Ultrastructural Changes of Hippocampus after Exhaustive Treadmill Running in Rats

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Abstract. Objective: Hippocampal CA1 (cornuammonis 1) pyramidal neurons are extremely sensitive to ischemia and hypoxia. Transient global cerebral ischemia can induce cell death in a particular form known as delayed neuronal death (DND). In this paper, the rats' brain hippocampal pyramidal cells and mitochondrial morphological changes were studied to further reveal the process of nerve degeneration, the impact of exercise on learning, memory and other functions. To observe the ultrastructural changes in hippocampal pyramidal cells and cerebral mitochondria of rats after endurance exercise training. Methods: The study was conducted during June 2015 to November 2017 in Zhengzhou University. Selected 40 eight-week old male SD rats, which were randomly divided into four groups: Group A (no training); Group B, the 24 h acute exhaustive exercise group (no training, acute exhaustive treadmill exercise the day before sacrificed); Group C, the endurance training group with immediate acute exhaustive exercise (endurance training implementation and acute exhaustive treadmill exercise and sacrificed immediately after exercise), and group D, endurance training and 24 hours later after acute exhaustive exercise groups (endurance training and acute exhaustive treadmill exercise, then sacrificed 24 hours later). Each group had 10 rats. For the training groups, exhaustion exercise started the next day at a rate of 10 m/min. Gradually the speed was increased up to a predetermined speed (medium and higher exhaustive exercise respectively at a speed of 20 m/min and 36 m/min), and maintained speed until exhaustion, then record the exhaustive exercise time. The training plan was rats in treadmill exercise trained once a day, 6 days per week, treadmill speed is gradually increased from the beginning of the fourth week of 10 m/min to 30 m/min, exercise time from 30 min/d to 40 min/d. Exhaustion standard for rats is driven out in the end to stay 2 s treadmill reluctant to run, and the loss of quick turn reflex. Main outcome measures: After being sacrificed, ultrastructural changes in the rats brain hippocampus pyramidal cells and their mitochondria were studied. Results: 40 SD rats completed the experimental trial and all results were analyzed. It showed that the number of apoptotic cells in the brain of rats increased significantly after endurance training and exhaustive exercise group. Exhaustive exercise increased the number of apoptotic cells, among which were mostly glial cells. The percentage of apoptotic cells in brain and the degeneration of pyramidal cell and mitochondria by transmission electron microscopy in rats' hippocampus increased significantly. Conclusion: We observed that endurance training and exhaustion exercise might cause certain damage to brain cells, the hippocampus neurons and mitochondria degeneration. This may be due to exhaustive training that caused brain tissue acidosis; meanwhile lack of oxygen caused some degeneration of brain cells that affecting the memory.

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1. Introduction

Exhaustive training induced hypoxia directly, which inevitably lead to different effects on brain function [1-4]. It has been found that prolonged high-intensity exercise, especially exhaustive exercise can cause apoptosis of skeletal muscle, cardiac cells; and their cellular damage after exercise is closely related [5-7]. Exercise induces human functional cells' apoptosis significantly, a normal physiological process of the body to clear damaged cells, and reflect the organization of self-protection function [8]. Continuous apoptosis result in a large decrease in tissues and organs' functions. Exhaustive exercise is one of the multipal mechanisms. Hippocampal CA1 pyramidal neurons are extremely sensitive to ischemia and hypoxia, and transient global cerebral ischemia can induce a special form of cell death, known as delayed neuronal death (DND).In this paper, after endurance training and exhaustive exercise, cells and morphological changes of hippocampal mitochondria were studied.

2. Materials and Methods

Design: A randomized controlled nerve morphology study. **Time and place:** The experiment was conducted in 2015-06/2017-11 at the Medical Research Center of Zhengzhou University. **Materials:** 40 male SD rats aged 8 weeks, body weight 325~452g, safe and healthy , provided by the Medical Experimental Animal Center of Henan (license number: Medical Activity No: 710126). Animals were bred and kept in ambient temperature (21 ± 2)°C, humidity 50% to 75%. A week before being sacrificed, each cage's experimental animal was checked to comply with animal ethics requirements. **Exercise protocol:** After regular feeding, 40 rats were exposed to an acute exhausted sport 3-5 d, the PT-98 -type animal treadmill for 2 days (running speed of 10 m/min, 1 time/d, time control within 10 min). After the end of the adaptation exercise, the rats were randomly divided into four groups of 10: Control group (Group A) is non-training. Group B is the 24 h after acute exercise which did not implement endurance training program, conducted only acute exhaustive treadmill exercise and after 24 h exercise were sacrificed. Group C is the acute exercise endurance training, sacrificed immediately after endurance training implementation and acute exhaustive treadmill exercise. The endurance training +24 h after acute exercise implementation (Group D) then finally acute exhaustive treadmill exercise and sacrificed 24 h after exercise. Speed of exhaustive exercise began 10 m/min, gradually increasing the speed and reached a predetermined speed in 3 min (medium strength, high speed exhaustive exercise were 20 and 36 m/min), keeping pace until exhaustion, and record Exhaustive exercise time. Endurance training programs: Rats in animal treadmill exercise training underwent it for 1/d, 6 d/week. Treadmill speed is gradually increased from the beginning of the fourth week from 10 m/min to 30 m/min, exercise time by a 30 min/d to 40 min/d. Exhaustion standard for rats driven at the end to stay 2 s treadmill as still reluctant to run, and the loss of quick turn reflex.

3. Main outcome Measures

Materials and Methods: conventional HE staining: after intraperitoneal 0.4% sodium pentobarbital anesthesia administered in rats, brain was removed immediately after decapitation and quickly placed in a 20°C precooling tray to be separated from the hippocampus. The remaining brain tissue fixed in 10% formalin solution, embedded in conventional paraffin, serially sectioned at 5μm slice, HE staining, wood dyeing liquid impregnation for 15 minutes, eosin staining for 1 minute. **Immunohistochemistry:** Bax or Bcl-2 a working fluid resistant 4°C overnight, then a second incubation for 30min at room temperature, SP working solution incubation at room temperature for 20min, DAB color, and wood grain after dyeing. **Cholinesterase staining:** Freshly prepared cholinesterases dip 1hour incubation at 37°C dye. **Nissle staining:** Frozen section 1% tar purple dye solution impregnation under 37°C for 1 hour. **The extraction of hippocampal mitochondria:** referring to the methods of Gross et al. [9] the hippocampus after cleaning, weighing, according to (W:V) to join the frozen mitochondrial extracts 4°C, 1000×g centrifugal 5

min, carefully taken the supernatant was placed on ice for later carefully transferred to another centrifuge tube 4°C 3500×g centrifugation for 10 min. The resulting mitochondrial pellet was resuspended in extraction solution (by weight join in proportion to the volume of 1:10) and repeat the centrifugation step to eventually get the precipitation is the extract of heavy hanging in the mitochondria (about 40 ml) per 100 mg of organization to join and placed in a hole on the ice of the transmission electron microscope. **TEM:** mitochondria immersed in solution containing 2.5% glutaraldehyde, O.1 mol/L acid sodium dimethyl, and fixed at 4°C for 12 h, and then 1% acid at room temperature fixed for 2 h. Transmission electron microscopy of ultrathin sections was produced by the method of dehydration, infiltration, embedding, thin sectioning and observation. The resulting purity of the preparation liquid is detected in mitochondria by the formula : purity (%) = N mito/N (100% of total N mito, and Ntotal represent an electron micrograph as seen in the total number of mitochondria and micro-organelles. **TUNEL apoptosis detection and apoptosis percentage method:** In situ apoptosis detection kit was purchased from Roche German Company operating in strict accordance with the instructions provided by the kit. Using OLYMPUS microscope counting in three high power fields (×400) under TLJNEIJ markers the number of positive nuclei (tan of nuclei having brown particles, form an irregular breaking point) and negative nuclei number , and calculated the percentage of apoptosis ([= number of positive nuclei/(number of positive nuclei tens negative nuclei)] × l0%). (Experimental design evaluators: authors have undergone formal training.).

4. Statistical analysis

All data were analyzed using SPSS13.0 statistical software for analysis and processing. The Kolmogorov-Smimov test used for data normality test; the application of single factor analysis of variance (ANOVA) and least significant difference test (LSD) data used to compare and analyze each. Each set of data are used as mean ± standard deviation and $P < 0.05$ as statistically significant difference in the standard.

5. Results

5.1 HE staining

Typical late-onset die of neurons showed cell body shrinkage, eosinophilic staining enhanced widened weak intracellular gap, karyopyknosis stain, chromatin condensation lumps, apoptotic bodies appear. Group and II hippocampal pyramidal cell layer arrangement was "C" shaped, CA1 neurons arranged neat; and a large bubbly lightly stained, interstitial dense area showed no significant pathological changes; III, IV, V hippocampal CA1 neurons derangement some appear delayed neuronal death, scattered in the pyramidal cell layer, but no significant pathological changes in CA3 region. Group V, CA1 region of delayed neuronal death was significantly reduced, and group III or IV differences were statistically significant (compared $P < 0.05$); compared III, IV group had no significant difference ($P > 0.05$). See Figure1～6.

5.2 TUNEL staining

TUNEL positive neurons scattered, nucleus colored brown. Group A and group B show no positive hippocampus neurons; C and D group CA1 area have positive neurons, mainly in the pyramidal cell layer, and CA2 area boundary, CA3 area did not show a positive neuron, See Figure7～8. Group D CA1 improved, there is significant difference compared with group B ($P < 0.05$), there was no significant difference between group C and D ($P > 0.05$). Each group's cell apoptosis after endurance training and exhaustion exercise has effect on the brain, is shown in **Table 1**. TUNEL labeling of each part of the brain's cell apoptosis, endurance training and the percentage of apoptosis compared with control group increased significantly, some parts of the apoptosis even for more than 30%. After the other 3 groups were compared with A group, the

difference was statistically significant, while among the exercise groups no significant differences, $P < 0.05$. See **Table 2.**

5.3 Endurance training and hippocampus of rats after exhaustion exercise ultrastructure comparison

Through image study analysis, endurance training and after exhaustion exercise group of rats' cerebral cortex and hippocampus neuron appeared to have an intracytoplasmic mitochondria swelling and cristae fracture and cavity, increase heterochromatin, degeneration phenomenon such as rough endoplasmic reticulum. In the cerebral cortex appeared glial cells chromatin gathered, surrounding the necrosis of neurons, engulfing neurons apoptosis phenomenon such as small body, glial cells having different degrees of swelling, endoplasmic reticulum expanded. In the hippocampus of rats in the after exhaustion exercise also appeared a lot of fat brown pigment. See Figure9-12.

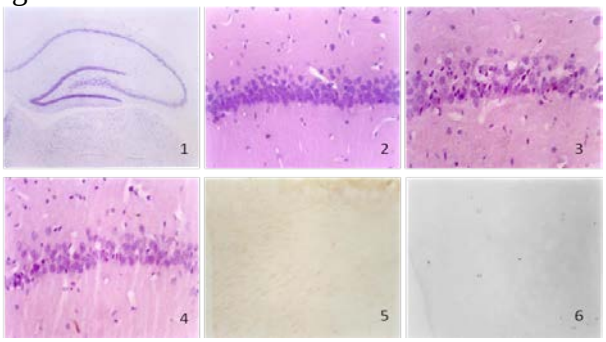

Figure 1～6

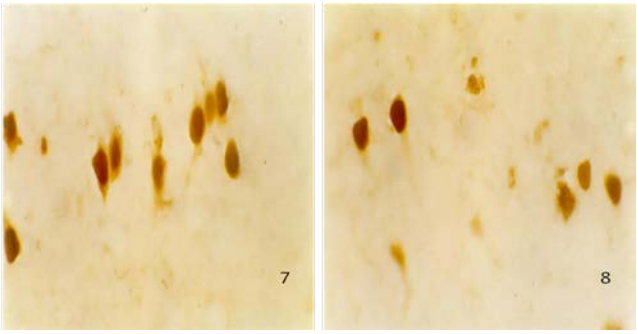

Figure 7～8

Figure 1. Group A, set of hippocampal Nissle dyeing ×200.

Figure 2. Group B, Hippocampal CA1 area HE staining ×400.

Figure 3. Group C, CA1 areaHE staining×400.

Figure 4. Group D, CA3 areaHE staining×400.

Figure 5. Group A, Hippocampal cholinesterase stain×200.

Figure 6. Group D Hippocampal umbrella cholinesterase stain×200.

Figure 7. Group C, CA1 region group TUNEL staining ×1000.

Figure 8. D group CA1 region TUNEL staining ×1000.

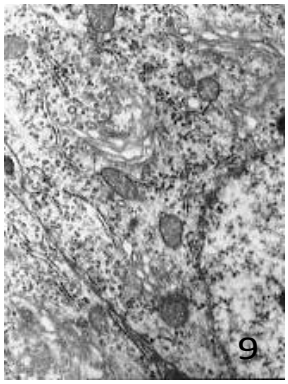

Figure9: Control group

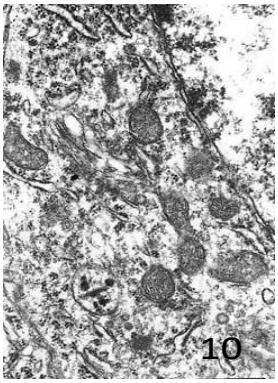

Figure 10: 24 h after acute exercise group

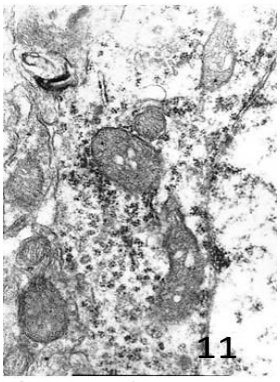

Figure 11: + after acute exercise endurance training Immediate group

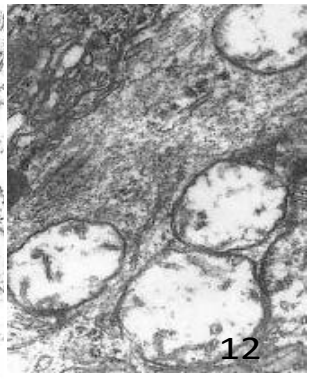

Figure 12: + after acute exercise endurance training 24 h group

Figures 9-12. Rats' hippocampus ultrastructure (×20000).

Table 1. Brain cell apoptosis of CA 1 after endurance training and exhaustion treadmill

GROUP	N	Br Aim Cell Apoptosis
Control	10	6.56+1.24
24h after acute exercise group	10	16.143.26a
Endurance training+immediately after an acute exercise group	10	29.78±1.96a
Endurance training+24 groups after acute exereise	10	32.43±2.35n

Table 2. The effect s of cortical necrotic neuron count of brain after endurance training and exhaustion treadmill running

CROUP	N	Cortical Necrotic Neuron Count of Brain
A group	10	4.90±1.20
B group	10	29.60±2.27
C group	10	20.30±1.90
D group	10	21.50±1.90

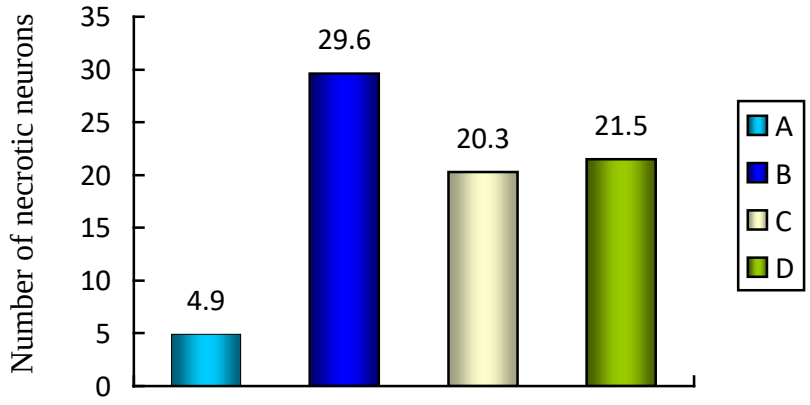

Fig 1. The effect s of cortical necrotic neuron count of brain after endurance training and exhaustion treadmill running

Compared with the A group, the difference had significant significance, while the difference between the groups was no significant. F=294.77 P<0.05

6. Discussion

This experiment showed that endurance training and exhaustive exercise could affect the rats brain cells, leading to protuberant enlargement of the cell body, and increase in the number of apoptosis, from the analysis on shape of glial cells. Acute brain cells apoptosis percentage was significantly increased after exhaustion exercise, some parts of the apoptosis number more than 30%. CA1 area scattered late-onset death of neurons, a typical cell shrinkage, acidophilic cytoplasm staining, widened cell weak gap, nuclear pyknosis thick dye, condensed chromatin clumps block or even rupture fragments, and visible apoptotic bodies. This physiological stimulation made the body to produce "two-way" adaptation, i.e. proper exercise can strengthen physique, improve health; and excessive or exhaustion exercise for a long time stress can lead to dysfunction of brain ischemic hypoxia which in turn caused apoptosis and dysfunction [10]. The experiment's results show that the exhaustion exercise can lead to a large number of brain cells apoptosis. This experiment found that endurance training and exhaustion exercise led to increase cell body, increase protuberant enlargement and apoptosis from the analysis on of glial cells shape. Endurance training and acute

brain cells apoptosis percentage were significantly increased after exhaustion exercise, some parts of the apoptosis account for more than 30%. CA1 area scattered late-onset death of neurons, a typical cell shrinkage, acidophilic cytoplasm staining, cell weak gap widened, nuclear pyknosis thick dye, condensed chromatin clumps block or even rupture fragments, visible apoptotic bodies.

Experiment observed in endurance training and after exhaustion exercise, causes cerebral cortex and hippocampus neuron degeneration, increased heterochromatin, rough endoplasmic reticulum expansion; and found a lot of fat in the hippocampus in brown neurons, cytoplasm of mitochondria swelling and cristae broken, hence cavitation phenomenon. Functionally, euchromatin is active, while heterochromatin is inactivation or less active. After exhaustion exercise, increase heterochromatin proved cell division or change in metabolic activity. Rough surface is the main function of endoplasmic reticulum protein synthesis; including copying organelles producing neurotransmitters related proteins and enzymes. The fatigue training cause of rough endoplasmic reticulum expansion is a sign of endoplasmic reticulum stress movement, the synthesis of proteins, lipids, glucose metabolism and detoxification and proteins secretions are affected. Fat brown pigment is a product of membrane unsaturated fatty acid peroxide, because its molecules contain unusual key, not easy to digest lysosome, and along with the increase and accumulation, it is considered to be the basic feature of cell aging [11]. In this paper, experiments showed that in the hippocampus of rats after exhaustion exercise, there was a lot of fat brown pigment, which suggests that the great fatigue strength training may cause nerve cell aging or cell metabolism disorder.

Fatigue due to long time training makes the energy supply concentrate in muscle tissue, and decreased accordingly for the supply of brain tissue. Brain tissues need oxygen and glucose and if no supply in time, causes hippocampal tissue metabolism imbalance, energy conversion efficiency and the accumulation and synthesis of ATP is abate, which may cause structural brain tissue micro damage. Mitochondria as a cell energy metabolism center, has a series of oxidase system able to form ATP. The oxidation of nutrients and the lining is to synthesize ATP required device. Intensive training causes hippocampus neurodegeneration, intracytoplasmic mitochondria swelling, forms of fracture, cavity phenomenon, inevitably cause ATP synthesis ability is abate, reduce the energy supply. This experiment observed that endurance training and exhaustion exercise cause certain damage to glial cells of cerebral, cortex chromatin gathered, glial cells surrounding the necrosis of neurons and engulfing neurons apoptosis phenomenon such as small body. The glial cells in the hippocampus surround the neuronecrotic cells, and glial cells shown in different degrees of swelling, and endoplasmic reticulum expansion.

As one of the two components of nerve tissue, brain astrocytes especially perform related microenvironment and neurotransmitter important functions, and provide nutrients and neurotrophic factors to neurons. The neurons survival, development, regeneration and differentiation play an important role. Astrocytes also play an important role in the neural plasticity; can support the synaptic sprouting, forming new synaptic connections, to maintain the existing neural circuits, etc. [12-15]. Astrocytes have a dual role in hypoxic ischemic brain damage; too much or too little can affect the function of neurons, thus influence the development of the whole process of cerebral ischemic hypoxia. These ultimately affect the learning and memory and other brain function [16, 17]. In the process of sport activities, the people without basic training should avoid acute exhaustion exercise, and prevent excessive apoptosis caused by the influence of the normal function of CNS.

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