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Original Research

Effect of Triptolide on retinal ganglion cell survival in an optic nerve crush model

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Abstract: Optic nerve crush model could be used to investigate the mechanism of neuronal survival and axonal regeneration in central nervous system. Triptolide, a Chinese herb extract with anti-inflammatory and immunosuppressive activities, has shown neuron protective functions in nervous system. In this study, we investigated the changes in retinal ganglion cell survival and axonal regeneration after administration of triptolide in optic nerve crush model. Triptolide treatment tended to promote retinal ganglion cell survival rather than optic nerve regeneration as well as inhibit the expression of tumor necrosis factor-α and activation of nuclear factor-kappa B. These findings suggested that intraperitoneal injection of triptolide may be an effective treatment for optic nerve injury and this effect was attributed at least in part to its anti-inflammatory actions.

Key words: Optic nerve crush; Neuroprotection; Triptolide; TNF-α; NF-κB.

Introduction

The regeneration failure following injury to the adult mammalian central nervous system (CNS) has been attributed to disruption of intrinsic growth pathways and the presence of growth inhibitory molecules in the CNS environment (1-3). Using an optic nerve crush (ONC) model could investigate the mechanism of neuronal survival and axonal regeneration in CNS. The optic nerve is a white matter tract, consisting of axons from a single cell type, the retinal ganglion cell (RGC), in the retina. The absence of any surrounding gray matter allows for the investigation of cell survival and neuronal regeneration as distinct events following injury (4). In addition, optic nerve injury models have been used extensively to investigate the potential factors on cell survival and regeneration (5-7). Therefore the optic nerve crush provides an ideal model to investigate the efficacy of Triptolide in neuroprotection and regeneration.

Triptolide, a diterpenoid epoxide, is an extract of the traditional Chinese medicine Tripterygium wilfordii Hook F, which has immunosuppressive, anti-inflammatory, and anti-proliferative effects (8-10) and has been used widely in China for treatment of a variety of inflammatory and autoimmune diseases, such as rheumatoid arthritis, glomerulonephritis and other autoimmune diseases (11-13). In addition, *In vitro* and *in vivo* trials have shown that triptolide has a neuron protective function in nervous system (14-17). However, it remains elusive how triptolide affects neuron survival and axon regeneration after CNS injury.

Tumor necrosis factor-α (TNF-α) is a pro-inflammatory factor with improving inflammation potential and works as an initiation factor of inflammatory reaction (18). It is reported that triptolide regulated the expression of TNF-α in primary microglial cultures. Nuclear factor-kappa B (NF-κB) is a nuclear transcription factor involved in the regulation of inflammation (19). Previous study has shown that triptolide attenuated cerebral ischemia and reperfusion injury in rats through the inhibition the NF-κB signaling pathway (20). However, it remains to be ascertained whether or not triptolide inhibits TNF-α and NF-κB following ONC injury *in vivo*.

In this study, we delivered triptolide to C57Bl/6J mice with ONC injury and investigated its effects on RGCs survival and axonal regeneration. We also examined the role of TNF-α and NF-κB on RGCs in ONC model.

Materials and Methods

Animals

All procedures involving animal experiments were in accordance with animal use protocols approved by the Committee for the Ethics of Animal Experiments, Shenzhen-Peking University-The Hong Kong University of Science and Technology Medical Center (SPHMC; protocol number 2011-004). C57BL/6J mice were purchased from Guangdong Medical Lab Animal Center. All the mice were raised in a temperature-controlled room with free access to food and water, and 12 h light–dark cycle.

Triptolide administration

Triptolide was purchased from Tocris, with purity greater than 99%. Triptolide was initially dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10mg/ml and diluted with 0.7% saline. C57BL/6J mice were divided randomly into two experimental groups (n=6) and a control group (n=6). We also chose 6 mice

as Naive control without drug treatment or optic nerve crush surgery. Beginning at 6-7 weeks of age, intraperitoneal injection of triptolide was administered to the experimental group for three days before performing ONC injury and for 2 weeks after surgery with a dose of 0.1mg/kg and 0.25mg/kg, respectively. Because of toxic side effects, the group with 0.5mg/kg of triptolide all died in the pre-experiment. The dose of 0.1mg/kg and 0.25mg/kg were selected. The control group received intraperitoneal injections of vehicle.

Animal model of ONC surgery

C57BL/6J mice were anesthetized with Avertin (2,2,2-tribromoethanol, 500mg/kg). ONC was performed as described previously (21). In brief, three days following triptolide intraperitoneal injection, the left optic nerve was exposed intraorbitally and crushed with jeweler's forceps (Dumont #5; FST) for 5 seconds approximately 0.5 mm behind the eyeball. Care was taken not to damage the underlying ophthalmic artery. Eye ointment containing atropine sulphate was applied to protect the cornea after surgery.

RGC axon anterograde labeling

For anterograde labeling of RGC axons, 2 µl of cholera toxin β subunit (CTB) (2 µg/µl, Invitrogen) was injected into the vitreous with a Hamilton syringe 2 days before sacrifice. Animals were given a lethal overdose of anesthesia and perfused with phosphate buffer saline (PBS) and 4% (wt/vol) polyformaldehyde (PFA). For the animals with an optic nerve injury, eyes with the nerve segment still attached were dissected out and post-fixed in the same fixative overnight at 4°C. Tissues were cryoprotected through increasing concentrations of sucrose and embedded into optimal cutting temperature compound (Tissue Tek). Optic nerves were cut longitudinally (8µm) and stored at −80°C until processed.

Immunofluorescence of whole-mount retina

Retinas were dissected out from 4% PFA fixed eyeballs and washed extensively in PBS before blocking in staining buffer (4% normal goat serum (NGS) and 1% Triton X-100 in PBS) for half an hour. All antibodies were diluted in the same staining buffer. Antibody used was mouse neuronal class β-III tubulin (clone Tuj1, 1:500 dilution, Sigma). Floating retinas were incubated with primary antibodies overnight at 4°C and washed 3 times for 30 minutes each with PBS. Secondary antibody Alexa Fluor 488 goat anti-mouse was then applied (1:500; Invitrogen) and incubated for 1 hour at room temperature. Retinas were again washed 3 times for 30 minutes each with PBS.

Immunofluorescence of retina frozen sections

PFA fixed eyes were dehydrated with increasing concentrations of sucrose solution (15%–30%) overnight and embedded in OCT on dry ice. Serial cross-sections (25µm) were cut and stored at −80°C. All sections were blocked in 4% normal goat serum and 1% Triton X-100 in PBS for 1h and incubated in the primary antibodies (TNF-α, 1:50, Proteintech; NF-κB, 1:300, Abcam) diluted in the same solution overnight at 4°C. After being washed three times by PBS, appropriate secondary antibodies (Invitrogen, 1:500) were applied for 1-2h at room temperature for staining. After being washed three times, sections were mounted onto glass slides (Superfrost Plus, Fisherbrand) and examined using confocal microscope (Zeiss, LSM Meta710).

Counting surviving RGCs and regenerated axons

For RGC counting, whole-mount retinas were immunostained with the Tuj1 antibody and 6–9 fields were randomly sampled and counted from peripheral regions of each retina. For axon counting, regenerating RGC axons in injured optic nerves distal to the crush site were quantified as described previously (22). The number of CTB labeled axons was estimated by counting the number of CTB-labeled fibers extending different distances from the end of the crush site in 5 sections (every 4th section) per animal.

Statistical analysis

Data are presented as means ± standard error of the mean (SEM) with 6 mice per experimental group. We used Oneway ANOVA with Bonferroni's post hoc test for multiple comparisons.

Results

Triptolide could not improve optic nerve regeneration

To assess whether any regenerating RGC axon were present, frozen sections of optic nerves were labeled using CTB-488 immunofluorescence. As shown in Figure 1, No CTB-488 labeling axon was present on neither side of the crush site in vehicle nor triptolide groups, indicating no axon regeneration distal or proximal to the crush site.

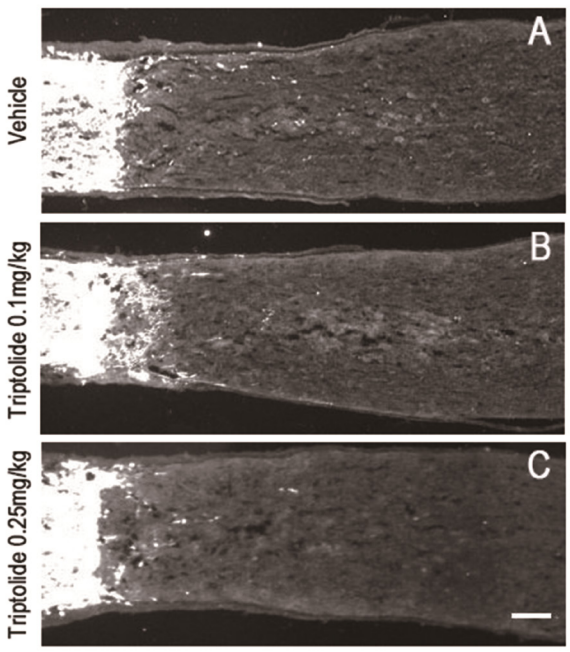

Figure 1. Triptolide could not improve axonal regeneration after optic nerve crush. A-C, representative pictures of CTB-labeled axons after optic nerve crush following 2 weeks of vehicle (A), 0.1mg/kg triptolide (B) or 0.25mg/kg triptolide (C) treatment. Scale bar= 20µm.

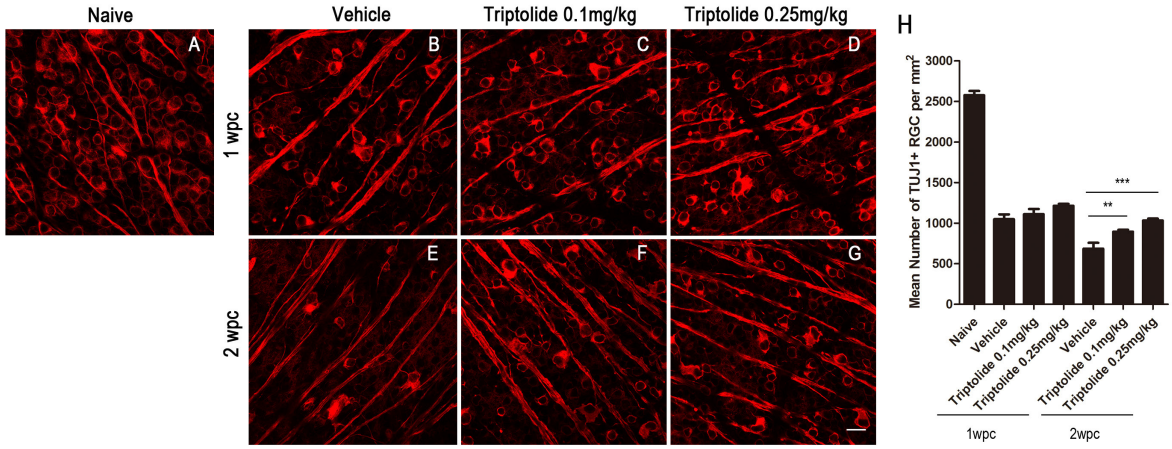

Figure 2. Triptolide pre-treatment could promote retinal ganglion cells survival after optic nerve crush. A, representative picture of RGC labeled with β-III tubulin (Tuj1) in Naive group. B-D, representative pictures of RGC labeled with Tuj1 in vehicle (B), 0.1mg/kg triptolide (C) and 0.25mg/kg triptolide (D) group 1 week after optic nerve crush. E-G, representative pictures of RGC labeled with Tuj1 in vehicle (E), 0.1mg/kg triptolide (F) and 0.25mg/kg triptolide (G) group 2 weeks after optic nerve crush. Scale bar=20μm (H) Quantification of Tuj1 positive RGCs. **p<0.01 compared to vehicle group 2 weeks after ONC. ***p<0.001 compared to vehicle group 2 weeks after ONC. (1wpc: 1 week post optic nerve crush; 2wpc: 2 weeks post optic nerve crush).

Triptolide promoted RGCs survival after optic nerve crush

To determine whether treatment with triptolide has protective effects on RGCs in the ONC retina, Tuj1, a specific neuron marker, was used to quantify the survival RGCs. we characterized the number of surviving RGCs at 1 week and 2 weeks post optic nerve crush injury. As shown in the Figure 2, both vehicle group and triptolide group had a dramatic decrease in the number of RGCs after optic nerve crush compared with Naive group. After 1 week of ONC injury, the average number of Tuj1+ cells/mm^2 in vehicle, 0.1mg/kg triptolide and 0.25mg/kg triptolide group was 1048±60.2 cells/mm^2, 1110±60.7 cells/mm^2, 1214±22.5 cells/mm^2, respectively (Figure 2B-2D). There was no significant difference in the number of RGCs compared the vehicle group with triptolide groups (Figure 2H). However, after 2 weeks of ONC injury, a dramatic decrease in the number of RGCs was observed in vehicle group but not in the triptolide groups, the RGC density in vehicle group decreased to 685±72.5 cells/mm^2 (Figure 2E). The number of survival RGCs showed a dose-dependent effect in triptolide treated groups, with 894±22.9 cells/mm^2 in 0.1mk/kg triptolide group (Figure 2F) and 1032±22.6 cells/mm^2 in 0.25mk/kg triptolide group (Figure 2G). These results suggest that administration of triptolide could induce neuroprotection and promote RGCs survival after ONC injury.

Triptolide decreased the activation of TNF-α in the retina

To investigate the anti-inflammatory effects of triptolide, the expression of TNF-α in retina was assessed by immunofluorescence with TNF-α in frozen sections of retina. The results revealed that TNF-α was mainly distributed in the inner retinal layers: including nerve fiber layer, ganglion cell layer and inner plexiform layer (Figure 3). As shown in Figure 3, a dramatic increase of immunofluorescence intensity of the TNF-α was observed in vehicle group in comparison to Naive group (Figure 3B and 3E). However, there was an obvious reduction of immunofluorescence intensity of the TNF-α in the retina treated with 0.1mg/kg and 0.25mg/kg triptolide (Figure 3H and 3K) compared with the retina of vehicle group (Figure 3E).

Triptolide inhibited the nuclear translocation of NF-κB p65 in RGCs.

The translocation of NF-κB p65 from cytoplasm to nucleus is known to induce NF-κB activation (19). Comparing with Naive group (Figure 4A-4D), the double immunofluorescence labeling showed that most of NF-κB p65 was mainly localized in the neuron nuclear in the vehicle treated ONC injury group (Figure 4E-4H). However, triptolide treated with 0.1mg/kg and 0.25mg/kg per day reduced the amount of nuclear NF-κB p65

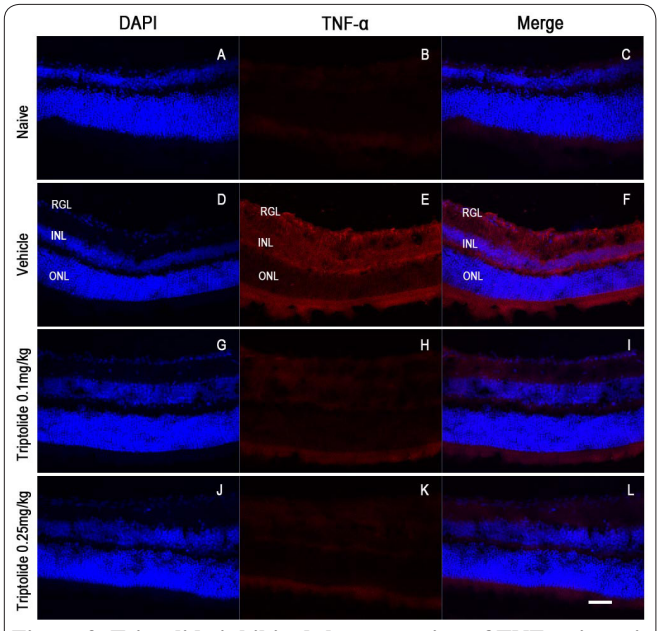

Figure 3. Triptolide inhibited the expression of TNF- α in retina 2 weeks after optic nerve crush. A, D, G and J, staining with 4'6-diamidino-2-phenylindole (DAPI); B, E, H and K, staining with anti-TNFα antibody in the retina treated with vehicle, 0.1mg/kg triptolide or 0.25mg/kg triptolide, respectively; C, F, I and L, show panels B, E and H merged with DAPI. Scale bar=50μm. Cell layers as marked in D, E and F (RGL, retinal ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer).

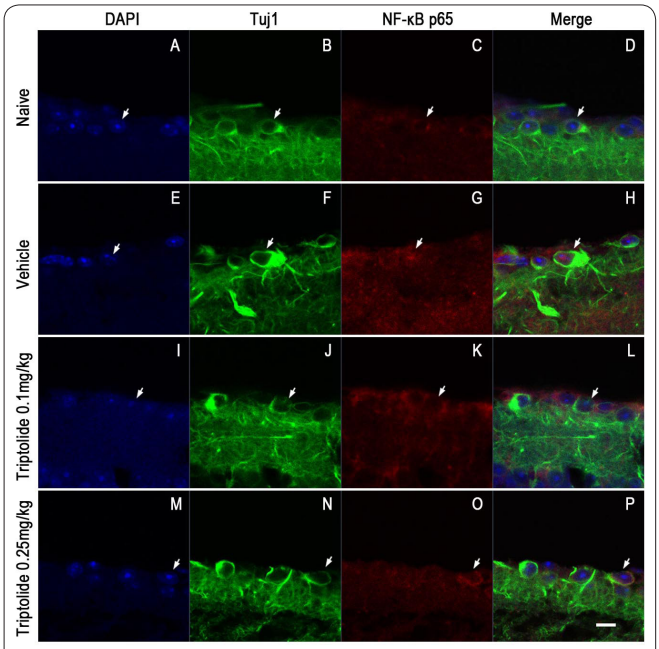

Figure 4. Triptolide inhibited the nuclear translocation of NF-κB p65 in neurons 2 weeks after optic nerve crush. Representative immunofluorescence images showing retina co-labeled with DAPI (A, E, I and M, blue), Tuj1 (B, F, J and N, green), and anti NF-κB p65 (C, G, K and O, red), and merged images (D, H, L and P). Arrow: representative neuron in each group. Scale bar=10μm. A-D: Naive group, E-H: vehicle group; I-L: 0.1mg/kg triptolide group; M-P: 0.25mg/kg triptolide group.

intensity observably compared with the vehicle treated ONC injury group 2 weeks after ONC injury (Figure 4I-4P).

Discussion

In this study, we demonstrated that intraperitoneal injection of triptolide significantly promoted RGCs survival rather than optic nerve regeneration in mice model of optic nerve crush as well as inhibited the expression of TNF-α and activation of NF-κB, suggesting that intraperitoneal injection of triptolide may be an effective treatment for optic nerve injury and this effect was attributed at least in part to its anti-inflammatory actions.

We tested the effects of different does of triptolide (0.1mg/kg, 0.25mg/kg) on RGCs survival after optic nerve injury. The highest concentration of triptolide (0.25mg/kg) significantly increased RGC numbers compared to the lower concentration of triptolide (0.1mg/kg) after 2 weeks of ONC injury, indicating that triptolide dose-dependently promoted RGCs survival. However, because we only tested two concentrations of triptolide, it remains to be determined whether higher concentrations are required to achieve maximum effects on RGCs survival. The effect of neuronal protection only occurred in triptolide pre-treatment. It means that triptolide was administered to the experimental group for three days before performing ONC surgery. We also did the experiment of triptolide post-treatment. Triptolide was delivered into experimental group for two weeks after ONC injury. As shown in Figure S1, There was no significant difference in the number of RGCs compared the vehicle group with triptolide groups. Thus, triptolide post-treatment could not promote RGCs survival after ONC injury. It could be explained by that triptolide pre-treatment may activate related signals which are important for the early injury. Pre-treatment could accumulate the signals which protect RGCs from loss after ONC injury, while triptolide post-treatment may not help RGCs to overcome the ONC injury. We did not find any significant optic nerve regeneration neither in vehicle group nor in the triptolide treatment group 2 weeks after optic nerve crush. A possible explanation is that the significant regeneration of optic nerve after ONC injury has been achieved by manipulating different signaling pathways such as phosphatidylinositol 3-kinase (PI3K)/akt, janus kinase/signal transducer and activator of transcription (JAK/STAT) and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK/ERK) pathways (6,23). Thus, only treating ONC injury eyes with triptolide may not enough to promote optic nerve regeneration.

Triptolide treatment improved neuron survival in ONC model, reducing the production of inflammatory mediators TNF-α and inhibiting NF-κB p65 nuclear translocation in the neurons. TNF-α is a proinflammatory cytokine that is rapidly up-regulated after ischemic and excitotoxic brain injury, suggesting that this cytokine is important in modifying the neurodegenerative process (24-27). There is growing evidence for the involvement of TNF-α can exert negative effects on neuronal integrity and survival (28). Intravitreal injections of TNF-α into rabbit eyes induced axonal degeneration in the optic nerve (29). In this study, there was an obvious decrease of TNF-α immunofluorescence intensity

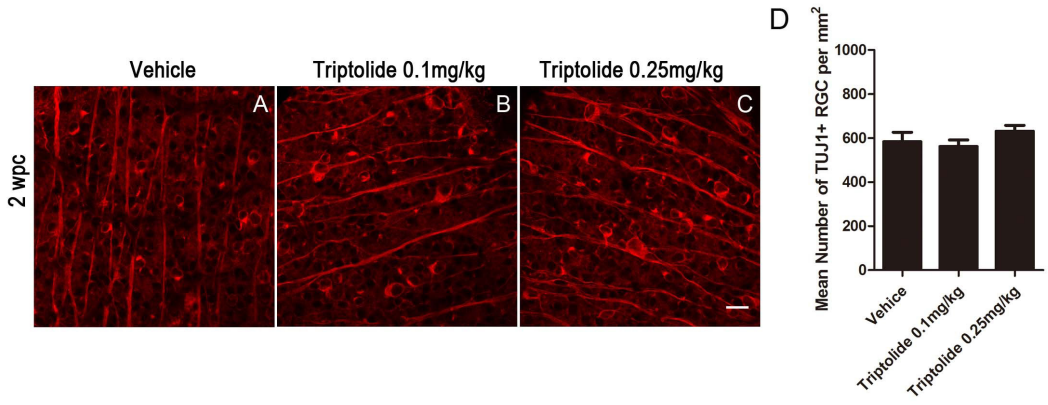

Figure S1. Triptolide post-treatment could not promote retinal ganglion cells survival after optic nerve crush. A, representative picture of RGC labeled with Tuj1 in vehicle group. B, C, representative pictures of RGC labeled with Tuj1 in 0.1mg/kg triptolide (B) and 0.25mg/kg triptolide (C) group 2 week after optic nerve crush. Scale bar=20μm (D) Quantification of Tuj1 positive RGCs. (2wpc: 2 weeks post optic nerve crush).

in the ONC injury eyes of the triptolide group compared with that in the vehicle group, suggesting that TNF-α production was inhibited by triptolide. Thus triptolide was suspected to protect RGCs in this ONC model by restraining the expression inflammatory cytokines, such as TNF-α. As a nuclear transcription factor, NF-κB p50/p65 could be activated and contributed to neuronal death in cerebral ischemia (30,31). The result of this study showed that the nuclear translocation of NF-κB p65 in neurons after ONC could be attenuated by triptolide, indicating involvement of suppression of NF-κB activation in the neuroprotective effects of triptolide. Previous findings have shown that triptolide could inhibit the inflammatory factors by suppressing the activation of glia cells. In the rat spinal cord injury model, triptolide treatment could inhibit reactive astrogliosis and SCI-induced inflammatory reaction (32). Triptolide could play a neuroprotective role in focal cerebral ischemia by reducing astrocyte numbers and NF-κB up-regulation (33). However, further research is still needed to investigate the specific mechanism of inflammatory suppression by triptolide. Taken together, the results suggest that the anti-inflammatory actions of triptolide may play a key role in its protective effects against ONC injury, highlighting triptolide as a promising preventive and therapeutic agent for optic nerve injury.

In summary, this study showed the neuroprotective effects of triptolide in the mice model of ONC. The neuroprotection by triptolide is attributed at least partially to its anti-inflammatory actions. Nevertheless, the contribution of other mechanism for the beneficial role of triptolide needs to be ruled out. Our findings make triptolide an attractive candidate as clinical therapeutic for optic nerve injury.

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