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Original Research

Physical Exercise and Wet Cupping Therapy Stimulate Neural Stem Cells in Animal Model of Ischemic Stroke

Imam Subadi[1,2]*, Nanda Aulya Ramadhan[1,2], Albert Setiawan[1,3], Nur Sulastri[1,2], Wibi Riawan[4], RA Meisy Andriana[1,5], Martha Kurnia Kusumawardani[1,5], Yudith Dian Prawitri[1,2], Sintia Dewi Septiani M.K[1,5]

[1]*Department of Physical Medicine and Rehabilitation, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia*
[2]*Department of Physical Medicine and Rehabilitation, Universitas Airlangga Hospital, Surabaya, Indonesia*
[3]*Department of Physical Medicine and Rehabilitation, Faculty of Medicine, Widya Mandala Catholic University Surabaya, Indonesia*
[4]*Department of Biomolecular Biochemistry, Faculty of Medicine, Brawijaya University, Malang, Indonesia*
[5]*Department of Physical Medicine and Rehabilitation, Dr. Soetomo General Academic Hospital, Surabaya, Indonesia*

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Abstract

The process of neuroplasticity in stroke recovery remains poorly understood. SRY-related HMG-box 2 (SOX2), a protein that plays a role in controlling neural stem cell (NSC) proliferation and differentiation, is of particular interest. This research seeks to investigate how exercise and wet cupping therapy affect SOX2 expression in animal models of stroke, which are created using unilateral common carotid artery occlusion (UCCAO). The study utilized a control group design with a post-test evaluation. Thirty-six Wistar rats, aged 3 months, were randomly divided into six groups of equal size: control (n=6), stroke (n=6), cupping (n=6), exercise (n=6), stroke plus cupping (n=6), and stroke plus exercise (n=6). Wet cupping therapy was performed twice a week for three weeks. The exercise included a 20-minute swimming activity scheduled daily. This swimming exercise was performed three times a week for three weeks. Analysis of multiple comparisons among groups employing the ANOVA test proved that SOX2 expression in both the stroke plus exercise group and the stroke plus cupping group was significantly elevated. The stroke group was significantly lower than the control group. This study highlights the potential of physical exercise and wet cupping therapy to upregulate SOX2 expression in a UCCAO-induced brain injury model, supporting their role as promising alternative neuroregenerative interventions.

Keywords: Exercise; Cupping therapy; SOX2 protein; Neural stem cells; Animal model; Ischemic stroke; Neuronal plasticity

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*Corresponding Author:Imam Subadi
Department of Physical Medicine and Rehabilitation, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia
Email: imam.subadi0322@gmail.com

Introduction

Globally, stroke ranks as a primary contributor to disability [1]. The prevalence of stroke globally reached 80.1 million, 87% of which was ischemic stroke [2]. In Indonesia, the prevalence of stroke amounted to 8.3 per mile [3]. Among all stroke cases worldwide, the most common form is ischemic stroke, accounting for 62.4% of occurrences [4]. The disability resulting from stroke has a significant impact on patient, affecting not only their physical capabilities, but also their social interactions. Post-stroke depression affects both survivors and their families [5]. Rehabilitation is essential for minimizing sequelae following stroke that stimulate the endogenous neural plasticity, but the mechanism is still unclear [6].

A range of interventions can be implemented for recovery, including bimanual training, constraint-induced movement therapy, repetitive task training, neuromuscular electrical stimulation, and robotic-assisted interventions [6]. Research has shown that engaging in physical exercise (PE) is associated with improved neuroplasticity, elevated levels of neurotrophic factors, and enhanced brain function [7]. The process of neuroplasticity involves the generation of neural stem cells (NSCs), which are responsible for producing neurons, astrocytes, and oligodendrocytes within the central nervous system [8].

Previous research has shown that brain-derived neurotrophic factor (BDNF) expression is enhanced following exercise and wet cupping therapy in an animal model of stroke [9]. A biomarker indicating neuroplasticity, known as BDNF, is associated with the outlook and rehabilitation of individuals who have experienced a stroke [10]. Wet cupping therapy is a method of therapy that applies negative pressure and punctures to the skin [11]. Research revealed that wet cupping therapy could improve the motor skills of stroke patients [12], yet the mechanism remains unclear.

The mature nervous system is composed of various neuronal and glial cell types, which are generated by NSCs. Generally, as NSCs differentiate, the expression of SRY-related HMG-box 2 (SOX2) decreases. SOX2 is an essential protein that plays a key role in regulating NSC proliferation and differentiation when injury occurs [13]. Nevertheless, studies examining protein expression (e.g., by immunofluorescence using anti-SOX2 antibodies) have demonstrated increased SOX2 levels in certain differentiated glial and neuronal cell types [14].

This research aims to investigate how exercise and wet cupping therapy affect SOX2 expression. This study hypothesizes that SOX2 expression will be elevated by exercise and wet cupping therapy. The increase in SOX2 expression is expected to trigger neural plasticity; thus, the motor function will increase as well.

Materials and Methods

Animal Housing and Stroke Induction

Protocols for animal experiments were approved by the Animal Experimental Ethics Committee of the Universitas Airlangga (Approval number 2.KE.073.06.2021). This randomized post-test only control group design involved 36 male Wistar rats (3 months old), randomly allocated using manual block randomization into six equal groups (n=6 per group): control, stroke (unilateral common carotid artery occlusion), wet cupping, exercise, stroke plus cupping, and stroke plus exercise.

Rats were housed in standard cages (4–5 rats per cage) under a 12:12-hour light-dark cycle (lights off at 7:30 PM) at a constant temperature of 24°C for 7 days. Food (Pellet HI-PRO-VITE 594, Charoen Pokphand Indonesia) and tap water were provided *ad libitum*. Before treatment, we performed a unilateral common carotid artery occlusion (UCCAO). First, the rats were administered anesthesia using ketamine (80 mg/kg BW) and xylazine (10 mg/kg BW). The rats were positioned supine and fixed to a rodent surgical table using adhesive tape. Afterwards, we shaved the hair on their necks and made a small incision of about 1-2 cm in the midline. The unilateral common carotid artery was isolated from the surrounding connective tissue and occluded with a bulldog clamp for three hours, after which the clamp was subsequently removed. The sham group was subjected to the same procedure without a carotid block. After surgery, the incision was then closed. Thereafter, we observed consciousness in rats and examined whether or not any stroke model emerged [15].

Interventions

Ten lancet punctures were made using aseptic technique as part of the wet cupping therapy procedure. To guarantee a good fit for the cupping therapy, a wide section of the back was chosen. For five minutes, negative pressure of -200 mmHg was applied to the right and left sides of the back using a 2 cm diameter. On the other hand, the exercise included a swimming activity scheduled for 20 minutes per day. Rats swam individually in 1.2 m diameter and 1.1 m height deep water tanks. Further, the water depth was set at 0.7 m, and the water temperature was maintained at 28- 30 °C. The wet cupping therapy and swimming exercise were performed twice weekly for three weeks. In the intervention group, wet cupping therapy and exercise were performed four days after UCCAO.

Immunohistochemistry

Brain tissues were collected and preserved in neutral buffer formalin at 4°C overnight. Afterward, they were embedded in paraffin and sliced into sections (4 microns) using a microtome (BQ-318D). The sections were subsequently heated at 75°C for 30 minutes, followed by 10-minute immersion in xylene and an additional 10-minute immersion in clean xylene.

Subsequently, the tissue samples were immersed in a series of ethanol solutions (100%, 95%, and 80%) and distilled water, with each immersion lasting 3 minutes. After this process, the samples were submerged in a citrate buffer solution (used for antigen retrieval) and then washed with phosphate buffer solution (PBS). Following this, the samples were placed in a humid chamber at ambient temperature and exposed to 3% hydrogen peroxide for 10 minutes. Afterward, normal goat serum was applied to the sections for 30 minutes, and any excess liquid was removed. A dissolved SOX2 antibody (1:200; Santacruz Biotech, USA) was then injected into each slice to be incubated afterwards at 4°C overnight, and the slices were rinsed again using PBS. Henceforth, the secondary antibody buffer (1:500) was injected into each slice. The slices were then incubated at room temperature for an hour. Afterward, the slices were rinsed, stained, and hydrated to be placed on and examined using a microscope. Further, the quantitative analysis of this study adopted the Image J software.

Statistical analysis

The statistical analysis was performed using IBM SPSS Statistics 21.0 (IBM Corp., Armonk, NY, USA). Descriptive tests were performed for all variables in the study. A one-way analysis of variance (ANOVA) was conducted, followed by Tukey's post hoc test for pairwise comparisons. Prior to analysis, the assumptions of normality and homogeneity of variances were assessed and met using the Shapiro–Wilk test (p=0.804) and Levene's test (p=0.774) to evaluate normality and homogeneity, respectively.

Results

SOX2 (SRY-related HMG-box 2) expression

The immunohistochemical technique applied in this study suggested that there was expression of SOX2 in the cytoplasm of nerve cells in the subventricular area. This expression was evidenced by a brown-colored reaction when exposed to the anti-SOX2 antibody, as depicted in figure 1. The mean of nerve cells expressing SOX2 in the control group (Control) was 6.83 ± 1.47; the stroke group (ST) was 2.50 1.05; the cupping group (B) was 9.00 ± 1.41, the exercise group (T) was 9.50 ± 1.05, the stroke with cupping treatment group (ST-B) was 11.83 ± 1.47; and the stroke with exercise treatment group (ST-T) was 12.33 ± 1.63 (Figure 2). Multiple group comparisons using ANOVA revealed significant differences in SOX2 expression across various conditions. The ST group exhibited substantially lower SOX2 expression compared to the Control group (p<0.05). In contrast, the B group and T group both showed noticeably elevated SOX2 expression in comparison to the Control group (p<0.05). Both ST-B and ST-T groups displayed markedly higher SOX2 expression than the ST group (p<0.05 for both comparisons). When comparing the T and B groups, the former showed a marginally higher, though statistically insignificant, SOX2 expression (p=0.987). The ST-T group exhibited significantly greater SOX2 expression than the ST-B group (p<0.05). Additionally, the ST-B group's SOX2 expression was significantly higher than

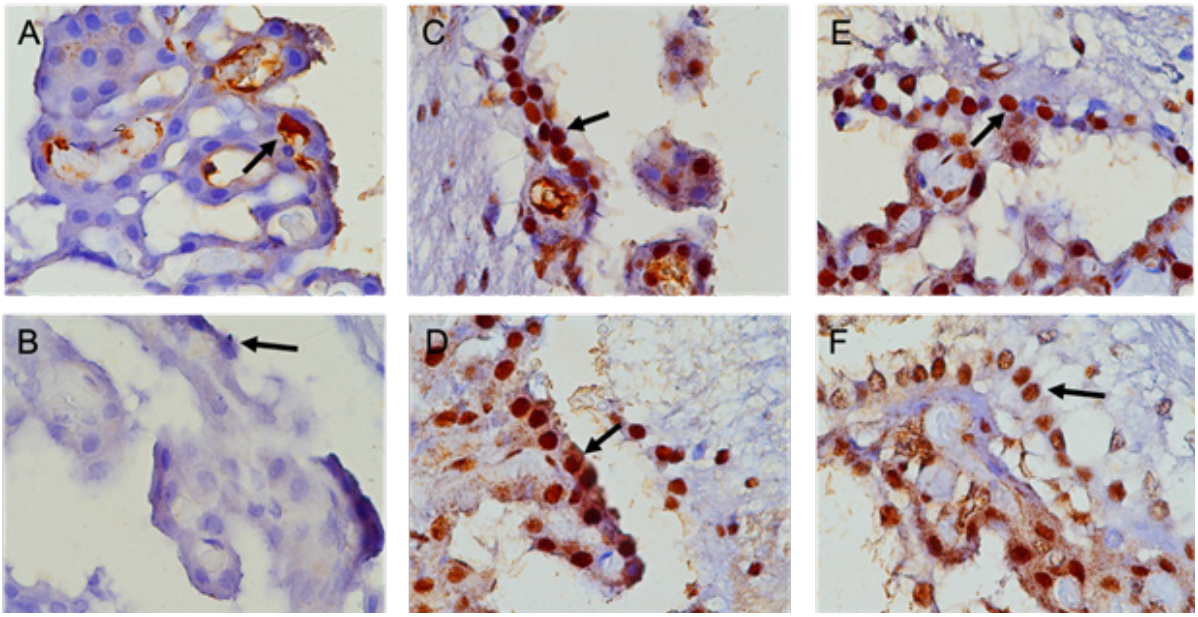

Figure 1. Immunohistochemistry, showing the distribution of SOX2 (light microscope, magnification 1000). A. Expression of SOX2 control group, B. Expression of SOX2 in the stroke group, C. Expression of SOX2 in the cupping group, D. Expression of SOX2 in the exercise group, E. Expression of SOX2 in the stroke-cupping group, F. Expression of SOX2 in the stroke-exercise group. Black arrow: SOX2 expression was lower in B compared to A; while C and D showed higher expression levels than A. Additionally, D and F exhibited higher SOX2 expression compared to B.

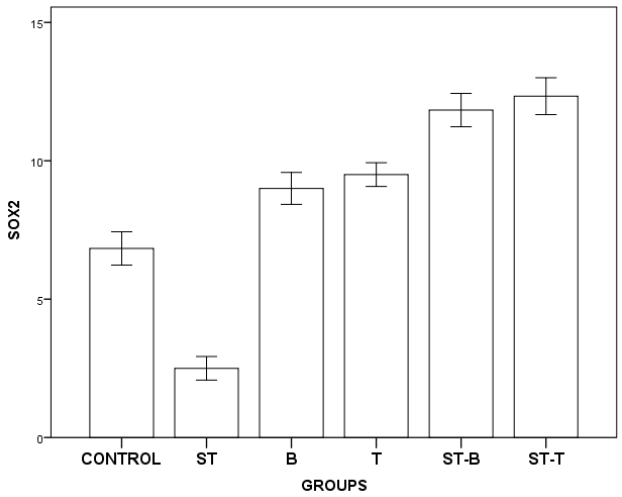

Figure 2. Number of neural stem cells expressing SOX2 in the control group, the stroke group (ST), the cupping group (B), the exercise group (T), the stroke-cupping group (ST-B), and the stroke-exercise group (ST-T). SOX2 expression was lower in group B compared to the control group; while groups B and T showed higher expression levels than the control group. Additionally, groups ST-B and ST-T exhibited higher SOX2 expression compared to the control group.

that of the B group (p<0.05). Lastly, the ST-T group demonstrated significantly elevated SOX2 expression compared to the T group (p<0.05).

Discussion

Understanding the mechanism underlying neural plasticity after stroke in animal models as well as clinical studies provides the foundation for evidence-based neurorehabilitation [16]. Research has identified two regions in the mature mammalian brain capable of ongoing neuron generation: the hippocampal dentate gyrus (DG) and the lateral ventricle's subventricular zone (SVZ). Research has shown that physical activity can boost both the multiplication of adult hippocampal neural progenitor cells (NPCs) and their transformation into neurons [17]. This study's observations were focused on the subventricular zone, which is one of the primary locations where adult neurogenesis occurs [18].

Our research revealed a reduction in SOX2 expression within the stroke group (Figure 1B and Figure 2). The underlying cause for this decrease remains uncertain. We hypothesize that the lack of glucose and oxygen during cerebral ischemia triggers neural cell depolarization and the release of glutamate. This process subsequently activates Na+ / Ca2+ channels associated with N-methyl-D-aspartate receptors (NMDRs)[19], resulting in the production of reactive oxygen species (ROS). These ROS induce oxidative stress, which disrupts mitochondrial function and leads to neuronal death. Furthermore, the overactivation of NMDA receptors interferes with neural plasticity [20].

Research indicates that physical activity may influence the growth and specialization of NSCs, but the precise cellular mechanisms behind this process remain unclear [21]. This research showed that exercise increased the expression of SOX2 in the SVZ, both in the UCCAO animal model and without UCCAO. As a transcription factor, SOX2 plays a crucial role in the regulatory system that maintains pluripotency in both cultured embryonic stem cells and early-stage embryos. In addition, SOX2 plays a pivotal role in neural stem cell formation and neurogenesis [22]. Nam et al. showed that nestin-immunoreactive cells, a biomarker of neural stem cells, increased significantly by 159.4% in the six-week running group compared sedentary group in mice [23]. Research by Wu and colleagues demonstrated that mice engaging in treadmill running for a five-week period experienced a significant increase in BrdU+ mitotic cells, DCX+ immature response, and BrdU+/DCX+ newborn neurons within the dentate gyrus [24]. Additionally, Liu et al. proposed that physical activity promotes the proliferation and differentiation of endogenous NSCs through the activation of the ERK signalling pathway[21].

This research showed that cupping therapy increased SOX2 expression in the UCCAO animal model. Wet cupping's mechanism of expressing SOX2 is still unclear. So far, there have been no studies that discuss the effects of wet cupping on NSCs. Previous research has demonstrated that wet cupping therapy increases BDNF levels [9]. BDNF is one of the primary growth factors with a crucial role in the development and function of the nervous system, as well as in the regulation of neural stem cells [25]. We suspect that the increase in SOX2 is related to the expression of BDNF.

The interesting thing about this study is that the effectiveness of exercise and wet cupping therapy is higher in the UCCAO animal model than without UCCAO. The effectiveness of wet cupping therapy is the same as exercise. We suspect that after injury, the brain undergoes more intensive reorganization than the normal brain. Research has demonstrated that the subventricular zone continues to produce NSCs or multipotent neural progenitor cells throughout an individual's lifespan. In response to neuronal damage, the brain has the ability to regenerate lost neurons through the proliferation and differentiation of these NSCs [26].

This research has limitations, including a restricted sample size and the use of animal subjects, necessitating additional human trials to validate the findings. Additionally, the post-only control design method used does not fully reflect the outcomes of the intervention.

Conclusion

This study demonstrates that both physical exercise

and wet cupping therapy enhance SOX2 expression, and injury to the brain decreases expression of SOX2. Exercise and wet cupping therapy's efficacy as alternative therapies is encouraging, as evidenced by the higher SOX2 levels in the UCCAO animal model compared to the standard one. Further studies in an alternative animal model are required to guarantee the consistency of the SOX2 increase in response to the UCCAO.

Conflict of Interests
None.

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