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***Research Article** (Open Access)*

TITANIUM DIOXIDE NANOPARTICLES INDUCED GENOTOXICITY AND FREE RADICAL GENERATION IN THE TISSUES OF *OREOCHROMIS MOSSAMBICUS*

Mubeen Sultana D.*, Lucky R. and Aneesh Nair
P.G and Research Department of Zoology,
J.B.A.S. College for Women, Teynampet, Chennai – 600018, India
**Author for correspondence: mubeenjbas2020@gmail.com*

ABSTRACT
The exploitation of Nanoparticles (NP) has increased in various fields such as drug delivery systems, cosmetics and electronics. With the increase in the utilization of these NPs, there is also an increase in the potential risks associated with the release of the NPs into the environment. Titanium dioxide nanoparticles (nano-TiO_2) are one of the most common materials used in nanotechnology. In the present study, nano-TiO_2 induced genotoxicity and free radical generation in the tissues of *Oreochromis mossambicus* has been investigated. Genotoxicity analysis was carried out by DNA fragmentation technique. The change in antioxidant potential of the fish treated with the nanoparticles was examined by FRAP assay and hydrogen peroxide scavenging assay. It was observed that the DNA fragmented upon treatment with the nanoparticles and increased with time. Nano-TiO_2 contributes to the generation of free radicals which in turn leads to toxicity of the fishes. The toxicity level increases as the stress increases.

***Keywords**: Titanium dioxide nanoparticles; Oreochromis mossambicus; Genotoxicity; DNA fragmentation; FRAP; Hydrogen peroxide scavenging*

INTRODUCTION
Nanotechnology means a new set of technologies that are used to develop nanoscale structures and devices with one dimension size of 1–100nm with special properties utilized in commercial applications (Murashov, 2009). In recent decades, advances in nanotechnology engineering have given rise to the rapid development of many novel applications for nanoparticles (NPs) in various industries. The main concerns that NPs create in the workplace are the adverse effects of acute or chronic exposure (Li *et al.,* 2008). With the development of nanotechnology, there has been a tremendous growth in the application of NPs for drug delivery systems, antibacterial materials, cosmetics, sunscreens and electronics (Elena *et al.*, 2007; Robertson *et al.*, 2010). As the interest in the potential benefits of nanoparticles has increased, there is also increasing concern over their potential toxic effect resulting from use or unintentional release into the environment (Dreher 2004; Andre, 2006; Bernd & Thomas, 2007).
Titanium dioxide nanoparticles (nano-TiO2) are one of the most common materials used in nanotechnology Project on Emerging Nanotechnologies (2011). In recent years, the threat of NPs and nanomaterials to biological systems has become widely recognized and the number of ecotoxicological studies on these emerging pollutants has increased (Oberdorster *et al.,* 2005; Carinci *et al.,* 2003; Lam *et al.,* 2004). *In vitro* studies performed to better understand the effects of TiO2 crystallinity on cells have revealed that the anatase crystal form causes a greater toxic response than the rutile form dose Braydich *et al.,* 2009; Wu *et al.,* 2010). Elucidation of the toxic response has involved the MTS (Wu *et al.,* 2010), MTT (Brian *et al.,* 2009; Barillet *et al.,* 2010; Simon-Deckers *et al.,* 2008; Sayes *et al.,* 2006), cell-staining, and cell-proliferation (Braydich-Stolle *et al.,* 2009) assays which revealed that TiO2 nanoparticles cause a dose and time-dependant decrease in cell viability, TiO2 nanoparticles, particularly

the anatase form, cause increased levels of inflammatory indicators such as LDH (Brian *et al.,* 2009 , Barillet *et al.,* 2010; Wu *et al.,* 2010., Sayes *et al.,* 2006) and IL-8 (Sayes *et al.,* 2006); again, anatase nanoparticles cause a greater inflammatory response than do rutile nanoparticles. The anatase crystal form may be more toxic because of its greater oxidizing potential, which would generate a greater number of reactive species (Jiang *et al.,* 2008). Wide application of TiO2 NPs confers substantial potential for human exposure and environmental release, which inevitably allows for a potential health risk to humans, livestock, and the eco-system (Long *et al.,* 2007). With significant advancements in genetics, proteomics, cellular and molecular biology, and biochemical engineering, separate safe exposure limits for micro particles and NPs may be set (Ahmed *et al.,* 2012).

The aquatic environment is particularly at risk of exposure to these nanoparticles, as it acts as a sink for most environmental contaminants. As an ultrafine-sized material, the TiO2 NPs may enter the fish body through respiratory tract, gastrointestinal tract and skin Hao *et al.,* 2009. It is estimated that the concentration of particles in the aquatic environment is between 0.0007 and 0.0245 µg/ml Muller and Nowack (2008); Perez *et al.,* (2009); consequently, there is increasing concern about its potential toxic effect on the marine and aquatic environments.In the present study, titanium dioxide nanoparticles induced genotoxicity and free radical generation in the tissues of *Oreochromis mossambicus* was investigated.

MATERIALS AND METHODS

Fish Collection and Maintenance:

Thirty Tilapia, *Oreochromis mossambicus* fishes weighing 5-10 gm were collected from Sholinganallur, Chennai, Tamil Nadu, India. *Oreochromis mossambicus* were acclimatized to laboratory conditions. The fishes were maintained in three plastic containers (Control, 10mg/L, 60mg/L of Titanium dioxide nanoparticle - TiO_2 NP) supplied with dechlorinated aged tap water under continuous aeration for 21 days. 10 fishes were introduced in each of the containers. During this period, fishes were fed daily with commercial fish food pellets.

Short term oral toxicity assay:

Ten fishes were introduced into each of the three containers and exposure studies to titanium dioxide were conducted for 21 days. The fishes introduced into the container with aged tap water were considered as controls. The water in the container was subjected to vigorous aeration continuously using air pumps in order to minimize aggregation of nanoparticles. Swimming pattern and opercular movement rates of the control fish and the fish exposed to TiO_2 NPs were recorded after one hour and two hours of the exposure. The fish samples were fed with commercially available food pellets twice a day. TiO_2 NPs was fed at an interval of 24 h. At 7 days of exposure, three fishes were randomly removed from each container and were subjected to genotoxicity and stress enzyme studies.

DNA fragmentation analysis:

The procedure for DNA isolation using CTAB was followed by the method of Saghai-Maroof *et al.,* (1984) with few modifications. 0.5g of tissue material was ground in a motor and pestle. To this, 1X CTAB buffer was added and incubated at 55°C for about 60 minutes. After mixing thoroughly, the tubes were centrifuged at 10,000 rpm for 10 minutes. The supernatant was transferred to a new sterile microfuge tube. Equal volume of ice cold (Phenol: chloroform: Isoamylalcohol) was added and mixed well by inversion. The tubes were then centrifuged at 10,000 rpm for 10 minutes. The aqueous phase was transferred to a new tube and 1ml of 70% ethanol was added to wash the pellet. The tubes were stored at -20°C for about 1 h. The pellet was washed with 70% ethanol twice and was air dried at room temperature. The pellet was dissolved in TE buffer and stored under -20°C. DNA samples were visualized via Agarose gel electrophoresis in a UV transilluminator.

Ferric Reducing Antioxidant Power (FRAP) assay

Total antioxidant activity is measured by ferric reducing antioxidant power (FRAP) assay of Benzie and Strain (1999). Stock solution of sample extracts was prepared to the concentration of 1mg/ml. From the stock solution, various concentration of the samples (250, 500, 750, 1000µg) were aliquoted and made up to 1ml with distilled water and was mixed with 1.5ml of FRAP reagent and incubated at 37°C for 4 minutes. After incubation the absorbance was measured at 593nm. Ferrous sulphate standard was processed in the same way and calibration curve was generated using various concentration of ferrous sulphate (200-1000µg). Blank consist of all the reagents except for the extract or standard solution and are substituted with water. The amount of ferrous released in µM of the sample can be calculated as:

$$\text{FRAP value of sample (µM)} = \frac{\text{Abs (sample)} \times \text{FRAP value of Std (µM)}}{\text{Abs (Std)}}$$

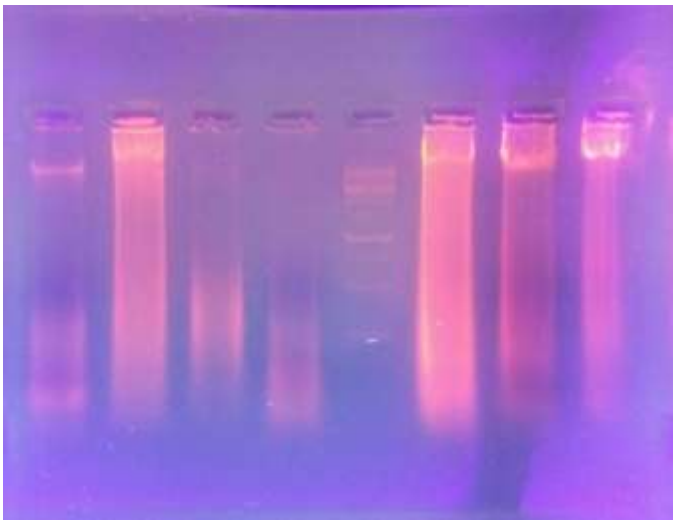
Where, the FRAP value of Std (Ascorbic acid) is 2.

Hydrogen peroxide scavenging assay

Antioxidant activity was also investigating by measuring the potential of hydrogen peroxide scavenging. 1g of ground sample was mixed with 5ml of 50mM phosphate buffer and centrifuged at 5000 rpm for 10 min. Stock solution of sample extracts (supernatant) was prepared at various concentrations of the sample (250, 500, 750,1000µg) were aliquoted and made up to 3 ml by adding hydrogen peroxide (1.8ml) and phosphate buffer (1.2 ml) in each tube and incubated for 10 minutes in room temperature. Blank consist of all the reagents except for the extract or standard solution and are substituted with water. After incubation the absorbance was measured at 240nm. The rate of absorbance change at 240 nm was recorded, which indicated the decomposition of H_2O_2. The percentage inhibition was calculated by the following formula

% inhibition = (Absorbance of control – Absorbance of test) /Absorbance of control × 100

RESULTS



LANE 1: DNA from Control fish
LANE 2: DNA from TiO_2 treated fish (10mg/L -21st day)
LANE 3: DNA from TiO_2 treated fish (10mg/L - 14th day)
LANE 4: DNA from TiO_2 treated fish (10mg/L -7th day)
LANE 5: Ladder
LANE 6: DNA from TiO_2 treated fish (60mg/L -21st day)
LANE 7: DNA from TiO_2 treated fish (60mg/L -14th day)
LANE 8: DNA from TiO_2 treated fish (60mg/L – 7th day)

Plate:1 DNA fragmentation analysis from *Oreochromis mossambicus*

Genotoxicity Studies

Genotoxicity of the NP was carried out by DNA fragmentation analysis in tissues. DNA isolated from the fish group- Control, 10mg/L and 60mg/L, where run on an agarose gel at 50V and visualized on a transilluminator is represented in (Plate 1). The image shows that the control fish sample had a sharp, crisp and a defined band of DNA corresponding to base pair of 2kb, while the DNA of the sample exposed to 10mg/L of NP were found to be fragmented and the number of fragments increased from day 7 to day 21 of the treatment. The fragmentation was found to be increased in the sample with 60mg/L of NP exposure. Thus, number of fragments in this sample (60mg/L) was more compared to the one with low NP exposure (10mg/L).

FRAP Assay

FRAP activity of the fish was determined by measuring the absorbance at 593nm and the value was calculated using the formula. The values were tabulated and it was found that the control fish had the maximum FRAP value of 0.4µM on all three days (7th day, 14th day and 21st day). FRAP value of the fish treated with 10mg/L decreased from 0.4µM to 0.12µM at the end of 21st day (Table 1, Fig 1(a), 1(b) and 1(c)). The value decreased from 0.42µM to 0.06µM for fishes exposed to 60mg/L. The decrease in the value shows that the TiO2 NPs releases number of free radicals into the body of the fishes. This reveals the stress caused in the fish models was due to the exposure of TiO2 NPs.

Table: 1 Frap Reducing Ability in the tissues of *Oreochromis mossambicus*

	FRAP Value of the sample (µM)									
	Day7			**Day14**			**Day21**			
Concentrations (µg)	**GROUP I**	**GROUP II**	**GROUP III**	**GROUP I**	**GROUP II**	**GROUP III**	**GROUP I**	**GROUP II**	**GROUP III**	**Control**
250	0.4401	0.4318	0.4206	0.4472	0.3586	0.2690	0.4453	0.1688	0.1322	0.0717
500	0.4593	0.3933	0.3890	0.4581	0.2356	0.1491	0.4563	0.1534	0.1165	0.0823
750	0.4682	0.3471	0.2051	0.4672	0.1613	0.0982	0.4661	0.1385	0.0762	0.0841
1000	0.4781	0.1995	0.1148	0.4774	0.0984	0.0765	0.4773	0.1294	0.0656	0.0875

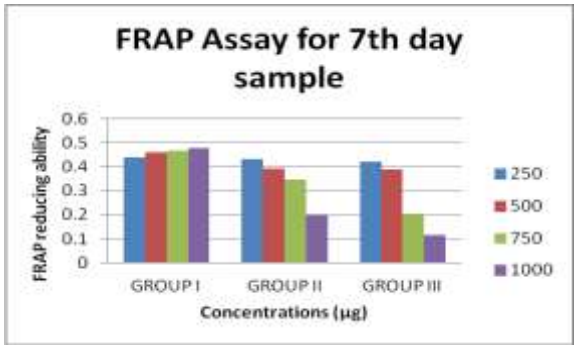


Figure 1 (a) FRAP ACTIVITY ON 7th DAY SAMPLE

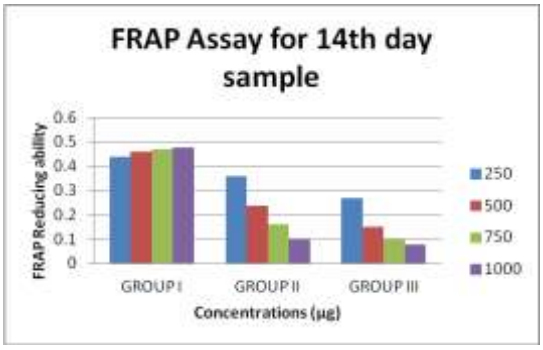


Figure 1 (b) FRAP ACTIVITY ON 14th DAY SAMPLE

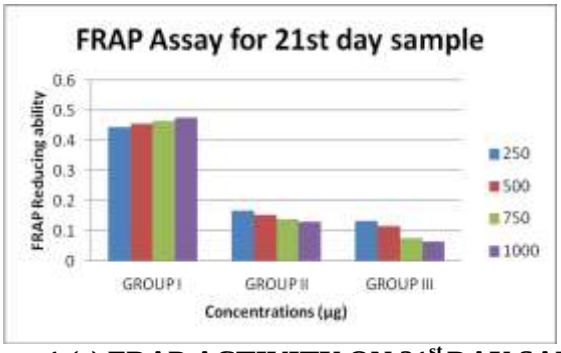


Figure 1 (c) FRAP ACTIVITY ON 21st DAY SAMPLE

H_2O_2 Scavenging Assay:

TiO2 Nanoparticles contributes to the generation of free radicals which in turn leads to toxicity of the fishes. The more the stress caused in the organism, the more the toxicity level. This correlates with the values tabulated in the (Table 2, Fig 2(a), 2(b) & 2(c)). Percentage of inhibition of control fish was found to be 81%, while the percentage decreased from 51 to 37% for the sample treated with 10mg/L. The inhibition decreased still more for the sample treated with 60 mg/L from 51% to 33%. Thus, the present study confirms that the increase in TiO_2 concentration and the time of exposure results in higher toxicity in fish models.

Table: 2 Percentage Inhibition for Hydrogen Peroxide Scavenging Activity in the tissues of *Oreochromis mossambicus*

	% Inhibition									
	Day7			Day14			Day21			
Concen-tration (µg)	GROUP I	GROUP II	GROUP III	GROUP I	GROUP II	GROUP III	GROUP I	GROUP II	GROUP III	Ascorbic Acid
250	53.09	51.15	52.43	54.07	45.72	40.45	52.20	37.12	33.34	76.10
500	59.50	51.92	51.17	59.07	43.42	38.41	59.61	30.95	28.38	82.51
750	67.18	50.36	50.19	67.24	41.06	35.71	65.31	17.53	17.14	88.32
1000	81.06	49.64	50.01	80.46	40.31	30.23	80.25	14.42	8.15	91.27

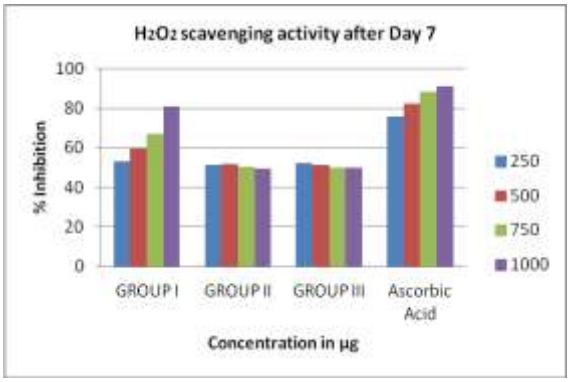


Figure 2 (a) H_2O_2 SCAVENGING ACTIVITY ON 7th DAY SAMPLE

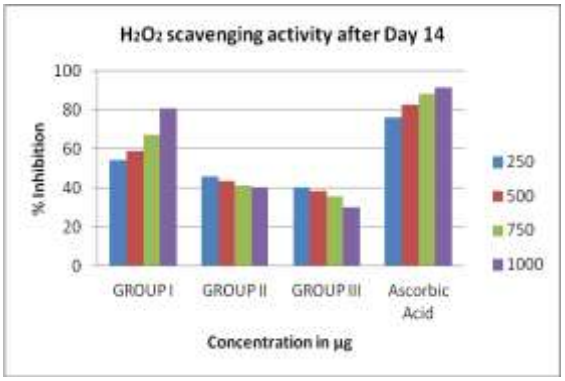


Figure 2 (b) H_2O_2 SCAVENGING ACTIVITY ON 14th DAY SAMPLE

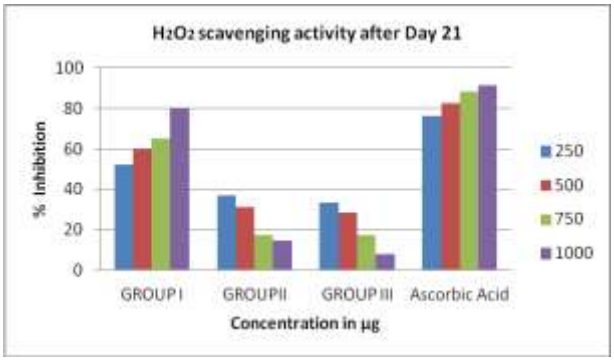


Figure 2 (c): H_2O_2 SCAVENGING ACTIVITY ON 21ST DAY SAMPLE

DISCUSSION

There is a growing concern regarding the safety of nanoparticles in relation to their toxicity. Several studies have reported the potential risk to human health from nanoparticles based on evidences of inflammatory reactions caused by ferric oxide nanoparticles in rats (Zhu *et al.*, 2008) and toxic effects of silica nanoparticles on fibroblast and tumor cells (Chang *et al.*, 2007). Engineered nanoparticles have wide range of application due to its unique properties when compared with their bulk counterparts (Andre *et al.*, 2006). Nanoparticles of titanium dioxide and Zinc oxide are widely used in suncare products (Serponr *et al.*, 2007) as well as on self-cleaning coatings (Cai *et al.*, 2006). During life cycle of three commercial products, nanoparticles mat be released into the environment and become a threat to environment and become a threat to ecosystem. A series of physiological effects induced by TiO_2 NPs have been observed in rainbow trout *Oncorhynchus mykiss* (Federici *et al.*, 2007). Several workers have proposed that the size effect seems more important to nanoparticle toxicity than the actual composition of the material (Kreuter *et al.*, 2002).In the present study short term oral toxicity of Titanium dioxide nanoparticle was analyzed in *Oreochromis mossambicus*. The toxicity studies include the effects of titanium dioxide nanoparticles on the DNA fragmentation analysis and the generation of reactive oxygen species in tissues. The dose of titanium dioxide nanoparticles administered orally was low and hence there was no death reported in the experimental group. Toxicity of TiO_2 nanoparticles increased significantly in the tissues of TiO_2-NP exposed fish. The increase was significant in fish exposed to 10 mg/L and highly significant in fish exposed to 60 mg/L, while the control fish sample had no toxicity due to nonexposure of NPs. Continuous treatment of fish to the NPs led to higher damage, the maximum found in 21st day fish model treated with 60mg/L. TiO_2 NPs induce phototoxicity upon UV irradiations. They have been shown to induce apoptosis. The genotoxicity of TiO_2 NPs attributes to ROS generation and oxidative stress in cells, which elicits signal transduction pathways leading to apoptosis or cellular death. They have been shown to induce DNA double-strand breakage leading to cell cycle arrest. The induction of ROS may reduce NADH levels, impairing mitochondrial membrane potential and causing mitochondrial dysfunction. The exposure of TiO_2 NPs may also cause genotoxic effect in 24 hours treatment. The genotoxicity, apoptosis, and mitotic arrest are caused by nanoparticles of TiO_2 in various tissues.

Damage to DNA is a fundamental example of cellular toxicity, and it is critical to assess such damage for any nanoparticle that is likely to come in contact with humans, given that damage to DNA is highly correlated with an increased risk of cancer. The present study referred to the most important biomolecule of the cell, the DNA that might be affected by TiO_2NPs administration. TiO_2NPs (10 and 60mg/L) treatment at different sampling time leads to DNA damage of the tissues that is highly affected at 21st days' sampling time. The DNA was isolated from all three types of fish model- Control and 10, 60mg/L. The isolated DNA was run on an agarose gel with a 2Kb DNA ladder to study the toxicity level. The

image obtained, depicted a sharp and a crisp band of control fish model and a sheared DNA (damaged one) for samples treated with 10mg and 60mg of NPs. An increase in the sharedness was observed visually with increase in the sampling time from 7th day to 21st day. More the fragments of the DNA sample in the gel, higher the toxicity level of the NPs. This conforms the genotoxic effect of TiO_2 NPs over the fish model.

The generation of ROS and oxidative injury plays a significant role in many of the observed biological responses to NPs. The size, surface area, and surface chemistry (e.g., reactive groups) of particular NPs contribute to the generation of ROS. In addition to their damaging effects on cellular proteins, lipids, and DNA, an increased level of ROS triggers the cell to respond by inducing proinflammatory signaling cascades, ultimately inducing apoptosis. Additionally, NPs may induce or aggravate inflammatory and allergic responses by directly influencing immune-related cell populations in the lung. The ability of NPs to generate oxidative stress has formed the basis of their hypothetical structure-toxicity relationship.

Exposure of fishes to TiO_2 NP caused a reduction of FRAP reducing activity in tissues, as well as a decrease in H_20_2 scavenging ability in tissues. In addition, NP exposure caused changes in the activity of antioxidant enzymes in tissues. FRAP reducing ability decreased from 0.43 of the 7th day sample to 0.16 for 21st day sample with 10mg/ml of TiO_2 treatment. The reducing ability decreased more for sample treated with 60mg/ml from 0.42 of day7 to 0.13 of day21. Hydrogen peroxide scavenging activity was also significantly affected (from 75.23% to 33%) by NP in both tissues, suggesting that antioxidant mechanisms are also a target for TiO_2 NP. Induction of oxidative stress represents a major cause of toxicity associated with these NP. Oxidative stress has also been implicated as the cause of toxicity, structural damage and mortality in response to NP exposure in the fish.

CONCLUSION

The environmental fate and behavior of Titanium dioxide nanoparticles is a rapidly expanding area of research. The process study focuses on the effect of TiO_2 nanoparticles on the free radicals in fishes. According to Oberdorster *et al.*, (2007), nanomaterials when released into the environment through industrial or domestic waste may tend to disrupt the microflora of soil and water. This in turns leads to alteration of the food chain and disrupt plant productivity by disrupting nitrogen assimilation and metabolism can damage aquatic organisms too. The results of the present study are also in concordance with the above findings. The free radicals were reduced on the last day (21st day) of the study when compared with the control. Our conclusion that oxidative stress is a major factor in the toxicity of NP in fish is supported by our data on the regulation of enzymes. The control fish had the highest FRAP reducing ability and H_2O_2 scavenging activity when compared to the fish models treated with 10 and 60mg/ml of titanium dioxide nanoparticles. This may be unique to *Oreochromis mossambicus* and a few other species of fish, which are known to be extremely hardy towards many different types of environmental stress.

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