

Effects of long-term post-ischemic treadmill exercise on gliosis in the aged gerbil hippocampus induced by transient cerebral ischemia

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Abstract. Therapeutic exercise is an integral component of the rehabilitation of patients who have suffered a stroke. The objective of the present study was to use immunohistochemistry to investigate the effects of post-ischemic exercise on neuronal damage or death and gliosis in the aged gerbil hippocampus following transient cerebral ischemia. Aged gerbils (male; age, 22-24 months) underwent ischemia and were subjected to treadmill exercise for 1 or 4 weeks. Neuronal death was detected in the stratum pyramidale of the hippocampal CA1 region and in the polymorphic layer of the dentate gyrus using cresyl violet and Fluoro-Jade B histo-fluorescence staining. No significant difference in neuronal death was identified following 1 or 4 weeks of post-ischemic treadmill exercise. However, post-ischemic treadmill exercise affected gliosis (the activation of astrocytes and microglia).

Glial fibrillary acidic protein-immunoreactive astrocytes and ionized calcium binding adaptor molecule 1-immunoreactive microglia were activated in the CA1 and polymorphic layer of the dentate gyrus of the group without treadmill exercise. Conversely, 4 weeks of treadmill exercise significantly alleviated ischemia-induced astrocyte and microglial activation; however, 1 week of treadmill exercise did not alleviate gliosis. These findings suggest that long-term post-ischemic treadmill exercise following transient cerebral ischemia does not influence neuronal protection; however, it may effectively alleviate transient cerebral ischemia-induced astrocyte and microglial activation in the aged hippocampus.

Introduction

Transient forebrain ischemia induces the damage/death of pyramidal neurons in the CA1 region of the hippocampus (1-3). It has previously been reported that aged animals are less vulnerable to ischemia, and ischemia-induced neuronal degeneration occurs much later than in adult animals (4-6).

Microglia, which are primary immune cells that are located in the central nervous system, and astrocytes, which act as important modulators of neuronal activity, are both involved in maintaining homeostasis of the brain microenvironment (7). Microglia and astrocytes maintain a resting phenotype under physiological conditions; however, in the process of aging or pathological conditions, including ischemia-reperfusion injury, they exhibit activation with morphological and functional alterations, including hypertrophy and the release of various factors, which have been reported to modulate the injury process (8-10). It is well known that glial cells serve complex roles in neuroinflammation and in the regeneration of brain tissue following ischemic insults (11,12).

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In the case of stroke, exercise treatment has been used in humans to aid the remaining functions (13). In experimental animals, exercise reduces astrocyte and microglial activation in the acute phase following transient focal ischemia in rats (14) and traumatic brain injury in mice (15). The effects of exercise on glial activation in animal models of brain injuries have previously been investigated; however, long-term alterations to glial activation in the ischemic hippocampus in aged animals have yet to be fully elucidated. Therefore, the present study aimed to investigate the effects of post-ischemic exercise on neuronal damage and gliosis in the hippocampus following transient cerebral ischemia in the aged gerbil, a useful animal model for transient cerebral ischemia and aging research (16-19).

Materials and methods

Experimental animals. A total of 35 male Mongolian gerbils (*Meriones unguiculatus*; age, 22-24 months; weight, 80-90 g) were supplied by the Experimental Animal Center, Kangwon National University (Chuncheon, South Korea). Gerbils were housed in a conventional facility, at a temperature of $23\pm 3^{\circ}\text{C}$ and relative humidity of $55\pm 5\%$, under 12/12 h light/dark cycles, and were allowed free access to food and water. Animal handling and experimental protocols were approved by the Institutional Animal Care and Use Committee of Kangwon National University (approval no. KW-130424-1). The gerbils were randomly divided into five groups: i) Sham group ($n=7$), which underwent sham surgery; ii) ischemia group ($n=7$), which underwent 5 min of transient forebrain ischemia; iii) ischemia-SD4 group ($n=7$), which had a sedentary routine for 4 weeks (SD4) from 5 days post-ischemia; iv) ischemia-TR1 group ($n=7$), which performed 1 week treadmill exercise (TR) from 5 days post-ischemia; and, v) ischemia-TR4 group ($n=7$), which performed 4 weeks TR from 5 days post-ischemia. The animals were sacrificed 31 days following ischemia; at which point, the TR training was concluded in the ischemia-TR4 group.

Induction of transient cerebral ischemia. Following the method described in our previous study (20), the gerbils were anesthetized with a mixture of 2.5% isoflurane (Baxter Healthcare Corporation, Deerfield, IL, USA) in 33% oxygen and 67% nitrous oxide. After a sagittal ventral midline incision, common carotid arteries were carefully separated from the respective vagal nerves and were occluded for 5 min using nontraumatic aneurysm clips (Yasargil FE 723K; Aesculap AG, Tuttlingen, Germany). Following occlusion for 5 min, the clips were removed and the wounds were sutured with wound clips (12022-09; Fine Science Tools, Inc., Foster City, CA, USA). Normothermic body (rectal) temperature ($37\pm 0.5^{\circ}\text{C}$) was monitored until the animals completely recovered from anesthesia. Sham surgery animals were subjected to the same surgical procedures without the occlusion of the bilateral common carotid arteries.

Treadmill exercise. The running speed and duration of treadmill exercise was determined according to Sim's protocol (21-23), with modification. Briefly, from 5 days post-ischemia, the gerbils in the TR groups were forced to run

on a motorized treadmill for 30 min/day and 5 days/week for 1 or 4 consecutive weeks. The exercise workload consisted of running at a speed of 5 m/min for the first 5 min, 7 m/min for the next 5 min and then 10 m/min for the last 20 min with 0° inclination. The animals in the SD group were placed on the treadmill for 30 min, without being induced to run.

Tissue processing for histology. Tissue processing was performed according to a previously published procedure (20). Briefly, animals ($n=7/\text{group}$) were anesthetized with sodium pentobarbital (40 mg/kg, i.p.; JW Pharmaceutical Co., Ltd., Seoul, Korea) and perfused transcardially with 4% paraformaldehyde. Brain tissues were serially sectioned into 30 μm coronal sections.

Cresyl violet (CV) staining. To investigate morphological alterations, CV staining was performed according to a previously published procedure (24). Briefly, the sections were stained with 1% CV acetate (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and immersed in serial ethanol baths. CV-stained structures were observed under an AxioM1 light microscope (Zeiss AG, Oberkochen, Germany) equipped with a camera (AxioCam; Zeiss AG) and photomicrographs were captured. The CV-stained structures were examined in a $250\times 250\ \mu\text{m}$ area that included the stratum pyramidale at the center of the hippocampal CA1 region, or in the whole dentate gyrus, using the image analysis system Optimas version 6.5 (CyberMetrics, Scottsdale, AZ, USA).

Fluoro-Jade B (F-J B) histofluorescence staining. Histo-fluorescence staining was performed according to a previously published procedure (25). F-J B (high-affinity fluorescent marker for the localization of neuronal degeneration) histo-fluorescence staining was performed to examine neuronal degeneration. Briefly, the sections were immersed in a solution containing 1% sodium hydroxide in 80% alcohol, transferred to a solution containing 0.06% potassium permanganate diluted in water, then transferred to an aqueous solution containing 0.0004% F-J B (Histo-Chem, Inc., Jefferson, AR, USA). After washing 3 times in water, the sections were placed on a slide warmer ($\sim 50^{\circ}\text{C}$) and examined using an epifluorescent microscope (Zeiss AG) with blue (450-490 nm) excitation source and a barrier filter.

Immunohistochemistry. Immunohistochemistry was performed according to our previously published procedure (24). Briefly, immunostaining was performed using mouse anti-glial fibrillary acidic protein (GFAP; 1:800; cat no. MAB360; EMD Millipore, Billerica, MA, USA) for astrocytes and rabbit anti-ionized calcium binding adaptor molecule 1 (Iba-1; 1:800; cat no. 019-19741; Wako Pure Chemical Industries, Ltd., Osaka, Japan) for microglia overnight at 4°C . Subsequently, samples were incubated with biotinylated horse anti-mouse immunoglobulin G (1:250; cat no. BA2000; Vector Laboratories, Inc., Burlingame, CA, USA) or goat anti-rabbit antibodies (1:250; cat no. BA1000; Vector Laboratories, Inc.) for 2 h at room temperature, and streptavidin peroxidase complex (1:200; Vector Laboratories, Inc.) for 1 h at room temperature. To establish the specificity of the

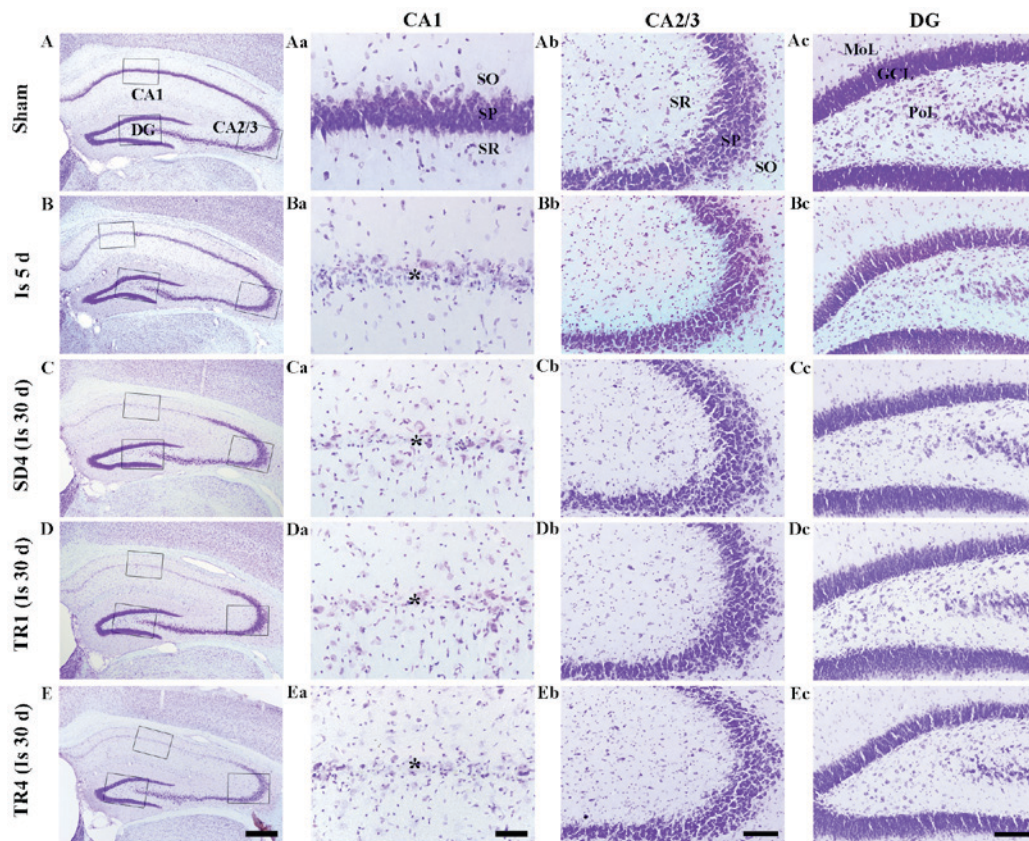


Figure 1. CV staining in the (A) sham, (B and C) ischemia and (Da and E) TR groups. Only a small number of CV-positive cells were detected in the SP (asterisk) of the CA1 region 5 days post-ischemia. In the SD4, TR1 and TR4 groups, the pattern of CV-positive cells distribution was similar to the ischemia group at 5 days post-ischemia. Scale bar: (Aa-Ea) 400 μ m, (Ab-Eb) 40 μ m and (Ac-Ec and Ad-Ee) 100 μ m. CV, cresyl violet; TR, treadmill exercise; SD, sedentary routine; DG, dentate gyrus; GCL, granule cell layer; MoL, molecular layer; PoL, polymorphic layer; SO, stratum oriens; SR, stratum radiatum; SP, stratum pyramidale.

immunostaining, a negative control test was performed and resulted in the absence of immunoreactivity in all structures.

Data analysis. In order to quantitatively analyze the number of F-J B-positive cells, digital images from seven sections per animal were captured using a light microscope (Axiom1; Zeiss AG) equipped with a digital camera (Axiocam; Zeiss AG) and connected to a PC monitor. The number of F-J B-positive cells was counted in a 250x250 μ m square including the stratum pyramidale at the center of the hippocampal CA1 region or in the whole dentate gyrus using the image analysis system Optimas version 6.5 (CyberMetrics). Cell counts were carried out by averaging the counts from each animal.

To quantitatively analyze the density of GFAP- and Iba-1-immunoreactive structures, the corresponding hippocampal areas were measured from seven sections per animal. Images of all GFAP- and Iba-1-immunoreactive structures were captured through an Axiom1 light microscope (Zeiss AG) equipped with a camera (Axiocam; Zeiss AG) and connected to a PC monitor. Densities of GFAP- and Iba-1-immunoreactive structures were evaluated on the basis of optical density (OD), obtained following the transformation of the mean gray level using the formula: $OD = \log(256/\text{mean gray level})$. The background was subtracted and the OD ratio for each image was calibrated as % relative optical density (ROD) using Adobe Photoshop version 8.0 (Adobe Systems, San Jose, CA, USA) and ImageJ software version 1.49 (National Institutes

of Health, Bethesda, MD, USA). The mean value of the OD of the sham group was designated as 100% and the ROD in each group was calibrated and expressed as a percentage of the sham group.

Statistical analysis. Data are expressed as the mean \pm standard error of the mean of at least 2 independent experiments. Data from F-J B immunofluorescence and immunohistochemical staining were analyzed using one-way analysis of variance, followed by a post hoc Bonferroni-Dunn Test using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

CV-positive cells

Sham group. CV staining is presented in Fig. 1. CV-positive cells were detected throughout the hippocampus; in particular, they were aggregated in the stratum pyramidale of the hippocampus proper (CA1-3 regions) and the granular cell layer of the dentate gyrus (Fig. 1Aa-d).

Ischemia groups. CV-positive cells were markedly decreased in the CA1 stratum pyramidale, but not in the other subregions, 5 days post-ischemia (Fig. 1Ba-d). In the SD4 group, the distribution pattern of CV-positive cells was similar to the ischemia group at 5 days post-ischemia (Fig. 1Ca-d).

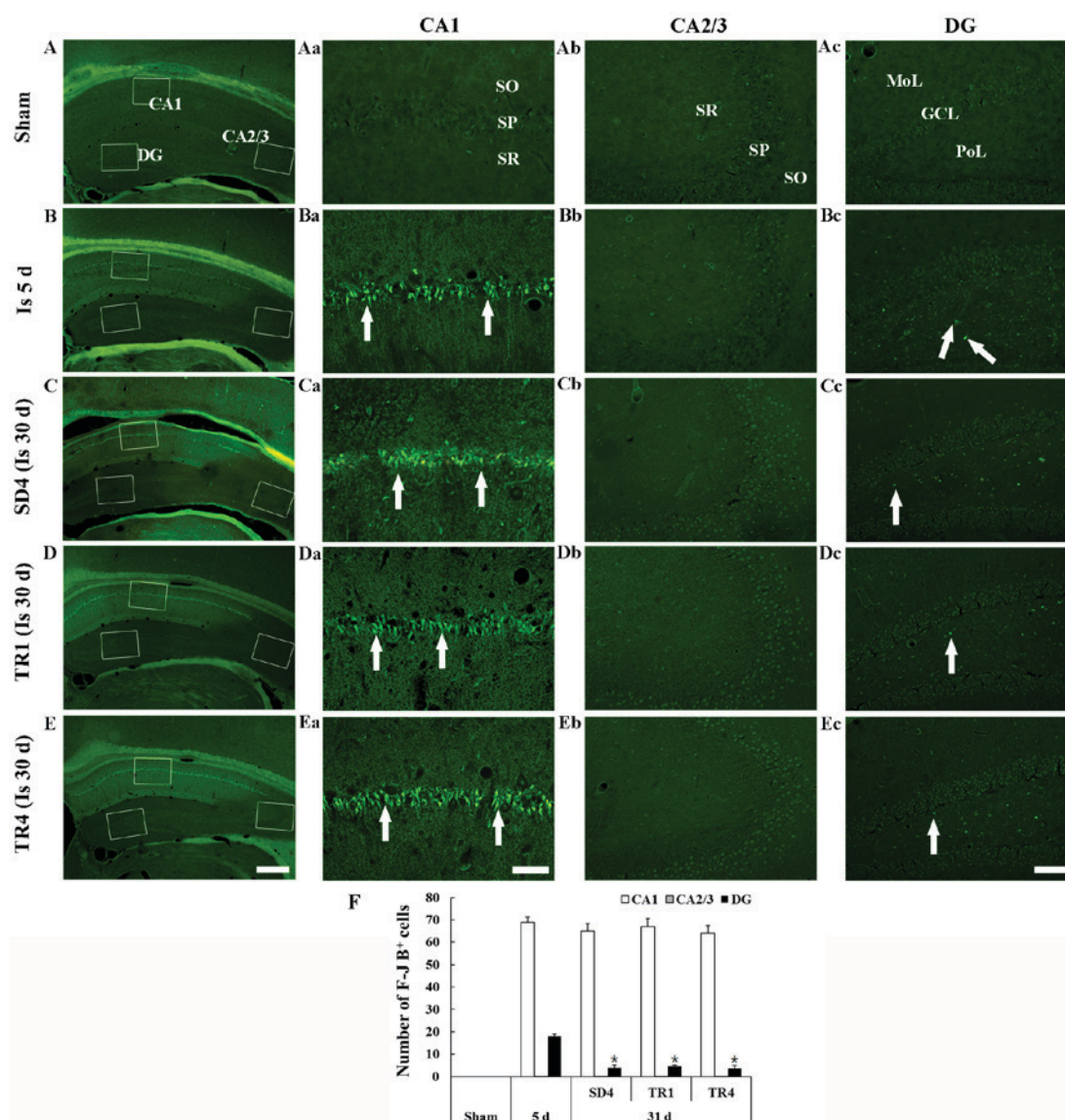


Figure 2. F-J B histofluorescence staining in the (A) sham, (B and C) ischemia and (D and E) TR groups. In the sham group, no F-J B-positive cells were detected. However, at 5 days post-ischemia, numerous F-J B-positive neurons (arrows) were detected in the SP of the CA1 region and in the PoL of the DG. In the SD4, TR1 and TR4 groups, the number of F-J B-positive neurons (arrows) in the SP of the CA1 region and in the PoL of the DG was similar between the groups. Scale bar: (Aa-Ea) 400 μ m, (Ab-Eb) 40 μ m and (Ac-Ec and Ad-Ed) 100 μ m. (F) Number of F-J B-positive cells in the SP of the CA1 and CA2/3 regions and in the PoL of the DG ($n=7$ /group). Data are presented as the mean \pm standard error of mean. * $P<0.05$ vs. ischemia group at 5 days post-ischemia. F-J B, Fluoro-Jade B; TR, treadmill exercise; SD, sedentary routine; DG, dentate gyrus; GCL, granule cell layer; MoL, molecular layer; PoL, polymorphic layer; SO, stratum oriens; SR, stratum radiatum; SP, stratum pyramidale.

TR-groups. In the TR1 and TR4 groups, the distribution pattern of CV-positive cells in the hippocampus was also similar to the SD4 group (Fig. 1Da-d and Ea-d).

F-J B-positive cells

Sham group. F-J B staining is presented in Fig. 2. F-J B-positive cells were not detected in any layers of the hippocampus proper and the dentate gyrus (Fig. 2Aa-d).

Ischemia groups. A total of 5 days post-ischemia, numerous F-J B-positive cells were detected in the stratum pyramidale of the CA1 region and a small number of F-J B-positive cells were detected in the polymorphic layer of the dentate gyrus (Fig. 2Ba-d and F). In the SD4 group, the distribution pattern and number of F-J B-positive cells in the CA1 stratum pyramidale was similar to the ischemia-group at 5 days post-ischemia;

however, the number of F-J B-positive cells was significantly decreased in the polymorphic layer of the dentate gyrus (Fig. 2Ca-d and F).

TR-groups. In the TR1 and TR4 groups, the number of F-J B-positive cells in the CA1 stratum pyramidale and in the polymorphic layer of the dentate gyrus was similar to the SD4 group and no significant difference in the number of F-J B-positive cells was observed between the TR1 and TR4 groups (Fig. 2Da-d, Ea-d and F).

GFAP-immunoreactive astrocytes

Sham group. GFAP staining is presented in Fig. 3. GFAP-immunoreactive astrocytes in the sham group were easily detected in all layers of the hippocampus proper and the dentate gyrus. The astrocytes appeared to be at resting

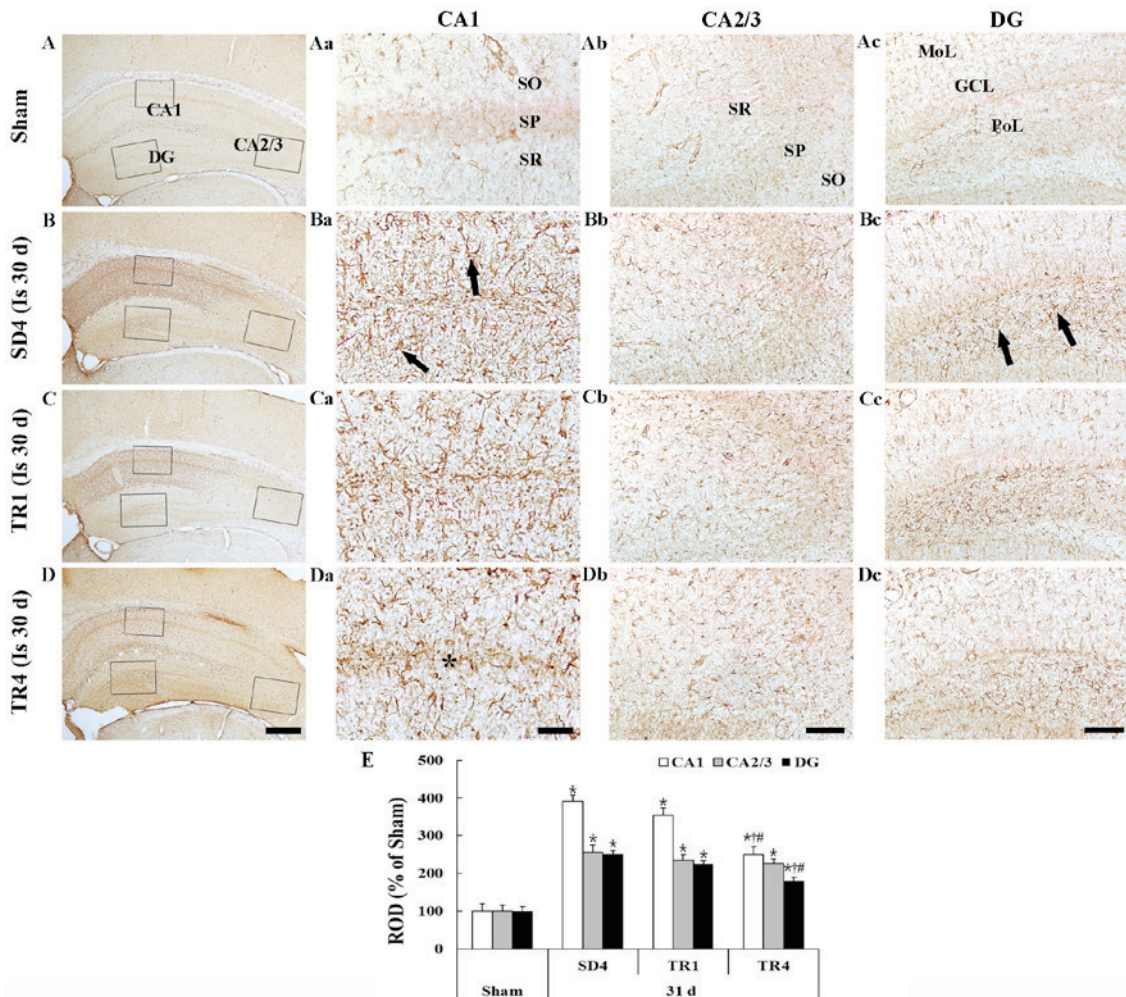


Figure 3. GFAP immunohistochemistry in the (A) sham, (B) SD4, (C) TR1 and (D) TR4 groups. In the SD4 group, GFAP-immunoreactive astrocytes (arrows) were activated in the CA1 region and in the PoL of the DG. In the TR1 group, the activation was similar to the SD4 group; however, in the TR4 group, the activation was significantly decreased compared with the SD4 group. Scale bar: (Aa-Da) 400 μ m, (Ab-Db) 40 μ m and (Ac-Dc and Ad-Dd) 100 μ m. (E) ROD expressed as a percentage of GFAP-immunoreactive structures (n=7/group). Data are presented as the mean \pm standard error of mean. *P<0.05 vs. sham group; †P<0.05 vs. SD4 group; ‡P<0.05 vs. TR1 group. GFAP, glial fibrillary acidic protein; SD, sedentary routine; TR, treadmill exercise; DG, dentate gyrus; GCL, granule cell layer; MoL, molecular layer; PoL, polymorphic layer; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; ROD, relative optical density.

form and had a small body with thread-like thin processes (Fig. 3Aa-d).

Ischemia group. In the SD4 group, numerous GFAP-immunoreactive astrocytes demonstrated a typical activated form that had a punctuated cytosol with thick processes (Fig. 3Ba-d). The density of the GFAP-immunoreactive structures (ROD) was significantly increased in all subregions compared with in the sham group (P<0.05; Fig. 3E); in particular, the activation was marked in the CA1 region and in the polymorphic layer of the dentate gyrus.

TR groups. In the TR1 group, the morphology of GFAP-immunoreactive astrocytes in the hippocampus proper and the dentate gyrus was similar to the SD4 group (Fig. 3Ca-d) and the ROD of GFAP-immunoreactive structures was not significantly different compared with the SD4 group (Fig. 3E). However, in the TR4 group the ROD was significantly decreased (P<0.05) compared with in the SD4 and TR1 groups (Fig. 3Da-d and E).

Iba-1-immunoreactive microglia

Sham group. Iba-1 staining is presented in Fig. 4. Iba-1-immunoreactive microglia were evenly distributed throughout the hippocampus. The microglia appeared to be at resting form and exhibited fine processes with web-like network characteristics (Fig. 4Aa-d).

Ischemia group. In the SD4 group, Iba-1-immunoreactive microglia were markedly altered in the CA1 region and in the polymorphic layer of the dentate gyrus; they exhibited bulky cytoplasm with short and thickened processes, which represents the activated form (Fig. 4Ba-d and E). In particular, activated Iba-1-immunoreactive microglia were aggregated in the stratum pyramidale of the CA1 region. The ROD of the Iba-1-immunoreactive structures was significantly increased (P<0.05) in the CA1 region and the polymorphic layer of the dentate gyrus compared with in the sham group (Fig. 4E).

TR groups. In the TR1 group, the distribution pattern of Iba-1-immunoreactive microglia in the hippocampus

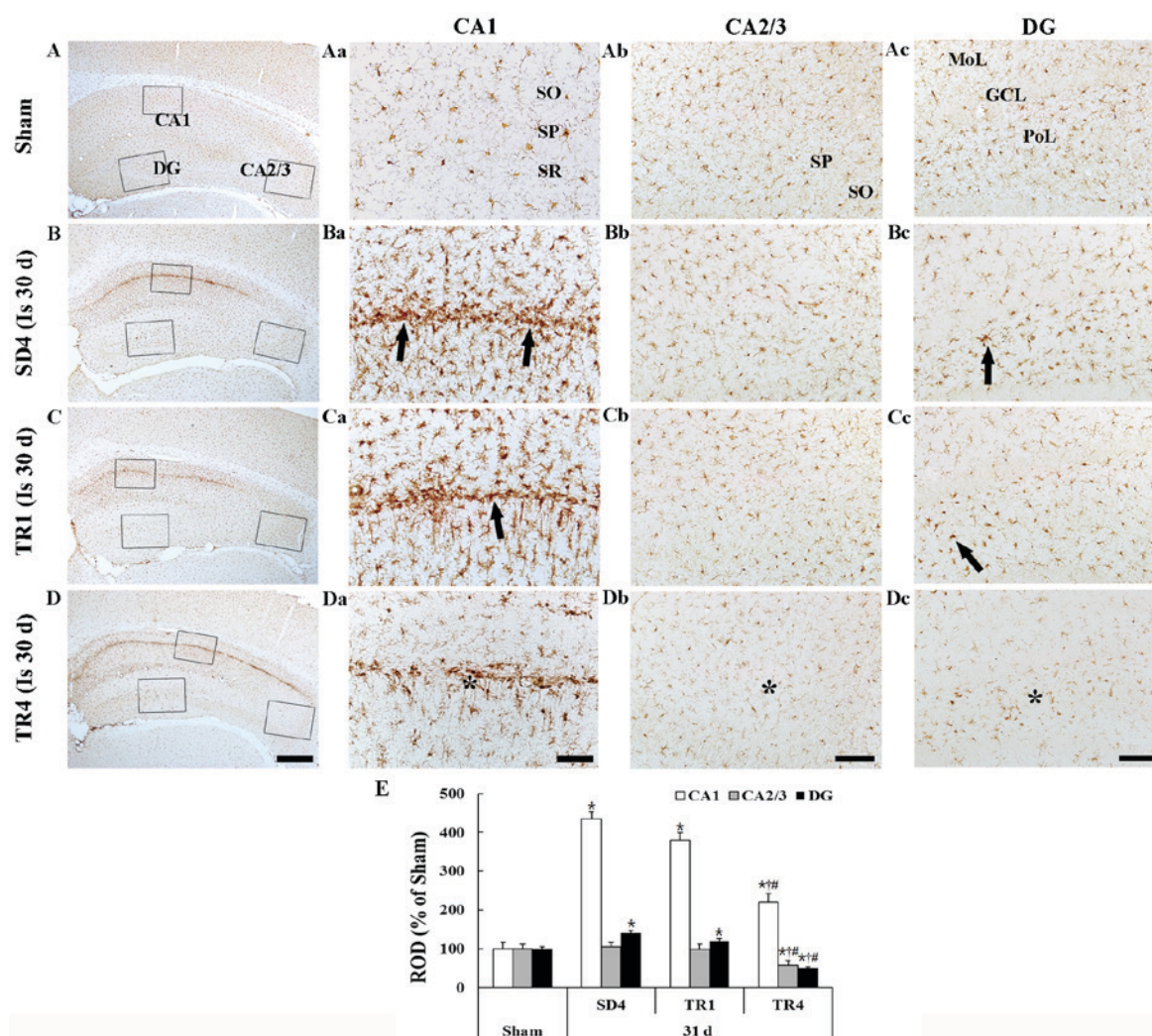


Figure 4. Iba-1 immunohistochemistry in the (A) sham, (B) SD4, (C) TR1 and (D) TR4 groups. Iba-1-immunoreactive microglia were activated (arrows) in the CA1 region and the PoL of the DG in the SD4 group. In the TR4 group, the activation of Iba-1-immunoreactive microglia was significantly decreased (asterisks) although the activation in the TR1 group was similar to the SD4 group. Scale bar: (Aa-Da) 400 μ m, (Ab-Db) 40 μ m and (Ac-Dc and Ad-Dd) 100 μ m. (E) ROD expressed as a percentage of Iba-1 immunoreactive structures ($n=7$ /group). Data are presented as the mean \pm standard error of the mean. * $P<0.05$ vs. the sham group; # $P<0.05$ vs. the SD4 group; *# $P<0.05$ vs. the TR1 group). Iba-1, ionized calcium binding adaptor molecule 1; SD, sedentary routine; TR, treadmill exercise; DG, dentate gyrus; GCL, granule cell layer; MoL, molecular layer; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; ROD, relative optical density.

was similar to the SD4 group; however, the activation of Iba-1-immunoreactive microglia was slightly decreased in the CA1 region and the dentate gyrus (Fig. 4Ca-d). In the TR4 group, the ROD of activated Iba-1-immunoreactive microglia was significantly decreased ($P<0.05$) in the CA1 region and the dentate gyrus compared with in the SD4 and TR1 groups (Fig. 4Da-c and E).

Discussion

Ischemic brain damage can lead to the development of neuronal damage and gliosis (26), and result in long-term functional disability (27,28). The present study investigated the effects of long- and short-term post-ischemic treadmill exercise on neuronal death and glial activation in the aged gerbil hippocampus induced by 5 min of transient cerebral ischemia.

In the present study, at 5 days post-ischemia, a distinct neuronal loss was observed in the CA1 stratum pyramidale

and in the polymorphic layer of the dentate gyrus in the aged gerbil hippocampus, as determined using CV and F-J B staining. This result is consistent with our previous findings, which demonstrated that a significant neuronal loss in the aged gerbil hippocampus was detected in the CA1 stratum pyramidale (5) and in the polymorphic layer of the dentate gyrus (29) 5 days after transient ischemia. At 31 days post-ischemia in the SD4 group, the number of F-J B-positive cells (dead neurons) in the CA1 region was similar to that at 5 days post-ischemia. Furthermore, the present study is the first, to the best of our knowledge, to report that short- and long-term post-ischemic treadmill exercise did not exhibit any neuroprotection in the TR1 and TR4 groups; the numbers of F-J B-positive neurons in the CA1 region and the dentate gyrus were no different compared with the SD4 group. It has previously been reported that short- and long-term treadmill exercise, initiated prior to ischemic neuronal death, exerted a neuroprotective effect by suppressing transient cerebral ischemia-induced apoptosis of the neurons in the CA1 region (21-23). Based on the findings

of the present study and previous studies, it may be concluded that treadmill exercise begun after transient cerebral ischemia-induced neuronal degeneration cannot protect neurons in the aged hippocampus.

In the present study, the significant activation of GFAP-immunoreactive astrocytes and Iba-1-immunoreactive microglia was observed in the CA1 region and the dentate gyrus of the SD4 group, and their ROD was significantly increased compared with in the sham group. However, 4 weeks of post-ischemic treadmill exercise significantly reduced the number of activated astrocytes and microglia in the CA1 region and in the dentate gyrus compared with the sedentary control (SD4 group). Conversely, 1 week of treadmill exercise did not effectively decrease their activation in the ischemic hippocampus. It is well known that ischemic hippocampus pathology is closely associated with an acute and prolonged inflammatory response, which is characterized by the production of inflammatory cytokines and the activation of resident glial cells (30,31). In this regard, previous studies have demonstrated that wheel-running exercise attenuated age-related astrocyte hypertrophy (32) and microglial proliferation (33). In addition, chronic exercise inhibited the activation of astrocytes and microglia, and other inflammatory-related factors, including inducible nitric oxide synthase, in murine models of Alzheimer's and Parkinson's diseases (34,35). The present results, along with the aforementioned findings, indicated that long-term treadmill exercise may alleviate increased neuroinflammation in the aged gerbil hippocampus induced by transient cerebral ischemia.

In conclusion, the present study suggested that 4 weeks of treadmill exercise, initiated after neuronal death, cannot influence neuronal protection; however, the exercise can effectively alleviate transient cerebral ischemia-induced gliosis in the hippocampus of aged gerbils.

Acknowledgements

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