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Original Article

Sublethal toxicity of TiO_2 nanoparticles to common carp (*Cyprinus carpio*, Linnaeus, 1758) under visible light and dark conditions

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Abstract: The objective of this study was to determine the sublethal toxicity of TiO_2 nanoparticles (TiO_2-NPs) on common carp (*Cyprinus carpio*) under visible light and dark conditions. Blood sampled was collected after 21 days and biochemical parameters, including glucose, total protein, albumin, globulin, creatinine, triglyceride and cholesterol levels, and aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transferase (GGT), lactate dehydrogenase (LDH), creatine kinase (CK), and alkaline phosphatase (ALP) activities were measured. The results showed that TiO_2-NPs is caused a significant effect on blood biochemical parameters of *C. carpio*. By changing lighting conditions from darkness to light, significant differences were observed in certain blood biochemical parameters, including AST, ALT, LDH, ALP and CK activities, glucose, cholesterol and triglyceride levels in fish exposed to TiO_2-NPs under light conditions as compared with fish exposed to TiO_2-NPs under dark conditions. Cholesterol and triglyceride levels in fish exposed to 0.0 mg L^{-1} TiO_2-NPs under darkness conditions were significantly higher than the control. The results revealed that toxicity of TiO_2-NPs under visible light conditions was more than darkness conditions.

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Introduction

The application of nanotechnology in industries such as biomedical sciences, pharmaceutical and cosmetics, wastewater treatments and electronic, and consequently the availability of nanoparticles into the environment are increasing. Therefore, nanoparticle products are rapidly accumulating in the aquatic ecosystems (Nowack and Bucheli, 2007) and their potential for exhibiting environmental toxicity is growing.

Titanium dioxide (TiO_2) nanoparticles (TiO_2-NPs) have the most industrial application compared to other nanoparticles. Ortlieb (2010) reported an annual amount of four million consumption of TiO_2 nanoparticle, used as pigments in a variety of products including food colorings, paper products, ink and plastic products (Ortlieb, 2010), cosmetics (as a UV filter), shampoos, soaps, toothpastes, sunscreens (Melquiades et al., 2008), building materials and paints (Chen and Poon, 2009) as well as many synthetic vitamin tablets, over-the-counter pain relievers, capsulated antidepressants and antibiotic products (Luft et al., 2010). In addition, the nanoparticles of TiO_2 are used in drinking water treatment especially for arsenic removal and also used as a photocatalyst in wastewater treatment plants for removal of contaminants. Therefore, TiO_2-NPs is one of the biggest environmental concern due to their easy transfer to aquatic ecosystems (Kaegi et al., 2008). According to the reports, the amount of TiO_2-NPs estimated to be 0.0007 to 0.0245 μg mL^{-1} is available in the environment (Mueller and Nowack, 2008; Pérez et al., 2009).

The widespread use of various nanoparticles in pharmaceutical and cosmetic industry, producing disinfectants, wastewater treatments and chemical removal from water have all contributed to increasing amount of nanoparticles to the surface

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water (Zou et al., 2014; Lei et al., 2016). Therefore, aquatic organisms are constantly exposed to a variety of potentially toxic nanoparticles (Li and Lenhart, 2012; Zou et al., 2014; Koetsem et al., 2015; Zhang et al., 2015; Lei et al., 2016). Exposure to TiO_2-NPs significantly induced the antioxidant enzymatic activity and increased the lipid peroxidation levels most evidently in liver, and caused histopathological damages in different tissues of common carp (Hao et al., 2009), zebrafish (Xiong et al., 2011) and rainbow trout (Federici et al., 2007). However, our knowledge on the potential toxic effects of TiO_2-NPs on the health of these organisms in regard to biochemical parameters is really limited. Many of the toxic effects of TiO_2-NPs on the environment depend on the interactions between the physico-chemical properties of TiO_2 and physical and chemical conditions of the water parameters. Since light exposure can play an active role in the photocatalytic activity of TiO_2, we hypothesize that there is a significant difference between the toxicity of TiO_2-NPs under visible light and darkness. Therefore, the aim of this study was to evaluate the toxicity of nanoparticles of TiO_2 on blood biochemical parameters of common carp (*Cyprinus carpio*) under light photoperiod (light: dark: 16: 8 h) and dark (no irradiation) conditions.

Materials and Methods

Nanoparticles characterization: Commercial TiO_2-NPs, with an average primary particle size of 20 nm in the powder form (Table 1), were purchased from Iranian Nano-materials Pioneers Company.

Fish: Juvenile common carp with average body weight of 30±5 g were maintained in the laboratory of the Department of Aquaculture, Khatam Al-anbia University of Technology, Iran. Fish were maintained in 80 L tanks with de-chlorinated tap water under controlled environmental conditions at 24±2°C on a 16:8 h (light: dark) photoperiod during two weeks acclimatization period. Fish were fed with commercial carp pellets (Beyza Fedd Mill Co. Iran) at the manufacturer's recommended rate.

Preparation of Test Nanoparticles: Stock solutions of TiO_2-NPs were prepared using distilled water and then ultrasonicated (10 min, 35 KHz, 100/400W) using an ultrasound bath (Elma, Germany) for dissolution. Then, solutions were added to 10 L of dechlorinated tap water in exposure tanks to obtain nominal concentrations of 0.125 mg L^{-1} TiO_2.

Sublethal toxicity: A total of 144 common carp were randomly distributed in twelve 80 L tanks (4 treatments with three replicates). The experiment was conducted for 21 day sublethal toxicity tests. Every tank contained 12 fish which were exposed to test solutions with the following concentrations of TiO_2-NPs: 0.0 mg L^{-1} TiO_2-NPs, and 0.125 mg L^{-1} TiO_2-NPs each under different dark and light conditions. Sublethal concentrations were selected according to Xiong et al. (2011), Hao et al. (2009) and Lee et al. (2012).

The experiments were carried out according to a factorial scheme considering two factors (at different concentrations of TiO_2 and photoperiod conditions) in two parallel sets. Visible light irradiation was provided by four white fluorescent lamps (600 lux, 40 W, Pars-Shahab, Iran). In darkness experiments, the tanks were wrapped with opaque sheets and were kept in darkness without intervention of any visible light. The photoperiod (16:8 h) condition was considered as light condition and dark (no irradiation) as dark condition throughout the experiment. Although continuous aeration of water may partly prevent deposition of nanoparticles on the bottom of tanks, nanoparticles tend to form agglomeration (Hao et al., 2009). The actual amount of TiO_2 NPs may decrease up to 50% after 3 days (Hao et al., 2009). Therefore, 50% of the water was exchanged every 12 hours and nanoparticles solution was added again to the tanks to maintain TiO_2-NPs concentrations in constant (equivalent to 0.125 mg per liter). This is necessary to inhibit the agglomeration of NPs and their absorption by fish feces and uneaten food.

Sampling and analysis of blood biochemical parameters: Fish were starved for 24 hrs before sampling. After the 21-day exposure period, 12 fish from each treatment (4 fish from each tank) were

Table 1. Physicochemical proprieties of TiO_2 nanoparticles according to the manufacturer.

Titanium Oxide	**TiO_2, 80 vol% anatase + 20 vol% rutile**
Purity	+99%
Average Primary Particle Size (D50)	20 nm
Specific surface area (SSA)	10-45 m^2 g^{-1}
Color	White
Bulk density	0.46 g ml^{-1}
pH	5.5-6.0
TiO_2	≥99%
Al	≤17 ppm
Mg	≤65 ppm
Si	≤120 ppm
Ca	≤75 ppm
S	≤130 ppm
Nb	≤80 ppm
Loss of weight in drying	0.48%
Loss of weight on ignition	0.99%

removed for sublethal toxicity studies. Fish were quickly netted from tanks and placed in 4 liter buckets filled half with 200 mg L^{-1} of clove powder solution. Blood sample was collected from the caudal vein using heparinized syringes, centrifuged at 6000×g for 10 min and stored at -25°C.

All blood biochemical parameters were determined using a UV-visible spectrophotometer (UNICO 2100) and standard biochemical reagents (Pars Azmun Company, Tehran, Iran). Each blood biochemical parameter was measured by a certain method. Total plasma protein was measured at 540 nm by the Biuret reaction. Albumin assay was based on the dye-binding properties of plasma albumin with bromocresol green. An increase in blue-green color was measured at 630 nm. The plasma globulin was estimated based on the ratio of albumin versus total protein (Johnson et al., 1999). Plasma glucose was measured by the glucose-oxidase method at 500 nm (Sacks, 1999). Plasma cholesterol levels were determined by the CHOD-PAP method at 510 nm, triglyceride levels by GPO-PAP method at 546 nm (Rifai et al., 1999) and creatinine by JAFFE method at 510 nm (Foster-Swanson et al., 1994). Urea is hydrolyzed enzymatically by urease to yield ammonia and carbon dioxide. The ammonia and α-oxoglutarate are converted to glutamate in a reaction catalyzed by L-glutamate dehydrogenase. Simultaneously, one molar equivalent of reduced NADH is oxidized. Two molecules of NADH are oxidized for each molecule of hydrolyzed urea. The rate of change in absorbance at 340 nm, due to the disappearance of NADH, is directly proportional to the blood urea concentration in the sample (Lumeij and Remple, 1991). The activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in plasma were determined by NADPH consumption and its conversion to NAD^+ at 340 nm. Gamma-glutamyl transferase (GGT) activity is determined by a coupled enzyme assay in which GGT transfers the γ-glutamyl group from the substrate L-γ-Glutamyl *p*-nitroanilide, liberating the chromogen *p*-nitroanilide at 418 nm proportional to GGT. Lactate dehydrogenase (LDH) in plasma was determined based on the conversion of pyruvate to lactate at 340 nm, alkaline phosphatase (ALP) based on converting nitrophenol phosphate into nitrophenol and phosphate at 405 nm, creatinine kinase (CK) based on the conversion of creatinine phosphate into creatinine at 340 nm and based on optical density (OD) absorption and the formula presented in the kits' manual (Moss and Henderson, 1999).

Statistical analysis: All data were examined for normality (Kolmogorov-Smirnov test) and then analyzed for significance using one-way analysis of variance (ANOVA). The significant means were compared by Duncan's test and a $P<0.05$ was

Table 2. Alterations in the blood biochemical parameters of common carp, *Cprinus carpio* exposed to TiO_2 nanoparticles (0.00 and 0.125 mg L^{-1}) under photoperiod (light: dark: 16: 8 h) and dark (no irradiation) conditions.

Blood biochemical parameters	0.00 mg Nano-TiO_2 (light)	0.00 mg Nano-TiO_2 (Dark)	0.125 mg Nano-TiO_2 (light)	0.125 mg Nano-TiO_2 (Dark)
AST (U L^{-1})	44.22±3.77^a	54.35±6.60^a	125.94±19.21^c	70.95±6.65^b
ALT (U L^{-1})	14.67±1.91^a	11.67±0.47^a	80.54±20.43^c	26.84±4.29^b
LDH (U L^{-1})	146.94±11.34^a	182.53±15.76^a	305.76±67.74^c	260.04±33.47^b
ALP (U L^{-1})	59.33±5.80^a	55.91±3.31^a	113.95±10.40^c	93.74±19.24^b
CPK (U L^{-1})	318.90±76.30^a	324.25±14.16^a	1421.37±250.52^c	1126.75±258.81^b
GGT (U L^{-1})	14.15±1.06^b	14.76±0.82^b	4.93±1.15^a	4.29±1.05^a
Glucose (mg L^{-1})	58.32±9.76^a	56.48±5.37^a	79.09±8.58^b	95.01±12.13^c
Creatinine (mg L^{-1})	0.14±0.02^a	0.17±0.02^a	1.17±0.42^b	1.08±0.24^b
Total protein (g dL^{-1})	4.06±0.35^b	4.29±0.25^b	2.82±0.49^a	2.94±0.54^a
Albumin (g dL^{-1})	2.03±0.28^a	2.15±0.37^a	2.22±0.29^{ab}	1.99±0.16^a
Globulins (g dL^{-1})	2.03±0.53^b	2.13±0.52^b	0.60±0.44^a	0.95±0.45^a
Cholesterol (mg L^{-1})	145.03±16.86^b	166.62±14.73^c	90.31±17.03^a	131.44±5.83^b
Triglycerides (mg L^{-}	222.85±16.62^c	251.51±30.27^d	99.06±10.63^a	139.91±19.46^b

Statistically significant differences comparatively to controls if * (P<0.05). Alphabet letters indicate significant differences between groups.

considered statistically significant. Statistical analyses were performed using SPSS 19 (IBM Corp.). Data are presented as mean ±SD.

Results

No significant mortality was observed in all treatments. During the assay, increased mucus secretion, color changes, a progressive loss of scales, behavioral changes such as tremors, lethargy, and erratic swimming in the surface water were important changes found in the individuals exposed to TiO_2-NPs.

The effects of sublethal concentration (0.125 mg L^{-1}) of TiO_2-NPs on the blood biochemical parameters of common carp under photoperiod (light: dark: 16: 8 h) and dark (no irradiation) conditions are shown in Table 2. Fish exposure to 0.0 mg L^{-1} TiO_2-NPs under dark conditions showed no significant effects on the activity of AST, ALT, LDH, ALP, CPK and GGT and levels of glucose, creatinine, total protein, albumin and globulin. However, cholesterol and triglyceride levels in these fish were significantly higher than the control group i.e. under light condition (P<0.05).

The plasma AST, ALT, LDH, ALP and CK activities showed a significant increase in fish exposed to TiO_2-NPs compared to control group (P<0.05). The plasma AST, ALT, LDH, ALP and CK activities in fish exposed to TiO_2-NPs under light conditions were significantly greater (P<0.05) than fish exposed to TiO_2-NPs under dark conditions. GGT activity, total protein and globulin levels in the plasma of fish exposed to TiO_2-NPs in both photoperiod conditions were significantly lower (P<0.05) compared to the respective control groups.

Hyperglycemic condition was significant (P<0.05) in both photoperiod conditions in fish exposed to TiO_2-NPs. Plasma glucose levels in fish exposed to 0.125 mg L^{-1} of TiO_2-NPs under light photoperiod conditions were significantly lower than fish exposed to TiO_2-NPs under dark conditions (P<0.05). There was a significant increase (P<0.05) in creatinine levels of fish exposed to TiO_2-NPs compared to control group. However, no significant changes were observed in levels of plasma albumin in fish treated with TiO_2-NPs compared to the control group (P>0.05). A significant decrease (P<0.05) was observed in cholesterol levels in fish exposed to 0.125 mg L^{-1} of TiO_2-NPs under light photoperiod conditions compared with other groups. Triglyceride levels in plasma of fish exposed to TiO_2-NPs were significantly lower than control group, and a significant decrease (P<0.05) was observed in triglyceride levels in fish exposed to TiO_2-NPs under light photoperiod conditions as compared to fish exposed to TiO_2-NPs under dark

conditions ($P<0.05$).

Discussion

The results have shown that damage to the skin and the consequent loss of fish scales may facilitate the penetration of TiO_2 into body of the fish. The attachment of TiO_2-NPs to cells might impair the physiological function of cell membranes. Since skin and gills are in direct contact with the aqueous pollutants, increased mucus may act as a barrier and defense mechanism in sensitive stratified gill epithelium and skin epithelium against TiO_2-NPs (Smith et al., 2007).

Biochemical data showed that TiO_2-NPs at sublethal concentrations exerted a significant effect on some of the studied blood biochemical parameters. Reeves et al. (2008) found that in addition to generation of reactive oxygen species (ROS), possible mechanisms of toxicity to organisms induced the adhesion of TiO_2 to cells and physical disruption of the cell membranes. There is strong evidence that TiO_2-NPs are able to generate ROS such as hydroxyl (OH) radical (Reeves et al., 2008), superoxide radical anions (O_2^-) and hydrogen peroxide (H_2O) (Dodd and Jha, 2009) not only in the presence of UV irradiation but also in the absence of photo-activation (Armelao et al., 2007; Gurr et al., 2005; Reeves et al., 2008). When cells are attacked by ROS, cell membrane happens to be the first target. In the present study, increased AST, ALT, LDH, ALP and CK activities were observed in fish exposed to TiO_2-NPs, which might be due to increased cellular membrane damage and enzymes leaking out of cells which are damaged by TiO_2-NPs. Similar results have been reported by Wang et al. (2007), Chen et al. (2009), Duan et al. (2009) and Liu et al. (2009). However, the activity of these enzymes in the photoperiod condition was found to be more than dark condition.

The results showed that damage to cell membrane in visible light conditions was more severe than that of darkness conditions. TiO_2 nanoparticles may react with oxygen and water molecules to produce reactive oxygen species under dark conditions (Fenoglio et al., 2009). In addition to the direct interaction of TiO_2-NPs with oxygen and water molecules under light conditions, TiO_2-NPs can absorb the light to produce hydroxyl radicals, which can provide the basis for the production of superoxide anion, super hydroxide radicals and peroxide hydrogen (Li et al., 2015). The results indicate that increased production of ROS in an environment treated with nanoparticles of TiO_2 under light conditions can cause more damage to cell membranes. ROS and its consequent oxidative stress may cause peroxidation of the phospholipid membranes and direct damage to proteins, and affect the physiological function of the cell membrane (Reeves et al., 2008; Dodd and Jha, 2009). Hao et al. (2009) confirmed that histopathological damage to various tissues of juvenile carps exposed to TiO_2-NPs was related to oxidative stress.

The hyperglycemic condition observed in the present study could be attributed to the mobilization of glucose or increased secretion of cortisol due to TiO_2 toxicity. Hyperglycemia or elevated blood glucose levels indicated the impaired glucose and lipid metabolism and degradation of glycogen in liver (Liu et al., 2009). Glycogen depletion has also been reported by Lourenço (2012) in Gold fish (*Carassius auratus*) exposed to concentrations of TiO_2-NPs within a range from 0.01 to 800 mg L^{-1}.

A decrease in total protein and globulin levels in fish exposed to TiO_2-NPs indicated reduced protein and globulin synthesis in hepatocytes. The decrease in protein levels may be related to malnutrition, the increased energy cost of homeostasis, tissue repair and the detoxification mechanism under stress conditions. Griffitt et al. (2009) found that TiO_2-NPs could affect the expression of genes involved in protein synthesis process. Also, decreased globulin levels may reduce the resistance of fish to pathogens. Increased photoperiod is reported to have a significant effect on the activity of enzymes involved in fatty acid synthesis and fat accumulation in tissues (Faulconnier et al., 2001). Moreover, the influence of photoperiod on the synthesis level and excretion of insulin, thyroid hormones and corticosteroids

which play an important role in fat metabolism is interesting (Marie et al., 2001). Therefore, an increase in cholesterol and triglycerides in the blood of fish kept in dark condition may be due to the effect of darkness on fatty acid biosynthesis, reduced storage capability of blood lipid in adipose tissue and alterations in hormones involved in blood lipid homeostasis in fish. Decreased cholesterol and triglyceride levels in the blood of fish exposed to TiO_2-NPs under normal photoperiod conditions might be attributed to the influence of TiO_2-NPs on the synthesis of cholesterol and triglycerides in liver or reduced intestinal uptake of lipids and fatty acids. Using cholesterol and triglycerides to deal with the toxicity of TiO_2-NPs under light conditions might be due to lower levels of cholesterol and triglycerides in blood. Liu et al. (2009) demonstrated that TiO_2-NPs have negative effects on metabolism of lipids in animals. Prochazka et al. (2012) found that TiO_2-NPs have the potential to breakdown lipids, especially cholesterol, in biological systems. An increase in creatinine was observed in fish exposed to TiO_2-NPs that might be due to kidney dysfunction and is considered as the physiological evidence of TiO_2-induced nephrotoxicity (Wang et al., 2007; Liu et al., 2009; Wu et al., 2016).

Our results showed that alterations in blood biochemical parameters in fish exposed to TiO_2-NPs under light photoperiod conditions were more acute than fish exposed to TiO_2-NPs under dark conditions. In fact, the possibility of having reactive oxygen species in aquatic environments by TiO_2-NPs under visible light is more than that under darkness.

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چکیده فارسی

تاثیر سمیت زیرکشنده نانوذرات دی‌اکسید تیتانیوم بر ماهی کپور معمولی (*Cyprinus carpio*) تحت شرایط نور مرئی و تاریکی

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گروه شیلات، دانشکده منابع طبیعی و محیط زیست، دانشگاه صنعتی خاتم الانبیاء (ص) بهبهان، بهبهان، ایران، کدپستی: ۴۷۱۸۹-۶۳۶۱۶

چکیده:

هدف از این مطالعه تعیین سمیت تحت کشنده نانوذرات TiO_2 در ماهی کپور معمولی (*Cyprinus carpio*) در زیر نور مرئی و شرایط تاریک است. پس از ۲۱ روز از ماهیان خون‌گیری شده و پارامترهای بیوشیمیایی از جمله گلوکز، پروتئین کل، آلبومین، گلوبولین، کراتینین، سطح تری گلیسیرید و کلسترول خون و آسپارتات آمینوترانسفراز (AST)، آلانین آمینوترانسفراز ((ALT، γ گلوتامیل ترانسفراز (GGT) و لاکتات دهیدروژناز (LDH)، کراتین کیناز (CK) و فعالیت‌های آلکالین فسفاتاز (ALP) اندازه‌گیری شد. نتایج نشان داد که نانوذرات TiO_2 اثر معنی‌داری بر پارامترهای بیوشیمیایی خون *C. Carpio* داشت. با تغییر شرایط نوری از تاریکی به روشنایی، تفاوت معنی‌داری در برخی از پارامترهای بیوشیمیایی خون، از جمله فعالیت آنزیم‌های AST، ALT، LDH، ALP و CK، و سطح گلوکز، کلسترول و تری‌گلیسرید در ماهی در معرض نانوذرات TiO_2 در شرایط نور در مقایسه با ماهی در معرض نانوذرات TiO_2 در شرایط تاریک مشاهده شد. نتایج نشان داد که سمیت نانوذرات TiO_2 در شرایط روشنایی بیشتر از شرایط تاریکی است.

کلمات کلیدی: نانوذرات TiO_2، کپور معمولی، پارامترهای بیوشیمیایی، شرایط دوره نوری.