

Effect of Different Exercise Intensity Preconditioning on Global Ischemia-Induced Neuronal Death and Expression of Gadd45 β and DNA-PKcs in Rats

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Abstract: *Background* Acute ischemic stroke causes long-term neurological and neurobehavioral dysfunctions. With the development of clinical medicine, the importance of pre-ischemic exercise intervention has been gradually recognized, but its mechanism remains to be further explored. *Objective* This study investigates the effects of different exercise intensity preconditioning in changes of hippocampal neurons and the expression of Gadd45 β and DNA-PKcs in the hippocampal region after cerebral ischemia-reperfusion in rats. *Method* 160 SD rats were divided into control group (n=40), cerebral ischemia reperfusion group (I/R group, n=40), middle intensity exercise preconditioning group (EI 1+I/R group, n=40), high intense exercise preconditioning group (EI 2+I/R group, n=40). Stroke was induced by improved Pulsinelli four blood vessel blocking after exercise preconditioning. Morphological changes of neurons in the hippocampal region of rats were observed by HE staining at 6 h, 1d, 3d and 7d after ischemia in each group. Immunohistochemistry method was used to detect the expression of Gadd45 β and DNA-PKcs in hippocampus CA1. The mRNA level of Gadd45 β and DNA-PKcs in hippocampal CA1 was detected by Real Time PCR. *Results* Compared with I/R group, the neuronal cell necrosis of was alleviated in EI 1+I/R group, but more serious in EI 2+I/R group; The expression of Gadd45 β and DNA-PKcs were significantly higher in the EI 1+ I/R group, but lower in EI 2+I/R group ($P<0.01$). *Conclusion* Moderate intensity exercise preconditioning can improve the survival of neurons after cerebral ischemia-reperfusion injury in rats. However, high-intensity motor preconditioning increased the damage and loss of neurons, and its mechanism may be related to the regulation of the expression of Gadd45 β and DNA-PKcs in the hippocampus of cerebral ischemia-reperfusion rats, thus protecting and promoting the function of DNA repair system.

Keywords: Exercise, Cerebral Ischemia/Reperfusion, Gadd45 β , DNA-PKcs

1. Introduction

About 75% of the existing patients with cerebrovascular diseases in China lose their ability to work to varying degrees. Cerebral ischemia or stroke is a leading cause of mortality and long-term disability worldwide and economic costs of post-stroke care and treatment are substantial [1-4]. Therefore, in addition to early detection and treatment, prevention of stroke is also of vital importance. Exercise is widely used as a

method of disease prevention and rehabilitation because of its convenience and economy. Studies have shown [5, 6] that extensive repeated exercise training before stroke can induce cerebral ischemia tolerance and has neuroprotective effects. However, the choice of exercise intensity and its complicated neuroprotective mechanisms have not yet been fully elucidated. Our previous studies have shown [7] that the role of exercise preconditioning on cerebral ischemia is related to the regulation of the projection and formation of hippocampal axons, the

regulation of oxygen free radical metabolism and the repair and regeneration of neuron cells. However, in addition to the above mechanisms, the DNA damage repair mechanism of brain tissue has been proved to be closely related to the occurrence and development of cerebral ischemia. When brain tissue is ischemia and hypoxia, the endogenous defense mechanism of the body will be activated, inducing the production of a series of DNA repair proteins and antioxidant enzymes to eliminate excessive free radicals. If this process is inhibited, DNA damage accumulation will be aggravated, cell damage will occur, and ischemia damage will be accumulated [8-10].

Growth arrest and DNA-damage gene β (Gadd45 β) and catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) were recognized as two genes involved in DNA damage repair system. Gadd45 β [9] can regulate and monitor cell proliferation, cell cycle, negative cell growth and other functions related to DNA damage repair. While DNA-PKcs [10] can bind Ku protein complex, activate DNA-PK enzyme activity, and further recruit DNA repair and connected proteins for DNA repair. So, is the protective effect of pre-ischemic exercise on the brain after stroke related to the damage repair mechanism that triggers the body's regulatory DNA? Based on the above problems, the present study investigated the differences and changes in the expression of Gadd45 β and DNA-PKcs in the hippocampal region of cerebral tissue during ischemia-reperfusion after two kinds of exercise preconditioning with different intensity, so as to find a new way for the protective mechanism of ischemic neurons.

2 Materials and Methods

2.1. Materials

160 SPF grade male SD rats with body mass of 300 \pm 20g (Beijing Viton Lever); Gadd45 β and DNA-PKcs rabbit anti-mouse polyclonal antibody, DAB color development kit (Beijing Boorson Biotechnology Co., LTD.); PV6001 secondary antibody kit, complex enzyme digestion solution (Beijing Zhongshan Jinqiao Biotechnology Co., LTD.); Real-time PCR kit, RNA extraction solution (Bao Bioengineering Co., LTD.); Primer synthesis (Shanghai Sangon Biological Engineering Co., LTD.); ZH-PT Computer controlled animal laboratory bench (Anhui Zhenghua Biological Instrument Equipment Co., LTD).

2.2. Methods

2.2.1. Animal Groups and Model Preparation

Experimental animals were randomly divided into 4 groups: control group (40), I/R group (40), EI1+ I/R group (40), EI 2+ I/R group (40). Each group was randomly divided into four sub-groups at 6h, 1d, 3d and 7d, with 10 at each time point (5 for pathological staining and 5 for PCR respectively). They are treated as follows:

Two groups of rats were selected to receive adaptive running table training. The slope of the treadmill was set as 0°, with a speed of 10/15/20m/min lasting 8-10min respectively for 7 days. Modeling was started when the rats were familiar with the

treadmill equipment and were able to maintain movement (at a speed of 15m/min for 30min), lasting for 21days, with a total time duration of 28days. The speed of EI 1+I/R group was set as 15m/min, for 30min (5min for warm up+20min for speed increasing+5min for relax), the intensity of exercise intervention was about 30% VO₂ max. The speed of EI 2+I/R group was set as 20m/min, for 90min (5min for warm up+80min for speed increasing+5min for relax), the intensity of exercise intervention was about 70% VO₂ max. The speed of warm-up and relaxation was 10m/min, and the intensity of electrical stimulation was 2mA. After 4 weeks of treadmill training, the cerebral ischemia-reperfusion model was established in both groups.

Cerebral ischemia-reperfusion model was induced by modified Pulsinelli four-vessel occlusion (4-vo) method. The rats were intraperitoneally injected with 50g/L chloral hydrate 30min before surgery for anesthesia (300-350mg/kg). A 3cm median incision was made in the neck. Bilateral common carotid arteries were separated and surgical lines were placed under the vessels. The rats were exposed to a stereotactic locator and had their head and neck fixed. 2cm incision was made in posterior central of the occipital. The muscle and fascia layer were obtuse to expose the transverse processes of the 2nd to 3rd cervical vertebrae on both sides, and the vertebral artery passing through the upper lamina foramen was electrocauterized about 3-5s/ time. The rats were treated with intraoperative fluorescent lamp to keep their body temperature constant. After the operation, the rats were placed in a single cage. After 24h, the left and right common carotid arteries were simultaneously clamped with a non-invasive microartery clamp, which was loosened 30min after ischemia for 3 times. After ischemia, the rats were in a coma within 30-60s, with bilateral pupils turning white, positive turn-over reflex disappearing and EEG detection showing a straight line. The above indications indicate the success of the modeling.

2.2.2. Collect Samples

At the end of the observed process, rats were anesthetized with Chloral Hydrate. 5 rats from each time point were fixed via the left ventricle for HE staining and immunohistochemistry staining. The rats were perfused with 4% paraformaldehyde until the limbs of rats got stiff. About 6mm thick serial sections were removed from the coronal dorsal hippocampus of the brain and fixed at 4°C with 4% paraformaldehyde. Besides, other 5 rats in the same group had their brains removed directly after anesthetization. The hippocampus were collected under aseptic conditions and conserved in liquid nitrogen for detecting the expression of Gadd45 β and DNA-PKcs mRNA.

2.2.3. HE Staining

The brain tissues were dehydrated with conventional gradient ethanol, hyalinized with xylene, immersed in wax, then sliced into 4 μ m thick pieces and pasted on object slide and dried in an incubator at 45°C for use. After 24h, the sections were dewaxed into water, stained with hematoxylin for 1min, washed with running water for 30min. Then, soaked with 1% hydrochloric acid alcohol for 3-8s, stained with 0.5% eosin for 1min, and sealed with gum after dehydration. The

changes of neurons in hippocampus were observed under high power (40*10) optical microscope.

2.2.4. Immunohistochemistry

Tissue and section preparation was the same as HE staining. After dewaxing and hydration, the sections were dropped with a compound digestion solution, incubated at 37°C for 30min, washed with PBS and immersed in 0.3% H_2O_2 for 15min. After washed with PBS, DNA-PKcs and Gadd45 β rabbit anti-rat polyclonal antibody were dropped (the best dilution ratio was 1:300 after pre-experiment), and incubated in a refrigerator at 4°C overnight. After washing with PBS, PV6001 secondary antibody was added, and incubated at 37°C for 40 min. After washing with PBS, DAB color solution was added for controlled staining under a microscope, followed by hematoxylin re-staining, dehydration, transparency and sealing. For the negative control, 0.01 mol/L PBS was used instead of primary antibody.

2.2.5. RNA Isolation and Real-Time PCR

For the hippocampus total RNA extration, frozen tissues was homogenized in RNAiso plus at low temperature according to the instructions of the kit (Takara, Japan), and was dissolved in 30ul DEPC water. The concentration and purify of RNA were determined at 260/280nm. Samples were store it at -80°C. Nucleotide sequences of specific primers for the selected genes were as follows: Gadd45 β forward primer (5'-TAACTGTCGGCGTGTACGAGGCA-3'), reverse primer

(5'-GCACCCACTGATCCATGAGCG-3'); DNA-PKcs forward primer (5'-ATGGACTATTTGCGGGACCT-3'), reverse primer (5'-GAAGGAAACTTGGCATCTGG-3'), GAPDH forward primer (5'-CTCCCATTCCRCCACCTTTG-3'), reverse primer (5'-CCACCACCCTGTTGCTGAG-3'). According to the Gadd45 β , DNA-PKcs and GAPDH sequences published by CenBank, the primers were synthesized by Shanghai Shengon Biotechnology Co., LTD., and the operating instructions of ABI PRISM®7000 and Applied BiosystEIs 7500 fast real-time PCR system were Applied. The Real Time PCR protocol was following procedures: Satge1, 2 (reverse transcription): 42°C 5min, 95°C 10sec, Reps: 1. Stage3 (real-time quantitative PCR): 95°C 5sec, 60°C 31sec, Reps: 40. Stage4: Dissolution curve analysis.

2.2.6. Image Processing and Statistical Analysis

Positive cells were counted by motic-6.0 image acquisition and analysis system, and observed by 400 times light microscope. 5 sections of CA1 area of hippocampal tissue were selected for each index of each animal, and 5 non-overlapping visual fields were randomly selected for each section. The number of positive cells in each visual field was calculated and the average value was taken. Statistical analysis was performed using SPSS16.0 for windows. Statistical differences among different groups were assessed with one-way analysis of variance (ANOVA). A value of $P < 0.05$ was considered statistical significant.

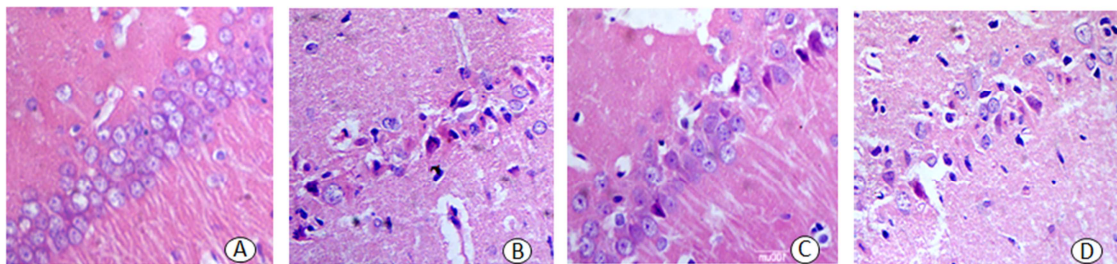


Figure 1. HE staining of neurons in the hippocampal CA1 region in each group at 1d.($\times 400$). Normal neurons observed in sham group (A); increased neurons necrosis observed in I/R group (B); compared with I/R group, decreased neurons necrosis observed in EI 1+I/R group (C) and further increased neurons necrosis observed in EI 2+I/R group (D).

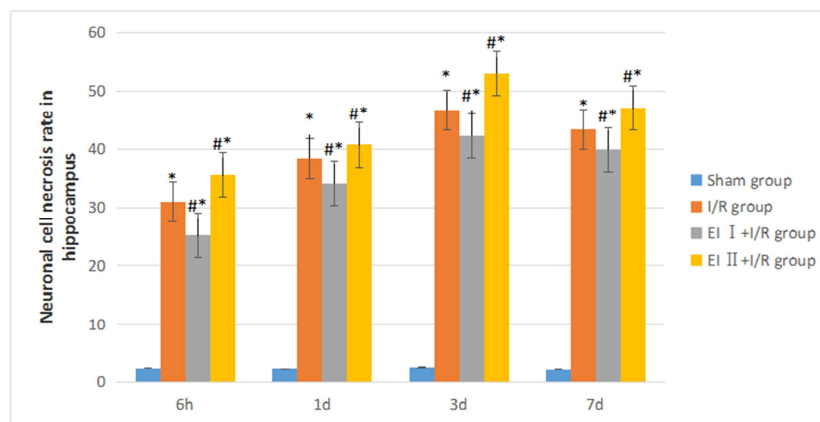


Figure 2. Neuronal cell necrosis rate in hippocampus in different groups. HE staining shows the neuronal cell necrosis rate, represented as folds relative to sham group, I/R group, EI 1+I/R group and EI 2+I/R group at 6h, 1d, 3d, 7d. Data were shown as means \pm SD of 5 rats (* $P < 0.01$ vs. sham group; # $P < 0.01$ vs. I/R group).

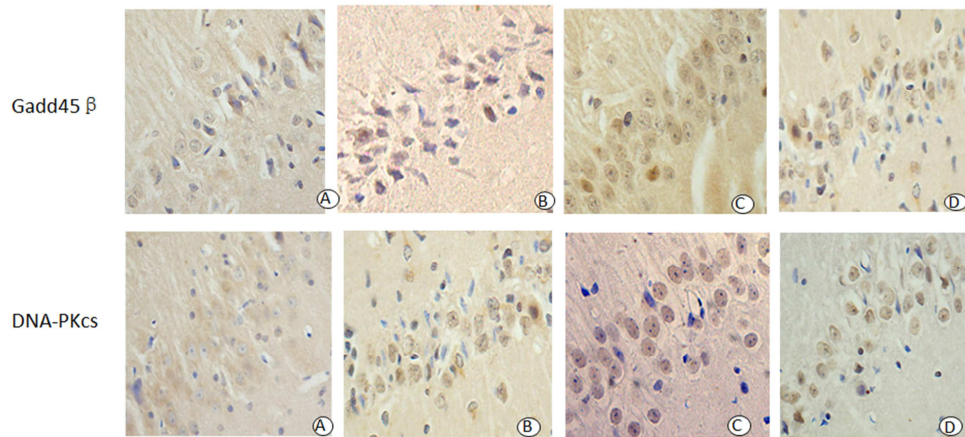


Figure 3. Immunohistochemistry staining of Gadd45 β and DNA-PKcs in the hippocampal CA1 region in each group at 1d.($\times 400$). Weak staining observed in sham group (A); increased staining observed in I/R group (B); Compared to I/R group, further increased staining and a circumferential nucleus expression observed in EI 1+I/R group (C) and decreased staining observed in 2+I/R group (D).

3. Results

3.1. Pathological Changes of Hippocampal Area in Each Group

Figure 1 and Figure 2 shows that, in the sham group, no obvious neurodegeneration, necrosis and other pathological changes were observed. Compared with the sham group, the neurons in I/R group presented triangular cell bodies, nuclear shrinkage and deep staining, plasma eosinophil, and the number of surviving neurons decreased with the extension of ischemia time, with the most significant neuronal necrosis at 3d. Compared with I/R group, the number of necrotic neurons in the EI 1+I/R group was significantly lower ($P<0.01$), and nuclear hyperchromatism and edema were reduced. But However, the number of necrotic neurons was significantly higher in EI 2+I/R group ($P<0.01$), and the neuronal cells were significantly condensed and hyperchromatic, with more nuclei lost and vacuolated abnormal morphological neurons.

3.2. The Expression of Gadd45 β and DNA-PKcs Protein

Figure 3 shows that, compare with sham group, the level of Gadd45 β and DNA-PKcs increased at 6h after ischemia then peaked at 1d and gradually decreased to a low level which was still higher ($P<0.01$) than the sham group at 7d. Compare with I/R group, the expression of Gadd45 β and DNA-PKcs was significantly upregulated ($P<0.01$) in EI 1+I/R group, but significantly downregulate ($P<0.01$) in EI 2+I/R group at all time points.

3.3. The Expression of Gadd45 β and DNA-PKcs mRNA

Figure 4 and Figure 5 shows that, compare with sham group, the level of Gadd45 β and DNA-PKcs protein increased at 6h after I/R, reached the maximum at 1d and gradually decreased to a low level which was still higher ($P<0.01$) than sham group at 7d. Compare with I/R group, the expression of Gadd45 β and DNA-PKcs was significantly upregulated ($P<0.01$) in EI 1+I/R group, but significantly downregulate ($P<0.01$) in EI 2+I/R group at all time points.

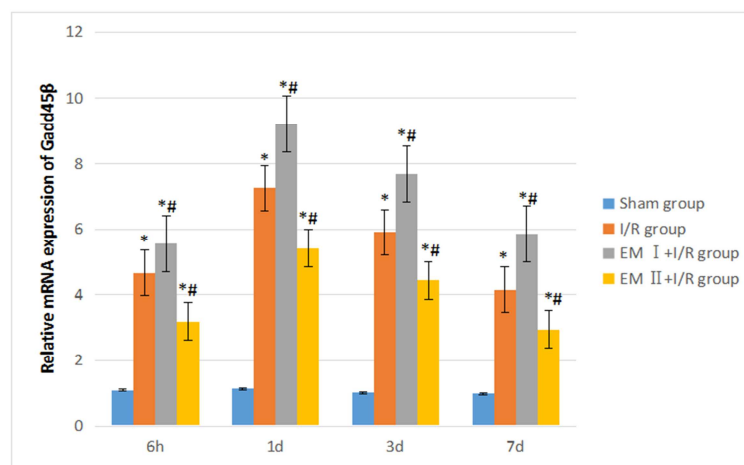


Figure 4. Gadd45 β mRNA expression in hippocampus in different groups. Real-time PCR shows the expression of Gadd45 β mRNA in sham group, I/R group, EI 1+I/R group, EI 2+I/R group at 6h, 1d, 3d, 7d after ischemia. Data were shown as means \pm SD of 5 rats (* $P<0.01$ vs. sham group; # $P<0.01$ vs. I/R group).

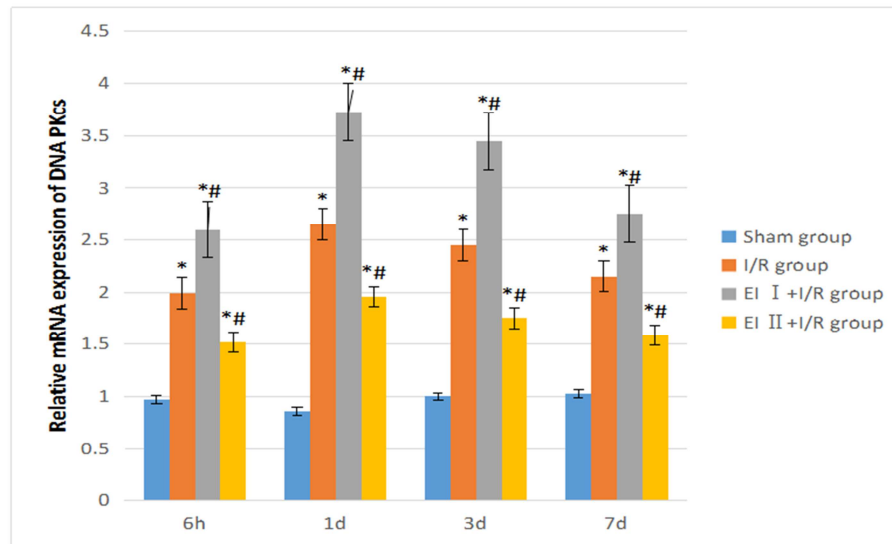


Figure 5. DNA-PKcs mRNA expression in hippocampus in different groups. Real-time PCR shows the expression of Gadd45 β mRNA in sham group, I/R group, EI I+I/R group, EI II+I/R group at 6h, 1d, 3d, 7d after ischemia. Data were shown as means \pm SD of 5 rats (* P <0.01 vs. sham group; ** P <0.01 vs. I/R group).

4. Discussion

The concept of exercise preconditioning was first proposed in 1999, and many studies have confirmed that exercise can trigger the protective mechanism of brain tissue and produce a protective effect [11, 12]. However, the issue of exercise intensity is still controversial. As Shown by Linder *et al.*, higher intensity exercise can promote functional recovery and neuroplasticity after stroke [13-15]. Other studies have shown that mild exercise can improve neuroprotection and synaptic plasticity after stroke [16]. These conflicting results underscore that exercise intensity is an important determinant of neurological outcomes after stroke. Therefore, this study selected two kinds of exercise preconditioning methods with medium and high intensity to conduct pre-ischemia exercise training in rats. The results showed that, compared with the I/R group, after four weeks of moderate exercise intensity training, there were still more viable and well-arranged neurons in the hippocampal area after cerebral I/R. However, rats preconditioned by high-intensity exercise not only suffered a large amount of damage to neurons, but also had sluggish behavior, dull response to electrical stimulation, and had a high mortality during the operation. To investigate the mechanism, we observed the expression of Gadd45 β and DNA-PKcs in the hippocampus of rats.

The accumulation of DNA damage and damaged DNA repair ability are the important mechanisms that make it difficult to recover nerve function after cerebral I/R. As a stress response gene, Gadd45 β affects cell apoptosis, growth and DNA repair [17, 18]. DNA-PKcs, as a catalytic subunit of DNA-dependent proteases, are mainly engaged in double-stranded DNA fracture repair and serve as the center of DNA repair in non-proliferating cells, limiting the accumulation of DNA damage [19]. In recent years, scholars have been exploring intervention methods to improve DNA repair function after ischemic stroke. Liu *et al.* [14] found that

electric stimulation of the cerebellar parietal nucleus in focal cerebral ischemia rats could enhance DNA repair ability by up-regulating the expression or activity of DNA repair protein Ku, and reduce neuronal apoptosis and cerebral infarction area. Zhang Keming found that ALK5 can activate Smad2/3 and promote the expression of Gadd45 β , exert the regulation of axonal plasticity after brain I/R injury, and improve the recovery of nerve function [20].

The results of this study showed that the expression of Gadd45 β and DNA-PKcs protein and mRNA in the hippocampus of rats with moderate intensity exercise training increased significantly after modeling compared with that of the I/R group. However, the expression of Gadd45 β and DNA-PKcs in the hippocampus of rats was further inhibited by high-intensity exercise. Results combined with morphology and the behavior of rats we can speculate that moderate intensity exercise can coordinate DNA damage repair genes and produce endogenous protective effect on ischemic injury. At the same time, the expression of a series of DNA repair proteins and antioxidant enzymes was induced to eliminate free radicals and enhance DNA repair ability, and the binding opportunity of Gadd45 β and DNA-PKcs with proliferating nuclear antigen PCNA was also given to enable the body to start DNA repair system more effectively, thus promoting cerebral ischemia tolerance and enabling more neurons to survive. However, in the process of high intensity exercise, as a source of environmental stimulus, exercise increased brain tissue oxygen free radical damage and substantial accumulation, cause the body lipid peroxidation of rats, the liquidity of biofilm, liquid, and the permeability change, thus affecting the cell metabolism and function, and increased the accumulation of DNA damage, protein aggregation and rupture, which interfere with the entire organs physiological function [21], it is also consistent with our previous findings.

5. Conclusion

In conclusion, exercise training should be selected at an appropriate intensity. Moderate intensity exercise can protect the neurons after brain I/R and generate cerebral ischemia tolerance, while high intensity exercise can aggravate the nerve function injury after brain I/R and cause the morphological changes of neurons in the hippocampus. The mechanism is involved in regulating the expression of Gadd45 β and DNA-PKcs in the hippocampus of brain tissue.

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