Original Article
Extracorporeal shock wave effectively attenuates brain infarct volume and improves neurological function in rat after acute ischemic stroke

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Abstract: Background: To investigate the effect of shock wave (SW) on brain-infarction volume (BIV) and neurological function in acute ischemic stroke (AIS) by left internal carotid artery occlusion in rats. Methods and results: SD rats (n=48) were divided into group 1 [sham-control (SC)], group 2 [SC-ECSW (energy dosage of 0.15 mJ/mm²/300 impulses)], group 3 (AIS), and group 4 (AIS-ECSW) and sacrificed by day 28 after IS induction. In normal rats, caspase-3, Bax and TNF-α biomarkers did not differ between animals with and without ECSW therapy, whereas Hsp70 was activated post-ECSW treatment. By day 21 after AIS, Sensorimotor-functional test identified a higher frequency of turning movement to left in group 3 than that in group 4 (P<0.05). By day 28, brain MRI demonstrated larger BIV in group 3 than that in group 4 (P<0.001). Angiogenesis biomarkers at cellular (CD31, α-SMA+) and protein (eNOS) levels and number of NeuN+ cells were higher in groups 1 and 2 than those in groups 3 and 4, and higher in group 4 than those in group 3, whereas VEGF and Hsp70 levels were progressively increased from groups 1 and 2 to group 4 (all P<0.001). Protein expressions of apoptosis (Bax, caspase 3, PARP), inflammation (MMP-9, TNF-α), oxidative stress (NOX-1, NOX-2, oxidized protein) and DNA-damage marker (γ-H2AX), and expressions of γ-H2AX+, GFAP+, AQP-4+ cells showed an opposite pattern compared to that of angiogenesis among the four groups (all P<0.001). Conclusion: ECSW therapy was safe and effective in reducing BIV and improved neurological function.

Keywords: Acute ischemic stroke, extracorporeal shock wave, angiogenesis, inflammation, oxidative stress

Introduction

Stroke is always a very critical issue in developed countries [1, 2]. Not only is it associated with a high mortality and prevalence in the elderly, but it also shows an increasing tendency to attack a younger cohort which is the main manpower in the economic structure of each country [3, 4]. In the aspect of cerebrovascular disorders, beside acute ischemic stroke, cerebrovascular insufficiency and its associated risk of dementia also contribute to the social economic burden [5, 6]. The global increase in the incidence of cerebrovascular disease and
lack of satisfactory treatment indicate the need for an effective therapeutic strategy to reduce both the mortality and neurologic sequelae [7].

Shock wave (SW) therapy is characterized by the deliverance of a sequence of transient pressure disturbances of high peak pressure (100 MPa), fast pressure rise (<10 ns), rapid propagation, and short lifecycle (10 µs) produced by an appropriate generator and directed to a specific target area with an energy density in the range of 0.003-0.890 mJ/mm² [8, 9]. Thus, SW is a kind of physiotherapy popular in initially treating bone nonunion and musculoskeletal disorder [10-12]. Recently, this therapeutic modality has been reported to be effective in treating ischemia-related organ disorders [13], including ischemic heart disease [14, 15]. The underlying mechanism of extracorporeal shock wave (ECSW) for improving ischemia-related organ dysfunction have been suggested to be increased angiogenesis via VEGF regulation, enhanced homing effect of progenitor cell via mechanical-activated pathway [16-18], and protective effect through augmented nitric oxide production. It may also reduce cell death by means of reduced oxidative stress, apoptosis, and inflammation [8, 19].

Surprisingly, while ECSW has been revealed to be effective for improving ischemia-related organ dysfunction in both experimental studies [14, 16-19] and in clinical trial [20], there has been no available data to address the impact of ECSW in the setting of cerebral ischemia. Based on the results of those studies [8, 14, 16-19], this study tested the hypothesis that low-energy ECSW might offer benefit in reducing brain infarct size and improving hypoperfusion-related neurological dysfunction through enhancing angiogenesis as well as inhibiting inflammatory reaction, cell apoptosis, and oxidative stress.

Materials and methods

Ethics

All animal experiments were approved by the Institute of Animal Care and Use Committee at Kaohsiung Chang Gung Memorial Hospital (Affidavit of Approval of Animal Use Protocol No. 2012051401) and performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, National Academy Press, Washington, DC, USA, revised 1996).

Experimental model of acute ischemic stroke and corner test for assessment of neurological function

The protocol and procedure of the rodent model of acute ischemic stroke (IS) have previously been described in details [21]. Adult male Sprague-Dawley rats, weighing 325-350 g, were utilized (Charles River Technology, BioLASCO Taiwan Co., Ltd., Taiwan). Each animal was anesthetized by 2% inhalational isoflurane in a supine position on a warming pad (37°C). After exposure of the left common carotid artery (LCCA) through a transverse neck incision, a small incision was made on the LCCA through which a nylon filament (0.28 mm in diameter) was carefully advanced into the distal left internal carotid artery for occlusion of the left middle cerebral artery (LMCA) to cause brain ischemia and infarction of its supplied area. The nylon filament was removed 120 min after occlusion, followed by closure of the muscle and skin in layers. The rats were then placed in a portable animal intensive care unit (ThermoCare®) for 24 hours. The sensorimotor functional test (Corner test) was conducted for each rat at baseline and on day 21 after acute IS induction as we previously described [21, 22]. Briefly, the rat was allowed to walk through a tunnel and then into a corner, the angle of which was 60 degrees. To exit the corner, the rat could turn either left or right. The results were recorded by a technician who was blind to the study design. This test was repeated 10 to 15 times with at least 30 seconds between each trial. We recorded the number of right and left turns from 10 successful trials for each animal and used the results for statistical analysis.

Animal grouping and the treatment protocol

Forty-eight rats were equally categorized into group 1 [sham control (SC), i.e., by cutting open the neck skin and exploring the LCCA only], group 2 [SC + ECSW (energy dosage of 0.15 mJ/mm²/300 impulses), group 3 (IS only) and group 4 [IS + ECSW (energy dosage of 0.15 mJ/mm²/300 impulses)] at 3 h after acute IS induction.

Rationale for ECSW [Eva Trode (Swaziland)] dosage was first tested based on our previous
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reports [13, 14] with modified energy dosage from (1) 0.15 mJ/mm²/300 impulses, (2) 0.22 mJ/mm²/300 impulses, (3) 0.26 mJ/mm²/300 impulses, (4) 0.30 mJ/mm²/300 impulses, and (5) 0.52 mJ/mm²/300 impulses. Additional three rats were used in each dosage test in the present study. The results showed that ECSW of energy below 0.12 mJ/mm²/300 impulses could not be transmitted through the skull into the brain tissue because the protein and cellular analyses by western blot and immunofluorescent staining showed no significant change after ECSW treatment with ECSW ≤0.12 mJ/mm²/300 impulses. On the other hand, 0.15 mJ/mm²/300 impulses showed no damage to the brain tissue and offer its therapeutic effect (please see Figure 1). Thus, in consideration of the effect and safety, the ECSW energy of 0.15 mJ/mm²/300 impulses was utilized in the present study.

In vitro studies for identification of effects of SW on enhancing angiogenesis and suppressing the oxidative stress and inflammation

To elucidate the impact of ECSW on enhancing angiogenesis, the human umbilical vein endothelial cells (HUVECs) were cultured in M199 culture medium (in 10-cm discs) with or without ECSW therapy (0.12 mJ/mm²/impulse for totally 200 impulses). The cells were continuously cultured in M199 medium for 36 h before being collected for the assessment of angiogenesis (i.e., Matrigel assay). The time for tubular formation was 16 h in the present study.

To determine the impact of ECSW on inhibiting oxidative stress, rat smooth muscle cells were cultured in MEM-α culture medium with or without subsequent ECSW treatment (0.12 mJ/mm²/impulse for 200 impulses). After ECSW treatment, the cells were cultured with menadione (25 µM) for 30 minutes (i.e., for assessing oxidative stress) for 30 minutes. The cells were washed with PBS and then continuously cultured for 48 hours after shock wave treatment.

Additionally, to measure the effect of ECSW on suppressing inflammation, mouse macrophage (7.0×10³) cell line (RAW 264.7; mouse macrophage; Bioresource Collection and Research Center) was cultured in DMEM-HG medium that contained 10% FBS and pretreated with without subsequent ECSW treatment (0.12 mJ/mm²/impulse for 200 impulses). Three hours after ECSW therapy, co-culture with lipopolysaccharide (LPS) (100 ng/mL) was done for 3 h (i.e., for measuring inflammation). The cells were then collected for the study.

Procedure and protocol of brain magnetic resonance imaging (MRI) study

Magnetic resonance imaging (MRI) was performed at days 3 and 28 after acute IS induction. Briefly, during MRI measurements, the rats were anesthetized by 3% inhalational isoflurane with room air and placed in an MRI-compatible holder (Biospec 94/20, Bruker, Ettingen, Germany). Rectal temperature and respiration were monitored throughout the procedure to ensure the maintenance of normal physiological conditions. MRI data were collected using a Varian 9.4T animal scanner (Biospec 94/20, Bruker, Ettingen, Germany) with a rat surface array. The MRI protocol consisted of 40 T2-weighted images. Forty continuous slice locations were imaged with a field-of-view of 30 mm × 30 mm and an acquisition matrix dimension of 256×256 and slice thickness of 0.5 mm. The repetition time (TR) and echo time (TE) for each fast spin-echo volume were 4200 ms and 30 ms, respectively. A custom software, ImageJ (1.43i, NIH, USA), was used to process the region of interest (ROI). Planimetric measurements of images from MRI T2 were performed to calculate the stroke volumes of cortex.

Specimen collection and preparation for individual study

For examination of protein expression, animals in all groups (n=6) were sacrificed on day 28 after brain MRI, and the brain of each rat was promptly removed, immersed in cold saline, snap-frozen in liquid nitrogen and then stored at -80°C for individual study. For immunofluorescent (IF) and immunohistochemical (IHC) staining studies, the brains of 6 other animals in each group were reperfused with normal saline via the carotid artery, removed, fixed with 4% paraformaldehyde in 1x PBS (pH 7.4), and soaked in 20% sucrose in 1x PBS (freshly prepared) until the brain took on a completely sunken appearance. The sucrose was then discarded and the brain soaked in 30% sucrose in 1x PBS (freshly prepared) for 48 h. The infarct-
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ed and non-infarcted parts were then collected. Finally, the OCT block (Tissue-Tek, Sakura, Netherlands) was prepared for IHC and IF staining.

Figure 1. Safety and efficacy of shock wave (SW) therapy using energy dosage of 0.15 mJ/mm²/300 Impulses to brain of living rats. (A and B) The protein expressions of tumor necrosis factor (TNF)-α, cleaved caspase 3 and mitochondrial Bax did not differ in SW therapy at energy of 0.15 mJ/mm²/300 impulse as compared with control group, whereas this energy augmented the protein expression of heat shock protein 70 (Hsp70). (C) Additionally, the grossly anatomy of the rat brain did not show an grossly pathological change as compare with normal control during time courses (1, 16, 24 and 48 h) of follow-up in this SW energy application. (D) Time courses of protein expression of Hsp70 showed that this parameter was enhanced in relatively late state (i.e., ≥16 h) after SW therapy. (E) Immunofluorescent microscopic finding (200 ×) revealed that as compared with control group, SW therapy notably augmented the Hsp70+ cell (green color) expression at 24 h. (F) Statistical analysis of (E), * vs. †, P<0.0001.
Immunofluorescent (IF) staining of brain specimens

The procedure and protocol of IF and IHC staining were based on our previous reports [21-23]. In details, frozen sections (4 µm thick) were obtained from BIA of each animal, permeated with 0.5% Triton X-100, and incubated with antibodies against NeuN (1:1000, Millipore), glial fibrillary acid protein (GFAP; 1:500, DAKO), aquaporin4 (AQP4; 1:200, Abcam), vascular endothelial growth factor (VEGF; 1:400, Abcam), and γ-H2AX (1:5000, Abcam) at 4°C overnight. Alexa Fluor488, Alexa Fluor568, or Alexa Fluor594-conjugated goat anti-mouse or rabbit IgG were used to localize signals. Sections were finally counterstained with DAPI and observed with a fluorescent microscope equipped with epifluorescence (Olympus IX-40).

Three brain sections were analyzed for each rat. For quantification, three randomly selected high-power fields (HPFs; 400× or 200× for IF study) were analyzed in each section. The mean number of positively-stained cells per HPF for each animal was then determined by summation of all numbers divided by 9.

Western blot analysis of brain specimens

The procedure and protocol of Western blot were based on our previous reports [21-23]. In details, equal amounts (50 µg) of protein extracts were loaded and separated by SDS-PAGE using 12% acrylamide gradients. After electrophoresis, the separated proteins were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences). Nonspecific sites were blocked by incubation of the membrane in blocking buffer [5% nonfat dry milk in T-TBS (TBS containing 0.05% Tween 20)] overnight. The membranes were incubated with monoclonal antibodies against CD31 (1:100, Abcam), VEGF (1:1000, Abcam), NOX-1 (1:1500, Sigma), NOX-2 (1:500, Sigma), Cox 2 (1:1000, Cell Signaling), matrix metalloproteinase (MMP)-9 (1:3000, Abcam), tumor necrosis factor alpha (TNF-α; 1:1000, Cell Signaling), macrophage inhibitor factor (MIF) (1:2000, Abcam), endothelial nitric oxide synthase (eNOS) (1:200, Abcam), poly (ADP-ribose) polymerase (PARP) (1:1000, Cell Signaling), mitochondrial Bax (1:1000, Abcam), Caspase 3 (1:3000, Abcam), and heat shock protein 70 (Hsp70) (1:1000, Abcam) for 1 h at room temperature. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin IgG (1:2000, Cell Signaling) was used as a second antibody for 1 h at room temperature. The washing procedure was repeated eight times within 1 h, and immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences) and exposure to Medical X-ray film (FUJI). For quantitation, ECL signals were digitized using Labwork software (UVP). A standard control sample was loaded on each gel.

Vessel density in brain infarct area

The procedure and protocol for identification of small vessel in brain infarct area were basic on our previous reports [13, 15]. In details, IHC staining of small blood vessels was performed with α-SMA (1:400) as primary antibody at room temperature for 1 hour, followed by washing with PBS thrice. Ten minutes after the addition of anti-mouse-HRP conjugated secondary antibody, the tissue sections were washed with PBS thrice. Then 3,3′ diaminobenzidine (DAB) (0.7 gm/tablet) (Sigma) was added, followed by washing with PBS thrice after one minute. Finally, hematoxylin was added as a counterstain for nuclei, followed by washing twice with PBS after one minute. Three brain sections were analyzed in each rat. For quantification, three randomly selected HPFs (100×) were analyzed in each section. The mean number per HPF for each animal was then determined by summation of all numbers divided by 9.

Statistical analyses

Quantitative data are expressed as mean ± SD. Statistical analysis was performed by ANOVA followed by Bonferroni multiple-comparison post hoc test. All analyses were conducted using SAS statistical software for Windows version 8.2 (SAS institute, Cary, NC). A probability value <0.05 was considered statistically significant.

Results

Application of ECSW with energy dose of 0.15 mJ/mm²/300 impulses to rat brain was safe and effective

The results of Figure 1 showed that ECSW therapy with energy dose of 0.15 mJ/mm²/300 impulses to the normal rat brain did not enhance the protein expressions (Figure 1A) of inflammation (TNF-α), and apoptosis (cleaved...
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Figure 2. Shock wave (SW) therapy promoted angiogenesis and suppressed inflammation and oxidative stress (n=6). A. Microscopic findings of matrigel assay results showing that as compared with control group, the angiogenesis was remarkably increased in human umbilical vein endothelial cells (UVECs) after SW therapy. B. Analytical results of total tubular length, control vs. SW, P<0.0001. C. Analytical results of branch point formation, control vs. SW, P<0.0001. D. Analytical results of loop formation, control vs. SW, P<0.0001. E. Protein expression of matrix metalloproteinase (MMP)-9, * vs. other group with difference symbols (*, †, ‡), P<0.001. F. Protein expression of Cox-2, * vs. other group with difference symbols (*, †, ‡), P<0.0001. G. Protein expression of macrophage inhibitor factor (MIF), * vs. other group with difference symbols (*, †, ‡), P<0.001. H. Protein expression of tumor necrosis factor (TNF)-α, * vs. other group with difference symbols (*, †, ‡), P<0.001. I. Oxidized protein expression, * vs. other groups with different symbols (*, †, ‡), P<0.001. J. Protein expression of NOX-1, * vs. other group with difference symbols (*, †, ‡), P<0.001. K. Protein expression of NOX-2, * vs. other group with difference symbols (*, †, ‡), P<0.001. All statistical analyses were performed by one-way ANOVA followed by Bonferroni multiple comparison post hoc test. Symbols (*, †, ‡) indicate significance (at 0.05 level). SC = sham control, SW = shock wave. N=6 for each group.

caspase 3, mitochondrial Bax) or the grossly anatomic brain change (Figure 1B). However, this therapy increased the protein (Figure 1A, 1C) and cellular (Figure 1D) expressions of Hsp70 (upregulated in response to ischemia/stress stimulation for protecting the ischemic organ) which occurred relative late state (i.e., ≥16 h) after ECSW therapy. These findings suggest that the energy dose of 0.15 mJ/mm²/300 impulses of ECSW was effective and safe to the rat brain.

ECSW therapy enhanced angiogenesis and inhibited inflammation and oxidative stress

The results of matrigel assay showed that as compared with control group, the ECSW therapy significantly enhanced angiogenesis of HUVECs (i.e., total tubular length, branch point formation, total network formation). Additionally, the protein expressions of MMP-9, MIF, TNF-α and Cox-2, four indicators of inflammation were significantly higher in LPS-treated group than in control group and control group-treated by ECSW that were reversed after ECSW treatment. On the other hand, these parameters did not differ between the control group and control group-treated by ECSW. Furthermore, the protein expressions of NOX-1, NOX-2 and oxidized protein, three indicators of oxidative stress, showed an identical pattern of inflammation among these four groups (Figure 2). These findings implicated that the ECSW therapy promoted angiogenesis and suppressed the inflammation and generation of oxidative stress.

ECSW therapy limited brain infarct size and improved recovery of neurological function

By day 3 after acute IS procedure, brain MRI findings demonstrated that the ratio of left brain infarct volume to total left brain volume (LIV/TLBV) was significantly higher in group 3 (IS) and group 4 (IS + ECSW) than in group 1 (SC) and group 2 (SC + ECSW), but it showed no difference between groups 1 and 2 or between groups 3 and 4. Additionally, by day 28, the ratio of LIV/TLBV was still higher in groups 3 and 4 than in that in groups 1 and 2, and it displayed no difference between groups 1 and 2. However, by day 28, this parameter was significantly lower in group 4 as compared with that in group 3 (Figure 3).

Corner test for determining neurological function showed no difference among the four groups at day 0 prior to acute IS induction. However, by day 21 after acute IS, the neurological function was significantly impaired in groups 3 and 4 as compared with that in groups 1 and 2, and significantly impaired in group 3 than in group 4, but it showed no difference between groups 1 and 2 (Figure 3). These findings (i.e., brain MRI and corner test results) imply that ECSW attenuated brain infarct size and improved neurological function in rats after acute IS.

ECSW therapy significantly augmented angiogenesis in brain infarct region by day 28 after acute IS

Cellular (CD31+ cells) and protein (CD31) expressions of angiogenesis were significantly lower in groups 3 and 4 than those in groups 1 and 2, and significantly lower in group 3 than those in group 4, but they exhibited no difference between groups 1 and 3 (Figure 4). In addition, the number of smooth muscle actin (SMA) + cells (i.e., number of small vessels), an indicator for angiogenesis/neovascularization, showed an identical pattern compared to that of CD31+ cell expression among the four groups.
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Figure 3. Brain magnetic resonance imaging (MRI) findings of brain infarction volume (n=6) and corner test for determining neurological function (n=12). A. 6 of 12 rats in each group were randomized into receive brain MRI study by days 3 and 28 after acute IS induction (i.e., same rat for twice). The yellow arrow indicated the infarction area in IS group and IS + SW group. B. Left brain infarct volume (LIV) divided by total left brain volume (TLBV) (%) by day 3, * vs. †, P<0.0001. C. LIV divided by TLBV (%) by day 28, * vs. other groups with different symbols (*, †, ‡), P<0.0001. D. Corner test showing the state of neurological functional did not differ among four groups at day 0 prior to acute IS. E. Significant impairment of neurological function was found in IS group than in IS + SW group on day 21 after acute IS, * vs. other groups with different symbols (*, †, ‡), P<0.001. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test. Symbols (*, †, ‡) indicate significance (at 0.05 level). SC = sham control, IS = ischemic stroke, SW = shock wave.

(Figure 4). Furthermore, the protein and cellular expressions of VEGF, another indicator of angiogenesis, was significantly progressively increased from groups 1 and 2 to group 4.
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Figure 4. Angiogenesis biomarkers of CD31 and α-smooth muscle actin-positively stained vessels in brain infarct area by day 28 after IS induction. A-D. The immunofluorescent microscopic findings (200×) of CD31+ cells (green color) in brain infarct area (BIA). E. Statistical analysis of numbers of CD31+ cells, * vs. other groups with different symbols (*, †, ‡), P<0.0001. The scale bars in right lower corner represent 50 µm. F. The protein expression of CD31 in BIA, * vs. other groups with different symbols (*, †, ‡), P<0.001. G-J. Microscopic finding (100×) of α-smooth muscle actin staining for identification of small vessels (yellow arrows). K. Statistical analysis of numbers of small vessels (i.e., diameter <25 µm) in BIA, * vs. other groups with different symbols (*, †, ‡), P<0.001. The scale bars in right lower corner represent 100 µm. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test. Symbols (*, †, ‡) indicate significance (at 0.05 level). SC = sham control, IS = ischemic stroke, SW = shock wave. N=6 for each group.

Figure 5. Angiogenesis biomarkers of VEGF and eNOS in brain infarct area by day 28 after IS induction. A-D. Immunofluorescent microscopic findings (400×) of vascular endothelial growth factor (VEGF)+ cells (green color) in brain infarct area (BIA). E. Statistical analysis of numbers of VEGF+ cells, * vs. other groups with different symbols (*, †, ‡), P<0.0001. The scale bars in right lower corner represent 20 µm. F. Protein expression of VEGF, * vs. other groups with different symbols (*, †, ‡), P<0.001. G. Protein expression of endothelial nitric oxide synthase (eNOS), * vs. other groups with different symbols (*, †, ‡), P<0.001. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test. Symbols (*, †, ‡) indicate significance (at 0.05 level). SC = sham control, IS = ischemic stroke, SW = shock wave. N=6 for each group.
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A  
Bax
(21 kDa)
CoIV
(17 kDa)

B  
c-Casp 3
(17 kDa)
Actin
(43 kDa)

C  
c-PARP
(89 kDa)
Actin
(43 kDa)

D  
r-H2AX
(15 kDa)
Actin
(43 kDa)

E  
SC

F  
SC + SW

G  
IS

H  
IS + SW

I  
% of r-H2AX+ cell

SC  
SW  
IS  
IS+SW

*  
†
ECSW reduces BIV and improves neurological function after IS

Figure 5. Moreover, the protein expression of eNOS, an angiogenesis biomarker, exhibited a pattern identical to that of CD31 protein expression among the four groups (Figure 5).

Figure 6. Apoptotic and DNA-damaged biomarkers in brain infarct area by day 28 after IS induction. A. Protein expression of mitochondrial Bax, * vs. other groups with different symbols (*, †, ‡), P<0.01. B. Protein expression cleaved caspase 3 (c-Casp 3), * vs. other groups with different symbols (*, †, ‡), P<0.001. C. Protein expression of cleaved poly (ADP-ribose) polymerase (c-PARP), * vs. other groups with different symbols (*, †, ‡), P<0.001. D. Protein expression of histone-2AX (H2AX) phosphorylation (γ-H2AX), * vs. other groups with different symbols (*, †, ‡), P<0.001. E-H. Immunofluorescent microscopic findings (400×) of γ-H2AX + cells (red color) in brain infarct area. I. Statistical analysis of numbers of γ-H2AX+ cells, * vs. other groups with different symbols (*, †, ‡), P<0.0001. The scale bars in right lower corner represent 20 µm. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test. Symbols (*, †, ‡) indicate significance (at 0.05 level). SC = sham control, IS = ischemic stroke, SW = shock wave. N=6 for each group.

Figure 7. Protein expression of reactive oxygen species and oxidative stress in brain infarct area by day 28 after IS induction. A. Protein expression of NOX-1, * vs. other groups with different symbols (*, †, ‡), P<0.01. B. Protein expression of NOX-2, * vs. other groups with different symbols (*, †, ‡), P<0.01. C. Protein expression of oxidative index (protein carbonyls), * vs. other group with different symbols, P<0.001. (Note: right and left lanes shown on the upper panel represent protein molecular weight marker and control oxidized molecular protein standard, respectively). DNP = 1-3 dinitrophenylhydrazone. Symbols (*, †, ‡) indicate significance (at 0.05 level). All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test. Symbols (*, †, ‡) indicate significance (at 0.05 level). SC = sham control, IS = ischemic stroke, SW = shock wave. N=6 for each group.
ECSW therapy significantly attenuated apoptosis and DNA damage in brain infarct region by day 28 after acute IS

The protein expressions of mitochondrial Bax, and cleaved caspase 3 and PARP, three indicators of apoptosis, was significantly higher in groups 3 and 4 than in groups 1 and 2, and significantly higher in group 3 than in group 4, but they showed no difference between groups 1 and 2. Besides, the protein and cellular expressions of γ-H2AX, a DNA damage marker, exhibited an identical pattern compared to that of apoptotic biomarkers among the four groups (Figure 6).

ECSW therapy significantly ameliorated generation of reactive oxygen species and oxidative stress in brain infarct region by day 28 after acute IS

The protein expression of NOX-1 and NOX-2, two indices of reactive oxygen species (ROS), was significantly increased in groups 3 and 4 than that in groups 1 and 2, and significantly increased in group 3 than that in group 4, but they exhibited no difference between groups 1 and 2. In addition, oxidized protein expression, an indicator of oxidative stress, demonstrated a pattern identical to that of ROS among the four groups (Figure 7).

ECSW therapy significantly suppressed expressions of inflammation, gliial fibrillary acid protein (GFAP) and aquaporin4 (AQP4) in brain infarct region by day 28 after acute IS

The protein expressions of TNF-α and MMP-9, two indicators of inflammation, were significantly higher in groups 3 and 4 than those in groups 1 and 2, and significantly higher in group 3 than that in group 4, but they showed no difference between groups 1 and 2. Moreover, immunofluorescent microscopy showed that the expression of GFAP, the principal intermediate filament of mature astrocytes, and AQP4, an indicator of brain edema, were significantly higher in groups 3 and 4 than those in groups 1 and 2, and significantly higher in group 3 than those in group 4, but they did not differ between groups 1 and 2, suggesting reduction in gliosis and edema after ECSW treatment (Figure 8).

ECSW therapy significantly preserved number of NeuN+ cells and enhanced Hsp70 expression in brain infarct region by day 28 after acute IS (Figure 9)

The IF stain showed that the number of neuN+ cells, an index of neuron, was significantly higher in groups 1 and 2 than in groups 3 and 4, and significantly higher in group 4 than in group 3, but it showed no difference between groups 1 and 2. On the other hand, the protein expression of Hsp70, a protective indicator in setting of ischemia, were highest in group 4 and lowest in group 1, and significantly higher in group 3 than in group 2 (Figure 9). This finding suggested that the Hsp70 was upregulated under the ischemic stimulation for cell survival that was further enhanced after ECSW therapy.

Discussion

This study tested the impact of ECSW therapy on rats after acute IS yielded several striking implications. First, the use of a standard method of brain MRI measurement showed that ECSW therapy significantly reduced rat brain infarct volume by day 28 after IS induction. Second, not only the image finding, but also the functional test (i.e., Sensorimotor function test), disclosed that neurological function was remarkably improved by day 21 in rats with IS after receiving ECSW. Third, the cellular proliferation marker of neuN+ cells (i.e., day 28, late phase of IS) were substantially increased as reflected in the IF microscopic findings. Fourth, ECSW therapy markedly reduced inflammatory response and apoptosis, and the generations of oxidative stress and ROS. Finally, ECSW therapy notably enhanced the angiogenesis in BIA and protective factor of Hsp70 in normal and acute IS animals.

Novel findings in the present study

This is the first study to address the use ECSW against acute IS in an experimental setting. Of importance in the present study is the significant reduction in brain infarct volume by day 28 after IS compared to those without ECSW therapy after IS induction as shown in the results of MRI studies. Therefore, our findings, based on the results of imaging analysis and neurological function test, suggest that ECSW may be an
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A. MMP-9 (92 kDa) and Actin (43 kDa) relative density (MMP-9/Actin) with significance markers.

B. TNF-α (25 kDa) and Actin (43 kDa) relative density (TNF-α/Actin) with significance markers.

C-D. Immunofluorescence images of SC and SC + SW conditions.

E-F. Images for IS and IS + SW conditions.

G. Graph showing percentage of GFAP positive cells with significance markers.

H-I. Images of SC and SC + SW conditions.

J-K. Images for IS and IS + SW conditions.

L. Graph showing percentage of AQP-4 positive cells with significance markers.

Legend:

- SC
- SW
- IS
- IS + SW

Significance markers:

* p < 0.05
† p < 0.01
‡ p < 0.001

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Figure 8. Portiien expressions of inflammatory biomarkers and Immunofluorescent staining for identification of GFAP+ and AQP-4+ cells in brain infarct area by day 28 after ischemic stroke induction. A. Protein expression of matrix metalloproteinase (MMP)-9, * vs. other groups with different symbols (*, †, ‡), $P<0.001$. B. Protein expression of tumor necrosis factor (TNF)-α, * vs. other groups with different symbols (*, †, ‡), $P<0.01$. C-F. Immunofluorescent staining (400×) of glial fibrillary acid protein (GFAP) (red color) in brain infarct area. G. Statistical analysis of numbers of GFAP+ cells, * vs. other groups with different symbols (*, †, ‡), $P<0.0001$. The scale bars in right lower corner represent 20 µm. H-K. Immunofluorescent staining (400×) of aquaporin 4 (AQP-4) (green color) in brain infarct area. L. Statistical analysis of numbers of AQP-4+ cells, * vs. other groups with different symbols (*, †, ‡), $P<0.0001$. The scale bars in right lower corner represent 20 µm. Symbols (*, †, ‡) indicate significance (at 0.05 level). All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test. Symbols (*, †, ‡) indicate significance (at 0.05 level). SC = sham control, IS = ischemic stroke, SW = shock wave. N=6 for each group.

Figure 9. NeuN+ cell and Hsp70 expression in brain infarct area by day 28 after IS induction. A-D. Immunofluorescent staining (400×) of neurons (red color stained by DAPI) in brain infarct area. E. Statistical analysis of numbers of neuN+ cells, * vs. other groups with different symbols (*, †, ‡), $P<0.0001$. The scale bars in right lower corner represent 20 µm. Symbols (*, †, ‡) indicate significance (at 0.05 level). F. The protein expression of heat shock protein 70 (Hsp70), * vs. other groups with different symbols (*, †, ‡, §), $P<0.001$. The scale bars in right lower corner represent 20 µm. Symbols (*, †, ‡, §) indicate significance (at 0.05 level). All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test. Symbols (*, †, ‡, §) indicate significance (at 0.05 level). SC = sham control, IS = ischemic stroke, SW = shock wave. N=6 for each group.
accessory therapeutic modality for patients after acute IS.

**Mechanism of ECSW therapy underlying BIV reduction and neurological functional improvement - role of angiogenesis, Hsp70 and NeuN+ cell proliferation**

A principal finding in the present study was that as compared with IS animals without ECSW treatment, the number of neuN+ cells was significantly increased in animals after ECSW therapy. This finding could partially explain why the BIV was significantly reduced the recovery of neurological function was notably improved in IS rats following ECSW treatment.

Another principal finding in the present study was that the number of neuN+ cells was significantly increased in animals after ECSW therapy. This finding could partially explain why the BIV was significantly reduced and the recovery of neurological function was notably improved in IS rats following ECSW treatment.

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**Mechanism of ECSW therapy underlying BIV reduction and neurological functional improvement - role of suppression of inflammation, apoptosis, DNA damage, and oxidative stress**

Undoubtedly, abundant previous studies [13, 14, 21-23, 31, 32] have supported the findings that ischemia elicits vigorous inflammatory response and the generation of oxidative stress and ROS which, in turn, cause DNA damage, cell apoptosis, and death, and ultimately organ dysfunction, and poor prognostic outcome [31]. The principal findings in the present study are that not only was the inflammatory biomarkers, but also the oxidative stress and ROS biomarkers were substantially increased in animals after IS. These findings could explain the significant increase in apoptosis and DNA damage biomarkers in IS animals compared with those without IS. Importantly, the expressions of these parameters were remarkably reduced in IS animals after ECSW treatment. In this way, our findings, in addition to reinforcing those of previous studies [13, 14, 21-23, 31, 32], could partially explain the notable decrease in BIV and improvement in neurological functional recovery in rats with IS following ECSW treatment.

**Mechanism of ECSW therapy underlying BIV reduction and neurological functional improvement - role of suppression of GFAP and AQP4 expressions**

Our previous studies have revealed that the expressions of GFAP, an indicator of glial cells that respond to stimulation and produce chemokines and perpetuate the local inflammatory process, and AQP4, an indicator of brain edema, were remarkably augmented in animals after IS [21, 22] and were notably reduced after stem cell treatment [16, 21]. Consistently, these two parameters were significantly upregulated in IS animals without treatment in the present study. However, these two biomarkers were significantly downregulated in animals with IS after ECSW treatment. Our previous studies have revealed that the expressions of GFAP, an indicator of glial cells that respond to stimulation and produce chemokines and perpetuate the local inflammatory process, and AQP4, an indicator of brain edema, were remarkably augmented in animals after IS [21, 22] and were notably reduced after stem cell treatment [16, 21]. Consistently, these two parameters were significantly upregulated in IS animals without treatment in the present study. However, these two biomarkers were significantly downregulated in animals with IS after ECSW treatment. These findings, perhaps, also explain the reduction of BIV and improvement of neurological function in the animal model of IS.
ECSW reduces BIV and improves neurological function after IS

In vitro study

<table>
<thead>
<tr>
<th>Angiogenic assay</th>
<th>Angiogenic factors</th>
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<td>Loops ↑</td>
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<tr>
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In Vivo study

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Cerebral structure integrity ↑

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<tr>
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<tr>
<td>AQP4+ cells ↓</td>
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Neurological function ↑

Sensorimotor function

Corner test score ↑

Figure 10. Proposed mechanisms underlying the effects of ECSW therapy on reducing brain infarct volume and improving neurological function in a rodent model of acute ischemic stroke based on findings of the present study. eNOS = endothelial nitric oxide synthase; VEGF = vascular endothelial growth factor; MMP-9 = matrix metalloproteinase 9; MIF = macrophage inhibitor factor; TNF-α = tumor necrosis factor-α; Hsp70 = heat shock protein 70; Mito-Bax = mitochondrial Bax; Csp 3 = caspase 3; PARP = poly (ADP-ribose) polymerase; gGFAP = glial fibrillary acid protein; AQP4 = aquaporin4.
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Study limitations

This study has limitations. First, although the 28-day short-term outcome was promising in the present study, the impact of ECSW on long-term outcome in rats with IS remains uncertain. Second, the exact mechanisms by which ECSW reduced BIV and improved neurological function are still unclear. The schematic illustration in Figure 10 shows our proposed mechanisms of the therapeutic effects of ECSW against acute IS based on our findings.

In conclusion, the results of our study support that ECSW treatment in rats after acute IS effectively inhibited neurological dysfunction and reduced BIV through the possible mechanisms of enriching angiogenesis and Hsp70 expression and preserving neuron cells, as well as attenuating inflammation, cell apoptosis, and oxidative stress.

Acknowledgements

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Disclosure of conflict of interest

None.

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References

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