The Endurance Training Effects on Apoptosis in the Rat Hippocampus Following Transient Common Carotid Artery Occlusion

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Abstract

**Introduction & Objective:** Apoptosis, one of the most important mechanisms explaining stroke-induced functional deficit severity, might be reduced by physical rehabilitation. The purpose of this study is to assess effects of an 8-week endurance training both on apoptosis-related genes and proteins expression levels after a transient common carotid artery occlusion (tCCAO) in rats.

**Methods:** Thirty Wistar male rats were randomly assigned into one of the 3 following groups: Control, tCCAO and tCCAO with endurance training (tCCAO+END). The tCCAO lasted 45 min. The endurance training was performed on treadmill over an 8-week period (5 days per week). BAX and BCL2 genes and proteins expression levels were used by qPCR, immunohistochemistry. Nissl staining was also carried out at the end of the training protocol.

**Results:** The BAX expression increased following tCCAO but decreased when endurance training was performed (p<0.05). The BCL2 expression decreased following tCCAO and increased with training (p<0.05). In parallel, the cell death in the hippocampus was reduced when rats were trained.

**Conclusion:** The endurance training can reduce the pro-apoptotic events after tCCAO suggesting a neuronal survival in the CA1 hippocampus. The link between apoptosis and functional recovery after training seems to be of clinical interest, and thus, should be more investigated.

1. Introduction

Transient common carotid artery occlusion (tCCAO) is one cause of cerebrovascular events with a prevalence of approximately 0.24–5% in stroke patients. It can strongly affect patient quality of life by leading to both vascular dementia (VD) and/or motor dysfunctions. The tCCAO is known to damage the hippocampus, a brain structure showing an important degree of synaptic plasticity and adult neurogenesis influencing the functional recovery. To limit such impairments, physical rehabilitation is the main strategy to improve recovery in the absence of pharmacological compounds. Surprisingly, there is little information regarding the effectiveness of functional rehabilitation strategies on brain health following tCCAO.

Among the different stroke rehabilitation strategies, endurance training is well known to improve cardiovascular, muscular and cerebral functions after brain injury (Pin-Barre et al., 2021). Indeed, it seems to be crucial to reduce the neurological deficit score and infarct volume, but also to promote neuronal survival in the rat brain by reducing the expression of pro-apoptotic factors (Cao et al., 2019). Interestingly, pre-ischemic moderate endurance training reduces apoptosis in the hippocampal CA3 cells after tCCAO by modulating the BAX/BCL2 protein ratio and reducing the caspase-3 activation (Aboutaleb et al., 2015). It suggests that endurance training induces a neuroprotective/preventive effect in the hippocampus by promoting neuronal survival with a reduction of pro-apoptotic events when a brain damage occurs (Chen et al., 2007). However, no study has yet reported an effect of endurance training on the hippocampal apoptosis following tCCAO although it is required to define the accurate role of...
endurance training during functional rehabilitation. The purpose of this study was thus to investigate the effects of an 8-week endurance program on apoptosis-related genes and proteins (BAX and BCL2) expression in the CA1 hippocampus following tCCAO in rats.

2. Materials And Methods

2.1. Animals

Thirty adult male Wistar rats (weighing 250–300 g) that were purchased from Shahid Mirghani Research Institute of Iran were included in the study. The rats were randomly assigned into one of the following groups: a) Control, which is composed of healthy rats, b) tCCAO, which is composed of rats with a 45 min global cerebral ischemia without training and c) tCCAO + END, in which rats with tCCAO performed an 8-week endurance training.

This study was initiated after receiving the approval code of ethics committee, IR.SEMUMS.REC.1397.057, by observing all points of the ethics guide for working with animals. As in clinical trial, all acquisitions and analyses were performed in a single-blind manner. Animal suffering was monitored from clinical signs of pain and discomfort (prostrated animals with absence of movement in the cage and hunched posture, ungroomed appearance, absence of weight recovery, dehydration, and decreased urine/fecal output, piloerection, chronic porphyrin staining around eyes, nose or forelimbs, rapid respirations). Animal welfare was ensured with enriched environment. Food and water were available ad libitum. Rats were maintained at 22°C with a 12 h light/dark cycle at the Shahid Mirghani Research Institute. Animals were housed by 3–4 per cage in order to provide sufficient space for essential aspects of rat behavior and preserve social interaction according to the safety conditions of interventions.

2.2. Transient common carotid artery occlusion

The tCCAO was induced by the common carotid artery obstruction surgery method (Farhadi Moghadam and Fereidoni, 2020). To summarize, after anesthesia with 100 and 20 mg/kg of ketamine and xylazine respectively by an intraperitoneal (IP) injection, occlusion of bilateral common carotid arteries was performed for 45 minutes using a surgical clamp. After removing the surgical clamp, surgical wounds were sutured. Rats remained in a separate cage for post surgery recovery. They were monitored for 96 hours and returned to their normal cages.

2.3. Endurance training protocol on treadmill

All rats were familiarized before the experiment with treadmill inclined at 0° at 10–15 m.min⁻¹ (5 min per day, 3 times every two days, 2 weeks before surgery) to both limit stress during training and detect if rats were able to run in these conditions. The endurance training lasted 8 weeks, including 5 sessions per week. Exercise intensity and duration increased gradually week to week to reach 50 min at a speed of 30 m.min⁻¹ with a 10° slope. Touching and rubbing the tail were performed by experimenters to force the
locomotor activity when necessary. Such external motivation might be less stressful than electrical shocks.

**2.4. Tissue preparation and Cresyl violet (Nissl) staining**

Rats (n = 5 per group) were sacrificed 48 hours after the last session and related evaluation under very deep anesthesia using pentobarbital sodium (100 mg/kg; IP). Brain were removed and stored (-80°C) for 3 days in 0.1M phosphate buffer (7.4 pH) containing 0.9% saline, 4% paraformaldehyde. The paraffin coronal sections were prepared for histological staining in the range from 3.3 to 7 mm behind Bregma in accordance with Paxinos protocol and necrotic cells evaluation was done according to the Kiernan method. In this session, the 7 mm cuts of paraffin blocks were fixed and mounted on the slides. Three slices (7 µm thickness) of each sample were chosen, stained with a 0.1% cresyl violet acetate solution and covered with Entellan (Merck, Germany). The cell counting in the hippocampal CA1 region was performed by Image J software (version 1.49, NIH) after taking pictures of samples at 40X magnification using a light microscope (AX-70 Olympus). The irregular and dark cells with nuclei or uncertain nuclei were considered as cell death.

**2.5. Immunofluorescence**

The thin cuts (3.3–4.2 mm) of paraffin blocks were fixed and mounted on glass slides and after that deparaffinized by absolute xylene. These samples were stained using H&E staining (ab245880, Abcam, United Kingdom) kit and the damaged cells were observed as neurons with dark red cytoplasm and wrinkled nucleus using a light microscope (AX-70 Olympus).

The protein level of BAX and BCL2 was evaluated in thin cuts (3.3–4.2 mm) of samples after deparaffinization and dehydration of fixed slide using Xylene and Serial dilution of absolute ethanol from 96–25%, respectively. After preparation of samples, the specific monoclonal antibody against BAX (ab53154, Abcam, USA) and BCL2 (ab196495, Abcam, USA) were added into slides at the dilution of 1:150 then incubated at 37°C for 90 minutes. The stained slides are washed 4 times then nucleus staining is performed with DAPI according to Hoffman's established method*. The BAX/BCL2 ratio was measured because it is considered as one of the most popular indexes of apoptosis state.

**2.6. mRNA extraction and complementary DNA (cDNA) synthesis**

After removing the hippocampus tissue, the CA1 hippocampus were homogenized using a homogenizer device (PRO250 homogenizer, USA) then mixed cells were exposed with nitrogen liquid to improve cell lysis of tissue. The RNA extraction solution (TRIZOL®, GIBCO-USA) was added to samples and total RNA was isolated from the CA1 hippocampus according to the protocol and Optical Density of the final product was measured using a Nano drop device (Thermo Fisher-USA) in 230, 260 and 280-nanometer wavelength.
The extracted RNA was synthesized into complementary DNA (cDNA) using MMLV (Moloney murine virus reverse transcriptase) enzyme, dNTP (Deoxynucleoside triphosphates), oligo-dT and random hexamer following the manufacturer's protocol (Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit #K1622 Lot, USA). The concentration of product was measured by Nano-drop device.

2.7. Real Time PCR

The mRNA level of Bcl2 and Bax was evaluated by real-time PCR test in the CA1 hippocampus. The real-Time PCR test was done using the Syber Green method and specific primers (see below). The PCR mix was prepared using 200 ng of cDNA and 0.5 microliters of each primer (10 pmol), which were added to 10 µL of 10X Syber Green master mix (Amplicon-Korea). The remaining volume of the master mix was completed by adding deionized water. The final reaction volume should be considered 20 µL. The Real-time PCR reactions were performed in ABI7200 for 40 cycles and the additive stage for melting curve analysis was executed. The folding change of genes was calculated based on genes expression level in the healthy rats using the $2^{-\Delta\Delta CT}$ formula.

| Primers | Sequences             |
|---------|-----------------------|
| Bax-F   | GCAAACCTGGTGCTCAAGG   |
| Bax-R   | CAGCCACAAAGATGGTCA    |
| Bcl2-F  | GAGTGGGGATCTGGAGATGAAG |
| Bcl2-R  | TGGTAGCGACGAGAGAAGTC  |
| GAPDH-F | AAGTTCACGGGACAGTAGAAGG |
| GAPDH-R | CATACTCAGCACCAGCATCAC |

2.8. Statistical Analysis

All data of 3 groups were collected after each test the inserted into SPSS software version 22 (SPSS Inc., Chicago, IL). All results were evaluated and the groups were compared with a one-way ANOVA (groups) and Tukey procedure for post hoc analysis. The results were considered as significant when p < 0.05.

3. Results

3.1. The post ischemia exercise impact on apoptosis related genes and proteins in the CA1 hippocampus

No difference was found for the Bax and Bcl2 expression in the tCCAO + END and tCCAO group using real-time qPCR despite that endurance training tended to increase Bcl2 expression and decrease Bax expression. However, the BAX protein expression significantly increased in tCCAO group (p < 0.001) while the BCL2 protein expression was significantly higher in the tCCAO + END group than tCCAO group (p <
Likewise, the BAX/BCL2 ratio was higher in tCCAO group compared with tCCAO + END group (p < 0.001) (Fig. 1D-F).

3.2. The exercise effect on damaged cells

In this study, dark cells (cell death) were counted by Cresyl violet staining. Quantitative analysis shows that the Control had the lowest number of dark cells (11.43 ± 5.5) which was significantly different from other groups. However, the dark cells in the tCCAO + END group (25.8 ± 6.5) group was significantly lower than tCCAO (68.07 ± 4.2; P < 0.05). The H&E staining results would be checked in case of morphology and cell population. Integrity and structure of Corpus callosum and neural pathway areas seem unaffected in all groups. Nucleus membrane and cytoplasm appearance of cells in the tCCAO group was deformed without any intervention. Exercise intervention preserved percentage of neuron with unaffected appearance up to 35–40%.

4. Discussion

This study was the first to show that endurance training induces an anti-apoptotic effect following tCCAO. The BAX/BCL2 ratio is one of the most popular indexes of apoptosis state in tissues because it indicates pro-apoptotic signaling cascade activation. The decrease of BAX/BCL2 ratio after endurance training suggests that such training could reduce the progression of apoptosis in the CA1 hippocampus, so that it might increase neuronal survival in rats with tCCAO. This effect is supported by our protein expression results showing that the BAX positive cells population is lower after endurance training whereas a lack of difference is observed between trained rats and Control. It is also reinforced by the increase of BCL2 expression after training, which is a major anti-apoptotic marker. In accordance with our results, early training can reduce apoptosis rate in the cerebral cortex after cerebral ischemia by assessing BAX, BCL2 and Caspase 3 genes (Zhang et al., 2013). Chen et al. used TUNEL assay for apoptosis evaluation, BAX/BCL2 ratio and caspase 3 for gene and protein expression to show a beneficial effect of endurance training on neuronal survival by reducing the apoptosis progression in cerebral cortex of ischemic animals that is in line with our results (Chen et al., 2017). The reduced cell death observed in trained rats strongly suggests that endurance training on treadmill might promote neuronal survival after tCCAO. Interestingly, it was found elsewhere a link between neuronal survival and functional recovery after stroke (Sánchez-Morán et al., 2020). For instance, individuals with stroke carrying the Wrap53 human nonsynonymous single-nucleotide polymorphism (leading to DNA repair) showed less infarct volume and better functional outcome after stroke (Sánchez-Morán et al., 2020). They also showed similar results in mice with cerebral ischemia. Taken together, neuronal survival should be more investigated when assessing the effectiveness of a given treatment on functional recovery after cerebral ischemia.

In order to use a model of tCCAO leading to severe brain impairments, it seems necessary to prolong ischemia duration that is considered in this study. Indeed, our tCCAO with 45 min of artery obstruction is suitable for that purpose because the longer carotid artery occlusion, the deeper and wider brain lesion
area. However, the rodent mortality rate is also more important in that context. Nevertheless, when the animal care is reinforced during and after surgery (ensuring food and water supply and social interaction for instance), the mortality rate decreases. It thus allows providing new information on neuronal survival after cerebral ischemia.

Despite the emerging role of apoptotic markers in recovery, no study has investigated how endurance training might influence them. Yet, endurance training is well known to promote neuroplasticity in both ipsi- and contralesional hippocampus and cortex after stroke by upregulating neurotrophic markers such as the phosphorylated form of tyrosine kinase TrkB (high affinity with the brain-derived neurotrophic factor or BDNF) and neurotrophin receptor p75 (p75NTR, low affinity with BDNF) as well as the vascular-endothelial growth factor (VEGF), among others (Pin-Barre et al., 2021). This study also suggests that the effect of training on the link between neurotrophic factors and apoptotic markers, which remains elusive, should be investigated following cerebral ischemia.

Limitations of the study are related to first the clinical relevance of the exercise intensity of training sessions. The training protocol used in the present study is based on prior works (Chen et al., 2007). To agree with the principle that physical training in rodents needs to show a translational relevance in humans, exercise protocol should consider physiological indicators such as the lactate threshold (Pin-Barre et al., 2021). Nevertheless, no study has assessed endurance training effects over a 8-week period following tCCAO, which is in accordance with the stroke rehabilitation recommendations (Fisher et al., 2009). Indeed, 8 weeks of training was frequently used to ensure that training is effective to assess neuroplasticity markers (Constans et al., 2021). It should now be relevant to investigate endurance-training effects after tCCAO on cognitive performance at the behavioral level, given that we show a beneficial influence of endurance on apoptosis in the hippocampus. In addition, we are unable to determine which mechanisms are specifically related to BAX and BCL2 expression and cell death. In this context, it remains difficult to conclude that BAX/BCL2 is directly related to neuronal survival after tCCAO despite that we found a beneficial effect of cell death in the hippocampus in parallel with the pro- and anti-apoptotic marker expression.

5. Conclusion

Endurance training reduces the apoptosis in the CA1 hippocampus in rats with tCCAO. Such study is encouraging to now investigate cognitive and motor recovery in further studies in regards with hippocampal neuroplasticity events. It should help to define the accurate role of training in the tCCAO rehabilitation. These results may be important, not only in tCCAO, but also in other neurological disorders associated with oxidative neuronal damage. Different types of exercise regimens (high-intensity interval training, combined exercise rehabilitation etc...) should be evaluated to define their complementarity on the pro-apoptosis events inhibition and functional recovery.

Declarations
Ethics approval and consent to participate

The study was performed in accordance with the “Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health (NIH publication No. 85–23, revised 1996), and the present protocol was approved by the local ethics committee (IR.SEMUMS.REC.1397.057). All efforts were made to minimize animal suffering and reduce the number of animals used.

Research involving Human Participants and/or Animals

Research involve animals

Disclosure of potential conflicts of interest

Not applicable

Consent for publication

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Availability of data and materials

The authors confirm that the data supporting the findings of this study are available within the article.

Competing interests

The authors stated that they have no conflicts of interest.

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Authors’ contributions

Study concept and design: Ali Golestani and Seyed Javad Mirghani, analysis and interpretation of data: Seyed Javad Mirghani, Abdorreza Eghbal Moghanlou and Shohreh Sharifian, drafting of the manuscript: Nicolas Hugues, Jerome Laurin and Zahra Eslami, statistical analysis: Shima Gholamalipor and Malihe Milani, collected the clinical data, interpreted them and revised the manuscript: Zahra Eslami

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References

1. Aboutaleb N, Shamsaei N, Khaksari M, Erfani S, Rajabi H, Nikbakht F (2015) Pre-ischemic exercise reduces apoptosis in hippocampal CA3 cells after cerebral ischemia by modulation of the Bax/Bcl-2 proteins ratio and prevention of caspase-3 activation. J Physiol Sci 65:435–443. https://doi.org/10.1007/s12576-015-0382-7

2. Cao L-M, Dong Z-Q, Li Q, Chen X (2019) Treadmill training improves neurological deficits and suppresses neuronal apoptosis in cerebral ischemic stroke rats. Neural Regen Res 14:1387. https://doi.org/10.4103/1673-5374.253523

3. Chen X, Zhang X, Xue L, Hao C, Liao W, Wan Q (2017) Treatment with Enriched Environment Reduces Neuronal Apoptosis in the Periinfarct Cortex after Cerebral Ischemia/Reperfusion Injury. Cell Physiol Biochem 41:1445–1456. https://doi.org/10.1159/000468368

4. Chen Y-W, Chen S-H, Chou W, Lo Y-M, Hung C-H, Lin M-T (2007) Exercise pretraining protects against cerebral ischaemia induced by heat stroke in rats. Br J Sports Med 41:597–602. https://doi.org/10.1136/bjsm.2006.033829

5. Constans A, Pin-Barre C, Molinari F, Temprado J-J, Brioche T, Pellegrino C, Laurin J (2021) High-intensity interval training is superior to moderate intensity training on aerobic capacity in rats: Impact on hippocampal plasticity markers. Behav Brain Res 398:112977. https://doi.org/10.1016/j.bbr.2020.112977

6. Farhadi Moghadam B, Fereidoni M (2020) Neuroprotective effect of menaquinone-4 (MK-4) on transient global cerebral ischemia/reperfusion injury in rat. PLoS ONE 15:e0229769. https://doi.org/10.1371/journal.pone.0229769

7. Fisher M, Feuerstein G, Howells DW, Hurn PD, Kent TA, Savitz SI, Lo EH (2009) Update of the Stroke Therapy Academic Industry Roundtable Preclinical Recommendations. Stroke 40:2244–2250. https://doi.org/10.1161/STROKEAHA.108.541128

8. Pin-Barre C, Hugues N, Constans A, Berton E, Pellegrino C, Laurin J (2021) Effects of Different High-Intensity Interval Training Regimens on Endurance and Neuroplasticity After Cerebral Ischemia. Stroke 52:1109–1114. https://doi.org/10.1161/STROKEAHA.120.031873

9. Sánchez-Morán I, Rodríguez C, Lapresa R, Agulla J, Sobrino T, Castillo J, Bolaños JP, Almeida A (2020) Nuclear WRAP53 promotes neuronal survival and functional recovery after stroke. Sci Adv 6:eabc5702. https://doi.org/10.1126/sciadv.abc5702

10. Zhang P, Zhang Y, Zhang J, Wu Y, Jia J, Wu J, Hu Y (2013) Early Exercise Protects against Cerebral Ischemic Injury through Inhibiting Neuron Apoptosis in Cortex in Rats. Int J Mol Sci 14:6074–6089. https://doi.org/10.3390/ijms14036074
Figures

Figure 1

Apoptosis related genes and proteins in the CA1 hippocampus. (A-C) Gene expression of BAX and BCL2 and the BAX/BCL2 ratio, (D-E) Significant difference between groups for hippocampal CA1 BAX protein expression (F(2,6)=77.73, P<0.001), hippocampal CA1 BCL2 protein expression (F(2,6)=84.81, P<0.001), (F) hippocampal CA1 BAX/BCL2 protein ratio (F(2,6)=23.58, P<0.05), (G-H) Representative images of BAX and BCL2 protein expression and (I) Representative image of coronal hippocampal CA1 region showing both dead neuronal cells (red arrows) and unaffected neurons (blue arrows), (J) Significant difference between groups for the percentage of CA1 dead cells counted by Cresyl violet staining (F(2,6)=77.73, P<0.001).