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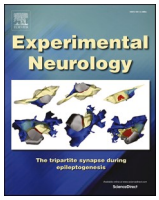

Research paper

Therapeutic effects of intracerebral transplantation of human modified bone marrow-derived stromal cells (SB623) with voluntary and forced exercise in a rat model of ischemic stroke

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ABSTRACT

Ischemic stroke results in significant long-term disability and mortality worldwide. Although existing therapies, such as recombinant tissue plasminogen activator and mechanical thrombectomy, have shown promise, their application is limited by stringent conditions. Mesenchymal stem cell (MSC) transplantation, especially using SB623 cells (modified human bone marrow-derived MSCs), has emerged as a promising alternative, promoting neurogenesis and recovery. This study evaluated the effects of voluntary and forced exercise, alone and in combination with SB623 cell transplantation, on neurological and psychological outcomes in a rat model of ischemic stroke. Male Wistar rats that had undergone middle cerebral artery occlusion (MCAO) were divided into six groups: control, voluntary exercise (V-Ex), forced exercise (F-Ex), SB623 transplantation, SB623 + V-Ex, and SB623 + F-Ex. Voluntary exercise was facilitated using running wheels, while forced exercise was conducted on treadmills. Neurological recovery was assessed using the modified neurological severity score (mNSS). Psychological symptoms were evaluated through the open field test (OFT) and forced swim test (FST), and neurogenesis was assessed via BrdU labeling. Both exercise groups exhibited significant changes in body weight post-MCAO. Both exercises enhanced the treatment effect of SB623 transplantation. The forced exercise showed a stronger treatment effect on ischemic stroke than voluntary exercise alone, and the sole voluntary exercise improved depression-like behavior. The SB623 + F-Ex group demonstrated the greatest improvements in motor function, infarct area reduction, and neurogenesis. The SB623 + V-Ex group was most effective in alleviating depression-like behavior. Future research should optimize these exercise protocols and elucidate the underlying mechanisms to develop tailored rehabilitation strategies for stroke patients.

1. Introduction

Ischemic stroke is one of the most significant causes of long-term disability and mortality worldwide (Goyal et al., 2015). Over the past several decades, novel therapeutic approaches such as recombinant tissue plasminogen activator and mechanical thrombectomy have been developed and advanced (Emberson et al., 2014; Nogueira et al., 2018). However, the conditions for their application, such as time after onset and infarct types, are still not broadly applicable, and the development of other new therapies is anticipated (Paul and Candelario-Jalil, 2021). Moreover, most of the currently available treatments for stroke do not promote neurogenesis and recovery (Chrostek et al., 2019).

Among the promising new therapies, stem cell transplantation is considered a leading candidate (Horie et al., 2015; Oshita et al., 2020; Ryu et al., 2016). There are various types of stem cells used in research for brain disorders, with mesenchymal stem cells (MSCs) being the most frequently utilized (Potdar, 2013). These cells are a heterogeneous population of fibroblast-like pluripotent cells in adults, capable of differentiating into various mesodermal lineages, and are found in numerous tissues such as bone marrow, umbilical cords, muscle, and

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adipose tissue (Caplan, 1991). They exhibit therapeutic effects through various mechanisms and have immunomodulatory functions, which make them widely used in medical research (Hasan et al., 2017; Rowart et al., 2015). In the field of brain disorders, the utility of stem cell transplantation has been evaluated in clinical trials, particularly for diseases such as Parkinson's disease and Alzheimer's disease (Bae et al., 2013; Venkataramana et al., 2010).

SB623 cells, human bone marrow-derived MSCs transiently transfected with the Notch-1 intracellular domain, have been shown to improve motor function, reduce infarct area, and promote neurogenesis in experimental ischemic stroke rats (Yasuhara et al., 2009). Several studies have demonstrated that treatment with SB623 cells upregulates neurotrophic factors (Tate et al., 2010), promotes angiogenesis (Dao et al., 2011), and enhances the migration and differentiation of neural stem cells to the site of injury (Tajiri et al., 2013). Furthermore, clinical trials have demonstrated that the intracerebral implantation of SB623 cells is safe in patients with chronic ischemic stroke (STR-01) (Steinberg et al., 2016; Steinberg et al., 2018) and patients with traumatic brain injury (STEMTRA trial) (Kawabori et al., 2021).

Rehabilitation therapy is one of the standard treatments for stroke patients (Langhorne et al., 2011). Rehabilitation includes both forced exercise, such as using treadmill devices, and voluntary exercise, such as using running wheels, and the therapeutic effects of both have been reported in animal models of ischemic stroke (Chen et al., 2019; Yabuno et al., 2023). However, there are very few reports comparing the therapeutic effects of these two types of exercises, and their conclusions are inconsistent (Alomari et al., 2013; Hayes et al., 2008; Ke et al., 2011a). Additionally, one of the challenges in rehabilitation therapy is post-stroke depression (PSD), which is a significant sequela seen in approximately 20 % of stroke patients. This sequela can hinder rehabilitation and delay discharge and return to home (Lenzi et al., 2008). There are reports suggesting that exercise, including forced exercise, can improve PSD (Zhang et al., 2017). Still, there are also reports indicating that forced exercise can increase stress and worsen PSD (Svensson et al., 2016).

Previously, we have reported the combination of transplantation of human modified bone marrow-derived stromal cells (SB623) and voluntary exercise therapy has a synergic effect in the rat model of ischemic stroke (Yabuno et al., 2023). Other studies have also reported the combination of stem cell therapy and exercise therapy improves post-stroke motor functions better than those of either only (Cho et al., 2016; Hicks and Jolkkonen, 2009; Sasaki et al., 2016; Seo et al., 2013). However, it has not been unveiled whether the combination of them improves the psychological symptoms better than those of either alone.

In this study, we investigated which type of exercise, voluntary exercise or forced exercise, had strong therapeutic effects for ischemic stroke. Moreover, we explored the synergistic effects of the combinations of transplantation of SB623 and each exercise for not only motor function, but psychological symptoms.

2. Materials and methods

2.1. Ethics statement

This study was conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Okayama University Graduate School of Medicine. The protocol was specifically approved by the Institutional Animal Care and Use Committee of Okayama University Graduate School of Medicine (protocol #OKU-2024331). At the end of the protocols, which is described below, all rats were euthanized by overdosing of intraperitoneal injection with a mixed solution of 0.3 mg/kg medetomidine, 4.0 mg/kg midazolam, and 5.0 mg/kg butorphanol. During middle cerebral artery occlusion (MCAO) surgery, anesthesia was induced by inhalation of 2.0 % sevoflurane in 30 % O_2 and 70 % N_2O via a facial mask. Every effort was made to minimize the distress of the animals. Measurements and analyses were conducted by examiners blinded to the treatment groups of the rats in this study.

2.2. Animals

In this study, 8-week-old male Wistar rats (Jackson Laboratory Japan, Inc., Yokohama, Japan) were used as subjects. The animal housing facility was maintained on a 12-h light/dark cycle, and the animals had free access to food and water. Body weight was recorded on days −7, 0, 1, 8, and 15 after MCAO. Due to the immunosuppressive properties of SB623 cells, immunosuppressants were not administered in this study (Caplan, 1991; Dao et al., 2011; Tate et al., 2010; Yasuhara et al., 2009).

2.3. Rats and experimental design

All rats were randomly assigned to one of six groups and subsequently underwent MCAO: Dulbecco's modified Eagle's medium (DMEM [Sigma-Aldrich, St. Louis, Missouri, USA]) injection group (control group: $n = 10$), DMEM injection + voluntary exercise group (V-Ex group: n = 10), DMEM injection + forced exercise group (F-Ex group: n = 10), SB623 cell transplantation group (SB623 group: n = 10), SB623 cell transplantation + voluntary exercise combination therapy group (SB623 + V-Ex group: n = 10), and SB623 cell transplantation + forced exercise combination therapy group (SB623 + F-Ex group: n = 10) (see study design in Fig. 1).

On day 7 before MCAO, rats in the control, F-Ex, SB623, and SB623 + F-Ex groups were housed individually in standard cages, while rats in the V-Ex and SB623 + V-Ex groups were housed in cages equipped with a running wheel (RW) (Fig. 2A). Consequently, voluntary RW exercise commenced for the V-Ex and SB623 + V-Ex groups 7 days before MCAO and continued for 22 days. Additionally, starting 3 days before MCAO, F-Ex and SB623 + F-Ex groups underwent mild forced exercise on a treadmill (10 m/min for 30 min per day), continuing until 1 day before MCAO. From 2 days after MCAO, these groups transitioned to intense forced exercise (20 m/min for 30 min per day, 5 days per week), which continued until day 15 after MCAO.

2.4. Running wheels apparatus

The V-Ex and SB623 + V-Ex groups were individually housed in plastic cages containing animal paper bedding and RW (SWY-30, Melquest, Toyama, Japan, 310 Φ × 84 mm, perimeter = 1 m) (Fig. 2A). The number of rotations of the RW was measured by a porcelain sensor and converted to distance (meters). The distance was displayed on an attached counter (CNT-10, Melquest, Toyama, Japan), and we recorded the distance covered over 24 h from 10:00 a.m. daily. The rats had free access to the RW, food and water.

2.5. Treadmill apparatus

The F-Ex and the SB623 + F-Ex groups were exercised on a five-lane treadmill (MK-680, Muromachi, Tokyo, Japan) (Fig. 2B). Prior to MCAO, the rats underwent 3 days of adaptive treadmill training at a 0° slope, 10 m/min, for 30 min per day. After MCAO, the treadmill training regimen was set at a 0° slope, 20 m/min, for 30 min per day, 5 days per week, for 2 weeks. After each session, the treadmill was cleaned with a 70 % ethanol solution. To encourage running, the rats received a mild electric shock stimulation (0.2–0.5 mA) upon contacting a metal grid.

2.6. SB623 cell production

SB623 cells, known as human modified bone marrow-derived MSCs, were produced by the transient transfection of a plasmid encoding the human Notch-1 intracellular domain cDNA into MSCs derived from the bone marrow of a healthy young adult human donor (SanBio, Inc.,

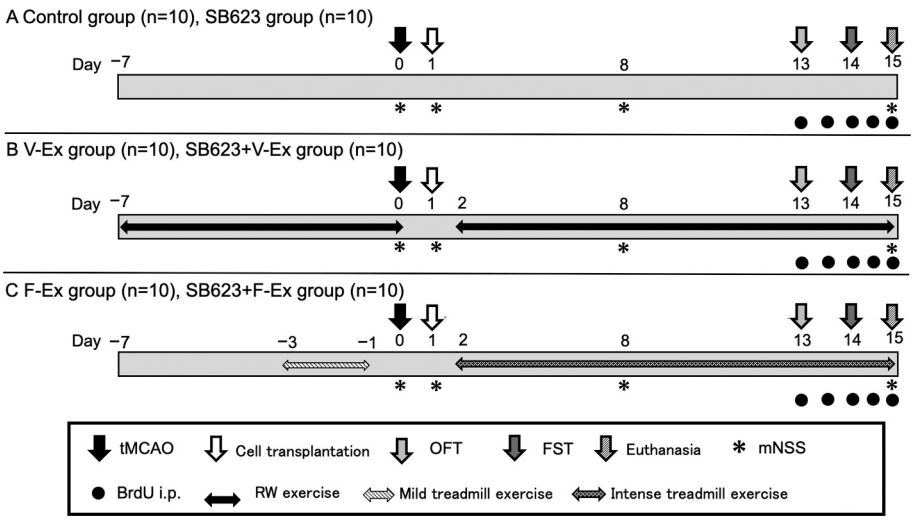

Fig. 1. Experimental protocol of this study.
(A) Study design for control group ($n = 10$) and SB623 group ($n = 10$).
(B) Study design for V-Ex group ($n = 10$) and SB623 + V-Ex group ($n = 10$).
(C) Study design for F-Ex group ($n = 10$) and SB623 + F-Ex group ($n = 10$).
The V-Ex group and the SB623 + V-Ex group trained in a cage with RW from 7 days before MCAO. The F-Ex group and the SB623 + F-Ex group trained on a treadmill for adaptation from 3 days to 1 day before MCAO. All rats were subjected to right MCAO. On day 1 after MCAO, all rats that were diagnosed with ischemic stroke by behavioral test were stereotactically transplanted with DMEM or SB623 cells into the right striatum. On day 2 after MCAO, the V-Ex group and the SB623 + V-Ex group restarted the rehabilitation and continued it for 13 days. The F-Ex group and SB623 + F-Ex group were trained on a treadmill with intense exercise from 2 days after MCAO and continued for 19 days. On day 15 after MCAO, all rats were euthanized, followed by histological analysis. Abbreviations: DMEM, Dulbecco's modified Eagle's medium; MCAO, middle cerebral artery occlusion; RW, running wheel.

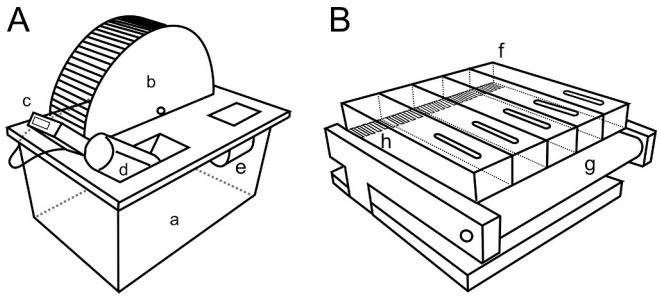

Fig. 2. Illustration of a running wheel apparatus and a treadmill apparatus.
(A) Running wheel used in this study. The V-Ex group and the SB623 + V-Ex group were housed individually in cages (a) with RW (b) for 7 days before MCAO and freely exercised. On day 2 after MCAO, they restarted the rehabilitation and continued until day 15 after MCAO. The distance run by the rats was displayed on a counter (c). Each cage was equipped with a water bottle (d) and a food container (e), allowing the rats free access to water and food within the cage.
(B) Treadmill used in this study. The F-Ex group and the SB623 + F-Ex group were trained on this apparatus with mild exercise for adaptation from 3 days to 1 day before MCAO. At 2 days after MCAO, the groups started intense exercise for 5 days per 1 week, and continued until day 15 after MCAO. The device had five running lanes (f), and the movement of the belt (g) forced the rats to exercise. Electrodes (h) were positioned at the back of each lane to deliver an electric shock when a rat's tail touched them. Abbreviations: MCAO, middle cerebral artery occlusion; RW, running wheel.

Mountain View, CA, USA). The characteristics of SB623 cells have been elucidated in previous studies (Dezawa et al., 2004; Savitz et al., 2014; Tate et al., 2010). Yasuhara et al. and Tate et al. applied these cells to animal models of ischemic stroke and Parkinson's disease, respectively (Tate et al., 2017; Yasuhara et al., 2009).

2.7. SB623 cell preparation

Frozen vials containing SB623 cells provided by SanBio, Inc., were gently thawed in a 37 °C water bath. Phosphate-buffered saline (PBS, 10 ml) was added to 1 ml of SB623 cell suspension and mixed by gentle pipetting. The suspension was then centrifuged at 1000 rpm (200 ×*g*) for 8 min at room temperature to form a cell pellet. The supernatant was carefully removed without disturbing the pellet, and the cells were resuspended in 100 μl of DMEM without serum and antibiotics. The viability of the cells was measured using the Trypan blue dye exclusion test, and the cell concentration was adjusted to 8.0×10^4 cells/μl for transplantation. The concentration of SB623 cells used in our study (8.0×10^4 cells/μl) was set close to that used in clinical trials (8.0×10^3 to 3.3×10^4 cells/μl), resulting in a cell solution containing 4.0×10^5 cells in 5 μl.

2.8. Surgical procedures to induce transient middle cerebral artery occlusion

Transient MCAO was conducted using the intraluminal suture technique as previously described in our studies (Kawauchi et al., 2022; Morimoto et al., 2018). During the surgery, body temperature was maintained at 37 °C using a heating pad. Under general anesthesia with 2.0 % sevoflurane in 30 % O_2 and 70 % N_2O, the bifurcation of the right common carotid artery was exposed. Following the severance of the right external carotid artery (ECA), a 4–0 monofilament nylon suture with a silicone-coated tip (Xantopren L blue and ACTIVATOR 2 Universal Liquid, Heraeus Kulzer GmbH & Co. KG, Hanau, Germany) was introduced 18–20 mm into the lumen of the internal carotid artery (ICA) and advanced toward the origin of the right MCA until slight resistance was encountered. After 90 min of occlusion, the filament was withdrawn and the ECA was cauterized. At the end of the operation, the skin was closed with 3–0 silk sutures. To alleviate postoperative pain and discomfort, 1 % lidocaine (10 mg/kg) was injected topically around the wound, and carprofen (5 mg/kg) was administered subcutaneously immediately after surgery. Upon recovery from anesthesia, the animals

were returned to their original cages.

2.9. SB623 intracerebral transplantation

All rats were anesthetized using an intraperitoneal injection of a mixed solution of 0.3 mg/kg of medetomidine, 4.0 mg/kg of midazolam, and 5.0 mg/kg of butorphanol. Each animal was then secured in a stereotactic apparatus (Narishige, Japan). A 22-gauge Hamilton syringe was used to inject either SB623 cells (8.0×10^4 cells/μl) or DMEM solution into the right striatum. The injection coordinates were set at 1.0 mm anterior to the bregma, 3.0 mm lateral to the sagittal suture, and 5.0 mm ventral to the brain surface, with the tooth bar at 0.0 mm. SB623 cells were prepared as a single-cell suspension at a concentration of 8.0×10^4 cells/μl, and a volume of 5 μl (4.0×10^5 cells in total) was injected at a rate of 1 μl/min. After the injection, the syringe was left in place for an additional 2 min, before being slowly retracted at a rate of 1 mm/min. The DMEM used for injection and cell preparation did not contain serum and antibiotics.

2.10. Behavioral tests

2.10.1. Modified neurological severity score (mNSS)

The mNSS was assessed on days −7, 0, 1, 8, and 15 after the MCAO. This scoring system evaluated motor function, sensory disturbance, balance, and reflexes. One point was assigned for an inability to perform a test or for the absence of a reflex. Therefore, a higher score indicates more severe injury. Neurological function was graded on a scale from 0 to 18 (normal score: 0; maximal deficit score: 18) (Chen et al., 2001; Morimoto et al., 2018; Shen et al., 2006). In this study, to standardize the postsurgical neurological severity, only rats that scored between 7 and 12 points on the mNSS on the day following MCAO were included.

2.10.2. Open field test (OFT)

To assess the degree of anxiety, OFT was performed on day 13 after MCAO. The OFT was conducted in a 100 cm × 100 cm × 80 cm black Plexiglas box with a black floor. At the beginning of the test, a rat was placed near the midpoint of the box and allowed to explore the apparatus for 10 min. The time spent in the center of the field and the total distance walked were measured using Ethovision® XT 9.0 video tracking software (Noldus, Wageningen, The Netherlands) set up with a digital video camera. The center of the field was defined as a 40 cm × 40 cm area (Niu et al., 2015). The time the rat spent in the center of the field was defined as any time when even a small portion of the rat entered the center.

2.10.3. Forced swim test (FST)

To assess the degree of despair, FST was performed. For the FST, the rats were placed individually in clear-plastic Plexiglas cylinders with dimensions of 45 cm in height and 20 cm in diameter. The cylinders were filled with water maintained at 25 °C to a depth of 30 cm. The FST was conducted over 2 days consecutively (days 14 and 15 after MCAO). On the first day, the rats were placed in the water for 15 min. Twenty-four hours later, the rats were returned to the water-filled cylinder and tested for 5 min (Porsolt et al., 1977). The water was changed after every swim session to ensure that every rat swam in clean water. Each rat's FST behavior was recorded with a digital video camera, and the percentage of time spent immobile was scored by the Ethovision® XT 9.0 software (Noldus). The immobility threshold in the detection settings of the Ethovision® XT 9.0 software (Noldus) was defined as 10 %, and the same threshold was applied to all rats. A rat was defined as immobile when fewer than 10 % of the pixels in one frame were identified as changed compared with the previous frame.

2.11. 5-Bromo-2′-deoxyuridine (BrdU) labeling

To evaluate endogenous neurogenesis in the subventricular zone (SVZ), 5-bromo-2′-deoxyuridine (BrdU, NACALAI TESQUE INC., Kyoto, Japan) was administered to all rats at a concentration of 50 mg/kg body weight, with five consecutive intraperitoneal injections every 12 h from day 13 to 15 after MCAO, before euthanasia to label proliferative cells (Greisen et al., 2005; Yasuhara et al., 2007).

2.12. Measurement of the cerebral infarct area

All rats were euthanized with an overdose of a mixed solution of 0.3 mg/kg of medetomidine, 4.0 mg/kg of midazolam, and 5.0 mg/kg of butorphanol on day 15 after MCAO. They were then perfused transcardially with 200 ml of cold PBS followed by 200 ml of 4 % paraformaldehyde (PFA) in PBS. Brains were removed and post-fixed in the same fixative overnight at 4 °C, and subsequently stored in 30 % sucrose in PBS until completely submerged. Coronal sections of 30 μm thickness were prepared with a freezing microtome (−20 °C). These sections were mounted onto slides. Five rats from each group were selected randomly. Nissl staining was performed to evaluate the cerebral infarct area. The cerebral infarct area was measured at the site of cell transplantation using computerized image analysis with ImageJ software (National Institutes of Health, Bethesda, USA). We evaluated the cerebral infarct area ratio by the following method: Cerebral infarct area ratio = [LT − (RT − RI)] × 100/LT (%), in which LT is the area of the left hemisphere in mm^2, RT is the area of the right hemisphere in mm^2, and RI is the infarct area in mm^2 (Morimoto et al., 2018; Toyoshima et al., 2015; Yasuhara et al., 2007). Fig. 3A depicts a standard coronal section identified at the level of 0.3 mm posterior to the bregma in the rat brain, which divides the right hemisphere into two subregions (ischemic core [IC] and ischemic boundary zone [IBZ]).

2.13. Immunofluorescence staining of BrdU

Five rats from each group were selected randomly. To demonstrate endogenous neurogenesis and cell proliferation in the SVZ and the DG, BrdU/Doublecortin (Dcx)/4,6-diamidino-2-phenylindole (DAPI) triple-immunofluorescence staining was performed based on methods from previous reports (Kin et al., 2017; Yasuhara et al., 2007). Briefly, free-floating sections were first incubated in HCl (2 N, 37 °C) for 20 min. This was followed by sodium borate incubation (pH 8.5) for 10 min. After rinsing four times with PBS, the sections were incubated for 24 h at 4 °C with rat anti-BrdU antibody (1:100, OBT0030G; Bio-Rad Laboratories Inc., Hercules, California, USA), rabbit anti-Dcx antibody (1:200, #4604; Cell Signaling Technology, Danvers, Massachusetts, USA), 10 % normal horse serum (Invitrogen, Carlsbad, California, USA), and 0.1 % Triton X-100 (NACALAI TESQUE INC., Kyoto, Japan). After rinsing several times in PBS, the sections were incubated for 90 min with

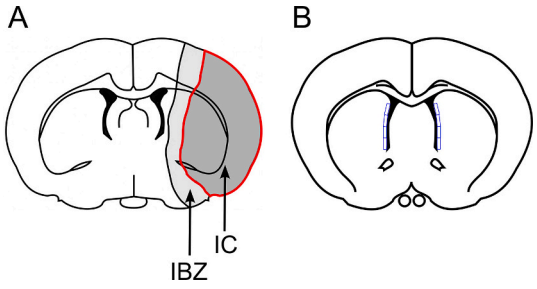

Fig. 3. Illustration of an ischemic lesion and SVZ.
(A) A coronal section identified at the level of 0.3 mm posterior from the bregma in rat brain that divides the right ischemic hemisphere into two subregions (ischemic core [IC]; ischemic boundary zone [IBZ]).
(B) Illustration of counting BrdU/Dcx double-positive cells in the SVZ. BrdU/Dcx double-positive cells were counted in 16 areas (4 areas × 2 sections × 2 hemispheres) in each rat. Abbreviations: BrdU, 5-bromo-2′-deoxyuridine, Dcx, Doublecortin; IBZ, ischemic boundary zone; SVZ, subventricular zone.

biotinylated anti-rat secondary antibody (1:100, 712–065-153; Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania, USA). Then, the sections were washed four times in PBS and incubated for 1 h with Streptavidin Alexa-488 (1:200, S11223; Invitrogen), goat anti-rabbit IgG Cy3 (1:200, ab97075; Abcam, Cambridge, UK), and DAPI (1:500, D3751; Thermo Fisher Scientific, Waltham, Massachusetts, USA). Finally, the sections were washed four times in PBS and mounted on albumin-coated glass slides.

2.14. Cell counting

Quantification of BrdU/Dcx double-positive cells in the SVZ was performed using methods according to previous studies (Kin et al., 2017; Yasuhara et al., 2007). Briefly, BrdU/Dcx double-positive cells were counted bilaterally in four defined areas (200 × 60 μm) of the lateral ventricle wall. For cell counting, the sections used for analyses were taken from the brain at the level of the SVZ in the area of 0.0–0.9 mm posterior to the bregma. The cell number was counted bilaterally and averaged over five sections (every 6th section). In summary, we counted 16 areas (4 areas × 2 sections × 2 hemispheres) for the SVZ (Fig. 3B) in each rat. Additionally, both Nissl staining and immunofluorescence staining were visualized using a BZ-X810 system (Keyence, Osaka, Japan).

2.15. Statistical analysis

We used the GraphPad Prism 8 (GraphPad Software, CA, USA). All data were tested for normality using the Shapiro–Wilk normality test to confirm whether they followed a normal distribution. The results of running distance were analyzed using a two-tailed *t*-test and Mann-Whitney *U* test. Comparisons between running distance and changes over time were analyzed using repeated measures analysis of variance (ANOVA) with the post hoc Bonferroni multiple comparisons test. Additionally, the results of body weight, immunofluorescence staining, the distance moved on OFT, and the immobility time on FST were analyzed using a one-way ANOVA with the Bonferroni's multiple comparisons test, and the time spent in the center zone on OFT was analyzed using the Kruskal–Wallis test to compare differences between. All quantitative results except running distance were presented as mean ± standard error (SE). The results of running distance were presented as mean ± standard deviation (SD). Statistical significance was present at a p-value <0.05.

3. Results

3.1. Voluntary exercise and forced exercise reduce body weight on day 0, day 1, and day 8 after MCAO

All groups gained body weight from day −7 to day 0 after MCAO, but the weight gain of the voluntary exercise groups (V-Ex and SB623 + V-Ex groups) was less than that of other groups. ANOVA revealed significant treatment effects, with post hoc Bonferroni's multiple comparisons test showing a significantly different weight gain between the voluntary exercise groups and other groups on day 0 before MCAO ($F(5, 54) = 7.271$, $p < 0.001$ on day 0) (day 0: control group: 349.28.0 ± 4.7 g; V-Ex group: 328.42 ± 3.2 g; F-Ex group: 353.5 ± 3.0 g; SB623 group: 344.6 ± 5.0 g; SB623 + V-Ex group: 323.5 ± 4.1 g; SB623 + F-Ex group: 350.6 ± 6.9 g, respectively). Comparing day 0 to day 1 after MCAO, body weight decreased in all groups on day 1 after MCAO. There was no significant difference in weight loss between the control group and the other groups, but ANOVA revealed significant treatment effects, with post hoc Bonferroni's multiple comparisons test showing a significantly different weight loss between the voluntary exercise groups and the F-Ex group, and between the SB623 group and the SB623 + V-Ex group on day 1 after MCAO ($F(5, 54) = 6.155$, $p < 0.001$ on day 1) (day 1: control group: 307.5 ± 5.5 g; V-Ex group: 296.3 ± 3.5 g; F-Ex group: 321.4 ± 3.0 g; SB623 group: 313.0 ± 5.1 g; SB623 + V-Ex group: 289.3 ± 3.7 g; SB623 + F-Ex group: 309.5 ± 6.3 g, respectively). Body weight increased from day 1 to day 8 after MCAO in all groups. There was no significant difference in weight gain on day 8 after MCAO among all groups ($F(5, 54) = 1.347$, $p < 0.001$ on day 8) (day 8: control group: 327.5 ± 4.1 g; V-Ex group: 327.2 ± 4.7 g; F-Ex group: 335.4 ± 3.7 g; SB623 group: 334.3 ± 4.8 g; SB623 + V-Ex group: 320.7 ± 4.3 g; SB623 + F-Ex group: 314.7 ± 10.8 g, respectively). Body weight increased from day 8 to day 15 after MCAO in all groups. There was no significant difference in weight gain on day 15 after MCAO among all groups ($F(5, 54) = 2.285$, $p = 0.0589$ on day 15) (day 15: control group: 350.9 ± 3.6 g; V-Ex group: 363.6 ± 4.0 g; F-Ex group: 365.1 ± 3.8 g; SB623 group: 358.5 ± 4.5 g; SB623 + V-Ex group: 355.5 ± 4.0 g; SB623 + F-Ex group: 348.4 ± 6.2 g, respectively) (Fig. 4A).

3.2. Treated stroke rats improve in mNSS score

In this protocol, only rats with scores between 7 and 12 on Day 1 were included in the study, and severe cases with mNSS scores ranging from 13 to 18 were excluded prior to the experiments. This decision was made because severe cases tend to have higher mortality rates, making it challenging to evaluate treatment effects and obtain reproducible results. ANOVA revealed that there were no significant treatment effects between the control group and the other groups in mNSS evaluation on day 1 after MCAO ($F(5, 54) = 5.177$, $p = 0.0006$ on day 1). However, ANOVA revealed significant treatment effects of the F-Ex, SB623, SB623 + V-Ex, and SB623 + F-Ex groups, respectively, in mNSS on day 8 after MCAO, compared with that of the control group ($F(5, 54) = 15.85$, $p < 0.001$ on day 8). In addition, ANOVA revealed significant treatment effects of the V-Ex, F-Ex, SB623, SB623 + V-Ex, and SB623 + F-Ex groups, respectively, in mNSS on day 15 after MCAO, compared with that of the control group ($F(5, 54) = 30.36$, $p < 0.001$ on day 15) (Table 1, Fig. 4B).

3.3. Running distance increases over time after stroke

There were no significant treatment effects among all groups on the average running distance from day −6 to day 15 after MCAO ($p = 0.15$) (Table 2). Similarly, there were no significant treatment effects among all groups on the average running distance for each post-MCAO testing period on day 3, day 8, and day 15 after MCAO, respectively (day 3: $p = 0.17$; day 15: $p = 0.11$). The highest average running distance in the V-Ex group was 2838 m on day 12 after MCAO ($p = 0.12$) and 3976 m in the SB623 + V-Ex group on day 12 after MCAO. Running distance increased in both groups from day 3 to day 12 after MCAO (Fig. 4C). We analyzed the correlation between the total running distance in the V-Ex group and SB623 + V-Ex group and other outcomes (body weight and mNSS on day 15, and infarct area); however, no significant correlations were observed.

3.4. Combination therapy of SB623 intracerebral transplantation and forced exercise with a treadmill most effectively reduces cerebral infarct area

Fig. 5A shows representative Nissl staining for the cerebral infarct area of all groups in our study protocol, respectively (control group: 64.59 ± 0.28 %; V-Ex group: 54.61 ± 1.12 %; F-Ex group: 43.67 ± 1.07 %; SB623 group: 54.06 ± 1.01 %; SB623 + V-Ex group: 47.13 ± 0.88 %; SB623 + F-Ex group: 36.10 ± 1.31 %, respectively). ANOVA detected significant treatment effects on cerebral infarct areas in Nissl-stained tissue sections, with significantly reduced infarct areas in the SB623 + F-Ex group compared with the control, V-Ex, F-Ex, SB623, and SB623 + V-Ex groups, respectively. The SB623 + V-Ex group showed significantly reduced infarct areas in Nissl-stained tissue sections compared with the control, V-Ex, and SB623 groups. The F-Ex group showed significantly reduced infarct areas in Nissl-stained tissue sections

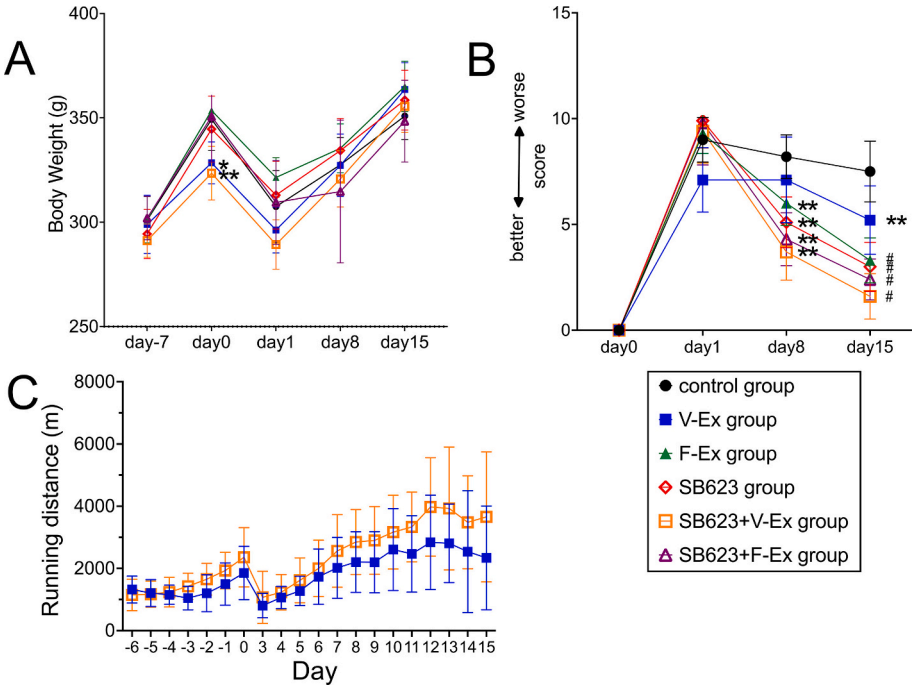

Fig. 4. The results of body weight, mNSS, daily running distance with RW.
(A) The weight gain of the voluntary exercise groups (V-Ex and SB623 + V-Ex groups) was less than that of other groups from day −7 to day 0 after MCAO. Comparing day 0 and day 1 after MCAO, body weight was decreased in all groups on day 1. There was no significant difference in weight loss between the control group and the other groups, but ANOVA revealed significant treatment effects, with post hoc Bonferroni's multiple comparisons test showing a significantly different weight loss between the voluntary exercise groups and the F-Ex group, and between the SB623 group and the SB623 + V-Ex group on day 1 after MCAO ($F(5, 54) = 6.155$, $p < 0.001$ on day 1) (mean ± SE, $^{*}p < 0.05$ vs. control group, $^{**}p < 0.01$ vs. control group). Body weight increased from day 1 to day 8 in all groups. Body weight increased from day 8 to day 15 in all groups.
(B) ANOVA revealed significant treatment effects, of the F-Ex, SB623, SB623 + V-Ex, and SB623 + F-Ex groups, respectively, in mNSS at later time points after MCAO, which improved significantly compared with that of control, and V-Ex groups on day 15 after MCAO ($F(5, 54) = 30.36$, $p < 0.001$ on day 15) (mean ± SE, $^{*}p < 0.05$ vs. control group, $^{**}p < 0.01$ vs. control group, $^{\#}p < 0.01$ vs. control group and $p < 0.05$ vs. V-Ex group).
(C) The running distance increased day by day between the V-Ex group and the SB623 + V-Ex groups. There was no significant difference in the average running distance from day −6 to day 15 after MCAO (mean ± SD, $p = 0.15$). There was no significant difference in the average running distance on day 3, day 8, and day 15 after MCAO, respectively (mean ± SD, day 3: $p = 0.38$; day 8: $p = 0.17$; day 15: $p = 0.11$, respectively). The highest average running distance in the V-Ex group was 2838 m on day 12 after MCAO and 3976 m in the SB623 + Ex group on day 13 after MCAO (mean ± SD, $p = 0.12$). Abbreviations: MCAO, middle cerebral artery occlusion; RW, running wheel.

Table 1
Modified neurological severity scores (mNSS) for each group at different time points (Day 1, Day 8, and Day 15) after MCAO. The scores reflect the degree of neurological impairment, with higher scores indicating more severe deficits. All values are expressed as mean ± SD.

Group	Day 1	Day 8	Day 15
Control	9.00 ± 0.33	8.20 ± 0.33	7.50 ± 0.45
V-Ex	7.10 ± 0.48	7.10 ± 0.64	5.20 ± 0.51
F-Ex	9.30 ± 0.30	6.00 ± 0.33	3.30 ± 0.33
SB623	9.90 ± 0.41	5.10 ± 0.38	3.00 ± 0.37
SB623 + V-Ex	9.40 ± 0.48	3.70 ± 0.42	1.60 ± 0.34
SB623 + F-Ex	9.80 ± 0.63	4.30 ± 0.40	2.40 ± 0.31

compared with the control, V-Ex, and SB623 groups. The V-Ex and SB623 groups showed significantly reduced infarct areas in Nissl-stained tissue sections compared with the control group. However, the infarct area did not differ significantly between the V-Ex group and the SB623 group ($p = 0.54$) (Fig. 5B).

3.5. Combination therapy of SB623 intracerebral transplantation and forced exercise with treadmill most effectively enhances endogenous neurogenesis in SVZ

Representative photographs of immunofluorescence staining for BrdU, Dcx, and DAPI in SVZ are shown in Fig. 6A,B. ANOVA detected

Table 2
Summary of running distances for V-Ex and SB623 + V-Ex groups. The table includes average daily distance, running distances on specific days, maximum distance achieved on Day 12, and total running distance from Day −6 to Day 15 after MCAO. All values are expressed as mean ± SD (m). Abbreviations: Avg, average.

Group	Avg Daily Distance	Day 3	Day 8	Day 15	Max Distance on Day 12	Total Distance
V-Ex	1808 ± 839	804 ± 372	2204 ± 926	2337 ± 1581	2838 ± 1440	26,892.8 ± 11,655.7
SB623 + V-Ex	2335 ± 933	1070 ± 795	2849 ± 991	3656 ± 1983	3976 ± 1501	35,772.8 ± 13,324.2

significant treatment effects on endogenous neurogenesis with significantly increased number of BrdU/Dcx double-positive cells in the SVZ in the SB623 + F-Ex group compared with all other groups, respectively ($F(5, 24) = 37.77$, $p < 0.001$) (control group: 133.40 ± 4.61/192,000 μm^2; V-Ex group: 163.20 ± 2.21/192,000 μm^2; F-Ex group: 200.80 ± 3.68/192,000 μm^2; SB623 group: 170.60 ± 4.32/192,000 μm^2; SB623 + V-Ex group: 201.40 ± 2.46/192,000 μm^2; SB623 + F-Ex group: 233.40 ± 5.84/192,000 μm^2, respectively). The F-Ex group and SB623 + V-Ex group significantly increased the number of BrdU/Dcx double-positive

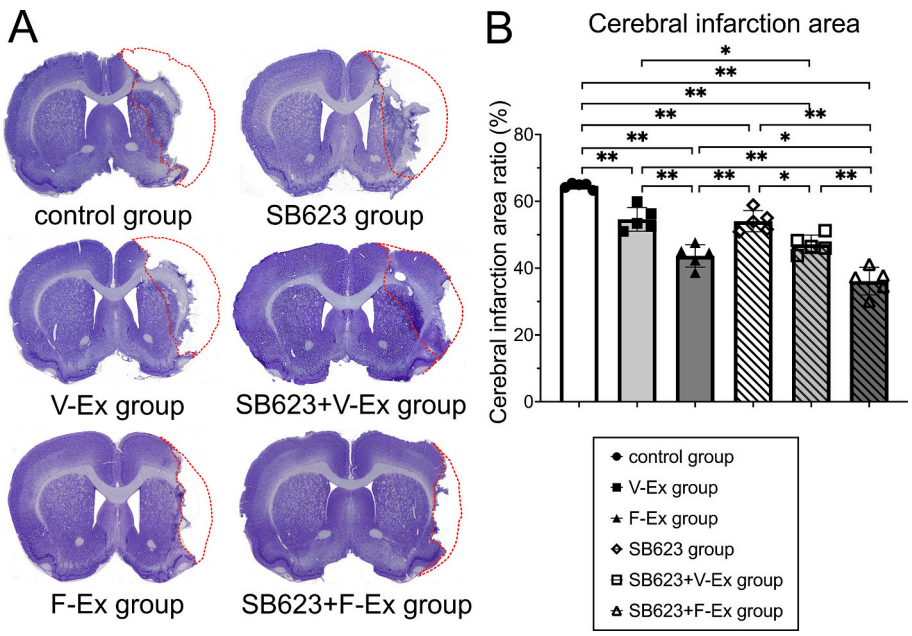

Fig. 5. Nissl staining for cerebral infarct area in each group.
(A) Cerebral infarct area is shown by representative Nissl staining. The tract of the red dotted line reveals the cerebral infarct area. We calculated the cerebral infarct area ratio by the following method: Cerebral infarct area ratio = [LT – (RT – RI)] × 100/LT (%), where LT is the area of the left hemisphere, RT is the area of the right hemisphere, and RI is the infarct area in mm2 at the level of SVZ.
(B) ANOVA revealed significant treatment effects, with the SB623 + F-Ex group showing significantly reduced infarct areas compared with all other groups. The SB623 + V-Ex group also showed significantly reduced infarct areas compared with the control, V-Ex, and SB623 groups. The F-Ex group exhibited significantly reduced infarct areas compared with the control, V-Ex, and SB623 groups. Both V-Ex and SB623 groups had significantly reduced infarct areas compared with the control group. There was no significant difference between the V-Ex and SB623 groups ($p = 0.54$). Abbreviation: SVZ, subventricular zone. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

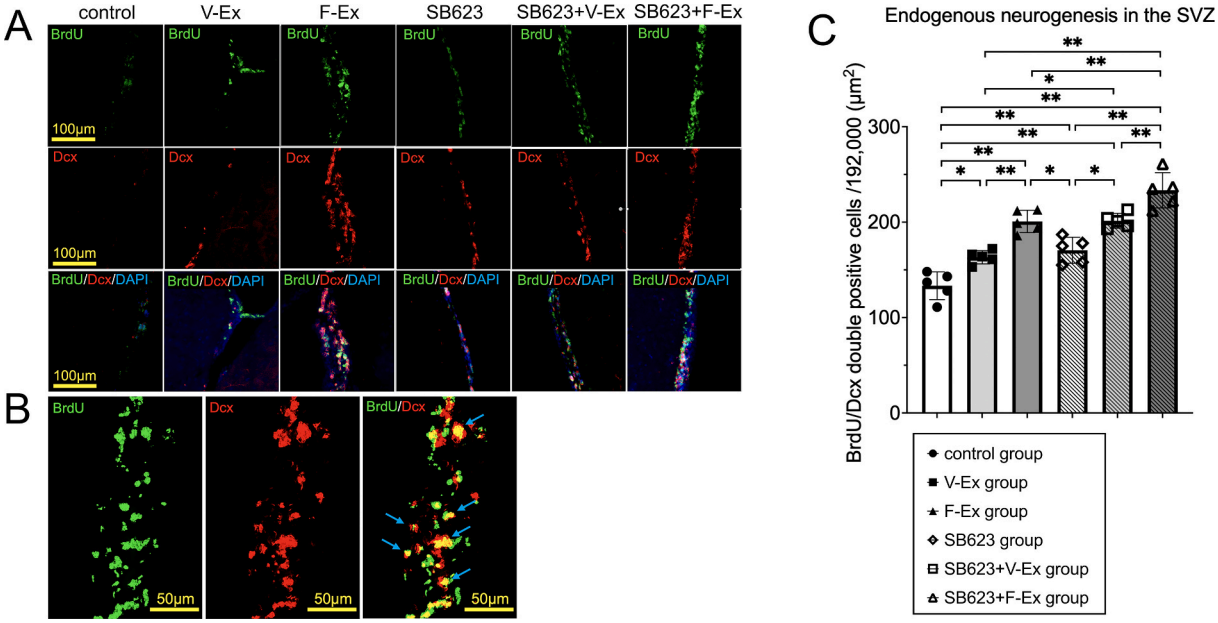

Fig. 6. Immunofluorescence staining for endogenous neurogenesis in SVZ.
(A) Immunofluorescence staining for BrdU, Dcx, and DAPI shows endogenous neurogenesis in SVZ. SVZ: Scale bar = 100 μm. DG.
(B) High-magnification photographs of representative immunofluorescence staining for BrdU/Dcx double-positive cells in the SVZ (blue arrows). Scale bar = 100 μm.
(C) ANOVA showed significant treatment effects on endogenous neurogenesis, with the SB623 + F-Ex group having significantly increased numbers of BrdU/Dcx double-positive cells in the SVZ compared with all other groups ($F(5, 24) = 37.77$, $p < 0.001$). The F-Ex and SB623 + V-Ex groups had significantly higher numbers than the control, V-Ex, and SB623 groups. The V-Ex and SB623 groups had higher numbers than the control group. No significant difference was found between V-Ex and SB623 ($p > 0.99$) or between F-Ex and SB623 + V-Ex ($p > 0.99$). Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; DAPI, 4′6-diamidino-2-phenylindole; Dcx, Doublecortin; SVZ, subventricular zone. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cells in the SVZ compared with the control, V-Ex, and SB623 groups. Additionally, the V-Ex group and SB623 group significantly increased the number of BrdU/Dcx double-positive cells in the SVZ compared with the control group. However, the levels of endogenous neurogenesis in the SVZ did not differ significantly between the V-Ex group and the SB623 group ($p > 0.99$), as well as between the F-Ex group and the SB623 + V-Ex group ($p > 0.99$) (Fig. 6C).

3.6. Combination therapy of SB623 intracerebral transplantation and voluntary exercise with RW most effectively improves the depression-like behavior after ischemic stroke

ANOVA revealed significant treatment effects on the time spent in the center zone during the OFT, with significantly increased time in the V-Ex and SB623 + V-Ex group compared with the control group, respectively ($p = 0.0018$) (Table 3, Fig. 7A). Additionally, the distance moved during the OFT was significantly increased in the V-Ex group compared with the F-Ex group, and in the SB623 + V-Ex group compared with the control, F-Ex and SB623 + F-Ex groups, respectively ($F(5, 54) = 6.83$, $p < 0.001$) (Table 3, Fig. 7B). During the FST, immobility time was significantly decreased in the V-Ex group and SB623 + V-Ex group compared with the control and F-Ex groups, respectively ($F(5, 54) = 10.67$, $p < 0.001$) (Table 3, Fig. 7C).

4. Discussion

In this study, we demonstrated that both voluntary and forced exercise, as well as intracerebral transplantation of human modified bone marrow-derived MSCs (SB623 cells), achieved neurological recovery and reduced cerebral infarct area in a rat model of ischemic stroke. The therapeutic effects of forced exercise were superior to voluntary exercise in terms of motor function improvement, infarct area reduction, and promotion of neurogenesis. However, voluntary exercise was more effective in improving depression-like behaviors. These results indicated that these combination therapies exerted strong therapeutic effects via neuroprotection and enhanced endogenous neurogenesis after ischemic insults, but each type of exercise therapy had its own advantages and disadvantages.

4.1. SB623 transplantation promotes neurogenesis after ischemic stroke

Stem cell transplantation therapy for animal models of cerebral ischemic stroke has been extensively studied. In particular, MSCs are widely utilized due to their relative ease of collection and potential for multifaceted therapeutic effects (Daadi et al., 2013; Horie et al., 2015). Various transplantation routes, including intracerebroventricular, intravenous, and intra-arterial administration, have been examined. Several basic studies have demonstrated that intracerebroventricular administration of MSCs is superior to intravenous administration in animal models of cerebral ischemia (Sasaki et al., 2016; Toyoshima et al., 2015; Yasuhara et al., 2009). Intracerebral transplantation of SB623 cells has also been shown to carry a low risk of adverse effects. A randomized, double-blind clinical trial, STEMTRA, conducted by Kawabori et al., demonstrated no significant difference in the incidence of surgical procedure-related adverse events between the SB623 transplantation group and the sham-control group. The sham-control group underwent only scalp incision and burr hole creation without intracerebral procedures, reinforcing the safety of intracerebral transplantation (Kawabori et al., 2021). The therapeutic mechanisms include the secretion of neurotrophic factors, promotion of angiogenesis, and enhancement of neurogenesis (Bao et al., 2011; Chrostek et al., 2019; Jin et al., 2019). In this study, we confirmed the promotion of neurogenesis by SB623 transplantation using BrdU/Dcx/DAPI multiple staining. Moreover, SB623 transplantation has been reported to elicit no immune responses (Dao et al., 2011), and no adverse effects on native brain cells have been observed. Our previous study has shown that SB623 cells can survive for up to 14 days post-transplantation, providing sustained therapeutic effects despite a gradual decline in cell numbers over time (Kawauchi et al., 2022). Taken together, these findings highlight the safety and therapeutic potential of SB623 cell transplantation for the treatment of cerebral ischemia.

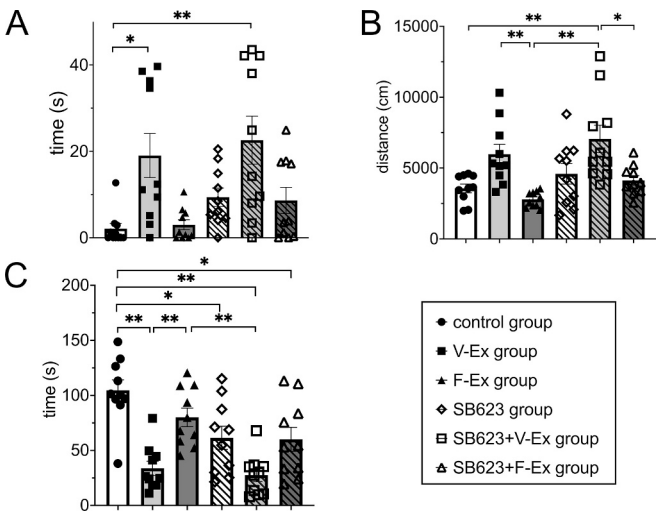

Fig. 7. The results of OFT and FST.
(A) ANOVA revealed significant treatment effects on time spent in the center zone during OFT, with the V-Ex and SB623 + V-Ex groups showing significantly increased time compared with the control group ($p = 0.0018$).
(B) The distance moved during OFT was significantly increased in the V-Ex group compared with the F-Ex group, and in the SB623 + V-Ex group compared with the control, F-Ex and SB623 + F-Ex groups ($F(5, 54) = 6.83$, $p \leq 0.001$).
(C) During FST, immobility time was significantly decreased in the V-Ex and SB623 + V-Ex groups compared with the control and F-Ex groups ($F(5, 54) = 10.67$, $p < 0.001$). Abbreviation: OFT, open field test; FST. forced swim test.

Table 3
Assessment of depression-like behaviors for each group using Open Field Test (OFT) and Forced Swim Test (FST). The table presents the time spent in the center zone, distance moved during OFT, and immobility time during FST. All values are expressed as mean ± SD.

Group	Time in Center Zone (s)	Distance Moved (m)	Immobility Time (s)
Control	2.11 ± 1.24	3566 ± 309	104.61 ± 9.46
V-Ex	19.03 ± 5.12	5978 ± 703	33.79 ± 6.41
F-Ex	3.00 ± 1.16	2788 ± 172	80.05 ± 8.40
SB623	9.37 ± 2.13	4595 ± 720	61.46 ± 10.58
SB623 + V-Ex	22.60 ± 5.56	7058 ± 971	27.49 ± 5.80
SB623 + F-Ex	8.63 ± 3.02	4134 ± 332	60.14 ± 10.84

4.2. Forced exercise enhances the treatment effect of SB623 transplantation more than voluntary exercise

SB623 has been reported to reduce infarct area, promote neurogenesis, and improve motor function in basic studies using a stroke model in rats (Dao et al., 2011; Kawauchi et al., 2022). In this study, SB623 transplantation showed similar results. SB623 cells have demonstrated favorable clinical outcomes in clinical trials involving patients with chronic ischemic stroke (STR-01) and traumatic brain injury (STEMTRA) (Kawabori et al., 2021; Steinberg et al., 2016; Steinberg et al., 2018). In both clinical trials, combining voluntary exercise with SB623 cell transplantation exhibited potent therapeutic effects. Previously, we reported that combining SB623 transplantation with voluntary exercise resulted in reduced infarct area compared with

SB623 transplantation or voluntary exercise alone in rat models of ischemic stroke (Yabuno et al., 2023). This study demonstrated that forced exercise also enhanced the therapeutic effects of SB623 cell transplantation, showing greater infarct reduction and promotion of neurogenesis compared with voluntary exercise. Although the detailed mechanisms by which voluntary and forced exercise enhance the therapeutic effects of SB623 transplantation have not been fully elucidated, increased blood flow and the secretion of neurotrophic factors due to exercise may improve the environment for the transplanted cells and maximize their therapeutic efficacy (Hicks and Jolkkonen, 2009).

4.3. Sole forced exercise showed a stronger treatment effect on ischemic stroke than sole voluntary exercise

Numerous studies have reported on the functional improvement effects of voluntary and forced exercise in animal models of ischemic stroke. While many studies have shown functional improvement for both types of exercise, it has also been reported that forced exercise can cause excessive stress, potentially inhibiting neurogenesis (Svensson et al., 2016). However, there have been few studies that directly compared these two exercise therapies. While these reports have evaluated outcomes such as infarct area, motor function, and cognitive function after stroke, the findings were inconsistent. One study reported no significant difference in the therapeutic effects between forced and voluntary exercise (Alomari et al., 2013; Endres et al., 2003), while others have reported that voluntary exercise was more effective (Ke et al., 2011b) or that forced exercise was superior (Hayes et al., 2008). Furthermore, Schmits et al. conducted a meta-analysis on the effectiveness of exercise therapy in stroke animal models and concluded that forced exercise had superior therapeutic effects (Schmidt et al., 2014). Although the variations in protocols and methods make direct comparisons challenging, there was no consensus about which type of exercise was superior. In this study, forced exercise showed superior effects on motor function, infarct area, and neurogenesis compared with voluntary exercise, although the specific animal model and exercise protocol used may have been particularly suited to forced exercise. Further research into these two exercise modalities in animal experiments to elucidate their relative effectiveness and underlying mechanisms, as well as to determine suitable exercise protocols for each, will clarify which exercise is more suitable for patients, enabling the formulation of individualized rehabilitation plans.

4.4. Voluntary exercise improves depression-like behavior after ischemic stroke

While numerous studies have reported the effects of stem cell transplantation on motor function improvement and neurogenesis, there have been fewer studies that reported improvements in depression-like behavior. Moriyama et al. reported that intravenous administration of neural precursor cells shortened the immobility time in the FST in post-stroke rats (Moriyama et al., 2011). Additionally, Lei et al. reported that MSC transplantation improved the outcomes in the OFT in post-stroke rats (Lei et al., 2022). In our study, the SB623 transplantation group exhibited a shortened immobility time in the FST compared with the control group, suggesting an effect on improving depression-like behavior. Conversely, no significant improvement was observed in the OFT. Regarding exercise therapy, it has been reported that voluntary exercise improved depression-like behavior in the OFT and FST (Luo et al., 2019; Mul, 2018). Early forced exercise has also been reported to improve depression-like behavior after stroke (Mul, 2018; Zhang et al., 2017), but there have been reports that treadmill exercise increased stress and worsens PSD (Svensson et al., 2016). In our study, both the V-Ex group and the SB623 + V-Ex group showed improvements in both the OFT and FST, consistent with previous reports. Forced exercise demonstrated superior therapeutic effects in terms of motor function improvement and infarct area reduction, but no improvement was observed in the OFT and FST compared with the control group. This lack of deterioration may suggest that the beneficial effects of forced exercise and the exacerbating effects of stress are counterbalanced.

4.5. The research design and the timing of starting exercises

In our study design, voluntary exercise was initiated 7 days prior to MCAO. This approach was based on reports indicating that cage changes can affect exercise activity, and we aimed to minimize this impact (Conour et al., 2006; Yoshizawa et al., 2019). In the studies by Mu and Hicks, voluntary exercise via an enriched environment (EE) was resumed on days 2 and 8 post-MCAO, respectively; however, their studies did not include pre-MCAO training (Hicks and Jolkkonen, 2009; Hicks et al., 2007; Hicks et al., 2008; Mu et al., 2019). Similarly, it has been reported that a habituation period is necessary for forced exercise prior to intervention. Although the exact number of days and protocol required for this habituation period are not uniformly agreed upon, previous studies often initiated forced exercise 3 days before MCAO, and we followed this protocol as well (Cheng et al., 2020; Pan et al., 2021). Additionally, in our study, there was a significant discrepancy in the total exercise volume between forced and voluntary exercise groups. The total running distance after MCAO was 6000 m in the F-Ex group and the SB623 + F-Ex group (20 m/min for 30 min per day, 5 days per week), whereas it was an average of 26,732.8 m in the V-Ex group and an average of 36,182.8 m in the SB623 + V-Ex group. We considered increasing the intensity of forced exercise or limiting voluntary exercise to correct this discrepancy. However, increasing the intensity posed a risk of excessive stress on the animals, and limiting voluntary exercise might deviate from the fundamental definition of voluntary exercise. Therefore, we adopted the current protocol. The relationship between exercise volume and therapeutic efficacy in ischemic stroke has been explored in studies involving forced exercise in animal models, for which it has been reported that excessive exercise may diminish therapeutic benefits (Modaberi et al., 2018; Ploughman et al., 2007). Additionally, a clinical study on stroke patients indicated that the volume of rehabilitation does not necessarily correlate with improved outcomes (Dromerick et al., 2009). No study has systematically compared the exercise volume between forced and voluntary exercise to assess its impact on therapeutic efficacy. Our study suggests that early intervention with both voluntary and forced exercise, as well as the differences in exercise volume, could have a decisive impact on behavioral outcomes and other histological improvements. Therefore, the timing of initiation and resumption of exercise remains a topic of discussion.

4.6. Future perspectives

This study demonstrated that the combination of intracerebral transplantation of SB623 cells and both voluntary and forced exercise exhibited strong therapeutic effects in a rat model of ischemic stroke. Forced exercise showed greater efficacy in reducing infarct area, improving motor function, and promoting neurogenesis, while only voluntary exercise improved depression-like behavior. Future research should focus on optimizing exercise protocols, elucidating underlying mechanisms, and assessing long-term safety and efficacy. Comparing various exercise protocols could provide valuable insights for individualized rehabilitation plans in clinical settings, supporting SB623 transplantation therapy as a novel stroke treatment option.

4.7. Limitations

There are several limitations to this study. First, we performed only intracerebral transplantation, and did not conduct intra-arterial or intravenous transplantation. This basic research aimed to approximate clinical studies using SB623 cells (Kawabori et al., 2021; Steinberg et al., 2016). Second, in the evaluation of motor function, no differences were observed among the F-Ex, SB623, SB623 + V-Ex, and SB623 + F-Ex

groups. However, in terms of infarct area and neurogenesis, the SB623 + F-Ex group showed significant improvement compared with the other groups. These differences in results may be related to natural recovery in rats post-MCAO and variability in behavioral outcomes. The mNSS can evaluate various functions, not just motor function, suggesting possible improvements in non-motor functions as well. Third, the total number of rats evaluated was small. A blinded examiner performed histological evaluations on five rats selected randomly from each group. All data were confirmed to follow a normal distribution using the Shapiro–Wilk test for normality. Fourth, this study did not include any female subjects. According to the 2016 NIH guidelines on experimental design, researchers should consider whether and how the estrous cycle of female subjects affects experimental planning (Cornelison and Clayton, 2017). However, estrogen is known to influence ischemic stroke (Céspedes Rubio et al., 2018; Liu and McCullough, 2011). In this study, we excluded the impact of sex differences. Additionally, from the perspective of animal welfare, it may be necessary to include both male and female rats to perform comprehensive behavioral and histological evaluations. Forth, the electrical stimulation applied to the tails of rats during forced exercise may have influenced the results of the behavioral evaluations. This method is commonly used in experiments involving forced exercise with a treadmill and was adopted in this study to ensure compliance with the forced exercise regimen. Although the intensity of the electrical shocks was minimized to reduce their impact, the possibility of an effect on the results of the behavioral evaluations cannot be ruled out. Finally, our study did not fully investigate the complex therapeutic mechanisms of the combination therapy of cell transplantation and exercise. Previously, we have reported the upregulation of brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) mRNA in combined therapy of SB623 transplantation and voluntary exercise. Future research should pursue direct probing and comprehensive mechanistic analysis of the signaling pathways of these neurotrophic factors.

5. Conclusions

This study demonstrates that combining intracerebral transplantation of SB623 cells with either voluntary or forced exercise significantly enhances neurological recovery and reduces cerebral infarct area in a rat model of ischemic stroke. The therapeutic effects on motor function, infarct area, and neurogenesis were most pronounced with the combination of SB623 transplantation and forced exercise, whereas the therapeutic effects on depression-like behavior were superior with the combination of SB623 transplantation and voluntary exercise.

These findings do not allow for a definitive conclusion on whether forced exercise or voluntary exercise is more appropriate to combine with SB623 transplantation. Future research should focus on optimizing exercise protocols and understanding the mechanisms underlying these combined therapies to develop effective, individualized rehabilitation strategies for stroke patients.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.expneurol.2025.115145.

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Ethics approval and consent to participate

This study was conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Okayama University Graduate School of Medicine. The protocol was specifically approved by the Institutional Animal Care and Use Committee of Okayama University Graduate School of Medicine (protocol #OKU-2024331).

Consent for publication

The authors declare that they consent to publication.

CRediT authorship contribution statement

Takayuki Nagase: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Takao Yasuhara:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Kyohei Kin:** Writing – review & editing, Supervision. **Susumu Sasada:** Writing – review & editing. **Satoshi Kawauchi:** Writing – review & editing. **Satoru Yabuno:** Writing – review & editing. **Chiaki Sugahara:** Writing – review & editing, Resources, Methodology. **Yuichi Hirata:** Writing – review & editing, Resources. **Hayato Miyake:** Writing – review & editing, Resources. **Tatsuya Sasaki:** Writing – review & editing. **Koji Kawai:** Writing – review & editing. **Shun Tanimoto:** Writing – review & editing, Methodology. **Tomoya Saijo:** Writing – review & editing. **Shota Tanaka:** Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no competing interests.

Data availability

Raw data and statistical data are published on mendeley, the DOI is DOI: 10.17632/bp6mcm3jhb.1.

All data generated or analyzed during this study are included in this published article.

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