Journal of Aquaculture Research & Development

Alkobaby and Abd El-Wahed, J Aquac Res Development 2017, 8:1
DOI: 10.4172/2155-9546.1000465

Research Article Open Access

The Acute Toxicity of Copper to Nile Tilapia *(Oreochromis niloticus)* Fingerlings and its Effects on Gill and Liver Histology

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Abstract

This study was carried out to evaluate the response of Nile tilapia, *Oreochromis niloticus* to acute copper toxicity. Nile tilapia fingerlings (2.97 g/f ± 0.37) were acclimated and randomly distributed at a rate of 10 fish per 60-L aquarium. In a series of static renewal-toxicity tests, fish were exposed to concentrations of 0, 5, 10, 15, 20, 25, 30, 35 and 40 mg L^{-1} copper sulfate ($CuSO_4 \cdot 5H_2O$). Fish not exposed to any chemical served as negative controls. Histological sections were done in fish gills and liver in all treatments. Estimates of mean 96-h LC_{50} values (median lethal concentration) value of copper sulfate was 31.2 mg L^{-1} (7.94 mg copper L^{-1}). In all exposure groups, some of the typical gill lesions are presented. The main alternations observed after the exposure to the cupper were epithelial hyperplasia, lifting of the lamellar epithelium, edema in the filamental epithelium, Curling, clubbed tips of secondary lamella and finally a complete fusion of several secondary lamellae at the 35 mg $CuSO_4$ concentration. The severity of the lesions detected increased with the increase of copper sulfate concentration. Exposure to concentrations of copper sulfate more than 10 mg L^{-1} increased the arithmetic thickness of secondary lamella epithelium in *O. niloticus* which was significantly higher ($P<0.001$) than the corresponding control. However, the liver of the Cu-treated fish showed histological alternations such as cytoplasmic rarefaction, an increase of cytoplasmic vacuolation, decreasing the number of hepatocytes nucleus in hepatic tissue and nuclear pyknosis.

Keywords: Nile tilapia; *Oreochromis niloticus;* Copper; Cu; $CuSO_4$; Toxicity; LC_{50}; Gills; Liver

Introduction

Copper is an essential trace metal which plays an important role in several fish metabolic functions. It represents a crucial role in several enzymatic processes (e.g. enzymes involved in cellular respiration, free radical defense, neurotransmitter function, connective tissue biosynthesis and other functions), as well as, into some structural proteins [1-3].

In latest years, environmental pollution from heavy metals has been intensively examined in freshwater ecosystems due to the bioaccumulation and toxicity of these metals [4]. Regardless of its important role in cellular metabolism, copper (Cu) is of particular interest because it is extremely toxic for aquatic animals if elevated concentrations are introduced into the water [5-8]. High copper levels can cause fast generation of reactive oxygen species [9]. It also binds histidine, cystein- and methionine-containing proteins, resulting in dysfunction [10].

The recommended Cu concentrations for fish therapeutic and control of algae and vascular plants in aquaria and fish ponds purposes usually range from 0.05 to 1.0 mg L^{-1} [11-13]. When copper is used in agriculture as fungicide and biofertilizer [14,15], the residual solution is eventually directed to the effluents. Therefore, copper is a pollutant found worldwide in aquatic ecosystems at concentrations ranging from 0.04 to 294 µg L^{-1} or, in extreme conditions, up to 20 mg L^{-1} [16].

Most of copper ions in natural waters are not free, since usually copper is associated with inorganic ions or organic substances. However, it is normal to use copper sulfate ($CuSO_4$) in Egypt and worldwide as an inorganic algaecide in commercial and recreational fish ponds to control the growth of phytoplankton and filamentous algae and to control many fish disease [12,17]. References [18-20] recommended a wide range of copper sulfate concentration (1 up to 100 mg L^{-1}) when it is used for therapeutic purposes according to the disease, such as reducing the incidence of fish endoparasites like protozoa, trematodes, and external fungi and bacteria. However, the concentrations of $CuSO_4$ used for phytoplankton control are seldom directly toxic to fish, but do kill large numbers of rotifers, cladocerans, and copepods [12].

Worldwide, tilapias (including all species) are the second most important group of farmed fish after carps, and the most widely grown of any farmed fish [21]. Nile tilapia (*Oreochromis niloticus*) is by far the most important species [22]. Egypt is the second largest producer of farmed Nile tilapia, which considered the most common fish currently being, cultured commercially [21]. However, Nile tilapia is an omnivorous fish which eats detritus, phytoplankton and zooplankton [23,24] and, consequently, can accumulate copper compounds.

To measure the effects of single or complex mixture of contaminants on the organisms, toxicity tests are conducted. In freshwater, the existence or absence of fish has been extensively used as a biological indicator of the level of pollution. In acute tests experiments, one of the commonly used measures is the lethal median concentration (LC_{50}) that causes mortality in 50% of the test organisms [25]. Nile tilapia is one of the most common freshwater fish used in toxicological studies [26-28]. Due to its easy handling, culture and maintenance in the laboratory, and because it promptly responds to environmental alterations, this species is also a well-established model for toxicological research [26,29].

Gills are considered as a critical organ to fish because they represent the primary place for gas exchange, ion regulation, and excretion of

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Received December 19, 2016; **Accepted** January 19, 2017; **Published** January 21, 2017

Citation: Alkobaby AI, Abd El Wahed RK (2017) The Acute Toxicity of Copper to Nile Tilapia *(Oreochromis niloticus)* Fingerlings and its Effects on Gill and Liver Histology. J Aquac Res Development 8: 465. doi: 10.4172/2155-9546.1000465

metabolic waste products. With a wide surface area open to the external milieu, gills are also the first target to waterborne pollutants [30,31]. When present at high concentrations, copper was reported to cause severe histopathological changes in gills of teleost fish [2,32-40]. Gill damage can be linked to impaired physiological function in fish [41]. Lease et al. [42] found gill histopathology is useful as an early-indicator to monitor fish health in the environment. To quantitatively characterize the histopathological changes caused by waterborne toxicants, several studies have used morphometric methods to analyze the thickness of the filamentary epithelium [34].

The liver plays a main role in the metabolism and excretion of xenobiotic compounds with morphological changes occurring in some toxic conditions [43]. It was reported that metals can increase or decrease the activities of hepatic enzyme and can lead to histopathological hepatic alterations, depending on fish species, the metal type and concentration, length of exposure and other factors [44]. The monitorization of histological changes in fish liver is a highly sensitive and accurate way to assess the effects of xenobiotic compounds in field and experimental studies. However, the copper poisoning can cause pathological injury in various tissues such as liver [38,45-47], kidneys [48] and cardiomyocytes structure [49].

Hence, the aim of this study was to investigate the effects of acute toxicity of copper as copper sulfate ($CuSO_4.5H_2O$) on histopathological aspects of gills and liver, and to determine the value of $CuSO_4$ lethal concentration (96 h-LC_{50}) for Nile tilapia fingerlings in order to determine specific safety concentrations.

Materials and Methods

Fish and maintenance

Healthy Nile tilapia fingerlings, *Oreochromis niloticus*, weighing 2.97 ± 0.37 g with total length of 5.39 ± 0.26 cm were collected from the concrete tanks in fish research Unit, Faculty of Agriculture, Cairo University. For acclimation to laboratory conditions, fish were placed into 60-L glass aquaria in the laboratory supplied with continuous aerated well-water for 3 weeks prior to the experiment. During this period supplemental aeration was provided to maintain the dissolved oxygen near saturation. Water temperature (23 ± 0.1°C), pH (8.2) and total alkalinity (195.66 mg L^{-1} as $CaCO_3$) were measured. Fish were fed "ad libitum" twice daily with pelleted 35% protein commercial tilapia food, and kept with a photoperiod of 12 h light -12 h darkness. Uneaten food was collected after 1 h and water exchanges were 100% every 24 h.

Acute toxicity test

The water used in the toxicity tests was the same as the acclimation period and feeding was stopped 24 hours prior the beginning of 96h-LC_{50} test. After acclimation period, the lethal concentration test of Cu after 96 h (96-h LC_{50}) of exposure was conducted in 60-L glass aquaria equipped with airstones to maintain dissolved oxygen levels greater than 75% saturation. Each aquarium contained 10 fish. Nile tilapia fingerlings were exposed to 0 (control), 5, 10, 15, 20, 25, 30, 35 and 40 mg L^{-1} copper sulfate ($CuSO_4 \cdot 5H_2O$) (the calculated copper concentration values were 1.25, 2.54, 3.82, 5.09, 6.36, 7.63, 8.91 and 10.18 mg Cu L^{-1}, respectively). Three replications per treatment in static renewal-toxicity test were performed to determine 96 h-LC_{50}. Copper sulfate was purchased from Sigma (Egyptian International Center for Import, Cairo, Egypt), and then stock solution was prepared with distilled water and renewed every 24 h to maintain water quality.

The amount of copper sulfate to be added in each aquarium was calculated after the volume of each aquarium was accurately determined. No food was provided to the fish during the test. The control group was submitted to the same protocol but without adding copper. All feces and residues were removed daily by suction, and the dead fish were recorded daily and removed from the aquaria. The mortality rate was determined at the end of the 96th hour. During the exposure to different concentrations of copper sulfate, the behavioral changes of the fishes were also recorded. However, Copper 96-h LC_{50} test was performed as described in the National Exposure Research Laboratory of the U.S. Environmental Protection Agency [50].

Histology

Three fish were taken randomly from each treatment at the final time point for dissection and histological examinations, then were anesthetized by immersion in clove oil solution (0.05 ml L^{-1}) for 5-10 min and euthanized by decapitation. Changes in some organs were observed by the naked eye. The second gill arches from both opercular cavities and then liver were harvested from control and exposed fish. Tissue specimens were first fixed in 10% buffered formalin for 24 h at 4°C, and then immediately dehydrated in a graded series of ethanol, immersed in xylol and embedded in paraffin wax. Sagittal sections (5 µm of thickness) were cut and mounted on glass slides. Sections were deparaffinized in xylene, hydratated in ethanol and was stained with hematoxylin-eosin (HE).

Changes induced by treatment in the gill and liver tissues were photographed and analyzed by light microscopy (LEICA DMI300 B microscope and LEICA DFC290 Digital Camera). Gill injuries were quantified by measuring the thickness of secondary lamella epithelium by using an image analysis system (Leica application suite V4, from leica Microsystems). Two primary filaments were selected from the middle of the second gill arch from each fish, and at least the thicknesses of 20 secondary lamellae were measured in each specimen.

Statistical analysis

Statistical analyses were carried out using statistical SPSS 16 package. A one-way analysis of variance (ANOVA) followed by a Duncan's post hoc test were performed to identify any significant differences between the treatments ($p<0.001$). All results were given in mean ± standard error of the mean (S.E.M).

Results

Fish behavior and anatomy

No control fish died during the experimental period. Mortality occurred only in the exposed fish and the mortality increased by increasing the concentration of copper sulfate in water. Before death, exposed fish showed unstable swimming with unbalanced movements, exhaustion, suspended in vertical position with the mouth up near the water surface and finally submerged in the bottom of water with no motion. Compared to the control, the treated fish showed pale gills, some damaged gills, flabby intestine, and swelling in liver, gall bladder and spleen.

Acute toxicity

Estimated mean 96-h LC_{50} value (median lethal concentration) determined in static renewal-toxicity test for Nile tilapia fingerlings was 31.2 mg copper sulfate L^{-1} (7.94 mg copper L^{-1}).

Gills histology and thickness of secondary lamella epithelium

The gill morphology of the untreated fish was similar to that of

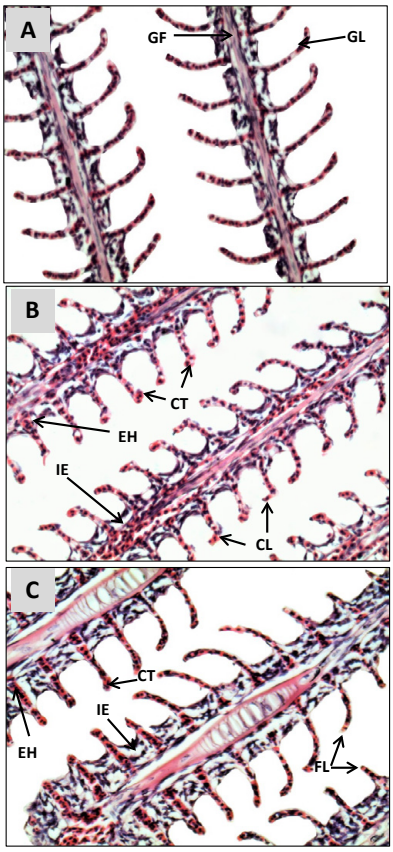

Figure 1: Histopathological changes in gills of *O. niloticus*. (A) Control treatment showing normal gill filaments (GF) and gill lamella (GL). (B) Gills of fish exposed to 25 mg L^{-1} copper sulfate, showing epithelial hyperplasia (EH), epithelial interstitial edema (IE), curling in secondary lamella (CL) and clubbed tips of secondary lamella (CT). (C) Gills of fish exposed to 35 mg L^{-1} copper sulfate, showing hyperplasia of epithelium, edema in the filamentary epithelium with intense lifting in the lamellar epithelium, curling and clubbed tips and finally a complete fusion of several secondary lamellae (FL). Sections (X400) were stained with hematoxylin and eosin.

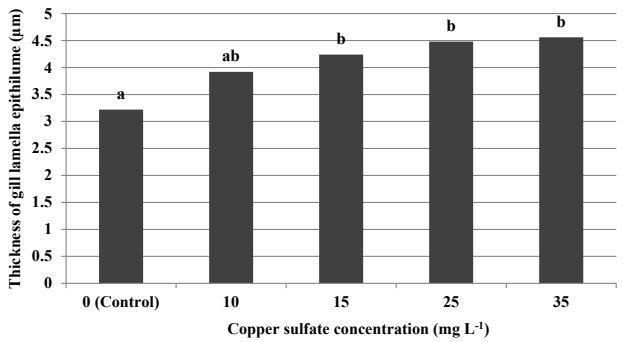

Figure 2: Arithmetic mean thickness of gill lamella epithelium of *O. niloticus* exposed to different concentrations of copper sulfate (mg L^{-1}). Different letters mean statistically significant differences (P<0.001) between copper-exposed and control fish.

other teleost fish species as reported by Wilson and Laurent in 2002 (Figure 1A). Fish which exposed to the different concentrations of $CuSO_4$ showed epithelial lesions. The main alternations observed after the exposure to the copper were epithelial hyperplasia of both primary and secondary lamellae epithelium, the severity of hyperplasia increased with the increase of copper sulfate concentration, leading to the complete fusion of several secondary lamellae at the 35 mg $CuSO_4$ L^{-1} concentration. Lifting of the lamellar epithelium and edema in the filamental epithelium were other lesions observed starting the 15 mg L^{-1} $CuSO_4$ exposure concentration. "Curling" and clubbed tips of secondary lamella were also detected (Figures 1B and C).

As shown in Figure 2, acute exposure to copper increased the arithmetic thickness of gill lamellae epithelium. Statistical analysis showed no significant ($p>0.001$) differences between thickness of gill lamellae epithelium of the control fish (3.22 ± 0.115 µm) and fish exposed to 10 mg L^{-1} copper sulfate (3.92 ± 0.204 µm). On the contrary, significant increase ($p<0.001$) in epithelial thickness occurred in fish exposed to 15, 25 and 35 mg L^{-1} copper sulfate, where the values of thickness were 4.24 ± 0.141 µm, 4.48 ± 0.174 µm and 4.56 ± 0.283, respectively.

Liver histology

Our study indicated that the hepatopancreas of control group showed a normal structure without any pathological lesions. The hepatocytes were polygonal cells with a homogenous cytoplasm, and a large central spherical heavily-stained nucleus, also the pancreatic area with its pancreatic acini was obviously normal along the portal vessels within the liver. The hepatic parenchyma of fish exposed to copper sulfate exhibited histological alternations such as cytoplasmic rarefaction and an increase of cytoplasmic vacuolation, the number of hepatocytes nucleus was decreased and nuclear pyknosis was observed (Figures 3A, 3B and 3C). These hepatic alterations were more evident in

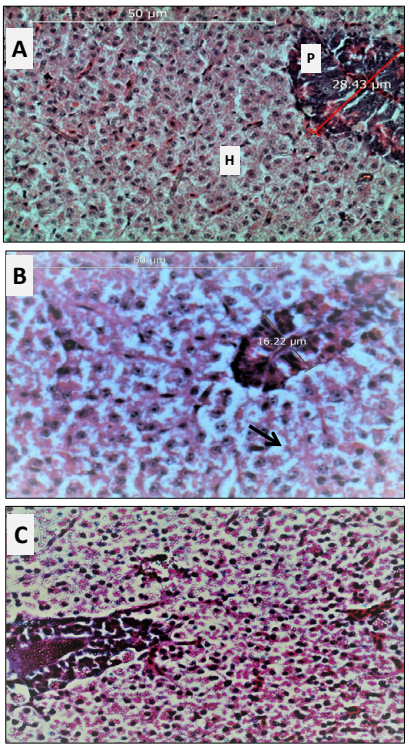

Figure 3: Histopathological changes in liver and pancreas of *O. niloticus*. (A) Control treatment showing normal hepatocytes (H) and pancreatic area (P) that corresponds to the acini of exocrine pancreas. (B) Liver of fish exposed to 25 mg L^{-1} copper sulfate, showing cytoplasmic vacuolation (arrow), nucleus pyknosis and partial atrophy in the pancreatic tissue. (C) Liver of fish exposed to 35 mg L^{-1} copper sulfate, showing deterioration of hepatic parenchyma and the acini in pancreatic tissue. Sections (X400) were stained with hematoxylin and eosin.

fish exposed to 25, 30, 35 and 40 mg L^{-1} copper sulfate concentrations. Furthermore, a partial atrophy in the pancreatic tissue was occurred in fish exposed to 25 mg L^{-1} copper sulfate, and deterioration of its acini and the acinar arrangement were observed in the fish exposed to higher concentrations of copper sulfate (35 and 40 mg L^{-1}).

Discussion

Acute toxicity

Our toxicity test result supports the observations of Abdel-Tawwab and Mousa [51] who stated that the range of 96-h LC_{50} for Nile tilapia was 5.03-14.27 mg copper L^{-1}. On the other hand, the 96-h LC_{50} determined in the present study was higher than other reported values of 1.09, 0.853 and 1.207 mg Cu L^{-1} for Nile tilapia [52-54]. This could be due to the differences of fish size and the relatively higher water alkalinity value of our case in comparison to their values. The toxicity of copper sulfate to fish decreases as pH, total alkalinity and total hardness increase, and as copper (Cu) binds to inorganic or organic substrates [17]. Abdel-Tawwab and Mousa [51] found that the pre-exposure to 50-100 mg Ca^{2+} L^{-1} might be effective in reducing the acute toxicity of copper to Nile tilapia fry. Copper sulfate treatments in low-alkalinity waters may be detrimental to the health of blue tilapia [55]. However, Kosai et al. [56] found copper LC_{50}-96 h value for Nile tilapia as 185.8 mg L^{-1}, which is extremely high compared to our results.

The impact of copper on the aquatic environment is complex and depends on the physicochemical characteristics of water and fish species [57-59]. However, previous researches have been carried out on the 96-h acute toxicity of Cu and showed many differences between species. These results reported 0.2 to 43.1 mg Cu L^{-1} for *O. aureus* [55], 0.24-6.23 mg Cu L^{-1} for *O. mossambicus* [60], 0.092 mg Cu L^{-1} for rainbow trout [61], 40.86 for *Clarias gariepinus* [62] and 0.77 mg Cu L^{-1} for *Synechogobius hasta* [39].

Gills histology and thickness of secondary lamella epithelium

In this study, histopathological alternations in gills were observed. The detected changes in tilapia gills as gill hyperplasia, Edema, lifting of lamellar epithelia, intense lamellar vasodilation and curling of secondary lamellae were generally accentuated to the lethal effect of copper. This study is in agreement with Mallatt and Fernandes et al. [30,38] who demonstrated that gill hyperplasia and interstitial edema are the more frequent lesions observed in gill epithelium of fish exposed to heavy metals, and with Al Bairuty et al. and Shaw et al. [47,63] who said that edema may result in osmotic imbalance. The results of this study showed the occurrence of edema independently of copper levels [2,64]. The lifting of lamellar epithelium is another histological change observed, probably induced by the incidence of severe edema. In the present study, increasing epithelial lifting and hyperplasia were depending on the increasing of waterborne Cu concentrations. The last observation is in agreement with other studies [39,65-67]. Edema with lifting of lamellar epithelium and different degrees of hyperplasia are typical defense mechanisms, because separation epithelial of the lamellae increases the pollutant-blood diffusion distance causing gaseous exchange [33]. These gill histological alterations have been observed by several authors in fish submitted to copper [33,38,40,65,66,68,69]. At the 25 mg L^{-1} $CuSO_4$ concentration applied, curling and clubbed tips of secondary lamella were occasionally detected and this finding is in agreement with References [47] and [67], as well as the studies of Gill et al. [70], Skidmore and Tovell [71] who used other types of metals (cadmium on *Puntius conchonius* and $ZnSO_4$ on *Oncorthynchus mykiss*, respectively). A complete fusion of several lamellae was recorded at the 35 mg L^{-1} $CuSO_4$. This could be attributed to the direct effect of heavy metal. The fusion of the secondary lamella was reported by Ostaszewska et al. [67], Al-Bairutya et al. [47]. Accordingly, all these histological changes resulting from the exposure to metals are found to be a compensatory response to keep metal from entering through gill cells [30,37,66,72].

In our study the mean of arithmetic thickness of gill lamellae epithelium was 3.22 ± 0.115 µm in control fish for *O. niloticus* fingerlings. Those values of thickness of gill lamellae epithelium differ between fish species. *O. mossambicus* has a mean value of 3.03 µm while *T. sparrmanni* has a mean value of 4.98 µm [73]. Studies done by Van Heerden et al. [36] and Lappivaara et al. [74] showed basal thickness of gill lamellae epithelium values for *O. mykiss* of 3.73 and 4.2 µm, respectively. The significant increase in epithelial thickness happened in this study when fish exposed to 15 mg L^{-1} copper sulfate (3.82 mg L^{-1} Cu), while Van Heerden and Tiedt [73] found the increasing in arithmetic thickness of gill epithelium in *Oreochromis mossambicus* and *Tilapia sparrmanii* was occurred when exposed to 600 µg L^{-1} and 4.4 mg L^{-1} copper, respectively. These variations may be occurred because of the differences of fish species, fish size and the conditions of the experiment. Thickening of epithelium in gills of fish exposed to copper was caused by hypertrophy in cells, beside lamellar telangiectasis. However, morphometric measurements on gills of fish such as the arithmetic thickness of secondary lamella epithelium could be a simple and real indicator of toxic exposure before permanent damage occurs.

Liver histology

Compared to control the histology showed some alternations in the liver of fish exposed to 15-40 mg L^{-1} copper sulfate such as cytoplasmic rarefaction and an increase of cytoplasmic vacuolation. The outcome of this study came in agreement with many References [38,39,44,66,75] who demonstrated an increase of cytoplasmic vacuolation. Degenerative-necrotic conditions are often associated with these histological changes [76]. These alternations could be explained by Cu-induced oxidative stress in the tissue of the liver [77]. Our study accentuated that the rate of karyolysis increases with the increase of copper concentration and also observed nuclear pyknosis. Many studies confirm the same alternations [38,39,66]. References [47,66,78] suggested that the alterations in size and shape of nucleus may be considered as signs of increasing the metabolic activity but probably of pathological origin.

Furthermore, a partial atrophy in the pancreatic tissue was observed in fish exposed to 25 mg L^{-1} $CuSO_4$ concentration, and deterioration of its acini and the acinus arrangement were observed in the exposed fish to high concentrations of copper sulfate (35 and 40 mg L^{-1}) and it could be attributed to the necrotic condition in the tissue. It could be concluded that the liver is a central compartment for the metabolism of Cu in fish [79,80] and also is the main organ for detoxification and more responsive for damage [66,75,81].

Conclusion

It could be concluded that although copper sulfate is a good ectoparasite therapeutant, it can be extremely toxic to Nile tilapia, which is relatively tolerant to copper sulfate when compared to other species. The results of this study indicate that exposure to 15 mg L^{-1} or more of $CuSO_4$ for 96 h causes significant injuries in gills, liver and panceriatic tissue of Nile tilapia. Furthermore, morphometric measurements on gills of fish such as the arithmetic thickness of secondary lamella epithelium could be a simple and real indicator of toxic exposure before permanent damage occurs.

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