Efficacy of different intensity of aquatic exercise in enhancing remyelination and neuronal plasticity using cuprizone model in male Wistar rats

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Abstract

Background. Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS). Most exercise studies concentrate on the impact of exercise on cardiovascular system; this study aims to present the effects of exercise of varying intensity on the nervous system. Most recently in MS, positive outcomes were obtained with resistance and high-intensity exercises. This study also analyzes the effects of a prior conditioning program before the induction of demyelination and subsequent neuroprotective effects of such program.

Objectives. To study and determine the neuroprotective and remyelinating effects of different intensity of aquatic exercise and a preconditioning exercise program on demyelination induced by oral administration of cuprizone (Cup).

Materials and methods. Six groups of animals, each containing 6 rats, were used in the study. The groups were as follows: group I – control group; group II – Cup group; group III – treated with methylprednisolone (MP); group IV – treated with low-intensity exercise (LIE), free swimming for 40 min and high-intensity exercise (HIE); group V – treated with a resistance of 9% body weight and free swimming for 40 min; group VI – treated with preconditioning exercise (free swimming for 40 min for 3 weeks) before Cup administration followed by the same exercise protocol as for group V. All data were analyzed using one-way analysis of variance (ANOVA) with Tukey's test, by means of SigmaPlot v. 14.5 software.

Results. Similarly to the MP group, group VI showed a positive outcome. A value of $p < 0.001$ was considered statistically significant. Also, group VI showed improved areas of remyelination in histopathology, an increased expression of myelin basic protein (MBP), reduced expression of glial fibrillary acidic protein (GFAP) in corpus callosum, and improved gene expression of brain-derived neurotrophic factor (BDNF) in the hippocampus region.

Conclusions. General fitness achieved through a preconditioning program combined with HIE showed neuroprotective effects, as evidenced by increased areas of remyelination and improved neuronal plasticity, observed mostly in group VI (conditioning+HIE).

Key words: demyelination, exercise, neuronal plasticity, cuprizone, conditioning

Background

The number of patients diagnosed with multiple sclerosis (MS) is increasing because of an increased access to magnetic resonance imaging (MRI) and early diagnosis. Multiple sclerosis is essentially an inflammatory disease of the central nervous system (CNS). The precise cause of the disease is still debatable and ascribed to autoimmune pathology. Genetic factors have also been taken into account in the literature.[1,2] Moreover, a disturbance in the reduction–oxidation metabolism has been pointed out as a causative factor for MS.[3] The treatment of MS involves the use of steroids and interferon therapy, and has greatly evolved over the past years. A combination of agents that modify the course of the disease and have various effects on immunity is used for treatment at different phases of the disease.[4,5] Vitamin plus interferon therapy and the use of medical cannabis have also been studied as methods of treatment in MS.[6,7] This disease leads to a widespread demyelination caused by selective involvement of oligodendrocytes and axonal loss. Additional psychobehavioral symptoms such as anxiety, dementia and depression are also observed.[8] At times, neurodegenerative disorders following substance abuse can mimic features of MS, which needs careful evaluation.[9] Potential biomarkers, such as myelin basic protein (MBP), glial fibrillary acidic protein (GFAP) and CNPase, can play a role in the diagnosis and assessment of therapeutic agents in MS.[10,11]

Cuprizone (bis-cyclohexanone oxaldihydrazone; Cup) has been successfully used for inducing demyelination.[12] It is a primary copper chelator which causes selective apoptosis of oligodendrocytes and induces CNS demyelination in rats. A 3-week exposure to Cup causes cerebral cortical demyelination and white matter damage in mice.[13] Cuprizone was the chosen method in the present study, as cessation of administration of Cup leads to spontaneous remyelination, which mimics the relapsing-remitting stage of MS (RRMS).[14]

Other methods for induction of demyelination include 1) experimental autoimmune encephalomyelitis (EAE) induced by myelin oligodendrocyte glycoprotein (MOG) antibodies, 2) viral model using Theiler's virus, and 3) toxic model of demyelination involving the use of lysolecithin, which leads to demyelination within 2 days of administration.[15–17]

Methylprednisolone (MP) is the standard drug of choice for MS.[18] It is a chemical modification of naturally occurring glucocorticosteroid – hydroxycortisone. This drug alleviates the inflammatory cycle by reducing the cytokine response and T cell inhibition and facilitating apoptosis of activated immune cells.

Exercise as a disease-modifying agent and a rehabilitation tool (which can slow down the effects of the disease) also plays a very important role. Exercise regimens that are commonly used in animal studies include free voluntary running in a wheel, forced running in the treadmill and resistance swimming models.[19] Also, it has been observed that in animal models of cerebral ischemia in MS, growth factors of neurons and factors inhibiting apoptosis of neurons were increased by treadmill exercise.[20] Exercise was also found to have a positive impact in dementia with cognitive disorder features and improved recovery from post-acute injuries of CNS.[21] In particular, exercises have positive correlations with improved neuronal plasticity and neuronal regeneration.[22] According to Rossi et al., exercise was found to confer neuronal protection in experimental autoimmune encephalomyelitis.[23] Exercise forms one of basis for treatment models of MS, reversing changes such as axonal demyelination and degradation of MBP.[24] This study employed a Wistar rat animal model using Cup to induce demyelination, which mimics the RRMS.

Objectives

The aim of this study was to determine the effects of aquatic exercise of varying intensity as a neuroprotective factor and a method of promoting neuronal plasticity using animal study. The authors aimed to determine the efficacy of varying intensity of exercise against demyelination induced by Cup, and to analyze the effects of exercise on oligodendrocyte regeneration in the corpus callosum using Luxol fast blue (LFB) staining and MBP immunohistochemistry, subsequent astrogliotic proliferation using glial fibrillary acidic protein (GFAP) immunofluorescence, and neuronal plasticity changes in the hippocampus region of the rat brain caused by brain-derived neurotrophic factor (BDNF) expression using quantitative real-time polymerase chain reaction (qRT-PCR), and finally to compare all the above results with the neuroprotective effects of MP administration.

Materials and methods

Animals and chemicals

Male Wistar rats weighing 150–200 g were used in the study. Animals were procured from Biogen Laboratory Animal Facility (Bangalore, India). The animals were maintained in an air-conditioned room with a 12-h light/12-h dark cycle; standard chow diet and water were provided ad libitum throughout the experimental period. The study was conducted between September and October 2020, after receiving proper Institutional Animal Ethical Clearance from the Saveetha University, Chennai, India (approval No. SU/CLAR/RD/010/12/2020).

The 0.2% Cup was purchased from Sigma-Aldrich (St. Louis, USA), and hydroxypropyl cellulose was obtained from Sisco Research Laboratories (Mumbai, India).

Experimental procedure and design

The animals were divided into 6 groups, with 6 rats in each group. After a week of acclimatization, the experiment was carried out for 8 weeks for groups I–V and 11 weeks for group VI. The animals in group I (control group) were administered 1.5 mL of 1% hydroxypropyl cellulose (HPC)/kg body weight (b.w.), per oral (p.o.) for 35 days. Group II (Cup group) was administered 450 mg of Cup/kg b.w. dissolved in 1.5 mL of 1% HPC p.o. for 5 weeks.[25] Group III (Cup+MP group) rats were administered 450 mg of Cup/kg b.w. p.o. for 5 weeks, and from the 3rd week, in addition to Cup administration, 20 mg of MP/kg b.w. were administered intraperitoneally for 3 weeks. Group IV (Cup+low-intensity exercise (LIE) group) rats were administered 450 mg of Cup/kg b.w. p.o. for 5 weeks, and from the 3rd week of Cup administration, free swimming with no resistance for 40 min for 5 weeks was included. Group V (Cup+high-intensity exercise (HIE) group) rats were administered 450 mg of Cup/kg b.w. p.o. for 5 weeks, and from the 3rd week of Cup administration, swimming with an added resistance of 9% b.w. for 5 weeks was included. Rats in group VI (conditioning (Cn)+Cup+HIE group) were made to swim 40 min a day without additional resistance for 3 weeks; after 3 weeks, the administration of 450 mg of Cup/kg b.w. p.o. commenced and was continued for 5 weeks; finally, from the 3rd week of Cup administration, an exercise program, the same as in group V, was initiated.

The Cup solution was prepared according the dosage precribed for a given day and administered through oral gavage.

Exercise regimen

The rats in group VI were preconditioned with free swimming in a circular tank with a depth of more than 50 cm, with a water temperature of 30 ±5°C for 40 min, 5 times a week, before Cup administration. After 3 weeks of Cup administration, rats in the Cup+LIE group were made to swim with no additional resistance for 40 min, 5 days a week, for 5 weeks.[26] For Cup+HIE and Cn+Cup+HIE groups, a resistance of 9% of b.w. in the form of a metal ring was tied around the tails of the rats, and they were made to swim for 40 min, 5 days a week for 5 weeks.[27] In case of slippage of the ring from the tail, the timer was paused, the ring was put back and timer continued for the remaining duration of the exercise. The intensity grading based on the load was adapted from a study performed by Gobatto et al.[28]

Induction of demyelination and methylprednisolone administration

Cuprizone, a primary copper chelator, induces selective oligodendrocyte apoptosis after 3 weeks of administration, which is followed by activation of innate neuroglial and immune cells in the brain by astrocytes and microglial proliferation. Finally, Cup administration leads to demyelination of distinct white and grey matter areas.[29] There is only a minimal involvement of the blood–brain barrier and cells of the immune system are believed to play a role in demyelination induced by Cup.[30]

Demyelination induction was achieved by administration of 450 mg of Cup/kg b.w. dissolved in 1.5 mL of 1% HPC.[31] The control group received 1.5 mL of 1% HPC. After 3 weeks of Cup administration, 20 mg of MP/kg b.w. were administered intraperitoneally for 3 weeks.[32]

Tissue preparation

After the assigned experimental period, the rats were euthanized by an overdose of 1% isoflurane. After the rats were perfused intracardially with 50 mM phosphate-buffered saline (PBS), the brain tissue was carefully dissected, washed in PBS and transferred to formalin containers for histopathology. The brain specimens intended for immunohistochemistry, immunofluorescence and qRT-PCR were wrapped in aluminum foil and kept in refrigeration at –80°C.

Luxol fast blue staining

This procedure was carried out as described in the literature.[33] The coronal sections of brain tissue were stained with LFB. Brain tissue slides were incubated in LFB solution (0.1%; Polysciences Inc., Warrington, USA) for 24 h at room temperature. The sections were rinsed thoroughly in distilled water and then dipped in lithium carbonate solution (0.05%; Polysciences Inc.) several times. The differentiation of the sections was then continued by repeatedly dipping the sections in alcohol reagent (70%) until the gray matter became colorless and the white matter remained blue. Then, the sections were rinsed in distilled water and incubated with cresyl echt violet (0.1%; Polysciences Inc.) for 2–5 min. Next, the sections were rinsed quickly in distilled water, dehydrated quickly in 3 changes of absolute alcohol, cleared in 3 changes of xylene, and mounted using a mounting medium. The slides were viewed under the Olympus binocular bright-field microscope (model DM 1000 LED; Olympus Corp., Tokyo, Japan) at ×60 magnification.

MBP immunohistochemistry

This procedure was carried out according to the method designed by Khodanovich et al.[34] The tissues were immersed in ice-cold PBS followed by immersion in freshly prepared filtered 4% paraformaldehyde (PFA) in PBS. After embedding, the tissues were cut using the Leica cryostat CM1850 (Leica Biosystems, Deer Park, USA) and stored in cryoprotective solution (25% glycerol, 25% ethylene glycol in PBS) at –20°C. The sections were incubated with polyclonal rabbit anti-MBP (1:1000; Abcam, Cambridge, UK)

overnight at 25°C, and then treated with streptavidin-peroxidase complex (1:200; Abcam). Sections were visualized using the reaction with 3,3-diaminobenzidine tetrachloride (Sigma-Aldrich) in 0.1 M Tris-HCl buffer (pH 7.2), and then dehydrated, mounted on a slide and visualized under a bright field microscope (Labomed Lx-500 HL Binocular Miscroscope; Labomed, Mumbai, India).

GFAP immunofluorescence

Chronic demyelination usually leads to astrocyte proliferation. This procedure was carried out with the method outlined by Madadi et al.[35] The frozen sections of corpus callosum (10 µm) were dried for 2 h at room temperature and then fixed with 4% PFA in PBS for 20 min. After blocking nonspecific antibody binding with 5% non-fat dried milk for 20 min, the sections were incubated overnight with rabbit anti-GFAP (1:4000; Enzo Life Sciences, Farmingdale, USA) diluted in blocking buffer at 4°C. Subsequently, the sections were washed in Tris-buffered saline (TBS) and incubated in a dark, humid chamber with the appropriate fluorescently labeled IgGs-Alexa Fluor 594 (1:400; Enzo Life Sciences) combined with donkey anti-rabbit IgG Alexa Fluor 488 (1:400; Enzo Life Sciences) diluted in TBS with Tween 20 (TBS-T) for 2 h at room temperature. After several washes in TBS, the sections were mounted using polyvinyl alcohol mounting medium. Immunofluorescence was examined using a Leica confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany).

Gene expression level of BDNF in the hippocampus detected with qRT-PCR

This procedure was carried out using the method previously described by Altieri et al.[36] The total RNA was prepared from the brain tissue using a TRIzol reagent (RNeasy® Mini kits; Qiagen, Hilden, Germany). The qPCR Master Mix Kit was purchased from Invitrogen (Waltham, USA). Complementary DNA was first synthesized from total RNA using reverse transcriptase. The qRT-PCR was performed using Applied Biosystems instrument QuantStudio 7 Pro (Waltham, USA). The operating conditions were as follows: for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) – 30 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 30 s; for BDNF – 27 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s and extension at 72°C for 30 s. The PCR products were separated on 1.2% agarose gels and stained with ethidium bromide. The density of each band was quantified using an image analyzing system (optical detection module in QuantStudio 7 Pro). The expression levels were compared with each other by calculating the relative density of the target band, such as BDNF, to that of GAPDH. The primer sequence is presented in Table 1.

Statistical analyses

All data are expressed as mean ± standard deviation (SD). Normality and equal variance of the data were analyzed using the Shapiro–Wilk test and the Brown–Forsythe test, respectively. Comparisons between groups were made using one-way analysis of variance (ANOVA) with Tukey's post hoc multiple comparison test. All statistical analyses were performed using SigmaPlot v. 14.5 (Systat Software Inc., Chicago, USA). A value of $p < 0.001$ was considered statistically significant. Immunostained slides were quantified using ImageJ software (National Institutes of Health (NIH), Bethesda, USA). The expression of the *BDNF* gene was measured using GAPDH as an internal control.

Results

Exercise improved remyelination as seen in LFB staining of the corpus callosum

The LFB staining was conducted to assess the myelin content in the corpus callosum. Widespread demyelination with increased degradation and vacuolation was evident in the Cup group (group II) compared to normal myelination observed in the control group. The Cn+Cup+HIE group showed better remyelination, as detected with blue staining of the myelinated areas visible in Fig. 1, similarly to the control and Cup+MP group images, with minimal areas of remyelination seen in Cup+LIE and Cup+HIE groups. Figure 1 presents the effect of exercise in Cup-induced changes on LFB staining in the corpus callosum region.

Distribution of data

The assumptions of ANOVA were based on normality and equal variance test. The data were found to be normally distributed in all groups. In the analysis of the percentage of cells that show MBP expression (MBP+ve cells %) and the percentage of the cells that show GFAP expression (GFAP+ve cells %), and the analysis of BDNF expression in the hippocampus, normal distribution was found, with $p = 0.893$, $p = 0.395$ and $p = 0.979$, respectively. Similarly, equal variance, determined using Brown–Forsythe test,

Table 1. Primer sequence for quantitative real-time polymerase chain reaction (qRT-PCR) brain-derived neurotrophic factor (BDNF) in the hippocampus region

Genes	Primer right	Primer left
BDNF (153bp)	5'-CAG GGG CAT AGA CAA AAG-3'	5'-CTT CCC CTT TTA ATG GTC-3'
GAPDH (409bp)	5'-ATC CCATCA CCA TCT TCC AG-3'	5'-CCT GCTTCA CCA CCT TCT TG-3'

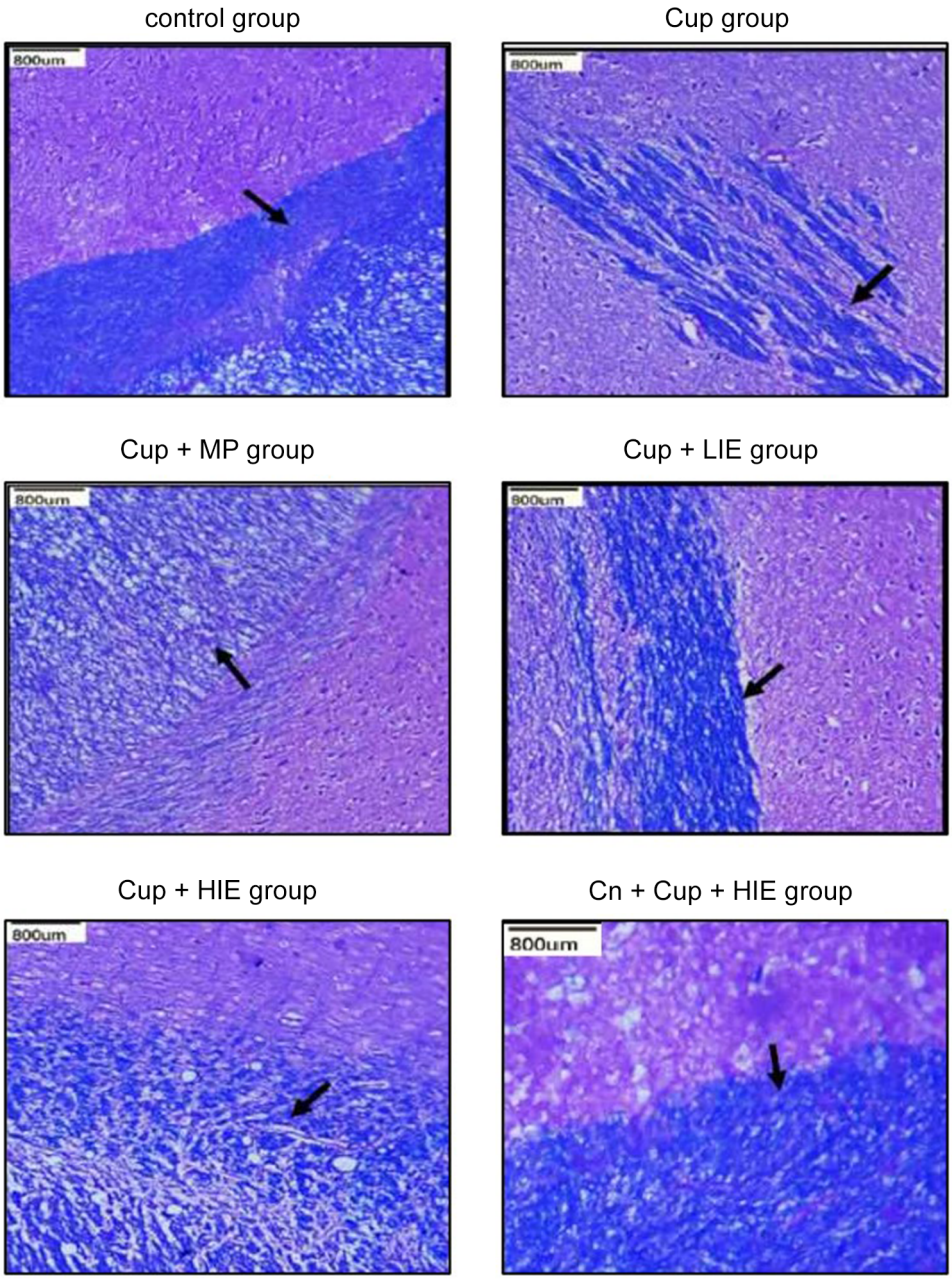


Fig. 1. Effect of exercise in Cup-induced changes on LFB staining. The control group showed normal myelination of the corpus callosum, while the Cup group showed more demyelination. The Cup+MP group showed improved myelin formation. The Cup+LIE and Cup+HIE groups showed remyelination in a minimum area. The Cn+Cup+HIE group showed an increased area of remyelination, which was almost as same as control

Cup – cuprizone; LIE – low-intensity exercise; HIE – high-intensity exercise; MP – methylprednisolone; LFB – Luxol fast blue; Cn – conditioning.

was also found, with p = 0.489, p = 0.883 and p = 0.270, respectively, for all 3 above parameters.

Exercise improves MBP expression in the corpus callosum

The MBP is the initial and reliable marker for remyelination. Therefore, MBP expression can act as a reliable guide to establish remylination criteria. MBP immunopositivity in the corpus callosum of the rat brain was drastically reduced in the Cup group and substantially improved in the Cup+MP group and Cn+Cup+HIE group. The Cup+LIE and Cup+HIE groups showed similar minimal expression. The quantitative analysis of cells expressing MBP showed a considerable decrease in MBP expression in the Cup group (8.267 ±0.351) compared to the control group (30.033 ±0.569%), with p < 0.001. It was found that MBP expression was increased in the Cup+MP group (22.967±0.473) and in Cn+Cup+HIE group (21.733 ±0.777); when these 2 groups were compared, p-value was found to be 0.128, with no statistical difference between Cup+MP (standard drug group) and Cn+Cup+HIE group, thereby showing that they produced similar results. The Cup+LIE group (13.333 ±0.306) and Cup+HIE group (17.467±0.153) presented moderate improvement, showing statistical significance when compared with control, Cup and Cup+MP groups.

Figure 2 presents the effect of exercise on Cup-induced changes, measured with MBP immunohistochemistry and quantitative analysis of MBP expression in corpus callosum.

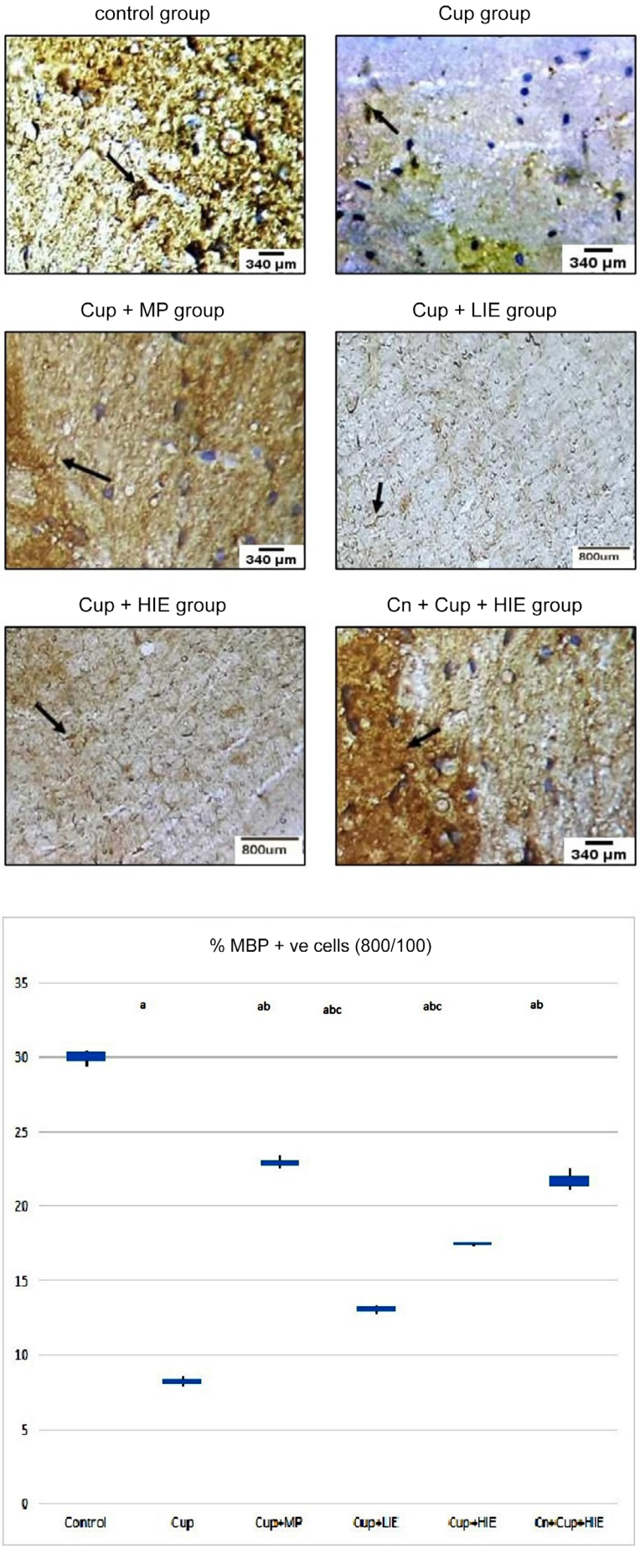


Fig. 2. Effect of exercise in Cup-induced changes measured with MBP immunohistochemistry and quantitative analysis of expression of cells expressing MBP in corpus callosum, analyzed with ImageJ software (National Institutes of Health (NIH), Bethesda, USA) and presented with a 800-µm scale bar and ×100 magnification. The MBP expression in the corpus callosum of the rat brain is shown with the arrow mark. The MBP immunopositive cells were less expressed in the Cup group compared to the control group. The highest expression was seen in the Cup+MP group, and only slightly lower the Cn+Cup+HIE group, even lower in the Cup+HIE group, and the lowest in Cup+LIE group. Values are presented as mean ± standard deviation (SD; n = 6 in each group). The F-values and p-values were determined using one-way ANOVA with Tukey's test

MBP – myelin basic protein; Cup – cuprizone; LIE – low-intensity exercise; HIE – high-intensity exercise; Cn – conditioning; MP – methylprednisolone; ANOVA – analysis of variance; a – significantly different from the control group; b – significantly different from the Cup group; c – significantly different from the MP group.

Exercise caused reduced GFAP expression measured with immunofluorescence

Active astrocytic proliferation is observed in chronic stages of demyelination. As presented in Fig. 3, high expression of GFAP was seen in the Cup group, while Cn+Cup+HIE group and Cup+MP group showed a reduced expression of GFAP in the corpus callosum. The Cup+HIE and Cup+LIE groups showed moderate GFAP expression. The quantitative analysis of cells expressing GFAP showed a definite increase in the Cup group (112.867 ±0.551) compared to the control group (30.550 ±1.054). This result was statistically significant ($p < 0.001$). The Cup+MP group showed a decline in GFAP expression (61.233 ±0.643), while the Cn+Cup+HIE group showed a better response than the Cup+MP group (50.367 ±0.513) ($p < 0.001$). The Cn+Cup+HIE group showed a 17.74% decrease of GFAP expression in comparison to Cup+MP (standard drug) group, which shows that there was less astrogliosis in Cn+Cup+HIE group compared to Cup+MP group. The Cup+LIE (80.900 ±1.127) and Cup+HIE (77.000 ±0.557) groups showed moderate response in comparison to the control, Cup and Cup+MP groups. This result was statistically significant ($p < 0.001$).

Figure 3 presents the effect of exercise on Cup-induced changes, measured with GFAP immunofluorescence and quantitative analysis of expression of cells expressing GFAP in the corpus callosum.

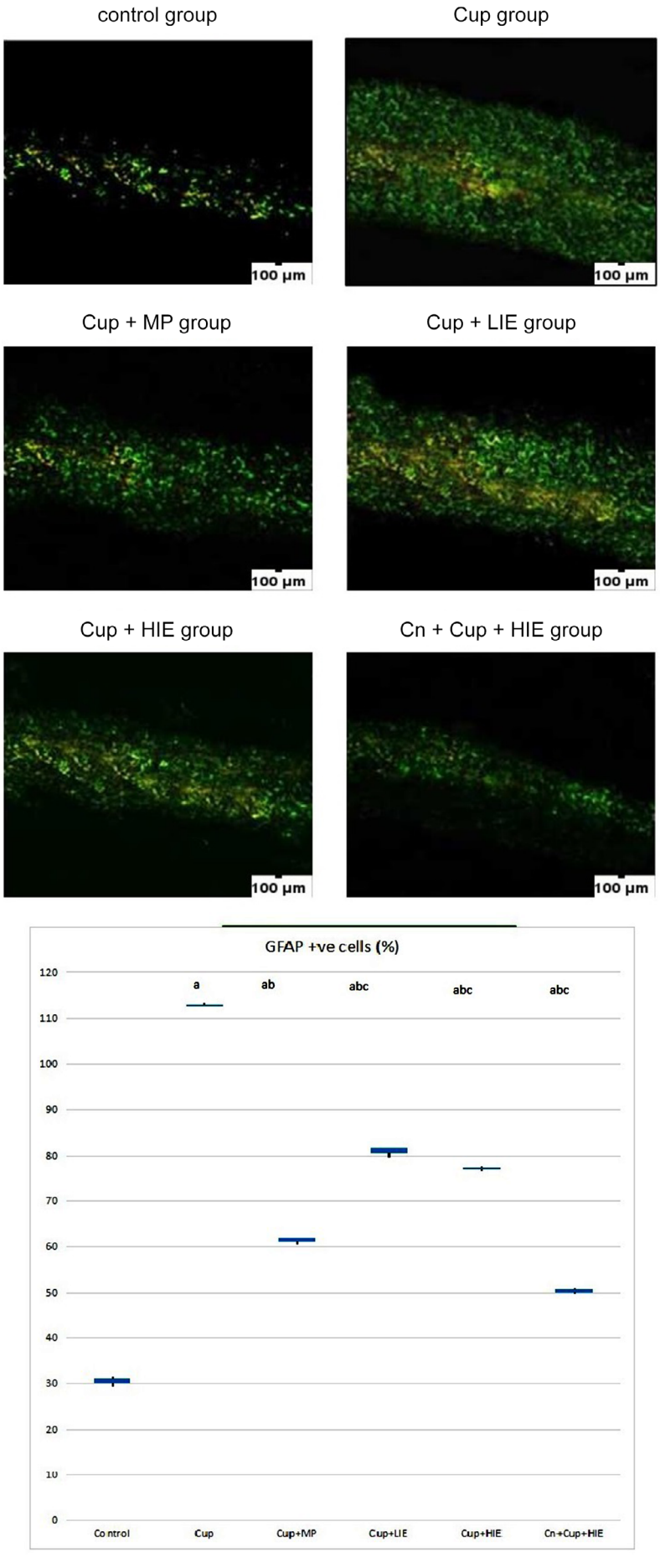
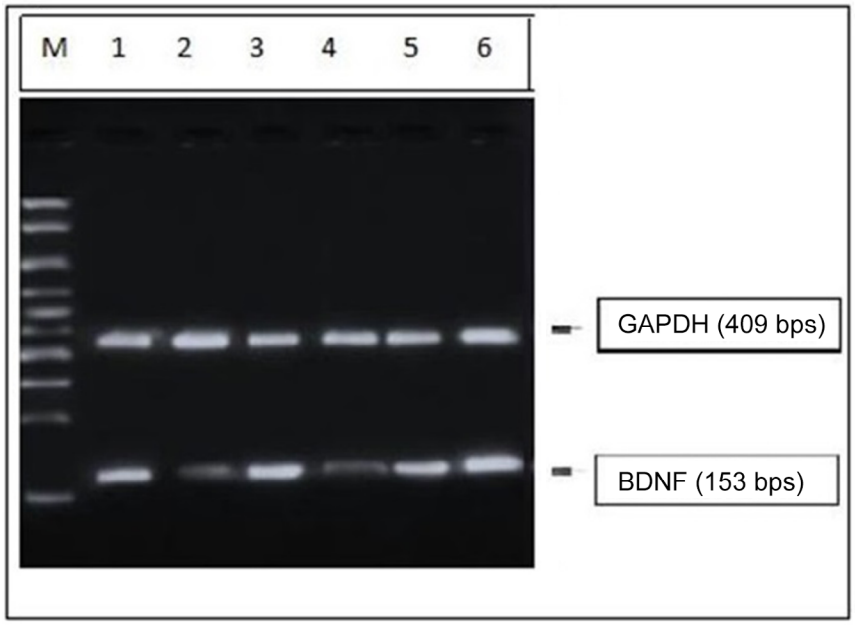


Fig. 3. Effect of exercise on Cup-induced changes measured with GFAP immunofluorescence and quantitative analysis of expression of cells expressing GFAP in the corpus callosum, analyzed using ImageJ software (National Institutes of Health (NIH), Bethesda, USA) and presented with a 100-µm scale bar and ×100 magnification. Immunopositivity to GFAP was higher in the Cup group compared to the control group. Lower expression was observed in the Cup+MP group, and the Cn+Cup+HIE group showed markedly declined expression, while Cup+HIE and Cup+LIE groups showed moderate expression. Values are presented as mean ± standard deviation (SD; n = 6 in each group). The F-values and p-values were determined using one-way ANOVA with Tukey's test

Cup – cuprizone; LIE – low-intensity exercise; HIE – high-intensity exercise; Cn – conditioning; MP – methylprednisolone; GFAP – glial fibrillary acidic protein; ANOVA – analysis of variance; a – significantly different from the control group; b – significantly different from the Cup group; c – significantly different from the MP group.

Gene expression levels of BDNF in hippocampus region measured with qRT-PCR

The BDNF is an important marker for neuroplasticity. Its expression was the lowest in the Cup group and the highest BDNF expression in the Cn+Cup+HIE group compared to Cup+MP and control groups. The second lowest BDNF expression was found in the Cup+LIE group, and it was comparably low in the Cup+HIE group, as seen in Fig. 4. The quantitative analysis revealed reduced BDNF expression in the Cup group (0.653 ±0.0651%) compared to the control group (2.693 ±0.179%) ($p < 0.001$). The highest expression was seen in the Cn+Cup+HIE group (3.470 ±0.0436%) followed by the Cup+MP group (3.123 ±0.0961%) ($p < 0.001$). The Cn+Cup+HIE group showed a 28.85% higher BDNF expression compared to the control group, whereas Cup+MP group showed a 15.96% higher expression compared to the control group. In comparison to the Cup+MP group, the Cn+Cup+HIE group showed an 11.11% increase in BDNF expression, whereas BDNF expression in the hippocampal region in the Cn+Cup+HIE group exceeded such expression in the standard drug Cup+MP group. The BDNF expression in the Cup+LIE (0.973 ±0.121) and Cup+HIE (1.823 ±0.0929) groups did not show much improvement. Cup+LIE group showed no statistical significance when compared to Cup group and showed decreased BDNF expression in comparison to the control and Cup+MP groups, which was statistically significant



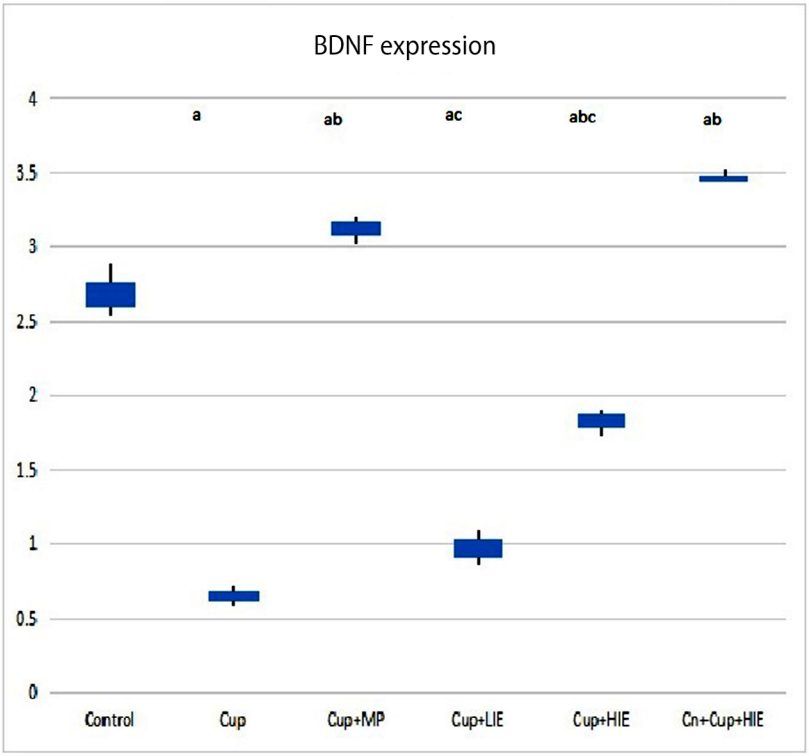


Fig. 4. Effect of exercise on Cup-induced changes of the gene expression, measured with BDNF and quantitative analysis of BDNF expression in the hippocampus region. Lane 1 – control; lane 2 – Cup group; lane 3 – Cup+MP group; lane 4 – Cup+LIE group; lane 5 – Cup+HIE group; lane 6 – Cn+Cup+HIE group. The BDNF expression was found to be the lowest in the Cup group and the second lowest in the Cup+LIE group. The highest expression of BDNF was found in the Cn+Cup+HIE group, followed by the Cup+MP group and Cup+HIE group. The BDNF expression in the Cn+Cup+HIE group was found to be higher than in the control group. Values are presented as mean ± standard deviation (SD; n = 6 in each group). The F-values and p-values were determined using one-way ANOVA with Tukey's test

Cup – cuprizone; LIE – low-intensity exercise; HIE – high-intensity exercise; Cn – conditioning; MP – methylprednisolone; BDNF – brain-derived neurotrophic factor; ANOVA – analysis of variance; GAPDH – glyceraldehyde-3-phosphate dehydrogenase; a – significantly different from the control group; b – significantly different from the Cup group; c – significantly different from the MP group.

($p < 0.001$). The expression of the Cup+LIE group showed no statistical difference when compared to the Cup group ($p = 0.053$). The expression of the Cup+HIE group (1.823 ±0.0929%) was found to be statistically significant in comparison with control, Cup and Cup+MP groups.

Figure 4 presents the effect of exercise on Cup-induced changes, measured with the gene expression of BDNF and quantitative analysis of BDNF expression, in the hippocampus region.

The summary of mean and SD levels of all groups with degrees of freedom (df), sum of squares (SS), mean of sum of squares (MS), and with F-values and p-values for all parameters mentioned is presented in Table 2.

Discussion

For the experimental study of MS pathology, a toxic model using Cup can work as an appropriate model to study the remyelination process.[37] In this model, the corpus callosum is the primary area showing white matter degeneration; other brain regions such as basal ganglia and

Table 2. Comparative effectiveness of MP, LIE, HIE, and Cn+HIE in Cup-induced demyelination, measured using quantitative analysis of 1) MBP+ve cells %, 2) GFAP+ve cells % in the corpus callosum region and 3) BDNF expression in hippocampal region

Sample	Parameters	Groups	Mean	SD	df	SS	MS	F-value	p-value
1	MBP+ve cells % in corpus callosum region, measured with ImageJ software and presented with a 800-µm scale bar and ×100 magnification	control Cup Cup+MP Cup+LIE Cup+HIE Cn+Cup+HIE	30.033 8.267 22.967 13.133 17.467 21.733	0.569 0.351 0.473 0.306 0.153 0.777	5	890.660	178.132	768.915	<0.001
2	GFAP+ve cells % in the corpus callosum region, measured with Image J software and presented with a 100-µm scale bar	control Cup Cup+MP Cup+LIE Cup+HIE Cn+Cup+HIE	30.500 112.867 61.233 80.900 77.000 50.367	1.054 0.551 0.643 1.127 0.557 0.513	5	12058.358	2411.672	3942.787	<0.001
3	quantitative analysis of BDNF expression in the hippocampus region	control Cup Cup+MP Cup+LIE Cup+HIE Cn+Cup+HIE	2.693 0.653 3.123 0.973 1.823 3.470	0.179 0.065 0.096 0.121 0.092 0.043	5	20.135	4.027	342.407	<0.001

SD – standard deviation; df – degrees of freedom; SS – sum of squares; MS – mean of sum of squares; BDNF – brain-derived neurotrophic factor; Cup – cuprizone; LIE – low-intensity exercise; HIE – high-intensity exercise; MP – methylprednisolone; MBP – myelin basic protein; GFAP – glial fibrillary acidic protein; MBP+ve cells % – percentage of cells that show MBP expression; GFAP+ve % cells – percentage of cells that show GFAP expression.

hippocampus are also affected.[38] In the present study, it was found that conditioned animals subjected to HIE encompassing swimming regimen showed improved areas of remyelination as seen in LFB staining, improved expression of MBP measured using immunohistochemistry, improved expression of GFAP measured using immunofluorescence, and improved genetic expression of BDNF in the hippocampal region analyzed using qRT-PCR. These results were concurrent with findings of Kim and Sung regarding MBP expression.[39] Selective ablation of astrocytes leads to a better remyelination response, as documented in a study by Madadi et al., with GFAP expression measured using immunofluorescence and improved areas of remyelination visualized with LFB staining.[35] In a study conducted by Gentile et al. and employing swimming protocol, BDNF levels were found to be significantly increased in mice with induced EAE.[40]

Aquatic exercise is particularly beneficial for MS patients due to its 3 vital effects: 1) buoyancy, whereby the load and stress on the joints are reduced; 2) viscosity, which leads to decreased drag and multiplanar movements; and, most importantly, 3) thermodynamics. As per the Uhthoff's phenomenon, symptoms of MS worsen in case of a setting that increases the body temperature.[41] Swimming exercise does not increase the temperature levels and maintains an optimum body temperature.[42] The LFB staining, one of the standard methods of visualizing white matter, showed improved effects of physical activity in the experimental groups (Cn+Cup+HIE and Cup+HIE groups), as was evidenced by the increased myelination in the exercise groups, which was in accordance with a study by Kim and Sung, where the demyelination in the spinal cord was studied.[39]

Myelin basic protein is important for maintaining the structural stability of myelin and is an essential component for efficient nerve conduction.[43] Prior regular exercise has been shown to have a positive impact on reducing demyelination and axonal damage in the spinal cord of EAE animals, as well as on dendritic damage in striatal neurons.[44] A decrease in MBP expression reflects the demyelinating status of MS.[45] The present study showed a decrease in MBP levels in the Cup group, and, conversely, improved MBP expression in Cup+MP and Cn+Cup+HIE gropus. The Cup+HIE and Cup+LIE groups showed only negligible improvement. Similar results regarding MBP expression were observed in the spinal cord, where the effects of free swimming were analyzed.[39] This study was the first to throw light on the neuroprotective effects of the preconditioning exercise program introduced together with the HIE regimen. Astrocytes are found to be promoters of demyelinating lesions, which are carried out by secretion of chemokines that recruit microglial inflammatory cells, which in turn restrict the process of remyelination.[46] Astrogliosis is a feature observed in chronic stages of MS.[47] The GFAP, a marker for astrogliosis, is found to be elevated in chronic stages of demyelination.[48] Better results with reduced astrocyte proliferation were observed in the Cn+Cup+HIE group. An improved remyelination was also reported in a study by Madadi et al., who performed selective astrocyte ablation with long-term Cup administration.[35]

Immunomodulatory effects of exercise (in the form of treadmill training) against neural damage and demyelination were analyzed and found to cause improvement in patients' condition.[49] One of the strongest and most reliable effects of exercise on the brain of treated rats was the upregulation of BDNF, an important marker for neuronal plasticity, which increases the number and synaptic uptake of α-amino-3-hydroxy-5-methyl-4-isoxazole

propionic acid (AMPA) receptors in experimental hippocampal slices and hippocampal neuronal cultures.[50]

According to Pencea et al., BDNF stimulates the recruitment of supraventricular zone cells and their migration, and facilitates the differentiation of neurons.[51] The present study showed an increase in expression of BDNF in the hippocampus of conditioned rats subjected to a HIE swimming protocol, which was in accordance with a study by Kim et al.[52] Motor activity in the form of aquatic exercise is found to have more neuroprotective and long-term effects regarding gene expression in the hippocampus of Cup-treated brains of Wistar rats.[53,54]

The fragmentation of DNA was seen in the dentate gyrus of the hippocampus in rats with induced MS. When treated with swimming exercise, reduced fragmentation of DNA and thereby, an improvement in short term memory was noticed.[55] Similar results were found in the present study, where the Cn+Cup+HIE group showed increased effects of neuroprotection with improved areas of remyelination in the corpus callosum and improved neuronal plasticity, as shown by BDNF expression in the hippocampal region. The expression of BDNF was found to be the highest in Cn+Cup+HIE group, even higher than in the standard drug Cup+MP group. It can be corelated with delaying or preventing secondary memory and functioning impairment in MS.

The current study shows the importance of a general exercise routine and thereby an improved fitness level, which, as shown by conditioning program in the current study, induced consistent protective effects on demyelination and also improved expression of BDNF in the hippocampal region. Providing and maintaing a standard and consistent environment for the exercise protocol were found to be challenging in this study. The scope of the study can be expanded by analyzing and comparing other modalities of exercise with aquatic therapy, also in other neurodegenerative disorders.

Limitations

This study examined the acute demyelination and remyelination changes caused by the administration of Cup. If the duration of Cup administration was prolonged, the chronic changes and subsequent effects could have been studied further.

Conclusions

Prior exercise conditioning program confers neuroprotective and remyelinating effects in rats subjected to induction of demyelination with Cup, in addition to leading to better fitness level. High-intensity exercise showed comparable significance, while the least significant effects were observed in LIE. Therefore, in MS, the HIE protocol can be applied, and in the long run, improved general fitness results in neuroprotection, in addition to the traditional cardioprotective effects documented so far, can be observed. The RRMS is observed in the initial stage. For the initial stage, the treatment involves the use of steroids and maintenance LIE only during relapses. Instead, a challenging HIE program can be the norm, keeping the basal body temperature constant throughout the disease course, irrespective of relapse or remission. Also, this study stresses the importance of an exercise program in general, even in an otherwise healthy population, to bring out better results when a neural pathology may be detected.

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