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Scientific Paper

FUMONISIN B_1 NEUROTOXICITY IN YOUNG CARP (*CYPRINUS CARPIO* L.)*

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For years scientists have suspected that the environment plays a role in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis. Mycotoxin fumonisin B_1 (FB_1) is produced by several *Fusarium* species, mainly by *Fusarium verticilioides*, which is one of the most common fungi associated with corn worldwide. Fumonisins are known to cause equine leukoencephalomalacia, a disease associated with the consumption of corn-based feeds contaminated with FB_1. Here we have reported chronic experimental toxicosis in one-year-old carp (*Cyprinus carpio* L.) receiving feed containing 100 mg kg^{-1} or 10 mg kg^{-1} of added FB_1 for 42 days. We focused on fumonisin toxicity in the fish brain. After staining with hemalaun-eosin, histology of the fish brain revealed vacuolated, degenerate, or necrotic neural cells, scattered around damaged blood capillaries and in the periventricular area. These findings suggest that fumonisin, although it is a hydrophilic molecule, permeated the blood-brain barrier of young carp and had a toxic effect on neuronal cells.

KEY WORDS: *blood-brain barrier, environment, mycotoxins, neurodegenerative diseases*

The old hypothesis that neurotoxins may play a role in neurodegenerative disorders has recently been reintroduced into the focus of scientific interest. There is provocative evidence that environmental exposure to certain toxins may affect the development of neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, parkinsonian syndromes, amyotrophic lateral sclerosis, and multiple sclerosis. Recently published works support the cycad hypothesis for Guamanian amyotrophic lateral sclerosis/parkinsonism dementia complex (ALS/PDC), based on the detection of [beta]-methylaminoalanine (BMAA) of cyanobacterial origin in cycad tissue (1). Some environmental factors (e.g. cigarette smoking, high serum cholesterol levels, infections, metals, industrial or other toxins) may trigger oxidation, inflammation, and disease processes, particularly in people with genetic susceptibility to Alzheimer disease (2-5). Administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces selective damage of the *substantia nigra* dopaminergic cells and causes the parkinsonian syndrome (6).

Mycotoxins are secondary fungal metabolites associated with severe toxic effects to vertebrates. They are produced by many phytopathogenic and food spoilage fungi including the *Aspergillus, Penicillium, Fusarium*, and *Alternaria* species. Food and feed contamination with mycotoxins is a worldwide problem. Beside their toxic effects on other organ systems, mycotoxins are neurotoxins that can produce a wide spectrum of behavioral and cognitive changes, ataxia, and convulsions. Systemic administration of harmaline and oxotremorine produces motor tremor

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in a variety of mammalian species (7, 8). Naturally occurring tremorogenic mycotoxins are synthesised by the *Aspergillus, Penicillium*, and *Claviceps* species.

Factors affecting the risk of toxin-induced neurodegenerative disorders include time and concentration in the organism, the ability to access the central nervous system, and the route by which a compound reaches the central nervous system or secondarily affects other organ systems leading to central nervous system disruption. The blood-brain barrier (BBB), which controls the passage of substances from the blood into the brain and spinal cord, lets essential metabolites, such as oxygen and glucose, pass from the blood to the brain and central nervous system (CNS), but blocks most molecules that are more massive than approximately 500 Da. BBB works as a filter, restricting access to many natural toxins, metals, antibodies, and biological complexes and, in many cases, actively removing these substances from the brain by energy-dependent efflux (9). Various factors and conditions can increase the permeability of BBB, including hypertension, hyperosmolarity, microwaves, radiation, infection, brain injury due to trauma, ischaemia, inflammation, pressure, and certain substances. Additionally, BBB is not fully formed at birth, which means that it is more permeable in young animals and humans (10, 11).

Fumonisins are toxins produced by the fungi *Fusarium verticiloides, Fusarium proliferatum*, and allied species under favourable conditions during infection of corn (12, 13). The most frequent and potent among them is fumonisin B1 (FB_1). Fumonisins contained in grain disrupt the sphingolipid metabolism by inhibiting ceramide synthase, which in turn rapidly increases intracellular sphinganine concentration (14). The primary cause of toxicity seems to be the accumulation of sphingoid bases, but the effects also involve many other biochemical events (14, 15). In addition to naturally occurring toxicoses in several species such as leukoencephalomalacia in horses (16) or pulmonary oedema in swine (17), laboratory experiments have demonstrated sensitivity to FB_1 in all tested animals. FB_1 has a molecular weight of 705 Da and is a highly hydrophilic chemical. Following gavage administration, it poorly absorbs from the gastrointestinal tract; trace amounts were detected in urine, liver, kidney, and in red blood cells, whereas none was detected in plasma, heart, or brain. It does not appear to cross the placenta, and there is little evidence that it crosses BBB (18-20).

The only fish species tested so far, channel catfish *(Ictalurus punctatus),* were exclusively fed on diet contaminated with FB_1 at concentrations of 35 mg kg^{-1}, 62 mg kg^{-1}, 170 mg kg^{-1}, or 313 mg kg^{-1} for 5 weeks (21). Here we have reported chronic experimental neurotoxicosis in one-year-old carp (*Cyprinus carpio* L.) receiving feed containing 100 mg kg^{-1} or 10 mg kg^{-1} of added FB_1 for 42 days.

MATERIALS AND METHODS

Fumonisin B_1

Fumonisins were biosynthesisedin a liquid culture medium (yeast extract 20 g; sucrose 40 g; 1000 mL sterile water, pH 7.4) inoculated with a *F. verticiloides* isolate from a corn sample from Northern Croatia. One-millilitre suspension of conidia was inoculated into 1000 mL of the medium and incubated at 25 °C for 15 days. All chemicals (pro analysis) used for the extraction of FB_1 were purchased from Kemika (Croatia). The *F. verticiloides* liquid culture medium was homogenised with a 100-mL acetonitrile:distilled water mixture (9:1) in an electric homogeniser (3500 rpm) for 10 minutes and then filtered. The filtrate was extracted with *n*-hexane (2x25 mL) to remove lipids. The upper hexane phase of the filtrate was removed and the water-soluble phase, adjusted to pH value 8 to 9 with 25 mL $NaHCO_3$ (saturated solution) was then shaken with 2x25 mL of chloroform. The water-soluble phase was partially evaporated at 80 °C and then concentrated under vacuum by lyophilisation (Freeze dryer Alpha 1-4, Martin Chirst Osterode/ Harz). The lyophilisate (100 mg) was dissolved in water (100 mL). The water solution of the sample (50 μL) and 10 μL of FB_1 standard (0.5 mg mL^{-1}) (Sigma Chemical Co., approx 98 %) were spotted on preparative GF254 silica plates (Sigma Aldrich Chemie Gmbh) (20 cm x 20 cm), preheated for an hour to 110 °C. The plates were developed with acetonitrile:toluene:water (93:5:2) and subsequently dried in warm air. FB_1 was visualised under 366 nm UV light as bright blue zones and identified by its retention factor (Rf) of 0.75. It was purified by thin layer chromatography (TLC). Different amounts of the lyophilisate water solution were spotted on preparative GF254 silica plates and developed with acetonitrile:toluene:water (93:5:2). The fumonisin was scraped from the plates, dissolved in water, filtered to remove the silica gel, and the filtrate was

lyophilised. This procedure was repeated to collect the necessary amount of FB_1. A stock solution of FB_1 was prepared indistilled water (5 mg mL^{-1}) and kept refrigerated at 4 °C. This stock solution was further diluted with distilled water for uniform admixing to the experimental feed.

Carp

Healthy one-year-old common carp *(Cyprinus carpio* L.) were obtained from a pond of the Topličica company (Novi Marof, Croatia). No obvious diseases or abnormalities were found by routine analyses before the experiment. The fish were randomly divided into four groups (mean weight in each group: 127 g; range: 70 g to 131 g) of eight fish each and placed in four wire mesh cages (0.7 m x 0.7 m x 0.7 m) immersed in a pond. The experiment started after 4 weeks of conditioning to the new environment and training to take manually given feed at or near the water surface.

Feed

Pellets were prepared weekly. Equal parts of crushed commercial pellets (PVA and Pellets Co., UK) for trout (protein content 45 %), wheat flour and an adequate amount of distilled water (with or without FB_1) were mixed to obtain the dough. A household machine was modified toform pellets from the dough. After drying, the feed was stored in a refrigerator. The concentration of FB_1 in the feed given to the experimental group I was 100 mg kg^{-1} of dry diet ingredients, and to group II 10 mg kg^{-1}.

Measurement of Evans blue dye (EBD) for BBB integrity

The permeability of Evans blue dye was evaluated for BBB integrity in accordance with a modified method by Rapoport et al. (22). BBB integrity was measured in eight control animals not treated with FB_1. The carp were anaesthetised with MS-222 (Sigma Aldrich Chemie Gmbh), and 2 mL kg^{-1} body weight of 2 % Evans blue dye saline (Sigma Aldrich Chemie Gmbh) was injected intracardially and allowed to circulate for a couple of minutes. The pericardial cavity of the carp was opened and perfused with saline solution through bulbus arteriosus, and then by 4 % formaldehyde in PBS. After the experiment, the anaesthetised carp were bled by cutting the gill arches. Their brains were removed immediately and post-fixed in 4 % formaldehyde in PBS solution. All brains were photographed (Fujifilm, FinePix A303, 2x) and the degree of Evans blue staining graded on a scale of 0 to 3, according to the modified method by Rapoport et al. (22). Zero denoted no stain uptake, 1 - light staining, 2 - darker staining, and 3 - deep blue staining.

Experimental procedure

The experiment started at the end of May and lasted 42 days. Mean water temperature during the experiment was (20±2.5) °C and the pH was 7 to 8. Three groups of fish were receiving appropriate pellets (control, 10 mg kg^{-1} of FB_1, or 100 mg kg^{-1} of FB_1) once a day in the amount equal to 5 % of their body weight. The fourth group was tested for BBB integrity with Evans blue dye. Once a week, the groups were examined visually by lifting the cages. We looked for dermal lesions, and followed fish behavior and vigilance.

Histopathology

At the end of the 42-day fumonisin B_1 administration, the animals were killed by overexposure to the anaesthetic MS-222 (Sigma Aldrich Chemie Gmbh) and resected. Samples of the brain were fixed in 10 % neutral buffered formalin (Kemika Ltd., Croatia). After fixation, the samples were automatically dehydrated (Reichert-Jung, histokinette 2000) in a series of ethanol solutions of increasing concentrations, and then impregnated in paraffin (termed hell-parafin). Paraffin blocks were cut to 5-µm thick slices using a microtome (Leitz 1512), and the slices were stained with haematoxylin and eosin according to the method of Lillie (23). Histological brain samples were examined under a light microscope (Olympus BH-2) at 200x to 700x magnification.

The degree of brain oedema was graded from 0 to 3, as follows: 0 - no oedema; 1 - slight oedema; 2 - moderate oedema; 3 - severe oedema. The grading was based of the generally accepted morphological criteria for cerebral oedema: pallor of myelin, distension of perivascular and pericellular spaces, a loose or sieve-like appearance of myelinated areas, rarefaction of subpial spaces, vacuolar appearance of the neuropil, and pools of protein-rich fluid (24, 25).

In the brain tissue, we determined the number of apoptotic cells, degenerative lesions, and the occurrence of inflammatory changes. The number of apoptotic cells was recorded in 10 randomly selected fields at 400x magnification. Degenerative lesions

(hyaline and/or vacuolar degeneration, necrosis) were counted in 10 randomly selected fields at 400x magnification. Inflammatory cells was counted in three randomly selected fields at 200x magnification and graded from 0 to 3, according to the number of fields affected.

Statistical analysis

The data were statistically analysed using SPSS® and Microsoft Excel® for Windows). The Kruskal-Wallis and Jonckheere Terpstra test were used for within-group comparisons of apoptotic cells, degenerative lesions, and inflammatory changes. Difference at $P<0.05$ was considered significant.

RESULTS

Clinical signs

We have observed no mortality in either experimental group. The final examinaiton revealed slower body-weight gain in both FB_1-exposed groups [(80±10) g per animal in the low-dose group and (81±5) g in the high-dose group] than in control (114±12) g. Furthermore, six of the eight fish in the high-dose fumonisin group had carp erythrodermatitis, and the group was markedly less vagile than other groups. However, no other clinical differences were observed among the three experimental groups.

BBB integrity testing

In the fourth group used exclusively for BBB integrity testing, the brains of six carps showed no Evans blue staining (Figure 1). In two carp, the spinal cord showed grade 1 staining (Figure 2), but the other parts of the brain were clear. Evans blue binds to albumin *in vivo*; its absence from the brain (with an exception of circumventricular organs) suggests that BBB is impermeable to proteins.

Histological examination

Five fish treated with higher FB_1 doses had a moderate (Figures 3 and 4), two a slight, and one severe brain oedema. The degree of brain edema differed significantly ($P>0.05$) from the group treated with lower FB_1 doses and control group. In both four carp had a slight brain oedema, two moderate, and two none.

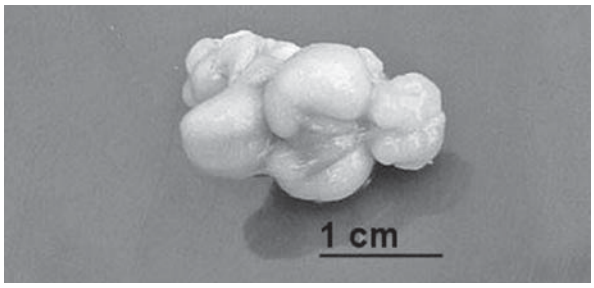

Figure 1 *Brain of control carp shows no Evans blue staining.*

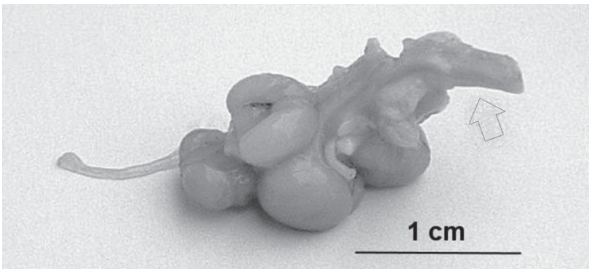

Figure 2 *Evans blue staining of the circumventricular structure in the caudal neurosecretory system (transparent arrow) of control carp*

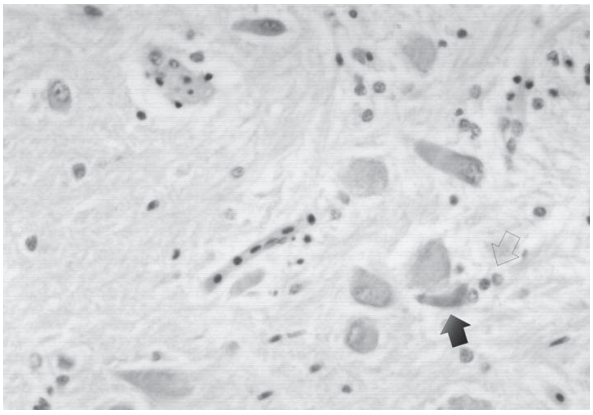

Figure 3 *Brain tissue shows accumulation of glial cells (open arrow) and some degenerate neural cells (black arrow) with histological signs of perivascular oedema in carp receiving diet with 100 mg kg^{-1} FB_1 (magnification 500x).*

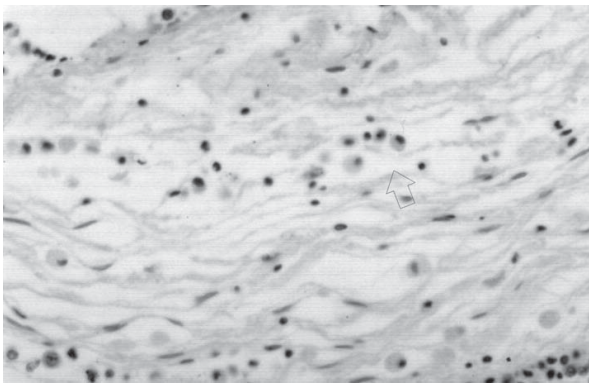

Figure 4 *Meningeal congestion and oedema with lymphomononuclear cells (transparent arrow) in carp receiving diet with 100 mg kg^{-1} FB_1 (magnification 400x).*

Apoptoses (Figure 5) varied in morphology from cell shrinks and chromatin condensation to apoptotic bodies. The mean number of apoptoses was (1.00±0.81) in the high-dose fumonisin group, (0.5±0.52) in the low-dose fumonisin group, and (0.1±0.31) in the control group. The difference between high-dose fumonisin group and low-dose fumonisin group and the difference between both treated groups and control was significant (P<0.05).

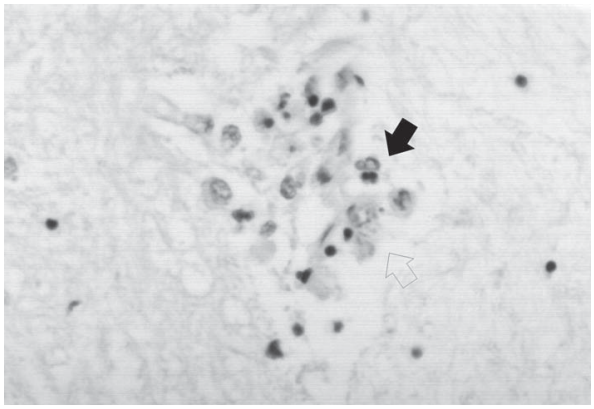

Figure 5 *Detail of focal neuronal apoptosis (black arrow) and necrosis (transparent arrow) with glial reaction in carp receiving diet with 100 mg kg^{-1} FB_1 (magnification 700x).*

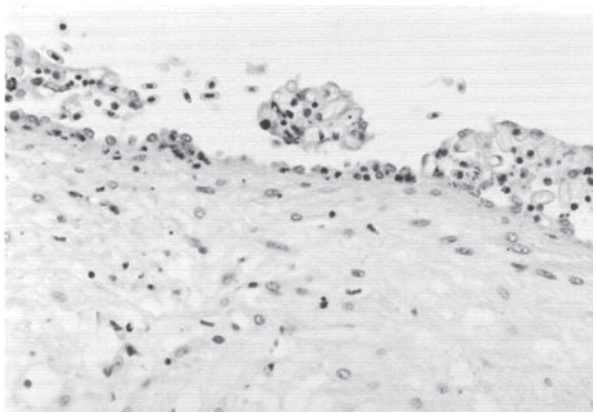

Figure 6 *Haematoxylin-eosin staining of coronal brain sections shows damaged, irregular, and ruptured structure of the ependymal cell layer. Cerebrospinal fluid in these areas contains cell debris and lymphocytes. (Carp receiving diet with 100 mg kg^{-1} FB_1; magnification 700x).*

The mean number of degenerative cells was (1.7±1.49) in the high-dose fumonisin group (Figures 3 and 6), (1.0±0.66) in the low-dose fumonisin group, and (0.2±0.42) in the control group (P<0.05).

Most of the fish treated with higher fumonisin doses had a moderate accumulation of inflammatory cells, and one showed severe inflammation (Figures 4 and 6). Other animals from this group showed no obvious signs of inflammation. The control group showed no inflammation. Accumulation of inflammatory cells differed significantly between the groups (P<0.05).

DISCUSSION

Depending on animal species, FB_1 can cause neurotoxicity, hepatotoxicity, nephrotoxicity, immunosuppression, developmental abnormalities, liver tumours, and other disorders (26). It can cause equine leukoencephalomalacia (27) and porcine pulmonary edema (28). Although FB_1 is evidently neurotoxic for horses and ponies, only a few investigations confirm its neurotoxicity in other animal species (29, 30).

Our results have shown dose-dependent histopathological changes in the brain and brain vasculature of carp treated with FB_1. They demonstrate the neurotoxicity of FB_1, which got across BBB.

An earlier study of adult channel catfish has shown that fumonisin causes perivascular lymphocyte infiltration in the brain (31). Our investigation suggests that FB_1 passed BBB in young carp. Previous findings indicate that the molecular weight of the compound plays an important role in its capability of crossing BBB, For example, albumin, with molecular weight of about 69,000 Da (32) did not get across BBB. Evans blue staining has shown only trace amounts of albumin in the grey matter of spinal cord of two fish. This suggests that FB_1 can probably pass BBB to a minor degree in lower vertebrate species, which calls for further investigation. This level of permeation may be more probable in young animals.

Even though our results indicate the transport of FB_1 to carp brain, it still remains unclear which mechanisms are involved. However, results on mammals suggest that FB_1 transport to the brain does not depend on multidrug transport system, in which P-glycoprotein plays a major role. This is supported by the findings of Sharma et al. (33), who demonstrated that knockout mice deficient in P-glycoprotein did not exhibit greater sensitivity to FB_1. Osuchovski et al. (34) reported that pre-treatment with lipopolysaccharides increased BBB permeability and allowed fumonisin to enter the brain of female BALB/c mice. Other studies showed that ochratoxin A (OTA) induced biochemical changes and cytotoxicity in rat brain (35, 36). Some *in vitro* studies suggest that ionophoric mycotoxin beauvericin (BEA) may be neurotoxic (37). In addition to fumonisins and

ochratoxin, *Fusarium* spp. can produce secondary toxic metabolites, the so-called emerging mycotoxins such as fusaproliferin, beauvericin, enniatins, and moniliformin (38). In other words, contamination with *Fusarium* mould could expose animals to several mycotoxin species at the same time.

Marcine et al. (39) showed that direct exposure of murine brain to FB_1 results in neurotoxicity, characterised by biochemical and pathological changes. Intraventricular injection of FB_1 in their study caused neurodegeneration, inhibition of *de novo* ceramide synthesis, stimulation of astrocytes, and upregulation of pro-inflammatory cytokines in the brain of BALB/C mice.

Kwon et al. (40) have shown that FB_1 alters sphinganine (Sa) levels and myelin synthesis in the central nervous system of developing rats. FB1 seems to cause pathological changes in the brain function and morphology by impairing sphingolipid metabolism.

Studies of equine leukoencephalomalacia raise the question why horses are particularly susceptible to neurotoxic effects of fumonisin than pther investigated animals. This is more likely due to lower BBB permeability to fumonisin-like molecules in these animals than due to greater fumonisin absorption from the gastrointestinal tract in horses. Further investigations should shed more light on this issue.

Our investigation also touches upon possible neurological consequences of fumonisin exposure early in life. Due to its specific mode of development, the central nervous system of a young animal is relatively unprotected and highly susceptible to damage, especially by environmental pollutants. Research conducted with several pollutants suggests that early-life exposure to chemicals, even at environmental levels can produce neurotoxic effects long after exposure (41). Our findings suggest that chronic environmental exposure in early life may play a major role in the development of neurodegenerative disorders later in life.

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Sažetak

NEUROTOKSIČNOST FUMONIZINA B_1 U ŠARANSKE MLAĐI (*Cyprinus carpio* L.)

Odavno je poznato da okoliš ima važnu ulogu u razvoju neurodegenerativnih bolesti kao što su Alzheimerova i Parkinsonova bolest te multipla skleroza. Mikotoksin fumonizin B_1 (FB_1) tvori nekoliko vrsta *Fusariuma*, najčešće *F. verticillioides*, koja je najučestaliji kontaminant kukuruza. Ovaj mikotoksin odgovoran je za leukoencefalomalaciju konja, mula i magaradi povezanu s konzumacijom kukuruza kontaminiranog s FB_1. U ovom su radu prikazani rezultati kronične eksperimentalne toksikoze mlađi šarana (*Cyprinus carpio* L.) koji su u hrani primali 100 mg kg^{-1} i 10 mg kg^{-1} FB_1 tijekom 42 dana. Nakon bojenja hemalaun-eozinom zabilježene su značajne histopatološke promjene na mozgu životinja uključujući vakuolizaciju, degeneraciju i nekrozu neurona, posebice u blizini oštećenih krvnih kapilara i u periventrikularnoj regiji. Ova saznanja pokazuju da FB_1, kao hidrofilna molekula, prolazi kroz krvno-moždanu barijeru mladih šarana uzrokujući oštećenje neurona.

KLJUČNE RIJEČI: *krvno-moždana barijera, mikotoksini, neurodegenerativne bolesti, okoliš*

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