• Basic Research •

Retinal ischemia-reperfusion injury and pretreatment with *Lycium barbarum* glycopeptide

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Abstract

● **AIM:** To investigate the antioxidant protective effect of *Lycium barbarum* glycopeptide (LbGP) pretreatment on retinal ischemia-reperfusion (I/R) injury (RIRI) in rats.

● **METHODS:** RIRI was induced in Sprague Dawley rats through anterior chamber perfusion, and pretreatment involved administering LbGP *via* gavage for 7d. After 24h of reperfusion, serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatinine (CREA) levels, retinal structure, expression of Caspase-3 and Caspase-8, superoxide dismutase (SOD) activity, and malondialdehyde (MDA) in the retina were measured.

● **RESULTS:** The pretreatment with LbGP effectively protected the retina and retinal tissue from edema and inflammation in the ganglion cell layer (GCL) and nerve fiber layer (NFL) of rats subjected to RIRI, as shown by light microscopy and optical coherence tomography (OCT). Serum AST was higher in the model group than in the blank group (P=0.042), but no difference was found in ALT, AST, and CREA across the LbGP groups and model group. Caspase-3 expression was higher in the model group than in the blank group (P=0.006), but no difference was found among LbGP groups and the model group. Caspase-8 expression was higher in the model group than in the blank group (P=0.000), and lower in the 400 mg/kg LbGP group than in the model group (P=0.016). SOD activity was lower in the model group than in the blank group (P=0.001), and the decrease was slower in the 400 mg/kg LbGP group than in the model group (P=0.003). MDA content was higher in the model group than in the blank group (P=0.001), and lower in the 400 mg/kg LbGP group than in the model group (P=0.016). The pretreatment with LbGP did not result in any observed liver or renal toxicity in the model.

● **CONCLUSION:** LbGP pretreatment exhibits dose-dependent anti-inflammatory, and antioxidative effects by reducing Caspase-8 expression, preventing declines of SOD activity, and decreasing MDA content in the RIRI rat model.

● **KEYWORDS:** retinal ischemia-reperfusion; *Lycium barbarum* glycopeptide; pretreatment; anti-inflammatory; antioxidative; rat

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INTRODUCTION

Retinal ischemia-reperfusion (I/R) injury (RIRI) is a prevalent pathological process observed in various ocular diseases, including glaucoma, diabetic retinopathy, and retinal vascular obstruction[1]. RIRI has the potential to induce irreversible damage to the retina, particularly affecting retinal ganglion cells (RGCs), which are essential for transmitting visual signals to the brain[2]. Therefore, revealing the underlying mechanisms of RIRI and developing effective strategies for protecting RGCs against RIRI-induced damage are crucial for ameliorating visual impairment and enhancing visual functionality. Despite extensive investigations into oxidative stress, inflammation, apoptosis, and autophagy involvement in RIRI[3-6], therapeutic options currently available remain limited and unsatisfactory.

Lycium barbarum (Gouqizi, *Fructus lycii*, Wolfberry) is a widely utilized herbal remedy in daily life. It possesses a pleasant taste and gentle properties that serve to regulate the liver and kidney meridians while nourishing these organs,

replenishing blood essence, and promoting ocular clarity. In daily use, *Lycium barbarum* can be consumed in various forms, such as dried berries, tea, or as an ingredient in soups and stews. Additionally, it is often taken as a supplement in the form of capsules or extracts to enhance its effectiveness. Previous studies have demonstrated that extracts derived from *Lycium barbarum* possess a diverse range of pharmacological properties, encompassing anti-inflammatory[7], antioxidant[8-10], anti-tumor[11-12], and neuroprotective effects[13-14]. However, the majority of investigations conducted thus far have primarily focused on exploring the therapeutic potential of *Lycium barbarum* polysaccharide (LBP)[15-17]. *Lycium barbarum* glycopeptide (LbGP) is the combination of LBP and polypeptides. In contrast to polysaccharides, glycopeptides not only provide specific oligosaccharides but also have specific information about the amino acid sequence. The bioactive peptide has undergone natural selection, resulting in enhanced *in vivo* stability[18]. The *in vitro* studies have demonstrated the inhibitory effect of LbGP on hypoxia-induced apoptosis in cardiomyocytes[19]. Furthermore, it also possesses notable anti-inflammation[20], antioxidant[21] and neuroprotective effects[22]. However, the pharmacological effects of LbGP *in vivo* are currently under-researched, particularly its role in rats with RIRI remains unclear.

In this study, we investigated the effects of LbGP pretreatment on RIRI-induced in rats. Our hypothesis posits that LbGP could prevent RIRI damage in rats. To validate this hypothesis, subsequent experiments were conducted.

MATERIALS AND METHODS

Ethical Approval All experimental procedures were conducted in the Laboratory of Ophthalmology College of Chengdu University of Traditional Chinese Medicine (Key Laboratory of Traditional Chinese Medicine Eye Disease Prevention and Visual Function Protection of Sichuan Province). Animal care and use complied with the American Association for Eye Research Statement on the use of animals in eye and vision research. This study was approved by the Ethical Committee for Experimental Animal Welfare of Chengdu University of Traditional Chinese Medicine (No.2020-02).

Animals Sixty male SPF rats (6-8 weeks old, weighing 180-220 g) were obtained from the Laboratory Animal Center of Chengdu University of Traditional Chinese Medicine and randomly allocated to five groups of 12 each using the random number table method. The five groups include: the blank group, the model group, the 50 mg/kg LbGP group, the 200 mg/kg LbGP group, and the 400 mg/kg LbGP group. The rats were maintained in the same center under the following conditions: temperature 22℃±1℃, humidity 40%±10%, 12:12h light-dark cycle, and standard chow and water *ad libitum*.

Pretreatment of LbGP The experimental drug LbGP was provided by Ningxia Tianren Goqi Biotechnology Co., Ltd., Zhongwei, China. Animals were pretreated for 1wk before induction of RIRI. The dried LbGP powder was dissolved in 0.9% saline to prepare fresh LbGP solutions of different concentrations. The LbGP solutions of 50, 200, and 400 mg/kg were then administered orally to the corresponding LbGP pretreatment groups from 15:30 to 16:30 every day. The model group received the same volume of normal saline by oral gavage.

Retinal Ischemia-reperfusion Injury The rat RIRI model was made with reference to other studies[23]. The rats were anesthetized with 2% sodium pentobarbital (30 mg/kg body weight) by intraperitoneal injection. The local corneal anesthesia was administered using aubucaine hydrochloride eye drops, while the pupil was dilated with compound tropicamide eye drops. Subsequently, an anterior chamber puncture of the right eye was performed utilizing a 27-gauge needle. The needle was attached to a container filled with 500 mL of sterile saline, which was then elevated 1.5 m above the eye to increase the intraocular pressure (IOP). I/R was induced for 1h. Retinal ischemia was confirmed by iris pallor and loss of fundus red reflex. Reperfusion was indicated by the return of fundus red reflex. The vital signs and IOP changes of rats were monitored closely during the surgery. IOP was measured by Tonolab (Icare®, Vantaa, Finland) to ensure consistent induction of I/R. The control group consisted of rats that did not undergo I/R surgery. After successful modeling, the rats were observed for a duration of 24h. Subsequently, abdominal aortic blood sampling was performed under anesthesia with 2% pentobarbital sodium (30 mg/kg body weight), and retinal tissue from the right eye of each rat was collected. Finally, euthanasia was conducted on the rats while they were in a deep anesthetic state using the cervical dislocation method.

Determination of Serum Alanine Aminotransferase, Aspartate Aminotransferase, and Creatinine in Rats To evaluate the liver and kidney toxicity of different doses of LbGP, blood samples were collected from the abdominal aorta of three randomly-selected rats in each group after 24h of reperfusion. The levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and creatinine (CREA) were measured using an animal biaochemical analyzer (BS-240Vet, Mindray, Shenzhen, China).

Hematoxylin and Eosin Staining Extracted eyes from three rats (right eye) per group were fixed in an automatic dehydrator (JT-12S, Wuhan Junjie Electronics Co., Ltd., Wuhan, China) for paraffin embedding (BMJ-A, Changzhou Zhongwei Electronic Instrument Co., Ltd., Changzhou, China) and hematoxylin and eosin (HE) staining (RS36, Changzhou

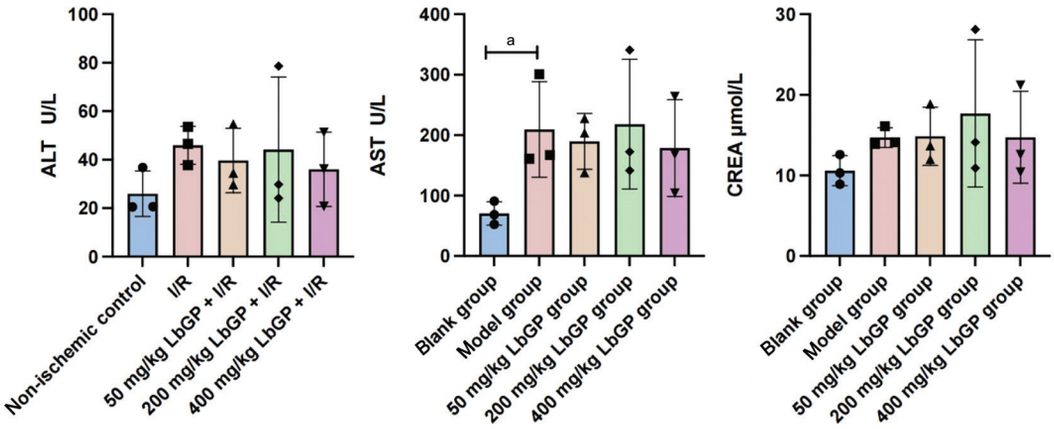

Figure 1 Serum levels of ALT, AST, and CREA LbGP: *Lycium barbarum* glycopeptide; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CREA: Creatinine. $^{a}P<0.05$.

Paisjie Medical Equipment Co., Ltd., Changzhou, China). The rat retina tissue was then stored at 4°C. Sections (5-μm thick) were cut from the paraffin block and dewaxed and rehydrated. The sections were stained with hematoxylin for 15min, differentiated and returned to blue, and then stained with eosin for 5min. After dehydration and transparency, the sections were sealed with neutral gum. The retinal tissue specimens were scanned by a digital slide scanner (Pannoramic 250, Jinan Tangier Electronics Co., Ltd., Jinan, China) and examined by microscope. The retinal condition of RIRI rats in each experimental group was evaluated by observing the gross and specific lesions at 400× magnification.

Western Blotting Retinal tissue from three rats (right eye) per group was lysed with RIPA buffer (P0013, Beyotime, China) and centrifuged (12 000 rpm, 10min). Protein concentration was measured using a BCA kit (P0009, Beyotime, Jiangsu, China) and samples were denatured with loading buffer. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to Immobilon-PSQ PVDF membrane (ISEQ00010, Sigma-Aldrich, Burlington, MA, USA). The membrane was blocked with skim milk and incubated with primary antibodies for Caspase-3 (1:1000), Caspase-8 (1:1000), and β-actin (1:100 000). After washing, the membrane was incubated with secondary antibodies and detected by enhanced chemiluminescence. Band intensities were analyzed by TianNeng GIS software and expressed as relative expression levels of target proteins.

Measurement of Superoxide Dismutase Activity and Malondialdehyde Levels in Retina Three retinal tissues were collected per group of rats (right eye). Superoxide dismutase (SOD) activity was determined by the WST-1 method and malondialdehyde (MDA) content was determined by the TBA method according to the kit instruction.

Statistical Analysis The data were presented as mean±standard deviation (SD). The data normally distributed were analyzed by one-way analysis of variance (ANOVA) with post hoc LSD test (for homogeneous variance) or Tamhane's T2 test (for heterogeneous variance) using SPSS 23.0 software. The *P* value less than 0.05 was considered statistically significant, and less than 0.01 was considered extremely significant.

RESULTS

Liver and Kidney Toxicity of LbGP Pretreatment in Rats The serum levels of ALT, AST, and CREA in each group were presented in Figure 1. Specifically, AST exhibited a significant increase in the model group (P=0.042).

Effect of LbGP Pretreatment on Tissue Structure of RIRI in Rats Figure 2 illustrated a representative retinal section stained with HE 24h after surgery. I/R causes severe damage to the retinal tissue, resulting in significant damage to the retinal nerve fiber layer (NFL) and ganglion cell layer (GCL). Specifically, the arrangement of the inner nuclear layer (INL) becomes disordered, the GCL is relatively sparse, and some regions show loss of nodal cells or ambiguous morphological structures. Severe lesions involved edema in the GCL and NFL, and the inner compound layer was also affected. The vessels proliferated and expanded to varying degrees inside the INL and GCL, and red blood cells were seen in some lumen. LbGP pretreatment (50 and 200 mg/kg) showed that there was still significant edema in the GCL and NFL of the rat retina compared with the model group, but vascular proliferation and dilation were slightly reduced. It should be noted that only mild edema was observed in the GCL and NFL of the 400 mg/kg LbGP group.

Pretreatment with LbGP Inhibited Apoptosis Induced by I/R Compared to the blank group, Caspase-3 and Caspase-8 protein expression in the retina significantly increased after 1h of ischemia and 24h after reperfusion in the model group (P=0.006, 0.000). However, the 400 mg/kg LbGP group

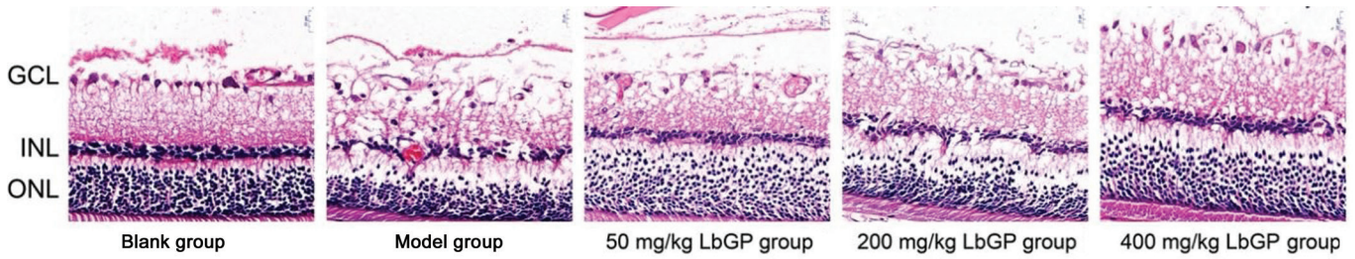

Figure 2 Retinal HE staining in each group (×400) GCL: Ganglion cell layer; INL: Inner nuclear layer; ONL: Outer plexiform layer; HE: Hematoxylin and eosin; LbGP: *Lycium barbarum* glycopeptide.

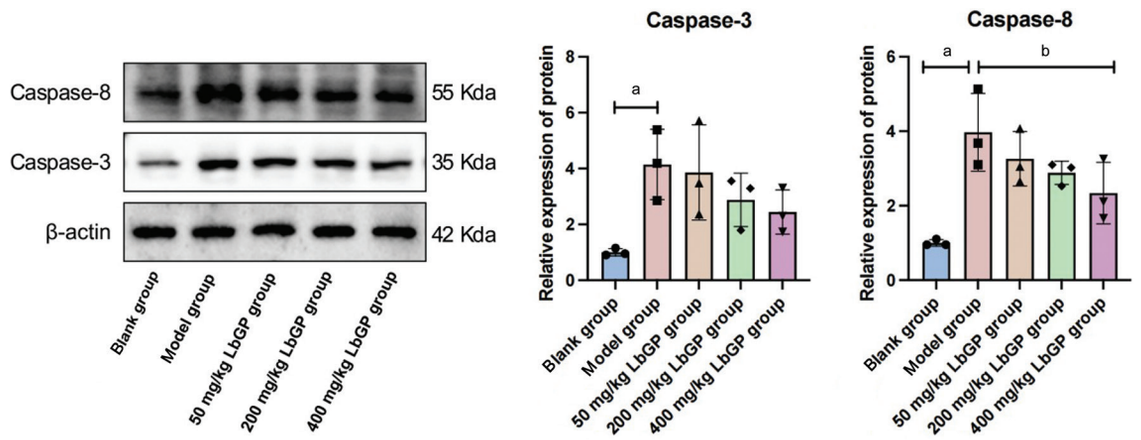

Figure 3 The expression of Caspase-3 and Caspase-8 proteins of each group LbGP: *Lycium barbarum* glycopeptide. [a]*P*<0.001; [b]*P*<0.05.

showed a significant decrease in Caspase-8 protein expression compared to the model group (*P*=0.016; Figure 3).

Pretreatment with LbGP-inhibited Oxidative Stress Induced by I/R The retinal homogenate of the model group showed a significant decrease in SOD activity compared to the blank group (*P*=0.001; Figure 4). The 400 mg/kg LbGP group demonstrated a significantly mitigated decline in SOD activity in retinal homogenate of rats compared to the model group (*P*=0.003; Figure 4). The SOD activity did not exhibit any significant disparity between the 50 and 200 mg/kg LbGP group (*P*=0.393, 0.197; Figure 4). Additionally, compared to the blank group, the retinal homogenate of the model group exhibited a significant increase in MDA content (*P*=0.001; Figure 4). However, the 400 mg/kg LbGP group showed a statistically significant reduction in MDA content in retinal homogenate compared to the model group (*P*=0.016; Figure 4).

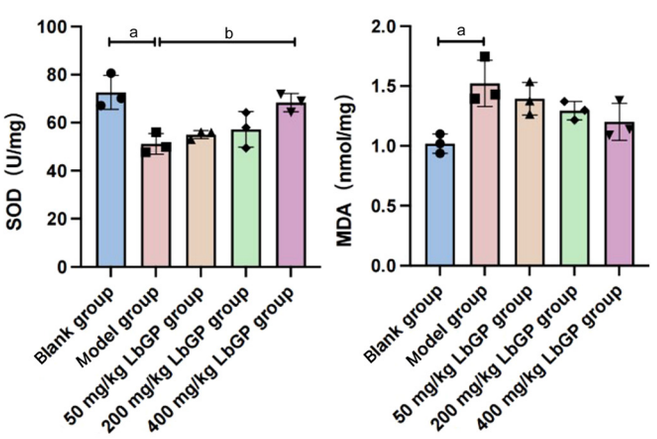

Figure 4 The SOD activity and quantification of MDA levels of each group LbGP: *Lycium barbarum* glycopeptide; SOD: Superoxide dismutase; MDA: Malondialdehyde. [a]*P*<0.001; [b]*P*<0.05.

DISCUSSION

The propagation of *Lycium barbarum* can be achieved through either seed germination or vegetative cuttings. This rare plant possesses significant nutritional and medicinal value, and serves as both as a source of food and traditional Chinese medicine. LbGP is the active ingredient extracted from LBP. The activity can reach one to two orders of magnitude of LBP. The aim of this study was to examine the preventive effect of LbGP on RIRI in an animal model.

Our research demonstrates that: 1) oral administration of 50, 200, and 400 mg/kg LbGP was not associated with hepatotoxicity, nephrotoxicity, or retinal toxicity in rats; 2) pretreatment with 400 mg/kg LbGP significantly mitigates retinal tissue damage caused by ischemia, and a dose-dependent response was observed; 3) pretreatment with 400 mg/kg LbGP also dose-dependently reduced retinal apoptosis and oxidative stress caused by retinal ischemia injury. These findings highlight the protective effect of LbGP on retinal degeneration caused by I/R and its potential role in the preventing and treating of eye diseases related to RIRI.

Repercussions in blood supply can give rise to morphological alterations and impairment of retinal cells[24]. The retinal degeneration observed following retinal I/R in rats is

characterized by significant cellular loss within the retinal GCL and INL of the inner retina[25]. It has also been found that RIRI following transient elevation of IOP can induce apoptosis in the GCL and INL cells, with disappearance of vacuoles in the INL observed after 18h of reperfusion, along with scattered condensed nuclei observed in both GCL and INL[26]. In this study, we observed significant damage to the retinal NFL and GCL following I/R. However, pretreatment with LbGP demonstrated a reduction in tissue edema of GCL and NFL as well as mitigating the degree of inflammation after I/R (Figure 1). Notably, among all groups, the 400 mg/kg LbGP group exhibited the lowest number of overall retinal tissue lesions. Furthermore, previous experiments reported that pretreatment with *Lycium barbarum* extracts also showed preventive effects against retinal tissue damage in a rat model of chronic ocular hypertension[27].

Additionally, we observed that escalating doses of LbGP pretreatment mitigated the upregulation of apoptosis induced by RIRI, including the expression of Caspase-3 and Caspase-8 proteins. The pathogenesis of RIRI involves a complex interplay between inflammation and oxidative stress[28]. Apoptosis can be triggered by activation of death receptors or mitochondria-mediated signaling pathways[29]. Caspase-3 is commonly used as a reliable indicator for both apoptotic pathways. Caspase-8 is an initiator in the death receptor-induced apoptosis signaling pathway and also regulates Caspase-3 cleavage, which plays a key role in mediating exogenous pathway apoptosis. Chi *et al*[30] demonstrated that Caspase-8 played a crucial role in regulating the activation of NLRP1/NLRP3 inflammasomes during RGCs death induced by IOP. Furthermore, the activation of TLR4/Caspase-8/interleukin (IL)-1β axis was also contingent upon Caspase-8 production[30]. Therefore, Caspase-8 acts as a mediator in IOP-induced RGCs death. LbGP may inhibit the activation of TLR4/Caspase-8/IL-1β axis by downregulating the expression of Caspase-8 and suppressing proinflammatory factor production to achieve retinal protection. Additionally, previous studies have demonstrated that Caspase-8 played a pivotal role in regulating Caspase-3 activity. Seong *et al*[31] demonstrated the inhibitory effect of resveratrol on Caspase-8 during I/R injury, which suppresses the expression of Caspase-3. Therefore, it can be postulated that the inhibitory effect of LbGP on Caspase-3 expression is mediated through the downregulation of Caspase-8 levels, thereby demonstrating its anti-inflammatory and anti-apoptotic properties.

The role of oxidative stress in the induction of damage during apoptosis is of paramount importance[23,32]. SOD activity decreased, whereas MDA content increased following retinal I/R in this study. However, pretreatment with 400 mg/kg LbGP effectively mitigated the decline in SOD activity and reduced MDA content. As SOD serves as an indicator of oxidative stress and MDA reflects the extent of accumulated damage caused by oxidative stress[23,33], I/R can trigger oxidative stress in the retina, while LbGP has the potential to mitigate I/R-induced oxidative stress. Due to the induction of oxidative stress, various antioxidation strategies have been investigated in the RIRI model. Green tea extract[34], baicalein[35], and ginkgo biloba extract[36] have demonstrated efficacy in mitigating RGCs loss in rodent models by attenuating oxidative stress .

The limitations of this study are evident in the absence of examination of apoptosis and oxidative stress pathways. Furthermore, only the changes in the levels of apoptotic proteins and antioxidant indicators 24h post-retinal I/R were observed. Additionally, no comparative analysis was conducted between LbGP and LBP. To more comprehensively understand the therapeutic protective effect of *Lycium barbarum* and its extracts on RIRI, future studies must use a larger sample size, investigate the underlying mechanism, and conduct comparative analysis between the two extracts.

In conclusion, LbGP is a potential therapeutic agent for RIRI, a condition that can cause vision loss and blindness. In this study, we investigated the effects of LbGP pretreatment on the retinal tissue of rats with RIRI. We found that pretreatment with LbGP protected the retinal tissue from injury by reducing the edema and inflammation of the GCL and NFL. Moreover, LbGP pretreatment exhibited anti-inflammatory effects by down-regulating the expression of Caspase-8 protein in the retina, and these effects were dose-dependent. Furthermore, LbGP pretreatment enhanced the antioxidative capacity of the retinal tissue by slowing the decline of SOD activity and reducing MDA content, and these effects were also dose-dependent. Importantly, we observed no liver and renal toxicity in rats with RIRI after LbGP pretreatment at any dose. Therefore, LBGP pretreatment may be a promising strategy for preventing and treating RIRI.

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Conflicts of Interest: Wu YX, None; **Yin S,** None; **Song SS,** None; **Liu X,** None; **Deng YX,** None; **Lu XJ,** None.

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