Frontiers in Bioscience Landmark

Front. Biosci. (Landmark Ed) **2022**; 27(12): 337
https://doi.org/10.31083/j.fbl2712337

Original Research

Brain Factor-7® Improves Cognitive Impairment Following Transient Ischemia and Reperfusion Injury in Gerbil Forebrain through Promoting Remyelination and Restoring Cholinergic and Glutamatergic Neurotransmission in the Hippocampus

Tae-Kyeong Lee[1,†], Sung-Su Kim[2,†], Ji-Won Lee[2], Dae Won Kim[3], Jae-Chul Lee[4], Jong-Dai Kim[5], Soo Young Choi[6], Seongkweon Hong[7], Moo-Ho Won[4,*], Yang Hee Kim[7,*]

[1]Department of Food Science and Nutrition, Hallym University, Chuncheon, 24252 Gangwon, Republic of Korea
[2]Precision Medicine R&D Center, Famenity Co., Ltd., Uiwang, 16006 Gyeonggi, Republic of Korea
[3]Department of Biochemistry and Molecular Biology, and Research Institute of Oral Sciences, College of Dentistry, Kangnung-Wonju National University, Gangneung, 25457 Gangwon, Republic of Korea
[4]Department of Neurobiology, School of Medicine, Kangwon National University, Chuncheon, 24341 Gangwon, Republic of Korea
[5]Division of Food Biotechnology, School of Biotechnology, Kangwon National University, 24341 Chuncheon, Republic of Korea
[6]Department of Biomedical Science, Research Institute for Bioscience and Biotechnology, Hallym University, Chuncheon, 24252 Gangwon, Republic of Korea
[7]Department of Surgery, Kangwon National University Hospital, School of Medicine, Kangwon National University, Chuncheon, 24289 Gangwon, Republic of Korea
*Correspondence: mhwon@kangwon.ac.kr (Moo-Ho Won); kyh1524@kangwon.ac.kr (Yang Hee Kim)
[†]These authors contributed equally.

Academic Editors: Mateusz Maciejczyk and Graham Pawelec
Submitted: 6 November 2022 Revised: 14 December 2022 Accepted: 22 December 2022 Published: 28 December 2022

Abstract

Background: Ischemia and reperfusion injury in the brain triggers cognitive impairment which are accompanied by neuronal death, loss of myelin sheath and decline in neurotransmission. In this study, we investigated whether therapeutic administration of Brain Factor-7® (BF-7®; a silk peptide) in ischemic gerbils which were developed by transient (five minutes) ischemia and reperfusion in the forebrain (tFI/R) improved cognitive impairment. **Methods**: Short-term memory and spatial memory functions were assessed by passive avoidance test and Barnes maze test, respectively. To examine neuronal change in the hippocampus, cresyl violet staining, immunohistochemistry for neuronal nuclei and fluoro Jade B histofluorescence were performed. We carried out immunohistochemistry for myelin basic protein (a marker for myelin) and receptor interacting protein (a marker for oligodendrocytes). Furthermore, immunohistochemistry for vesicular acetylcholine transporter (as a cholinergic transporter) and vesicular glutamate transporter 1 (as a glutamatergic synapse) was done. **Results**: Administration of BF-7® significantly improved tFI/R-induced cognitive impairment. tFI/R-induced neuronal death was found in the Cornu Ammonis 1 (CA1) subfield of the hippocampus from five days after tFI/R. Treatment with BF-7® following tFI/R did not restore the death (loss) of CA1 neurons following tFI/R. However, BF-7® treatment to the ischemic gerbils significantly improved remyelination and proliferation of oligodendrocytes in the hippocampus with ischemic injury. Treatment with BF-7® to the ischemic gerbils significantly restored vesicular acetylcholine transporter-immunoreactive and vesicular glutamate transporter 1-immunoreactive structures in the hippocampus with ischemic injury. **Conclusions**: Based on these results, we suggest that BF-7® can be utilized for improving cognitive impairments induced by ischemic injury as an additive for health/functional foods and/or medicines.

Keywords: cornu Ammonis 1; memory function; neurotransmission; oligodendrocytes; pyramidal neuron; silk peptide

1. Introduction

A temporary blockage of blood supply in the brain leads to ischemia and reperfusion injury [1]. It has been well addressed that ischemia and reperfusion injury in the brain triggers diverse neurological and behavioral alterations including vertigo, memory decline and cognitive impairment [2–4].

It has been well acknowledged that transient forebrain ischemia and reperfusion (tFI/R) induces selective neuronal death (loss) in the specific regions of the forebrain, such as hippocampus, neocortex and striatum, which are vulnerable to ischemia and reperfusion [5–7]. In particular, pyramidal neurons located in the Cornu Ammonis 1 (CA1) field of hippocampus, as principal cells, are prone to die following five-minute tFI/R in gerbils [8,9]. Since the hippocampus plays important roles in cognitive and memory functions, tFI/R-induced neuronal loss in the hippocampus brings cognitive impairment accompanied by demyelination, axonal damage and decline of neurotransmission [4,10].

Myelin sheath, an insulator enwrapping around axons in the central nervous system, is formed by oligodendrocytes and facilitates neural transmission by salta-

tory conduction [11,12]. It has been reported that, in vitamin B_{12}-deficient nonhuman primates, surviving adult (mature) oligodendrocytes extend their processes and ensheath demyelinated (damaged) axons [13]. In particular, a number of previous researches have demonstrated that the facilitation of remyelination in the hippocampus with ischemia and reperfusion injury is crucial to recover memory and cognitive functions [2,14,15]. In addition, previously reported data have shown that deficiencies in glutamatergic and cholinergic neurotransmission may be closely connected to cognitive dysfunction due to ischemic insults [16,17].

Brain Factor-7® (BF-7®; a silk peptide) (>85%, peptide; alanine and tryptophan included as major ingredients) has been developed and produced by Famenity Co., Ltd. (Uiwang, Gyeonggi, Republic of Korea) and reported to show various biological activities [18–20]. For example, pretreatment with BF-7® reduces infarct volume in the forebrain following focal cerebral ischemia induced by middle cerebral artery occlusion (MCAO) in rats and protects neuronal loss in the hippocampus of ischemic gerbil induced by tFI/R [20]. Furthermore, it has been demonstrated that treatment with BF-7® protects dopaminergic neurons in the substantia nigra and dopaminergic terminals in the striatum of a mouse model of Parkinson's disease induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [19].

However, to the best of our knowledge, it has been poorly reported whether therapeutic administration of BF-7® for a long time after hippocampal neuronal loss following tFI/R improves IRI-induced cognitive impairment. Therefore, the aim of this study was to examine whether BF-7® therapy can improve tFI/R-induced cognitive impairment in gerbils. Moreover, we investigated the effects of BF-7® therapy on remyelination and restoration of neurotransmission in the hippocampus with tFI/R injury.

2. Materials and Methods

2.1 Animals

Male gerbils (total $n = 84$; body weight, 80 ± 5 g) were secured from the Experimental Animal Center, an affiliated organization, of Kangwon National University (Chuncheon, Kangwon, Korea). Five gerbils were accommodated for the maximum per cage and they were housed in a convention room under optimum conditions: 24 ± 1 °C of room temperature, 50 ± 5% of relative humidity and twelve hours of dark/light cycle. Pellet feed (DBL Co. Ltd., Eumseong, Korea) and water were freely provided to the gerbils.

All experimental processes using the animals adhered to the guidelines included in the "Current International Laws and Policies", a part of the "Guide for the Care and Use of Laboratory Animals" [21]. Approval for the experimental protocol was sanctioned (approval no., KW-200113-1; approval date, 18th Feb. 2020) by Institutional Animal Care and Use Committee (IACUC), an affiliated organization, of Kangwon National University.

2.2 Experimental Groups

Gerbils were randomly grouped as follow: (1) sham+vehicle group ($n = 21$) which was given sham operation and administered vehicle (saline; 0.85% NaCl), (2) tFI/R+vehicle group ($n = 21$) which was subjected to tFI/R operation and treated with vehicle, (3) sham+BF-7® group ($n = 21$) which was given sham operation and administered BF-7®, and (4) tFI/R+BF-7® group ($n = 21$) which was subjected to tFI/R operation and treated with BF-7®.

2.3 tFI/R Operation and BF-7® Administration

In order to develop ischemic insult-induced cognitive impairment, the gerbils were given tFI/R operation in accordance with our published method [2,4]. In short, anesthesia was induced with 2.5% isoflurane (Hana Pharmaceutical Co. Ltd., Seoul, Korea) in mixture gas of 67% nitrous oxide (N_2O) and 33% oxygen (O_2). Under the anesthesia, the ventral neck of the gerbil was shaved and a midline incision (about 15 mm) was made. Both (right and left) common carotid arteries (CCA) were isolated and each CCA was ligated (0.69 N) with aneurysm clip (0.69 N; Yasargil FE 723K; Aesculap Inc., Tuttlingen, Germany) for five minutes to induce transient forebrain ischemia. Using ophthalmoscope (HEINE K180, Heine Optotechnik; Gilching, Germany), complete occlusion of CCA was confirmed via observing stop of blood circulation in the central retinal artery. The clips were removed after then, and the incision was sutured using 3-0 silk (Ethicon Inc, Somerville, NJ, USA). The gerbils belonging to the sham group were given identical surgical procedure without the ligation of CCA. During the ischemic surgery, body temperature was monitored via the rectum in real-time and maintained normothermic condition (37 ± 0.2 °C) using homeothermic system (Harvard Apparatus™, Holliston, MA, USA) which consists of three parts (heating pad, flexible temperature probe and control unit).

As shown in Fig. 1, using a curved feeding needle (16 gauge, 100 mm of length; Fine Science Tools, Inc. Foster City, CA, USA), vehicle or BF-7® (10 mg/kg) was orally administrated from five days after sham or tFI/R operation once a day for 25 days.

2.4 Passive Avoidance Test (PAT)

To evaluate short-term memory following tFI/R operation, PAT was performed at zero, five, 15 and 30 days after tFI/R (Fig. 1). As described previously [4], we used GEM 392 apparatus for PAT (San Diego Instruments Inc., San Diego, CA, USA). The apparatus consists of two compartments (dark and light) which communicate through a sliding gate. The evaluation was processed by two trials (training and substantial trial). The training trial was carried out one day before each substantial trial. For training, individual gerbil was placed in the dark compartment

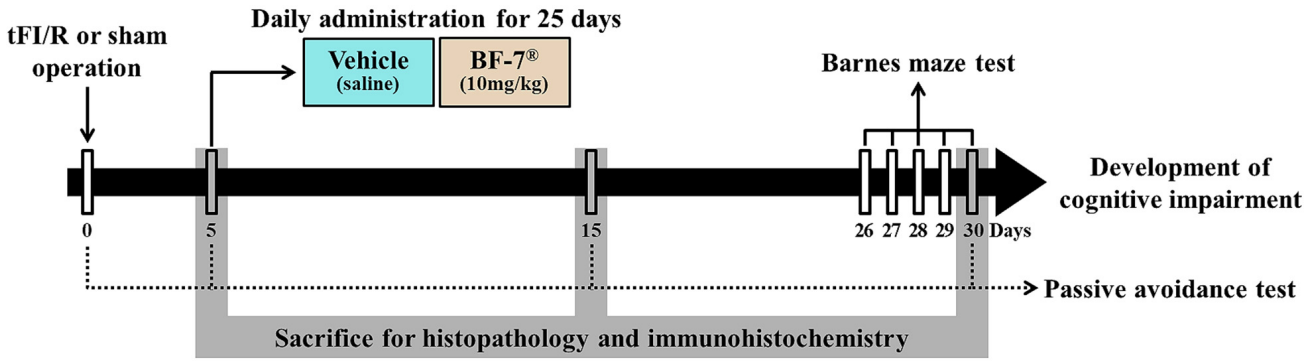

Fig. 1. Experimental schedule tFI/R-induced cognitive impairment is developed for 25 days from five days after tFI/R operation. Each vehicle and BF-7® (10 mg/kg) is orally administrated once a day from five to thirty days after tFI/R operation. Passive avoidance test is carried out at zero, five, 15 and 30 days after tFI/R. Barnes maze test is daily performed from 26 to 30 days after tFI/R. The gerbils are sacrificed at five, 15 and 30 days after tFI/R in order to histologically analyze.

and allowed to freely explore both compartments for one minute. When the gerbil entered the dark compartment, the gate was closed and the gerbil received electric foot-shock (0.5 mA) for five seconds from the steel grid floor. For substantial trial, the gerbil was placed in the light compartment, and then the latency time was recorded until the gerbil went into the dark compartment within three minutes.

2.5 Barnes Maze Test (BMT)

To examine spatial memory following tFI/R operation, BMT was conducted at 26, 27, 28 and 29 days after tFI/R operation (Fig. 1). Briefly, in accordance with previous studies [2,4], visual signs were placed around the maze at a height which is perceivable for the gerbil. To maintain steady brightness (220 lx) and background noise (85 dB), illumination and stereo speaker were respectively installed onto the ceiling of the maze. For training, individual gerbil was trained three times per day with 15 minutes of intervals for consecutive four days (on day 26–29 after tFI/R). The gerbil freely explored the maze until the gerbil found an escape which is linked to the refuge. Once the gerbil entered the refuge, the gerbil stayed there for 30 seconds. When the gerbil failed to find the refuge within three minutes, we carefully guided the gerbil toward the refuge. The substantial test was performed at one day after the final training (on day 30 after tFI/R). The refuge was removed, and, when the gerbil went to the entry area where the refuge had been previously located, the latency time was recorded within 90 seconds.

2.6 Tissue Preparation for Histology

The brain tissue sections were prepared according to our previously described method [22]. Shortly, the gerbils were deeply anesthetized with pentobarbital sodium (intraperitoneal injection, 150 mg/kg; JW pharm. Co., Ltd., Seoul, Korea). Under the anesthesia, the gerbils were perfused (flow rate, six mL/min; total perfused volume, 70 mL) with saline through the left ventricle of the heart. Subsequently, the gerbils were fixed with 4% paraformaldehyde (pH 7.4) with the same flow rate and perfused volume. Next, their brains were harvested and further fixed with the same fixative for six hours at room temperature and infiltrated with 30% sucrose (pH 7.4) to protect the brains from cryodamage for 24 hours at room temperature. Lastly, the brains were serially and coronally sectioned into 30 μm of thickness using sliding microtome (SM2010 R; Leica Biosystems, Wetzlar, Germany) attached with freezing stage (BFS-40MP; Physitemp Instruments Inc., Clifton, NJ, USA).

2.7 Histochemistry with Cresyl Violet (CV)

CV staining was carried out to investigate cellular distribution in the hippocampus according to our previous study [23]. In brief, the brain sections were mounted onto the microscopy slides coated with gelatin. After confirming the adherence of the sections to the slides, the sections were immersed in 0.1% CV acetate (Sigma-Aldrich Co, St. Louis, MO, USA) for 30 minutes at room temperature. And they were briefly washed in distilled water and decolorized in 50% ethyl alcohol for a few seconds and dehydrated by consecutively incubating in the serial ethyl alcohol (70%, 80%, 90%, 95% and 100%) for seven minutes, respectively, at room temperature. Lastly, the stained sections were cleared in xylene (Junsei Chemical Co., Ltd., Tokyo, Japan) and coverslipped with Canada balsam (Kanto Chemical Co Inc, Tokyo, Japan).

Hippocampal cells stained with CV were observed and captured using light microscope (BX53; Olympus, Tokyo, Japan) which is equipped with digital camera (DP72; Olympus, Tokyo, Japan).

2.8 Histofluorescence with Fluoro-Jade B (FJB)

In order to examine neuronal loss (death) in the hippocampus, histofluorescence with FJB was conducted. In short, as described in previous studies [24,25], the brain sections were mounted onto the gelatin-coated microscopy

slides. The sections were incubated in 0.06% potassium permanganate ($KMnO_4$; Sigma-Aldrich Co., St. Louis, MO, USA) for 20 minutes at room temperature, briefly rinsed with distilled water and stained with 0.0004% FJB (Histochem, Jefferson, AR, USA) on an orbital shaker (85 rpm; SW-250S; Gaon Science Co., Bucheon, Korea) for 40 minutes at room temperature. Thereafter, the sections were washed with distilled water three times (for two minutes, respectively) and dried in dry oven (45 °C; WiseVen® WOC High Clean Air Oven; Daihan Scientific Co., Ltd., Wonju, Korea) for eight hours. Finally, the sections were cleared by immersing in xylene (Junsei Chemical Co., Ltd., Tokyo, Japan) and coverslipped with dibutylphthalate polystyrene xylene (DPX; Fluka, Milwaukee, WI, USA).

FJB-stained cells were observed using epifluorescent microscope (BX53; Olympus, Tokyo, Japan) with a blue excitation light (450–490 nm of wavelength), and their digital images were taken using image capture software (cellSens Standard; Olympus, Tokyo, Japan). The FJB-stained cells were counted in 250 μm^2 at the same area containing FJB-stained cells using Image J software (version 1.46; National Institutes of Health, Bethesda, Rockville, MD, USA). The mean number of FJB-stained cells was calculated.

2.9 Immunohistochemistry

In this study, immunohistochemical staining was performed using avidin-biotin complex (ABC) method. In accordance with precedent studies [4,26], the brain sections were rinsed with 100 mM phosphate-buffered saline (PBS, pH 7.4), reacted in 0.3% hydrogen peroxide (in 100 mM PBS, pH 7.4) in order to block endogenous peroxidase activity for 35 minutes at room temperature and incubated in 5% normal horse, goat or rabbit serum (in 100 mM PBS, pH 7.4) in order to block non-specific immunoreaction for 40 minutes at room temperature. Thereafter, the sections were immunoreacted with each primary antibody: mouse anti-neuronal nuclei (NeuN; dilution, 1:1000; Chemicon, Temecula, CA, USA), rabbit anti-myelin basic protein (MBP; dilution, 1:200; Abcam, Cambridge, UK), Mouse anti-receptor interacting protein (Rip; dilution, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-vesicular acetylcholine transporter (VAChT; dilution, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-vesicular glutamate transporter 1 (VGLUT-1; dilution, 1:500; Synaptic Systems GmbH, Göttingen, Germany) for 48 hours at 4 °C, washed with 100 mM PBS (pH 7.4) and reacted with each biotinylated secondary antibody: horse anti-mouse IgG (dilution, 1:250; Vector Laboratories Inc., Burlingame, CA, USA), goat anti-rabbit IgG (dilution, 1:250; Vector Laboratories Inc., Burlingame, CA, USA) and rabbit anti-goat IgG (dilution, 1:250; Vector Laboratories Inc., Burlingame, CA, USA) for two hours at room temperature. After each immunoreaction, the sections were incubated in ABC (diluted, 1:250; Vector Laboratories, Burlingame, CA, USA) for one and a half hours at room temperature and washed with 100 mM PBS (pH 7.4). To make the sections visualized, 0.06% 3, 3′-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich Co, St Louis, MO, USA) (in 100 mM PBS, containing 0.1% H_2O_2, pH 7.4) was reacted under microscopic observation. As soon as the immunoreaction was confirmed, the sections were briefly washed with 100 mM PBS (pH 7.4), dehydrated in 70%, 80%, 90%, 95% and 100% ethyl alcohol, and cleared in xylene (Junsei Chemical Co., Ltd., Tokyo, Japan). Lastly, the sections were mounted with cover glasses and Canada balsam (Kanto Chemical Co., Inc., Tokyo, Japan).

NeuN-immunoreactive neurons and Rip-immunoreactive oligodendrocytes were observed using light microscope (BX53) and analyzed like the method described in the "*2.8. Histofluorescence with FJB*" section.

MBP (a protein believed to be important in the process of myelination of nerves)-immunoreactive structures and neurotransmitter VAChT and VGLUT-1-immunoreactive structures were analyzed according to previously published methods [4,27]. Briefly, digital images of those structures were captured using microscope (BX53). The captured images were converted to gray scale (8 bits; 0 to 255 of range from black to white) to evaluate grey scale intensities. Optical density of the immunoreactive structures was calculated in average using Image J software (version 1.46; National Institutes of Health, Bethesda, Rockville, MD, USA). The optical density of each immunoreactive structure was presented as relative optical density (ROD), as percentage considering the ROD of sham+vehicle group as 100%.

2.10 Statistical Analysis

In this experiment, we used SPSS software (version 15.0; SPSS Inc., Chicago, IL, USA) to carry out statistical analysis. For evaluation of normal distributions and identical standard error of the mean (SEM), we respectively performed Kolmogorov and Smirnov test and Bartlett test. The statistical significances of the mean among the groups were established by two-way analysis of variance (ANOVA) followed by *post hoc* Tukey's test for all pairwise multiple comparisons. All presented data were exhibited as the mean $\pm$ SEM. When p-value was less than 0.05, statistical significances were determined.

3. Results

3.1 Changes in Cognitive Functions

3.1.1 Short-Term Memory

In all of the groups, there was no significant difference in latency time at zero day after tFI/R operation (Fig. 2A). In both sham groups, latency time at each point in time following sham operation was similar to that shown at zero day (Fig. 2A). In the tFI+vehicle group, latency time was significantly shortened when compared with that in the sham+vehicle group, but the latency time was grad-

ually lengthened with time after tFI/R (Fig. 2A). In the tFI+BF-7® group, latency time at five days after tFI/R did not significantly differ from that in the tFI+vehicle group (Fig. 2A). However, latency time at 15 and 30 days after tFI/R was significantly lengthened when compared with that in the tFI+vehicle group (Fig. 2A).

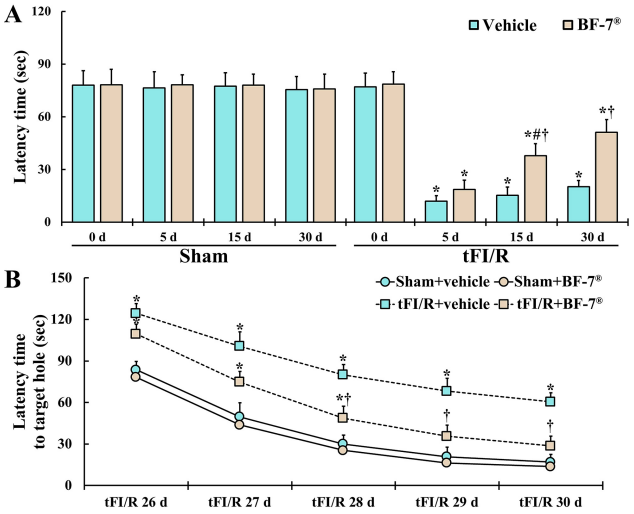

Fig. 2. Behavioral changes due to tFI/R. (A) Short-term memory function assessed by passive avoidance test. In the tFI/R+BF-7® group, latency time at 15 and 30 days after tFI/R increases significantly when compared with that in the tFI/R+vehicle group. (B) Spatial memory function measured by Barnes mase test. In the tFI/R+BF-7® group, latency time at 28, 29 and 30 days after tFI/R is significantly shortened compared to that in the tFI/R+vehicle group. The bars indicate mean ± SEM ($n = 7$, respectively; $^{*}p <$ 0.05 versus sham+vehicle group, $^{\#}p < 0.05$ versus prior time point of each group, $^{\dagger}p < 0.05$ versus corresponding time tFI/R+vehicle group).

3.1.2 Spatial Memory

As shown in Fig. 2B, latency time in all groups to find the target hole measured from 26 days to 30 days after tFI/R was gradually shortened (Fig. 2B). Latency time was not significantly different between the two sham groups (Fig. 2B). In the tFI+vehicle group, latency time was significantly longer than that in the sham groups (Fig. 2B). In the tFI+BF-7® group, latency time was also longer than that in the sham groups, but the latency time measured at 28, 29 and 30 days after tFI/R was significantly shortened when compared with that in the tFI+vehicle group R (Fig. 2B).

3.2 Changes in Hippocampal Cells

3.2.1 CV-Stained Cells (CV-Cells)

In all sham groups, CV-cells were obviously identified in the hippocampus (Fig. 3A,E). Particularly, CV-cells formed the stratum pyramidale (SP) which consists of pyramidal cells (neurons), as principal cells (Fig. 3A,E). In the tFI/R+vehicle and tFI/R+BF-7® groups, CV dyeability was reduced in the SP of the CA1 field, not the CA2/3 field at five days after tFI/R (Fig. 3B,F). This finding implies that tFI/R triggers neuronal damage or death in the CA1 field. In the two groups, the distribution pattern of CV-cells was not changed untill 30 days after tFI/R (Fig. 3C,D,G,H).

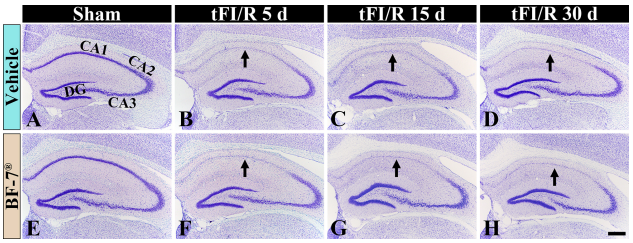

Fig. 3. tFI/R-induced change of cellular distribution in the hippocampus. CV staining in gerbil hippocampus of the sham+vehicle (A), sham+BF-7® (E), tFI/R+vehicle (B–D) and tFI/R+BF-7® (F–H) groups at five, 15 and 30 days after tFI/R. In both tFI/R+vehicle and tFI/R+BF-7®tFI/R groups, CV dyeability is apparently decreased in the stratum pyramidale (SP, arrows) of the CA1 field: the distribution pattern of CV-cells is not changed untill 30 days after tFI/R. DG, dentate gyrus. Scale bar = 400 μm. DG, dentate gyrus.

3.2.2 NeuN-Immunoreactive Neurons (NeuN-Neurons)

In both sham groups, pyramidal neurons located in the CA1 field showed strong immunoreactivity to NeuN (about 83 cells/250 μm^2) (Fig. 4Aa,Ae,B). In the tFI/R+vehicle and tFI/R+BF-7® groups, NeuN-neurons were scarcely detected (about 8 cells/250 μm^2) at five days after tFI/R (Fig. 4Ab,Af,B). In the two groups, the numbers of NeuN-neurons were not significantly changed until 30 days after tFI/R (Fig. 4Ac,Ad,Ag,Ah,B).

3.2.3 FJB-Positive Cells (FJB-Cells)

In all sham groups, FJB-cells were not observed in the CA1 field (Fig. 4Ca,Ce). In the tFI+vehicle and tFI/R+BF-7® groups, numerous FJB-cells were detected (about 75 cells/250 μm^2) in the SP at five days after tFI/R (Fig. 4Cb,Cf,D). In the two groups, the numbers of FJB-cells found at 15 and 30 days after tFI were not significantly different from those shown at 5 days after tFI/R (Fig. 4Cc,Cd,Cg,Ch,D).

Based on the results of NeuN immunohistochemistry and FJB histofluorescence, the administration of BF-7® did not affect tFI/R-induced neuronal death in the hippocampal CA1 field.

3.3 MBP-Immunoreactive Structures (MBP-Structures)

In both sham groups, MBP-structures, which covers axons, were distributed throughout all layers in the CA1

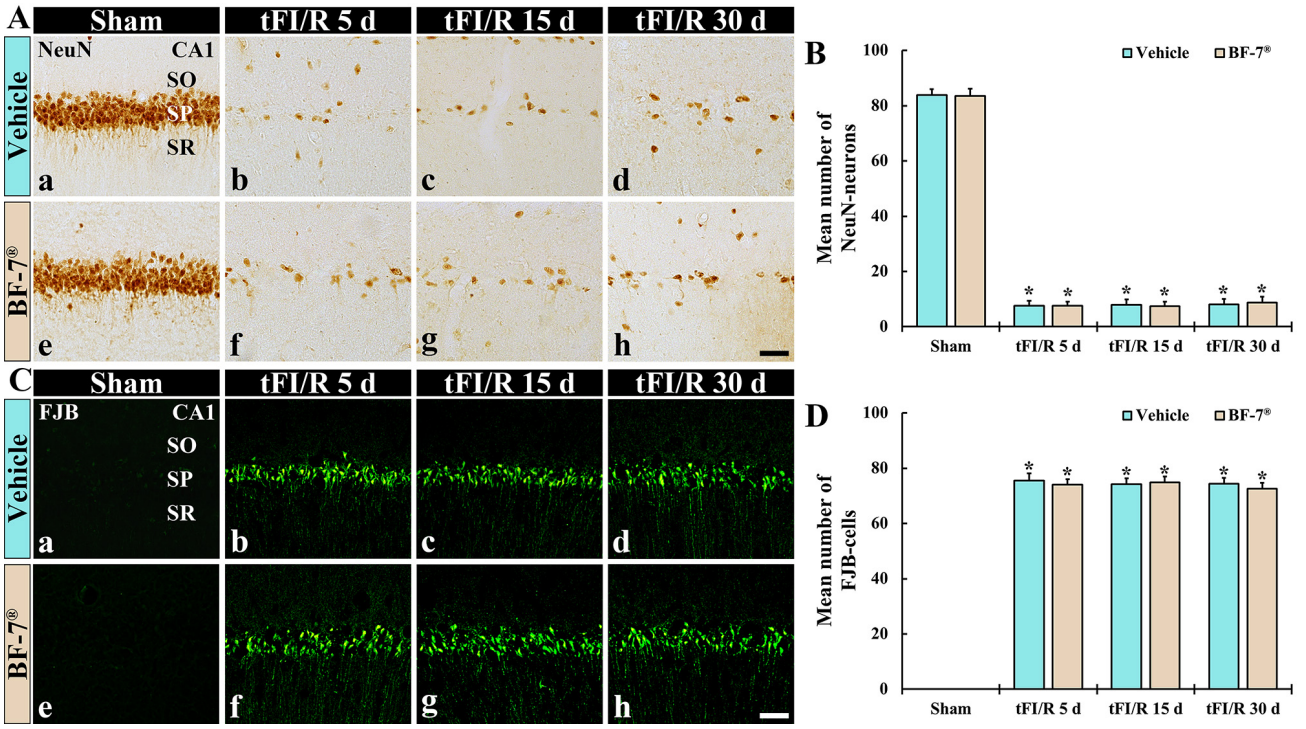

Fig. 4. tFI/R-induced neuronal loss in the hippocampal CA1 field. (A,C) NeuN immunohistochemistry (A) and FJB histofluorescence (C) in the CA1 field of the sham+vehicle (Aa,Ca), sham+BF-7® (Ae,Ce), tFI/R+vehicle (Ab–Ad,Cb–Cd) and tFI/R+ BF-7® (Af–Ah, Cf–Ch) groups at five, 15) and 30 days after tFI/R. In all tFI/R groups, NeuN-neurons are hardly observed and numerous FJB-cells are detected in the stratum pyramidale (SP). SO, stratum oriens; SR, stratum radiatum. Scale bar = 100 μm. (B,D) Mean numbers of NeuN-neurons (B) and mean numbers of FJB-cells (D). The bars indicate mean ± SEM (n = 7, respectively; $^{*}p < 0.05$ versus sham+vehicle group).

field (Fig. 5Aa,Ad). In the tFI+vehicle group, the density of MBP-structures was significantly decreased when compared with that in the sham+vehicle group (ROD: about 22% at 15 days and about 30% at 30 days after tFI/R versus sham+vehicle group) (Fig. 5Ab,Ac,B). However, in the tFI/R+BF-7® group, the density of MBP-structures was significantly increased when compared with that assessed in the corresponding time tFI/R+vehicle group (ROD: about 52% at 15 days and about 66% at 30 days after tFI/R versus sham+vehicle group) (Fig. 5Ae,Af,B).

3.4 Rip-Immunoreactive Oligodendrocytes (Rip-Oligodendrocytes)

In all sham groups, Rip-oligodendrocytes, which are responsible for myelination of nerves, were obviously observed in the CA1 field: they were scattered throughout all hippocampal layers (Fig. 5Ca,Cd). In the tFI/R+vehicle group, the numbers of Rip^{+} oligodendrocytes were significantly increased when compared with those of the sham+vehicle group (about 18 cells/250 μm^2 at 15 days and about 29 cells/250 μm^2 at 30 days after tFI/R) (Fig. 5Cb,Cc,D). On the other hand, in the tFI/R+BF-7® group, the numbers of Rip^{+} oligodendrocytes were significantly increased when compared with those of the corresponding time tFI/R+vehicle group (about 26 cells/250 μm^2 at 15 days and about 37 cells/250 μm^2 at 30 days after tFI) (Fig. 5Ce,Cf,D).

3.5 VAChT-Immunoreactive Structures (VAChT-Structures)

In both sham groups, VAChT-structures (responsible for loading acetylcholine into secretory organelles in neurons) were fundamentally observed in all layers of the CA1 field (Fig. 6Aa,Ad). In the tFI/R+vehicle group, VAChT-structures were significantly reduced when compared with those of the sham+vehicle group (ROD: about 26% at 15 days and about 31% at 30 days after tFI/R versus sham+vehicle group) (Fig. 6Ab,Ac,B). On the other hand, in the tFI/R+BF-7® group, VAChT-structures were significantly increased when compared with those measured in the corresponding time tFI/R+vehicle group (ROD: about 68% at 15 days and about 73% at 30 days after tFI/R versus sham+vehicle group) (Fig. 6Ae,Af,B).

3.6. VGLUT-1-Immunoreactive Structures (VGLUT-1-Structures)

In all sham groups, VGLUT-1-structures (associated with the membranes of synaptic vesicles and functions in glutamate transport) were easily identified in the CA1 field; the structures were not located in the SP (Fig. 6Ca,Cd): In the tFI/R+vehicle group, VGLUT-1-strutures were sig-

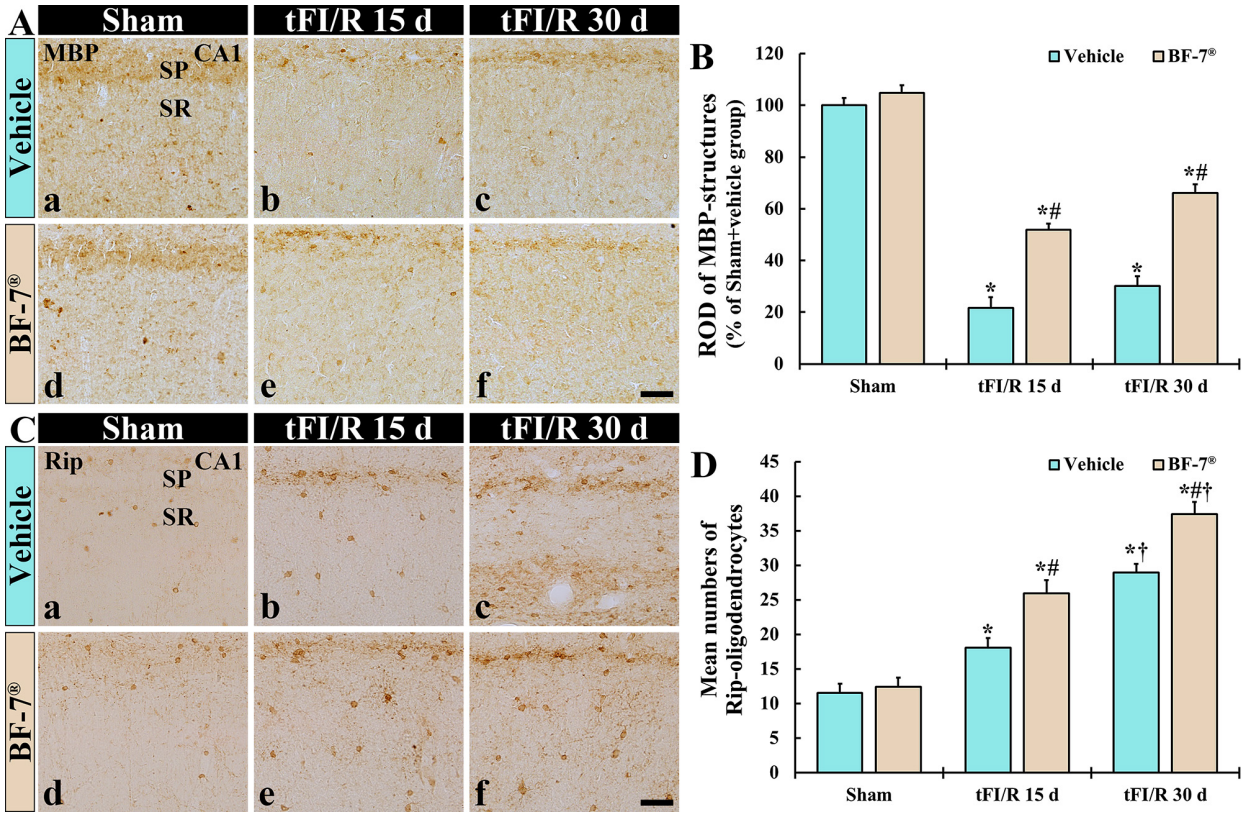

Fig. 5. Changes in myelination and oligodendrocytes in the hippocampal CA1 field following BF-7® treatment. (A,B) Immunohistochemistry for MBP (A) and Rip (C) in the CA1 field of the sham+vehicle (Aa,Ca), sham+BF-7® (Ad,Cd), tFI/R+vehicle (Ab,Ac,Cb,Cc) and tFI/R+BF-7® (Ae,Af,Ce,Cf) groups at 15 and 30 days after tFI/R. In the tFI/R+vehicle group, the density of MBP-structures and the numbers of Rip-oligodendrocytes are significantly low and high, respectively, when compared with the sham+vehicle group. In the tFI/R+BF-7® group, the density of MBP-structures and the numbers of Rip-oligodendrocytes are significantly high, respectively, when compared with the tFI/R+vehicle group. SP, stratum pyramidale; SR, stratum radiatum. Scale bar = 100 μm. (B,D) ROD of MBP-structures (B), and mean numbers of Rip-oligodendrocytes (D) The bars indicate mean ± SEM (n = 7, respectively; $^{*}p < 0.05$ versus sham+vehicle group, $^{\#}p < 0.05$ versus group before each group, $^{\dagger}p < 0.05$ versus corresponding time tFI/R+vehicle group).

nificantly reduced when compared with those of the sham+vehicle group (ROD: about 36% at 15 days and about 45% at 30 days after tFI/R versus sham+vehicle group) (Fig. 6Cb,Cc,D). However, in the tFI/R+BF-7® group, VGLUT-1-structures were significantly higher than those evaluated in the corresponding time tFI/R+vehicle group (ROD: about 72% at 15 days and about 80% at 30 days after tFI/R versus sham+vehicle group) (Fig. 6Ce,Cf,D).

4. Discussion

Researchers have established a gerbil model of tFI/R in order to investigate the mechanisms of tFI/R-induced neuronal death and search its neuroprotective and/or therapeutic materials because the model has simple surgical procedure and high reproducibility [22,23,28,29]. In the model, selective neuronal loss is triggered in the CA1 field of the hippocampus at four to five days after tFI/R, and the death is termed as “delayed neuronal death” [9,30]. It has been well acknowledged that the hippocampus plays pivotal roles in memory and learning, thus, the loss of pyramidal neurons (as principal cells in the hippocampus) due to tFI/R injury leads to changes in behavioral outcomes [7,23,31–33].

Previous studies on materials possessing beneficial properties against ischemia and reperfusion injury-induced cognitive dysfunction have shown that behavioral improvement including learning and spatial memory functions in rodent models of ischemic stroke is accomplished [2,4,34,35]. For example, Yan *et al.* [35] have reported that treatment with dimethyl fumarate (an FDA-approved therapeutic for multiple sclerosis) to rats with chronic cerebral hypoperfusion injury can improve cognitive impairment possibly via alleviating oxidative stress damage and neuroinflammation, and inhibiting ferroptosis of neurons in the hippocampus. Our present findings in the PAT and BMT showed that therapeutic treatment with BF-7® containing alanine and tryptophan (24% and 8%, respectively) as major ingredients apparently improved the impairment of learning and spatial memory functions following tFI/R. When we observed the hippocampus using CV staining, NeuN immunohisto-

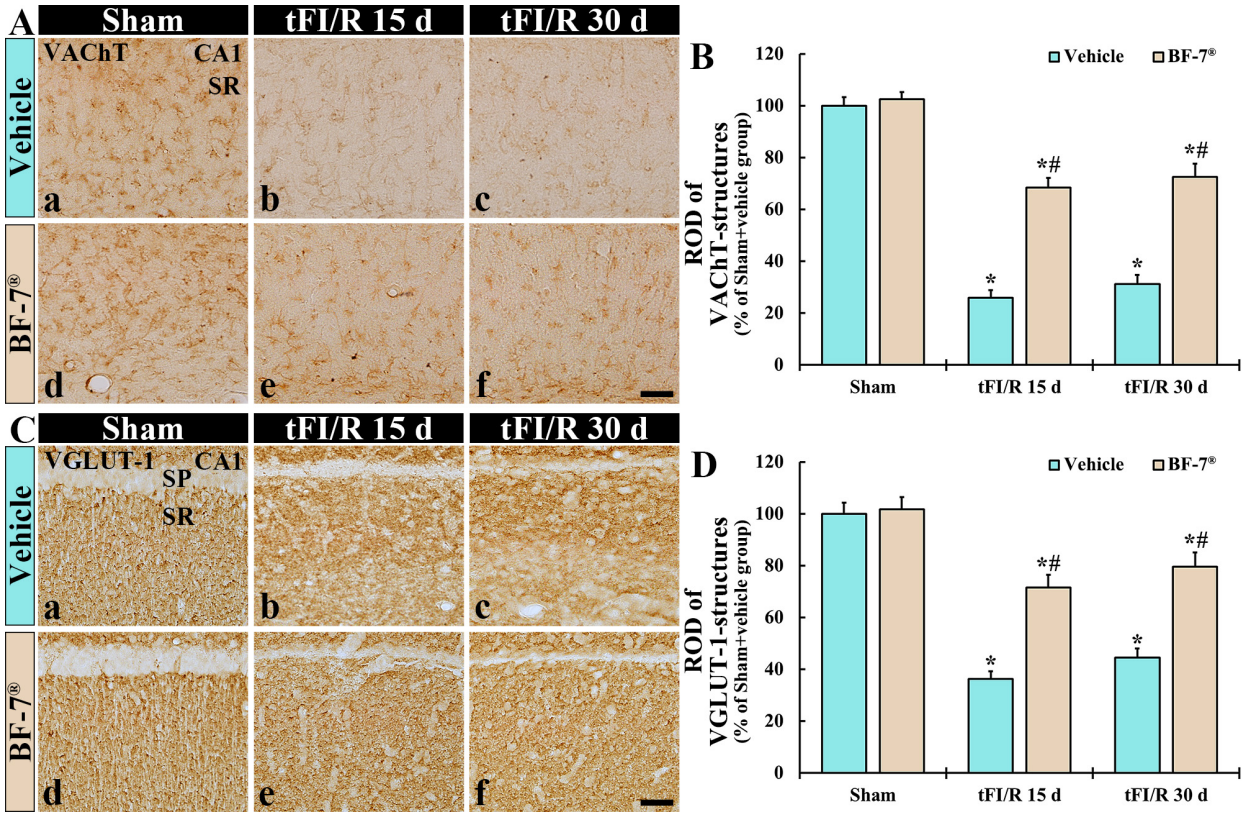

Fig. 6. Changes in cholinergic and glutamatergic neurotransmission in the hippocampal CA1 field following BF-7® treatment. (A,C) Immunohistochemistry for VAChT (A) and VGLUT-1 (C) in the CA1 field of the sham+vehicle (Aa,Ca), sham+BF-7® (Ad,Cd), tFI/R+vehicle (Ab,Ac,Cb,Cc) and tFI/R+ BF-7® (Ae,Af,Ce,Cf) groups at 15 and 30 days after tFI/R. In the tFI/R+vehicle group, VAChT and VGLUT-1-structures are reduced after tFI/R. In contrast, in the tFI/R+ BF-7® group, VAChT and VGLUT-1-strutures are significantly increased at 15 and 30 days after tFI/R compared to the tFI/R+vehicle group. SP, stratum pyramidale; SR, stratum radiatum. Scale bar = 100 μm. (B,D) ROD of VAChT- (B) and VGLUT-1- (D) structures. The bars indicate mean ± SEM (n = 7, respectively; $^{*}p < 0.05$ versus sham+vehicle group, $^{\#}p < 0.05$ versus group before each group, $^{\dagger}p < 0.05$ versus corresponding time tFI/R+vehicle group).

chemistry and FJB histofluorescence, pyramidal neurons (as principal cells) located in the hippocampus of the tFI/R group died in the CA1 field alone; pyramidal neurons of the CA2/3 field survived. In the tFI/R+ BF-7® group, the administration of BF-7® failed to reserve CA1 pyramidal neurons. In contrast, it has been demonstrated that pretreatment with BF-7® before tFI/R in gerbils confers neuroprotective effect (survival of CA1 pyramidal neurons) against tFI/R [20]. It is considered that the difference in neuroprotective consequence against tFI/R is attributed to the time of BF-7® administration (pretreatment or posttreatment). Furthermore, it is very important to select the time (immediately or late) of therapeutic drug administration after ischemia and reperfusion. In our current study, we administrated BF-7® for 25 days from 5 days after tFI/R when the CA1 pyramidal neuronal death could not be had already occurred.

In this study, MBP-structures (as myelin sheath) were significantly reduced in the tFI/R+vehicle group, but, in the tFI/R+ BF-7® group, MBP-structures were significantly increased when compared with the tFI/R+vehicle group. In addition, BF-7® administration after tFI/R significantly increased the proliferation of Rip-structures (as oligodendrocytes) as compared with the tFI/R+vehicle group. It has been accepted that loss of myelin sheath, which is attributed to diverse pathological processes in the central nervous system, delays axonal conduction and may arrest neurotransmission, passing through the demyelinated segments [36]. Axons lost myelin sheath by injuries undergo remyelination in order to recover neural functions which can be facilitated by newly produced oligodendrocytes [36,37]. Accumulating experimental data have shown that amelioration of cognitive dysfunctions induced by ischemic insults is attributed to promoted remyelination and proliferation of oligodendrocytes. For instance, administration of quercetin (a flavonoid abundantly contained in various plants) improves cognitive impairment following brain injury induced by cerebral hypoxia-ischemia in neonatal rats through promoting remyelination and proliferation of oligodendrocyte progenitor cells [15]. In addition, it has been reported that treatment with melatonin (a lipophilic hormone secreted from pineal body) after tFI/R in gerbils significantly im-

proves tFI/R-induced cognitive impairment, accompanied by improved remyelination and proliferation of oligodendrocytes [2].

Finally, VAChT (as a cholinergic transporter) and VGLUT-1 (as a glutamatergic synapse) were examined by immunohistochemistry in the tFI/R+vehicle and tFI/R+ BF-7® groups: therapeutic treatment with BF-7® significantly restored VAChT- and VGLUT-1-structures in the CA1 field when compared with the tFI/R+vehicle group. in the hippocampus with ischemic injury. A study has reported that regulating cholinergic and glutamatergic levels in the hippocampus may bring beneficial effects on cognitive dysfunctions induced by ischemic insult in rats [17]. Especially, Sun *et al*. [38] have shown that treatment with Dl-3-n-butylphthalide (a major ingredient derived from seeds of *Apium graveolens* (L.)) ameliorates memory function by increase of VAChT in a rat model of vascular dementia. Additionally, it has been reported that treatment with COG-up®, (a combined extract of *Erigeron annuus* (L.) Pers and *Brassica oleracea* Var.) improves tFI/R-induced cognitive impairment in gerbils via increasing VGLUT-1 [4].

5. Conclusions

The results of behavioral tests obviously showed that tFI/R-induced learning and spatial memory impairment was apparently improved by therapeutic treatment with BF-7® after tFI/R. However, therapeutic treatment with BF-7® did not protect or alleviate tFI/R-induced death of hippocampal CA1 neurons. Instead, our current findings revealed that treatment with BF-7® promoted remyelination and proliferation of oligodendrocytes. Moreover, treatment with BF-7® increased glutamatergic and cholinergic neurotransmissions. Based on the present results, we strongly suggest that BF-7® can be utilized for improving cognitive impairment following ischemia and reperfusion injury or ischemic stroke, as an additive for medicines and health/functional foods, promising that it can eventually contribute to improving national health.

Availability of Data and Materials

The data presented in this study are available on request from the corresponding authors.

Author Contributions

T-KL, J-WL, DWK and J-CL conducted experiments and data analysis. S-SK, J-DK, SH, SYC and YHK performed data curation and validation. T-KL and S-SK wrote the manuscript (original draft). M-HW wrote the manuscript (review and editing). M-HW and YHK supervised and administrated the project. S-SK, J-DK and SYC carried out funding acquisition.

Ethics Approval and Consent to Participate

All experimental processes using the animals adhered to the guidelines described in the "Current International Laws and Policies" a part of the "Guide for the Care and Use of Laboratory Animals". Approval for the experimental protocols was sanctioned (approval no., KW-200113-1; approval date, 18th Feb. 2020) by Institutional Animal Care and Use Committee (IACUC) of Kangwon National University (Chuncheon, Republic of Korea).

Acknowledgment

Not applicable.

Funding

This work was supported by the Technology development Program (S3212784) funded by the Ministry of SMEs and Startups (MSS, Korea), and by Basic Science Research Program through the National Research Foundation (NRF) of Korea funded by the Ministry of Education (NRF-2020R1F1A1062633 and NRF-2019R1A6A1A11036849).

Conflict of Interest

For declaring the conflict of interest, I inform that all authors of the present study have made a partnership with Famenity Co., Ltd., which produced the BF-7® used in this study.

References

[1] Lin L, Wang X, Yu Z. Ischemia-reperfusion Injury in the Brain: Mechanisms and Potential Therapeutic Strategies. Biochemistry & Pharmacology: Open Access. 2016; 5: 213.

[2] Chen BH, Park JH, Lee YL, Kang IJ, Kim DW, Hwang IK, *et al*. Melatonin improves vascular cognitive impairment induced by ischemic stroke by remyelination via activation of ERK1/2 signaling and restoration of glutamatergic synapses in the gerbil hippocampus. Biomedicine & Pharmacotherapy. 2018; 108: 687–697.

[3] Della-Morte D, Rundek T. Dizziness and vertigo. Frontiers of Neurology and Neuroscience. 2012; 30: 22–25.

[4] Lee T, Hong J, Lee J, Kim S, Sim H, Lee J, *et al*. Ischemia-Induced Cognitive Impairment Is Improved via Remyelination and Restoration of Synaptic Density in the Hippocampus after Treatment with COG-Up® in a Gerbil Model of Ischemic Stroke. Veterinary Sciences. 2021; 8: 321.

[5] Lee T, Lee J, Kim J, Kim D, Ahn J, Park J, *et al*. *Populus tomentiglandulosa* Extract Is Rich in Polyphenols and Protects Neurons, Astrocytes, and the Blood-Brain Barrier in Gerbil Striatum Following Ischemia-Reperfusion Injury. Molecules (Basel, Switzerland). 2021; 26: 5430.

[6] Seo WJ, Ahn JH, Lee T, Kim B, Lee J, Park JH, *et al*. High fat diet accelerates and exacerbates microgliosis and neuronal damage/death in the somatosensory cortex after transient forebrain ischemia in gerbils. Laboratory Animal Research. 2020; 36: 28.

[7] Kondo T, Yoshida S, Nagai H, Takeshita A, Mino M, Morioka H, *et al*. Transient forebrain ischemia induces impairment in cognitive performance prior to extensive neuronal cell death in Mongolian gerbil (*Meriones unguiculatus*). Journal of Veterinary Science. 2018; 19: 505–511.

[8] Kirino T, Sano K. Selective vulnerability in the gerbil hippocam-

pus following transient ischemia. Acta Neuropathologica. 1984; 62: 201–208.
[9] Lee T, Kim H, Song M, Lee J, Park JH, Ahn JH, *et al*. Time-course pattern of neuronal loss and gliosis in gerbil hippocampi following mild, severe, or lethal transient global cerebral ischemia. Neural Regeneration Research. 2019; 14: 1394–1403.
[10] Ojo OB, Amoo ZA, Saliu IO, Olaleye MT, Farombi EO, Akinmoladun AC. Neurotherapeutic potential of kolaviron on neurotransmitter dysregulation, excitotoxicity, mitochondrial electron transport chain dysfunction and redox imbalance in 2-VO brain ischemia/reperfusion injury. Biomedicine & Pharmacotherapy. 2019; 111: 859–872.
[11] Domingues HS, Portugal CC, Socodato R, Relvas JB. Oligodendrocyte, Astrocyte, and Microglia Crosstalk in Myelin Development, Damage, and Repair. Frontiers in Cell and Developmental Biology. 2016; 4: 71.
[12] Nave K, Werner HB. Myelination of the nervous system: mechanisms and functions. Annual Review of Cell and Developmental Biology. 2014; 30: 503–533.
[13] Duncan ID, Radcliff AB, Heidari M, Kidd G, August BK, Wierenga LA. The adult oligodendrocyte can participate in remyelination. Proceedings of the National Academy of Sciences of the United States of America. 2018; 115: E11807–E11816.
[14] Ahn JH, Chen BH, Shin BN, Cho JH, Kim IH, Park JH, *et al*. Intravenously Infused F3.Olig2 Improves Memory Deficits via Restoring Myelination in the Aged Hippocampus Following Experimental Ischemic Stroke. Cell Transplantation. 2016; 25: 2129–2144.
[15] Qu X, Qi D, Dong F, Wang B, Guo R, Luo M, *et al*. Quercetin improves hypoxia-ischemia induced cognitive deficits via promoting remyelination in neonatal rat. Brain Research. 2014; 1553: 31–40.
[16] Kugaya A, Sanacora G. Beyond monoamines: glutamatergic function in mood disorders. CNS Spectrums. 2005; 10: 808–819.
[17] Cao Y, Gou Z, Du Y, Fan Y, Liang L, Yan Y, *et al*. Glutamatergic and central cholinergic dysfunction in the CA1, CA2 and CA3 fields on spatial learning and memory in chronic cerebral ischemia-Induced vascular dementia of rats. Neuroscience Letters. 2016; 620: 169–176.
[18] Chae H-S, Kang Y-K, Shin Y-K, Lee H-J, Yu J-I, Lee K-G, *et al*. The role of BF-7 on neuroprotection and enhancement of cognitive function. The Korean Journal of Physiology and Pharmacology. 2004; 8: 173–179.
[19] Lee T, Lee J, Kim DW, Lee J, Kim S, Kim H, *et al*. Effects of Brain Factor 7® against motor deficit and oxidative stress in a mouse model of MPTP induced Parkinson's disease. Experimental and Therapeutic Medicine. 2022; 24: 635.
[20] Noh Y, Ahn JH, Lee J, Hong J, Lee T, Kim B, *et al*. Brain Factor-7® improves learning and memory deficits and attenuates ischemic brain damage by reduction of ROS generation in stroke in vivo and in vitro. Laboratory Animal Research. 2020; 36: 24.
[21] Janet CR, Wayne B, Joseph TB, Leigh Ann C, John CD, Dennis FK, *et al*. Council NR. Guide for the care and use of laboratory animals. 8th Edition. National Academies Press: Washington D.C., USA. 2010.
[22] Lee C, Lee T, Kim DW, Lim SS, Kang IJ, Ahn JH, *et al*. Relationship between Neuronal Damage/Death and Astrogliosis in the Cerebral Motor Cortex of Gerbil Models of Mild and Severe Ischemia and Reperfusion Injury. International Journal of Molecular Sciences. 2022; 23: 5096.
[23] Lee T, Kang I, Sim H, Lee J, Ahn J, Kim D, *et al*. Therapeutic Effects of Decursin and *Angelica gigas* Nakai Root Extract in Gerbil Brain after Transient Ischemia via Protecting BBB Leakage and Astrocyte Endfeet Damage. Molecules (Basel, Switzerland). 2021; 26: 2161.
[24] Schmued LC, Hopkins KJ. Fluoro-Jade B: a high affinity fluorescent marker for the localization of neuronal degeneration. Brain Research. 2000; 874: 123–130.
[25] Shin MC, Lee T, Lee J, Kim HI, Park CW, Cho JH, *et al*. Therapeutic effects of stiripentol against ischemia-reperfusion injury in gerbils focusing on cognitive deficit, neuronal death, astrocyte damage and blood brain barrier leakage in the hippocampus. The Korean Journal of Physiology & Pharmacology: Official Journal of the Korean Physiological Society and the Korean Society of Pharmacology. 2022; 26: 47–57.
[26] Zhu Y, Liu F, Zou X, Torbey M. Comparison of unbiased estimation of neuronal number in the rat hippocampus with different staining methods. Journal of Neuroscience Methods. 2015; 254: 73–79.
[27] Paizs M, Engelhardt JI, Siklós L. Quantitative assessment of relative changes of immunohistochemical staining by light microscopy in specified anatomical regions. Journal of Microscopy. 2009; 234: 103–112.
[28] Klisch J, Bode-Greuel KM. Ketanserin reduces neuronal calcium accumulation and cell death in the hippocampus of the Mongolian gerbil after transient forebrain ischemia. Brain Research. 1992; 578: 1–7.
[29] Kawano T, Fukunaga K, Takeuchi Y, Morioka M, Yano S, Hamada J, *et al*. Neuroprotective effect of sodium orthovanadate on delayed neuronal death after transient forebrain ischemia in gerbil hippocampus. Journal of Cerebral Blood Flow and Metabolism: Official Journal of the International Society of Cerebral Blood Flow and Metabolism. 2001; 21: 1268–1280.
[30] Kirino T. Delayed neuronal death in the gerbil hippocampus following ischemia. Brain Research. 1982; 239: 57–69.
[31] Sweatt JD. Hippocampal function in cognition. Psychopharmacology. 2004; 174: 99–110.
[32] Bird CM, Burgess N. The hippocampus and memory: insights from spatial processing. Nature Reviews. Neuroscience. 2008; 9: 182–194.
[33] Dhar A, Kaundal RK, Sharma SS. Neuroprotective effects of FeTMPyP: a peroxynitrite decomposition catalyst in global cerebral ischemia model in gerbils. Pharmacological Research. 2006; 54: 311–316.
[34] Kang Z, Wang H, Yang Y, Zhao X, Zhou Q, Yang Y, *et al*. Pinocembrin Ameliorates Cognitive Impairment Induced by Vascular Dementia: Contribution of Reelin-dab1 Signaling Pathway. Drug Design, Development and Therapy. 2020; 14: 3577–3587.
[35] Yan N, Xu Z, Qu C, Zhang J. Dimethyl fumarate improves cognitive deficits in chronic cerebral hypoperfusion rats by alleviating inflammation, oxidative stress, and ferroptosis via NRF2/ARE/NF-κB signal pathway. International Immunopharmacology. 2021; 98: 107844.
[36] Baaklini CS, Rawji KS, Duncan GJ, Ho MFS, Plemel JR. Central Nervous System Remyelination: Roles of Glia and Innate Immune Cells. Frontiers in Molecular Neuroscience. 2019; 12: 225.
[37] Jia W, Kamen Y, Pivonkova H, Káradóttir RT. Neuronal activity-dependent myelin repair after stroke. Neuroscience Letters. 2019; 703: 139–144.
[38] Sun Y, Zhao Z, Li Q, Wang C, Ge X, Wang X, *et al*. Dl-3-n-butylphthalide regulates cholinergic dysfunction in chronic cerebral hypoperfusion rats. The Journal of International Medical Research. 2020; 48: 300060520936177.