Carvacrol Protect Hippocampal Neurons Against Hydroxychloroquine-Induced Damage: In Vitro Study

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Abstract

The use of hydroxychloroquine, an antimalarial drug, in the treatment of Covid-19 disease, which has turned into a worldwide epidemic, was initially viewed positively. However, the lack of evidence for its use in treatment and even neuronal side effects caused hydroxychloroquine to be approached with suspicion. Carvacrol, on the other hand, is a very interesting ingredient with its anti-oxidant, anti-inflammatory, and anti-cancer properties. Primary neuron culture was prepared for our study. Carvacrol (10, 25, 50, and 100 mg/L), hydroxychloroquine (10,20,40, and 80µM), hydroxychloroquine+carvacrol groups (10µM+10mg/L, 20µM+25mg/L, 40µM+50mg/L, 80µM+100mg/L) were applied to neuron culture for 24 and 48 hours. After the application, results were obtained with MTT, TAS, TOS, Thiol analyses, and acetylcholinesterase (AChE), butyrylcholinesterase (BChE) activities. According to our MTT results, carvacrol (100mg/mL) increased neuronal viability by ~10% in the combined group compared to pure hydroxychloroquine (80µM). The same dose of carvacrol reduced the antioxidant level 1.3 times. Doses of carvacrol alone did not affect thiol levels but increased in combination with hydroxychloroquine(431µmol/L). It is now known that Covid-19 is associated with neurodegenerative diseases. Recent studies have shown that hydroxychloroquine, which is seen as a hope for the global epidemic, causes oxidative stress on neurons. In our study, we designed to both provide protection and prevent the occurrence of side effects by using carvacrol against the neurodegenerative effects of hydroxychloroquine.

Keywords: Carvacrol, Covid-19, Hydroxychloroquine, Neuron, Neuroprotective

Introduction

SARS-CoV-2, which emerged at the end of 2019, surpassed many diseases in the past in terms of both the number of infected people and the rate of spread of the epidemic (Hu et al., 2021). Complications caused by COVID-19 mainly target an immune-inflammatory pathway. Although there is still no proven cure, in vitro studies have shown that hydroxychloroquine, used to treat malaria, is effective in COVID-19 (Cortegiani et al., 2020). Effect of hydroxychloroquine on COVID-19; It manifests by interfering with the endocytic pathway, blocking sialic acid receptors, restricting pH-mediated spike (S) protein cleavage at the angiotensin converting enzyme 2 (ACE2) binding site, and inhibiting cytokine storm (Satarker et al., 2020). Although it is seen as beneficial for COVID-19, Sieb (Sieb et al., 1996) et al. showed in their studies that hydroxychloroquine negatively affects both the presynaptic and postsynaptic aspects of neuromuscular transmission. Bruinink (Bruinink et al., 1991) et al. also found that hydroxychloroquine negatively affected neuronal viability in the primary neuron cultures they used. Giovanella (Giovanella et al., 2015) et al. reported that hydroxychloroquine caused DNA damage in rat brain. While its effect for COVID-19 is still not fully proven, its effects especially on neuronal damage and oxidative stress limit the use of hydroxychloroquine.

Today, many researches are still carried out to find more effective treatment methods and natural products attract a lot of attention due to their anti-inflammatory, antioxidant and immunomodulatory properties (Gandhi et al., 2020). Carvacrol, one of the essential oil compounds, has received special attention due to its specific binding to nonstructural proteins in the viral genome (Javed et al., 2020). In fact, Abdelli (Abdelli et al., 2021) et al. reported that carvacrol can inhibit the entry of COVID-19 into the host cell by inhibiting ACE2 activity. Kulkarni (Kulkarni et al., 2020) et al found that many monoterpenoid phenols, including carvacrol, can inhibit the binding of virus spike (S) glycoprotein to the host cell. In addition, Guana (Guan et al., 2019) et al. showed that carvacrol provides protection against neuronal damage by reducing ROS production. In another study (Wang et al., 2017), carvacrol was shown to attenuate ethanol-mediated hippocampal neuronal dysfunction with antioxidative and antiapoptotic effectsIt has been reported that it exerts its protective effect by reducing neuronal oxidative stress.

The therapeutic use of essential oils in infectious, acute and chronic diseases has become clear. In our study, we aim to eliminate or improve the neuronal effects of hydroxychloroquine by using carvacrol.

Materials and Methods

This study was conducted at the Medical Experimental Research Center at Ataturk University (Erzurum, Turkiye). The Ethical Committee of Ataturk University approved the study protocol (04-2100138265/31.5.2021).

Chemicals

Hydroxychloroquine, Carvacrol, Dulbecco's Modified Eagles Medium (DMEM), Fetal calf serum (FBS), neurobasal medium (NBM), phosphate buffer solution (PBS), antibiotic antimitotic solution (100 x), L glutamine and trypsin-EDTA obtained from Sigma. (St Louis, MO, ABD).

Cell culture

Primary Neuron Culture

22 Sprague Dawley rats less than 24 hours of birth will be used to obtain cortex neurons in the study. Briefly, after the rats are rapidly decapitated, the removed cortexes will be transferred to 5 mL of Hanks' Balanced Salt solution (HBSS), macro fragmentation with the help of a scalpel, and then micro fragmentation with Trypsin-Ethylenediaminetetraacetic acid (EDTA) (0.25% trypsin-0.02% EDTA). Then the cells will be centrifuged at 1200 rpm for 5 min. Cells settled as pellets cellular medium (88% NBM (Neurobasal medium, Gibco, USA), 10% FBS (Fetal bovine serum, Gibco, USA), 2% B-27 (Supplement, Thermo Fisher, Germany), 0.1% It will be added to the environment containing antibiotics (Penicillin-Streptomycin) and amphotericin B (Thermo Fisher, Germany). Cells will be incubated at 5% CO_2 and 37^{o}C for 10 days by changing the medium every 3 days.

Drug Preparation

Drugs were applied after reaching the desired rate (80%) of cell density in neuron culture plates. For this purpose, the platelets were treated with drugs such as hydroxychloroquine (10,20,40 and 80 µM), carvacrol (10, 25, 50 and 100 mg/L), hydroxychloroquine + carvacrol (10µM + 10 mg/L, 20µM + 25mg/L, 40µM + 50mg/L, 80µM + 100mg/L). After application, it was incubated for 24 and 48 hours (5% CO_2, 95% humidity and 37°C).

MTT Tetrazolium Assay Concept

The MTT assay was performed with a commercially available kit (Sigma Aldrich, USA). Briefly, an MTT reagent (10µL at a concentration of 5 mg/ml) was added to each well in the plated and then incubated for 4 hours (5% CO_2; 37°C) (Ali Taghizadehghalehjoughi et al., 2019). After applications, the medium was removed, and 100 µL of dimethylsulfoxide (DMSO) (Sigma, USA) was added to each well to dissolve formazan crystals. Cell viability (%) was calculated by optical density read at 570nm using the Multiskan ™ GO Microplate Spectrophotometer reader (Thermo Scientific, Canada, USA). The control group was accepted as 100, other groups were calculated according to the formula below.

Viability Rate (%) = (O.D of groups/Control O.D.)X 100

Total Antioxidant Capacity (TAC) Assay

The antioxidant capacity was determined by inhibition of the 2-2'-azinobis (3-ethylbenzothiazoline 6-sulfonate = ABTS +) radical cation in the TAC assay (Rel Assay Diagnostics® Company (Gaziantep, Turkiye)). Briefly, to determine the TAC level, the wells are respectively; 30µL sample and 500µL Reagent 1 solution were

added, the initial absorbance was measured at 660nm. Then, 75µL Reagent 2 solution was added to the same wells, and after 10 minutes, the second measurement was made at 660nm. Absorbance values were replaced according to the formula specified in the procedure, and TAC values were calculated as Trolox Equiv/mmol L^{-1} (Aysegul YILMAZ et al., 2021).

A2-A1= ΔAbsorbance (Standard, sample, or H_2O)

$$\text{Result} = \frac{(H_2O\ \Delta Abs - \text{Sample}\ \Delta Abs)}{(H_2O\ \Delta Abs - \text{Standard}\ \Delta Abs)}$$

Total Oxidant Status (TOS) Assay

TOS assay is called the evaluation of color density with spectrophotometric properties depending on the number of oxidants in the sample (from Rel Assay Diagnostics® Company (Gaziantep, Turkiye)). For this purpose, to determine the TOS level, briefly, 500 µL of reagent1 solution was added to the wells containing 75 µL of a sample, and the initial absorbance value was read at 530nm. Then 25 µL of Reagent2 solution was added to the same well. After 10 minutes at room temperature, the second absorbance value was read. Absorbance values were replaced according to the formula specified in the procedure, and TOS values were calculated as H_2O_2 Equiv/mmol L^{-1}.

A2-A1= ΔAbsorbance (Standard or sample)

$$\text{Result} = \frac{\text{Sample}\ \Delta Abs}{\text{Standard}\ \Delta Abs} \times 10$$

The Cholinesterase Activity Assay

Following a 24-h and 48-h treatment with hydroxychloroquine, ginseng, and ginseng + hydroxychloroquine combinations drug, cells were scraped into 0,2 mL of ice-cold homogenization buffer (50 mm Tris–HCl, 1 m NaCl and 50 mm $MgCl_2$, pH 7.4 containing 1%, w/v, Triton X-100). The cells were sonicated on ice for 20 min and then centrifuged at 100.000 g at 4°C and the supernatant fraction collected. Enzymes activity have determined using a colorimetric assay employing acetylthiocholine iodide (ASChI) for AChE and butyrylthiocholine iodide (BTChI) for BChE as substrate (Ellman et al., 1961; Sáez-Valero et al. 1999; Fodero et al 2004). After a 3-min equilibration, the reaction was started with the addition of substrate (ASChI/BTChI). The substrates hydrolysis were determined by monitoring the change in absorbance at 412 nm.

Measurement of Total Thiol Amount

Total thiols were estimated according to the method of Sedlak and Raymond (Reddy et al 2004). Plasma samples (0.1 mL) were mixed with 1.5 mL of 0.2 M Tris buffer (pH 8.2) and 0.1 mL of 0.01 M DTNB. The mixture was made up to 10 mL with methanol and incubated for 30 min subsequently, it was centrifuged 15 min at 3000 rpm and assayed at 412 nm. Standard graphs were used to calculate total thiols.

Statistical Analyses

Statistical comparison between groups was calculated using One-way ANOVA and Tukey HSD

method. All calculations were performed using SPSS 20 software for statistical analysis, and P<0.05 was considered a statistically significant difference in all tests. Results are presented as mean ± standard error.

Results

MTT Tetrazolium Assay Concept

According to our MTT analysis results (figure 1), in which we measured cellular viability at the end of 24 and 48 hours, there was no significant difference even though carvacrol decreased viability with increasing dose and time. In fact, similar to the study results of Wang (Wang et al., 2017) et al., our results showed that carvacrol is protective in neuron cells (~90% protection). Hydroxychloroquine, which negatively affects viability depending on the increasing dose, was especially effective at 20, 40 and 80 µM doses. Hydroxychloroquine 80 µM dose caused a 33% decrease in viability. It was observed that carvacrol was effective even though there was a dose- and time-dependent decrease in viability in the combination groups. While pure hydroxychloroquine (80 µM) causes a 33% decrease in viability, a 24% decrease is observed when co-administered with carvacrol (100 mg/mL). This shows that carvacrol is effective in preserving neuronal vitality.

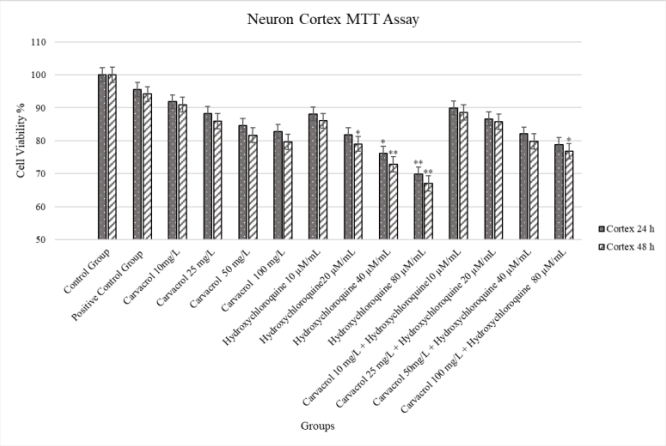
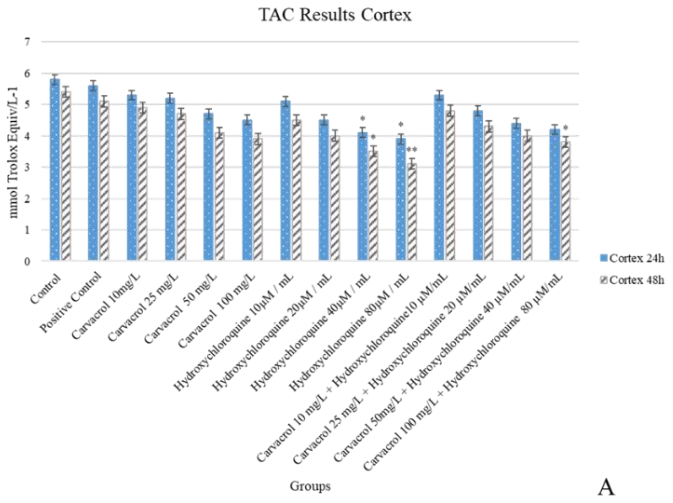


Figure 1. MTT assay results for Cortex cell line after 24 h and 48h Hydroxychloroquine, Carvacrol and combination treatment. (*Significant differences at P < 0.05 compared to control group; **Significant differences at P < 0.001 compared to control group)

Total Antioxidant and Oxidant Analysis

When we examined the antioxidant effects of the components we used in Figure 2A, it was observed that carvacrol decreased the antioxidant effect depending on the increasing dose and time. This decrease did not make a significant difference except for the dose of carvacrol 100 mg/mL at the 48th hour. Carvacrol 100 mg/mL dose reduced the antioxidant level 1.3 times compared to the control. This decrease may be due to the fact that carvacrol provides more effective protection at low doses, as Llana-Ruiz-Cabello(Llana-Ruiz-Cabello et al., 2015) et al. In hydroxychloroquine, the antioxidant level decreases with increasing dose and time. The most significant difference occurred at the hydroxychloroquine 80 µM dose (1.7fold decrease). The combination of carvacrol 100 mg/mL + hydroxychloroquine 80 µM caused a 1.4-times decrease in antioxidant levels (3.8 Trolox

Equiv/mmol L-1). When we examined the oxidant results in Figure 2B, no significant increase was detected at other doses, except for the carvacrol 100 mg/mL dose (3.9 H_2O_2 Equiv/mmol L-1). Hydroxychloroquine, which causes an increase in the oxidant level depending on the dose and time, on the contrary of the antioxidant level, however, a significant increase was observed only at the dose of 80 µM (1.5-times increase in 24 hours; 1.6-times increase in 48 hours). In the combination groups, the most significant difference was observed at the dose of carvacrol 100 mg/mL + hydroxychloroquine µM (1.5-times increase at 48 hours; 4.1 H_2O_2 Equiv/mmol L^{-1}).



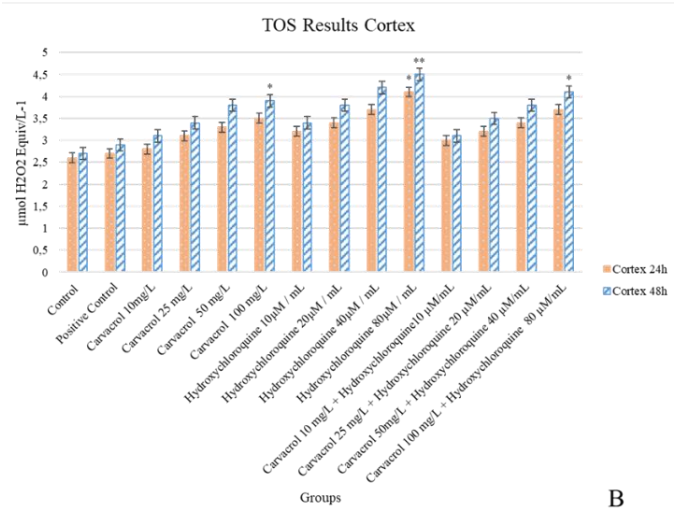


Figure 2. TAC and TOS Levels for Cortex cell line after 24 h and 48h Chloroquine, Carvacrol and combination treatment. A) Cortex cell line TAC Results, B) Cortex cell line TOS Results. (*Significant differences at $P < 0.05$ compared to control group; **Significant differences at $P < 0.001$ compared to control group).

Total Thiol Analysis

Thiol, which plays an important role in preventing the formation of oxidative stress in cells, is very effective in antioxidant defense. Disruption of dynamic thiol/disulfide balance is also an undesirable effect in antioxidant defense (Kundi et al., 2015). When we examined our results in Figure 3, it was seen that carvacrol did not affect the thiol level with increasing dose and time (excluding the dose of carvacrol 100 mg/mL). Hydroxychloroquine, on the other hand, caused an increase in thiol level with increasing dose and time. The most significant increase was seen at 48 hours at hydroxychloroquine 40 and 80 µM doses (respectively; 410 ve 456 µmol/L). In our thiol results, which also showed an increase in the combination groups, we found that the carvacrol 100 mg/mL + hydroxychloroquine 80 µM

group caused the most significant increase at the 48th hour (431 µmol/L).

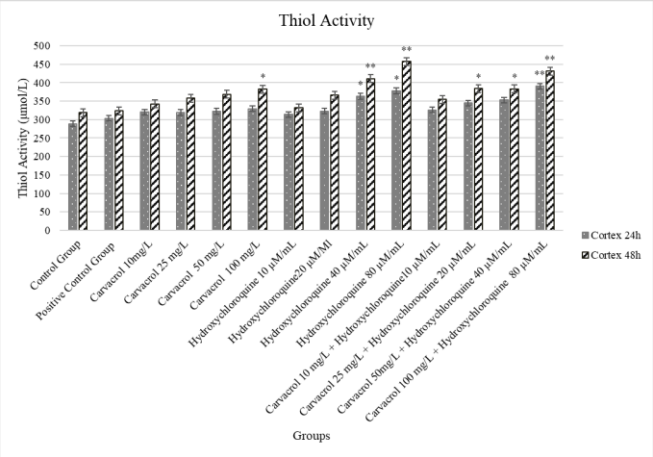


Figure 3. Total Thiol Analysis for Cortex cell line after 24 h and 48h Chloroquine, Carvacrol and combination treatment. (*Significant differences at P < 0.05 compared to control group; **Significant differences at P < 0.001 compared to control group).

Acetylcholinesterase and Butyrylcholinesterase Activity Assays

As shown in Figure 4A, it was shown that carvacrol did not significantly affect acetylcholinesterase (AChE) activity with increasing dose and time. When we look at the effects of carvacrol on butyrylcholinesterase (BChE), we see similar results (figure 4B). Hydroxychloroquine, on the other hand, increased both AChE and BChE activity in a time- and dose-dependent manner. The most significant increase occurred in hydroxychloroquine 80 µM dose, which provided a 1.3-times increase in both activities (0,46 EU/ml AChE and 81,7 EU/ml BChE).

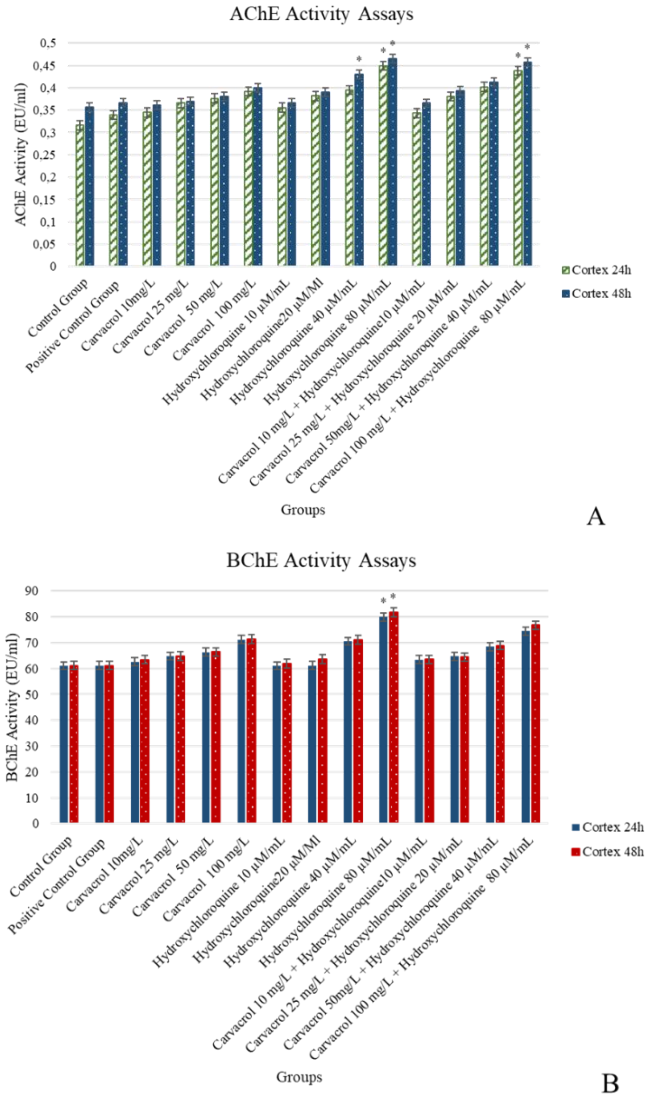


Figure 4. Acetylcholinesterase and Butyrylcholinesterase Activity Assays for Cortex cell line after 24 h and 48h Chloroquine, Carvacrol and combination treatment. A) Acetylcholinesterase activity assays for cortex cell line, B) Butyrylcholinesterase activity assays for cortex cell line (*Significant differences at P < 0.05 compared to control group; **Significant differences at P < 0.001 compared to control group).

Discussion

In general, neurodegenerative diseases are characterized by slowly progressive neuronal loss. Although the etiology of neurodegenerative diseases

has not been fully elucidated yet, oxidative stress is thought to be one of the main sources (Kim et al., 2015). Numerous studies have shown that hydroxychloroquine causes oxidative stress, especially on neurons (Fang et al., 2015; Giovanella et al., 2015; Klouda & Stone, 2020). In our study, we are investigating the effects of carvacrol against neuronal damage caused by hydroxychloroquine used for Covid-19.

Guan (Guan et al., 2019) et al., in their study with carvacrol, showed that carvacrol at 1.2mM and 2.4mM doses protects neuronal vitality, in parallel with ~90% protection in our study. In the same study, it was shown that carvacrol provides protection against neuronal damage and reduces reactive oxygen species (ROS) with an antioxidant mechanism. Guimaraes (Guimaraes et al., 2010) et al. used TBARS (thiobarbituric acid reactive species) and lipoperoxidation (oxidative damage to lipids) methods to measure the antioxidant effects of carvacrol (1µg/ml-1mg/ml). It can be shown to be similar to the result we found that carvacrol 100mg/mL reduced the antioxidant level by 1.4 times in the combined groups. Guimaraes et al. also supported our study findings by finding significant results with increasing dose. Hakimi (Hakimi et al., 2020) et al. showed that treatment with carvacrol was associated with increased thiol, SOD and CAT activity in brain tissue. They support our conclusion that 100 mg/mL dose of carvacrol causes an increase in thiol versus hydroxychloroquine with the combination of lipopolysaccharide (LPS) and carvacrol. Aazza (Aazza et al., 2011) et al. also showed that carvacrol is an important acetylcholinesterase inhibitor in their study and supports the inhibitory effect of carvacrol at low doses in our study. In their in vivo study, Bianchini (Bianchini et al., 2017) et al. observed that carvacrol (50-100mg/L) increased AChE activity in the brain, while Lopez (Lopez et al., 2015) et al. observed that compounds of the same class activated AChE at lower doses and inhibited at higher doses in vitro. According to these results, it can be said that the effects of carvacrol or, in general terms, monoterpenoids on AChE activity depend on concentration and tissue.

Conclusion

Hydroxychloroquine is one of the drugs used to protect against the Covid-19 disease, which has turned into a global epidemic, and it is one of the undesirable effects that it causes neurodegenerative diseases. It is one of the justifications for people to seek alternative ways to avoid being exposed to side effects while fighting the disease. Carvacrol is one of the components that have been used for many years and whose positive effects are known. Anti-oxidant, anti-inflammatory, anti-cancer properties have always made carvacrol interesting. In our study, we aimed to reveal the protective effect of hydroxychloroquine by drawing attention to the antioxidant system on neurons. We think that with the development and advancement of the positive results we have achieved, it can be quite effective in preventive medicine.

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