF was assessed using a color-coded laser perfusion

imager, and there were significant differences in CBF between the control group and group I on days 12 ($9.82\pm0.13\%$ vs. $13.54\pm1.22\%$, respectively; $P=0.039$) and 30 ($2.88\pm0.59\%$ vs. $12.55\pm0.98\%$; $P=0.0055$) (Figure 3).

VEGF, NSE, nestin, Wnt3a, and β -catenin expression

VEGF, NSE, and nestin levels in ischemic brain tissues were measured by immunofluorescence, western blot, and real-time PCR. Expression levels were not different between group I and the control group on day 3. However, VEGF expression significantly increased on days 12 (group I, 2.017 ± 0.05 vs. control group, 1.661 ± 0.05 ; $P=0.0097$) and 30 (group I, 0.56 ± 0.08 vs. control group, 0.27 ± 0.06 ; $P=0.027$) (Figure 4) after rESWT was used on the head of rats after MCAO. Furthermore, the protein and mRNA expression levels of NSE, the marker of neuronal cells, were higher in group I than those in the control group on day 12 (GI, 0.91 ± 0.05 vs. control group, 0.75 ± 0.03 ; $P=0.026$) and day 30 (GI, 2.13 ± 0.17 vs. control group 1.32 ± 0.44 ; $P=0.005$) (Figure 5). Nestin expression, an NSC marker, was significantly increased in group I on days 12 (0.69 ± 0.19 vs. control group, 0.42 ± 0.07 ; $P=0.018$) and 30 (1.46 ± 0.63 vs. control group, 0.20 ± 0.06 ; $P=0.002$) (Figure 6). To examine the effects of rESWT on the Wnt/ β -catenin pathway, we evaluated Wnt3a and β -catenin mRNA and protein levels in ischemic brain tissues by real-time PCR and western blot. rESWT resulted in higher Wnt3a and β -catenin expression on days 12 and 30 (Figure 7) compared to the control group.

Discussion

In the past 20 years, ESWT has proven to be an effective, safe, noninvasive treatment option for tendonitis and other musculoskeletal pathologies based on high-quality randomized controlled trials [8, 22]. In contrast to conventional focused ESWT (fESWT), rESWT disperses eccentrically from the applicator tip without concentrating the shock wave field on the targeted tissue, and ultrasound or fluoroscopy is not required [23]. There is no consensus in the literature regarding the energy differences between fESWT and rESWT [24]. The recent trend of increasing research interest in rESWT is due to its advantages of being low cost and relatively simple to administer [25]. During this

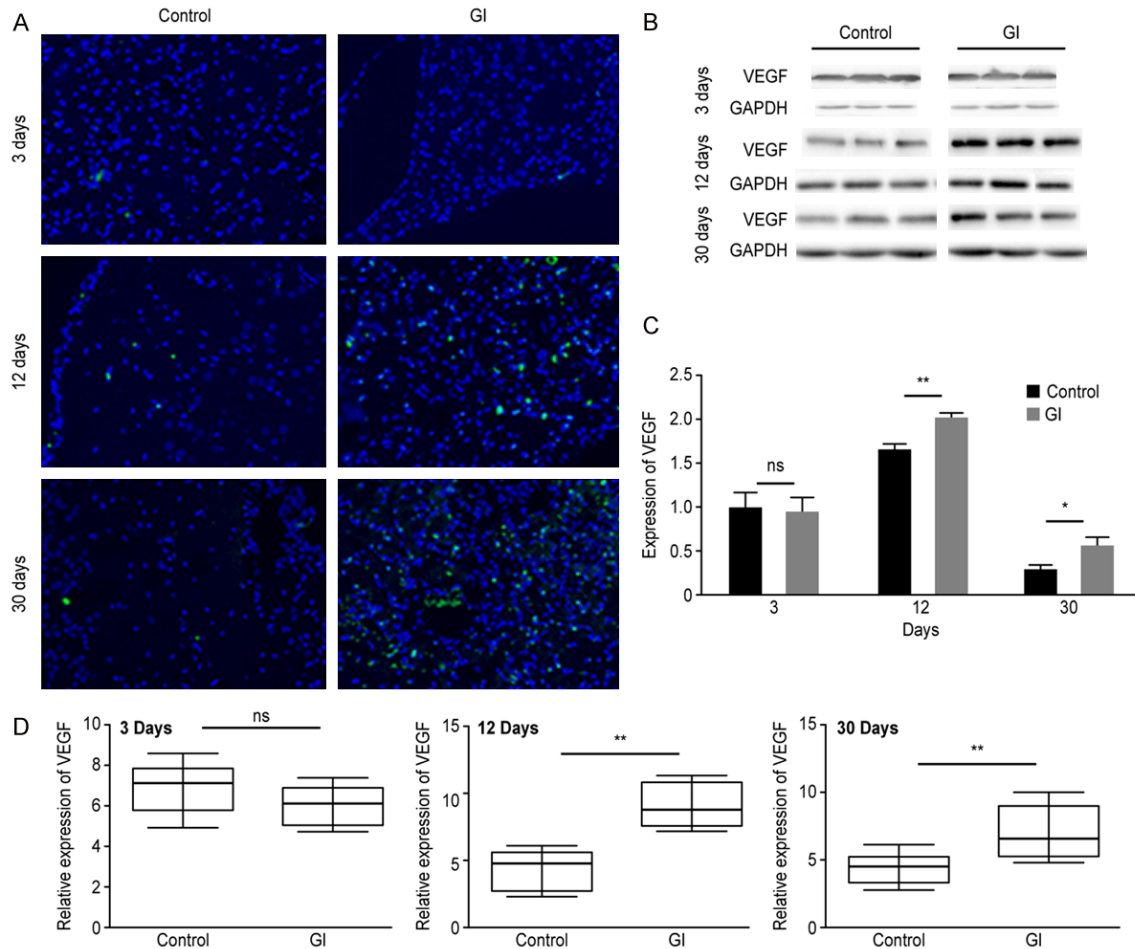


Figure 4. VEGF immunofluorescence, western blot, and real-time PCR findings. Immunofluorescence microscope images (200×) of the post-stroke cortex showed increased VEGF expression (green) compared with the control group (A). Western blot findings of VEGF (B). Densitometry results indicated a marked increase in group I compared with control at days 12 (** $P < 0.01$) and 30 (* $P < 0.05$) (C). Real-time PCR of VEGF mRNA expression showed significant increases in group I on days 12 (** $P < 0.01$) and 30 (** $P < 0.01$) after MCAO (D).

study, we chose an appropriate shock wave dose effect for rats with cerebral infarction that can quickly improve CBF to the infarcted area and contribute to neural function recovery.

In our study, rESWT significantly increased CBF, as measured with a full-field laser perfusion imager, and reduced brain infarct volume in a short period of time; such remarkable differences were not observed with other interventions, such as early exercise [26, 27]. The effect of rESWT in CBF may be associated with its ability to augment VEGF expression. At approximately 12 days, VEGF expression peaked and continued to increase 30 days after MCAO. A previous study reported that VEGF induced endothelial cell proliferation, migration, and survival [28], thereby lead to capillary for-

mation [29]. New capillaries may be the main reason for blood perfusion restoration, as shown by laser perfusion imaging. Another group suggested that increased blood perfusion enhances tissue oxygen uptake and results in less necrosis [29], which probably reduces infarct volume. ESWT may also regulate the inflammatory response and the expression of inflammatory factors [30], which would also reduce infarct volume.

Improved CBF is also related to neurological recovery. Our findings confirmed that mNSS were significantly ameliorated by rESWT, and the expression of the neuronal marker NSE also markedly increased. rESWT also strikingly enhanced expression of the NSC marker nestin. Notably, endogenous NSCs reside in many

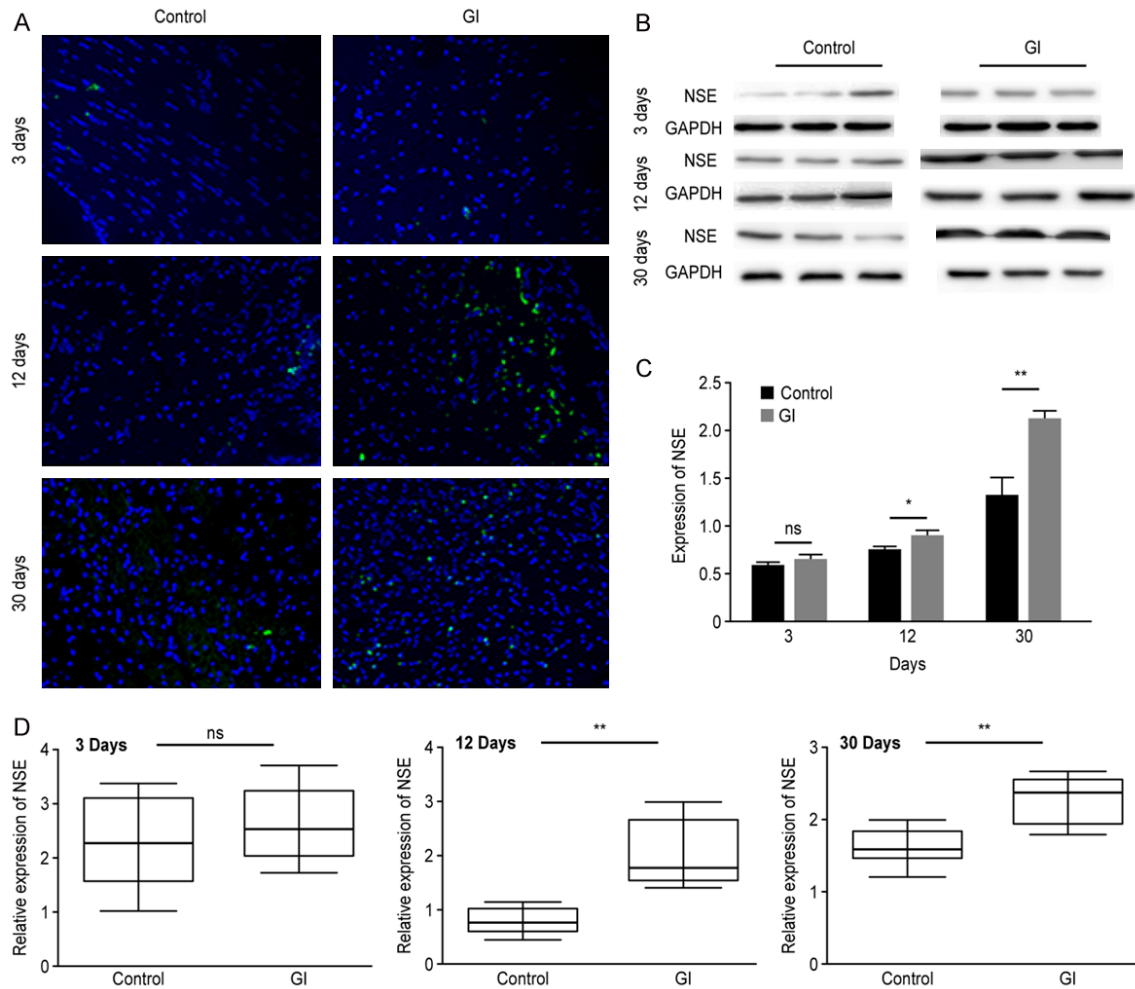


Figure 5. NSE immunofluorescence, western blot, and real-time PCR findings. NSE (green) immunofluorescence in the rat brain post-stroke cortex (A). Western blot of NSE (B) showing higher expression on days 12 (* $P < 0.05$) and 30 (** $P < 0.01$) compared with the control group (C). NSE mRNA expression as measured by real-time PCR was increased on days 12 (** $P < 0.01$) and 30 (** $P < 0.01$) after rESWT (D).

regions of the adult mammalian brain [31], and cerebral ischemia activates their proliferation, differentiation, and migration [32-34], thereby improving neurological function [35]. However, these NSCs have a limited effect on neurological recovery, likely due to a deleterious micro-environment [36]. Based on the above results, rESWT increases VEGF expression and augments CBF, which promotes the microenvironment and reduces neuronal apoptosis [30]. This effect augments the numbers of neurons and NSCs. In addition, VEGF has been shown to directly influence cell generation, which promotes NSC self-renewal [37]. Because ESWT induces the differentiation of human tendon-derived stem/progenitor cells in vitro [38], we deduced that rESWT directly contributes to NSC proliferation and differentiation.

Wnt/ β -catenin signaling is profoundly involved in the regulation of NSC proliferation and differentiation [39, 40]. The conditional expression of stabilized β -catenin in NSCs enlarges the cortical surface area by expanding the NSC population [40]. Wnt3 overexpression increases neurogenesis in adult hippocampal precursor cells [41]. The Wnt/ β -catenin pathway is activated when a Wnt ligand binds to its receptors, and stabilized β -catenin then translocates into the nucleus to activate downstream genes such as cyclin D1 and c-myc [42, 43]. Normally, β -catenin is degraded in the peri-infarct area of the brain following focal cerebral ischemia [44], but applying rESWT on the right side of the head critically induced Wnt3a and β -catenin expression and continued 30 days after MCAO. These data prove that rESWT has a

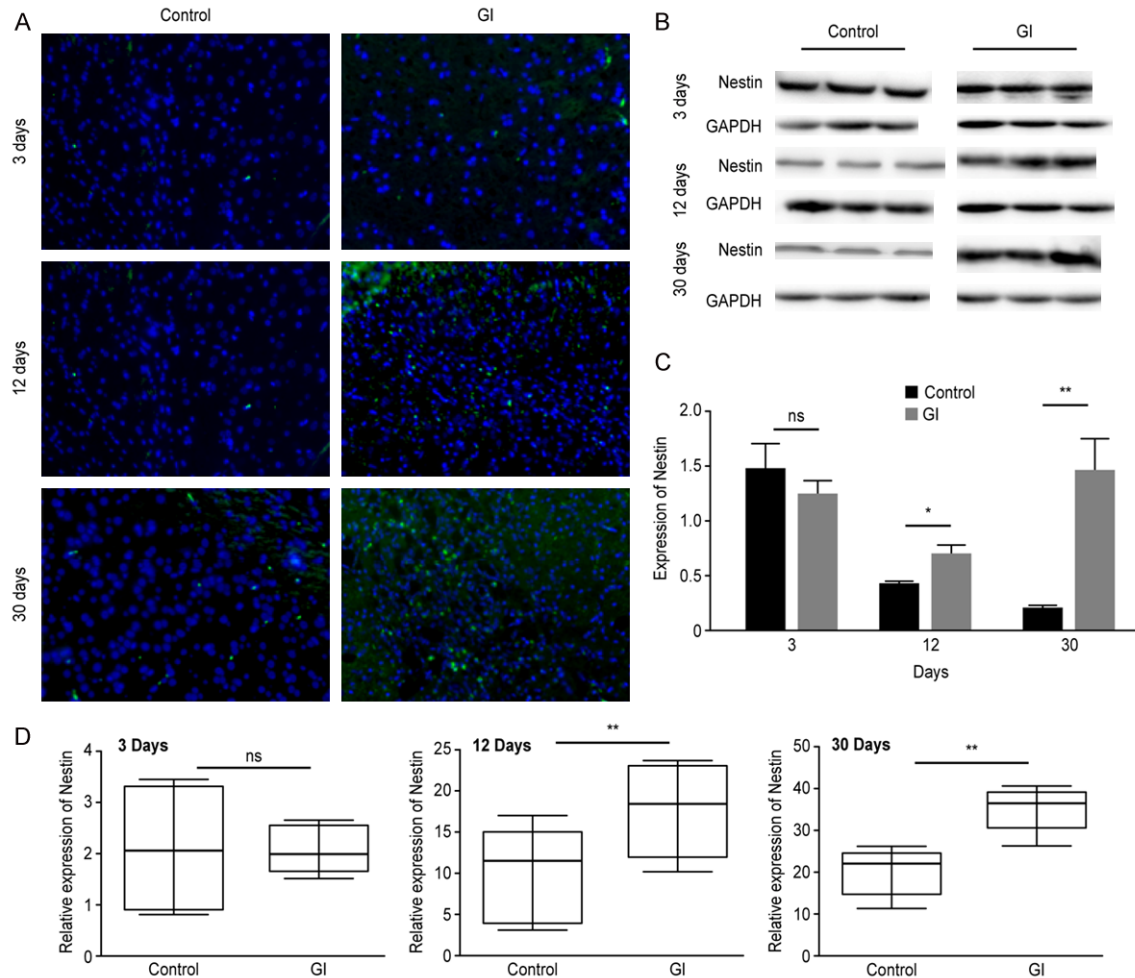


Figure 6. Nestin immunofluorescence, western blot, and real-time PCR findings. Nestin expression in the post-stroke cortex by immunofluorescence microscopy (200×) (A) and western blot (B). The green color represents nestin, and expression was strongly enhanced in group I as shown by western blot (C) on days 12 (* $P < 0.05$) and 30 (** $P < 0.01$) after MCAO. Compared with the control group, nestin mRNA expression was improved at 12 (* $P < 0.05$) and 30 days (** $P < 0.01$) (D).

powerful effect on NSC proliferation and differentiation via the Wnt/ β -catenin pathway to increase the number of neurons and improve neurological function.

Moreover, our results demonstrated that neurological function significantly increased in group II on day 12, indicating that the addition of rESWT to the limbs had a short-term effect because ESWT is an especially effective treatment for spasticity after brain injury [45]. Although there was no difference in mNSS on day 30, the scores in group II were still lower than those in group I, which might be due to the almost normal neurological function in both groups at that time point. Because rESWT deliv-

ered to the limbs will have little effect on the brain, we did not repeatedly analyze the indexes in group II.

Although ESWT is a promising treatment for various diseases, research on its application in the treatment of cerebral infarction is limited. In this study, we aimed to identify the proper rESWT dose that would not induce damage and could help restore normal function when applied to head. Rapid improvement in neurological function is related to revascularization of the infarcted area and an increase in the number of neurons, a process that might involve the Wnt/ β -catenin pathway. Future studies could investigate how ESWT affects

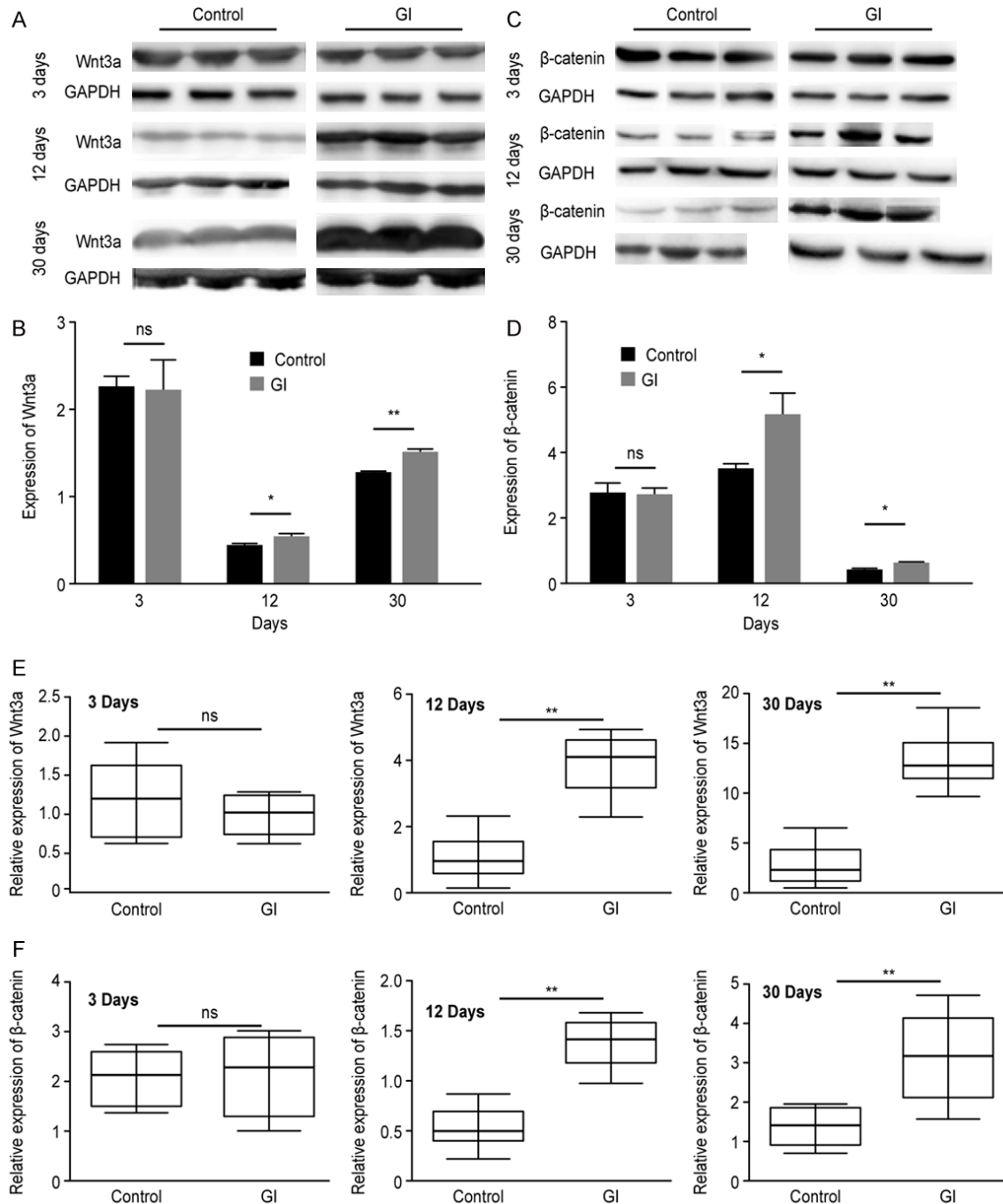


Figure 7. Wnt3a and β-catenin expression levels were evaluated by western blot and real-time PCR. Wnt3a and β-catenin expression levels were evaluated using western blot (A, C). Compared with the control group, Wnt3a expression was remarkably induced in group I (* $P < 0.05$ on day 12, ** $P < 0.01$ on day 30; B), and β-catenin expression increased after rESWT on days 12 and 30 (* $P < 0.05$, D). Wnt3a and β-catenin mRNA levels were measured with real-time PCR on days 12 and 30 (** $P < 0.01$; E, F).

different brain regions in a cerebral ischemia rat model. Moreover, clarification of the mechanism of increased CBF and amelioration of nerve function is needed, and further experiments on animals closer to humans are neces-

sary. Given the different rESWT transmitters available and their various depths, this method could open up excellent clinical opportunities and have beneficial effects in patients affected by cerebral ischemia.

Acknowledgements

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Disclosure of conflict of interest

None.

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